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GENE GENEALOGIES REVEAL DIFFERENTIATION AT SEX PHEROMONE OLFACTORY RECEPTOR LOCI IN PHEROMONE STRAINS OF THE EUROPEAN CORN BORER, *OSTRINIA NUBILALIS*

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Males of the E and Z strains of the European corn borer *Ostrinia nubilalis* (Lepidoptera: Crambidae) are attracted to different blends of the same pheromone components. The difference in male behavioral response is controlled by the sex-linked locus *Resp*. The two types of males have identical neuroanatomy but their physiological specificity is reversed, suggesting that variation at the periphery results in behavioral change. Differences in the olfactory receptors (ORs) could explain the strain-specific antennal response and blend preference. Gene genealogies can provide insights into the processes involved in speciation and allow delineation of genome regions that contribute to reproductive barriers. We used intronic DNA sequences from five OR-encoding genes to investigate whether they exhibit fixed differences between strains and therefore might contribute to reproductive isolation. Although two genealogies revealed shared polymorphism, molecular polymorphism at three genes revealed nearly fixed differences between strains. These three OR genes map to the sex chromosome, but our data indicate that the distance between *Resp* and the ORs is >20 cM, making it unlikely that variation in pheromone-sensitive OR genes is directly responsible for the difference in behavioral response. However, differences in male antennal response may have their origin in the selection of strain-specific alleles.

KEY WORDS: Communication, mate finding, olfactory receptors, sex chromosome, speciation.

Animals have evolved intricate mating systems in which visual, acoustic, and/or chemical signals are used to recognize conspecifics. In most moths (Insecta: Lepidoptera), mate finding is based on the detection of female sex pheromones by males and the stereotypic orientation behavior it elicits. Moths, therefore, provide important models for understanding mechanisms at play during speciation. In many moth lineages, divergence in pheromones is a key element in the origin of novel mate recognition systems (Cardé and Haynes 2004; Smadja and Butlin 2009). However, one of the conundrums in evolutionary biology lies in explaining how evolutionary diversification of sexual communication traits can occur in the face of stabilizing selection to maintain the integrity of signal and response (Symonds and Elgar 2008; Smadja and Butlin 2009). The theory of asymmetric tracking, which proposes that males and females experience different selection regimes, provides an appealing explanation for the evolution of pheromone diversity over time and space (Phelan 1997; Bengtsson and Löfstedt 2007). Weaker selection on female traits

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might permit the persistence of alleles that cause females to produce deviant pheromone blends, which could be tracked by males with a broad response window (Phelan 1997). Therefore, demonstrating how male response is determined and characterizing the nature of variation in this trait may be key to understanding the evolution of pheromone diversity.

In moths, the primary centers for pheromone detection, the antennae, are covered with thousands of sensillae housing olfactory sensory neurons (OSN) that express olfactory receptors (OR) at the surface of their dendrites (Hansson 1995; Touhara and Vosshall 2009). The olfactory information reaches the central nervous system via the OSN axons that project into the antennal lobe where they converge with the dendrites of second-order projection neurons and local interneurons to form glomeruli (Hansson 1995; Touhara and Vosshall 2009). Differences in behavioral responses may be the consequences of genetic changes altering the peripheral level and/or the central nervous system (Smadja and Butlin 2009; Touhara and Vosshall 2009).

Ostrinia nubilalis, the European corn borer (ECB), has become a model for studying the genetics of pheromone signaling and identifying genes underlying the origin of reproductive barriers (Cardé et al. 1978; Roelofs et al. 1987; Dopman et al. 2004, 2005; Lassance et al. 2010). ECB consists of two pheromone strains in which females produce and males respond to different blends of the *E* (trans) and *Z* (cis) isomers of Δ11-tetradecenyl acetate. The E race uses a 99:1 *E*/*Z* blend, whereas the Z race uses a 3:97 *E*/*Z* mixture (Klun et al. 1973; Kochansky et al. 1975). Sexual isolation results in part from stereotypic differences in male response to the female blend (Linn et al. 1997; Dopman et al. 2010). Data from interstrain crosses have revealed that female pheromone production and male behavioral response are each controlled by a single major locus (Roelofs et al. 1987). The autosomal gene determining pheromone production has recently been shown to encode a fatty-acyl reductase with strain-specific substrate specificity (Lassance et al. 2010). Difference in male behavior is encoded by a locus called *Resp* that maps to the sex chromosome (Roelofs et al. 1987; Dopman et al. 2004, 2005). In addition, variation in pheromone reception (antennal response) by ECB males can be attributed to the effects of both autosomal and sex-linked loci (Hansson et al. 1987; Roelofs et al. 1987; Olsson et al. 2010). The gene(s) underlying intraspecific differences in behavior and reception are yet to be identified. When it comes to neurophysiology, males of the two pheromone strains have a reversed functional topology of the antennal lobes (Karpati et al. 2008). This conclusion comes from the observation that the projections of OSNs responding to Z and E pheromone isomers are swapped so that in each case the neurons responding to the major and minor components of the pheromone always project to the same macroglomeruli. These findings suggest that changes in behavior may be the consequence of variation upstream of the central nervous system, that is, at the peripheral level. In particular, differences in expression or function of pheromone ORs could produce differential male olfactory and behavioral response, as suggested recently for sexual isolation between the moths *Heliothis virescens* and *H. subflexa* (Gould et al. 2010). Because pheromone-specific ORs tuned to the major pheromone components in *Bombyx mori* and *H. virescens* map to the sex chromosome (Sakurai et al. 2004; Gould et al. 2010), it is possible that the sex-linked locus *Resp* represents one or more pheromone ORs. In addition, pheromone OR genes may represent the sex-linked component affecting male peripheral olfactory response (Olsson et al. 2010).

Here, we perform a comparative genealogical analysis of sequence data from five OR genes that have been shown to be male-biased in their expression profiles and to respond to *Ostrinia* female sex-pheromone components (Wanner et al. 2010). We show that three OR genes are strongly differentiated between E and Z moths. Finally, we map these OR genes to the Z chromosome and evaluate their map positions relative to that of the *Resp* locus.

Materials and Methods

INSECT SAMPLES

For genealogical analysis, ECB moths were sampled from USA, France, Great Britain, Italy, Slovenia, Greece, Hungary, and Kazakhstan (Table 1 and Fig. S1). We started with genomic DNA from the panel of ECB moths used by Dopman et al. (2005). To augment this sample, moths were obtained from cultures derived from field collections and from field-collected individuals (fifth instar larvae). With the exception of ECB samples from France (Aquitaine, Loiret, Ardèche), all samples consisted of adult individuals. Where larvae were sampled, the sex of the insect was determined with molecular markers specific for the Z and W chromosomes (Coates and Hellmich 2003). Only females were used in genealogical analysis; females are the heterogametic sex in Lepidoptera (ZW), and are therefore hemizygous at all Z-linked loci. The pheromone type of ECB individuals was determined by gas chromatographic analyses of female pheromone gland extracts or on the basis of previous characterization of local populations.

DATA COLLECTION STRATEGY AND PRIMER DESIGN

Our primary goal is to construct and compare the pattern of gene genealogies for ECB pheromone ORs; therefore, we chose to examine intron sequences because of their tendency to be more variable. At the same time, intron sequences reflect selective pressures affecting adjacent sites in coding regions. Furthermore, because OR genes in *Ostrinia* are large, with many short exons, obtaining substantial amounts of coding sequence from genomic (as opposed to cDNA) would not be efficient.

Sequences for ECB ORs were obtained from GenBank (FJ385012-15; GQ844881). The exon–intron boundaries in the

Table 1. Sampled ECB populations.

Population	Abbreviation	Pheromone strain	Number of individuals
USA, Iowa	IOWA	Z	4
USA, North Carolina	NC	Z	2
		E	7
USA, New York	NY	Z	6
		E	5
France, Aquitaine	AQ	Z	11
France, Ardèche	AR	Z	7
France, Poitou-Charentes	PC	Z	2
France, Loiret	LO	Z	8
France, Picardie	PI	E	2
Great Britain	GB	E	8
Italy	CAS, GAZ, POD	Z	3
	CAS, GAZ	E	4
Slovenia	SLO	E	2
Greece	GR	E	8
Hungary	HUNG	Z	4
Kazakhstan	KAZ	E	3

OR sequences were predicted by comparison with the pheromone-specific OR genomic sequences retrieved from the *B. mori* genome (www.silkdb.org; (Wang et al. 2005)). Primers were designed using AlleleID 7.0 (Table 2).

DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

DNA was extracted from larvae or adult female moths using the DNeasy tissue kit (Qiagen, Sollentuna, Sweden), or the CTAB or Chelex methods. PCR reactions for genotyping individuals were performed using the following touchdown profile: 2 min at 92°C, 15 cycles of 50 s at 94°C, 45 s of primer annealing at an initial temperature of 60°C dropped 0.3°C per cycle, and 1 min at 72°C, followed by 25 cycles of 50 s at 94°C, 45 s at 55°C, and 1 min at 72°C. PCR products were treated with a mixture containing EXO (Exonuclease I) and SAP (shrimp alkaline phosphatase) and sequenced in both directions using the corresponding gene-specific primers with the BigDye terminator kit version 1.1 (Applied Biosystems) followed by analysis on a ABI PRISM 3130×l genetic analyzer (Applied Biosystems). Sequences of all loci were submitted to GenBank (accession nos. HQ873964-HQ874281).

DATA ANALYSIS

Sequences were aligned using MAFFT 6 (Katoh and Toh 2008) and manipulated with BioEdit version 7 (Hall 1999). Sequences were blasted using the BLASTN function to confirm that each corresponded to the targeted locus. For *OnOR3-6*, several indels were observed and the E-INS-i strategy used with MAFFT proved to give extremely reliable alignments.

We used DNASP 5 (Librado and Rozas 2009) to perform polymorphism analyses and calculate the following summary statistics: number of haplotypes (H), haplotype diversity (H_d), π and θ nucleotide diversity, and minimum number of recombination events (R_m). Indels were not included in the analyses. These descriptive parameters were estimated for the entire dataset (all ECB) and for data subsets corresponding to populations of the same pheromone type.

For each locus, we used Tajima's D test (Tajima 1989) and Fu and Li's D^* and F^* tests (Fu and Li 1993) to detect possible departures from a neutral model of molecular evolution. The analyses were performed using DNASP 5 on the entire dataset and on subsets consisting of individuals of the same pheromone type. Finally, the average net number of nucleotide differences between populations (D_a) was calculated.

Table 2. Marker primers and their position in the cDNA sequence.

Locus		Primer sequence (5′ to 3′)	Position in cDNA[1]
OnOR1	Forward:	GATGCAGTACAAGATCAGCCTCAAG	1155–1275
	Reverse:	TGACAAACCACAAATATAATAGATAGAAAC	
OnOR3	Forward:	ATCAAAGCGTTGGGTCTG	1168–1260
	Reverse:	AACTTATAAATATAACATGGTGCGTAG	
OnOR4	Forward:	CTGTTCCTGCTGCATAACG	1144–1258
	Reverse:	TTCGCAGTAGCATGAAGTAG	
OnOR5	Forward:	GTGCTGTTCCTGCTCTAC	1138–1266
	Reverse:	CGTTCGCAAGAACATGAAG	
OnOR6	Forward:	CGATACGGACCTTTGACTATGATCG	976–1126
	Reverse:	AAGCAGTTCGCTGGTTGCTGTTG	

[1]Base pairs of the cDNA sequence.

Analyses of molecular variance (AMOVA) (Excoffier et al. 1992) were conducted using Arlequin version 3.5 (Excoffier and Lischer 2010). The *P*-values associated with the fixation indices were obtained by permutation tests (10,000).

PHYLOGENETIC ANALYSES

We reconstructed OR gene trees using the Neighbor-Joining method in MEGA 4.0 software (Tamura et al. 2007). The maximum composite likelihood algorithm was used for determining the evolutionary distance between sequences. Alignment gaps were eliminated only in pairwise sequence comparisons. The inferred trees were tested by a bootstrap procedure with 1500 replications.

GENETIC MAPPING

To map *OnOR1, OnOR3* and *OnOR6*, we used genomic DNA from the male backcross progeny used by Dopman et al. (2004, 2005) (ExF_1; 78 male individuals). These males had been phenotyped for male behavioral response. For mapping *OnOR4* and *OnOR5*, 24 females from the same backcross progeny were genotyped. Details of crosses and phenotype determination of male behavioral response can be found in Dopman et al. (2004). Following PCR amplification of genomic DNA using the OR-specific primers, we could identify the segregating alleles of *OnOR3* and *OnOR6* on the basis of size differences on agarose gels, and thereby define BC individuals as homo-or heterozygote for a particular OR gene. PCR products were sequenced to distinguish the alleles of *OnOR1* and *OnOR5* and determine the genotype of BC individuals at the *OnOR1* and *OnOR5* loci. Finally, inheritance pattern for *OnOR4* was determined on the basis of presence/absence of a PCR product on agarose gel.

To map *OnOR1*, *OnOR3*, and *OnOR6*, genotype data for the OR genes were added to the published dataset of five Z chromosome markers: *Resp, Kettin* (*Ket*), *Lactate dehydrogenase* (*Ldh*), *Triose-phosphate isomerase* (*Tpi*), and the microsatellite marker ma169 (Dopman et al. 2005). Mapping was performed in JoinMap 4 using the Maximum likelihood mapping algorithm with default parameter settings (Van Ooijen 2006).

Results

DIFFERENTIATION BETWEEN POPULATIONS

For the five targeted OR genes, we amplified and sequenced genomic DNA fragments ranging from ≈410 bp to 1415 bp. The regions amplified encompassed ≈100 bp of coding region, and one intron of variable size. In each case, we confirmed that the resulting sequences corresponded to the target OR gene. Because of variation in intron size and the presence of insertion/deletion polymorphisms, the size of the amplification products varied among genes as well as within a particular gene. All fragments were ≈410-bp long for *OnOR1*, ranged from 480 to 703 bp for *OnOR3*, from 870 to 1415 bp for *OnOR4*, from 625 to 1047 bp for *OnOR5*, and from 850 to 1140 bp for *OnOR6*. All loci were polymorphic within ECB, but the extent of polymorphism varied among OR genes (Table 3). Both haplotype diversity and number of segregating sites were lower for *OnOR1, OnOR3,* and *OnOR6* compared to *OnOR4* and *OnOR5* (Table 3). For *OnOR1* and *OnOR3,* moths of the Z pheromone strain exhibited very little variation, and in general, there was more nucleotide variation in the E strain than

Table 3. Polymorphism statistics for each gene, for entire dataset and pheromone strain subsets.

Locus	Group	*n*	L	S	*H*	H_d	π	θ	R_m
OnOR1	All ECB	73	406	26	16	0.646	0.02022	0.01317	3
	E	32	406	26	15	0.861	0.01551	0.01590	3
	Z	41	410	14	2	0.049	0.00167	0.00798	0
OnOR3	All ECB	79	469	12	9	0.701	0.00643	0.00518	0
	E	35	470	11	8	0.618	0.00358	0.00568	0
	Z	44	479	3	3	0.348	0.00133	0.00144	0
OnOR4	All ECB	52	835	120	24	0.819	0.01173	0.03180	2
	E	18	921	29	9	0.863	0.00632	0.00915	0
	Z	34	837	111	17	0.783	0.01424	0.03243	2
OnOR5	All ECB	38	614	76	21	0.952	0.02810	0.02946	14
	E	17	614	48	12	0.963	0.02546	0.02312	10
	Z	21	616	71	13	0.938	0.03003	0.03204	10
OnOR6	All ECB	76	812	46	10	0.736	0.02253	0.01156	0
	E	38	815	37	5	0.666	0.01716	0.01081	0
	Z	38	819	15	6	0.614	0.00632	0.00439	0

n=number of sequences; L=length of sequence analyzed; S=Total number of polymorphic sites; *H*=number of haplotypes; H_d=haplotype diversity; π=pi nucleotide diversity; θ=theta nucleotide diversity; R_m=minimum number of recombination.

in the Z strain (Table 3). Intragenic recombination events (R_m) (Hudson and Kaplan 1985) were only detected at three loci and at very different frequencies (Table 3). Although recombination events were detected in both strains at *OnOR5*, recombination events were detected only in the E strain at *OnOR1* and in the Z strain at *OnOR3*.

Gene genealogies showed that most of the observed variation is partitioned among pheromone races for *OnOR1, OnOR3,* and *OnOR6,* whereas strain-specific variation was absent for *OnOR4* and *OnOR5* (Fig. 1). In the *OnOR1* and *OnOR3* genealogies, the few individuals that fall in the "wrong" clade (with respect to pheromone strain) all come from populations collected in Northern Italy, a geographic region in which hybridization between the pheromone strains is known to occur (Peña et al. 1988). In the *OnOR6* tree, in addition to Italian moths, E moths from France and Great Britain also cluster in the Z clade.

AMOVA analyses confirmed that much of the variation at *OnOR1, OnOR3,* and *OnOR6* is due to between strain variation (60–80% of the total variation), with the associated covariance component F_{CT} being significant (Table 4). In contrast, for *OnOR4* and *OnOR5*, haplotypes are commonly shared between strains and more than 80% of the variation can be attributed to differences within populations (Table 4).

GENETIC DISTANCE BETWEEN THE STRAINS AND DEVIATION FROM EQUILIBRIUM NEUTRAL EXPECTATIONS

We estimated whether the OR genes were at mutation–drift equilibrium and whether selection can explain some patterns of variation. The net distance between the strains was high for *OnOR1, OnOR3,* and *OnOR6* and low for *OnOR4* and *OnOR5* (Table 5). Neutrality tests performed on the entire dataset showed that *OnOR1, OnOR4,* and *OnOR6* deviate from equilibrium neutral expectations. Values of Tajima's D and Fu and Li's D^* and F^* were consistently positive for *OnOR1* and *OnOR6* and significantly negative for *OnOR4* (Table 5). For *OnOR3* and *OnOR5*, none of the values were significantly different from zero. Results of the analyses performed independently for the Z and E strains were consistent with the global trends for *OnOR3* and *OnOR5* (Table 5). In Z strain populations of *OnOR1*, negative values of Tajima's D and Fu and Li's D^* and F^* reflect the fact that, with the exception of one individual that falls in the E clade, all Z moths have identical haplotypes. In contrast, the negative values of D, D^*, and F^* for *OnOR4* in the Z strain are associated with a lack of reduction in nucleotide diversity and indicate an excess of rare variants and low frequency polymorphisms. In E strain populations, the neutrality tests have significant positive values for *OnOR6*, a consequence of mutations in external branches and an excess of intermediate frequency alleles. Recent demographic history alone can be excluded as a cause for the patterns that we see. If population bottlenecks or expansions had occurred, but selection was absent, there should be a consistent and similar trend toward positive or negative values across all loci, because demographic events would affect the entire genome. We do not observe such consistency in the results from our tests, nor when we compare our genealogies to those for other Z-linked loci (Dopman et al. 2005; Dopman 2010).

Insertion/deletion polymorphism in *OnOR3* and *OnOR6* was analyzed with Tajima's D statistic. The tests were not significant, although they showed excess of intermediate frequency sites (*OnOR3*: 819 sites, $D = 1.46294$; *OnOR6*: 1215 sites, $D = 1.64560$). Indeed, the major allelic classes revealed by the genealogical analyses of *OnOR3* and *OnOR6* differ in the presence/absence of large indels.

GENETIC MAPPING

For *OnOR1, OnOR3,* and *OnOR6* genes, genotypes were obtained for 78 male individuals of the BC1 mapping population. These three OR genes are sex-linked, and in our limited sample, we found no evidence of recombination among them (Fig. 2). They map close to a region of reduced recombination in the ECB genome that includes the *Tpi* gene and reveals the two pheromone strains to be exclusive groups (Dopman et al. 2004, 2005). The position of the OR genes on the ECB Z chromosome corresponds to the position of BmOR1 on the *Bombyx* Z chromosome.

OnOR4 and *OnOR5* are clearly not sex-linked and exhibit an inheritance pattern identical to that of AFLP marker p25–226, which is situated on an autosome (chromosome 5 in Dopman et al. 2004).

Discussion

In genomic regions that harbor genes essential for the maintenance of species boundaries, we expect to find fixed differences (or nearly fixed differences) between reproductively isolated lineages (Wu 2001). Therefore, identifying regions that exhibit a lack of shared polymorphism and/or strain/species exclusivity has the potential to uncover so-called speciation or barrier genes (Dopman et al. 2005; Maroja et al. 2009). Barrier genes encode phenotypes that are directly involved in reproductive isolation and are likely to evolve under selection. The two strains of *O. nubilalis* offer excellent opportunities to unravel the origins of barriers to gene exchange that result from divergence in pheromone communication. Indeed, the ECB has been the focus of recent studies investigating the extent of molecular differentiation between the E and Z pheromone strains at a number of nuclear loci (Willett and Harrison 1999; Dopman et al. 2005; Malausa et al. 2007; Geiler and Harrison 2010). Although genetic differentiation exists, gene genealogies have revealed that the two strains share an extensive amount of polymorphism at all loci investigated thus far with the

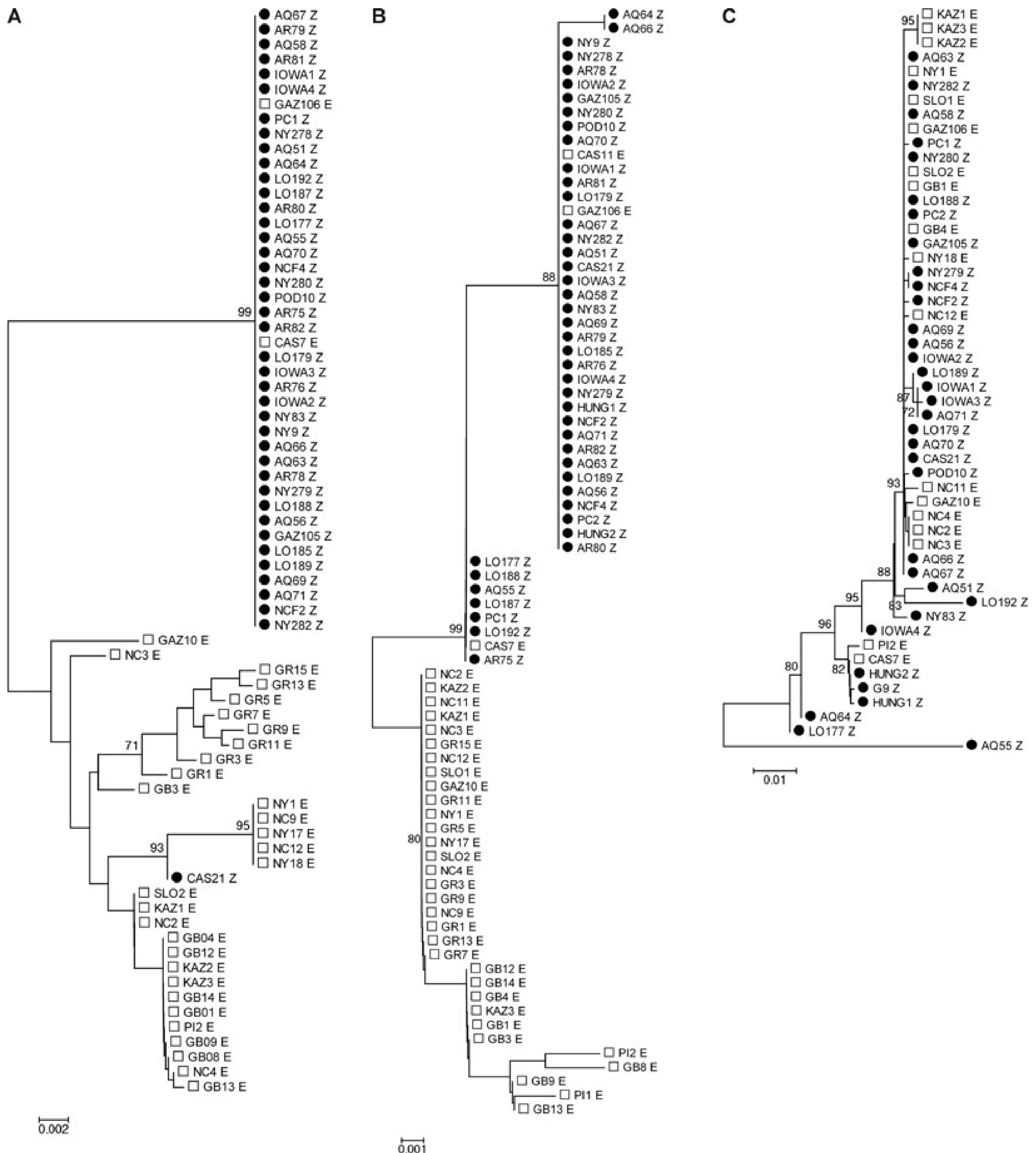

Figure 1. Unrooted neighbor joining trees for the five OR loci: (A) *OnOR1*, (B) *OnOR3*, (C) *OnOR4*, (D) *OnOR5*, (E) *OnOR6*. The genealogies are derived from moths collected in USA (IOWA, NC, NY), France (AQ, AR, PC, LO, PI), Great Britain (GB), Italy (GAZ, CAS, POD), Slovenia (SLO), Greece (GR), Hungary (HUNG), and Kazakhstan (KAZ). The symbols • and □ identify moths of the Z and E pheromone strain, respectively. Collection localities are reported in Figure S1. Details on collection sites are available from JML and RGH.

exception of *pgFAR* (the locus encoding a fatty acid reductase responsible for differences in pheromone production between E and Z moths) and the sex-linked marker *Tpi*. *Tpi* shows near exclusivity between strains and maps to a genomic region containing a major factor determining postdiapause development (*Pdd*) and ultimately voltinism differences (Glover et al. 1992; Dopman et al. 2005). However, whereas North American representatives of the pheromone strains appeared differentiated at *Tpi*

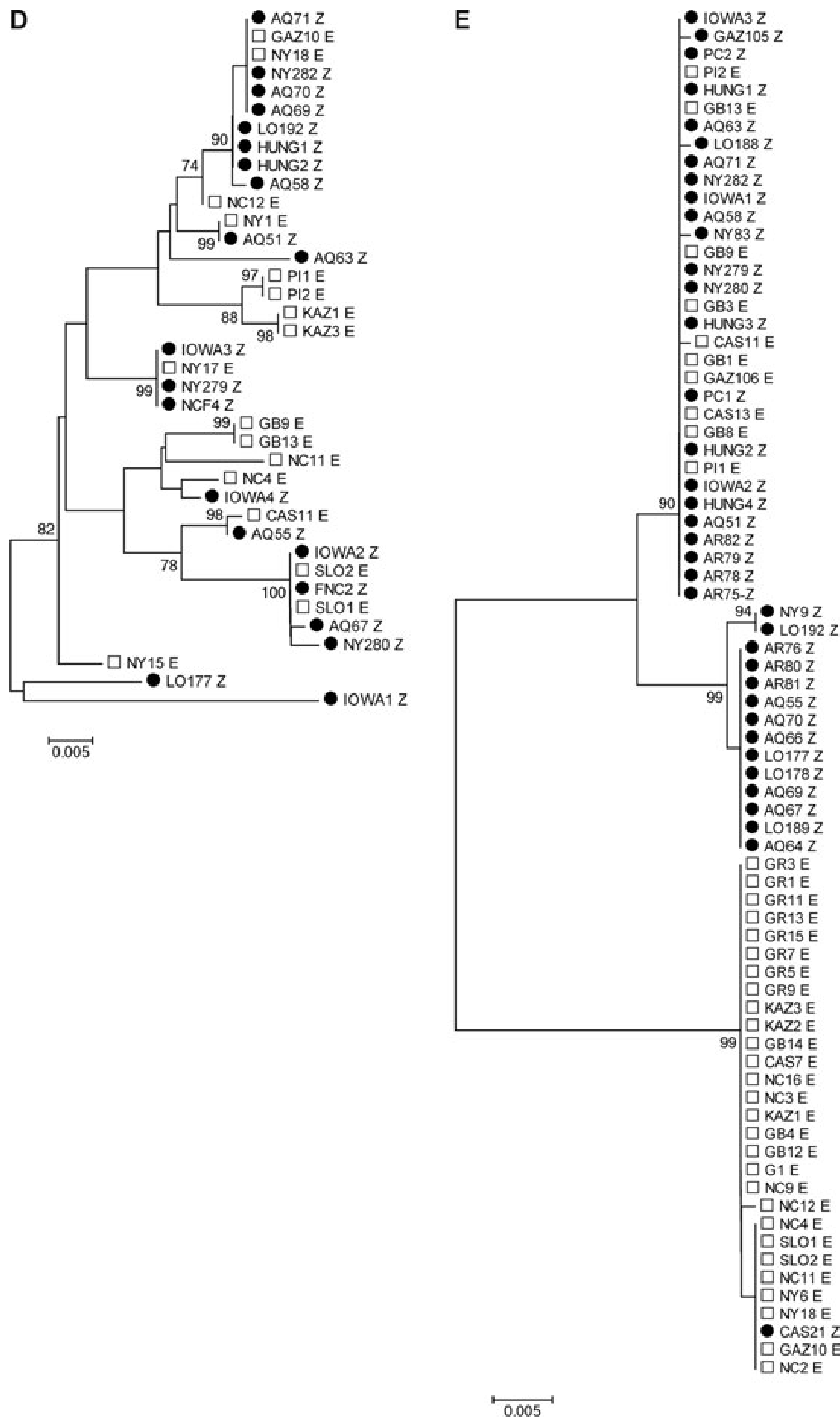

Figure 1. Continued.

(Dopman et al. 2005), differentiation at this locus is low between E and Z moths in France (Malausa et al. 2007), in spite of the fact that the two pheromone strains in France use different host plants and have been suggested to be distinct species (Frolov et al. 2007). ECB populations have multiple trait differences that restrict gene flow, and some barriers may operate throughout the range, whereas others may vary geographically (Dopman et al. 2010). Temporal isolation caused by differences in postdiapause development may therefore operate in northern North America but not necessarily everywhere else.

Table 4. Analysis of molecular variance (AMOVA) for populations of the E and Z strains.

Source of variation (%)	*OnOR1*	*OnOR3*	*OnOR4*	*OnOR5*	*OnOR6*
Among pheromone strains	73.46	78.06	0.14	0.44	59.30
Among populations within strains	15.74	9.60	5.79	17.64	17.59
Within populations	10.80	12.35	94.08	81.92	23.12
Fixation indices					
F_{CT} (strains/total)	0.735***	0.781***	0.001	0.004	0.593**
F_{SC} (population/strain)	0.593***	0.437***	0.058	0.177*	0.432***
F_{ST} (population/total)	0.892***	0.877***	0.059	0.181*	0.769***

*$P<0.05$; **$P<0.01$; ***$P<0.001$.

Patterns of variation and gene genealogies that we observe for *OnOR4* and *OnOR5* are similar to what has been seen for most other loci in ECB, including the sex-linked loci *Ldh* and *Kettin* (*Ket*) (Dopman et al. 2005; Geiler and Harrison 2010), and can be explained by recognizing that E and Z strain ECB not only share an extensive amount of ancestral polymorphism, but that patterns of variation may also be influenced by contemporary gene flow. Polymorphism data suggest that there are diverse alleles in both races, and neutrality tests even show an excess of rare variants for *OnOR4* in our sample, which could be a consequence of diversifying selection acting on that locus. This supports the idea that allelic variation might be responsible for observed differences in sequence and responsiveness for *OnOR4* and *OnOR5* orthologs functionally characterized by Wanner et al. (2010) and Miura et al. (2009, 2010).

In contrast, the presence of quasi-exclusive clades for *OnOR1, OnOR3,* and *OnOR6*, as well as the patterns of reduced variation at these three loci suggest that selective sweeps may have resulted in distinct haplotypes in the two pheromone strains and that ongoing gene flow is not sufficient to homogenize allele frequencies. We note that sex linkage is not itself an explanation for exclusivity because not all sex-linked genes for which gene genealogies are available show such a pattern. Indeed, *Ldh* and *Ket* show genealogies similar to those of *OnOR4* and *OnOR5* with extensive haplotype sharing between pheromone strains. Our mapping data reveal that the *OnOR1, OnOR3,* and *OnOR6* genes map to a region of the Z chromosome that experiences low recombination. It is tempting to argue that selection at the OR loci may be responsible for the origin and maintenance of differentiation in that genomic region. Such a scenario suggests that these loci are responsible for a reproductive barrier associated with differentiation of the E and Z pheromone systems in *O. nubilalis.* However, the map position of *OnOR1, OnOR3,* and *OnOR6* appears to be far from *Resp,* making it implausible that the origin of differentiation at these loci is a consequence of selection acting at the locus responsible for differences in male behavioral response. Because

Table 5. Tests of selection for each gene, for entire dataset, and pheromone strain subsets.

Locus	Group	Tajima's D	Fu and Li's D^*	Fu and Li's F^*	D_a
OnOR1	All ECB	1.66444	1.49839*	1.86191*	
	E	−0.08731	1.45181*	1.12009	E vs. Z: 0.02498
	Z	−2.48585**	−4.87423**	−4.82310**	
OnOR3	All ECB	0.66385	−0.32341	0.02525	
	E	−1.15135	−0.13116	−0.53254	E vs. Z: 0.00817
	Z	−0.16454	0.90523	0.68171	
OnOR4	All ECB	−2.24424**	−5.09386**	−4.80567**	
	E	−1.24286	0.16977	−0.27761	E vs. Z: 0.00016
	Z	−2.10793*	−4.01372**	−3.98579**	
OnOR5	All ECB	−0.16822	−1.31485	−1.08624	
	E	0.42094	0.56780	0.60870	E vs. Z: 0.00100
	Z	−0.25232	−1.13281	−1.01024	
OnOR6	All ECB	3.09197**	1.20022	2.3176**	
	E	2.07456*	1.42567*	1.95305**	E vs. Z: 0.02077
	Z	1.42271	0.29198	0.77666	

D_a=net divergence between strains.

*$P<0.05$; **$P<0.01$ (Tajima's D) or $P<0.02$ (Fu and Li's D^* and F^*).

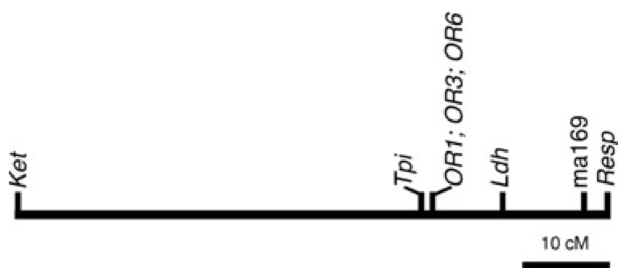

Figure 2. **Genetic linkage map of the Z chromosome in the ECB. The map shows the mapping position of the *OnOR1, OnOR3,* and *OnOR6* genes relative to the sex-linked genetic markers identified previously (Dopman et al. 2005).**

hybridization between E and Z moths occurs in natural populations, linkage disequilibrium between the ORs and *Resp* would erode over time. It is important to bear in mind that determining the phenotype of individual males for behavioral response probably has a higher error rate than scoring microsatellites, AFLPs, or other molecular markers. Therefore, the placement of *Resp* on the linkage map is less certain than the placement of other markers. However, reexamination of the data upon which male phenotypes were determined does not suggest that phenotyping errors could be frequent enough to imagine that *Resp* has been mapped >20 cM away from its actual position. Alternatively, it is also possible that a trait distinct from *Resp* is acting as an impediment to gene flow in the region containing the *OnOR1, OnOR3,* and *OnOR6* genes. In fact, selection may be acting on one or more of these genes. Interestingly, recent data on genetic determination of male antennal response suggest that a sex-linked locus may be involved (Olsson et al. 2010), which contrasts with earlier data that were interpreted as support for strictly single-locus autosomal inheritance. It is therefore possible that male response has a polygenic basis. However, selection at a yet unidentified locus, together with genetic hitchhiking, cannot be excluded.

The map position of the three sex-linked *O. nubilalis* OR genes corresponds closely to the map position in *B. mori* of a pheromone receptor that appears to be expressed only in male antennae and is tuned to the domesticated silkmoth pheromone (bombykol) (Sakurai et al. 2004). The three tightly linked ECB OR genes may reflect duplication events in the ECB lineage. All of these ORs appear to be pheromone receptors for which transcripts are far more abundant in male than in female antennae (Wanner et al. 2010). Furthermore, *OnOR6* is highly selective for (*Z*)-11-tetradecenyl acetate, one of the two components of ECB female pheromone. In addition, *OnOR4* and *OnOR5* appear to be closely linked genes, suggesting that tandem gene duplication may be an important mechanism contributing to the functional diversification of OR genes in *Ostrinia*. In general, the extent of variation in OR gene copy number and its consequence needs to be investigated further. In principle, variation in copy number via duplication or deletion can potentially result in modification of the expression pattern of an OR gene (Nei et al. 2008).

After encountering a female pheromone plume, a male moth may decide to take flight and start his quest for the pheromone source. Whether he does depends on his interpretation of the signal, which is defined by his sensitivity and selectivity. Variation in peripheral or central sensory physiology parallels variation in male response window and could enable a change in preference from one pheromone blend to another (Linn and Roelofs 1995; Heckel 2010). Under the asymmetric tracking hypothesis, variant female pheromones can be tracked by males with a wide window of response (Phelan 1997). Such a wide response window could be due to changes in some of their ORNs, which in turn could be the consequence of expressing broadly tuned ORs (Gould et al. 2010; Heckel 2010). In the case of *O. nubilalis,* given the similar organization of the central nervous system in both E and Z males, changes in the peripheral rather than in the central nervous system may account for the differences in behavioral attraction to the E and Z blends (Karpati et al. 2008). Therefore, transitions must have occurred in the antennal sensilla housing the pheromone-specific ORNs. Functional characterization of pheromone-specific ORs revealed that *Ostrinia* males express both broadly and narrowly tuned receptors (Miura et al. 2010; Wanner et al. 2010). Although the specificity of OSN is mainly determined by ORs, pheromone binding proteins (PBPs) present in the sensillar lymph might also be involved in the differential peripheral perception of species-specific pheromones, as demonstrated in *B. mori* and *H. virescens* (Grosse-Wilde et al. 2006, 2007). However, previous genealogical analyses included a PBP, and examination of variation at that PBP locus revealed no fixed differences between the races (Willett and Harrison 1999; Dopman et al. 2005). Further investigations are required to evaluate whether other PBP genes are significantly differentiated between the strains (Allen and Wanner 2010).

Interestingly, our data indicate that there exist strain-specific allelic variations in the ORs, which provide a basis for variation in both male moth antennal and behavioral response. First, the alleles may differ in their sensitivity to the pheromone components, which would directly alter the antennal response and the profile of the neuronal impulses that are transmitted and interpreted by the central nervous system of the insect. The limited functional data available suggest that the functionality of the ORs is altered, although additional work is required to fully explore this possibility. Second, it is possible that the allelic classes we have uncovered do not differ greatly in specificity but in the ORN classes expressing them. In insects, each OR gene possess a regulatory zip code located in the upstream region of the gene; the zip code determines in which ORN class the OR gene is expressed (Ray et al. 2008; Fuss and Ray 2009). OR gene choice is controlled by positive and negative regulatory elements and their interactions with

transcription factors (Fuss and Ray 2009). Positive selection acting at the level of the regulatory region of a particular OR gene could lead to loss of variation at the neighboring sites of that gene as well as adjacent loci, resulting in the pattern of genetic variability we report here. The reorganization of the periphery suggested by Karpati et al. (2008) would need a change in the regulatory region and/or in the transcription factors that bind to the regulatory sequence motifs.

Receptor gene choice combined with variation in the OR gene repertoire might be the key elements in the evolution of male response in *O. nubilalis*, and in male moths in general. Future work targeting the genome region revealed in the present study combined with detailed sequence analyses of ECB antennal libraries should help to unravel this puzzle.

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