LETTERS

Allelic variation in a fatty-acyl reductase gene causes divergence in moth sex pheromones

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Pheromone-based behaviours are crucial in animals from insects to mammals[1,2], and reproductive isolation is often based on pheromone differences[1–4]. However, the genetic mechanisms by which pheromone signals change during the evolution of new species are largely unknown[4]. In the sexual communication system of moths (Insecta: Lepidoptera), females emit a species-specific pheromone blend that attracts males over long distances[1,2,4]. The European corn borer, *Ostrinia nubilalis*, consists of two sex pheromone races, *Z* and *E*, that use different ratios of the *cis* and *trans* isomers of acetate pheromone components[5]. This subtle difference leads to strong reproductive isolation in the field between the two races[6,7], which could represent a first step in speciation. Female sex pheromone production and male behavioural response are under the control of different major genes[8,9], but the identity of these genes is unknown. Here we show that allelic variation in a fatty-acyl reductase gene essential for pheromone biosynthesis accounts for the phenotypic variation in female pheromone production, leading to race-specific signals. Both the *cis* and *trans* isomers of the pheromone precursors are produced by both races, but the precursors are differentially reduced to yield opposite ratios in the final pheromone blend as a result of the substrate specificity of the enzymes encoded by the *Z* and *E* alleles. This is the first functional characterization of a gene contributing to intraspecific behavioural reproductive isolation in moths, highlighting the importance of evolutionary diversification in a lepidopteran-specific family of reductases. Accumulation of substitutions in the coding region of a single biosynthetic enzyme can produce pheromone differences resulting in reproductive isolation, with speciation as a potential end result.

Lineages that are in the early stages of speciation through behavioural isolation can serve as important model systems for identifying genetic changes that underlie new signals or responses. Comparison of closely related *Drosophila* species has provided important examples[10–13]. In addition to such interspecific variation, the lepidopteran genus *Ostrinia* possesses species with intraspecific polymorphisms and has become a focus for studying the genetics of pheromone signalling and identifying the genes that contribute to reproductive barriers[6,8,9,14,15]. Two races of *O. nubilalis* occur in which females produce, and males fly to, different blends of the same components: the *E* race uses a 98:2 blend of (*E*)-11-tetradecenyl acetate and (*Z*)-11-tetradecenyl acetate, whereas the *Z* race uses a 3:97 *E*/*Z* mixture[5,16]. Differences in pheromones are important as barriers to gene flow, because they lead to positive assortative mating in the field[6,7]. Yet the races are completely interfertile and can be crossed to investigate the inheritance of their differences. Crossing studies have shown that sex pheromone production in females is controlled primarily by a single autosomal factor[8,17], and that male antennal chemosensory response is controlled by a different, unlinked autosomal gene[8,18,19], whereas male behavioural response is determined by a sex-linked genetic factor[8]. A genetic linkage map of *O. nubilalis* confirmed that variations in signal and response are genetically independent in this system[9]. What has remained unknown is the nature of the gene or genes.

We sought to identify the gene accounting for pheromone differences between the two strains by using a candidate-gene approach in combination with quantitative trait locus (QTL) mapping. The *O. nubilalis* female sex pheromone is biosynthesized from palmitic acid, which is chain-shortened through β-oxidation into myristate (Supplementary Fig. 1); Δ11 desaturation then occurs to produce the (*E*)-11-tetradecenoyl and (*Z*)-11-tetradecenoyl precursors. These intermediates are subsequently reduced to fatty alcohols and acetylated to give the (*E*)-11-tetradecenyl and (*Z*)-11-tetradecenyl acetates. Variation in the fatty-acyl desaturase gene family[20] has been shown to be important in determining pheromone differences among *Ostrinia* species[21]. However, both *E* and *Z* strains have similar ratios of unsaturated acyl intermediates[8,22]. The selective reduction of deuterium-labelled precursors *in vivo* suggested that the reduction step is more likely to define the final blend of pheromone components[23], because *in vivo* experiments demonstrated that the acetylation step seems to be unselective in *O. nubilalis*[23].

The enzymes converting fatty-acyl precursors into their corresponding alcohols are fatty-acyl reductases (FARs)[24]. All FARs possess the Pfam domains *NAD binding 4* and *Sterile*, and the gene family is particularly diverse in insects (that is, 13 subtypes in *Drosophila melanogaster*, compared with two in *Homo sapiens*). Thirteen putative FARs were identified from the closely related congener *O. scapulalis*, one expressed only in the pheromone gland[25]. We cloned and characterized the orthologue of this latter gene from *O. nubilalis*; sequences *pgFAR-Z* and *pgFAR-E* from the two strains showed 3.8% nucleotide divergence (7.5% amino-acid divergence), which is at the upper limit of the amount of sequence divergence found at other *O. nubilalis* loci[15] (Supplementary Fig. 2). In contrast, we found less than 0.5% intrastrain sequence divergence in this gene (Supplementary Table 1). The ratio of non-synonymous to synonymous (dN/dS) nucleotide substitution was 0.98; however, the level of divergence indicates that the coding region of the gene might be under selection. Indeed, several regions in the open reading frame deviate significantly from the neutral expectation (Supplementary Fig. 3), suggesting divergently adaptive evolution.

To determine whether these differences affected the reductase substrate specificity, we expressed both forms in yeast (*Saccharomyces cerevisiae*) supplied with (*E*)-11-tetradecenoyl and (*Z*)-11-tetradecenoyl precursors. In the presence of equal amounts of both fatty-acyl substrates, yeasts expressing *pgFAR-Z* converted almost exclusively the *Z*

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isomer into the corresponding alcohol, producing only very small amounts of the *E* alcohol (Fig. 1). Conversely, *pgFAR-E* conferred on the yeast the ability to convert mainly the *E* precursor, with only a minute amount of the *Z* isomer being reduced (Fig. 1). These observed ratios of fatty alcohols were identical to the final ratios of the corresponding acetates normally found in females of the *Z* and *E* strains, respectively. In addition, we assayed a series of substrates found as pheromone precursors in related *Ostrinia* species, namely the (*Z*)-9-tetradecenoyl, (*E*)-12-tetradecenoyl and (*Z*)-12-tetradecenoyl precursors, as well as the 16-carbon homologues that are precursors of the male pheromone[26], namely the (*Z*)-9-hexadecenoyl, (*Z*)-11-hexadecenoyl and hexadecanoyl precursors. Only the (*E*)-12-tetradecenoyl precursor was converted to the corresponding alcohol, and only by *pgFAR-Z*, which is consistent with previous *in vivo* experiments[23]. In all cases, yeast carrying the empty vector control produced no detectable fatty alcohols. Thus *pgFAR* is a functional FAR with distinct strain-specific substrate preferences that could account for the final ratio of pheromone components found in the *E* and *Z* strains.

To assess whether the *pgFAR* gene maps to the genomic location affecting pheromone production, we performed a QTL analysis based on a new linkage map created by crosses between moths of the *E* and *Z* strains. Analysis of a female-informative set of backcrosses allowed us to identify the 30 autosomes plus the sex chromosomes of *O. nubilalis*[27] with 234 amplified fragment length polymorphism (AFLP) markers. Autosomal linkage group 31 (LG31) accounted for 99% of the variance in the pheromone ratios produced by the female-backcross progeny (Supplementary Fig. 4) and thus contained the major QTL for pheromone phenotype ($P < 0.0001$). We found a perfect correlation between the genotype for *pgFAR*, the phenotype for pheromone production ratio among backcross female offspring (Fig. 2 and Supplementary Fig. 5) and LG31. In contrast, the gene *Onub-E/Z11* encoding a Δ11 fatty-acyl desaturase responsible for forming the (*Z*)-11-tetradecenoyl and (*E*)-11-tetradecenoyl precursors[21] mapped to LG23, which had no significant effect on pheromone differences (Supplementary Fig. 4).

Reverse transcriptase (RT)–PCR on pheromone gland RNA showed that, whereas only one allele of *pgFAR* is expressed in pure strain females, both *pgFAR-Z* and *pgFAR-E* are present in reciprocal F_1 hybrids (Fig. 3). With an isomeric composition that approximates 65:35 *E*/*Z*, female F_1 hybrids are not perfectly intermediate with respect to the parental strain[17]. The 65:35 ratio corresponds to the ratio of monounsaturated intermediates produced by the Δ11 fatty-acyl desaturase[8,22]. As hybrids possess and express both the *pgFAR Z* and *E* alleles, both the (*E*)-11-tetradecenoyl and (*Z*)-11-tetradecenoyl precursors would be reduced in hybrid females, and the ratio of the intermediate precursors would determine the proportion of pheromone components.

With the identification of the gene responsible for intraspecific pheromone variation in *O. nubilalis*, we can examine its relationship to interspecific pheromone divergence in the genus *Ostrinia*, and in moths in general. The typical moth pheromone biosynthetic pathway is derived from fatty-acid biosynthesis with extensive embellishments. The early evolution of Lepidoptera was accompanied by the recruitment and/or neofunctionalization of gene duplicates involved in fatty-acid synthesis, and by changes in activity and tissue specificity. For pheromone production, most moth species rely on different members of a diverse desaturase family, which has undergone great diversification within Lepidoptera[20]. Diversification of desaturases is also important in interspecific pheromone variation in *Ostrinia*[21,26]. In *O. nubilalis*, the absence of a strain-specific effect of the Δ11 fatty-acyl desaturase is consistent with the presence of similar ratios of unsaturated acyl intermediates in the *E* and *Z* strains[8,22]. Our finding that the intraspecific difference between the *E* and *Z* strains of *O. nubilalis* is determined not by a desaturase but by a FAR shows

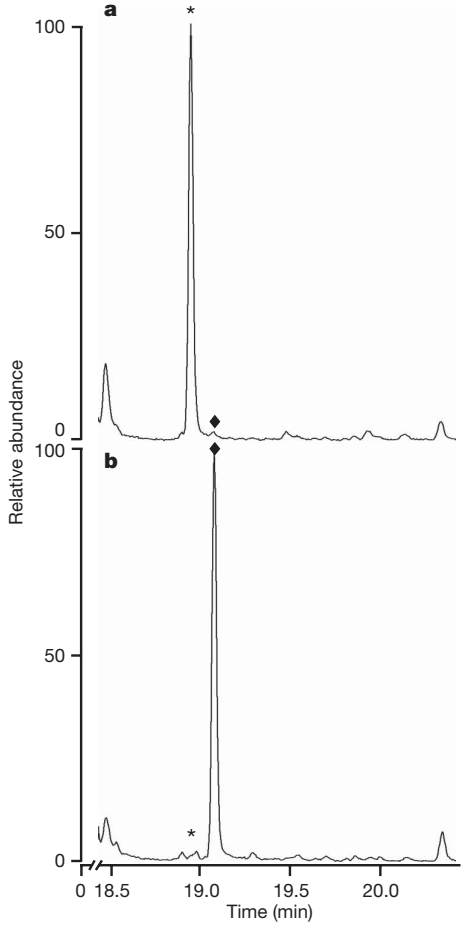

Figure 1 | Functional assay of the *pgFAR E* and *Z* alleles in yeast. Partial total-ion chromatograms of extracts from yeast expressing *pgFAR-E* (**a**) and *pgFAR-Z* (**b**). The yeast medium was supplemented with a 1:1 mixture of methyl (*E*)-11-tetradecenoate and methyl (*Z*)-11-tetradecenoate. The asterisk and diamond indicate peaks corresponding to (*E*)-11-tetradecenol and (*Z*)-11-tetradecenol, respectively.

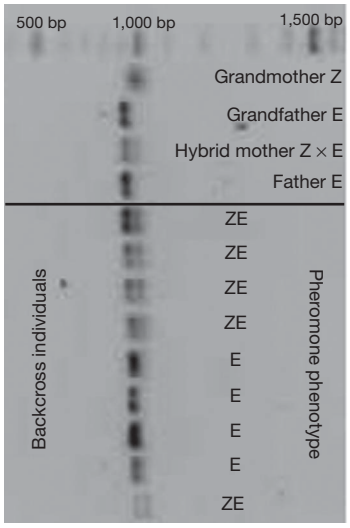

Figure 2 | Segregation of the *E* and *Z* alleles of *pgFAR*. Intron 7 is longer in the *Z* allele and allowed discrimination between the alleles in pure-strain individuals and the progeny from the F_1 cross and parental backcrosses. Pheromone phenotype was determined by gas chromatographic analysis of pheromone gland extracts (Supplementary Table 2). We found a perfect correspondence between genotype at *pgFAR* and phenotype for pheromone production: individuals with both *E* and *Z* allelic copies of *pgFAR* were intermediate in their pheromone phenotype, whereas individuals with a single allelic type had a pheromone corresponding to that of pure-strain individuals.

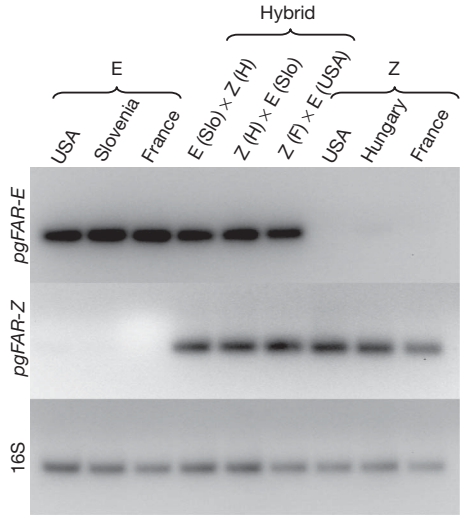

Figure 3 | Detection of *pgFAR* RNA in female pheromone glands. Allele-specific RT–PCR for *pgFAR-E* and *-Z* in pure strain individuals from different populations and their hybrids. The figure shows the results of agarose-gel electrophoresis of amplification products. For *pgFAR-E*, products were observed when the RNA source was from *E*-strain and F_1 individuals but no amplicon was detected with material originating from *Z*-strain individuals. Conversely, RT–PCR analysis for *pgFAR-Z* shows no expression in the *E* strain but expression in hybrid and *Z*-strain individuals. Results of RT–PCR for 16S RNA are shown as a control for input RNA amounts. Slo, Slovenia; H, Hungary; F, France.

that FARs should be included in modelling evolutionary pathways of sex pheromones.

When reconstructing a gene phylogeny, we found that the FAR genes involved in female moth pheromone production—the *pgFAR* in *O. nubilalis* and *O. scapulalis*[25] as well as the FAR producing bombykol in *Bombyx mori*[24]—form a Lepidoptera-specific cluster; no apparent orthologue to these genes was found in any other insect orders for which data are available (Fig. 4). This suggests that the production of novel substrates as the result of the recruitment of a new type of fatty-acyl desaturase[28] was associated with the evolution of a novel type of FAR, acting downstream of the desaturase. Our findings highlight the notion that the reduction step can be particularly conducive to functional changes, and support the idea that mutations in genes encoding biosynthetic enzymes can generate the great diversity of pheromones used in moths, permitting the coexistence of thousands of species belonging to one of the most speciose insect groups on Earth today.

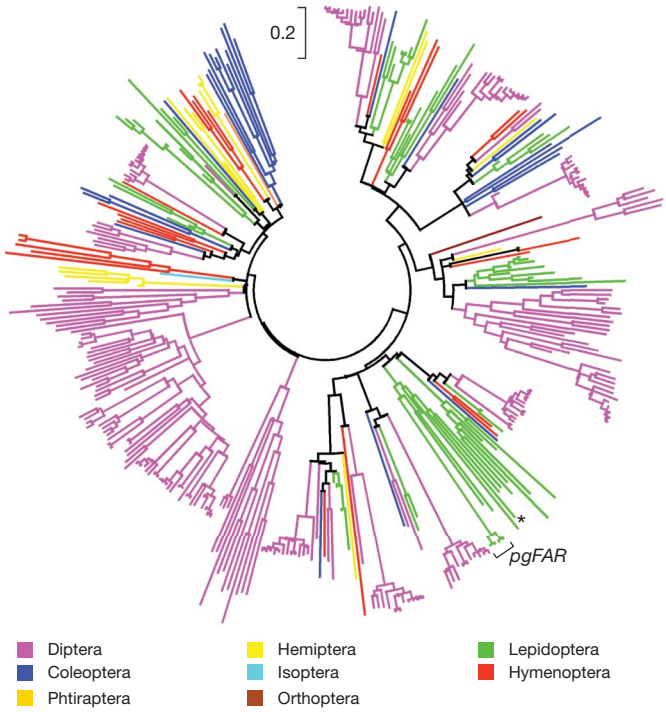

Figure 4 | Phylogenetic relationships of insect FARs. A branch specific to each insect species is coloured according to the colour code at the bottom. The scale bar represents 0.2 amino-acid substitutions per site. The bracket indicates *pgFAR-E* and *pgFAR-Z*; the asterisk indicates the FAR gene implicated in the pheromone biosynthesis of *Bombyx mori*[24]. The clade containing the *pgFAR* genes seems to be specific to Lepidoptera.

We have identified functionally important allelic variation at a gene responsible for pheromone-race difference in a moth species. Unravelling the genetic changes that cause modification of signals responsible for reproductive isolation is a necessary step in understanding the genetics of speciation. Speciation genes are those genes that are critical in the evolution of reproductive isolation between diverging lineages. If the *E* and *Z* races represent incipient species, then *pgFAR* may represent such a gene within *Ostrinia* and potentially other lepidopteran groups as well. Whereas modifications in chemical signals of drosophilids are caused by changes in the expression level of the *desatF* desaturase[13], our results show that divergence in pheromonal cues can also occur through changes within coding regions, by modifying the specificity of an enzyme affecting the signal emitted by the female. For a full understanding of the development of reproductive isolation by this mechanism, what remains to be learned is the nature of the alteration affecting the signal reception and acceptance by the male, and what determines the coevolution of signal and response.

METHODS SUMMARY

Complementary DNAs were synthesized from 0-day-old female pheromone gland and used for the molecular cloning of *pgFAR Z* and *E* alleles performed by using rapid amplification of cDNA ends (RACE) PCR with gene-specific primers (pheromone gland-specific FAR in *O. scapulalis*[25]; GenBank accession number EU817405). For the genetic mapping we used an approach similar to that described previously[9], with the exception that we crossed *Z* strain females to *E* strain males, and backcrossed F_1 female offspring to *E* males. Gene expression analyses were performed by RT–PCR with gene-specific primers. To collect homologous sequences of FAR genes in insects we conducted searches against GenBank protein and expressed sequence tag (EST) databases. In addition, we retrieved from the silkworm genome all paralogues of *Bombyx mori pgFAR*.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions J.M.L., A.T.G., D.G.H. and C.L. performed project design and interpretation. J.M.L., M.A.L. and B.A. conducted cloning of *pgFAR* cDNAs. F.A. and E.H. were responsible for the synthesis of precursors. J.M.L. and M.A.L. performed the functional assays. J.M.L. conducted the gas chromatography–mass spectrometry analyses. A.T.G., C.B. and D.G.H. performed QTL analyses and gene mapping. D.G.H. was responsible for the genomic sequence analysis. J.M.L. performed the transcriptional analysis. J.M.L. was responsible for bioinformatics. J.M.L., A.T.G., M.A.L., D.G.H. and C.L. prepared the manuscript.

Author Information The sequences reported in this article are deposited in GenBank under accession numbers FJ807735–FJ807736, GU808256–GU808276 and GU733831–GU733832. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to J.M.L. (jean-marc.lassance@ekol.lu.se) or C.L. (christer.lofstedt@ekol.lu.se).

METHODS

Moth populations. Insects of the *E* and *Z* strains of the European corn borer *Ostrinia nubilalis* (Lepidoptera: Crambidae) were obtained from laboratory cultures, and reared on artificial diet. Eggs or pupae from the different strains were thus obtained as follows: *O. nubilalis Z*, France (French National Institute for Agricultural Research (INRA)), Hungary (Swedish University of Agricultural Sciences (SLU)) and the USA (Cornell University); *O. nubilalis E*, USA (Cornell University) and Slovenia (SLU). The founders of these laboratory cultures originated from collections from cornfields. The *O. nubilalis E* from France originated from a collection of larvae from stalks of mugwort, *Artemisia vulgaris* (Picardie, France). Males and females were separated before eclosion and placed in climate chambers maintained at 23 ± 1 °C and a 17 h:7 h light–dark photoperiod. Newly emerged adults were separated daily and considered to be 0 days old.

Amplification of *pgFAR* transcripts. We extracted total RNA from pheromone glands of 20–25 0-day-old virgin females by using TRIzol (Invitrogen). We synthesized first-strand cDNA using Stratascript reverse transcriptase (Stratagene). Using gene-specific primers designed after the pheromone-gland-specific orthologue from *O. scapulalis* (FARXIII in ref. 25; GenBank accession number EU817405) and pheromone-gland cDNA from the *E* and *Z* strains as template, we amplified a 290-base-pair (bp) fragment with the Advantage 2 PCR kit (Clontech). The PCR conditions were as follows: 94 °C for 1 min followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The PCR products were gel-purified with the Wizard SV Gel and PCR Clean-up System (Promega), ligated into the pGEM-T easy vector (Promega), and transformed into *Escherichia coli* TOPO10 chemically competent cells (Invitrogen). Plasmids were purified and subsequently used as templates for sequencing reactions performed with universal primers and the BigDye Terminator cycle sequencing kit v.1.1 (Applied Biosystems) followed by analysis on a capillary ABI PRISM 3100 sequencer instrument (Applied Biosystems). Gene-specific primers ($T_a = 60$ °C) were designed on the basis of the curated sequence information and used in PCR reactions to amplify the 5′ and 3′ cDNA ends in combination with the SMART RACE cDNA amplification kit (Clontech) in accordance with the manufacturer's instructions. The 5′ and 3′ RACE PCR products were cloned and sequenced as described above, and the information was used to compile the full-length sequences. To investigate the variation within *O. nubilalis* we PCR-amplified the full-length cDNA with a set of primers amplifying the *Z* or *E* allele non-specifically.

RT–PCR was used to estimate the relative expression level of *pgFAR* in individuals from pure strains and reciprocal crosses. Total RNAs (50 ng) were used to amplify fragments of the *pgFAR* genes, using the Superscript III One-Step RT–PCR kit (Invitrogen) in accordance with the manufacturer's instructions.

Primers. Primers used for amplification of PCR products, RT–PCR, full-length open-reading-frame amplification products, genotyping and obtaining genomic sequences are listed in Supplementary Table 3.

Yeast expression. The strain of *Saccharomyces cerevisiae* used was INVSc1 (Invitrogen), and the expression vector was pYES2.1/V5-His TOPO (Invitrogen). Yeasts were transformed with recombinant expression vector alone or containing either the *pgFAR-E* or *pgFAR-Z* open reading frame with the *S. c.* EasyComp Transformation kit (Invitrogen) and propagated on SC-U (minimal medium lacking uracil) selective plates or in liquid culture containing 2% glucose. Yeast growth was measured by determining the attenuance at 600 nm of aliquots of cell suspensions with a spectrophotometer. Expression was induced by incubating yeast in SC-U medium containing 2% galactose. For precursor assays, aliquots of yeast cultures in the mid-exponential phase of growth were suspended in induction medium containing 0.5 mM of the methyl-ester precursor diluted in ethanol. Cells were incubated for 24 h at 30 °C with shaking at 300 r.p.m., and harvested by centrifugation. Before extraction with hexane, the cells were washed in pure water. After washing, the cells were extracted for 1 h at 21–22 °C in 1 ml of hexane containing 150 ng of (*Z*)-11-tridecenyl alcohol used as an internal standard. Samples were stored at −20 °C until analysis.

Gas chromatography–mass spectrometry analyses. Before analysis, samples were concentrated under a gentle flow of pure nitrogen to a final volume of 50 µl. Samples were analysed on a gas chromatograph (HP 5890 GC system; Hewlett Packard) coupled to a mass-selective detector (HP 5972; Hewlett Packard). The GC was equipped with a polar HP-INNOWax capillary column (100% polyethylene glycol; 30 m × 0.25 mm × 0.25 µm; Agilent Technologies). The GC–MS was operated in electron impact mode (70 eV). Helium was used as carrier gas (velocity 30 cm s^{-1}). The injection port was configured in splitless mode and its temperature set to 220 °C; the detector temperature was 280 °C. The oven temperature was held at 50 °C for 2 min and programmed to increase at 10 °C min^{-1} to 220 °C, then held for 20 min.

Genetic mapping. Moths used for initiating mapping families were from the Hungarian *Z* culture and the Slovenian *E* culture, and were maintained under a 14 h:10 h light–dark photoperiod. Single-pair matings were set up, hybridizing *Z* females with *E* males. The families that produced most offspring were used for backcrossing. In single-pair matings, individual female F_1 hybrids were backcrossed to *E* males to produce the backcross family used as mapping family. For the construction of the linkage map, the grandparents, parents and all backcross offspring of the mapping family were genotyped with AFLP markers, which were separated on a LiCOR 4300 sequencer. The markers were grouped into linkage groups with MAPMAKER/EXP 3.0 (ref. 29). Because there is no crossing-over in female Lepidoptera[30] and *Ostrinia* spp. contain 31 chromosomes[27], a linkage group can be considered a chromosome corresponding to about 3% of the insect genome.

Before extracting DNA, the backcross females were phenotyped by extracting their pheromone gland content for 1–2 h. Because mated females usually produce small amounts of pheromone, females were injected in the photophase with pheromone biosynthesis activating neuropeptide (PBAN) before gland extraction. A solution of 7.5 pmol of Hez-PBAN (Peninsula Laboratories) in 2 µl of saline was injected by using a 10-µl syringe with a 31-gauge needle (Hamilton) that was inserted ventrally between the eighth and the ninth abdominal segments. Pheromone gland extracts were analysed with a 7683 automatic injector and an HP7890 gas chromatograph equipped with a splitless inlet, a polar capillary column (DB-WAXetr (extended temperature range); 30 m × 0.25 mm × 0.5 µm) and a flame-ionization detector (FID), programmed from 60 °C with a 2-min hold, to 180 °C at 30 °C min^{-1}, then to 230 °C at 5 °C min^{-1}, during which the two pheromone components were eluted. The column was then heated to 245 °C at 20 °C min^{-1} and held at this temperature for 15 min to clean the column before the next analysis. The FID detector was held at 250 °C. To assess the phenotypic effect of each chromosome at the 0.05 level of significance, data on the pheromone components were analysed by analysis of variance (ANOVA; PROC GLM in SAS, version 9.1, 2002–2003). The R^2 values from these ANOVAs provided an estimate of the amount of phenotypic variation in pheromone composition in backcross females that could be explained by the presence of one copy of the *Z* chromosome.

Genotyping experiments. We developed a PCR-based assay to study the segregation of *pgFAR* alleles in individuals obtained from reciprocal crosses and maternal backcrosses. Using intron-spanning primers located within exons of the coding sequence, we amplified the entire gene from genomic DNA of parents and offspring of the mapping cross. The *pgFAR* gene consists of ten exons varying in size between 99 and 216 bp, and nine introns of 365–1,459 bp. The nucleotide-coding sequences of the alleles from the *Z* and *E* strains in this cross are, respectively, 99.1% and 99.5% identical to the cDNAs of *pgFAR-Z* and *pgFAR-E* that we expressed in yeast. Several introns show size variation between the two strains, and a substantial difference in the length of intron 7 (about 70 bp) allowed us to generate amplification products that could be size-discriminated by electrophoresis on an agarose gel (912 bp for *pgFAR-E*, compared with 981 bp for *pgFAR-Z*). The segregation pattern corresponded perfectly to AFLP linkage group 31 in the backcross.

To study the segregation of Δ11 fatty-acyl desaturase alleles in the same crosses, we designed primers D11-e1-F1 and D11-e2-R2 to span the first intron in the gene *Onu-Z/E11* (GenBank accession number EF113391)[31]. This gene produces the only one of three desaturase transcripts in the pheromone gland that encodes a functional product; its product was previously shown to encode a functional Δ11 fatty-acyl desaturase[21]. The 460-bp amplicon was sequenced and a diagnostic single nucleotide polymorphism in the intron (C marking the *Z*-strain allele (GenBank accession number GU733832), T for the *E*-strain allele (GenBank accession number GU733831)) was used to score granddaughters in the backcross that had also been scored for pheromone production, AFLPs and *pgFAR* genotypes. The segregation pattern corresponded perfectly to AFLP linkage group 23 in the backcross.

Sequence analysis. We used Molecular Evolutionary Genetics Analysis (MEGA) 4 (ref. 32) and DNASP 5 (ref. 33) to analyse DNA polymorphism parameters and tests of selection (Tajima's *D* and Fu and Li's *F**). To collect homologous sequences of FAR genes in insects we conducted BLASTP and TBLASTN searches against the GenBank protein and EST databases, respectively. In addition, TBLASTN searches were made against the clustered ESTs database on butterflybase (http://www.butterflybase.org; ref. 34). ESTs sequences were assembled manually into contigs when possible and translated into amino-acid sequences with the use of the translation tool on the Expasy proteomics server (http://www.expasy.ch; ref. 35). For all searches, the *pgFAR* gene identified as active in the pheromone biosynthesis of *Bombyx mori* (GenBank accession number BAC79425) was used as query. Finally, we retrieved from the Silkworm Genome Database (http://www.silkdb.org; ref. 36) all predicted paralogues of *Bombyx mori pgFAR*. Multiple protein sequences were aligned by using MAFFT 6 (ref. 37) followed by manual inspection. The aligned sequences are available from the authors on request.

Phylogenetic reconstruction was performed with MEGA. The tree was generated by using the Neighbour-Joining algorithm, and the implemented JTT model was

used as a substitution model for amino acids. To maximize the use of the available information and decrease the impact of short sequences predicted from ESTs, all positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. To estimate the confidence limits of nodes, 1,000 bootstrap samples were generated.

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