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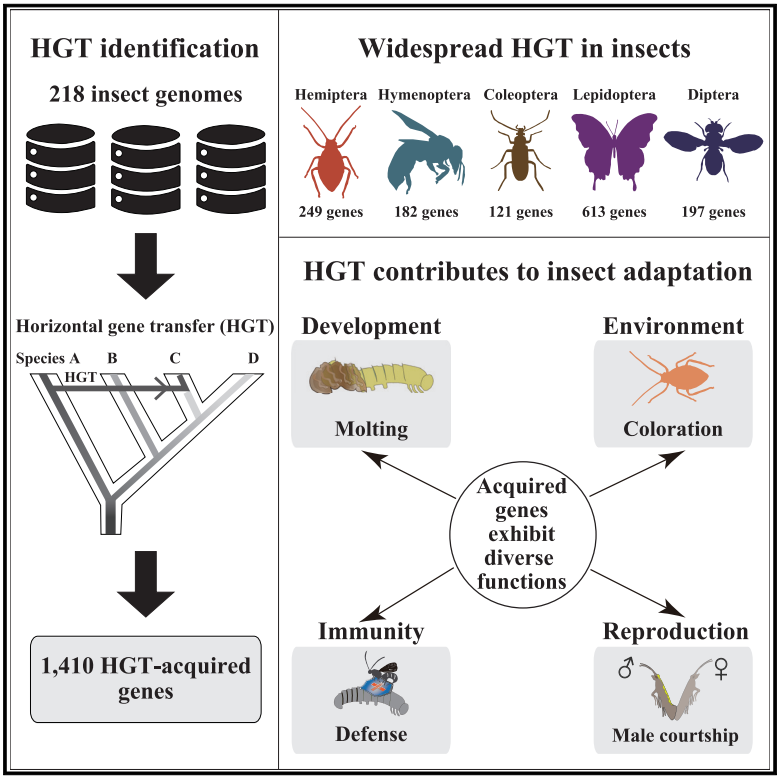
HGT is widespread in insects and contributes to male courtship in lepidopterans

Graphical abstract



Highlights

- Genome-scale screening of HGT in 218 insect genomes
- Intron gains from native insect genomes were likely involved in HGT adaptation
- Lepidopterans acquired, on average, the highest number of HGT-acquired genes
- HGT-acquired gene enhances male courtship behavior in lepidopterans

Authors

Yang Li, Zhiguo Liu, Chao Liu, ..., Antonis Rokas, Jianhua Huang, Xing-Xing Shen

Correspondence

antonis.rokas@vanderbilt.edu (A.R.), jhhuang@zju.edu.cn (J.H.), xingxingshen@zju.edu.cn (X.-X.S.)

In brief

A comprehensive resource of horizontal gene transfer (HGT) events in 218 insects acquired from non-metazoan sources provides insight into the adaptation of HGTs in insect genomes with the discovery of a functional role for the gene *LOC105383139* in male courtship behavior in lepidopterans.

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HGT is widespread in insects and contributes to male courtship in lepidopterans

Yang Li,[1,6] Zhiguo Liu,[1,6] Chao Liu,[2,3,6] Zheyi Shi,[1] Lan Pang,[1] Chuzhen Chen,[1] Yun Chen,[2] Ronghui Pan,[3] Wenwu Zhou,[1] Xue-xin Chen,[1] Antonis Rokas,[4,*] Jianhua Huang,[1,*] and Xing-Xing Shen[1,5,7,*]

[1]Key Laboratory of Biology of Crop Pathogens and Insects of Zhejiang Province, Institute of Insect Sciences, Zhejiang University, Hangzhou 310058, China

[2]Institute of Biotechnology, Zhejiang University, Hangzhou 310058, China

[3]ZJU-Hangzhou Global Scientific and Technological Innovation Center, Zhejiang University, Hangzhou 310027, China

[4]Department of Biological Sciences and Evolutionary Studies Initiative, Vanderbilt University, Nashville, TN 37235, USA

[5]Evolutionary & Organismal Biology Research Center, Zhejiang University, Hangzhou 310058, China

[6]These authors contributed equally

[7]Lead contact

*Correspondence: antonis.rokas@vanderbilt.edu (A.R.), jhhuang@zju.edu.cn (J.H.), xingxingshen@zju.edu.cn (X.-X.S.)

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SUMMARY

Horizontal gene transfer (HGT) is an important evolutionary force shaping prokaryotic and eukaryotic genomes. HGT-acquired genes have been sporadically reported in insects, a lineage containing >50% of animals. We systematically examined HGT in 218 high-quality genomes of diverse insects and found that they acquired 1,410 genes exhibiting diverse functions, including many not previously reported, via 741 distinct transfers from non-metazoan donors. Lepidopterans had the highest average number of HGT-acquired genes. HGT-acquired genes containing introns exhibited substantially higher expression levels than genes lacking introns, suggesting that intron gains were likely involved in HGT adaptation. Lastly, we used the CRISPR-Cas9 system to edit the prevalent unreported gene *LOC105383139*, which was transferred into the last common ancestor of moths and butterflies. In diamondback moths, males lacking *LOC105383139* courted females significantly less. We conclude that HGT has been a major contributor to insect adaptation.

INTRODUCTION

Insects originated in the Early Ordovician (~479 million years ago) (Misof et al., 2014) and comprise over 50% of all described living animals on Earth (Stork, 2018). This ancient lineage exhibits remarkable diversity in relation to, but not limited to, development, behavior, social organization, and ecology (Smith et al., 2008; Stork, 2018). Some studies have argued that the symbionts of host insects are important contributors to insect diversification (e.g., Archibald, 2015; Blondel et al., 2020; Bublitz et al., 2019; Degnan, 2014; Eleftherianos et al., 2013; Engel and Moran, 2013; Hotopp et al., 2007; Husnik et al., 2013; Paniagua Voirol et al., 2018; Perreau and Moran, 2022). For example, at least 20% of insect species harbor *Wolbachia* bacterial endosymbionts, whose genes have been found to be horizontally transmitted into host insect genomes (Boto, 2014). *Drosophila ananassae*, for instance, has acquired nearly the entire genome of *Wolbachia pipientis* via horizontal gene transfer (HGT) (Hotopp et al., 2007).

In addition to pieces of symbiont genomes introduced into insects via HGT, some studies have reported the transfer of a single or few genes from fungi, bacteria, plants, and viruses (e.g., Boto, 2014; Husnik and McCutcheon, 2018; Irwin et al., 2022; Perreau and Moran, 2022). The functions of these transferred genes appear ecologically important; for example, carotenoid biosynthesis genes transferred from fungi to aphids contribute to aphid body coloration (Moran and Jarvik, 2010), genes that neutralize phenolic glucosides acquired by whiteflies from plants contribute to whitefly detoxification capabilities (Xia et al., 2021), and a parasitoid killing factor gene transferred from a virus to lepidopterans contributes to lepidopteran defense (Gasmi et al., 2021).

Given the ecological importance of the few known examples of insect HGT and the enormous magnitude of insect diversity, we undertook a systematic investigation of HGT-acquired genes in insect genomes, including their functions and contributions to insect adaptation. Using a robust and conservative phylogeny-based approach, we systematically identified and characterized horizontally acquired genes in the high-quality genomes of 218 insects, representing 11/19 species-rich orders (i.e., orders with >1,000 described species) (Stork, 2018). Then, we asked three questions: (1) what is the distribution of horizontally acquired genes across major insect groups? (2) What factors contribute to the adaptation of HGTs in insect genomes? (3) What are the biological functions of HGTs in insects?

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RESULTS

Numerous horizontal gene transfers into insects

To systematically identify putative HGT-acquired genes in insects, we downloaded 218 publicly available genomes from GenBank and Lepbase (Challis et al., 2016) (see STAR Methods). The genomes of these 218 insects represent 11 of 19 species-rich orders (i.e., orders with >1,000 described species) (Table S1; Stork, 2018), including Ephemeroptera (2), Orthoptera (1), Blattodea (4), Thysanoptera (2), Hemiptera (19), Phthiraptera (1), Hymenoptera (68), Coleoptera (19), Lepidoptera (39), Siphonaptera (1), and Diptera (62). We used a robust and conservative phylogeny-based approach to examine the protein sequence of each of the 2,806,851 genes present in the contigs with a length of $\geq$100 kb from 218 insect genomes for evidence of HGT (e.g., Shen et al., 2018; Wisecaver et al., 2016) (Figure S1). We found a total of 1,410 genes in 192 insect genomes that were likely acquired via 741 distinct events from non-metazoan sources (Figures 1 and S2; Table S2), including 1,115 (79.0%) genes from bacteria, 194 (13.8%) genes from fungi, 43 (3.0%) genes from plants, 36 (2.6%) genes from viruses, and 22 (1.6%) genes from other lineages.

To gauge the reliability of the inference of these 1,410 HGT-acquired genes, we first used Conterminator v1.c74b5 (Steinegger and Salzberg, 2020) to detect contamination and found that none of these 1,410 HGT-acquired genes was identified as a potential contaminant. Second, we examined the recovery rate of 54 randomly selected genes from 16 insects representing 8/11 orders, using PCR and Sanger sequencing (see STAR Methods). Our results show that the rate of PCR success varied between 33.3% and 100% across 16 tested insects, with an average value of 83.3% (45/54 genes) (Figure 2A). Third, we compared our list of 1,410 HGT-acquired genes with a list of 193 previously published genes and found that 164/193 (85%) HGT-acquired genes in previous studies were also found in our study. Fourth, we examined the distribution of sequence lengths of the 928 genomic contigs that contain the 1,410 HGT-acquired genes alongside the distribution of sequence lengths of the 89,481 genomic contigs that do not contain HGT-acquired genes. We found that contigs containing the HGT-acquired genes were typically longer than contigs lacking them (Figure 2B). Fifth, we examined the distribution of proportions of 1,410 HGT-acquired genes that reside in the 928 contigs and found that none of the 928 contigs contained HGT-acquired genes in frequencies greater than 5% (Figure 2C). Finally, we examined the protein sequence similarity between the HGT-acquired genes in the insect recipients and their closest homologs in non-metazoan donors for all 1,410 HGT-acquired genes and found that similarity values ranged between 12% and 89%, with an average value of 39% (Figure 2D). Collectively, these results suggest that the list of 1,410 HGT-acquired genes present in the contigs with a length of $\geq$100 kb is reliable.

When evaluating the 1,410 HGT-acquired genes by the number of genomes included from each order, we found that the order Lepidoptera acquired by far the highest average number of HGT-acquired genes (16 genes per species), followed by the orders Hemiptera (13 genes per species), Coleoptera (6 genes per species), Hymenoptera (3 genes per species), and Diptera (2 genes per species) (Table S2). From the 741 distinct HGT events, 588 were species-specific, whereas the remaining 153 involved two or more species (Figure S2). Of the 153 distinct HGT events that involved two or more species, 63, 20, 15, 12, and 8 were found in the five largest orders Lepidoptera, Diptera, Hymenoptera, Coleoptera, and Hemiptera, respectively (Figure S2). When examining genome size, gene content, species divergence time, and genome completeness, we found that the number of HGT-acquired genes exhibited low levels of correlations with genome size, gene content, species divergence time, and genome completeness.

By examining putative donor organisms for 1,410 HGT-acquired genes using a combination of BLAST and phylogenetics, we found that the 1,410 HGT-acquired genes were likely acquired from 670 putative donor species (bacteria: 533/670 [80%], fungi: 74/670 [11%]; plants: 25/670 [4%], viruses: 29/670 [4%], and others: 9/670 [1%]) (Figure 3A). Overall, in addition to the common endosymbiotic bacterial genus *Wolbachia* (3%), the bacterial genera *Serratia* (3%), *Bacillus* (2%), *Pseudomonas* (2%), and *Paenibacillus* (2%) were also prevalent donor organisms (Figure 3A). In addition, some HGT donors were order-specific. For example, the fungal genera *Exophiala* and *Encephalitozoon* were Hemiptera-specific and Hymenoptera-specific donors, respectively; the bacterial genera *Streptomyces*, *Listeria*, and *Erwinia* were Coleoptera-specific, Lepidoptera-specific, and Diptera-specific donors, respectively.

Since some studies have argued that the genes in symbionts of the host insects have been horizontally transmitted into the host insect genomes (e.g., Archibald, 2015; Blondel et al., 2020; Perreau and Moran, 2022), we investigated the association between putative HGT donor organisms and known insect symbionts. Specifically, we calculated the relative abundance of each HGT donor genus as well as the relative abundance of each known symbiont genus in 20 insects (from 7/11 orders) in SymGenDB (Reyes-Prieto et al., 2015). We found that the correlation in relative abundance between HGT donor genera and known insect symbiont genera was significant ($r = 0.68$, p value $= 7.6 \times 10^{-9}$) (Figure 3B). This strong correlation still held when we examined the association between all putative HGT donor species and all insect symbiont species, without considering one-to-one corresponding relationships between HGT recipient insects and host insects of the symbionts. These results raise the possibility that symbionts of host insects—especially symbionts in the *Rickettsia* and *Wolbachia* lineages—might be involved in transitions of foreign genes into insect genomes.

Gene ontology (GO) analysis of the 1,410 HGT-acquired genes shows that most were associated with metabolism- and cellular-related terms (Figure 3C). In addition to previously reported functions (e.g., detoxification, body coloration, and defense) (Gasmi et al., 2021; Moran and Jarvik, 2010; Xia et al., 2021), we found diverse functions that include but are not limited to immunity, courtship behavior, metabolism, nutrition, adaptation to extreme environments, growth, and development. We found similar functional distributions of the GO categories (BP, biological process; CC, cellular component; and MF, molecular function) in the five largest orders in our study.

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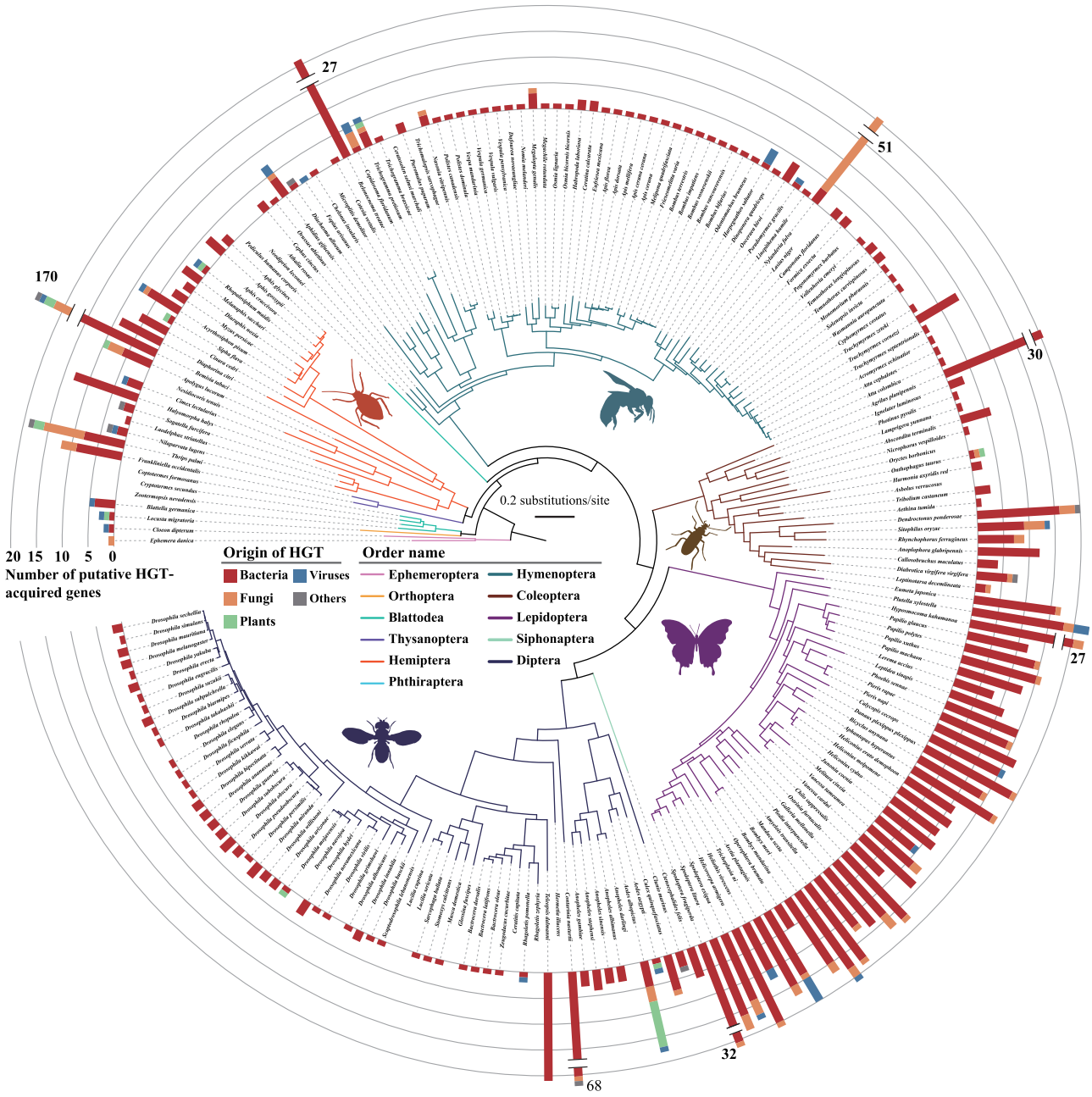

Figure 1. Distribution of the 1,410 putative HGT-acquired genes on the maximum likelihood phylogeny of 218 insects

We sampled 218 insects representing 11/19 species-rich orders (i.e., orders with >1,000 described species) (Stork, 2018). The phylogeny was a concatenated ML tree inferred from analysis of 1,367 single-copy BUSCO genes. These 1,410 putative HGT-acquired genes were likely acquired through 741 distinct HGT events of which 588 were species-specific and the other 153 were present in two or more species. The stacked bars indicate the number of HGT-acquired genes from the different HGT donor resources (red: bacteria, orange: fungi, green: plants, blue: viruses, and gray: others). Images representing taxa were taken from PhyloPic (http://phylopic.org).

See also Figures S1 and S2 and Tables S1 and S2.

Origin of introns in HGT-acquired genes and adaptation of HGT in insects

From the 1,410 HGT-acquired genes, 849 contain 1,534 introns ≥100 bp in length, whereas the remaining 561 lack introns (Figure 4A). Comparisons of introns between the genes of putative HGT donor organisms and their HGT insect recipients showed that all 1,534 introns present in the 849 genes were gained after these genes were inserted into insect genomes. Specifically, of the 1,410 HGT-acquired genes, 519 did not contain any introns in HGT donor organisms and recipient insects (i.e., no intron

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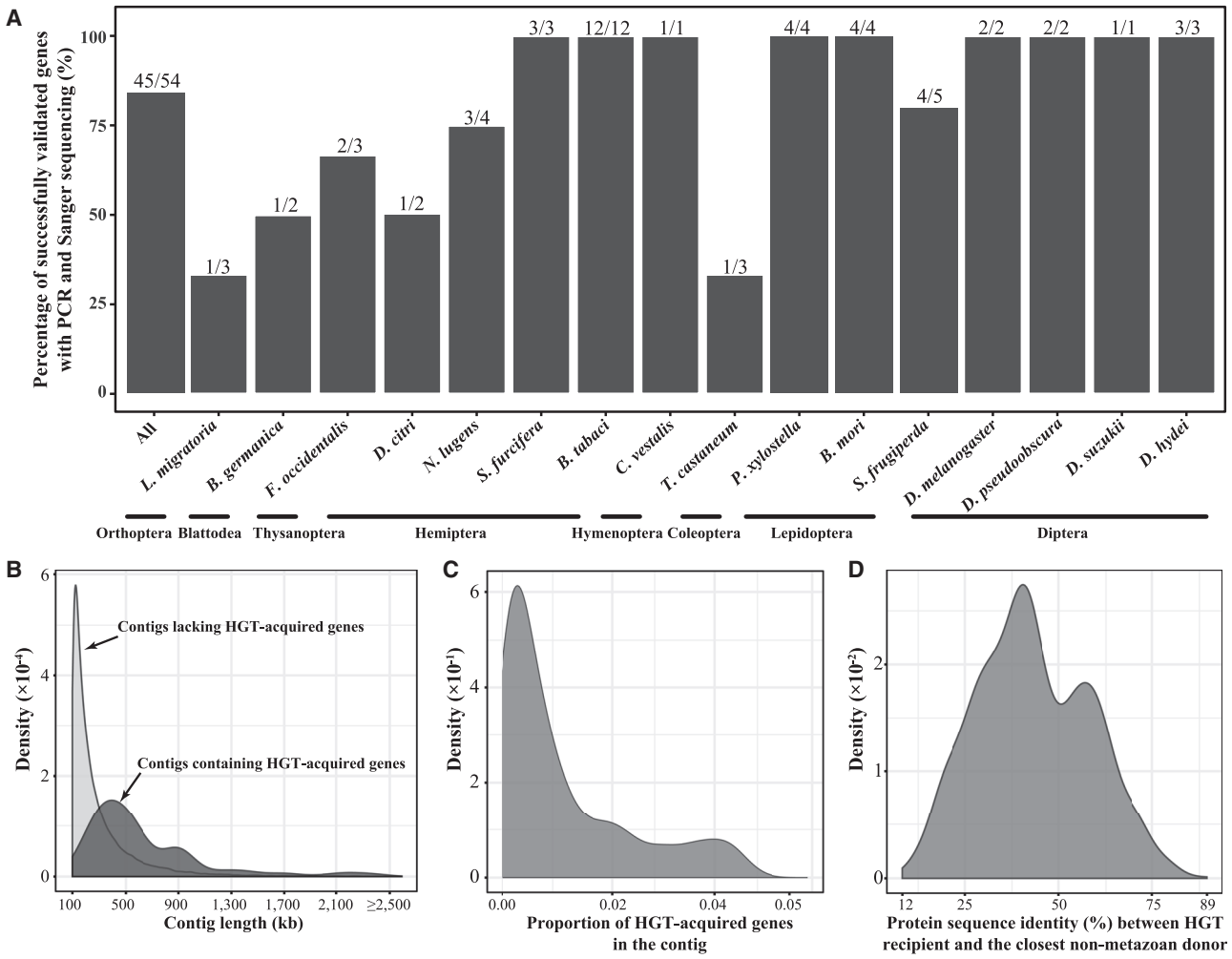

Figure 2. Robustness of HGT inference

(A) Validation of HGT-acquired genes using PCR and Sanger sequencing experiments. Since it is challenging to validate all 1,410 HGT-acquired genes in 218 insects due to limitation of insect genomic DNA, we examined 54 HGT-acquired genes in 16 insect species representing 8 of 11 orders. Note that the uneven sampling of insects for this analysis might not fully reflect the accuracy of HGT inference across orders. For each of the 54 HGT-acquired genes, two separate PCR reactions followed by Sanger sequencing of the amplicons were used to validate the presence of the HGT-acquired gene in the insect genome (see details in STAR Methods).

(B) Distributions of sequence lengths of genomic contigs with and without HGT-acquired genes. The darker distribution is that of the sequence lengths of the 928 contigs that contain the 1,410 HGT-acquired genes, and the lighter distribution is that of the sequence lengths of the 89,481 contigs that do not contain HGT-acquired genes.

(C) Distribution of proportions of HGT-acquired genes in each of the 928 contigs that harbors the 1,410 inferred HGT-acquired genes.

(D) Distribution of the protein sequence similarity between the sequence in the insect recipient genome and its closest hit in a non-metazoan donor genome for all 1,410 HGT-acquired genes.

gain or loss); 42 contained 53 introns in HGT donor organisms but did not retain them in recipient insects (i.e., intron loss); 849 lost 245 introns (loss is inferred based on the observation that 245 introns are present in the corresponding genes in HGT donor organisms) but gained 1,534 introns in recipient insects after gene transfers (i.e., intron gain) (Figure 4A).

Since the identifications of HGT-acquired genes were based on the protein sequences, the origins of these 1,534 gained introns in the 849 HGT-acquired genes were unknown. To address this question, we carried out BLASTN searches of DNA sequences of introns against a custom database consisting of nucleotide (nt) database at the NCBI as of 20 April 2022, and 218 insect genomes, with an e value cutoff of 1e−5 and the option "-task blastn-short." We found that 1,013/1,534 (66%) introns had BLAST hits, with an average identity of 86%, whereas 521/1,534 (34%) had no BLAST hits (Figure 4B). Further analyses of best hits for the 1,013 introns showed that all best hits came from their native insect genomes. Characterizing the features of these 1,013 gained introns from native insect genomes, we found that 891/1,013 (88%) introns are repeat-rich DNA sequences, including DNA transposons (51.6%), LTR transposons (25.8%), and unclassified repeats (10.6%) (Figure 4C).

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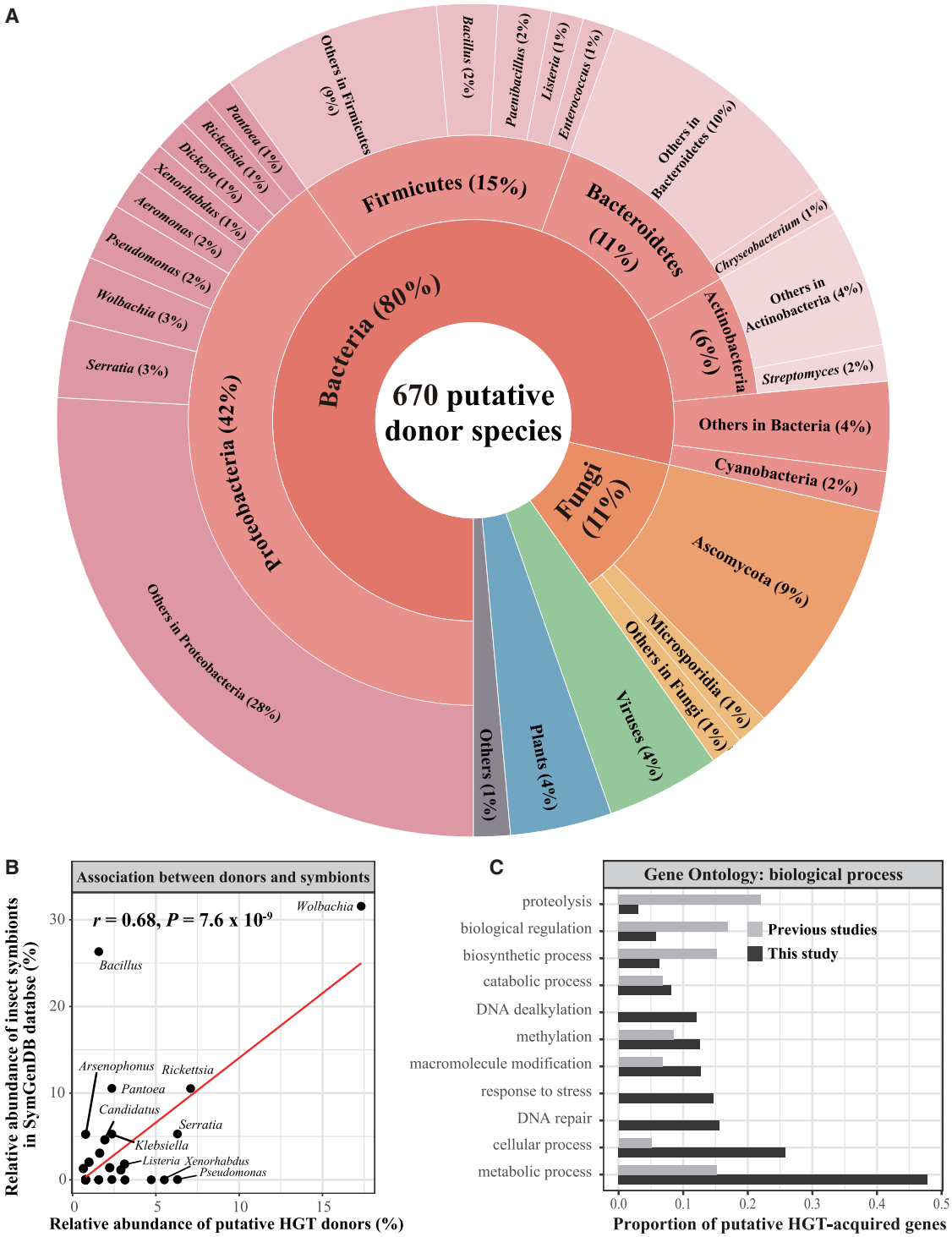

Figure 3. Symbionts of host insects were likely to be involved in the transitions of foreign genes into insect genomes

These 1,410 foreign genes were likely horizontally acquired through 741 distinct HGT events from 670 putative HGT donor species.

(A) The distribution of 670 putative donor species.

(B) The association in abundance between HGT donor species and known insect symbionts. We calculated the relative abundance of each HGT donor genus as well as the relative abundance of each known symbiont genus in 20 insects (from 7/11 orders) in SymGenDB. Pearson's correlation coefficient was used to test whether these two variables are significantly correlated.

(C) Gene ontology (GO) term analysis of 1,410 HGT-acquired genes in our study and of 193 HGT-acquired genes reported in previous studies in terms of biological processes.

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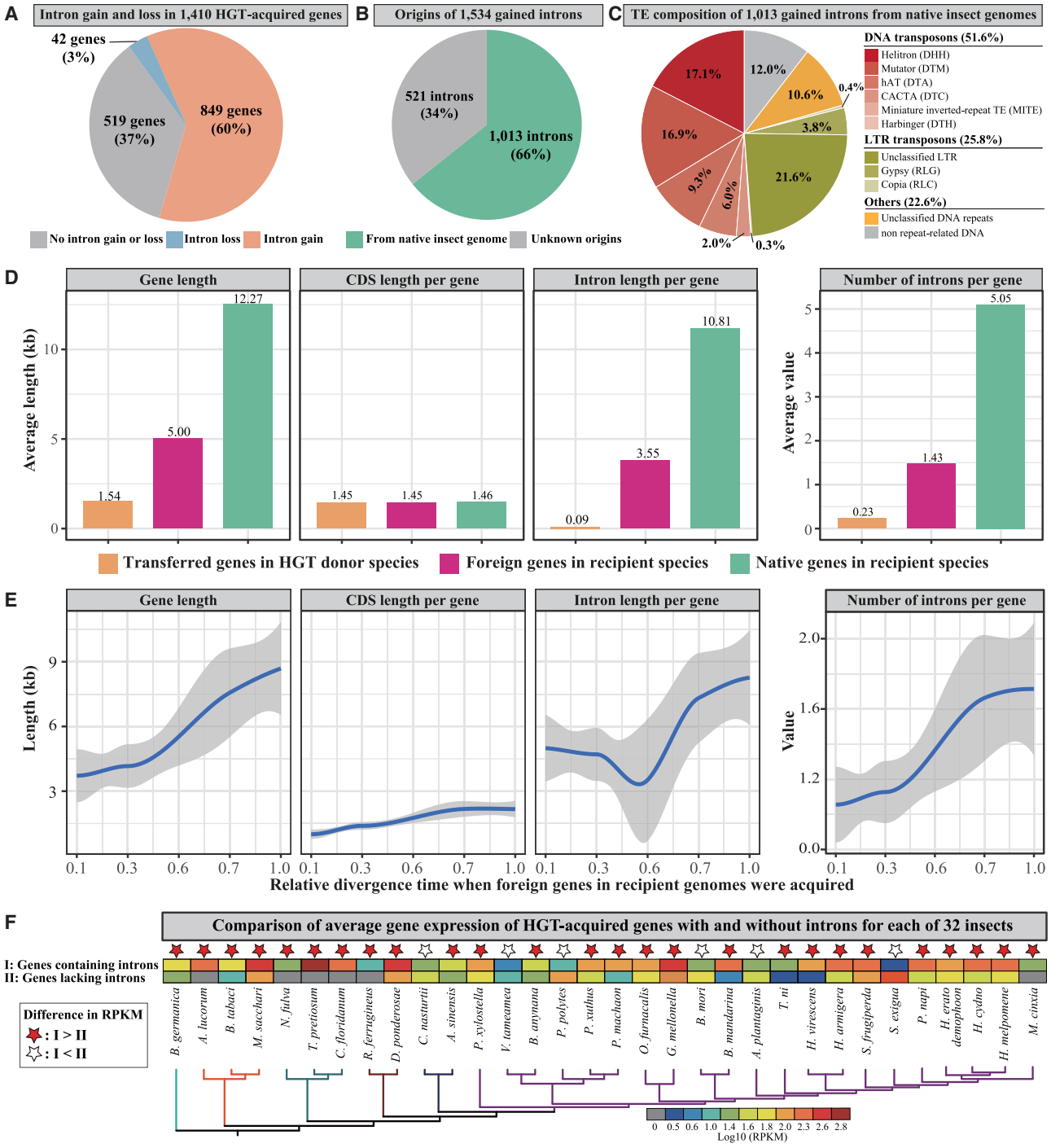

Figure 4. Repeat-rich intron gains from native insect genomes were likely involved in the adaptation of HGTs in insects

(A) After the integration of the 1,410 HGT-acquired genes into insect genomes, 849 gained 1,534 introns ≥100 bp in length (orange); 42 lost 53 introns (blue); and 519 had no intron gain or loss (gray).

(B) The origins of 1,534 gained introns. 1,013 introns are highly similar to sequences present in native insect genomes (green), whereas the sequences of the remaining 521 introns do not show similarity to the native insect genomes and were likely acquired from other organisms (gray).

(C) The transposable element (TE) compositions of 1,013 introns gained from native insect genomes.

(D) Comparisons of characteristics between transferred genes in HGT donor species (orange), foreign genes in recipient species (red), and native genes in recipient species (green). The left three boxes correspond to gene length, CDS length, and intron length, respectively. The right box corresponds to the number of introns per gene.

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By characterizing the gene structures of 1,410 HGT-acquired genes in the putative donor and recipient genomes as well as of all native insect genes, we found that the length of HGT-acquired genes in insects was significantly longer than that of their counterparts in HGT donor species, but it was significantly shorter than that of native genes in insects (on average, length of acquired genes in recipient genomes: 5.00 kb, length of transferred genes in HGT donor genomes: 1.54 kb, and length of native genes in recipient genomes: 12.27 kb) (Figure 4D). The 1,410 HGT-acquired genes in the putative donor and recipient genomes had similar coding sequence (CDS) lengths compared with all native insect genes (on average, length of CDS in foreign genes in recipient genomes: 1.45 kb, length of CDS in transferred genes in HGT donor genomes: 1.45 kb, and length of CDS in native genes in recipient genomes: 1.46 kb) (Figure 4D). By contrast, the length of introns in the 1,410 HGT-acquired genes in the recipient genomes was substantially longer than that in the putative donor genomes but was significantly shorter than that of introns in native genes (on average, length of introns in foreign genes in recipient genomes: 3.55 kb, length of introns in transferred genes in HGT donor genomes: 0.09 kb, and length of introns in native genes in recipient genomes: 10.81 kb) (Figure 4D). This trend can be explained by intron gain events (on average, number of introns in foreign genes in recipient insects: 1.43, number of introns in transferred genes in HGT donor species: 0.23, and number of introns in native genes in percipient insects: 5.05) (Figure 4D).

To further explore whether intron gains were involved in the adaptation of HGT-acquired genes to insect genomes, we conducted two separate analyses. First, we examined the changes of gene structures of HGT-acquired genes over evolutionary time (Figure 4E). Since inferring the time of evolutionary divergence of species-specific HGT-acquired genes is infeasible, we focused on the 822/1,410 HGT-acquired genes that were present in two or more insect species. We found that gene length, intron length, and the number of introns apparently increased over evolutionary time, whereas CDS length did not significantly change over evolutionary time (Figure 4E). Second, we compared expression levels of HGT-acquired genes containing introns and HGT-acquired genes lacking introns for transcriptome datasets from each of the 32 insects representing 6/11 orders using 90 publicly available transcriptome data (Table S3). Note that we compared HGT-acquired genes with and without introns only within each transcriptomic dataset (e.g., only using transcriptome data from the same stage and the same tissue for a given species). Of the 32 insect datasets, 26 (81.3%) had on average ~11-fold higher gene expression levels of HGT-acquired genes containing introns compared with HGT-acquired genes lacking introns, whereas only six (18.7%) had on average ~4-fold lower gene expression levels of HGT-acquired genes containing introns compared with HGT-acquired genes lacking introns (Figure 4F). Collectively, our results show that repeat-rich intron gains from native insect genomes, which enabled these foreign genes to increase their lengths toward the average length of native genes, were likely involved in adaptation of HGTs in insect genomes.

The last common ancestor of moths and butterflies horizontally acquired a foreign gene that enhances male courtship behavior from a donor in the bacterial genus *Listeria*

We evaluated the genetic function of the prevalent HGT-acquired gene *LOC105383139* in our list, which was acquired by the last common ancestor of moths and butterflies from a donor in the bacterial genus *Listeria* (Figure 5A). This gene belongs to the zinc-binding alcohol dehydrogenase family predicted by the Pfam database (Mistry et al., 2021) and the Alphafold2 (Jumper et al., 2021), but little is known about its function in either the donor organisms or the recipient insects. The gene family phylogeny of *LOC105383139*, which includes sequences of the gene from nearly all examined moths and butterflies, except for the moth *Chilo suppressalis* and the butterfly *Leptidea sinapis*, shows that 12 species contain multiple-copy homologs (e.g., *Papilio machaon*), whereas 25 species contain only a single-copy homolog (e.g., *Plutella xylostella*). After searching all 37 publicly available lepidopteran genomes, we found only six moth and two butterfly genomes that reported sex chromosomes. In all eight genomes, we found that the gene *LOC105383139* resides in the autosomes rather than in the sex chromosomes. To evaluate the gene's function, we used the CRISPR-Cas9 system to create homozygous mutants (MT-139) at generation 2 (G2) with two sgRNAs in the diamondback moth *Plutella xylostella*, a serious agricultural pest of crucifer vegetables (Figure 5B).

During rearing diamondback moths, we initially found that knockout (MT-139) moths have a ~5- to 6-fold lower number of offspring but have no significant differences in five developmental phenotypes, including body size, feeding, movement, testis size, and sperm activity (Figure S3), compared with wild-type (WT) moths. To identify underlying causes of the lower number of offspring in MT-139 moths, we first measured the number of eggs produced by emerging MT-139 and WT moths in 48 h.

(E) Characterizing changes in gene structures (gene length, CDS length, intron length, and number of introns) for the HGT-acquired genes in the context of relative divergence times. For these analyses, we examined the 822/1,410 HGT-acquired genes that were inferred to have been acquired in the common ancestor of two or more of the 218 species included in our study. The relative divergence times were inferred by the RelTime in MEGA7 (Kumar et al., 2016) using the ML tree in Figure 1.

(F) Comparison of average expression level between HGT-acquired genes containing introns (I: first row) and HGT-acquired genes lacking introns (II: second row) for each of the 32 insects representing 6 of 11 orders in our study. Note that we compared HGT-acquired genes with and without introns only within each transcriptomic dataset (e.g., only using transcriptome data from the same stage and the same tissue for a given species). We used 90 publicly available transcriptome datasets to calculate the expression levels of HGT-acquired genes containing introns and HGT-acquired genes lacking introns within each transcriptomic dataset. The information of developmental stage and tissue for the transcriptome data for each species are given in Table S3. The phylogeny of 32 insects was taken from the full phylogeny of 218 insects in Figure 1. For a given species, a red star indicates that the average expression level of HGT-acquired genes containing introns is higher than that of HGT-acquired genes lacking introns, while a white star indicates that the average expression level of HGT-acquired genes containing introns is lower than that of HGT-acquired genes lacking introns.
See also Table S3.

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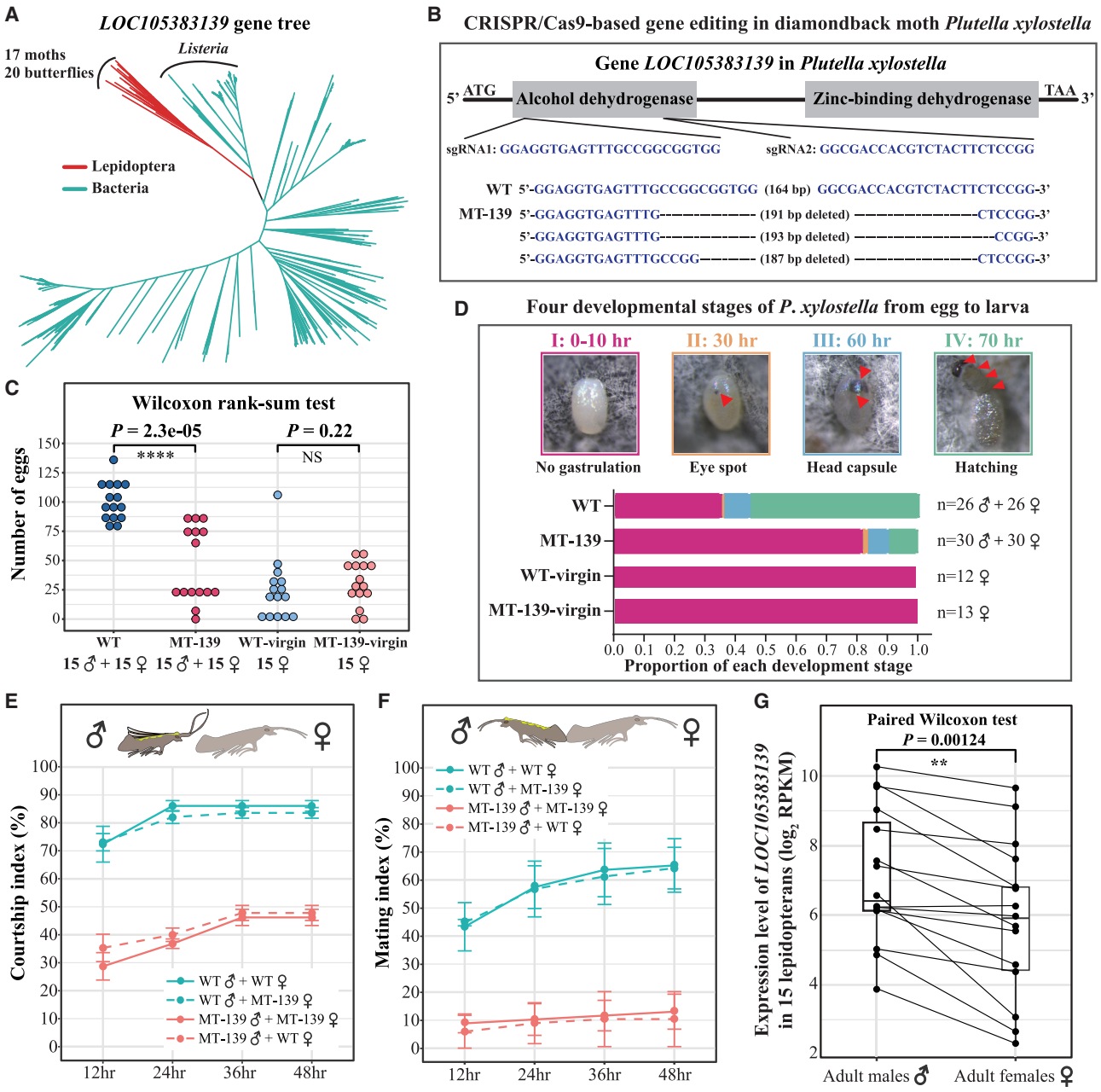

Figure 5. The prevalent HGT-acquired gene *LOC105383139* enhances male courtship behavior in lepidopterans

The prevalent HGT-acquired gene *LOC105383139*, which belongs to the large protein family of zinc-binding alcohol dehydrogenases, is present in nearly all moths and butterflies in our study, except for the moth *Chilo suppressalis* and the butterfly *Leptidea sinapis*.

(A) A simplified gene family phylogeny of the gene *LOC105383139*. Red branches indicate moths and butterflies, while green branches indicate bacteria.

(B) A simplified schematic diagram of the generation of the homozygous mutant line (MT-139) using the CRISPR-Cas9 system with two sgRNAs to edit single-copy gene *LOC105383139* in *Plutella xylostella*. Three representative mutant lines are given in the box below.

(C) Comparison of numbers of eggs produced by wild-type males + wild-type females (WT, n = 15 pairs), knockout males + knockout females (MT-139, n = 15 pairs), wild-type virgin females (WT-virgin, n = 15 females), and knockout virgin (MT-139-virgin, n = 15 females) in 48 h.

(D) Characterizing four developmental stages of *P. xylostella* from egg to hatching within 70 h. Red arrows in the upper box indicate changes through four developmental stages. Stacked bars in the box below indicate the proportions of each of the four stages for four different treatments. Note that all eggs produced by 4/26 (15.4%) pairs of wild-type moths (WT) and by 20/30 (66.7%) pairs of knockout moths (MT-139) were completely stuck in stage I (no gastrulation).

(E) Percentage of successfully courted pairs of adult females and adult males during 48 h. Courtship index is the percentage of successfully courted pairs, in which the male moves toward the female with flapping wings and tipping the abdomen, in a given time period.

(F) Percentage of successfully mated pairs of adult females and adult males during 48 h. Mating index is the percentage of successfully mated pairs in which the male copulates with the female for approximately 1 h. We used four treatments to conduct behavioral assays: wild-type males (WT♂) + wild-type females (WT♀),

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We found that the number of eggs produced by 15 pairs of knockout males (MT-139♂) + knockout females (MT-139♀) was significantly lower than that of eggs produced by 15 pairs of wild-type males (WT♂) + wild-type females (WT♀) (on average, MT-139: 46 eggs and WT: 101 eggs) (Figure 5C). However, the number of eggs produced by 15 knockout virgin females (MT-139-virgin) was similar to the number of eggs produced by 15 wild-type virgin females (WT-virgin) (on average, MT-139-virgin: 28 eggs and WT-virgin: 25 eggs) (Figure 5C). Next, we examined four developmental stages from eggs to hatching during 70 h. We found that the rate of successfully hatched eggs (stage IV in Figure 5D) in MT-139 moths was substantially lower than that of successfully hatched eggs in WT moths (MT-139: rate of hatched eggs = 9.5% and WT: rate of hatched eggs = 56%). Among these unsuccessfully hatched eggs in MT-139moths, over 80% of eggs were stuck in stage I (no gastrulation) for 70 h (Figure 5D), whereas in WT moths only 35% of eggs were stuck in stage I for 70 h. Strikingly, of the 30 examined pairs of MT-139 moths, 20 (66.7%) were found to have all their eggs stuck in stage I (no gastrulation). However, in WT moths, there were only 4/26 (15.4%) pairs whose eggs were stuck in the stage I. We also examined the developmental stages of unfertilized eggs produced by MT-139-virgin and WT-virgin. We found that all unfertilized eggs were completely stuck in stage I from MT-139-virgin and WT-virgin. These results suggest that the higher proportion of eggs that were stuck in stage I (no gastrulation) from MT-139 moths were likely due to the higher rate of unfertilized eggs in MT-139 moths (Figure 5D).

Through further observations, we found that MT-139 moths had apparently lower mating rates than WT moths. To precisely quantify mating behavior, we evaluated courtship index and mating index for WT and MT-139 moths that were 1 day old after emergence for 48 consecutive hours, respectively. Courtship index is the percentage of successfully courted pairs, in which the male moves toward the female with flapping wings and tipping the abdomen, in a given time period (Xu et al., 2020). Mating index is the percentage of successfully mated pairs in which the male copulates with the female for approximately 1 h (Song et al., 2014). We used four treatments for behavioral experiments: WT♂ + WT♀, WT♂ + MT-139♀, MT-139♂ + MT-139♀, and MT-139♂ + WT♀ (each treatment had three replicates using 24 pairs of 1-day-old male and female adult moths) (Figures 5E and 5F). Strikingly, we found that MT-139♂ had a significantly lower percentage of courting attempts toward MT-139♀ and WT♀ than WT♂ (on average, percentage of courted pairs, MT-139♂ + MT-139♀: 46%; MT-139♂ + WT♀: 48%; WT♂ + WT♀: 86%; and WT♂ + MT-139♀: 84%) (Figure 5E). Moreover, MT-139♂ had a significantly lower percentage of mating with MT-139♀ and WT♀ than WT♂ (on average, percentage of mating pairs, MT-139♂ + MT-139♀: 13%; MT-139♂ + WT♀: 10%; WT♂ + WT♀: 65%; and WT♂ + MT-139♀: 64%) (Figure 5F; Table S4; Video S1).

We also examined the role of the gene *LOC105383139* in two closely related butterflies (*Heliconius melpomene* and *Heliconius cydno*) with publicly available courtship data and transcriptome data (eye and brain) in courtship situations (Merrill et al., 2019; Rossi et al., 2020) (Figure S4). We found that *H. melpomene* males, which courted females significantly more than *H. cydno* males (on average, number of courting episodes toward females in five trials, *H. melpomene*: 18 and *H. cydno*: 6) (Figure S4A), had a 6.5-fold higher expression level of the gene *LOC105383139* compared with *H. cydno* males (on average, the expression level of the gene *LOC105383139* in five male adults, *H. melpomene*: 130 reads per kilobase of exon model per million mapped reads [RPKM] and *H. cydno*: 20 RPKM) (Figure S4B). Further analyses of the publicly available transcriptome data of 15 pairs of adult male and female lepidopterans revealed that males had significantly higher expression levels of the gene *LOC105383139* than females as well (Figure 5G). Collectively, these results suggest that one of the functions of the gene *LOC105383139*, acquired by the last common ancestor of moths and butterflies via HGT, is the enhancement of male courtship behavior.

The question then arises, what genes interact with the male courtship-associated foreign gene *LOC105383139*? We first quantified the gene's expression levels at 13 different developmental stages from egg to adult in diamondback moths (see STAR Methods). Consistent with the results of 15 pairs of publicly available lepidopteran transcriptome data (Figure 5G), the qRT-PCR results showed that adult males had the highest expression levels when compared with different developmental stages in male and female moths. In addition, we also examined the expression levels of the foreign gene *LOC105383139* in five tissues (antennae, head, thorax, abdomen, and reproductive system) in male moths. Interestingly, we found that the foreign gene was highly expressed in the abdomen and reproductive system but was lowly expressed in the antennae, head, and thorax. Next, we generated transcriptome data of the whole bodies for 1-day-old wild-type male adult (WT male), wild-type female adult (WT female), knockout male adult (MT-139 male), and knockout female adult (MT-139 female). We found that 462 genes were significantly under-expressed and 359 were significantly over-expressed in the MT-139 male versus WT male analysis.

The GO term enrichment analysis reveals that in the MT-139♂ versus WT♂ analysis, the terms courtship behavior, reproductive process, metabolic process, biological regulation, and response to stimulus were significantly enriched in the 462 under-expressed genes, whereas the terms developmental process, localization, metabolic process, and immune system process were significantly enriched in the 359 over-expressed genes (Figure S5A). Examination of the biological process of the 462 under-expressed genes in the MT-139♂ versus WT♂ analysis identified nine genes (*FBgn0028572*: quick-to-court, *FBgn0003068*: period, *FBgn0000535*: ether a go-go, *FBgn0263111*: cacophony,

wild-type males (WT♂) + knockout females (MT-139♀), knockout males (MT-139♂) + knockout females (MT-139♀), and knockout males (MT-139♂) + wild-type females (WT♀). Each treatment had three replicates using 24 pairs of 1-day-old male and female adult moths.
(G) Comparison of gene expression of the gene *LOC105383139* in 15 pairs (males♂ and females♀) of adult lepidopterans.
See also Figures S3–S5, Table S4, and Video S1.

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FBgn0020277: lush, *FBgn0011279*: odorant-binding protein 69a, *FBgn0283510*: peptidyl-α-hydroxyglycine-α-amidating lyase 1, *FBgn0004573*: 5-hydroxytryptamine receptor 7, and *FBgn0005626*: tyrosine 3-monooxygenase in *Drosophila melanogaster*), which could potentially be involved in courtship behavior (GO term: 0007619). By contrast, courtship-associated genes were not found from the set of the 359 over-expressed genes (Figure S5A). These results suggest that these nine courtship-associated genes, and possibly other differentially expressed genes, that interact with the foreign gene *LOC105383139* might be involved in male courtship behavior, but their roles in diamondback moths deserve further experimental investigations. We also performed analysis of differential gene expression and GO term enrichment for MT-139♀ versus WT♀, and identified 348 genes that were significantly under-expressed and 375 that were significantly over-expressed. However, these differentially under-/over-expressed genes were mostly involved in the metabolic process, developmental process, cellular process, biological regulation, locomotion, response to stimulus, and signaling, but none of them were associated with female mating behaviors, including mate choice (mate recognition and acceptance) and oviposition (Figure S5B).

DISCUSSION

In this study, taking advantage of the high-quality genomes of 218 insects representing 11 of 19 species-rich orders (i.e., orders with >1,000 described species) (Stork, 2018), we systematically inferred that 1,410 genes were transmitted via 741 distinct HGT events into insects from non-metazoan (mostly bacterial) sources.

What is the distribution of HGT-acquired genes across insects?

Many previous studies have shown the occurrence of HGT in insects, but their taxon sampling strategies focused on either a few insects of interest or on a specific order of insects (e.g., Crisp et al., 2015; Daimon et al., 2005; Dhaygude et al., 2019; Irwin et al., 2022; Di Lelio et al., 2019; McKenna et al., 2019; Moran and Jarvik, 2010; Parker and Brisson, 2019; Sun et al., 2013; Woolfit et al., 2009; Xia et al., 2021; Zhu et al., 2011). To date, McKenna et al. (2019) carried out a comprehensive investigation of HGTs in the order Coleoptera (beetles) in which they used 154 transcriptomes or genomes to specifically study the 10 plant cell wall-degrading enzymes (PCWDEs) that were acquired from bacteria and fungi via HGT.

Although these previous efforts are significant in establishing the occurrence and ecological importance of HGT in insects, the use of sparse and sporadic sampling of insect genomes has hampered better understanding of the distribution of HGT-acquired genes across the insect lineage, the largest and most diverse clade comprising >50% of all described animals. Our systematic identification of 1,410 HGTs shows that the order Lepidoptera acquired by far the highest average number of HGT-acquired genes (16 genes per species), followed by the orders Hemiptera (13 genes per species), Coleoptera (6 genes per species), Hymenoptera (3 genes per species), and Diptera (2 genes per species) (Figure 1). In addition, examination of putative HGT donor organisms and known symbionts of host insects revealed that genes in insect symbionts were likely to be horizontally transferred into the host insects (Figures 3A and 3B), which is consistent with previous findings (Archibald, 2015; Blondel et al., 2020; Bublitz et al., 2019; Degnan, 2014; Eleftherianos et al., 2013; Engel and Moran, 2013; Hotopp et al., 2007; Husnik et al., 2013; Paniagua Voirol et al., 2018; Perreau and Moran, 2022).

What factors contribute to adaptation of foreign genes in insects?

In general, many studies agree that HGT-acquired genes were involved in adaptation to recipient genomes, but views vary on factors that contribute to the adaptation of these foreign genes in insect genomes (e.g., Arnold et al., 2022; Husnik and McCutcheon, 2018). For example, codon usage is an important factor that determines the fate of transferred genes due to the need for compatibility with the transfer RNA (tRNA) pool in the host (Husnik and McCutcheon, 2018). Selection is also considered as the dominant force for the adaptation of HGTs in bacteria, but there is still much debate on whether most transfers are beneficial, neutral, or even deleterious to the recipients (Arnold et al., 2022). In addition, a recent study argued that introns did not give rise to the significant difference in gene length between foreign genes and native genes and did not play an important role in adaptations of foreign genes to recipient phytoplankton genomes (Fan et al., 2020). In our study, we found that 849/1,410 HGT-acquired genes contain repeat-rich introns, which were likely acquired from the native insect genomes after the initial gene transfers (Figures 4A–4C). Moreover, a comparison of gene structures between HGT-acquired genes in the context of divergence times shows that intron gain events occurred over evolutionary time, which enabled these foreign genes to increase their lengths toward the average length of native genes (Figures 4D and 4E). More importantly, HGT-acquired genes containing introns exhibited substantially higher expression levels than genes lacking introns (Figure 4F), which is consistent with previous studies in diverse organisms (green microalgae, plants, and insects) showing that intron gains can enhance gene expression levels (Baier et al., 2018; Husnik et al., 2013; Rose et al., 2011). Overall, our results suggest that the repeat-rich introns acquired from native insect genomes were likely involved in adaptation of HGTs to recipient genomes.

What are the biological functions of foreign genes in insects?

Many previous studies have reported instances of HGT-acquired genes contributing to important traits in insects, although only some of them constructed the mutants to verify function due to the challenge in genome editing for non-model insects (Dai et al., 2021; Gasmi et al., 2021; Di Lelio et al., 2019; Meng et al., 2009; Moran and Jarvik, 2010; Parker and Brisson, 2019; Xia et al., 2021). Among these previously reported HGTs, three are well studied and have shown that transferred genes can have ecologically diverse functions, including in body coloration (Moran and Jarvik, 2010), detoxification (Xia et al., 2021), and defense (Gasmi et al., 2021). In our study, the set of 1,410 HGT-acquired genes not only included the majority (~85%) of previously reported cases (including the three examples cited in the

previous sentence) but also provided additional diverse functions, which include but are not limited to metabolism, courtship behavior, nutrition, adaptation to extreme environment, growth, and development. In the list of 1,410 HGT-acquired genes, the prevalent gene *LOC105383139*, which was horizontally introduced into nearly all moths and butterflies from a donor in the bacterial genus *Listeria*, was validated by the CRISPR-Cas9 system and a series of behavioral experiments in the diamondback moth *Plutella xylostella*. Surprisingly, we found that male diamondback moths lacking the gene *LOC105383139* courted female ones significantly less, showing a reduced level of mating behavior. The master genes *fruitless* and *doublesex* were well studied in courtship behavior in the fruit fly and silkworm (Anderson, 2016; Greenspan and Ferveur, 2000; Pan and Baker, 2014; Xu et al., 2020; Yamamoto and Koganezawa, 2013), but none of the previous studies reported that a foreign gene can be also associated with courtship behavior.

In summary, our results provide a resource of HGT-acquired genes in insects. This resource will enable users to study the functions of these foreign genes not only in our examined species but also in other insects. Moreover, the tempo and mode of evolution of these HGT-acquired genes insects also provide guidelines for insect biological science, insect pest control, and insect biodiversity.

Limitations of the study

Our study suggests that HGT is widespread in insect genomes and has likely contributed to insect adaptation. There are a few limitations to our study. First, the disproportional genome sampling across different insect orders could potentially influence the precision of our estimates of the number of HGT-acquired genes and HGT donor sources (e.g., symbionts) between orders. Second, although we deemed that intron gains from insect native genomes were likely involved in adaptation of HGTs in insect genomes, we cannot exclude alternative explanations of how foreign genes evolved in insect genomes, such as selective constraint under rapid adaptation to environmental change (Woods et al., 2020). Third, the disruption of the gene *LOC105383139* in diamondback moths significantly reduces males' courting of females, but its functional role in other lepidopteran species has not yet been validated with genome editing technology. As more insect genomes are sequenced and genome editing techniques enable genetic manipulation experiments in lepidopterans (e.g., butterflies) at higher levels of efficiency, these limitations could be experimentally tested.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cell.2022.06.014.

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AUTHOR CONTRIBUTIONS

X.-X.S., J.H., and A.R. conceived and designed the study. X.-X.S., Y.L., Z.L., C.L., Z.S., L.P., C.C., and W.Z. performed computational analyses and experiments. X.-X.S., J.H., A.R., Y.L., Z.L., C.L., Y.C., R.P., and X.-x.C. interpreted results. X.-X.S., A.R., and J.H. wrote the paper with input from all authors.

DECLARATION OF INTERESTS

A.R. is a scientific consultant for LifeMine Therapeutics, Inc.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0	TaKaRa	Cat# 9762
T7 High Yield RNA Transcription Kit	Vazyme	Cat# TR101-01
GenCrispr NLS-cas9-NLS nuclease	GenScript	Cat# Z03469
FastPure Cell/Tissue DNA Isolation Mini Kit	Vazyme	Cat# DC102
FastPure Cell/Tissue Total RNA Isolation Kit	Vazyme	Cat# RC101-01
HiScript III RT SuperMix for qPCR	Vazyme	Cat# R323-01
ChamQ SYBR qPCR Master Mix Kit	Vazyme	Cat# Q311-02
Brilliant blue	Tokyo Chemical Industry	Cat# F0147
LIVE/DEAD Sperm Viability Kit	Invitrogen	Cat# L-7011
Bovine serum albumin	Sigma	Cat# V900933-100G
1M Hepes Solution	BBI Life Sciences	Cat# E607018-0100
Deposited data		
Data matrices	This study	Figshare data repository: 10.6084/m9.figshare.18094172
Phylogenetic trees	This study	Figshare data repository: 10.6084/m9.figshare.18094172
Alignments and ML trees for horizontally acquired genes	This study	Figshare data repository: 10.6084/m9.figshare.18094172
Experimental models: Organisms/strains		
218 sampled insect species	This study	See Table S1
Software and algorithms		
HGTfinder v1.0	Shen et al., 2018	https://github.com/xingxingshen/HGTfinder/
MAFFT v7.299	Katoh and Standley, 2013	https://mafft.cbrc.jp/alignment/software/
DIAMOND v 2.0.9	Buchfink et al., 2021	https://github.com/bbuchfink/diamond/
trimAl v1.4	Capella-Gutierrez et al., 2009	http://trimal.cgenomics.org/
IQ-TREE v1.6.12	Nguyen et al., 2015	http://www.iqtree.org/
iTOL v4	Letunic and Bork, 2019	https://itol.embl.de/
BUSCO v5.2.2	Manni et al., 2021	https://busco.ezlab.org/
MEGAv7	Kumar et al., 2016	https://www.megasoftware.net/
Conterminator v1.c74b5	Steinegger and Salzberg, 2020	https://github.com/martin-steinegger/conterminator
OrthoDB V10	Kriventseva et al., 2019	www.orthodb.org
featureCounts v1.6.0	Liao et al., 2014	https://rnnh.github.io/bioinfo-notebook/
EDTA v1.9.4	Ou et al., 2019	https://github.com/oushujun/EDTA/
Trimmomatic v0.39	Bolger et al., 2014	http://www.usadellab.org/cms/?page=trimmomatic
R package edgeR v3.360	Robinson et al., 2010	http://bioconductor.org/packages/release/bioc/html/edgeR.html
R package limma v3.50.0	Ritchie et al., 2015	https://bioconductor.org/packages/release/bioc/html/limma.html
R package ggplot2	Wickham, 2009	https://cran.r-project.org/web/packages/ggplot2/index.html
Metascape v3.5	Zhou et al., 2019	https://metascape.org/

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RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact: Xing-Xing Shen (xingxingshen@zju.edu.cn).

Materials availability

This study did not generate any new unique reagents or materials to report. All reagents or materials used are commercially available.

Data and code availability

- All gene alignments, gene trees, additional figures and tables, and summary statistics, are publicly available on the figshare repository (https://doi.org/10.6084/m9.figshare.18094172). Raw RNA sequencing data has been deposited in GenBank under Bioproject ID: PRJNA801500 and are publicly available as of the date of publication.
- All original code is publicly available on Github (https://github.com/xingxingshen/HGTfinder).
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plutella xylostella rearing

Plutella xylostella that were originally collected in 2015 from the cabbage field in Hangzhou (30°30'N, 120°09'E), Zhejiang Provence, China, were reared at 25 ± 1°C and 65 ± 5% relative humidity under a 16-hour light and 8-hour dark photoperiod. Larvae were fed with cabbage, while adults were supplied with 10% honey solution. Both one day old male and female adults after emergence were used in this study.

METHOD DETAILS

Taxon sampling

To collect the greatest possible set of genome representatives of the class Insecta as of 17 November 2020, we used "insects" as search term in NCBI's Genome Browser (https://www.ncbi.nlm.nih.gov/genome/browse!/eukaryotes/insects) to obtain the basic information of species name, assembly accession number, assembly release date, assembly level (e.g., contig, scaffold, etc.), and GenBank FTP access number. For species with multiple genomes sequenced, we only included the genome that has publicly available annotation, the highest assembly level, and the latest release date. In addition, we also included high-quality genomes of all 12 butterflies in Lepbase (http://lepbase.org/) (Challis et al., 2016). Collectively, we included 218 insects representing 11 of 19 species-rich orders (i.e., orders with >1,000 described species) (Stork, 2018), including Ephemeroptera (2), Orthoptera (1), Blattodea (4), Thysanoptera (2), Hemiptera (19), Phthiraptera (1), Hymenoptera (68), Coleoptera (19), Lepidoptera (39), Siphonaptera (1), and Diptera (62). Analysis of genome assembly completeness reveals that 212 of 218 (~97%) genomes have ≥ 90% of the 1,367 preselected genes that are single-copy in at least 90% of the 75 reference insect genomes in OrthoDB v10 (Kriventseva et al., 2019; Manni et al., 2021; Waterhouse et al., 2013). Detailed information is given in Table S1.

Identification of HGTs into insects

To detect insect genes that may have been horizontally acquired from non-metazoan organisms, we employed a robust and conservative phylogeny-based approach (Figure S1; Gonçalves et al., 2018; Shen et al., 2018; Wisecaver et al., 2016). Our approach incorporated the information from each gene's Alien Index (AI) score, which compared the similarity of the gene between specified ingroup and outgroup taxa (e.g., insects and bacteria, respectively), the distribution of outgroup taxa in the list of each gene's top 1,000 blast hits against the Refseq database (last accessed January 10, 2021), as well as each gene's placement in a maximum likelihood phylogenic tree with its 1,000 most similar homologs. To avoid spurious results due to the presence of small genomic fragments of contaminant organisms in our genome assemblies, we limited our analyses to those genes that resided in genomic contigs or scaffolds that were ≥ 100 kb, which was adopted from our previous study (Shen et al., 2018). This filter resulted in the analysis of 2,806,851 of 3,185,017 (88.1%) protein-coding genes in 218 insect genomes.

For each gene's protein sequence, we evaluated whether it had been horizontally acquired using a two-step workflow following the pipeline provided by Shen et al., (Shen et al., 2018; Figure S1). Briefly, in step 1 we first carried out a BLASTP in DIAMOND v 2.0.9 (Buchfink et al., 2021) search against a custom database (Refseq+) consisting of the reference protein sequences (Refseq) (last accessed January 10, 2021) and all insect protein sequences, with an e-value cutoff of 10^{-10}. We next used HGTfinder v1 (Shen et al., 2018) to: (a) assign taxonomic information to each BLAST hit from the NCBI Taxonomy database, and then (b) parse the BLAST hits, based on their taxonomic information, into three different lineages (RECIPIENT: insects; GROUP: other metazoans; OUTGROUP: non-metazoan) so as to obtain three values: **bbhO** (BLAST bitscore of the best hit in OUTGROUP lineage), **bbhG** (bitscore of the

best hit in GROUP lineage but not in RECIPIENT lineage), and **maxB** (bitscore of the query to itself). Using this information, we next calculated: (a) the Alien Index: **AI** = (*bbhO* /*maxB*) − (*bbhG* /*maxB*), and (b) the percentage of species from OUTGROUP lineage (**outg_pct**) in the list of the top 1,000 hits that have different taxonomic species names. From the 2,806,851 genes analyzed, 28,822 genes passed the cutoffs AI value > 0 and outg_pct ≥ 80%. In step 2, we retrieved the 1,000 most similar homologs from the Refseq+ database (see above), aligned them by the MAFFT, version 7.299 (Katoh and Standley, 2013), with "–auto" option, and trimmed ambiguously aligned regions using trimAl v1.4 (Capella-Gutiérrez et al., 2009) with "-automated1" option. We then used the resulting alignment to infer the ML tree using IQ-TREE 1.6.12 (Nguyen et al., 2015) with its best-fitting model of amino acid evolution and 1000 ultrafast bootstrapping replicates (Minh et al., 2013). Lastly, we rooted each ML tree at the midpoint using the ape and phangorn R packages (Ihaka and Gentleman, 1996; Paradis et al., 2004; Schliep, 2011) and visualized it using the command version of iTOL v4 (Letunic and Bork, 2019). After manually inspecting all 28,822 ML trees, we identified 1,410 putative HGT-acquired genes. We compared the number of HGT-acquired genes between 165 genomes annotated by NCBI pipeline and 53 genomes annotated by the authors of the original studies and found that the number of HGT-acquired genes did not differ significantly between the two types of genome annotations (Wilcoxon rank-sum test; *P*-value = 0.16).

Validation of HGT-acquired genes

To evaluate the reliability of 1,410 putative HGT-acquired genes, we carried out three separate analyses: PCR assays, comparison of our putative HGT-acquired genes with previously published genes, and gene expression assays of HGT-acquired genes.

PCR assays

We randomly sampled 54 genes acquired by 16 different insects, representing 8 / 11 orders in this study. For each gene, we first used two separate PCR reactions to amplify upstream and downstream regions that flanked the foreign gene. Each PCR target size was ~ 1,500 bp. We used agarose gel electrophoretic analysis to judge whether PCR products were expected or not. If the PCR product matched our expected size, we then sequenced the PCR product using Sanger sequence technology. Here, we considered that the HGT-acquired gene was successfully validated if its upstream and downstream regions were successfully amplified and their Sanger sequences were nearly identical (identity of ≥ 98%) to DNA sequences in our contigs or scaffolds.

Comparison of our putative HGT-acquired genes with previously published genes

To collect the greatest possible set of previously published HGT-acquired genes as of 20 June 2021, we used "insect and HGT" and "insect and horizontal gene transfer" as search terms in NCBI's PubMed Browser (https://pubmed.ncbi.nlm.nih.gov/). As a results, 68 studies were found. For each study, we manually checked whether the insects mentioned in the published study were also included in the list of our 218 insects. This filter resulted in the analysis of 193 HGT-acquired genes from 14 previously published studies.

Gene expressions of HGT-acquired genes

To retrieve the transcriptome data for 218 insects, we used each species name as search term in NCBI's Sequence Read Archive (SRA) Browser (https://www.ncbi.nlm.nih.gov/sra) to get RNA-seq SRA accession numbers. As a result, 90 transcriptome datasets for 32 insects representing 6 / 11 orders were downloaded. The detailed information of stage and tissue for the transcriptome data for each species is given in Table S3. For each of 699 genes acquired by 32 insects via HGT, we calculated its gene expression by the reads per kilobase of exon model per million mapped reads (RPKM) using featureCounts v1.6.0 (Liao et al., 2014). We found that at least 478 / 699 (68%) HGT-acquired genes had expression values ≥ 5 RPKM. Average expression of each of 32 insects varies between 11 and 4,082 RPKM. Compared to the average expression level of native genes (608 RPKM), the average expression level of HGT-acquired genes was moderately lower (350 RPKM), which is consistent with the observations in phytoplankton genomes (Fan et al., 2020) and fungal genomes (Shen et al., 2018).

Analyses of the origins of introns in HGT-acquired genes

Origins of introns

From 1,410 HGT-acquired genes, 849 gained 1,534 introns ≥ 100 bp in length. To identify the origin of each of 1,534 introns, we first carried out a BLASTN search against a custom database consisting of the Nucleotide (nt) database at the NCBI as of 20 April 2022 and 218 insect genomes (note that DNA sequences of HGT-acquired genes were masked in insect genomes to avoid self-hits), with an e-value cutoff of 1e-5 and the option "-task blastn-short". We next parsed all hits for each intron and determined the putative origin of the intron.

Feature of introns

To characterize the features of DNA sequences in introns, we first used the Extensive de novo TE Annotator (EDTA v1.9.4) to annotate the whole genome for each of 218 insects with the default settings (Ou et al., 2019). Next, we retrieved the features of introns according to their positions in genome.

Generation of the gene *LOC105383139* mutants using CRISPR/Cas9 system

CRISPR/Cas9 genome editing

Two single guide RNAs (sgRNAs) were designed to the *P. xylostella* gene *LOC105383139* via the CRISPdirect online tool (http://crispr.dbcls.jp) based on the N_{20}NGG rule. After searching against the *P. xylostella* genome, we selected two sgRNAs with the lowest probability of off-target effects: sgRNA1: 5'-GGAGGTGAGTTTGCCGGCGGTGG-3' and sgRNA2: 5'-GGCGACCACGTCTACTT

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CTCCGG-3'. Note that 3' end TGG for sgRNA1 and 3' end CGG for sgRNA2 are the protospacer adjacent motif (PAM) sequences. DNA templates used for in vitro sgRNA synthesis were amplified with forward primers (Px139-sg1-F: 5' TAATACGACTCAC TATAGGAGGTGAGTTTGCCGGCGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCC-3'; Px139-sg2-F: 5'-TAATAC GACTCACTATAGGCGACCACGTCTACTTCTCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCC-3') and the common reverse primer (sgRNA-R: 5'-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTC-TAGCTCTAAAA-3'), respectively. PCR products were purified using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa) and then transcribed using T7 High Yield RNA Transcription Kit (Vazyme) according to the manufacturer's protocol. For preparing injection solution, a mixture of sgRNA1 (500 ng/μl), sgRNA2 (500 ng/μl) and Cas9 protein (500 ng/μl, GenCrispr NLS-cas9-NLS nuclease, GenScript) was incubated at 37 °C for 10 min to form a stable sgRNAs/Cas9 complex. Given concentrations are at final volume of the injection solution.

Fresh eggs collected within 1 h post oviposition were injected with the sgRNAs/Cas9 solution using a FemtoJet 4i and an InjectMan 4 microinjection system (Eppendorf). The injected eggs were immediately returned to normal rearing conditions and were allowed to develop to adult as the initial generation (G0). Then, a serial crossing scheme was designed to establish a stable homozygous mutant strain of *LOC105383139* gene. Briefly, the virgin G0 adults were mated with virgin wild-type (WT) adults in single pairs to produce the G1 progeny. After that, the genomic DNA was extracted from G0 individuals using the FastPure Cell/Tissue DNA Isolation Mini Kit (Vazyme). PCR was performed to amplify the region containing the sgRNA target sites with the genomic DNA from a plucked leg in adult moth, and the generated PCR products were sequenced to examine the mutation. After genotyping, we focused on G1 progeny derived from mutated G0. Retained G1 siblings were crossed in single pairs to generate G2 progeny. Then, single-pair crosses between G2 siblings were performed and kept only G3 progeny from homozygous mutant G2 parents by PCR-based genotyping to establish the *LOC105383139* knockout strain (MT-139).

Evaluation of reproductive success

Number of eggs

For the egg-laying assay, four different treatments of *P. xylostella* were set up: 15 pairs of emerged WT female and male adults (WT mated group), 15 pairs of emerged MT-139 female and male adults (MT-139 mated group), 15 emerged WT female adults (WT virgin group), and 15 emerged MT-139 female adults (MT-139 virgin group). Each pair or single female was separately kept in a plastic box. 24 hours later, the *P. xylostella* females were moved to a new box with a parafilm sheet containing the cabbage leaf extract for egg laying. The *P. xylostella* females were allowed to lay eggs for 48 hours, and the number of eggs was recorded individually. 10% honey solution was provided for nutrition.

Development of eggs

To investigate the development of the eggs laid by the above different groups of *P. xylostella* females, the percent of eggs terminated at four stages were carefully monitored. The four stages are: 0-10 h (Stage I, no gastrulation), 30 h (Stage II, eye spot is visible), 60 h (Stage III, head capsule is visible), and 70 h (Stage IV, hatching). The morphological characteristics of the developing *P. xylostella* eggs were photographed by digital microscope SZX2-ILLT (OLYMPUS).

Behavioral experiments

We conducted four treatments: wild-type males (WT♂) + wild-type females (WT♀), wild-type males (WT♂) + knockout females (MT-139♀), knockout males (MT-139♂) + knockout females (MT-139♀), and knockout males (MT-139♂) + wild-type females (WT♀). Each treatment had three replicates using 24 pairs of male and female moths. We sampled adults that were one day old after emergence because by this stage males are mature and frequently court females. For each individual pair no-choice assay, one 1-day-old male adult and one 1-day-old female adult were loaded into round chambers (diameter: 1.6 cm; height: 1.6 cm). The behavioral assays were performed at 25 ± 1 °C and 65 ± 5% relative humidity under the full spectrum LED light (400 lux); the assays started at 10am of 4 January 2022 and were recorded by digital video camera (FDR-AX700, SONY) for 48 consecutive hours of constant light. For a given time period, courtship index is the percentage of successfully courted pairs, in which the male moves toward the female with flapping wings and tipping the abdomen (Xu et al., 2020). Mating index is the percentage of successfully mated pairs in which the male copulates with the female for approximately one hour (Song et al., 2014).

Quantitative real-time PCR

P. xylostella from different stages of development were sampled, including egg, L1 (day 1-2 larvae), L2 (day 3 larvae), L3 (day 4 larvae), L4E (day 5 larvae), L4L-M (day 7 male larvae), L4L-F (day 7 female larvae), PE-M (day 1-2 male pupae; early pupal stage), PE-F (day 1-2 female pupae; early pupal stage), PL-M (day 4-5 male pupae; late pupal stage), PL-F (day 4-5 female pupae; late pupal stage), A-M (day 1-2 male adults) and A-F (day 1-2 female adults). In addition to 13 different stages, we also sampled five different tissues (antennae, head, thorax, abdomen, and reproductive system) in 1-day-old male adults. Total RNA was extracted using the FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme) and then reverse transcribed into cDNA using HiScript III RT SuperMix for qPCR (Vazyme) according to the manufacturer's protocol. qRT-PCR was performed in the AriaMx real-time PCR system (Agilent Technologies) with the ChamQ SYBR qPCR Master Mix Kit (Vazyme). Reactions were carried out for 30s at 95°C, followed by 45 cycles of three-step PCR for 10s at 95 °C, 20 s at 55°C, and 20s at 72°C. The RNA levels of the target gene *LOC105383139* were normalized to that of tubulin mRNA, and the relative concentration was determined using the $2^{-\Delta\Delta Ct}$ method.

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Transcriptome data

RNA sequencing

P. xylostella total RNA was isolated from the whole body from 1-day-old MT-139 male adult, MT-139 female adult, WT male adult, and WT female adult using FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme), and the residual DNA was removed according to the manufacturer's protocol. Each treatment had three replicates using 10 moths. For RNA-seq data, library construction and sequencing were performed on an Illumina HiSeq2000 (pair ends).

Transcriptome analysis

Raw RNA-seq reads were removed of low-quality reads and adapter sequences using Trimmomatic v0.39 (Bolger et al., 2014) with default parameters. Clean reads were mapped to the reference *P. xylostella* genome using STAR v2.7.6a (Dobin et al., 2013). The reads numbers mapped to each gene was counted by featureCounts v1.6.0 (Liao et al., 2014) and the resulting transcript count tables were subjected using R packages edgeR v3.360 (Robinson et al., 2010) and limma v3.50.0 (Ritchie et al., 2015) for differential expression analysis. Transcripts with an adjusted P value of $\leq$ 0.05 and $\log_2$ fold change of $\geq$1 or $\leq$ -1 were determined as differentially expressed genes. Gene Ontology (GO) enrichment analysis of differentially under- or over-expressed genes was conducted using Metascape v3.5 (https://metascape.org/) (Zhou et al., 2019) in knockout male vs wild-type male and knockout female vs wild-type female, respectively.

The role of the gene *LOC105383139* in butterflies

To investigate the role of the gene *LOC105383139* in butterflies, two closely related *Heliconius* butterflies (*H. melpomene* and *H. cydno*) were examined (Merrill et al., 2019; Rossi et al., 2020). First, we counted the number of male courting episodes toward females in five trials (each trial lasted 15 minutes) for *H. melpomene* and *H. cydno*, respectively. The publicly available courtship data were retrieved from Merrill et al. (Merrill et al., 2019). Second, we calculated the expression level of the homolog of the gene *LOC105383139* in *H. melpomene* and *H. cydno* males using the publicly available transcriptomic data (Rossi et al., 2020), which were mapped to the *H. melpomene* and *H. cydno* genomes in Lepbase (http://lepbase.org/) (Challis et al., 2016), respectively. These publicly available transcriptomic data were generated in 2019 from the tissues (eye and brain) from 10-day-old male adults. By this stage males are mature and frequently court females.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of assessment of genome assemblies

We used the Benchmarking Universal Single-Copy Orthologs (BUSCO), version 5.2.2 (Manni et al., 2021) to assess the quality of each of the 218 insect genome assemblies. Each assembly's completeness was assessed based on the presence / absence of a set of 1,367 predefined orthologs that are single-copy in at least 90% of the 75 reference insect genomes in OrthoDB v10 (Kriventseva et al., 2019; Waterhouse et al., 2013). Further details of these analyses are provided in STAR Methods.

Analysis of developmental and behavioral phenotypes

We used the Wilcoxon rank-sum test in R v. 3.6.3 (R Core Team, 2021) to test whether the sets of values in two groups (knockout vs wild-type) are significantly different (NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Data are shown as the mean ± SD. Further details of number of samples and replicates are provided in STAR Methods.

Supplemental figures

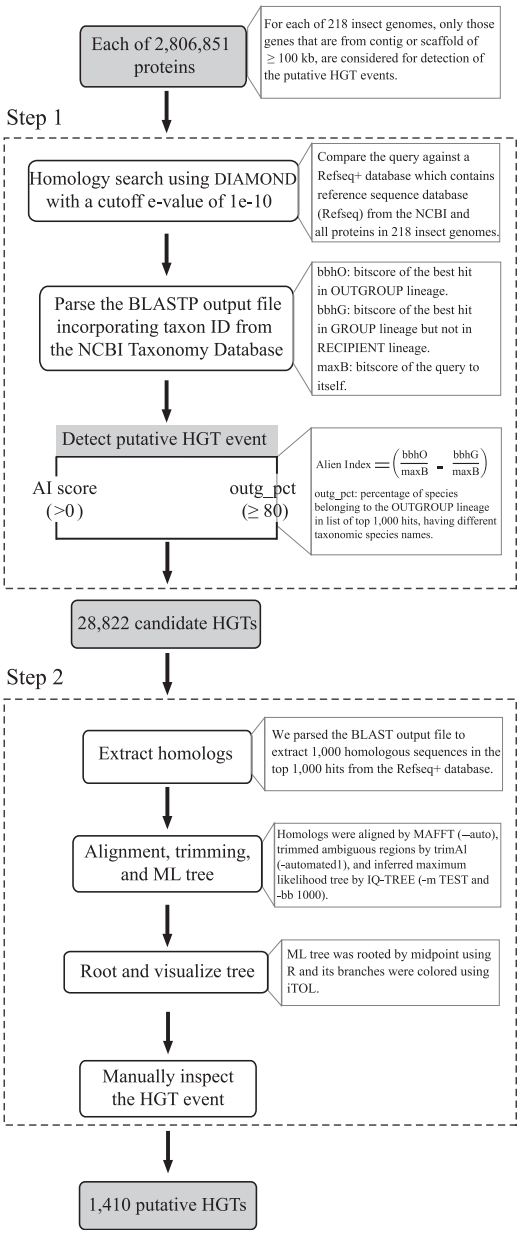

Figure S1. The workflow used for the identification of genes found in insects that were likely acquired by horizontal gene transfer (HGT) from non-metazoan species, related to Figure 1

A detailed description of the analyses performed in each step of the workflow is provided in the "identification of HGTs into insects" section of the STAR Methods. RECIPIENT: insects, GROUP: other metazoans, OUTGROUP: non-metazoan species.

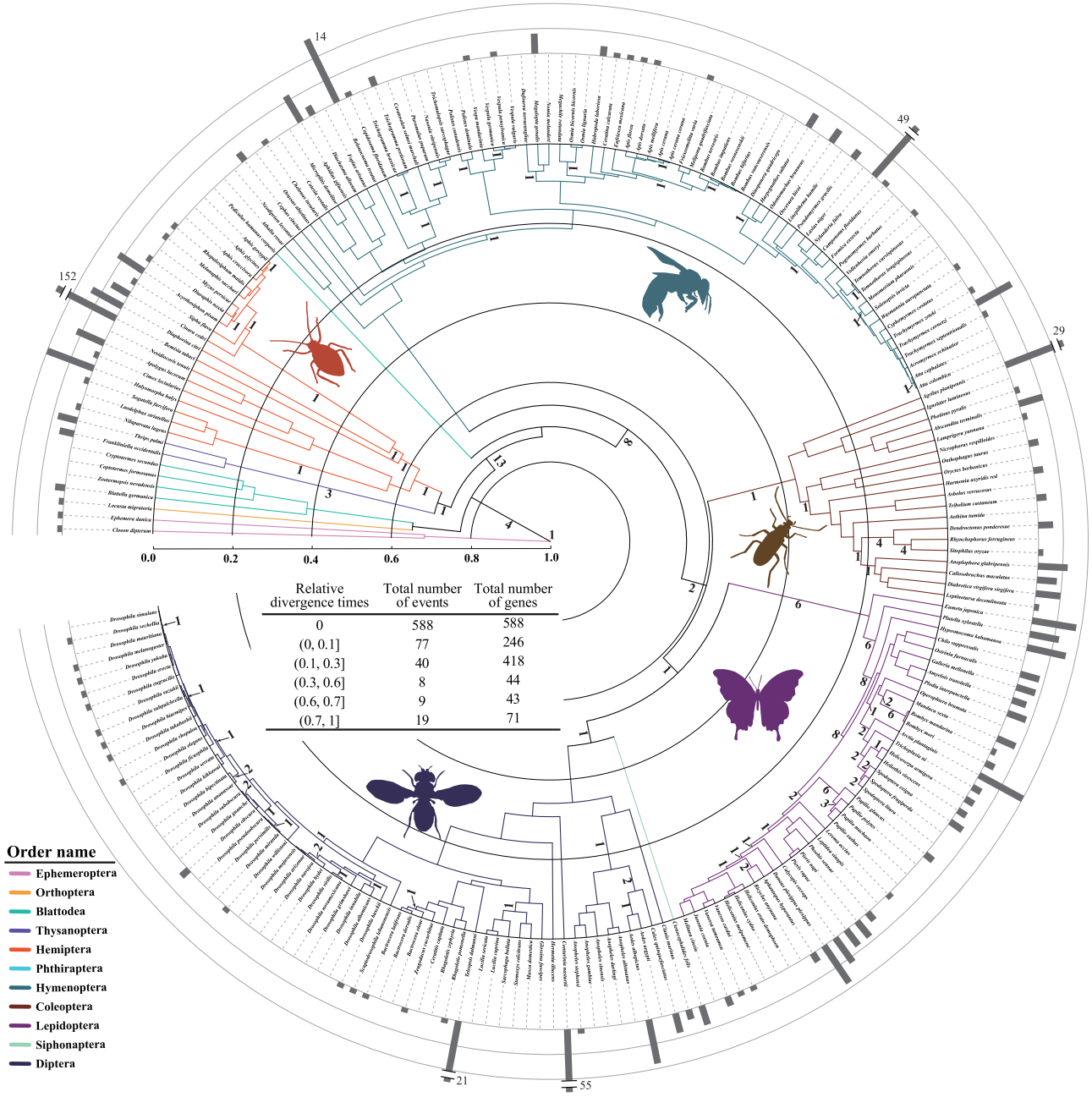

Figure S2. Distribution of 741 HGT events on the insect phylogeny, related to Figure 1

Examination of the phylogenetic trees of the 1,410 HGT-acquired genes showed that they stem from 741 distinct transfer events. 588 of these transfer events appear to be species-specific, whereas the remaining 153 are inferred to have occurred in the common ancestor of two or more species included in our study. Bars next to species names denote numbers of species-specific HGT events. Numbers near internodes denote numbers of HGT events that led to HGT-acquired genes found in two or more species. The RelTime algorithm employed in the command line version of MEGA7 was used to infer the relative divergence times. Note that detained numbers of HGT events given a range of the relative divergence times are given in the inset in the middle of the timetree.

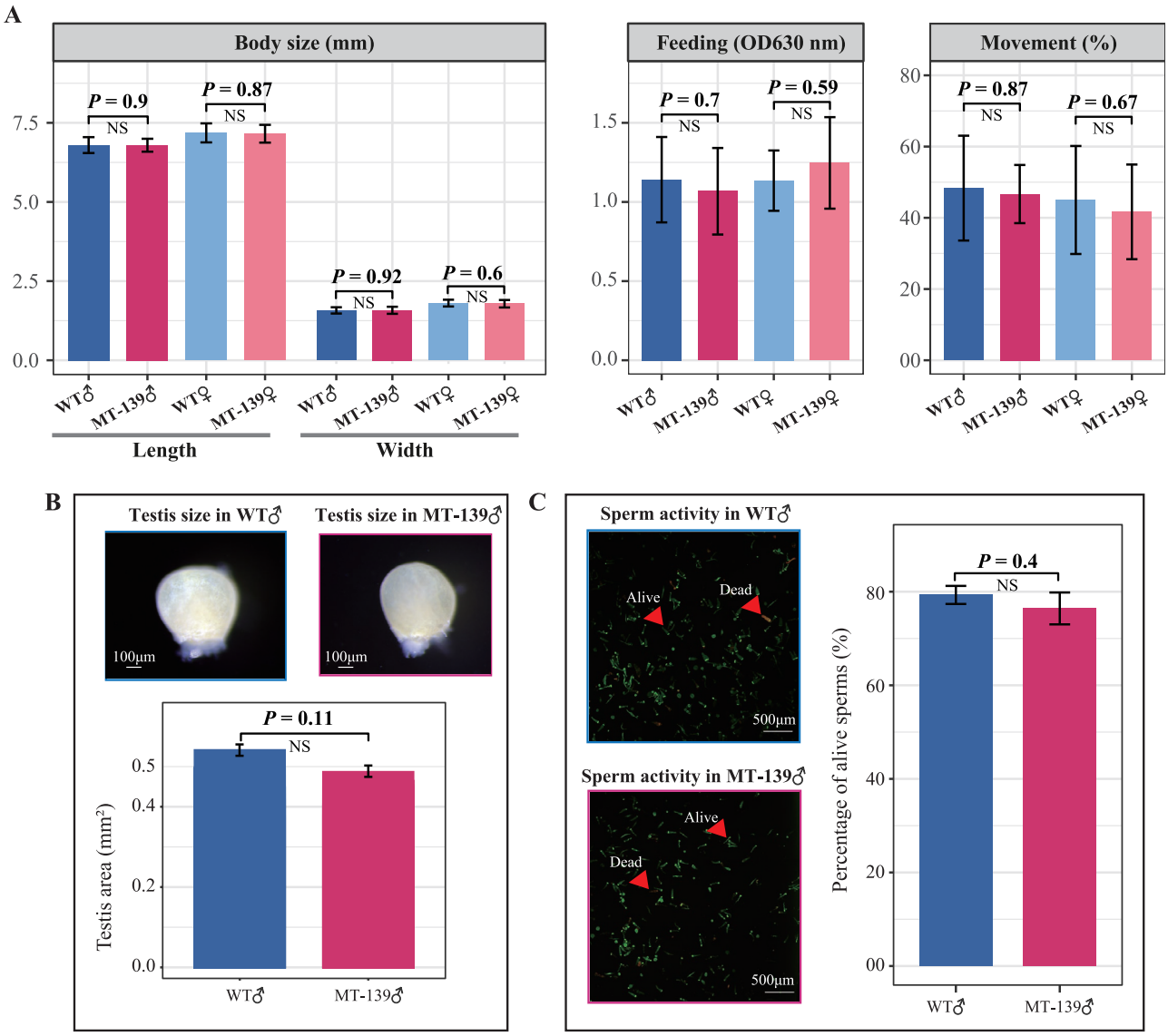

Figure S3. Comparisons of developmental phenotypes between WT and MT-139 *Plutella xylostella* diamondback moths, related to Figure 5

(A) Body size (length and width), feeding (measured by the concentration of brilliant blue in extracts from abdomens of five moths after feeding with the mixture of brilliant blue and honey water), and movement (measured by percentage of 10 moths climbing over half a bottle [height: 20 cm; diameter 2 cm] in 5 min) were examined in wild-type (WT) and knockout (MT-139) male and female adults.

(B) Testis size (testis area) in wild-type (WT) and knockout (MT-139) male adults.

(C) Percentage of alive sperms in wild-type (WT) and knockout (MT-139) sperm bundles. GFP (SYBR 14 dye) and RFP (propidium iodide) were used to transfect sperms (LIVE/DEAD Sperm Viability Kit). Red arrows indicate examples of sperms that were alive or dead. Number of replicates for examining body size is 15; number of replicates for examining feeding and movement is 6; number of replicates for examining testis size and sperm activity is 3. All diamondback moths that were used to examine the developmental phenotypes were 1 day old after emergence. Each bar denotes mean value with standard deviation. The Wilcoxon rank-sum test was used to test whether the sets of values in two groups are significantly different (NS, $p > 0.05$). Our results show wild-type (WT) and knockout (MT-139) diamondback moths had no significant differences in any of the developmental phenotypes examined.

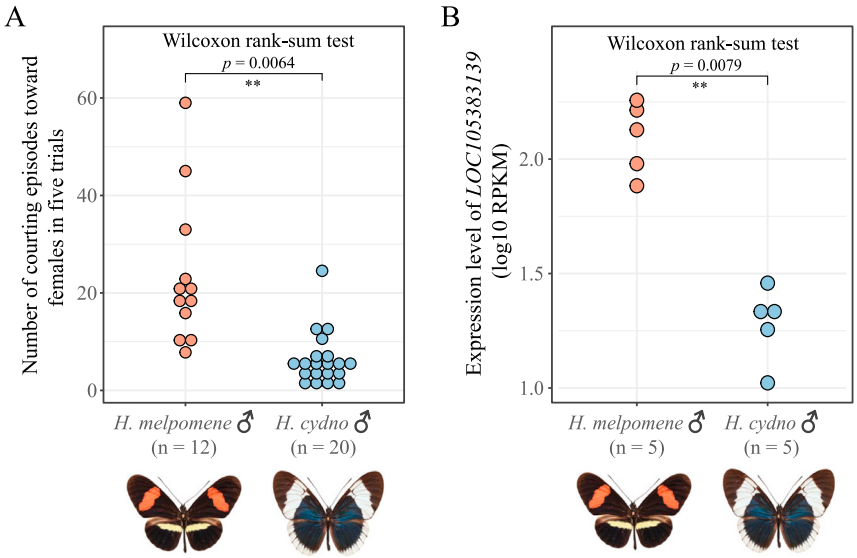

Figure S4. The horizontally acquired gene *LOC105383139* might be involved in male courtship behavior in butterflies, related to Figure 5

(A) Two closely related *Heliconius* male butterflies (*H. melpomene* and *H. cydno*) were used to examine the number of courting episodes toward females in five trials (each trial lasted 15 min), respectively. The publicly available courtship data were retrieved from Merrill et al. (2019).

(B) The expression level of the horizontally acquired gene *LOC105383139* in *H. melpomene* and *H. cydno* males during courting. The publicly available transcriptome datasets were generated in 2019 from the combined tissues (eye and brain) from 10-day-old male adults because by this stage males are mature and frequently court females (Rossi et al., 2020). These data suggest that *H. melpomene* males had a significantly higher number of courting episodes toward females than *H. cydno* males as well as higher expression levels of the gene *LOC105383139* than *H. cydno* males. These results are consistent with our hypothesis that the foreign gene *LOC105383139* might be involved in male courtship behavior in lepidopterans.

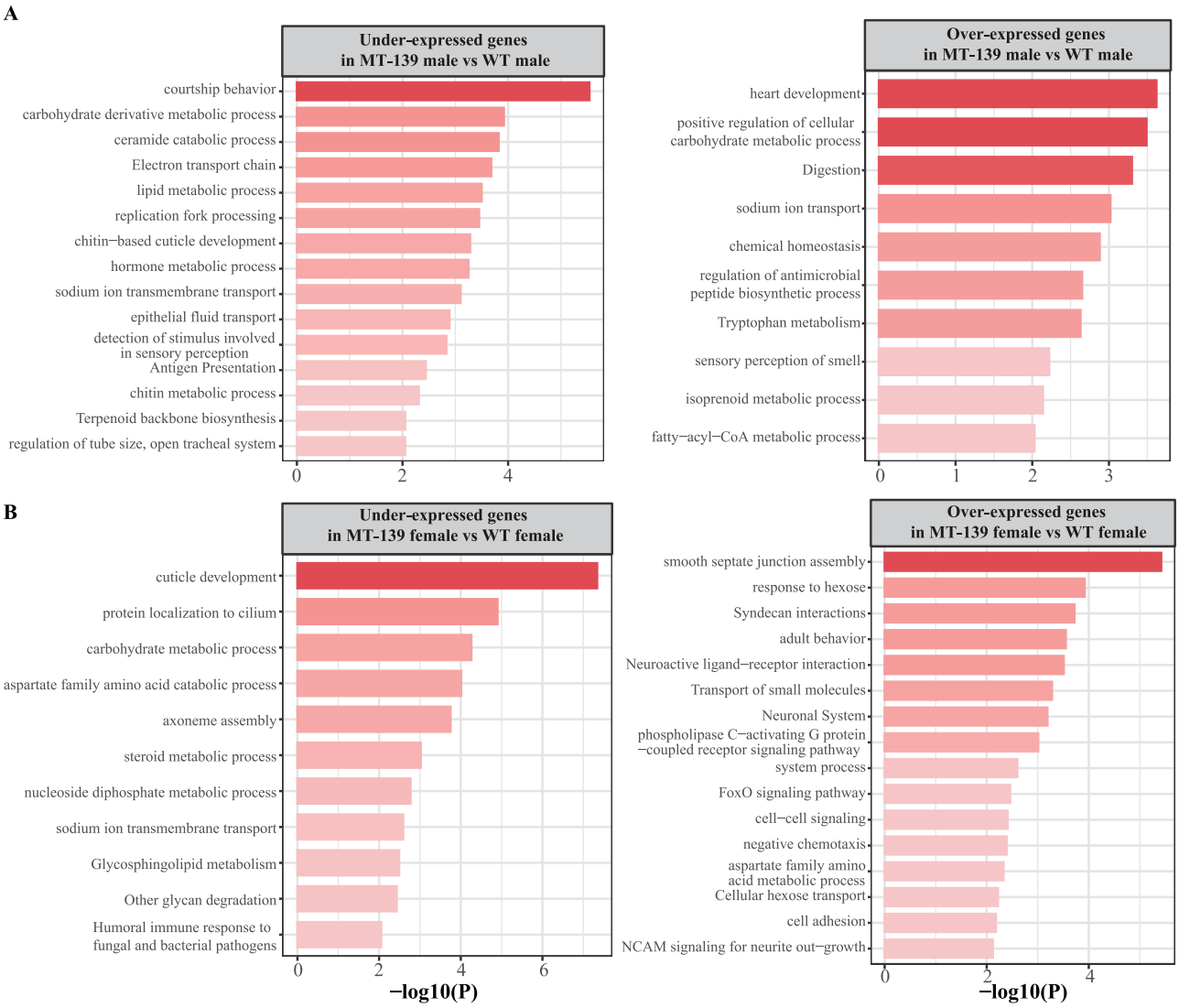

Figure S5. Functional analysis of differentially expressed genes in MT-139 and WT diamondback moths, related to Figure 5

(A and B) GO term enrichment analysis of genes differentially expressed in knockout (MT-139) male (A) and female (B) relative to wild-type (WT) male and female. Statistically overrepresented GO categories in under-expressed and over-expressed gene sets in MT-139 versus WT are shown in left panel and right panel, respectively. $-\text{Log}_{10}(p)$ is the p value in −log base 10. Bar graph of enriched terms across input gene lists, colored by p values. The transcriptome data were generated from the whole bodies of ten 1-day-old wild-type male adults (WT male), ten 1-day-old wild-type female adults (WT female), ten 1-day-old knockout male adults (MT-139 male), and ten 1-day-old knockout female adults (MT-139 female), respectively.