Insects use sex pheromones as a reproductive isolating mechanism to attract conspecifics and repel heterospecifics. Despite the profound knowledge of sex pheromones, little is known about the coevolutionary mechanisms and constraints on their production and detection. Using whole-genome sequences to infer the kinship among 99 drosophilids, we investigate how phylogenetic and chemical traits have interacted at a wide evolutionary timescale. Through a series of chemical synthesizes and electrophysiological recordings, we identify 52 sex-specific compounds, many of which are detected via olfaction. Behavioral analyses reveal that many of the 43 male-specific compounds are transferred to the female during copulation and mediate female receptivity and/or male courtship inhibition. Measurement of phylogenetic signals demonstrates that sex pheromones and their cognate olfactory channels evolve rapidly and independently over evolutionary time to guarantee efficient intra- and interspecific communication systems. Our results show how sexual isolation barriers between species can be reinforced by species-specific olfactory signals.
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rganisms communicate with each other through exchanging signals that include visual, acoustic, tactile, and chemical (smell and taste) senses. The chemical sense is common in all organisms, from bacteria to mammals, and therefore, regarded from an evolutionary perspective as the oldest one. Animals are surrounded by a world full of odors emitted from conspecific or heterospecific individuals, as well as from the environment. The ability to exchange and decipher these signals has a significant impact on a species’ success as odors help to avoid imminent threats and localize and judge food or potential mates. Olfactory systems have, therefore, evolved in a sophisticated way to meet the challenge of detecting and discriminating a countless number of odorants. While it is well established how animals use odors for intra- and interspecific communication, the evolution of olfactory systems with respect to signal production and perception is poorly understood.

One of the most crucial channels that have been suggested to contribute to speciation is the sex pheromone-sensing channels. Volatile sex pheromones—airborne chemicals that stimulate sexual behaviors in the opposite sex—are the primary signals that reinforce the isolation barriers between different species. These species-specific signals often provide a full biography written in scent molecules about the sender, such as information about the reproductive and internal status. Diversification of sex pheromones among species arises via sexual, and/or natural selection. Closely related species tend to use different pheromone blends of shared chemical compounds as a result of genetic similarities and biosynthetic pathways shared by ancestry. This diversity in sex pheromone communication can become further affected by factors like geographical or host variations. For example, sympatric species develop pronounced divergent communication systems to overcome the risk of hybridization, while the unimpeded divergence due to geographic barriers may lead to relaxed accumulation of differences. Moreover, colonization of a different host plant—an ecological adaptation—could also lead to differential sex pheromones and new ways of signal transmission and perception. Although many studies have reported the diversity of sex pheromones among related species, the evolutionary phylogenetic history of these traits and their detection systems remains obscure.

Flies, like most animals, rely on chemical cues to locate and choose an appropriate mating partner. For several reasons, flies within the genus Drosophila represent ideal species to study the evolution and diversity of sex pheromones, as well as their associated behaviors. First, Drosophila species live in an extensive range of diverse habitats across all climatic conditions, from deserts and caves to mountains and forests. In these environments, drosophilids feed and breed on varied hosts such as decaying fruits, slime fluxes, mushrooms, flowers, as well as frog spawn. Second, sexual behaviors of drosophilids feed and breed on varied hosts such as decaying fruits, slime fluxes, mushrooms, flowers, as well as frog spawn. Third, the neural processing of pheromones in the brain of some drosophilids, especially D. melanogaster, is largely understood. Fourth, pheromone receptors are narrowly tuned to fly odors and expected to evolve at fast rates to match the dramatic diversity of pheromones among closely related species. Lastly, out of the 52 classes of olfactory sensory neurons (OSNs) in D. melanogaster, only four respond to fly odors and are localized in a specific sensillum type. Hence, the restricted number of orthologues, that are expressed in an easily identifiable and accessible sensillum type, represent promising candidates to study the coevolution of Drosophila pheromones and their corresponding odorant receptors (Or).

Olfactory sexual communication in D. melanogaster is arguably one of the best-studied systems in animals, and is carried out through limited chemical signals, including cis-vaccenyl acetate (cVA). This compound is produced exclusively by males and transferred to females during copulation, which then reduces the attractiveness of the freshly mated females. Moreover, cVA regulates multiple behaviors: it induces sexual receptivity in virgin females, elicits aggression in males, modulates oviposition behaviors, and acts as aggregation pheromone in presence of food. Despite the profound knowledge of cVA-induced behaviors in D. melanogaster, little is known about analogous stimuli that regulate social and sexual behaviors in other drosophilids.

Here, we identify the sex pheromones and their roles in 99 species within the family Drosophilidae, explore the evolution of pheromone signaling systems with respect to phylogenetic relationships, and highlight how sexual isolation barriers between species are reinforced by olfactory signals.

Results

Whole-genome information-based phylogeny of 99 drosophilids. The genus Drosophila is arguably one of the most extensively studied model systems in evolutionary biology. The phylogenetic relationships among drosophilids have suffered from low supports. We, therefore, investigated the relationships of 99 species within the family Drosophilidae, explore the evolution of pheromone signaling systems with respect to phylogenetic relationships, and highlight how sexual isolation barriers between species are reinforced by olfactory signals.
high correlation coefficients to chemical profiles of other male species of their own group, but negative correlation coefficients to chemical profiles of males of different groups (i.e., blue cells are frequently present around the diagonal) (Fig. 1c). However, female species generally display high correlation coefficients (>0.75) randomly to each other apart from their phylogenetic relationships (Fig. 1c'). Likewise, measurement of the phylogenetic signal using Pagel’s λ (a measurement of the statistical dependence among species’ trait values due to their phylogenetic relationships) revealed that males of related species tend to chemically resemble each other more than females \( (p = 0.006) \) (Supplementary Fig. 1C; Supplementary Data 2). Together, our data reveal that male chemical profiles exhibit a stronger phylogenetic signal than female chemical profiles.
Previously unidentified potential sex pheromones underwent rapid evolution. In drosophilid sex-specific compounds typically serve as short-range communication signals that induce or inhibit sexual behaviors\(^{23}\). For example, in the *mojavensis* complex, (Z)-10-heptadecen-2-yl acetate, the male-specific sex pheromone, is detected by all populations, but only induces female receptivity in the populations that produce it\(^{22}\). Similarly, in the *melanogaster* group, 7,11-heptacosadiene, a female-specific compound, induces male courtship in the producing species, but serves as an isolation barrier for the closely related non-producing species\(^{44-46}\). In search for analogous compounds all along the *Drosophila* genus, we analyzed the chemical profiles of the 99 species and compared the chromatograms of both sexes within each species (Fig. 2a). Males and females of only 18 species exhibited sexually monomorphic chemical profiles (i.e., same compounds were found in both sexes, regardless of differences in the compounds’ quantity), while 81 species exhibited sexually dimorphic cuticular chemicals (a dimorphic chemical is identified as a compound that is present only in one sex) (Fig. 2b). All the 81 dimorphic species unveiled male-specific compounds (in total 43 compounds), while only 15 species exhibited female-specific ones (in total 9 compounds) (Fig. 2b; Supplementary Fig. 2A, B). Of note, most of the female-specific compounds, are long-chain unsaturated hydrocarbons (Supplementary Fig. 2A), display high boiling temperature (Supplementary Data 3), and hence are likely to be non-volatile\(^{44}\). However, male-specific compounds range between 10 to 32 carbon atoms, and belong to different chemical classes such as esters, ketones, and alkenes, as well as ether and alcohol (Fig. 2c; Supplementary Data 3). Notably, when analyzing the chemical profiles of freshly mated females, we found that many of the male-specific compounds were transferred to females during mating (green cells in Fig. 2b), reminiscent of the transfer of male-specific compounds in *D. melanogaster* and *D. mojavensis*\(^{22,47,48}\). On the contrary, none of the female-specific compounds was transferred to males during mating (Supplementary Fig. 2B).

Many of the male-specific chemicals exhibited low phyl genetic signals, and thus often are not conserved among closely related species, but sometimes present across distant species (Supplementary Fig. 2C; Supplementary Data 2). Indeed, mapping the sex-specific compounds onto the phylogenetic tree revealed that many distant species use the same male-specific compounds (Fig. 2b). However, few compounds are exclusively species- or group-specific compounds (Fig. 2b; Supplementary Fig. 2B). For example, several male-specific compounds, including cVA that has been thought to be restricted to the melanogaster and the immigrans groups\(^{45}\), are present across several species in different groups in both subgenera Sophophora and Drosophila, while only methyl myristoleate is specific for the willistoni group (Fig. 2b). Similarly, consistent with the rapid evolution of pheromone-producing enzymes in drosophilid females\(^{49}\), 7,11-heptacosadiene and 7,11-nonacosadiene, the female-specific compounds in *D. melanogaster*\(^{23,45}\), are not restricted to a specific group (Supplementary Fig. 2B). This pattern supports the presence of strong selection on the sex-specific compounds to evolve fast and deviate from expectations based on stabilizing selection. In addition, our analyses revealed that 58 of the 81 dimorphic species have a blend of multiple male-specific compounds that could reach up to seven compounds, as in *D. mercatorum*, while the other 23 species employ single male-specific compounds (Fig. 2b). Overall, we identified 52 potential sex pheromones (Supplementary Data 3), which seem to evolve independently from phylogenetic constraints across drosophilids.

Drosophilids communicate intra- and inter-specifically through rapidly evolving olfactory channels. The volatility (i.e., low boiling points due to their shorter chain length compared to female-specific compounds; Supplementary Data 3) of most male-specific compounds suggests that they could be potential olfactory signals. We, therefore, screened for OSNs that detect male-specific compounds in *Drosophila* species via single sensillum recordings (SSR). We focused our attention on 54 species—49 dimorphic and 5 monomorphic species—because they could be successfully reared on artificial food under our lab conditions. In *D. melanogaster*, olfactory sex pheromone-responsive neurons are localized in antennal trichoid (at) sensilla, which are morphologically distinct from other sensillum types and belong to two classes (at1 and at4) that are known to be located on different antennal regions\(^{30}\). The at1 sensillum houses a single neuron (Or67d) that responds to cVA\(^{32}\), while at4 houses 3 neurons (Or47b, Or65a/b/c, and Or88a) that respond to methyl laurate, cVA, and methyl palmitate, respectively\(^{24,51,52}\). Indeed, we found the at1-like and at4-like sensillum classes in all tested species except *D. pseudothepa* and *D. robusta*, whose at1-like sensilla could not be identified (Supplementary Fig. 3A; for identification, see Methods). We next recorded the responses of both trichoid sensillum classes in the females of 54 species to an array of chemicals (Fig. 3a), which includes 28 male-specific compounds and 8 compounds that were previously described as
drosophilid sex pheromones (Supplementary Data 4). The electrophysiological recording revealed that females of 36 of 49 dimorphic species detect their conspecific males’ compounds (Fig. 2b) by olfactory neurons (Fig. 3b, c). Of note, flies are also able to detect many male-specific compounds of other species (Fig. 3b; Supplementary Fig. 3A, B’; Supplementary Data 5). One should note here, that our analysis focuses on the a1 and a4 sensilla that have been shown to be involved in the detection of volatile pheromones in D. melanogaster and close relatives, as well as in D. mojavensis. We, however, cannot exclude that further compounds are detected by other olfactory or even gustatory sensilla types. To analyze the olfactory-based interactions between the different species, we performed network analyses, which revealed a higher olfactory

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**Diagram Description**

The diagram (Fig. 3c) illustrates the detection pattern of various Drosophila species for different compounds. The green squares represent the detection of a compound, while the red squares indicate that the compound was not detected. The numbers next to each compound refer to the compound's identification number in the list provided. The diagram is divided into two main sections: the male-specific compounds and the olfactory or gustatory sensilla types. The compounds detected by each species are color-coded, with each compound represented by a square. The detection pattern is shown for 36 of 49 dimorphic species, indicating whether they are able to detect a compound (green) or not (red).
clustering coefficient (i.e., the number of olfactory interactions between the species divided by the number of interactions that could possibly exist) of interspecific interactions through at1-like (for identification, see “Methods”) compared to at4-like sensilla (Fig. 3d; Supplementary Fig. 3B, B’). However, self-loops, which signify the number of the intraspecific olfactory interactions (i.e., the ability of females to detect their conspecific male compounds), are comparable through at1-like and at4-like sensilla (Supplementary Fig. 3B, B’). Pairwise correlation and statistical analyses revealed that electrophysiological responses at1 and at4 neurons of the different species have low phylogenetic signals (Fig. 3e and Supplementary Fig. 3C).

We further asked whether pheromone receptors in drosophilids have evolved under positive selection. We, therefore, queried the genomic data for the orthologs of the known pheromone receptors in drosophilid flies. Of these receptors, we found in our WGS data 42, 41, and 36 orthologs of Or47b, Or67d, and Or88a, respectively, which displayed full-length sequences. We next assessed the selection pressures on these genes by computing the ratio of nonsynonymous (dN) to synonymous (dS) substitutions across the whole gene (see Methods). Statistical analyses revealed evidence of positive selection on all tested pheromone receptors with the highest pressures on the Or47b and the Or67d loci (Or47b locus, p-value < 0.0001; Or67d locus, p-value < 0.0001; Or88a locus, p-value = 0.014), indicating that pheromone receptors of the different species evolve rapidly apart from their phylogenetic relationships.

Lastly, using two different model-tuning criteria, we performed a phylogenetically corrected correlation between the evolution of male chemical phenotypes and the associated females’ olfactory responses of different species and between the closely related species. Despite the high intraspecific match—females of 36 out of 49 species detect their males’ compounds (Fig. 3c), the evolution of females’ responses among closely related species (limited to findings pertaining to at1 and at4 responses) does not correlate with the evolution of their male-specific compounds (Supplementary Data 6, 8–16). This implies the presence of a low interspecific correlation between detection and production. Indeed, for example, females of 19 out of 20 species, whose males produce cVA, detect this compound (i.e., cVA functions as a conspecific signal), while females of 21 out of 34 species are still able to detect cVA (Fig. 3b), although their males do not produce it (i.e., cVA functions as a heterospecific signal).

Male-specific compounds regulate intra and interspecific sexual behaviors. To examine the intra- and interspecific behaviors governed by the male-specific olfactory signals and to gain a better understanding of the courtship rituals of these 54 species, we recorded the sexual behaviors of conspecific couples in a single-pair courtship arena. Many species displayed different species-specific behaviors (Movies 1 to 427, available on https://doi.org/10.17617/3.5w; in total 1467 replicates, 16–48 replicates per species). For example, males of D. elegans and D. suzukii dance and spread their wings in front of females35; D. mojavensis and D. virilis males release fluidic droplets while courting the females22; D. subobscura males extend their proboscis to gift females with regurgitated drop of their gut contents19, and D. nannoptera couples tend to re-mate as many as two to three times within the recording time frame of 60 min56. We further quantified copulation success, latency, and duration (Supplementary Fig. 4A), which varied largely among different species. Unlike the prolonged copulation time in the species of the melanogaster group, copulation lasts for < 2 min in members of the repleta group (Supplementary Fig. 4A). Together, courtship recordings (available on https://doi.org/10.17617/3.5w) reveal numerous quantitative and qualitative differences in sexual behaviors among the Drosophila species.

We next focused on Drosophila species that detect their male-specific compounds via olfaction—36 out of 49 species (Figs. 3c, 4a)—and asked whether these compounds induce female receptivity. Drosophila females exhibit a preference to copulate with older males22,51,52, which mostly possess higher amounts of male-specific compound. Therefore, we hypothesized that males perfumed with single male-specific compounds would have a higher copulation advantage than the solvent (DCM) perfumed males, which carry the same compound but a lower amount of it. In a competition-mating assay, virgin females of each species were allowed to choose between two conspecific males perfumed with a male-specific compound (Fig. 4b) or solvent [consistency of perfuming and correspondence to biologically relevant amounts were confirmed by chemical analyses; see “Methods”]. In 11 instances, females displayed a preference to copulate with males perfumed with the male-specific compound over the control ones (Fig. 4b; Supplementary Data 7). However, females of six species avoided copulating with the males perfumed with the male-specific compound (Fig. 4b).

Notably, perfuming an additional amount of cVA on males of the melanogaster clade did not increase the males’ copulation success (Fig. 4b), indicating that the built-in amount of cVA in the control males is already sufficient for females’ acceptance. To assure that the high copulation success of perfumed males was not due to an increased intensity of male courtship38, we recorded their courtship activities. Courtship indices did not differ between perfumed and control males (Supplementary Fig. 4b), indicating that these compounds influence exclusively the females’ sexual decisions.

Many of these olfactory-detected male-specific compounds are transferred to females during copulation (Fig. 2b; 28 out of the
D. melanogaster guarding strategy [as described for transferred pheromones in these compounds contributes to a general post-copulation mate-species choice to court two headless females perfumed with a male-specific compound, and not detected by at1 detected by at4 detected by both. To distinguish male sexual behaviors from the female acceptance, males were offered the choice to court two headless females perfumed with a male-specific compound or solvent. We scored the first attempt to copulate with one of the rival females as a choice. However, we ensured that males do choose between perfumed females and not simply attempt copulation with the first female they court. Males in almost half of the tested species that exhibit male-transferred compounds displayed a preference to copulate with the solvent-perfumed females, including males of many species of the melanogaster group (Fig. 4c; Supplementary Data 7). Perfuming
with single male-specific compounds in numerous instances has no impact on the male courtship preference. This suggests that these single compounds either are not involved in the mating decision or work synergistically in combination with other compounds. On the contrary, males of *D. hydei* exhibited copulation preference for the perfumed females over the control ones, indicating that in different species male-transferred compounds can result in reverse effects. Together, male-specific compounds of 24 species regulate sexual behaviors via olfaction.

Lastly, we examined why females are still able to detect the chemical signals of heterospecific males and whether these heterospecific signals could act as reproduction isolation barriers. We focused our analysis on eVA due to its presence in many cosmopolitan species (*e.g.*, *D. melanogaster*, *D. simulans*, *D. funebris*, and *D. immigrans*) that have a high chance to meet other non-eVA-producing species (Fig. 4d). Females of *Drosophila* species, which are able to detect eVA as a heterospecific signal (i.e., their males do not produce it), had the choice to mate with two conspecific males perfumed with eVA or solvent. Each of these females detected eVA with OSNs that did not detect the compounds of their conspecific males (Fig. 3b). Notably, females’ preference for their conspecific males in 8 out of 13 species was significantly reduced by eVA (Fig. 4d; Supplementary Data 7). Indeed, perfuming males with eVA-like compounds—which activate the same sensillum type that eVA activate—resulted in comparable results (Supplementary Fig. 4C), suggesting that the activation of a eVA-responding neuron in this sensillum governs avoidance of heterospecific males. Overall, many male-specific compounds seem to regulate intra-sexual behaviors all along the *Drosophila* phylogeny and promote sexual isolation for heterospecific species.

**Discussion**

Sexual selection imposed by the coevolution of female preferences to particular male traits leads to rapid and dramatic evolutionary divergence and potentially contributes to speciation processes. Using whole-genome sequences of 99 drosophilid species, we investigated how phylogenetic constraints impact the evolution of cuticular hydrocarbons and potential sex pheromones per se. By linking the chemical variations and phylogenetic relationships on the one hand with the physiological responses and behavioral functions on the other, we provide large-scale evidence for the rapid coevolution of sex pheromone production and detection among drosophilid flies. The characterizations of sex pheromones, their cognate olfactory channels, and behavioral significances provide several insights into the evolution of chemical communication systems and their role in speciation.

In general, cuticular chemistry varies between closely related species in relation to their genetic relationships, geographical locations, and environmental factors. Environmental factors are thought to have a stronger impact on the evolution of cuticular chemicals than genetic relatedness. Our findings reveal that many of the male-specific compounds display low phylogenetic signals, i.e., less conserved in the closely related species) (Fig. 2b; Supplementary Fig. 2B, C), which might result in divergence of sexual communication among the sibling species. However, compared to females, males have significantly more chemicals with higher phylogenetic signals, i.e. there is a better correlation between genetic and chemical distance in males (Supplementary Fig. 1C). Notably, Consistent with our results, nonsexual chemical hydrocarbons in ants, aphids, ladybird beetles, moths, and drosophilids exhibit gradual evolution, while aggregation pheromones in beetles display saltational (i.e., sudden and large) shifts. By contrast, our observed saltational shifts in male-specific compounds contradict previous studies on the gradual mode of evolution of some of the sex pheromones in *Bactrocera* and some aggregation pheromones in *Drosophila*. We identified some of these previously identified aggregation pheromones as potential sex pheromones (Figs. 2b and 4b, c). The discrepancy of the mode of evolution of these sex pheromones could be explained due to binary encoding (i.e., presence or absence) of these traits among a limited number of species. The salutational changes of sexual signaling are likely to occur between closely related sympatric species to overcome the homogenizing effects of gene flow.
The high proportion—approximately 82%—of species that exhibit sexually dimorphic chemical profiles (Fig. 2b) indicates the significance of chemical communication in the genus Drosophila. This sexual dimorphism seems to positively correlate with flies’ inability to mate under low light conditions, while many of the chemically monomorphic species cannot mate in the dark. The latter often display sexually dimorphic color patterns, implying that they rely on visual cues during sexual communication. Of note, many of the sex-specific compounds exist across many species in different groups, indicating that the modification of a sexual chemical trait has occurred independently multiple times during the evolution of drosophilid flies (Fig. 2b; Supplementary Fig. 2B). For example, cVA, (Z)-11-eicosen-1-yl acetate, and palmityl acetate are present in 34, 13, and 12 species that belong to...
Females of more than 75% of the dimorphic species are able to the evolution of sex pheromones in conspecific cuticular hydrocarbons are modulated more easily by lab-induced that pheromone-responsive olfactory channels of at1 and females are less receptive to oe host selection and principally occurred to match chemical divergence associated among the 54 species. Note that in 11 instances, females displayed a preference to copulate with the male-specific compound-perfumed males over the control ones, while 6 compounds resulted in avoidance, and 29 turned out to be neutral. See Supplementary Data 7 for raw data and statistical analyses.

See Supplementary Fig. 4B for the effect of perfuming on the males’ courtship behavior. Drawings made by Mohammed A. Khalaf. c Top: Competition courtship arenas where a male of each species had the choice to court two decapitated conspecific females perfumed with the male-transferred compound. Note that we tested only transformed compounds (green). Below: bar plots represent the percentage of the first copulation attempts towards perfumed and control females. Results from males that only mated one female were excluded; see Methods. Note that 15 compounds inhibited courtship, 1 compound increased courtship and 16 compounds turned out to be neutral. Drawings made by Mohammed A. Khalaf. d Top: Schematic of a mating arena where a female of each species had the choice to mate with two conspecific males perfumed with olfactory-detected heterospecific cVA or solvent (DCM). Note that we only tested the species that do not produce but still detect cVA. Below: bar plots represent the percentages of copulation success of the rival males. Results from females that were only mated by one male were excluded. Drawings made by Mohammed A. Khalaf.

One key observation of our study is the diversity and abundance of male-specific compounds compared to female ones—43 compared to 9, respectively—across the dimorphic species (Fig. 2b; Supplementary Fig. 2B). Surprisingly, 81 dimorphic species exhibit male-specific compounds, while only 15 species have female-specific compounds (Fig. 2b; Supplementary Fig. 2B). This could be attributed to the fact that drosophilid females are regarded as “the choosy sex”, which rely on volatile male sex pheromones to find a high-quality conspecific male22,36,53,81 and to avoid costly interspecific mating22,32,82. Moreover, males are found to court heterospecific females in equal vigor as conspecific females22,45, even after learning the conspecific females’ chemical profiles85. Similarly, males exhibit a higher preference for females that exhibit no cuticular hydrocarbons (i.e., females lacking oenocytes (“oe−” females)) over wild-type females, while females are less receptive to oe− males86. Furthermore, male cuticular hydrocarbons are modulated more easily by lab-induced natural and sexual selection than female cuticular hydrocarbons87. All these reasons, aside from the females’ strong preferences for male sexual traits, seem to have resulted in stronger selection pressures on the cuticular hydrocarbons of drosophilid males.

To match the diverse male chemical traits, females are expected to coevolve cognate sensory detection systems to permit mate recognition1. Drosophilid chemoreceptor genes evolve rapidly88 and single point mutations can result in species-specific variance of receptor tuning89. Such specificity has been shown to be not random and principally occurred to match chemical divergence associated to host selection88–92 or mate recognition93. Similarly, we found that pheromone-responsive olfactory channels of at1 and at4 sensilla evolve high selectivity that permits an extreme fit to the evolution of sex pheromones in conspecific partners (Fig. 3b). Females of more than 75% of the dimorphic species are able to detect their diverse conspecific male-specific compounds through the same olfactory channels (at1 and at4 neurons) (Fig. 3b), suggesting that their cognate olfactory receptors are under positive selection that has acted strongly to modify their functional capabilities. Similar to the evolution of male-specific compounds, the functional divergence of these olfactory channels among the closely related drosophilids is not correlated with their phylogeny nor with the evolutionary changes in male chemical profiles (Fig. 3e, Supplementary Data 6, and ref. 90). Moreover, we found that many species detect other heterospecific male compounds, highlighting the broad potential for interspecific olfactory communication among the different drosophilids (Fig. 3b). Behavioral experiments revealed that heterospecific signals reduce the likelihood of hybridization through different olfactory channels from those specialized to detect conspecific pheromones (Fig. 3b). For example, the subspecies of D. mojavensis, as well as D. arizonae and D. navajoa detect their own pheromones through their at4-like sensilla, while they detect the heterospecific cVA through the at1-like sensilla (Fig. 4d). Interestingly, contrastingly to that, at1-sensilla are used in D. melanogaster to detect conspecific pheromones32. These results reveal that species retain—at the peripheral level—the ability to detect the chemicals no longer produced by conspecifics, but a change in valence is likely encoded at the level of central circuits94. In line with our findings, previous studies have shown that heterospecific sex pheromones could reinforce the sexual isolation among sympatric species or recently diverged populations through conserved peripheral olfactory pathways22,45.

Unlike cVA-induced behaviors in D. melanogaster, which are encoded mainly through a single olfactory channel32, sexual behaviors of many other drosophilids seem to be mediated by different compounds through multiple channels (Figs. 3b, 4b). The lack of genetic tools for most of the drosophilid species currently precludes further investigations of the genetic and neuronal correlates of intraspecific sexual behaviors and interspecific sexual isolation. A future challenge will be to investigate the genetic basis of the rapid evolutionary rate of sex pheromone production and detection and how these chemicals, together with the other sensory signals, collaborate to result in the birth of new species.

**Methods**

*Drosophila* lines and chemicals

Fly stocks. Wild-type flies used in this study were obtained from the National *Drosophila* Species Stock Centre (NDSSC; http://blogs.cornell.edu/drosophila/) and Kyoto stock center (Kyoto DGG; https://kyotofly.kit.cji/cgi-bin/stocks/index.cgi).
Stock numbers and breeding were listed in Supplementary Data 1. All odors were reared at 25 °C, 12 h Light:12 h Dark and 50% relative humidity. For more details on the rearing conditions see Drosophila Species Stock Centre (http://blogs.cornell.edu/drosophila/recipes/). Care and treatment of all flies complied with all relevant ethical regulations.

**Chemicals.** Male- and female-specific compounds are listed in Supplementary Data 3, while compounds used for SSR and behavior, their sources and CAS numbers are listed in Supplementary Data 4. All odors were diluted in dichloromethane (DCM) for SSR and behavioral experiments.

**Whole-genome sequencing and phylogenetics.** Sequencing library preparation. Genomic DNA was extracted from a single fly per each species (for details see Supplementary Data 1) using Qiangen DNeasy blood and tissue kit (cat. nos. 69504). Extracted DNA (~20 ng/µl) was quantified with Qubit broad range dsDNA kit, and diluted to a concentration of 1 ng/µl. Tagmentation was performed with in-house T5s transposase prepared with a previously described method (Picelli et al.15; Genome Research). Tagmented fragments were purified with 1 volume of SPRI beads (1 mL, SeraMag GL LNP-20 BioHealthcare, 651521050250 beads in 100 mL of PEG8000 20%, NaCl 2.5 M, Tris-HCl pH = 8.0 10 mM, EDTA 1 mM, Tween20 0.05%), and subjected to 20 cycles of Kapa HiFi enrichment with barcoded primers using the following cycling conditions: 72 °C 3 min, 98 °C 1 min, 20 cycles of 98 °C 5 s, 65 °C 30 s, 72 °C 30 s. An equal volume of PCR products was pooled and purified with SPRI beads with a two-sided size selection protocol, using 0.5X (of the PCR pool volume) SPRI beads for the first selection and 0.2X SPRI beads for the second. Library pool was quantified with Qubit broad range dsDNA kit and sized with TapeStation® D1000. Sequencing was performed on two HiSeq X lanes. Genomes are available on NCBI with accession number: PRJNA696909.

**Gene annotation and determination of orthology.** Nine draft assemblies deposited on NCBI genbank (D. albomicans, D. americana, D. montana, D. nasuta, D. pseudoobscura, D. robusta, D. subobscura, S. lebanonensis, P. variegata) were not annotated. We lifted over annotation information from Drosophila melanogaster for these genomes by performing blast, followed by exonerate and geneview alignments as previously described. We classified annotated genes by clustering protein-coding sequences from 31 species using UPHO as previously described. Together, 11575 orthologs were identified from the annotated genomes. Together with already annotated genomes (n = 22), they serve as reference genomes to which short reads from other species were mapped. ORFs were identified from the UPHO ortholog assignment pipeline by requiring the ortholog to include an annotated D. melanogaster OR. The gene name of an ortholog is then assigned by the D. melanogaster gene name. For re-sequenced species, coverage filters were applied as described for the genes used for phylogenetics. Genes with excessive coverage in re-sequenced species were completely discarded.

**Read processing and generation of pseudogene assemblies.** Raw reads were demultiplexed with dual barcodes by the sequencing facility, and trimmed to remove any adapter sequences using Trimmomatic version 0.32 using the following parameters:illumina-adapters.fa,3:3:7:1:true,LEADING:25 TRAILING:25 SLIDINGWINDOW:6:20 MINLEN:50. We next determined the optimal reference genome to use by mapping the first 10,000 paired-reads with BWA-MEM to each of the 31 reference genomes, followed by computing the proportion of properly mapped read pairs Pprop and the averaged mapping quality MAPQ. We designed an ad hoc index to maximize data usage, reference quality index = (completeness of reference genome annotation) + Pprop * (1-100%MAPQ). The reference with the highest reference quality index was chosen for each short-read dataset. Pseudogenomes were produced as previously described, by mapping reads to the best reference, realigning around gaps, and substituting bases of the reference genome and masking regions with no mapped reads (MAPQ > 20).

**Alignment and phylogenetics.** Orthologous protein-coding sequences were extracted from reference genomes and pseudogenomes by using the GFF annotations of the corresponding reference species. TranslatorX was used to align the coding-sequence by codon, and cleaned with GBReads (flank size = 0.5, MinSeq-Flank = 0.55). Aligned protein-coded sequences were concatenated for each species, resulting in the final alignment matrix with 11,479 genes, 13,433,544 sites in 99 species (5 species were excluded based on a preliminary tree, due to their clear contradiction with well-established taxonomy, suggesting potential problems in mislabelling or strain contamination). Data completeness ranges from 4.46–97.27% (mean = 58.59%). Partitioning the full alignment into 3 codon positions, we inferred a maximum likelihood tree by using RAxML 8.2.4 with 100 rapid bootstrap supports. Because branch length may not be accurate with extensive missing data, we then further optimized the branch lengths with ForeSeq using a branch-length optimization parameter-“branches s-threshold 0.5”. Due to computational constraints, only the top 500 most informative genes were used to re-optimize branch lengths.
and did not simply copulate or court with the first partner they encountered. Results from females that were only courted by one male, or males that only courted one female were excluded. All artwork and copulation data were acquired by a researcher blind to the treatment.

Electrophysiological experiments

Single sensillum recording (SSR). Female flies were immobilized in pipette tips, and the third antennal segment was placed in a stable position onto a glass coverslip98. Trichoid sensilla were identified based on their sensillum morphology under a microscope (BX51WI; Olympus) at ×100 magnification. The two different classes of trichoid sensilla were identified on the basis of their anatomical location (at1 sensilla in the central region, while at4 sensilla in the distolateral region of the antenna) and spontaneous activities (at1 sensilla house less neurons than at4 sensilla), which are known from D. melanogaster99. The extracellular signals originating from the OSNs were measured by inserting a tungsten wire electrode in the base of a sensillum and a reference electrode into the eye. Signals were amplified (Syntech Universal AC/DC Probe; Syntech), sampled (10,667.0 samples/s), and filtered (300–3000 Hz with 50/60 Hz suppression) via USB-IDAC connection to a computer (Syntech). Action potentials were extracted using AutoSpikewhite software, version 3.7 (Syntech). Synthetic compounds were diluted in dichloromethane, DCM, (Sigma-Aldrich, Steinheim, Germany). Prior to each experiment, 10 µl of the diluted odor was freshly loaded onto a small piece of filter paper (1 cm2, Whatman, Dassel, Germany), and placed inside a glass Pasteur pipette. Similar to ref. 23, our preliminary electrophysiological recordings revealed that high concentrations of odors (e.g., 10−5 dilution (v/v)) elicited strong responses that might saturate or kill the olfactory neurons, while low concentration of odors (e.g., 10−5 dilution (v/v)) elicited no or low responses. Therefore, an intermediate concentration (10−5) has been used for all odors. The odorant was delivered by placing the tip of the pipette a few millimeters away from the antennae to ensure the delivery of the low volatile chemicals98. Neuron activities were recorded for 10 s, starting 2 s before a stimulation period of 0.5 s. Responses from individual neurons were calculated as the increase (or decrease) in the action potential frequency (spikes/s) relative to the pre-stimulus frequency. Traces were processed by sorting spike amplitudes in AutoSpikewhite analysis in Excel and illustration in Adobe Illustrator (Adobe systems, San Jose, CA). Note that number of neurons per same sensillum type is not conserved in the different Drosophila species—as revealed by number of at1 neurons across the different species in Supplementary Fig. 3A. Moreover, sorting the number of neurons based on the spike amplitudes in all at4 and some at1 sensilla is technically challenging due to the close spike amplitudes of the sensillum neurons.

Statistical analyses

Estimating phylogenetic signal with Pagel’s λ. Raw peak signals were first standardized by dividing the area under each peak by the sum of areas under all peaks. For each taxon, the corresponding peaks were aligned, and the standardized signals across samples were log-transformed to approximate normality, followed by standardization with a z-transformation. The phylogenetic signals contained in each chemical component were estimated by combining the transformed peak intensity with the DNA phylogeny, using the phylolog function in the phylotools R package (Version 1.1.447). We compared the distribution of Pagel’s λ between sexes using the unpaired Wilcoxon rank sum test. In order to test whether correlations exist between chemical production and neuronal responses, we applied phylogenetic generalized linear models (PGLS). Raw neuronal response values were used as independent variables, and only z-transformed because statistical test (Shapiro–Wilks test) revealed normal distribution of the dataset. Chemical levels were transformed as described in the previous section, and two encoding methods were used for the chemical levels—binary for presence or absence, or continuous. When the chemical levels were binary-encoded, we used phylogenetic logistic regression implemented in the R package phyloseq and 2000 bootstraps to determine statistical significance. For continuous encoding of the chemical levels, we used the PGLS method implemented in the R package caper, with the optimal branch transformation model determined by model selection with BIC as previously described100.

Selection pressure analysis. BUSTED (Branch-Site Unrestricted Statistical Test for Episodic Diversification) was used to assess if a gene has experienced a positive selection at one site at the gene-wide level. BUSTED approach is available at the datamonkey web server (https://www.datamonkey.org)151. All branches of the three phylogenetic trees—including 42, 41, and 36 orthologs of Or47b, Or67d, and Or88a, respectively—were entirely tested for positive selection.

Statistics and figure preparations. The normality test was first assessed on datasets using a Shapiro test. Statistical analyses (see the corresponding legends of each figure) and preliminary figures were conducted using GraphPad Prism 8 v. (https://www.graphpad.com). Figures were then processed with Adobe Illustrator CS5.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All relevant data supporting the findings of this study and all unique biological materials generated in this study are available https://doi.org/10.17617/3.5tw. The whole-genome sequences are available via the accession code PRJNA669609.

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Author contributions
M.A.K., H.K.M.D., B.S.H. and M.K. conceived the project. All authors contributed to the experimental design, analysis, and interpretation of results. M.A.K. prepared all figures and collected all experimental data. R.C. and D.R.V. reconstructed the phylogeny and performed all phylogenetic analyses. A.S. identified and J.W. synthesized the sex-specific compounds. Other experimental contributions were as follows: M.A.K. (Figs. 1, 2, 3a–d, 4, Supplementary Figs. 1B–E, 2, 3, 4, movies 1–427, Supplementary Data 1, 3–5, and 7, and Supplementary Data 8–16), R.C. (Fig. 1a, 3e, Supplementary Figs. 1A, A’, and C, D, 2C, and Supplementary Data 2, 4), J.W. (Fig. 2c, and Supplementary Fig. 2A). M.A.K. wrote the original manuscript, and M.K., R.C., D.R.V. and B.S.H. contributed to the final manuscript. All coauthors contributed to the subsequent revisions.

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