Large-scale characterization of sex pheromone communication systems in *Drosophila*

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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 syntheses and electrophysiological recordings, we identify 51 sex-specific compounds, many of which are detected via olfaction. Behavioral analyses reveal that many of the 42 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 inter-specific communication systems. Our results show how sexual isolation barriers between species can be reinforced by species-specific olfactory signals.

Main

Organisms 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 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 that, and 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 are the sex pheromone-sensing channels [1]. 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 reproductive and internal status. Diversification of sex pheromones among species arises either via neutral drift, or via sexual, and/or natural selection [2-6]. 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 [7,8]. 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 [9]. Moreover, colonization of a different host – an ecological adaptation – could also lead to differential sex pheromones and new ways of signal transmission and perception [10,11]. 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

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cues to locate and choose an appropriate mating partner [1,12-14]. 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 [15]. In these environments, drosophilids feed and breed on varied hosts such as decaying fruits, slime fluxes, mushrooms, flowers, as well as frog spawn [16]. Second, sexual behaviors of drosophilid flies differ quantitatively and qualitatively [17], which may include nuptial gift donation [18], partners' song duet [19,20], territorial dating [20], or the release of an anal fluidic droplet [21]. Third, the neural processing of pheromones in the brain of some drosophilids, especially *D. melanogaster*, is well understood [22]. Fourth, pheromone receptors are narrowly tuned to fly odors [23] and expected to evolve at fast rates to match the dramatic diversity of pheromones among closely related species [5,24]. Lastly, out of the 52 classes of olfactory sensory neurons (OSNs) in *D. melanogaster* [25], only four respond to fly odors and are localized in a specific sensillum type [26]. Hence, the restricted number of orthologues, that are always expressed in an easily identifiable and accessible sensillum type, represent promising candidates to study the coevolution *of Drosophila* pheromones and their corresponding receptors.

Olfactory sexual communication in *D. melanogaster* is arguably one of the best studied systems in animals [27], and is by a large part carried out through a single molecule, *cis*-vaccenyl acetate (*c*VA) [28]. This compound is produced exclusively by males and transferred to females during copulation, which then reduces the attractiveness of the freshly mated females [29]. Moreover, *c*VA regulates multiple behaviors: it induces sexual receptivity in virgin females [30-32], elicits aggression in males [33], modulates oviposition behaviors [34], and acts as aggregation pheromone in presence of food [30,35]. Despite the profound knowledge of *c*VA-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 [16,36-38]. However, the phylogenetic relationships among drosophilids have suffered from low supports [39-41]. We therefore investigated the relationships of 99 species within the family Drosophilidae, 95 of which span the diversity of flies across the genus *Drosophila*.

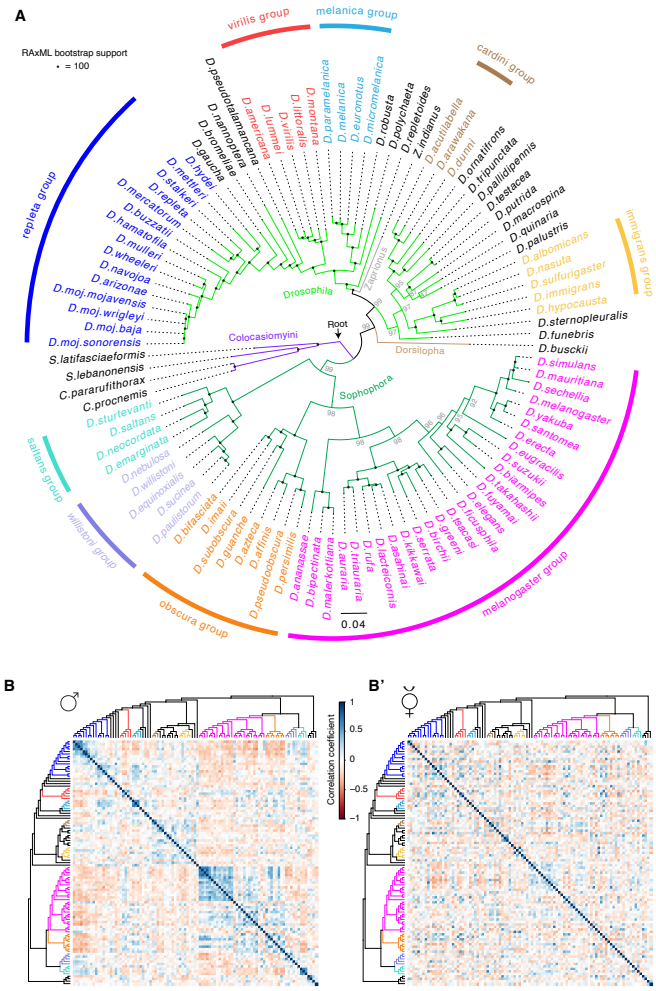

Fig. 1. Phylogenetic relationships and chemical variations among drosophilids

(A) Phylogeny of 99 species within the family Drosophilidae inferred from 13,433,544 amino acids sites that represent 11,479 genes (See Table S1 and Materials and Methods for details). Using 4 species in the Colocasiomyini subgenus as outgroups (purple), 95 species are distributed in four subgenera belonging to the Drosophilini tribe (Drosophila, light green branches; Zaprionus, grey branch; Dorsilopha, brown branch; Sophophora, dark green branches). Species names are color coded according to their relationships in nine different species groups, with black species depicting individual representatives of species

groups. Scale bar for branch length represents the number of substitutions per site. Maximum likelihood (ML) phylogenetic analyses display strong rapid bootstrap support (100% support indicated by black circle at the nodes) for most relationships among the different species.
(B) Heat map showing pairwise correlations between male chemical profiles of the 99 species (ordered on each axis according to their phylogenetic relationships from Fig. 1A). Overall peak areas of 248 male chemical features across the 99 species were compared using Pearson correlation coefficient (R^2); Color codes in the heat map illustrate the pairwise correlations, which range from dark blue (Perfect correlation between chemical profiles) through white (no correlation) to dark red (perfect anticorrelation). The diagonal of the correlation matrix is the correlations between each species and itself (values of 1). Note that the male correlation matrix displays frequent dark blue cells mainly around the diagonal, i.e., high correlation coefficients are observed mostly between closely related species.
(B') Pairwise correlation analysis between female chemical profiles of the 99 species arranged according to their phylogeny from Fig. 1A. Overall peak areas of 256 female chemical features across the 99 species were compared using Pearson correlation coefficient (R^2). See Table S2 for the statistical Pagel's lambda correlation analysis.

Whole-genome sequences (WGS) for 41 of these 99 species are available (Table S1), thus, we generated WGS for the other 58 species (See Material and Methods; accession number: PRJNA669609). We, then, reconstructed the phylogeny of these 99 species using 13,433,544 amino acids sites from 11,479 genes (Fig. 1A). Maximum likelihood (ML) phylogenetic analyses revealed strong support for the relationships among the different species (Fig. 1A). Using the four species in the Colocasiomyini subgenus as outgroups, we recovered four main clusters within the genus *Drosophila*. First, the Drosophila subgenus that contains five main groups (repleta, virilis, melanica, cardini, and immigrans groups); second, the Zaprionus subgenus that includes *Zaprionus indianus*; third, the Dorsilopha subgenus that includes *D. busckii*; fourth, the Sophophora subgenus that includes melanogaster, obscura, willistoni, and saltans groups (Fig. 1A).

Closely related drosophilids exhibit highly divergent chemical profiles

We then asked whether the phylogenetic relationships could reflect the differences in cuticular chemicals among these species. We, therefore, analyzed the chemical profiles of males and virgin females of all 99 species, with five replicates or more of each sex yielding more than 580 and 520 replicates, respectively. Chemical analyses recognized the presence of 248 and 256 compounds (i.e., features with distinct *m/z* (mass-to-charge ratios)) across male and female replicates, respectively (Fig. S1A and A'). Principal component analyses revealed that females of the different species groups exhibit closer distances than male species groups, indicating that females exhibit more similar chemical profiles across the species groups (Fig. S1B and B'). Similarly, in a cluster analyses of the chemical signals most males belonging to the same species group are clustered, while species groups in females are dispersed across the chemometric tree (Fig. S1C and C'). Next, we assessed whether the phylogenetic and chemometric distances are correlated. Pairwise correlation analyses imply that indeed closely related species exhibit more similar chemical profiles in males (Fig. 1B and B'). For example, male species of the *repleta* and the *melanogaster* group display high correlation coefficients to other male species of their own group, but negative correlation coefficients to males of heterospecific groups (i.e., blue cells are frequently present around the diagonal) (Fig. 1B). However, female species generally display high correlation coefficients (>0.75) randomly to each other apart from their phylogenetic relationships (Fig. 1B'). Indeed, males have significantly more chemicals with higher phylogenetic signals (i.e., males of related species tend to resemble each other more than males of unrelated species) than females as measured by Pagel's λ (p = 0.006) (Fig. S1D and Table S2). Together, our data suggests that a subset of the male chemicals evolve neutrally in relation to phylogeny, while other male chemicals diversify rapidly and could be under positive selection.

Previously unidentified potential sex pheromones undergo rapid evolution

In drosophilids sex-specific compounds typically serve as short range communication signals that induce or inhibit sexual behaviors [22]. 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 [21]. 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 [42,43]. 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

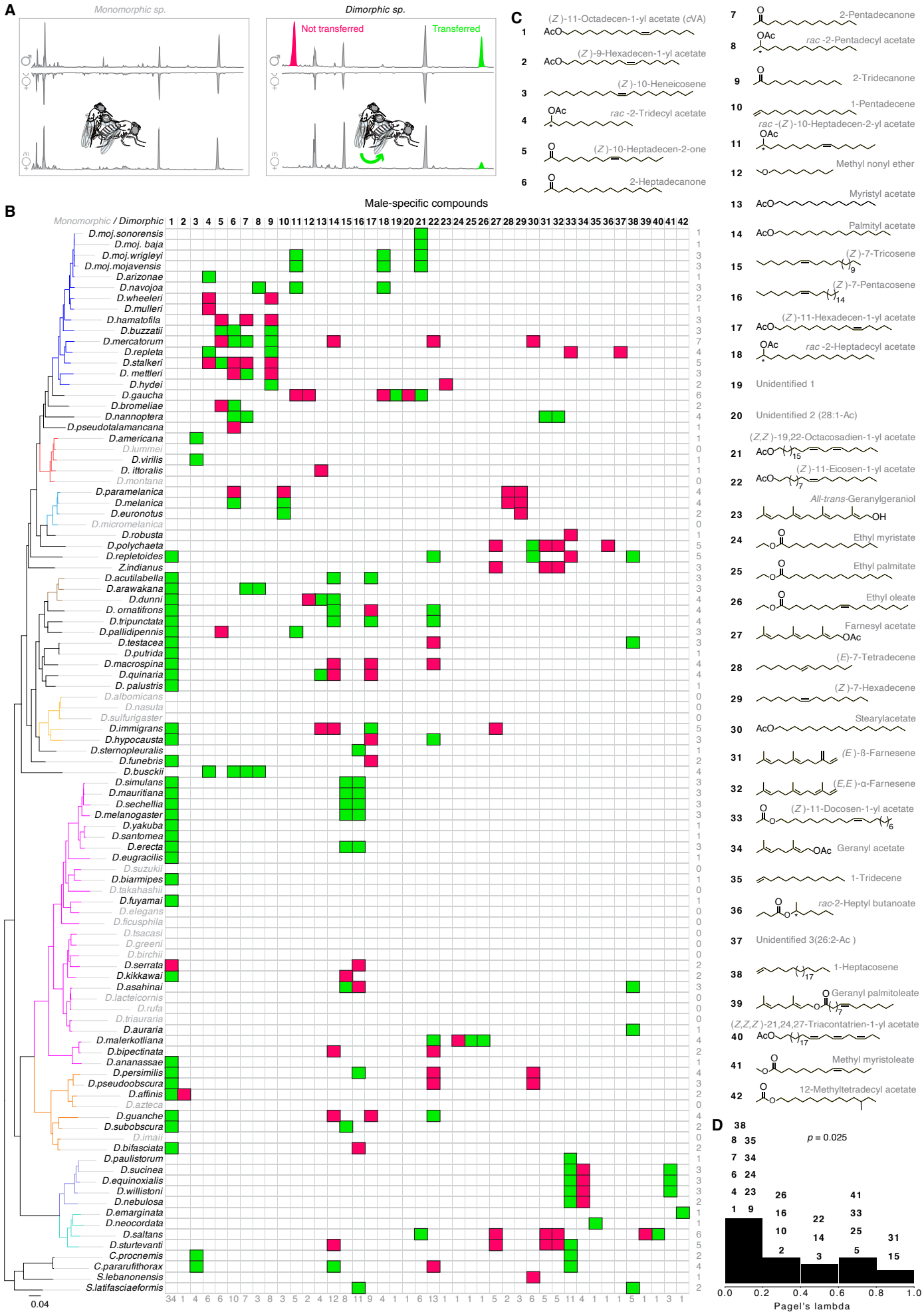

Fig. 2. Newly-identified potential sex pheromones display rapid evolution

(A) Representative gas chromatograms of virgin male (♂), and virgin (v♀) and mated (m♀) female flies obtained by solvent-free thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) [48]. Five replicates or more of each

sex were analyzed, yielding more than 580, 520, and 500 replicates of males, and virgin and mated females of all 99 species, respectively. Left panel, example of a monomorphic species, whose males exhibit a chemical profile identical to that of virgin and mated females. Right panel, example of a dimorphic species that displays sexually dimorphic profiles. Colored peaks indicate male-specific compounds (green, compounds transferred to females during mating; red, non-transferred compounds).
(B) Distribution of 42 male-specific compounds among different drosophilids; 81 species are dimorphic species (in black), while 18 species (in grey) are monomorphic species. Phylogeny on the left side is identical to the tree in Fig. 1A; the branches are colored according to group identities. Numbers on the right side represents the sum of male-specific compounds present per species, while numbers at the bottom of the table represent number of times each male-specific compound appeared in the different species. Cell colors refer to transferred (green) and non-transferred (red) compounds. See Fig. S2 for female-specific compounds.
(C) Chemical structures and names of the male-specific compounds according to the International Union of Pure and Applied Chemistry (IUPAC). Out of 42 male-specific compounds, 39 compounds were chemically identified. Compound size ranges between 10 to 32 carbon atoms, with 23 esters, 4 ketones, 8 alkenes, 2 terpenes, 1 ether and 1 alcohol. See Table S3 for Kovat's Index, chemical formula, exact mass, mass spectrum (*M/Z*), and boiling temperature of these compounds.
(D) Frequency histogram of Pagel's lambda estimates, which measures the phylogenetic signal exhibited by the chemical trait [94]. A value of 0 suggests the male chemical trait cannot be explained by phylogenetic relatedness, while a value close to 1 suggests strong phylogenetic signal consistent with neutral evolution. See Materials and Methods for details on statistical analyses. Note that the proportion of male-specific chemicals with low phylogenetic signals is significantly higher than those with high phylogenetic signals; * $P = 0.025$, one-sample Wilcoxon signed rank test ($n = 24$). See Table S2 and Fig. S1C for Pagel's lambda estimates for male and female chemicals.

chemical profiles, while 81 species exhibited sexually dimorphic cuticular chemicals (Fig. 2B). All the 81 dimorphic species unveiled male-specific compounds (in total 42 compounds), while only 15 species exhibited female-specific ones (9 compounds) (Fig. 2B and Fig. S2A and B). Of note, all the female-specific compounds, are long-chain unsaturated hydrocarbons (Fig. S2A), display high boiling temperature (Table S3), and hence are likely to be non-volatile [42]. 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 an ether and an alcohol (Fig. 2C and Table S3). Notably, when analyzing, in addition, 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* [21,44,45]. On the contrary, none of the female-specific compounds was transferred to males during mating (Fig. S2B).

The proportion of male-specific chemicals with low phylogenetic signals is significantly higher than those with high phylogenetic signals ($p = 0.025$), signifying that closely related species possess dissimilar male-specific compounds (Fig. 2D and Table S2). Indeed, mapping the sex-specific compounds onto the phylogenetic tree revealed that many of them are repeatedly present across different species in different groups, while few are exclusively species- or group-specific compounds (Fig. 2B and Fig. S2B). For example, several male-specific compounds, including *c*VA that has been thought to be restricted to the melanogaster and the immigrans groups [24], 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 [46], 7,11-heptacosadiene and 7,11-nonacosadiene, the female-specific compounds in *D. melanogaster* [22,43], are not restricted to a specific group (Fig. S2B). This pattern supports the presence of strong selection on the sex-specific compounds to evolve fast and deviate from expectations based on neutral evolution. Additionally, our analyses revealed that 58 of the 81 dimorphic species have a blend of multiple 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 51 potential sex pheromones (Table S3), which seem to evolve rapidly and 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; Table S3) of most male-specific compounds suggests that they could be potential olfactory signals. We, therefore, screened for olfactory sensory neurons (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*, 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

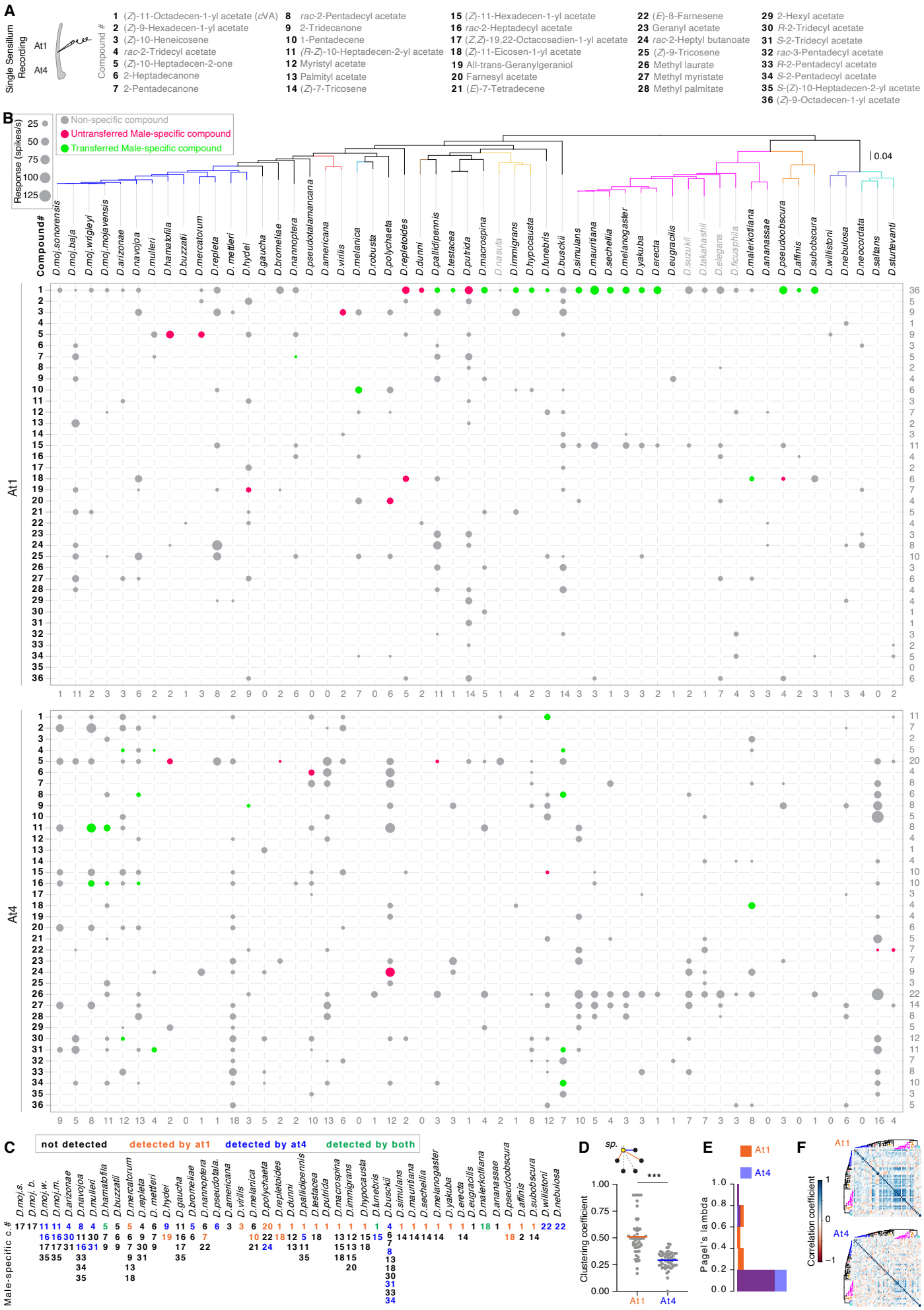

Fig. 3. Drosophilids communicate intra- and inter-specifically through rapidly evolving olfactory channels
(A) Left: schematics of single sensillum recordings (SSR) from the antennal trichoid (at1 and at4) sensilla. Right: Names of the different chemicals used to screen the trichoid sensilla. Note that all chemicals are male-specific compounds

identified in this study (Fig. 2B), except compounds# 25, 26, 27, 28, 29, 32, and 36, which were described as flies' pheromones in [48-50].

(B) Color-coded electrophysiological responses towards heterospecific compounds (grey bubbles) and conspecific compounds (colored bubbles) in at1 (top) and at4 (bottom) sensilla of 54 species. Compound names are depicted in Fig. 3A. Red and green bubbles represent species-specific male untransferred and transferred compounds, respectively. Bubble size corresponds to the response values ranging from 25 to 125 spikes per second. Responses less than or equal to 10 spikes per second were excluded from the bubble chart (see Fig. S3A and A', and Materials and Methods for more details). Species names are arranged on the top according to their phylogenetic relationship; the tree branches are colored according to the group identities. Numbers on the right side represent the sum of species that can detect each of the male-specific compounds, while the number below the table represent the sum of chemicals that can be detected by each species.

(C) A summary of female's abilities to detect their own male-specific compounds through olfaction in 47 dimorphic species (two species, *D. robusta* and *D. neocordata*, whose compounds were not included among the 36 compounds, were excluded). Black numbers, undetected male-specific compounds; orange, detected by at1 neuron(s); blue, detected by at4 neurons; green, detected by both. See Fig. S3A for more details about the number of neurons in at1 sensillum. Note that, out of 47, females of 36 species detect their conspecific male cues through at1 and/or at4.

(D) Top: A schematic example of how to calculate the olfactory clustering coefficient of a given species (yellow circle) to communicate with heterospecific species (black circles) through at1 (orange lines) and at4 (blue lines). The olfactory clustering coefficient is the number of other species that are detected by a given species through at1 or at4 (colored lines) divided by the total number of detected and undetected species (colored + grey dashed lines). The clustering coefficient of a species is a number between 0 (i.e., no species detected) and 1 (i.e., all species detected). Below: scatter plot indicates olfactory clustering coefficients of the 54 species and their mean through at1 (orange) and at4 (blue); Mann Whitney U test, *** $P < 0.001$ ($n = 54$ species). Note that species exhibit more olfactory intra- and inter-specific communication through at1 than at4. See Fig. S3B and B' and Materials and Methods for more details on communication network analyses.

(E) Frequency histogram of Pagel's lambda estimates, which explain the correlation between the olfactory responses of at1 (orange) and at4 (blue) among the different species and their phylogenetic relationships. Note that responses of both at1 and at4 display low phylogenetic signals (i.e., do not correlate with the phylogeny). Additionally, their phylogenetic signals are comparable to each other; Mann Whitney U test, ns $P = 0.27$ between at1 and at4 responses.

(F) Heat map showing the pairwise correlations between the electrophysiological responses of at1 (top) and at4 (bottom) sensilla in 54 species, which are ordered on each axis according to their phylogenetic relationships in Fig. 3B. Overall responses to 36 compounds across the 54 species were compared using Pearson correlation coefficient (R^2); Color codes in the heat map illustrate the pairwise correlations, which range from dark blue (Perfect correlation between species' responses) through white (no correlation) to dark red (perfect anticorrelation). The diagonal of the correlation matrix depicts the correlations between each species and itself (values of 1). The phylogenetic trees on each axis are similar to Fig. 3B; the branches are colored according to the group identities. Note that the male correlation matrix displays frequent dark blue cells around the diagonal and overall the matrix, i.e., high correlation coefficients only between the closely related species. Unlike the at1 correlation matrix, species' responses display lower correlation coefficients, indicating that olfactory channels in at4 evolve rapidly and independently from the phylogeny.

different antennal regions [47]. The at1 sensillum houses a single neuron (Or67d) that responds to *c*VA [31], while at4 houses 3 neurons (Or47b, Or65a/b/c, and Or88a) that respond to methyl laurate, *c*VA, and methyl palmitate, respectively [23,48]. Indeed, we found the at1-like and at4-like sensillum classes in all tested species except *D. pseudotalamancana* and *D. robusta*, whose at1-like sensilla could not be identified (Fig. S3A; for identification, see Materials and 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 (Table S4) [48-50]. Electrophysiological recording revealed that females of 36 of 49 dimorphic species detect their conspecific males' compounds (Fig. 2B) by olfactory neurons (Fig. 3B and C). Of note, flies are also able to detect many male-specific compounds of other species (Fig. 3B, Fig. S3A and B', and Table S5). To analyze the olfactory-based communication between the different species, we performed network analyses, which revealed a higher olfactory clustering coefficient (i.e., the number of olfactory communications between the species divided by number of communications that could possibly exist) of interspecific communication through at1-like (for identification, see Materials and Methods) compared to at4-like sensilla (Fig.3D, and Fig. S3B and B'). However, self-loops of the intraspecific communication (i.e., the ability of a female to detect her conspecific male) are comparable through at1-like and at4-like sensilla (Fig. S3B and B'). Pairwise correlation and statistical analyses revealed that electrophysiological responses of the different species exhibited low phylogenetic signals (Fig. 3E-F), indicating that olfactory channels of the different species evolve rapidly apart from their phylogenetic relationships.

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 per gene (see Materials and Methods). Statistical analyses revealed an 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).

Lastly, using two different model-tuning criteria, we performed a phylogenetically

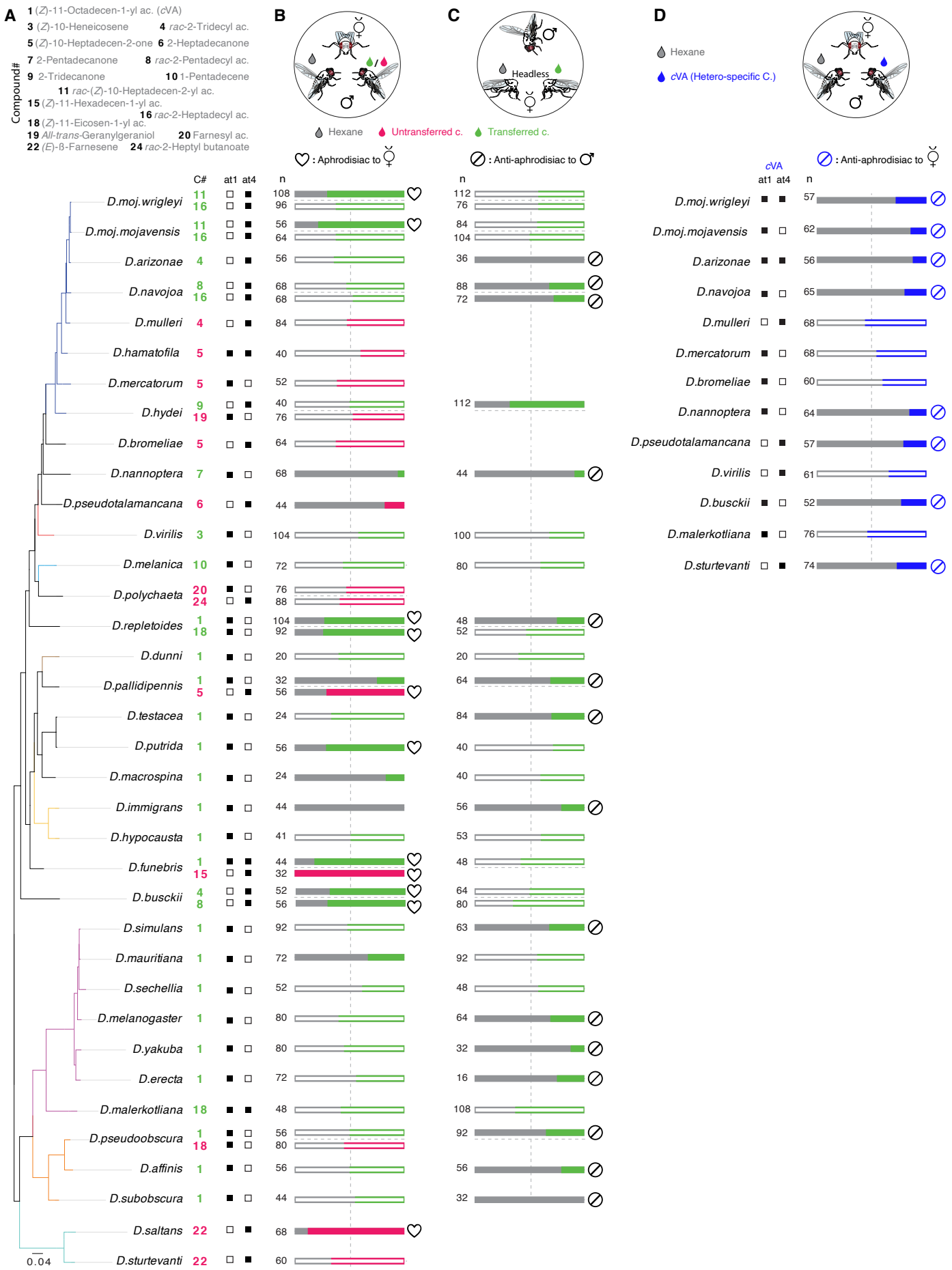

Fig. 4. Male-specific compounds regulate intra- and inter-specific sexual behaviors

(A) Left: Names of the compounds (top) that are exclusively produced by males of 54 species (left below) and detected by conspecific females through at1 or/and at4 (right below). These compounds were used for the behavioral experiments in Fig. 4B, C, and D.

(B) Top: Schematic of a mating arena where females of each species had the choice to mate with two conspecific males perfumed with their olfactory-detected male-specific compound (indicated, in Fig. 4A, on the left side of the horizontal dashed stoke) or solvent (dichloromethane, DCM). For consistency of perfuming and correspondence to biologically relevant amounts see Materials and Methods. Below: bar plots represent the percentages of copulation success of the rival males. Results

from females that were only courted by one male were excluded. In this and other panels, filled bars indicate significant difference between the tested groups; ns $P > 0.05$; ** $P < 0.01$; *** $P < 0.001$, chi-square test. Number of replicates are stated on the left side on the bar plots. See Fig. S4A for details regarding the differences and similarities of sexual behaviors 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 Table S7 for raw data and statistical analyses. See Fig. S4B for the effect of perfuming on the males' courtship behavior.

(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 transferred compounds (green). Below: bar plots represent the percentage of the first copulation attempts towards perfumed and control females. Results from males that only courted one female were excluded; see Materials and Methods. Note that 15 compounds inhibited courtship, 1 compound increased courtship and 16 compounds turned out to be neutral.

(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 *c*VA or solvent (DCM). Note that we only tested the species that do not produce but still detect *c*VA. Below: bar plots represent the percentages of copulation success of the rival males. Results from females that were only courted by one male were excluded.

corrected correlation between the evolution of male chemical phenotypes and the associated olfactory responses of their females. Despite the high intraspecific match – females of 36 out of 49 species detect their males – (Fig. 3C), the evolution of females' responses does not fit the evolution of their male-specific compounds (Table S6). This implies presence of low interspecific correlation between detection and production. Indeed, for example, females of 19 out of 20 species, whose males produce *c*VA, detect this compound (i.e., *c*VA functions as a conspecific signal), while females of 21 out of 34 species are still able to detect *c*VA (Fig. 3B), although their males do not produce it (i.e., *c*VA functions as a heterospecific signal).

Male-specific compounds regulate intra- and inter-specific sexual behaviors

To examine the intra- and inter-specific 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 (Movies1 to 427, available on ftp://ftp.ice.mpg.de/outgoing/sessions/20201001-eiCh2neeng; 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 females [51], *D. mojavensis* and *D. virilis* males release fluidic droplets while courting the females [21], *D. subobscura* males extend their proboscis to gift females with regurgitated drop of their gut contents [18], and *D. nannoptera* couples tend to re-mate as many as two to three times within the recording time frame of 60 min [52]. We further quantified copulation success, latency, and duration (Fig. S4A), which varied largely among different species. Unlike the prolonged copulation time in the species of the *melanogaster* group, copulation lasts for less than two minutes in members of the *repleta* group (Fig. S4A). Together, courtship recordings (available on ftp://ftp.ice.mpg.de/outgoing/sessions/20201001-eiCh2neeng) 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 (Fig. 3C and Fig. 4A) – and asked whether these compounds induce female receptivity. 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 Materials and Methods]. In 11 instances, females displayed a preference to copulate with males perfumed with the male-specific compound over the control ones (Fig. 4B and Table S7). However, females of six species avoided copulating with the males perfumed with the male-specific compound (Fig. 4B). Notably, perfuming an additional amount of *c*VA on males of the melanogaster clade did not increase the males' copulation success (Fig. 4B), indicating that the built-in amount of *c*VA in the control males is already sufficient for females' acceptance. To assure that high copulation success of perfumed males was not due to an increased intensity of male courtship [53], we recoded their courtship activities. Courtship indices did not differ between perfumed and control males (Fig. S4B), 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 36 species in Fig. 4B). We, therefore, asked whether transfer of these compounds contributes to a general post-copulation mate-guarding strategy [as described for transferred pheromones in *D. melanogaster* and *D. mojavensis* [21,44,54]]. To

distinguish male sexual behaviors from 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 and Table S7). 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, out of the 36 *Drosophila* species that produce and detect male-specific compounds, the compounds of 24 species regulated sexual behaviors in our experiments.

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 *c*VA 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-*c*VA-producing species (Fig. 4D). Females of *Drosophila* species whose females detect, but their males don't produce *c*VA, had the choice to mate with two conspecific males perfumed with *c*VA or solvent. All these females detected cVA 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 *c*VA (Fig. 4D and Table S7). Overall, many male-specific compounds seem to regulate intra-sexual behaviors all along the *Drosophila* phylogeny and promote inter-sexual isolation for heterospecific species.

Discussion

Sexual selection imposed by coevolution of female preferences to particular male traits leads to rapid and dramatic evolutionary divergence [55,56] and potentially contributes to speciation processes [57]. 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 its role in speciation.

In general, cuticular chemistry varies between closely related species in relation to their genetic relationships [58,59], geographical locations [60], and environmental factors [61]. Environmental factors are thought to have stronger impact on the evolution of cuticular chemicals than genetic relatedness [58,62]. Our findings reveal that males have significantly more chemicals with higher phylogenetic signals than females, i.e. there is a better correlation between genetic and chemical distance in males (Fig. S1C and D). Notably, the picture changes when we focus on sex-specific compounds, which evolve at rapid rates (i.e., display low phylogenetic signals) resulting in dramatic differences between closely related species (Fig. 2B and D, and Fig. S2B) and indicating the divergence of sexual communication among them. Consistent with our results, nonsexual chemical hydrocarbons in ants [58], aphids [63], ladybird beetles [64], moths [65], and drosophilids [24] exhibit gradual evolution, while aggregation pheromones in beetles – used as sexual signals – display saltational (i.e., sudden and large) shifts [66]{Symonds, 2016 #13123}. By contrast, our observed saltational shifts in male-specific compounds contradicts previous studies on the gradual mode of evolution of some of the sex pheromones in *Bactrocera* [67] and some aggregation pheromones in *Drosophila* [24]. We identified some of these previously-identified aggregation pheromones as potential sex pheromones (Figs. 2B and 4B). 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 [24]. The saltational changes of sexual signaling are likely to occur between closely-related sympatric species [68,69] to overcome the homogenizing effects of gene flow.

The high proportion – ~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' ability to mate under low light conditions [15,70], while many of the chemically monomorphic species cannot mate in the dark [15]. The latter often display sexually-dimorphic color patterns, implying that they rely on visual cues during sexual communication [70,71]. Of note, many of the sex-specific compounds exist across many species in different groups, indicating that the gaining of a sexual chemical trait has occurred independently multiple times during the evolution of drosophilid flies (Fig. 2B and Fig. S2B). For example, *c*VA, (*Z*)-11-eicosen-1-yl acetate, and palmityl acetate are present in 34, 13, and 12 species that belong to different groups, respectively. Instead, the production of 2-heptadecanone and (*Z*)-7-pentacosene are present in different groups but restricted to higher taxonomic levels (i.e., subgenre Drosophila and Sophophora, respectively). Moreover, 13 compounds have appeared on only one occasion across the 99 species. The observed saltational changes are not necessarily unexpected as minor mutations suffice to induce large-scale changes in the biosynthetic pathways of sex pheromones [65,72,73]. Likewise, gene families involved in biosynthesis of cuticular chemicals have been shown to evolve rapidly and independently among closely related drosophilids [74].

One key observation of our study is the diversity and abundance of male-specific compounds compared to female ones – 42 compared to 9, respectively – across the dimorphic species (Fig. 2B and Fig. S2B). Surprisingly, all the 81 dimorphic species exhibit male-specific compounds, while only 15 species have female specific compounds (Fig. 2B and Fig. S2B). 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 male [31,35,49,75] and to avoid costly interspecific mating [21,31,76]. Moreover, males are found to court heterospecific females in equal vigor as conspecific females [21,77,78], even after learning the conspecific females' chemical profiles [79]. Similarly, males exhibit higher preference for females that exhibit no cuticular hydrocarbons (i.e., females lacking oenocytes (" oe⁻" females)) over wildtype females, while females are less receptive to oe⁻ males [80]. Furthermore, male cuticular hydrocarbons are modulated more easily by lab-induced natural and sexual selection than female cuticular hydrocarbons [81]. 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 recognition [1]. Drosophilid chemoreceptor genes evolve rapidly [82] and single point mutations can result in species-specific variance of receptor tuning [83]. Such specificity has shown to be not random and principally occurred to match chemical divergence associated to host selection [82-86] or mate recognition [87]. Similarly, we found that pheromone-responsive olfactory channels 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 (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 different drosophilids is not correlated with their phylogeny (Fig. 3E and [84]). 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 (Figs. 3B and D). For example, the subspecies of *D. mojavensis*, as well as *D. arizonae* and *D. navojoa* detect their own pheromones through their at4-like sensilla, while they detect the heterospecific *c*VA through the at1-like sensilla (Fig. 4D). Interestingly, contrary to that, at1-sensilla are used in *D. melanogaster* to detect conspecific pheromones [31]. These results reveal that species retain – at the peripheral level – the ability to detect the chemicals no longer produced by conspecifics, but reverse – at the central level – the hedonic value of these signals

[88]. 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 pathways [21,43].

Unlike *c*VA-induced behaviors in *D. melanogaster*, which are encoded mainly through a single olfactory channel [31], sexual behaviors of many other drosophilids seem to be mediated by different compounds through multiple channels (Figs. 3B and 4B). The lack of genetic tools for most of the drosophilid species currently precludes further investigations of the genetic and neuronal correlates of intra- and inter-sexual communication. 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.

Materials and 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 DGGR; https://kyotofly.kit.jp/cgi-bin/stocks/index.cgi). Stock numbers and breeding diets are listed in Table S1. All flies were reared at 25°C, 12 h Light:12 h Dark and 50% relative humidity. For more details on the food recipes see *Drosophila* Species Stock Centre (http://blogs.cornell.edu/drosophila/recipes/).

Chemicals. Male- and female-specific compounds are listed in Table S3, while compounds used for SSR and behavior, their sources and CAS numbers are listed in Table S4. 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 more details see Table S1) using qiagen DNeasy blood and tissue kit (cat. nos. 69504). Extracted DNA (~20ng/µl) was quantified with Qubit broad range dsDNA kit, and diluted to a concentration of 1ng/µL. Tagmentation was performed with in-house Tn5 transposase prepared with a previously described method (Picelli et al., 2014; Genome Research). Tagmented fragments were purified with 1 volume of SPRI beads (1mL SeraMag GE Healthcare, 65152105050250 beads in 100mL of PEG8000 20%, NaCl 2.5M, Tris-HCl pH=8.0 10mM, EDTA 1mM, Tween20 0.05%), and subjected to 20 cycles of Kapa HiFi PCR enrichment with barcoded primers using the following cycling conditions: 72°C 3min, 98°C 1min, 20 cycles of 98°C 45s, 65°C 30s, 72°C 30s. An equal volume of PCR products was pooled and purified with SPRI beads with a two-sided size selection protocol, using 0.55X (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: PRJNA669609.

Gene annotations and determination of orthologs

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 genewise 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.

Read processing and generation of pseudogenome assemblies

Raw reads were demultiplexed with dual barcodes by the sequencing facility, and trimmed to remove any adapter sequences using Trimmomatic 0.32 using the following parameters: ILLUMINACLIP:illumina-adaptors.fa:3:7:7:1:true LEADING:25 TRAILING:25 SLIDINGWINDOW:4:20 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 *Pproper* and the averaged mapping quality *MAPQ*. We designed an *ad*

hoc index to maximize data usage, reference quality index = (completeness of reference genome annotation) * *Pproper* * (1-10^*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-sequencings by codon, and cleaned with GBlocks (MinSeqConsv=0.5, MinSeqFlank = 0.55). Aligned protein-coding sequences were concatenated for each species, resulting in the final alignment matrix with 11,479 genes, 13,433,544 sites in 99 species (5 samples were excluded based on a preliminary tree, due to their clear contradiction with well-established taxonomy, suggesting potential problems in mislabeling 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 ForeSeqs using a branch-length stealing algorithm using the parameters "--branches s --threshold 0.5". Due to computational constraints, only the top 500 most informative genes were used to re-optimize branch lengths.

Chemical analyses

Thermal Desorption-Gas Chromatography-Mass Spectrometry (TD-GC-MS). Individual headless male and female flies in different mating status (virgin or freshly-mated) were prepared for chemical profile collection as described previously [48], with some modifications. Briefly, the GC-MS device (Agilent GC 7890A fitted with an MS 5975C inert XL MSD unit; www.agilent.com) was equipped with an HP5-MS UI column (19091S-433UI; Agilent Technologies). After desorption at 250°C for 3 min, the volatiles were trapped at -50°C using liquid nitrogen for cooling. In order to transfer the components to the GC column, the vaporizer injector was heated gradually to 270°C (12°C/s) and held for 5 min. The temperature of the GC oven was held at 50°C for 3 min, gradually increased (15°C/min) to 250°C and held for 3 min, and then to 280°C (20°C/min) and held for 30 min. For MS, the transfer line, source and quad were held at 260°C, 230°C and 150°C, respectively. Eluted compounds for this and the following analyses were ionized in electron ionization (EI) source using electron beam operating at 70 eV energy and their mass spectra were recorded in positive ion mode in the range from *m/z* 33 to 500. The structures of the newly identified compounds were confirmed by comparing their mass spectrum with synthesized or commercially available standards (for more details see Table S4. Age of males and females is 10 days.

Body extract analysis by GC-MS. Fly body extracts were obtained by washing single flies of the respective sex and mating status in 10 µl of hexane for 30 min. For GC stimulation, 1 µl of the odor sample was injected in a DB5 column (Agilent Technologies; www.agilent.com), fitted in an Agilent 6890 gas chromatograph, and operated as described previously [89]. The inlet temperature was set to 250°C. The temperature of the GC oven was held at 50°C for 2 min, increased gradually (15°C/min) to 250°C, which was held for 3 min, and then to 280°C (20°C/min) and held for 30 min. The MS transfer-line, source and quad were held at 280°C, 230°C and 150°C, respectively.

Chiral chromatography. To check the presence of different stereoisomers of some compounds, hexane body extracts of male flies were injected into a CycloSil B column (112-6632, Agilent Technologies; www.agilent.com) fitted in Agilent 6890 gas chromatograph and operated as follows: The temperature of the GC oven was held at 40°C for 2 min and then increased gradually (10°C/min) to 170°C, then to 200°C (1°C/min), and finally to 230°C (15°C/min) which was held for 3 min.

Perfuming flies with hexane or male-specific compounds. Male and female flies were perfumed with the compounds singly diluted in DCM or DCM alone as previously described [21]. Briefly, 10 µL of a 50 ng/µL stock solution was pipetted into a 1.5-mL glass vial. After evaporating the DCM under nitrogen gas flow, ten flies were transferred to the vial and

subjected to three medium vortex pulses lasting for 30 s, with a 30-s pause between each pulse. Flies were transferred to fresh food to recover for 2 h and then introduced to the courtship arenas or subjected to GC-MS analysis to confirm the increased amount of the perfumed acetate. Each fly was coated with ~2-10 ng of the compound of interest.

Chemical identification and synthesis (provided as a separate word file).

Behavioral experiments

Single and competitive mating assays. Males and females were collected after eclosion and raised individually and in groups, respectively. For each experiment, courtship assays were performed in a chamber (1 cm diameter × 0.5 cm depth) covered with a plastic slide. Courtship behaviors were recorded for 60 min using a GoPro Camera 4 or Logitech C615 as stated in the figure legends. All single mating experiments were performed under normal white light at 25°C and 70% humidity. Each video was analyzed manually for copulation success, which was measured by the percentage of males that copulated successfully, copulation latency, which was measured as the time taken by each male until the onset of copulation, and copulation duration. In all competition experiments, copulation success was manually monitored for 1 h. Decapitated females were used in the courtship assays to disentangle male sexual behaviors from female acceptance.

In the competition mating assays, rival flies were marked by UV-fluorescent powder of different colors (red: UVXPBR; yellow: UVXPBB; green: UVXPBY; purchased from Maxmax.com; https://maxmax.com) 24 h before the experiments. Competition assays were manually observed for 1 h and copulation success was scored identifying the successful rival under UV light. Decapitated females were used to observe the first copulation attempt of males in presence of the different compounds and DCM perfumed conspecific females. Data from competition experiments represents either females courted by both rival males or males courted with both rival females to ensure that females or males chose between rival pairs 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 courtship and copulation data were acquired by a researcher blind to the treatment.

Electrophysiological experiments

Single sensillum recording (SSR). Adult flies were immobilized in pipette tips, and the third antennal segment was placed in a stable position onto a glass coverslip [90]. 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. melanogaster* [47]. 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 – 3,000 Hz with 50/60 Hz suppression) via USB-IDAC connection to a computer (Syntech). Action potentials were extracted using AutoSpike software, version 3.7 (Syntech). Synthetic compounds were diluted in dichloromethane, DCM, (Sigma-Aldrich, Steinheim, Germany). Prior to each experiment, 10 µl of diluted odor was freshly loaded onto a small piece of filter paper (1 cm^2, Whatman, Dassel, Germany), and placed inside a glass Pasteur pipette. The odorant was delivered by placing the tip of the pipette few millimeters away from the antennae, to ensure the delivery of the low volatile chemicals [91]. 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 AutoSpike, analysis in Excel and illustration in Adobe Illustrator CS (Adobe systems, San Jose, CA).

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 sex, the corresponding peaks were aligned, and

the standardized signals across samples were logarithm-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 phylosig function in the phytools R package. 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 visual inspection showed an approximately 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 phylolm 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 described [92]

Selection pressure analysis

BUSTED (Branch-Site Unrestricted Statistical Test for Episodic Diversification) was used to asses if a gene has experienced a positive selection at any site at the gene-wide level. BUSTED approach is available at the datamonkey web server (https://www.datamonkey.org/) [93]. 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

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 v. 8 (https://www.graphpad.com). Figures were then processed with Adobe Illustrator CS5.

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Author Contributions: M.A.K, H.K.M.D., B.S.H., and M.K. conceived of the project. All authors contributed to 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 (Fig.1, Fig.2A-C, Fig. 3A-D and F, Fig. 4, Fig. S1B-C', Fig. S2, Fig. S3, Fig. S4, movies S1-427, and Tables S1,3-5, and 7), R.C. (Fig. 1A, Fig.2D, Fig.3E, Fig.S1A-A', and D, Tables S2, and 4), J.W. (Fig.2C, Fig. S2A). 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.

Competing interests: the authors declare that they have no competing interests.

Data and biological material availability: All relevant data supporting the findings of this study and all unique biological materials generated in this study are available from the corresponding authors upon request.

Supplementary Figures and Tables

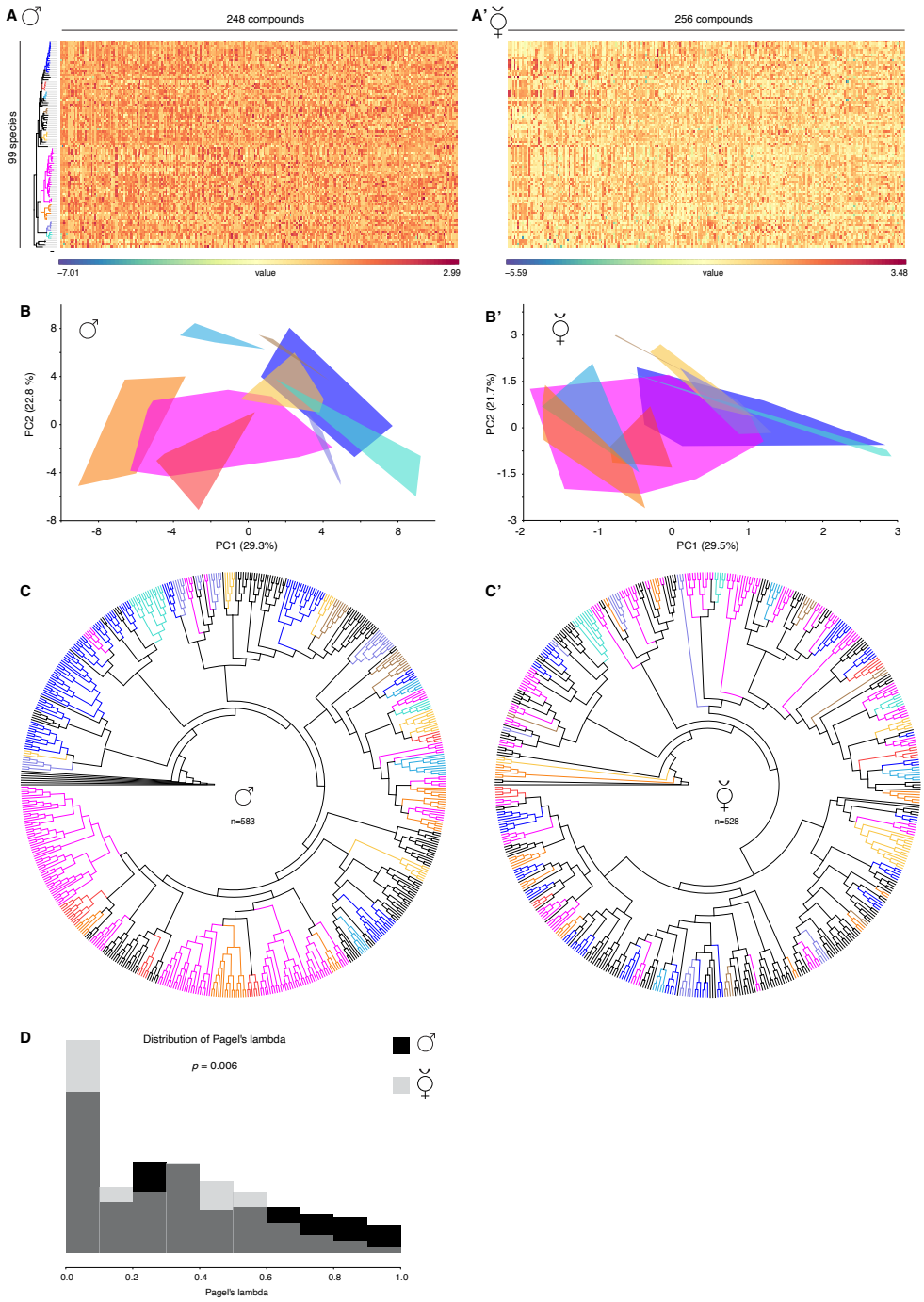

Fig. S1. Chemical analyses of 99 species within the family Drosophilidae
(A) Heat map of normalized percentages of peak areas of 248 features as detected by XCMS across the males of 99 species (See Materials and Methods for details). Rows represent the species, which are ordered on each axis according their DNA phylogenetic relationships shown on the left side, while columns represent the 248 male chemical features present across the species sorted by lambda, highest Pagel's lambda values to the left side of the panel (i.e., most correlated with phylogeny). Each cell represents the mean of the normalized percentages of the feature's peak area in the different replicates of the same species. The feature's percentage is calculated by dividing the peak area of a particular feature by the sum of areas of all features in the replicates of the same species.
(A') Normalized percentages of peak areas of 256 female chemical features across 99 female species. Similar to Fig. S1A, rows and columns represent the species and chemical features, respectively.
(B) The first two principal components of male chemical profiles (data in Fig. S1A) of the 583 replicates across the 99 male species (>5 replicates per species) based on difference in peak areas of 248 male chemical features present across these replicates (see Materials and Methods for details). Data points of each group are enclosed within line. The lines' fill is colored according to the group identities in Fig. 1A.
(B') The first two principal components of male chemical profiles (data in Fig. S1A') of the 528 replicates across the 99 male species (>5 replicates per species) based on difference in peak areas of 256 male chemical features present across these replicates.
(C) Chemometric hierarchical cluster analysis of 583 replicates across the 99 male species. Replicates are color coded according their species group in Fig. 1A. Cluster analysis was performed using neighbor joining (NJ) and correlation similarity index based on peaks' quantities. *S. latifasciaeformis* was used to root the Chemometric tree.
(C') Chemometric hierarchical cluster analysis of 528 females (≥5 replicates per species) based on 256 female chemical features (see Materials and Methods). Algorithm and parameter settings are similar to Fig.S1C. Note that male chemometric tree has recovered more monophyletic groups than female chemometric tree (i.e., species groups cluster in males more than in females).
(D) Frequency histogram of Pagel's lambda estimates, showing more male chemicals (black) to be concordant with the phylogeny than in females (grey). * $P = 0.006$, Mann Whitney U test ($n = 248$ and 256 for male and female chemical compounds, respectively).

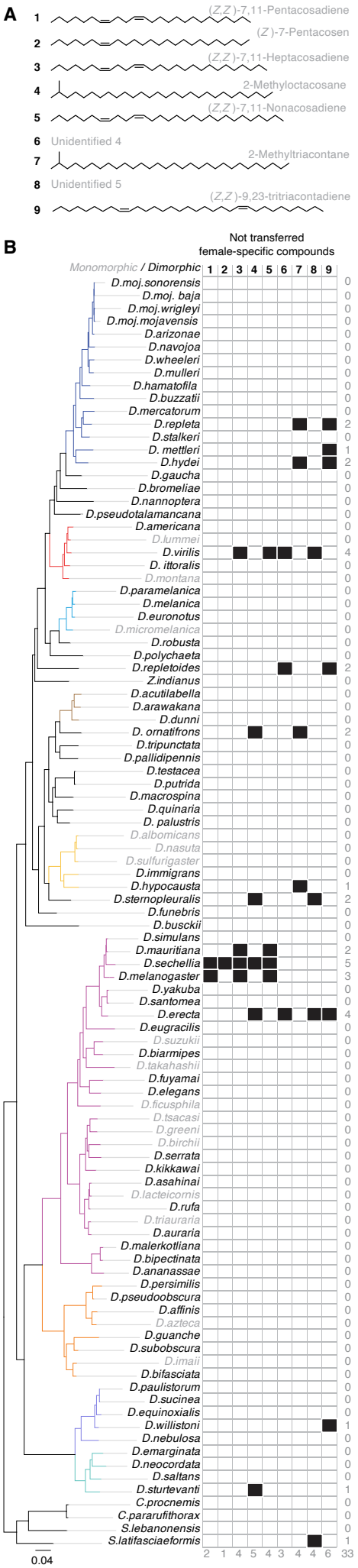

Fig. S2. The female-specific compounds in 99 species within the family Drosophilidae

(A) The chemical structure and names of the female-specific compounds according to the International Union of Pure and Applied Chemistry (IUPAC). Out of 9 female-specific compounds, 6 were identified.

(B) Distribution of 9 female-specific compounds among different drosophilids. Only 15 species display female-specific compounds. Species in black are dimorphic species, while in grey are monomorphic species (See Fig. 2B for more details). Phylogeny on the left side is identical to the tree in Fig. 1A; the branches are colored according to the group identities. Numbers on the right side represent the sum of female-specific compounds present per species, while numbers at the bottom of the table represent number of times each female-specific compound appeared in the different drosophilids.

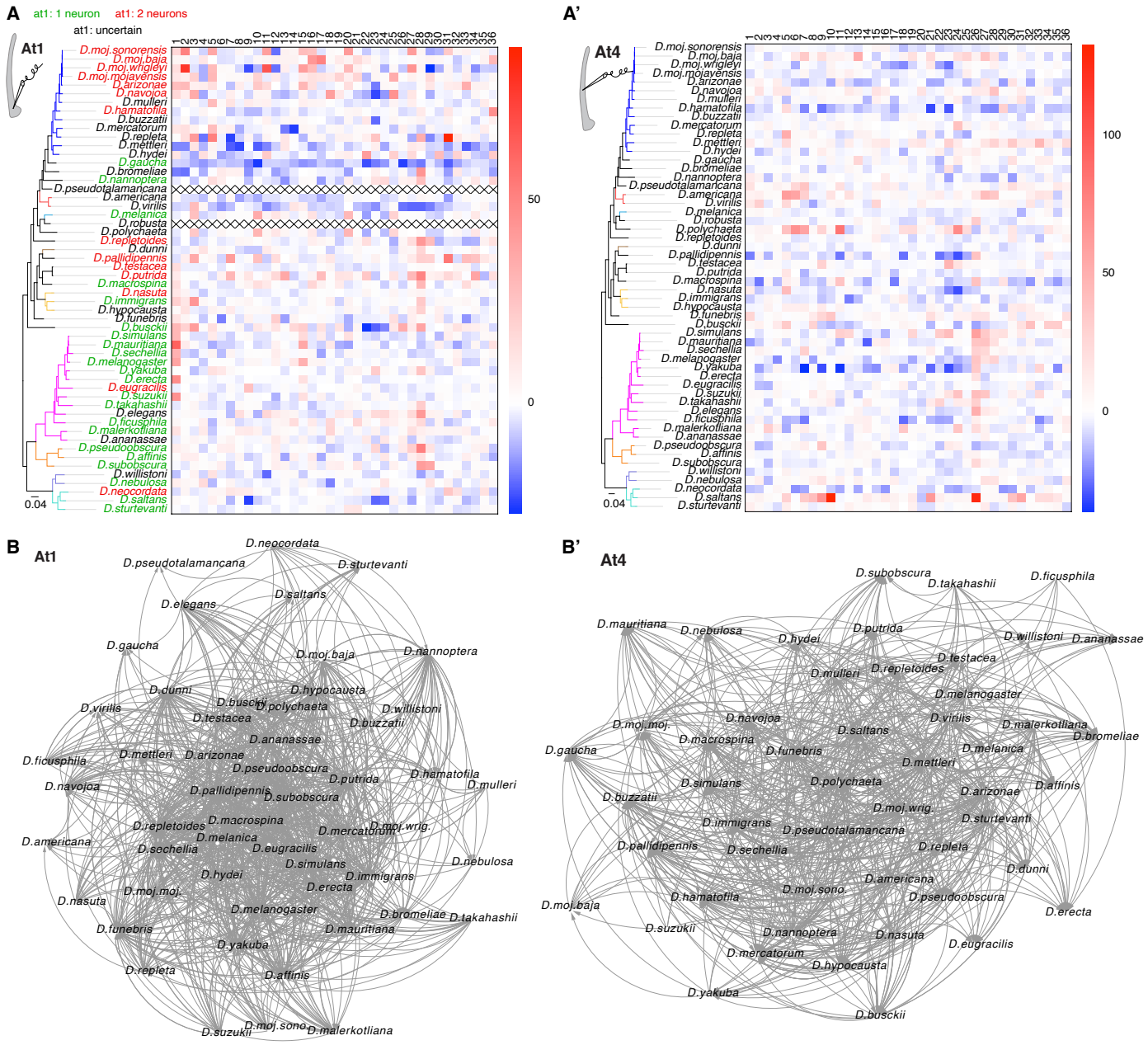

Fig. S3. The intra- and inter-specific communication through trichoid sensilla

(A) Responses of single sensillum recordings of at1 sensillum (Left: schematic drawing) in 54 species to 36 compounds used in Fig. 3B. Species names are arranged according to their phylogenetic relationship; the tree branches are colored according to the group identities. Species names are colored to reflect the presence of one (green) or two (red) neurons in at1 sensilla [95]. Due to technical difficulties, neurons in at1 of species colored in black could not be counted by spike sorting. Note that at1 in *D. pseudotalamancana* and *D. robusta* could not be found. Color codes in the heat map illustrate the response values, which range from blue (inhibition) through white (no response) to red (activation).

(A') Electrophysiological responses of at4 neurons in 54 species to 36 compounds used in Fig. 3B.

(B) Species-species interaction network through at1 sensillum. The directed edges (i.e., arrows that connect the 53 species (i.e., nodes)) represent the olfactory interaction between species pairs (2076 pairs). Direction of the arrow points to the species which is detected. Number of species that detect itself through at1 (i.e., self-loops) is 27 out of 49. The average of correlation coefficient is 0.51 (for more details see Fig. 3D and Table S5). The network was analyzed by Cytoscape (for more details see Materials and Methods).

(B') Species-species interaction network through at4 sensillum. Number of edges is 1559 that connect 52 species, 21 of them are able to detect their own odors by at4 (i.e., self-loops). The average of correlation coefficient is 0.292.

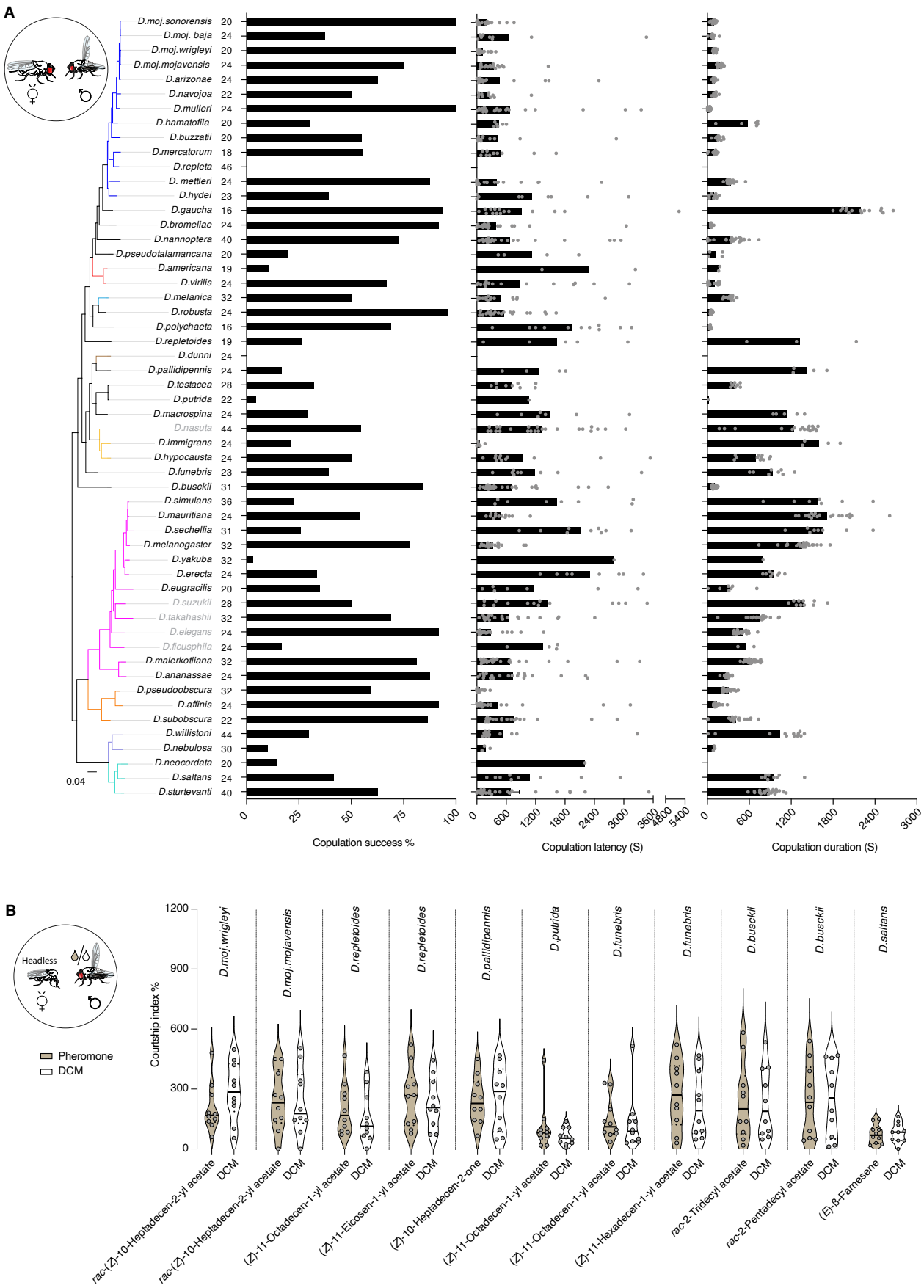

Fig. S4. Sexual behaviors of 54 species within the genus *Drosophila*

(A) Left: Copulation success [%] of virgin couples in the different species (illustrated in schematics on left side) within a 1-hour time window. For more details on the courtship behaviors of a particular species, watch its movies (Movies1 to 427, available on ftp://ftp.ice.mpg.de/outgoing/sessions/20201001-eiCh2neeng). Species are arranged in respect to phylogeny; color of the tree branches corresponds to the species group. Number of replicates is indicated right of species names. Middle: Copulation latency in seconds (s). Males exhibiting no courtship behavior were excluded from analysis. Right: Copulation duration of the different species in seconds (s). Unlike the prolonged copulation time in *melanogaster* group (≥ 15 min) [52], copulation lasts for ~2-3 min in most species of *repleta* group. In this and the below panels, age of males and females is 10 days.

(B) Left: Schematic of courtship arena where a decapitated female is courted by a conspecific male perfumed with DCM or one of the other compounds. Right, y-axis represents courtship index [%] (equal the time a male exhibits courtship behaviors / total amount of recording time (10 minutes)). Ns $P > 0.05$, Mann Whitney U test (n = 10 replicates).

Table Legends

Table S1: List of the 99 species, their classification according to NCBI, their stock number, and their breeding media. Whole-genome sequences (WGS) for 58 of theses 99 species (written in blue) are generated in this study, while the genomes of the other 41 species (written in green) were available from the corresponding reference.

Table S2: Pagel's lambda estimates that explain the correlation between the chemicals among the different male and female species and their phylogenetic relationships. Lambda values are plotted in Fig. S1D; male-specific compounds are identified and shown in Fig. 2D.

Table S3: List of the names (according to IUPAC), appearance times, Kovat's Index, chemical formula, exact mass, and *m*/*z* of male and female-specific compounds.

Table S4: Names and chemical classes of the compounds used in the SSR experiments. 21 of these compounds are synthesized in this study, while the other 15 are commercially available through the listed vendors and CAS numbers.

Table S5: Species-species interaction network through at1 and at4 sensillum. The number of edges represent the olfactory interaction between species pairs, while the number of self-loops represent the intraspecific interactions. Correlation coefficient of each species is given and indicate the number of edges between the species divided by number of edges that could possibly exist.

Table S6: Pagel's lambda estimates that explain the phylogenetic corrected correlations between male-specific compounds and their neuronal response in at1 and at4.

Table S7: Values of sexual preferences of males and females of the different species. Statistical analysis between the tested groups is conducted via chi-square test.

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