Evolutionary Genetics of Reproductive Behavior in *Drosophila*: Connecting the Dots

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Annu. Rev. Genet.
2005. 39:263–91

doi: 10.1146/
annurev.genet.39.073003.112454

0066-4197/05/1215-
0263$20.00

Key Words

evolution, genomics, sexual behavior, ecological genetics, sensory signals

Abstract

Species of the genus *Drosophila* exhibit enormous variation in all of their reproductive behaviors: resource use and specialization, courtship signaling, sperm utilization, and female remating. The genetic bases of this variability and its evolution are poorly understood. At the same time, *Drosophila* comparative genomics now has developed to a point at which approaches previously only possible with *D. melanogaster* can be exploited to address these questions. We have taken advantage of the known phylogenetic relationships of this group of flies not only to place these behaviors in an evolutionary framework, but to provide a roadmap for future genetic studies.

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INTRODUCTION

Researchers using *Drosophila* as a model system to address evolutionary questions are standing on the threshold of a new era. One species, *D. melanogaster*, has emerged as a premier model organism for elucidating basic principles of eukaryotic genetics. Research utilizing *D. melanogaster* has been primarily laboratory based, relying increasingly on sophisticated molecular tools to understand the genetic bases of fundamental biological processes. A major milestone in the history of this model system was the sequencing of its entire genome in 2000 (1), the annotation of which is still being fine-tuned.

The wealth of interspecific diversity in this genus typically has long been attractive to evolutionary researchers hoping to understand the genetic basis of sexual selection and the process of speciation (18–20, 111, 137, 144; see also 35). Observations and experiments over nearly a century have revealed an astounding breadth of morphological, ecological, and behavioral diversity from hundreds of *Drosophila* species. Issues such as the role of reproductive behavior, including ecological and sexual isolation, relative to postzygotic incompatibilities or barriers, remain at the heart of many controversies about the process of speciation.

Evolutionary genetics now sits at the confluence of several biological disciplines, and advances in each will enable the next generation of researchers to ask and answer specific questions about *Drosophila* reproductive behavior. Given the whole genome sequence, the bulk of ecological and behavioral data, and the refinement in phylogenetic relationships of *Drosophila* species, the wealth of interspecific diversity in reproductive traits can now be placed in contexts that allow hypotheses to be generated not only about their evolution, but about the genetic mechanisms underlying them. Recently, these disciplines have begun to cross-fertilize and yield detailed hypotheses about the evolutionary genetics of morphological diversity, ecological adaptations, and reproductive isolation (61, 75, 76, 78, 157). As DNA sequencing technology has become more efficient, the potential for comparative genome sequencing from a number of closely related taxa has been realized. By the end of 2005, sequencing of the genomes of 12 *Drosophila* species will have been completed and the ability to employ molecular techniques previously available only for *D. melanogaster* will become increasingly accessible for these 12 species and their relatives. This means that we can now finally examine the interspecific diversity in such a way as to understand its origins and genetic bases.

Reproductive behavior actually represents a broad array of traits. For this review, reproductive behaviors are organized into two subgroups, premating and postmating. Premating reproductive behaviors include the full range of behaviors of both sexes, including mate location as well as courtship itself, which lead to successful copulation. Postmating reproductive behaviors refer to behaviors of inseminated females, primarily oviposition

and receptivity to remating. Within these broad subdivisions of behavior, each category is itself a complex set of behaviors. This review has two parts. In the first section, we examine interspecific variability in reproductive behaviors in a broad and comprehensive evolutionary framework. All behaviors with which we are concerned involve the detection of signals, either from the environment or from another fly, and the responses of individuals to those signals. Thus in the second part of the review, we explore the potential sources and organization of genetic variability in the signals and the sensory systems that receive and process them in order to frame future experiments to elucidate their evolution.

REPRODUCTIVE BEHAVIORS IN DROSOPHILA

Extensive studies have demonstrated that the pre- and postmating reproductive behaviors referred to briefly above exhibit great interspecific variability in *Drosophila*. It is this variability that is treated here, accompanied by discussion of approaches for its study. Space limitations prevent the inclusion of all categories of variability. We therefore focus upon behavioral variants that appear to be more or less discrete phenotypes and for which a sufficient number of species have been characterized. In order to present the changes in reproductive behaviors in an evolutionary framework, we capitalize upon the known phylogenetic relationships among species. The genus *Drosophila* is divided into a number of species groups, radiations, and subgenera (156). Recent phylogenetic work (e.g., 119, 147) has provided a framework of evolutionary relationships within this genus that can be used to examine behaviors important to reproduction. The fine points of many relationships among groups are continually being refined, but the lack of resolution regarding those details should not detract from the goals of this review.

Premating Reproductive Behaviors

Locating breeding sites. For any given species, mating takes place at particular locations and at specific times of the year and/or day. Thus, an important part of premating behavior involves locating sites, via long-range signal detection-response systems, where prospective mates will be encountered. Signaling and response mechanisms underlying the location and utilization of such sites constitute an important reproductive behavioral process. *Drosophila* species vary widely in the resources they use, and thus the signaling mechanisms are expected to exhibit genetically based differences.

For most species of *Drosophila*, resources used for adult feeding are at or near the oviposition sites. Host or resource use thus can be considered to be both a premating and a postmating behavior and is treated as such here for the sake of economy. *Drosophila* species range from generalists (oligophagy, polyphagous) to specialists (monophagy). In layman's terms, *Drosophila* are referred to as fruit flies. However, many *Drosophila* species have become associated with decaying plant material including cacti, flowers, mushrooms or other fungi, tree sap or slime fluxes, and even with the excretory organs of land crabs (118). Most of these resources are associated with a unique microbe fauna that provides both larval and adult *Drosophila* with nutrition. This ecological diversity raises questions about the phylogenetic distribution of resource localization strategies and the genetic mechanisms that control their identification and utilization by flies.

Figure 1 is an overview of resource use by different species groups in the genus *Drosophila*. There is a considerable degree of conservation of resource type within *virilis-repleta* and *immigrans-tripunctata* radiations. Based on this phylogeny, the ancestor of the *virilis-repleta* radiation bred in sap or slime fluxes. There was then a switch to flowers and small, dry fruits in the lineage leading to the *repleta* radiation, with a subsequent switch to

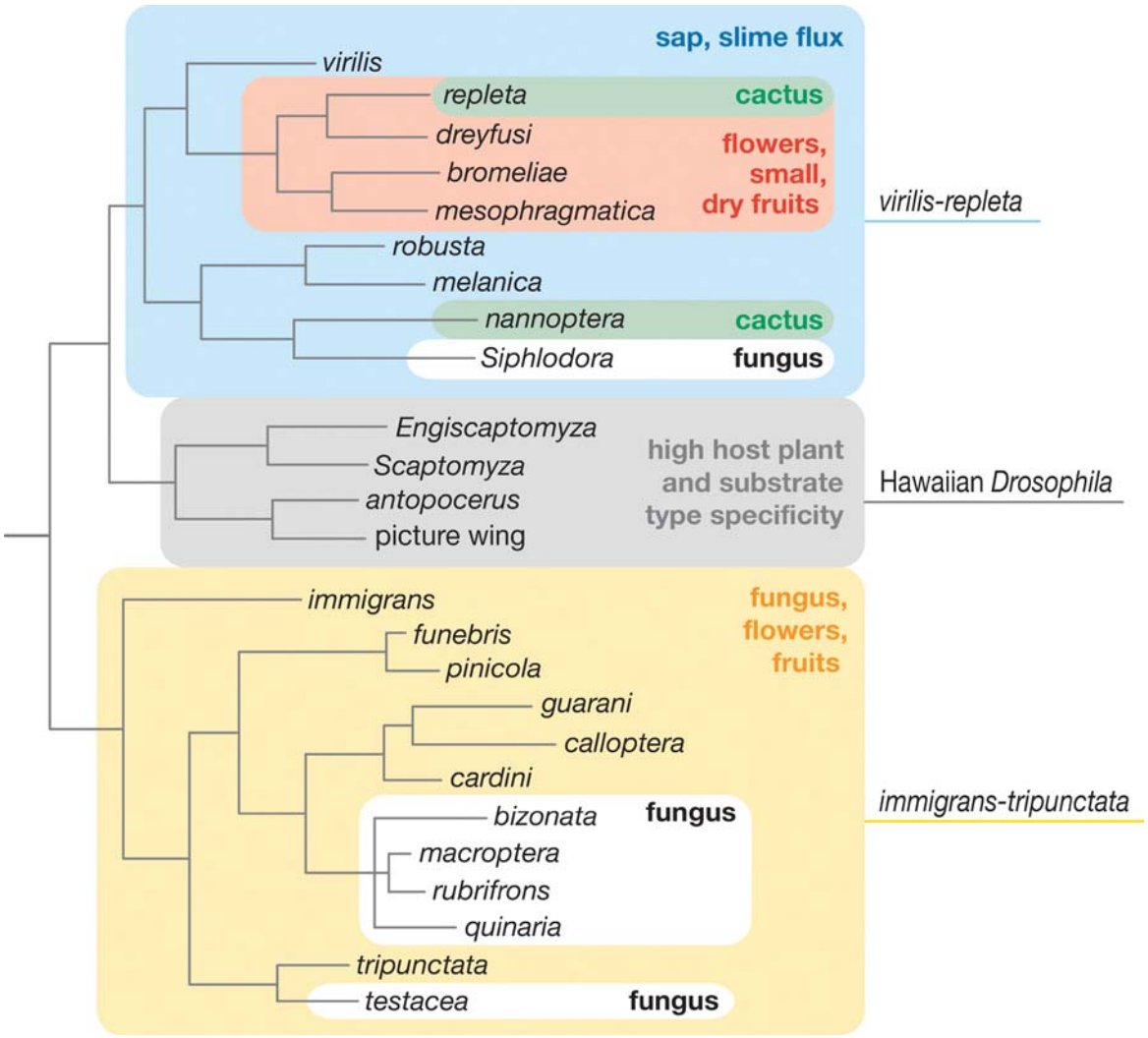


Figure 1

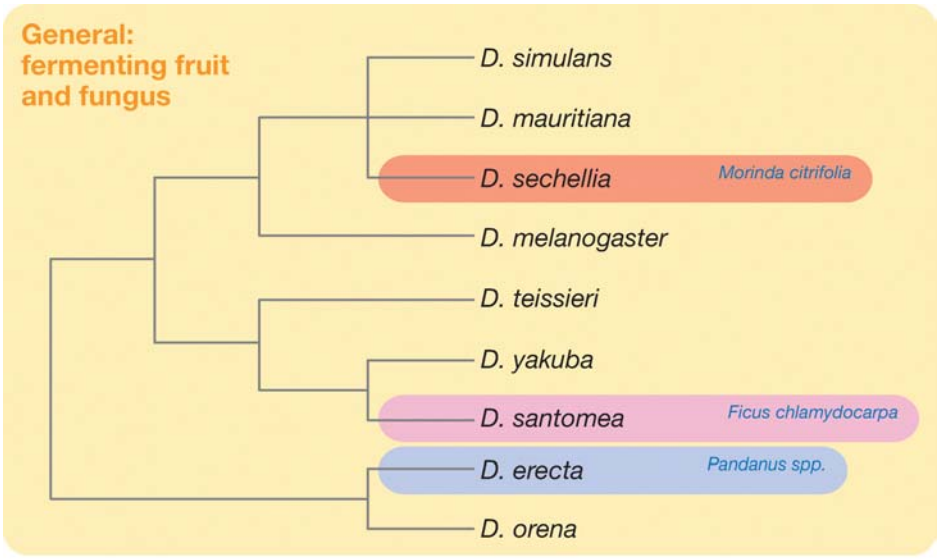
Phylogenetic distribution of host-resource mapped onto the phylogeny of *Drosophila*. The ancestor of the *virilis-repleta* radiation bred in sap and slime flux (blue). One lineage then evolved to use flowers and small, dry fruits (orange). Cactus use (green) evolved independently at least twice and fungal specialization (white) at least once. The ancestor of the *immigrans-tripunctata* radiation was a generalist on fungus, flowers and fruits (yellow). Specialization on fungi (white) evolved independently at least twice. (Modified after 119, 136, 156)

cactus in the *repleta* species group. Based on this phylogeny, the *nannoptera* group represents an independent exploitation of cactus as a host substrate. Patterns of host switching within the *immigrans-tripunctata* radiation are less clear. Many of these species are fungus specialists, but several taxa also are regularly associated with flowers or small fruits, suggesting that this group is able to utilize smaller, more temporally restricted substrates. The Hawaiian Drosophilidae, in contrast to the remainder of the genus *Drosophila*, are highly host plant specific, with roughly 80% of picture wing species utilizing a

single family as oviposition substrate (67, 77, 104).

A number of *Drosophila* species are known to specialize on a single host resource (**Figure 2*a*,*b***). In some cases, the genetics of this phenomenon is quite well understood. *Drosophila sechellia*, for example, has specialized on morinda fruit (*Morinda citrifolia*), a resource that is toxic to all other members of the *melanogaster* species group (**Figure 2*a***). *Drosophila sechellia* is resistant to these toxins and, in fact, requires them for full stimulation of oviposition behavior. Similarly, *D. pachea* of the *nannoptera* group has specialized on senita (*Lophocereus schottii)*, which is highly toxic to other *Drosophila* (**Figure 2*b***). Thus evolution of specialization seems to have taken place on two separate scales. First, the radiation of an entire group of species onto a certain type of host has taken place. Within these clades, however, species are able to further specialize on particular types of resources within the host taxon.

Once they are at the breeding site, however, not all species orchestrate their courtship activities in the same way. Some species



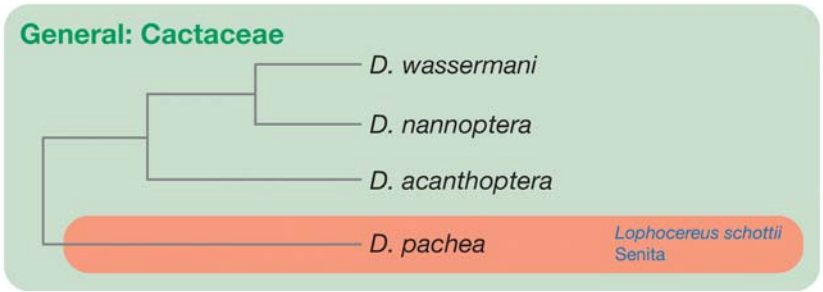


Figure 2

Examples of host specialization within the *melanogaster* and *nannoptera* species groups. (*a*) In the *melanogaster* subgroup the ancestral condition is the general use of rotting fruit and fungus (*yellow*) Subsequent specializations on *Morinda* (*red*), *Ficus* (*green*), and *Pandanus* (*violet*) evolved separately in three species. (*b*) The nannoptera species are all cactophilic and use a suite of host species. One taxon, D. pachea, has specialized on senita (*Lophocereus)*. (Modified from 68, 122, 163)

display at sites away from where they feed or oviposit (67, 104, 138). In others, such as *D. nigrospiracula* (93), males defend mating territories on parts of the plant that are away from the feeding locations. Species such as *D. melanogaster* and *D. simulans*, on the other hand, mate right on the rotting fruit where they feed. Mating location, relative to food resources, has not yet been sufficiently documented in enough species to permit any meaningful phylogenetic mapping.

Regardless of where courtship takes place, it will involve signaling between individuals that takes place at shorter range than those signals that attract flies to aggregation sites. Courtship has several components, starting with the identification, at the mating site, of conspecific members of the opposite sex, following which courtship and all of its various components can proceed. For species of *Drosophila* that have an exclusive association with a particular resource, once they arrive at the feeding site, the only other *Drosophila* they will encounter will be conspecific females and males. In such specialist taxa, courtship involves discriminating between conspecific males and females, and engaging in species-specific courtship processes in ways that will ensure their reproductive success. For *Drosophila* species that mate at resources utilized by congeners, additional systems must be present that allow them to discriminate members of their own from other species prior to investing energy and time in the courtship process. Reproductive behavior includes male-male interactions as well as those between the sexes, although the former are less well studied. Sexual signaling takes place in three sensory modalities: visual, auditory, and chemosensory.

Visual sexual signals. The role of visual signals during courtship can be inferred from several observational studies. In order for signals to have a visual component, they must be performed in the light and conducted within the visual field (i.e., in front) of the receiving individual. For many species, laboratory and field observations have documented the relative positions of males and females with respect to each other during courtship. For species in which courtship has not been observed directly, morphological or coloration patterns may be such that visual signaling can be inferred. For some *Drosophila* species, the sexes differ with respect to the potential for certain aspects of visual signaling. This difference is a function of the fact that, during the specific part of courtship involving male attempts to mount, or his licking of the female's genitalia, the male is behind the female and can receive, but not transmit visual information. Male visual information, then, will only be transmitted when males leave this position and move in front of females, or, as in some lekking Hawaiian species, perform ritualized displays to attract females to a mating site. For this reason, we examine the phylogenetic distribution of visual displays separately for males and females.

Figure 3 illustrates the phylogenetic distribution of whether or not males tend to position themselves in front of females during courtship as opposed to remaining behind them, out of view. There is a tendency for males of species in the *virilis-repleta* radiation to court behind the females, suggesting that male visual displays are not the primary form of sexual signaling in these taxa. This interpretation is consistent with the fact that these species show effectively no sexual dimorphism in coloration, wing pattern, or other visible morphological traits. It is also supported by the existence of exceptional taxa, such as *D. acanthoptera* in the *nannoptera* group, in which there is a sexual dimorphism for body color and in which males court in front of females.

Females are not merely recipients of visual signals during courtship. In a large number of species, females indicate their receptivity to males by a characteristic spreading of their wings (**Figure 4*a***). This behavior is typical of species in the *virilis* (162) and *repleta* groups (99), but has also has arisen in several other groups of *Drosophila* (137). The distribution of this behavior is variable,

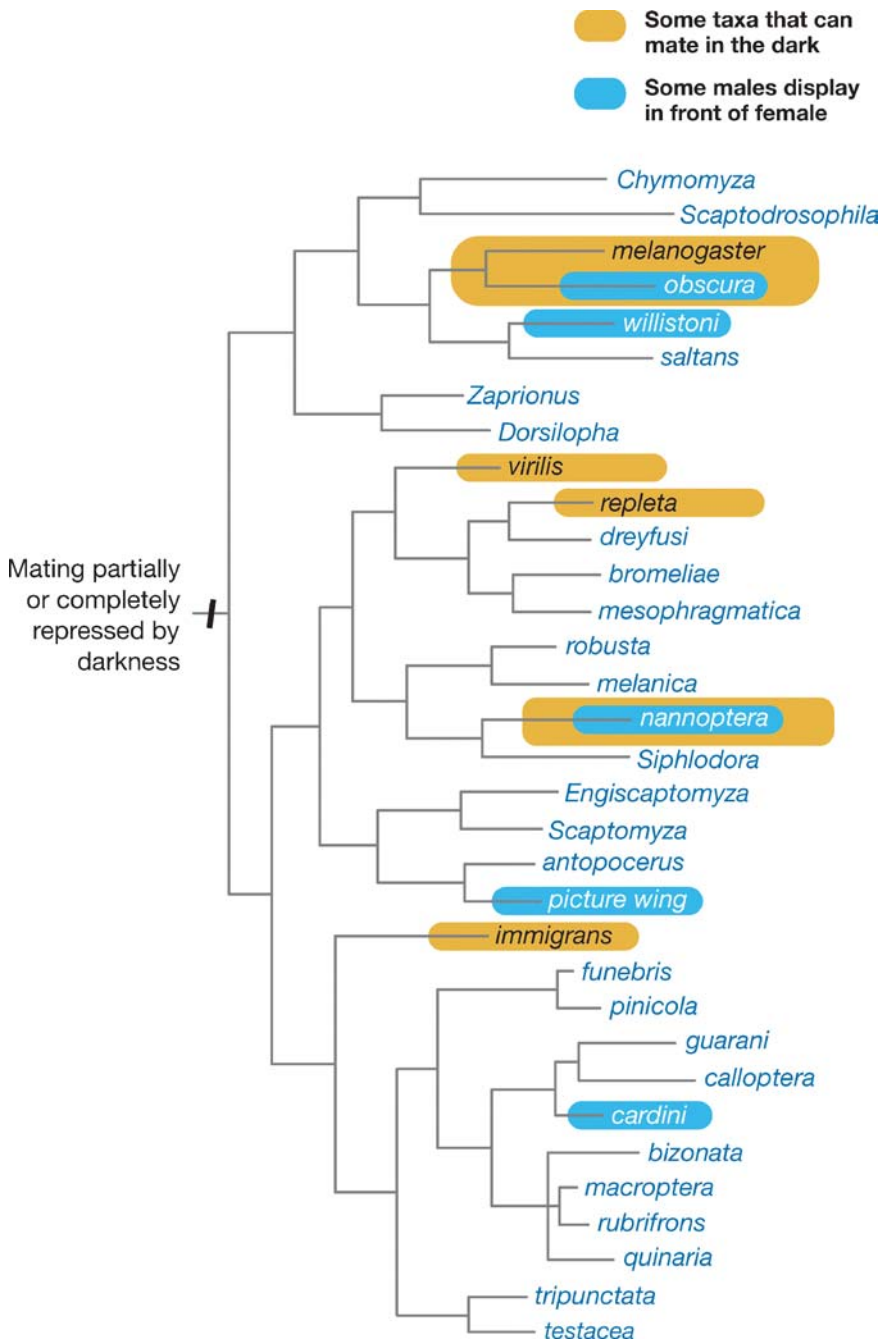


Figure 3

The evolution of male visual signals in courtship behavior in response to light availability. The ancestral condition is to have mating that is either partially or completely repressed by darkness. Those taxa that can mate in the dark are shown in tan. Taxa where males display in front of females are indicated in blue (Modified from 63, 65, 137).

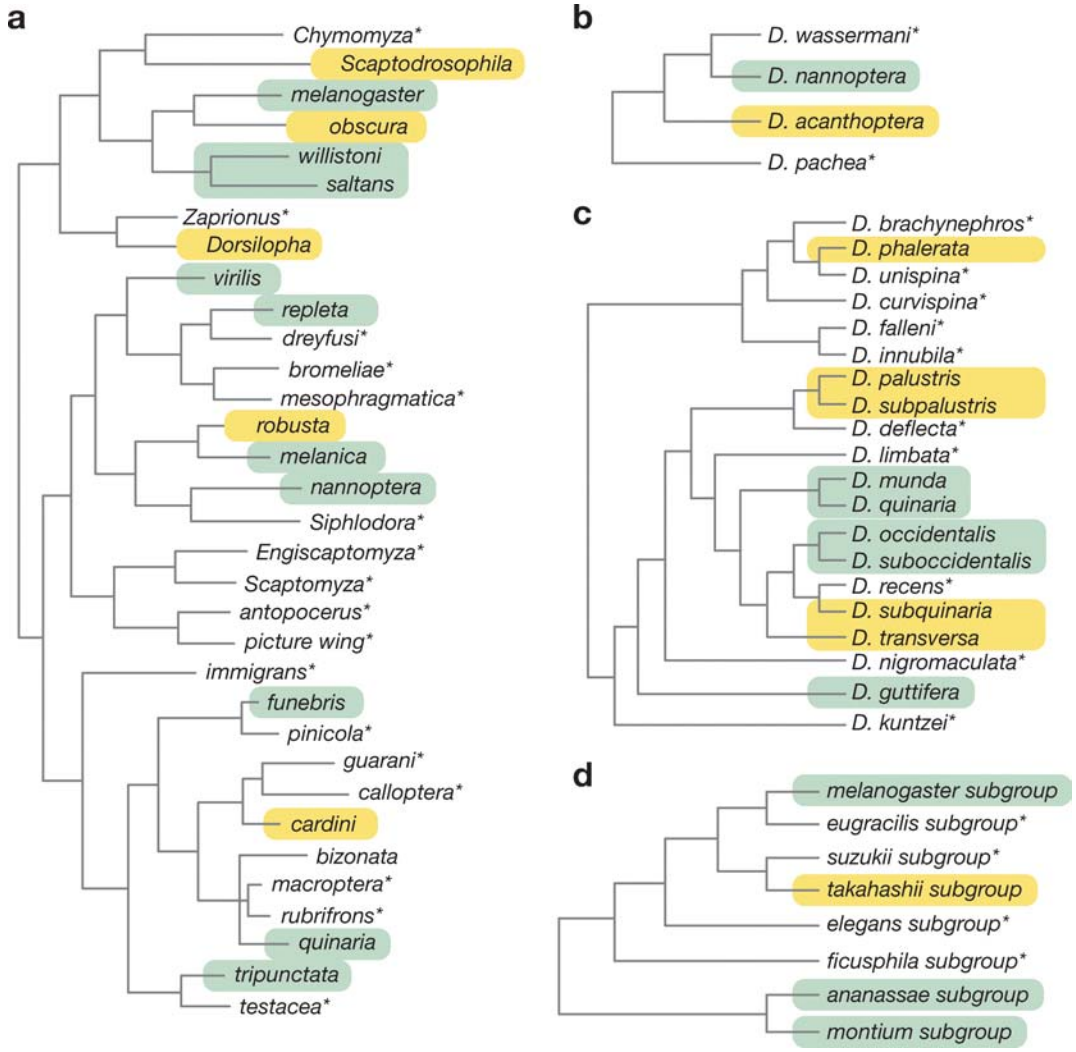


Figure 4

Phylogenetic distribution of female wing spreading display during courtship (modified from 137). No data are available for taxa with an asterisk. (*A*) Overview of the evolution of female wing spreading displays in the genus *Drosophila* and relatives. Clades where females of some species display (*green*) and do not display (*yellow*) are shown. (*B*) Data from only two taxa, *D. nannoptera* and *D. acanthoptera*, are available in the *nannoptera* group so the exact character transition is uncertain (163), (*C*) The wing spreading display has evolved at least three times in the *quinaria* species group (phylogeny modified after 128). (*D*) Data in the large *melanogaster* species groupare highly variable, particularly within the *melanogaster* and *montium* species group, where female wing spreading may have been gained or lost several times.

particularly in the *nannoptera*, *quinaria*, and *melanogaster* groups (**Figure 4*b–d***). Likewise, males of these species typically will not attempt to mount a female until she has given this signal, whereas males in those species groups where female wing-spreading is absent will attempt intromission on a repeated and constant basis. Females of any species may perform other behaviors that indicate their receptivity, such as simply slowing down their

locomotion (154), or spreading their vaginal plates (137), but these behaviors are not always strictly visual or even discrete, and thus less comparative information is available about them.

In addition to those visual behaviors that can be directly scored, the importance of unspecified visual cues can be inferred from studies in which insemination rates have been compared between pairs of the same species placed together in darkness and in light (63–65). Based upon the outcomes of this type of study, species can be considered to be either light independent, partially light dependent, or completely light dependent in their mating behavior (**Figure 3**).

Auditory signals. Auditory signals are utilized in the courtship of most *Drosophila* species. Courtship songs have been studied in over a hundred *Drosophila* species, and the majority of these have focused upon male songs and upon song variability at the inter- and intraspecific levels (70). Females of species in the *virilis-repleta* radiation and the *nannoptera* group also regularly produce songs while being courted (36, 48, 109, 124), and in a number of these species, an actual dialogue occurs between the sexes during courtship, referred to as "dueting" (10, 43). Males of many species also produce songs that are unlike courtship songs, but rather appear to be utilized to dissuade the amorous advances of other males (145).

The diversity observed among *Drosophila* species in male courtship song is so variable that it is difficult to describe in manageable terms. Most songs are composed of various pulses or bursts that have different structural and temporal features that distinguish them at the species level. Some species utilize one "type" of song, whereas others may perform four or five. Some song types are performed earlier in courtship than others. One caution should be entertained when examining songs that occur later in courtship. Recordings of courtship songs are conducted in small chambers in which female decamping is not one of the options available to unreceptive females. Courtships observed under these conditions are thus likely to last longer than those in nature where uninterested females can depart. Under confined conditions, therefore, males may become frustrated, and as courtships continue, exhibit behavioral components rarely observed in nature. A further complication is that different investigators have employed different terminologies to describe song parameters. There do not appear to be any homologies between lineages for particular song elements. In rare cases, a species will produce no sounds at all. Nonetheless, we have attempted to capture this variation in a meaningful way. Because there is no simple way to reduce all of the variation to character states that then can be placed in a phylogenetic context, we have scored members of a group or subgroup as to the number of the courtship song components typical of the group.

For each species group, we have summarized the number of different song types a species has been reported to produce (**Figures 5*a*–*e***). The *melanogaster* species group is a large clade that shows a highly variable array of auditory mating strategies. **Figure 5*a*** shows the distribution of song number within the *melanogaster* subgroup. Males of most species produce two song types, a sine song (158) and a pulse song (48), the latter of which exhibits important interspecific differences. Interestingly, males of one species, *D. yakuba*, have lost the sine song and only rely on the pulse song for identification of conspecific individuals. Males of the *montium* subgroup (not shown) produce one type of song, which varies among species, but is largely produced once copulation has begun (150, 151). This has reached its extreme proportion in two species of this subgroup, *D. birchii* and *D. serrata*, where males produce song only during copulation itself (71). In males of *D. ananassae* and others of the subgroup, males produce either one or two types of pulse songs (36, 168).

The *obscura* group is the sister clade of the *melanogaster* group yet produces completely

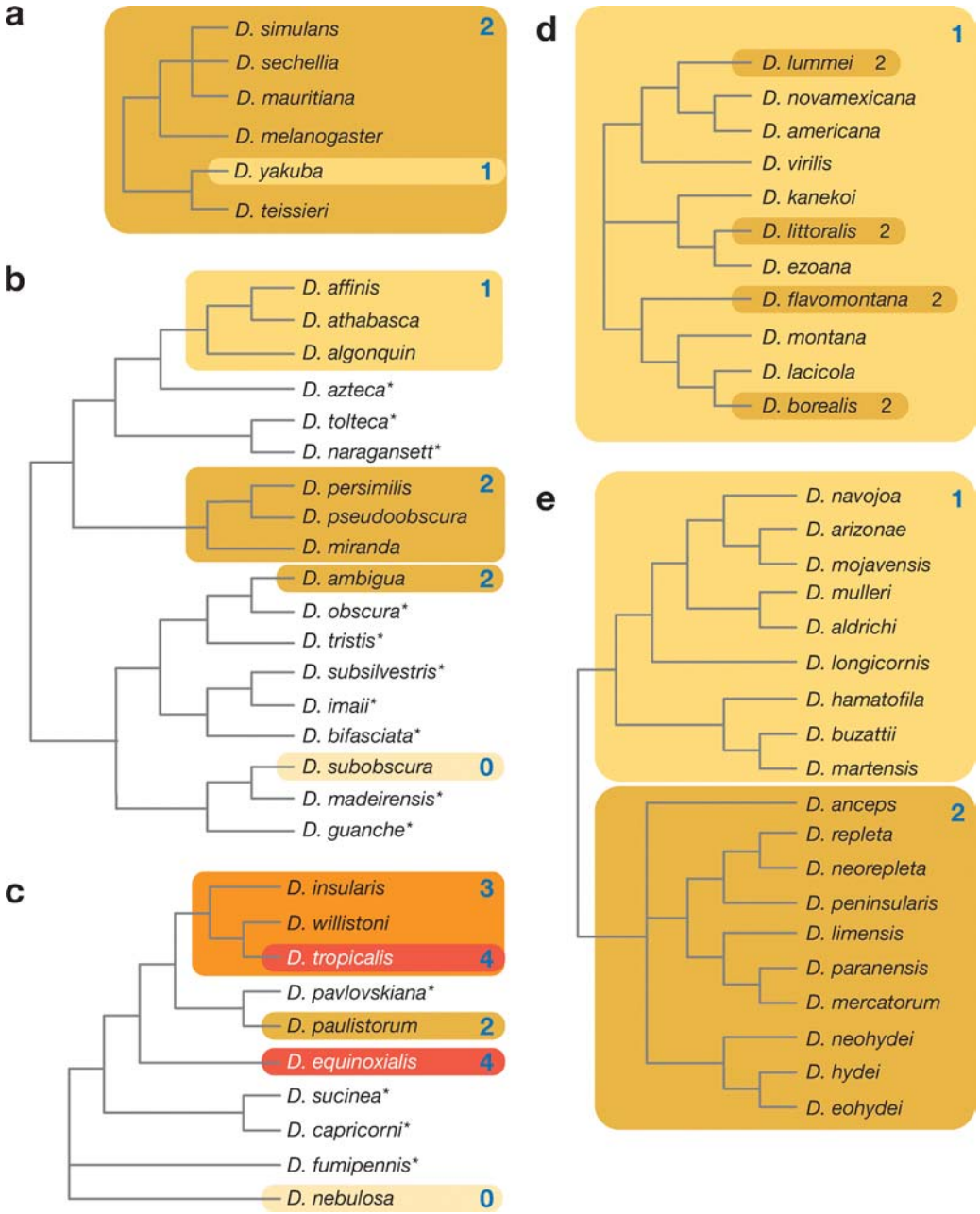


Figure 5

Evolution of the number of male courtship songs in the (*a*) the *melanogaster* species subgroup (modified from 48, 128); (*b*) the *obscura* species group (48, 108); (*c*) the *willistoni* species group (modified from 58); (*d*) the *virilis* species group (modified from 70, 135); and (*e*) the *repleta* species group (modified from 45, 49). Increasing color intensity from pale tan (0) to red (4) indicate number of male courtship songs observed. Information is lacking for species with an asterisk.

different types of song (**Figure 5*b***), referred to as high- and low-repetition songs (107). Neither of these is homologous to the pulse and sine songs observed in the *melanogaster* species. It is difficult to determine how these different signaling strategies may have evolved. Some species in the *affinis* subgroup (*D. affinis*, *D. athabasca*, *D. algonquin*) seem to use only a single type of song, whereas others in the *pseudoobscura* (*D. pseudoobscura*, *D. persimilis*, *D. miranda*) and *obscura* (*D. ambigua*) subgroup use two. Based on the current data, however, it is not clear whether the ancestral condition in the *obscura* group was one or two song types or whether the two-song strategy seen in the *pseudoobscura* and *obscura* subgroups was derived once or twice. Perhaps the most interesting song strategy observed in this group is found in *D. subobscura*, where males do not sing at all (48). It is likely that *D. subobscura* has shifted to an entirely visual mate recognition strategy since these species do not mate in the dark and males display in front of females (**Figure 3**).

Males of *willistoni* group species can produce up to four types of song (**Figure 5*c***), although the exact evolutionary history of song loss and acquisition is not completely clear (120). Although they are not sister taxa, both *D. tropicalis* and *D. equinoxialis* utilize four courtship songs, suggesting a complex series of gains and losses of song type and number for the intervening taxa. Interestingly, here again, there is a species, *D. nebulosa*, that does not sing at all. This species may rely on visual more than on auditory signaling. *Drosophila nebulosa* and *D. fumipennis*, the other basal member of this group, both have pigmented wings, a character not seen in the other *willistoni* taxa. Both of these species also display in front of the female, unlike all other *willistoni* taxa (**Figure 3**).

Males in the *virilis* group produce either one or two types of pulse songs. The evolution of this behavior, however, is quite complex. It appears that the use of a second pulse song has evolved at least four times in this group (**Figure 5*d***). In males of the *repleta* group (49), we also see a pattern in which there are either one or two types of songs produced (**Figure 5*e***).

Chemosensory signals. Chemical communication during courtship in *Drosophila* is thought to be mediated by the hydrocarbons (HCs) found in the adult epicuticle. Because they consist largely of long chain compounds that are not volatile, these HCs likely function at short range, through contact. Some HCs serve as aggregation pheromones (7–9, 66, 103, 127). Hydrocarbons can exhibit a remarkable degree of variability. They can differ in chain length, in the presence or absence of double bonds, and in the positions of the double bonds. Among *Drosophila* species, chain lengths range from between 20 and 40 carbons and for the most part are composed of various alkanes and alkenes (single and double bonds). Most *Drosophila* species produce a blend of HCs, and the characteristics of this blend can vary with age, sex, diet, and geographic origin within a species. Sexual dimorphisms in HCs can range from subtle differences in relative quantities of one or more molecules to the presence of completely different HCs between the sexes. Interspecific differences are also both quantitative and qualitative in nature. Considerable evidence exists that HCs play a role in sexual signaling within a species as well as for species recognition (reviewed in 52). Furthermore, because HCs are known to be important in water balance, these molecules and the genes controlling their production can be under both sexual and natural selection (99).

HC length is fairly well conserved in the genus *Drosophila* and can be roughly divided into three classes: short, intermediate, and long chains. The short chain morphology (23–29 carbons) is found in two groups, the subgenus *Sophophora* (*melanogaster* and *willistoni* species) and the Hawaiian *Drosophila*. Intermediate length chains (22–31 carbons) are seen in the *virilis* group. The *repleta* group has the longest chains, from 28–40 carbons. Where these HC differ is in degree of

sexual dimorphism and presence of unique molecules. The Hawaiian *Drosophila*, for example, have a unique, sex-specific HC profile. Other groups, such as the *repleta* species, show very small quantitative differences between the sexes.

HC variability in the *virilis* group roughly follows the phylogenetic relationships of the phylads (**Figure 6*a***). The *virilis* phylad, with the exception of *D. lummei*, shows some dimorphism in HCs (8). The *littoralis* and *montana* phylads, with the exception of *D. kanekoi*, are not dimorphic. The pattern of dimorphism in HC profiles is not as simple in the *melanogaster* subgroup (**Figure 6*b***). Although the ancestral reconstructions are equivocal, it is clear that dimorphism in HC characters is highly plastic in the *melanogaster* group and has shifted back and forth several times.

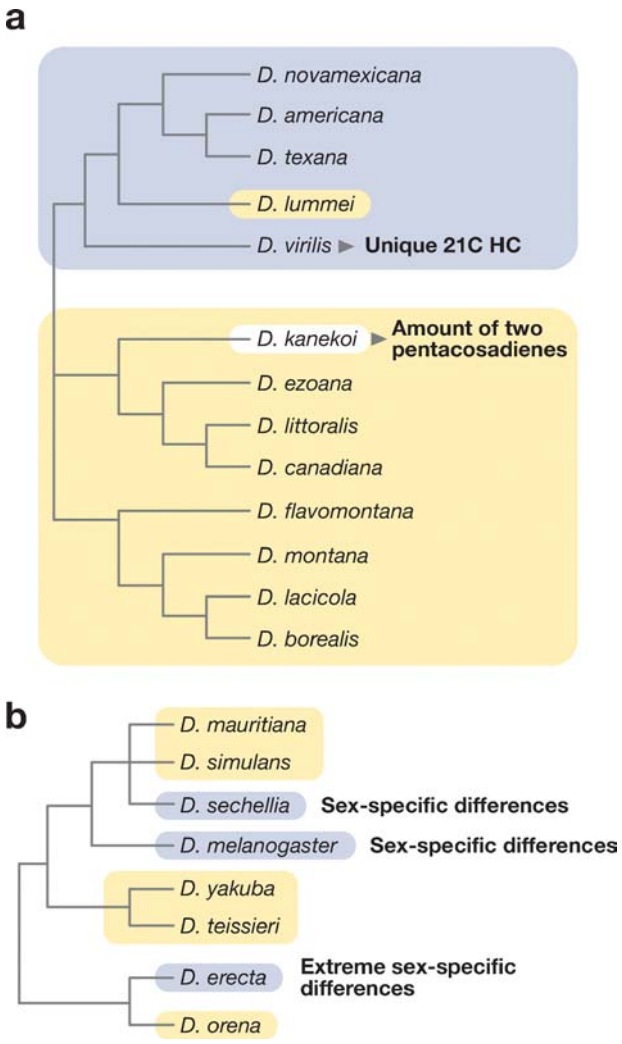


Figure 6

Evolution of hydrocarbon profiles in (*a*) the *virilis* species group (modified from 6, 135) and (*b*) the *melanogaster* species group (modified from 52, 74, 128). Species dimorphic for HC characters are shown in blue, those shown in yellow do not display sexual dimorphism in HC profile. Whether HC profiles are dimorphic or not seems to track phylogenetic relationships in the *virilis* group but are much more variable in the *melanogaster* subgroup.

Postmating Reproductive Behaviors

How do the reproductive behaviors of various species differ once mating has occurred? Processes occurring within the mated female can have significant effects on the reproductive success of both the female and male. For example, if females remate, it may create the opportunity for sperm competition. The ultimate fate of sperm inside the female reproductive tract can be the product of the continuing influence of a mate on the female's behavior, as can the propensity to remate and to oviposit, utilizing the sperm of that specific male. Finally, mated females must locate and utilize suitable oviposition sites.

Female remating. Species of *Drosophila* exhibit enormous variation in the frequency at which females remate (reviewed in 94, 96). In some species such as *D. subobscura*, *D. acanthoptera*, and *D. silvestris*, females effectively mate only once in their lifetimes, whereas in other species such as *D. hydei* or *D. nigrospiracula*, they have been observed to remate up to four times in a given morning. Frequencies at which females remate are presented in **Figure 7**.

The frequency at which females remate has important implications for their own reproductive fitness as well as that of their mates. In *D. melanogaster*, substances transferred to females during mating have been shown to reduce lifespan (21, 55), and it has been proposed, though not demonstrated, that

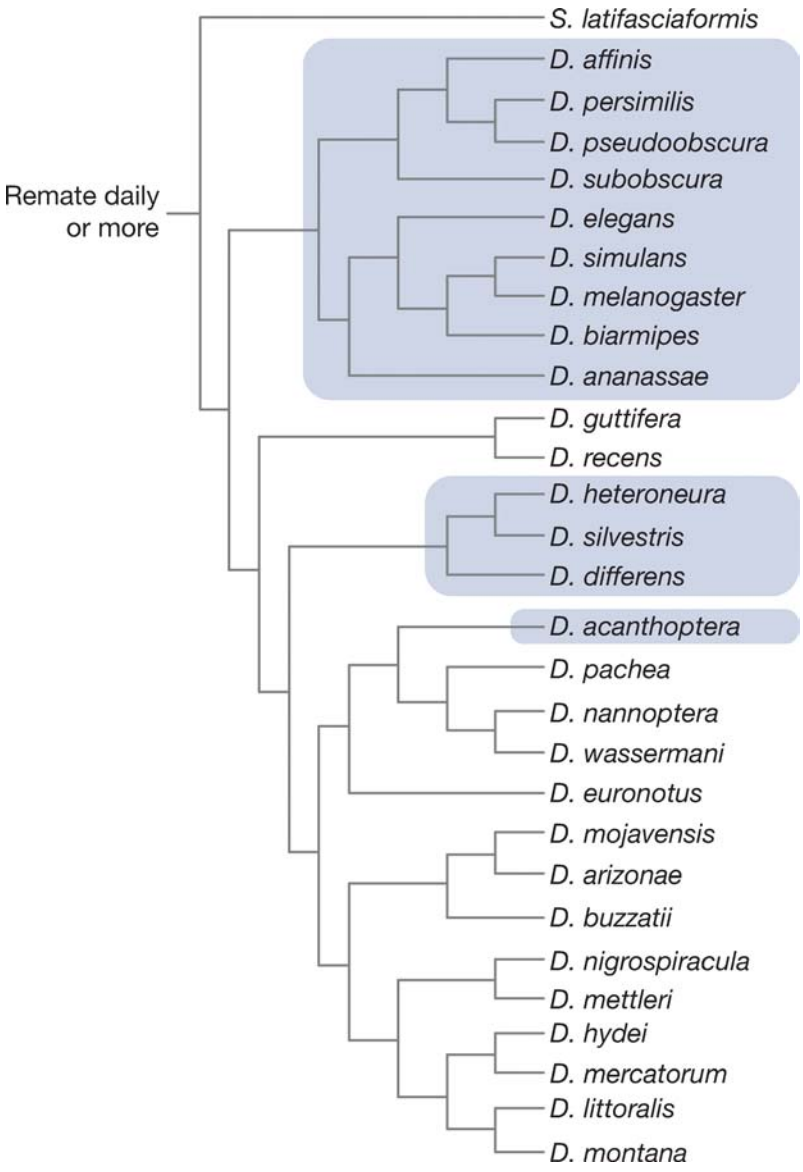


Figure 7

The evolution of female remating frequency. Although the ancestral condition in the group is to remate daily or more often several lineages have evolved taxa where remating occurs less than daily (blue). (Modified from 96, 132)

copulating pairs are more vulnerable to predation and parasitism (117).

Remating and sperm utilization appear to be under the control of many factors: the characteristics of the ejaculate typically passed to females on a given mating and the interaction between the ejaculate components and the female's reproductive system. While these factors have been most widely studied in *D. melanogaster*, this species turns out not to be representative of the nature of these factors in the other species (94, 96). *Drosophila* species exhibit tremendous variation in their sperm-storage organs and in the use of these organs for the storage and retrieval of sperm (116). Species differ, as well, in the number of sperm males typically transfer during a single mating, from as few as 14, in *D. pachea* (114),

to 25,000, in *D. pseudoobscura* (133). For *D. melanogaster*, female remating is considered to be at least in part under the influence of the physical presence of sperm in the storage organs (89), but more recently, the effects of a number of different male accessory gland products have been demonstrated to influence not only female remating latency but the onset of oviposition and differential sperm utilization as well (26, 82, 167).

Sperm utilization patterns are such that a tendency toward sperm mixing exists in species in which females remate rapidly, but last male precedence exists in those species where females remate less often. This is likely to be a function of the numbers of sperm involved, as in species characterized by rapid female remating, where females receive few sperm on any given copulation, whereas relatively large numbers are delivered during a single copulation in species where females take longer to remate (94, 96, 113).

Seminal fluid components other than sperm appear to be pivotal to many of the processes that occur inside the female after she mates. A rapidly evolving group of approximately 80 proteins (25) are transferred to females in *D. melanogaster*. As these become characterized, it is clear that they play important roles in sperm storage and recovery, in female remating latency, and in the onset of oviposition (82, 167). In *D. melanogaster*, a species in which females remate after approximately 5 days, the receipt of certain seminal fluid components has been found to be responsible for shortening the life spans of females (21, 55). These studies suggest that seminal fluid components have evolved in ways that permit males to control female behavior long after the copulation is over. Mating systems of other *Drosophila*, however, suggest that females of some species have evolved different processes for dealing with seminal fluid compared with *D. melanogaster* (94, 96). Females of a number of *Drosophila* species actually extract large quantities of ejaculatory proteins (97, 98, 114, 115) and phosphorus (100) and use them to produce eggs. Because accessory gland proteins in *Drosophila* are rapidly evolving (25, 79), the identification and characterization of seminal fluid proteins in these other species has lagged behind *D. melanogaster*, and thus there are few comparative data at this time.

One obvious question, given the detrimental effect of mating on life span in *D. melanogaster* females, is whether females in rapidly remating species have even greater reductions in their life spans, given their frequent mating, or whether they have evolved some mechanism to escape this cost of mating. We addressed this question by examining the longevities of females from a set of other *Drosophila* species in which female mating frequencies are greater, as well as less, than those of *D. melanogaster*; *D. nigrospiracula*, in which the remating frequency is four times a day (91); *D. pachea*, *D. mettleri*, and *D. mojavensis*, in which females remate daily (91, 113); and *D. acanthoptera*, in which they mate once in their lifetime (114). We also included *D. melanogaster* as a control. The influence of mating was examined by placing females in food vials, which were changed daily, at which time any dead flies were counted. Females were placed in three treatment groups, with 25 individuals per treatment: single virgin females, females paired continually with males, and females paired with other females. In the female-female group, survival of focal females was scored. When either a male or a companion female died, it was replaced until the death of the focal fly was observed. The experiment was repeated twice. Mean age at death is presented in **Table 1**. The survival rates of mated versus virgin or control *D. melanogaster* females are consistent with earlier studies showing a significant effect of mating in reducing female longevity. Of the other species, for *D. acanthoptera*, in which females only mate once in their life times, survival was significantly reduced in mated females. In the remaining four species, however, females did not suffer any reduction in longevity, despite continual mating. These four species, *D. pachea*, *D. mettleri*, *D. nigrospiracula*, and *D. mojavensis*, are

unrelated, and their mating systems have very different characteristics. For example, *D. mojavensis* females incorporate large amounts of seminal proteins from males into their somatic tissues and ovarian acolytes, whereas females of the other two do not. *Drosophila pachea* males pass very few sperm per mating and females are highly sperm limited. Therefore, in each lineage, different mechanisms have likely evolved to overcome or disarm the detrimental effects of mating, but the nature of those mechanisms remains a mystery for now.

Oviposition behavior. The basis for oviposition site selection may overlap, in part, with identification of mating sites, but not necessarily completely. Long-range signals, largely volatile but possibly also visual, as well as microclimatic factors, attract the flies to potential sites. Females use contact signals, however, to make oviposition decisions, and genetic variation exists within a species (4, 73, 88) as well as among related species (50, 122) for oviposition preferences. It is largely assumed that at a gross level, female oviposition preferences, in terms of resource type, will be governed by long-range resource location, discussed above, which probably explains the paucity of data at the oviposition level. Other aspects of oviposition, such as periodicity, have not been studied in enough species to examine this reproductive character in a comparative or evolutionary context (50, 131).

Table 1 Mean age at death for six species of *Drosophila* with different female remating frequencies. Flies were housed either alone (SF), with another female (FF), or with a male (MF) and scored daily for survival. Significant differences are shown with an asterisk. Flies were changed every other day to fresh food vials until the focal female died

		Mean age at death		
Species	**Rep**	**SF**	**FF**	**MF**
D. melanogaster	1	70.9 ± 3.8	71.9 ± 3.6	53.3 ± 5.1*
	2	74.6 ± 3.3	67.5 ± 7.9	53.1 ± 5.0*
	3	63.5 ± 4.7	59.8 ± 5.8	43.6 ± 7.1*
D. nigrospiracula	1	83.0 ± 4.7	76.4 ± 7.7	81.0 ± 4.6
	2	72.0 ± 5.5	75.8 ± 2.8	68.8 ± 7.1
D. acanthoptera	1	47.3 ± 2.0	44.9 ± 1.31	36.2 ± 1.3*
	2	46.7 ± 2.9	43.2 ± 1.5	37.4 ± 2.2*
D. pachea	1	32.2 ± 3.0	43.5 ± 2.6	35.0 ± 3.0
	2	51.4 ± 3.7	52.6 ± 3.2	47.7 ± 4.1
	3	33.6 ± 2.8	42.2 ± 3.4	42.2 ± 3.2
D. mojavensis	1	50.4 ± 3.4	50.0 ± 3.0	57.9 ± 3.5
	2	44.7 ± 4.4	46.7 ± 2.8	41.1 ± 3.4
D. mettleri	1	57.0 ± 2.9	46.9 ± 6.8	57.4 ± 2.5
	2	36.7 ± 2.9	39.1 ± 3.2	41.4 ± 2.6

EVOLUTIONARY GENETICS OF REPRODUCTIVE BEHAVIOR

From the foregoing descriptions, several conclusions can be drawn. One is that *Drosophila* species exhibit considerable variability in all aspects of their reproductive behaviors. Some of the divergence occurred at the time major lineages were forming, but some involves more recent differentiation. In all cases, the patterns necessarily involve shifts in the signals different species use to successfully reproduce. These shifts have occurred both within particular sensory modalities, for example from attraction to one type of host versus another, or using one type of courtship song versus two, as well as with respect to the relative importance of multiple sensory modalities, such as vision versus olfaction during courtship. What this indicates is that, if signals themselves vary, the systems involved in their reception and response must also vary. What remains unclear, however, is how the differences among species and species groups have arisen and what their genetic, cellular, and physiological bases are. Historically, approaches to understanding the genetics of particular traits have used the tools of classical genetics: intra- and interspecific crosses, QTL mapping, and mutagenesis. These approaches have been useful in demonstrating genetic bases to intra and interspecific differences in behaviors and in some cases, in identifying the genetic architecture or an area of the nervous system involved. In terms of identifying specific signals and signal detection systems,

and having the ability to examine their coevolution, however, we have far to go. Given recent developments in genomics, including the sequencing of the genomes of multiple species of *Drosophila*, new approaches now can be exploited to address the evolutionary genetics of reproductive behaviors.

Host Use

Host use, in terms of both resource location and oviposition site preference, is mediated by the chemosensory system. We do not mean to imply that other factors, such host microclimate, play no role in resource location, but these variables are beyond the scope of this review. Chemosensory information is classified as either olfactory or gustatory, depending upon whether the signals are volatile or contact. Although there may be some degree of functional overlap, the olfactory system is more likely to be involved in longer-range location of resources, whereas the gustatory system figures more prominently in close-range signaling between individuals during courtship and in oviposition decisions.

Olfactory information is received and processed by olfactory receptor neurons (ORNs), which are found in the two olfactory organs, the antennae and the maxillary palps. Antennae contain approximately 1200 ORNs whereas the maxillary palps contain only about 120. The ORNs fall into 16 functional classes based upon the their odor response spectra (38), which are thought to depend, in turn, upon the expression of approximately 60 different odor receptor genes (160). Gustatory or taste receptor neurons (GRNs) most likely to be involved with oviposition and with sexual behavior are those on the abdomen, forelegs, and mouthparts, which are in contact with substrates and with flies of the opposite sex during the tapping and licking phases of courtship. With respect to the bristles on the forelegs, male *D. melanogaster* have nearly twice as many taste bristles on their forelegs as do females (102, 106, 143). Based upon the degrees of sequence similarity, the olfactory and gustatory receptor genes are likely to have a common evolutionary origin. Chemical information received by flies has two origins: the host resources and other flies. Candidate sensory processes for host location, therefore, are likely to be associated with the ORNs, whereas those mediating sexual behavior and oviposition are more likely associated with morphological structures in contact with other flies and food, the GRNs.

Each type of host resource provides a different chemical profile based not only upon the host's own chemistry, but upon the microbial community responsible for its breakdown, making it suitable as a *Drosophila* breeding site. Volatile profiles have been characterized for several *Drosophila* resources (51, 54, 87, 141). Stensmyr et al. (142) utilized the ecologically relevant volatiles to examine evolutionary conservation and divergence in the olfactory code among nine members of the *D. melanogaster* group of species. The group includes *D. sechellia*, which, in addition to being an island endemic, has specialized upon the fruit of *Morninda citrofolia*, which has a distinct chemical profile compared with the broader range of fruits utilized by other members of the group. The ab2 type (38) sensillum and its neurons were found to be missing in *D. sechellia*, apparently replaced by a higher number of the ab3 type, such that the overall number of sensilla of the large basiconic (LB) class was the same among the species in the group. There was also a shift in the key ligand for the ab3A-type neuron, from ethyl to methyl hexanoate. The bases for sensitivity shifts within given ORNs is unclear, but could be due to substitutions in their receptors. For example, ab3A ORNs in *D. melanogaster* express the receptor *Or22a*. *Drosophila simulans* has an orthologous counterpart, *DsOr22a*, whose sequence homology with that of *D. melanogaster* is 94% (40). Another group of proteins, the odorant binding proteins (161), which are thought to bind and present the odorants to the receptors, may also be found to contribute to observed species differences.

Long-distance location of appropriate hosts is only the first step in host utilization. Inseminated females then must make decisions about oviposition. When host shifts involve novel compounds, there is the potential for a mismatch between oviposition site and larval performance or fitness. The evolution of preference-performance correlations (31, 73) is a long-standing problem that can potentially be resolved using the *Drosophila* model system. An obvious approach is to identify the genes involved in oviposition decisions as well as in performance on a specific host and look for genetic and physiological relationships between the two processes. Oviposition preferences of a number of *Drosophila* species have been characterized (3, 88, 136). In most cases, there is a clear preference for certain types of hosts, and even for the yeasts associated with those hosts (4). Where interspecific crosses have been possible, as in the case of *D. sechellia*, the genetics of oviposition preference also has been analyzed (3). With respect to oviposition decisions, the mechanisms are likely to involve the gustatory reception system described under *Chemosensory signals and courtship*. Jones (75, 76) and Cariou et al. (17) have examined the genetic basis for resistance to *Morinda* toxins (octanoic acid and other compounds) by *D. sechellia* adults and larvae, revealing that resistance is due to a few semidominant alleles present only in this species. Two other taxa in the *melanogaster* subgroup, *D. santomea* and *D. erecta*, also appear to be restricted to single hosts (*Ficus* and *Pandanus*, respectively), in spite of more generalist sister species (**Figure 3*a***). The *melanogaster* subgroup offers an excellent opportunity to resolve genetic questions concerning preference-performance correlations and the evolution of host shifts (31).

Chemosensory Signals and Courtship

Contact chemoreceptors on other adult structures mediate the signaling involved in courtship and mating. A family of about 70 gustatory receptors, coded for by gustatory receptor genes (Gr), are found in sensilla on the proboscis, legs, and anterior wing margins (27, 44, 130). Some of these sensilla are male specific, occurring in twice the number on male forelegs as on those of females (106). One of the taste receptor genes, Gr68a, is expressed in 10 of the males-specific foreleg bristles of *D. melanogaster* (14). When expression of this gene is disrupted (14), male courtship is interrupted in a way that suggests the gene product is a putative receptor of female pheromones in this species.

Chemical signals received from other flies could come from several sources. The most likely, however, are the hydrocarbons associated with the epicuticle of flies of both sexes. As seen earlier, these can vary in a wide range of ways within and between species. Epicuticular hydrocarbons originate in the large polyploidy oenocytes located in the subepidermal layer (123). In most cases, once flies are sexually mature, their hydrocarbon profiles are fairly constant. In some species, it appears that females may emit pulses of pheromones by extruding their ovipositors, as this behavior can result in the inhibition of male courtship (137). In some species, such as *D. subobscura* (140) or *D. adiastola* (139), males present a liquid drop to females during courtship, the pheromonal properties of which remain unclear.

Many genetic studies of intra- and interspecific hydrocarbon variability strongly support not only the role of these compounds in sexual selection as well as in behavioral isolation, but also give an idea of the genetic architecture of the variability (reviewed in 52). For example, crosses among species of the *melanogaster* group indicate that female-specific pheromones are controlled by at least five different genes in chromosome 3, whereas male differences are interspersed across all three major chromosomes (32–34). Differences in the hydrocarbon profiles of *D. virilis* and *D. novamexicana* reside in two chromosomes, including one gene of major effect (41).

In *D. melanogaster*, mutants and other genetic manipulations have revealed that sex differences in the hydrocarbon profile are ultimately under the control of the sex determination hierarchy of genes (53, 125, 152, 153). With respect to species and sex differences in characteristics like HC chain length and double bond positions, the specific biosynthetic pathways and the genes controlling them are still largely unknown (reviewed in 52). Two desaturase genes, *desat1* and *desat2*, discovered in mutagenesis screens, are involved in hydrocarbon biosynthesis (37, 90, 146), and mutations in these genes disrupt courtship.

Visual Signals and Courtship

Visual signals and their reception and processing may prove more complicated to study. For chemical or auditory communication, the signals can be categorized as to features such as chemical composition or sound wavelength and nature and frequency of pulses. In addition, chemical and auditory communication can be manipulated at the levels of the signal and signal detection not only through genetic manipulation, but by nongenetic means as well. Although a role for visual signaling can be assumed with confidence for some species, the existence of variability in visual signaling within and between species is based largely upon inference. It may be that for many species the role of visual signals is less specific than the roles of chemosensation or audition. For example, when a given species will not mate in the dark (**Figure 3**), visual signals can be assumed to be critical to the process. Whether the critical signals are produced by the female, the male, or both, is not known. Also unknown is whether the relevant signals are the same as in a related species, but simply are less critical to the process or are perceived in a different way. Clues could be obtained through observations under infrared light, or by utilizing genetically blind flies of one sex or the other and determining the stage at which courtship fails.

Auditory Signals and Courtship Behavior

Courtship songs of most *Drosophila* species are generated by vibrations of the wings, through mechanisms related to the flight neuromuscular circuitry. The signals, produced by way of air displacement (46), give rise to "near-field sound," perceived only within a short distance of the source. The motor patterns producing the songs are different, however, from those for flight, and for species producing more than one type of courtship sound, there may be multiple motor patterns involved (149). Furthermore, it appears that the motor patterns involved in song production involve a feedback component (148).

Audition, the receipt and processing of auditory information, is thought to have evolved in species-specific ways in *Drosophila*. The auditory apparatus in *Drosophila* is the Johnson's organ, a type I mechanoreceptor (46) in the antenna, which functions though particle displacement generated by wing vibrations. What is known of the process for *Drosophila* is based upon studies with *D. melanogaster* (62). The antennal complex is comprised of three segments. The segment most proximal to the head is the scape, the middle segment is the pedicel, and the most distal segment, the funiculus gives rise to the arista, a long structure with multiple branches. The arista receives sound-induced vibrations and behaves like a stiff rod, which, because it is tightly connected to the funiculus, also stimulates this structure (62). The two in fact function as one mechanoreceptor unit. The auditory receptor itself, the Johnson's organ, lies within the pedicel. A process from the funiculus inserts into the pedicel, transferring the vibrations it receives via the arista to the auditory receptors (62).

The importance of these structures in audition has been verified by mutations in *D. melanogaster* that have modified or eliminated some aspect of their structure or function. Mutants such as *aristless (al)* and *thread (th)* alter the perception of courtship songs and

courtship behavior (92) by modifying the external structures (15, 16), whereas others, such as *atonal (ato)*, *beethoven (btv)*, and *touch-insensitive-larvae-B (tilB)* affect neural structure and function as well as other developmental processes (47).

A considerable number of genes have been discovered to influence song in *D. melanogaster*, but none exclusively so. These genes and their actions are nicely reviewed by Gleason (57), who groups them by function into regulatory, ion channel, sex determination, and flight genes. Regulatory genes include *period (per)* (85) and *no-on-transient-A (nonA)*, of which *dissonance* is an allele (84). Ion channel genes include *cacophony (cac)* (83, 158, 159, 165) and *slowpoke* (112). Genes in the sex-determination hierarchy, *transformer (tra)*, *doublesex (dsx)*, *fruitless (fru)*, also have been found to influence song (reviewed in 11). Finally, some of the genes affecting flight, such as *croaker (cro)* (169) and *ariadne (ari-1)* (2) because of the role of the flight musculature in sound production, also influence songs, whereas some flightless mutants do not (5).

The relationship between the loci for which song aberrations have been found and those underlying naturally occurring variation in song production having evolutionary potential is unknown. Genetic variability for interpulse interval (IPI) in natural populations of *D. melanogaster* responds rapidly to directional selection and is thought to have an additive polygenic basis (121). Naturally occurring intraspecific variability in IPI has been localized to chromosome 3 of *D. melanogaster* (30), and QTL analysis of inbred lines revealed three significant QTLs (59). Similar studies have been undertaken in *D. virilis* and *D. littoralis* (72), *D. pseudoobscura* (134, 166) and *D. polios*, and its sibling *D. ananassae* (42). A major difference between song production in the Sophophoran subgenus and that observed for many flies in the subgenus *Drosophila* is that males of many species in the latter vibrate both wings simultaneous when singing (137; T.A. Markow, unpublished observations). Examining the effects of genes identified in *D. melanogaster* that modify wing position or cause the simultaneous use of both wings should provide attractive candidates for evolutionary studies.

Postmating Control of Reproductive Behavior

Copulation produces specific changes in *D. melanogaster* that have been well studied.

The two principal effects of mating in this species are the stimulation of oviposition and the delay of female remating. These are not identical in other species, as shown above. In a number of species, female remating is not delayed. Although there is less comparative information on the onset of oviposition, the few data that there are suggest that species differ in this character as well. For example, unlike *D. melanogaster*, where females begin to lay eggs within a few hours of mating, in some species a large mass, called the insemination reaction, forms in the uterus after mating, and oviposition does not commence until the mass subsides, which in some cases is the next day (164).

In *D. melanogaster*, postmating effects have been firmly connected with various male accessory gland proteins (Acps) passed to the female (reviewed in 82, 167). Some of these Acps also have been examined in the *D. simulans* complex of species (79). One protein in particular, the sex peptide, or Acp 70a, has been characterized in the greatest number of species (23, 24, 129), and its function and sites of action are becoming better known than those for other Acps. For example, by incubation of cryostat tissue sections of *D. melanogaster* females with a radioactive synthetic form of the sex peptide, binding was observed in parts of both the central and peripheral nervous systems as well as in the female genital tract (39, 82). Similar observations were also made for another seminal protein, DUP99B, which is made in the male's ejaculatory duct (82). In addition, the sex peptide has been found bound to the tails of

sperm (82), which would explain the importance of sperm, as well as accessory gland proteins to the postmating behaviors of females (22). Male-derived proteins can be thought of in the same way as other signals that pass from one sex to the other. In this case, the signal is internal, but can be traced to sites of action in the female, including the CNS. Unlike in marine invertebrates where the female receptor is known (56) the mode of action of these Acps is not yet well understood. Given their number, in *D. melanogaster*, at least, there is likely to be either some redundancy in their action or specialization in function (22). Because of the number of processes that actually occur between copulation and oviposition, male-derived substances are likely to have a role in a variety of them. For example, sperm must find their way into storage, and they must be recovered, a process which, even within a species, is not random with respect to the male and female genotype (95). Females must recognize that they are inseminated, and then begin to release oocytes. Sperm must remain viable until an oviposition site is found, and they may also need to resist displacement or preferential use by females. The idea that the Acps are at least in part specialized in their functions is supported by the data: some Acps have been localized to certain parts of the female reproductive tract involved with sperm storage, and there are indications that they are involved in the sperm storage or utilization process (167). The sex peptide seems to have multiple functions, as indicated above. Still another set of putative postmating behavior control genes has been identified from mutagenesis screens designed to disrupt oviposition. These genes are likely to act downstream of any male-induced signal, but until further study their functional role in the mating-oviposition cascade will not be known. The action of downstream genes, such as the oviposition mutants *dissatisfaction* (*dsf*) and Tyrosine Beta Hydroxylase (TBH), required for production of octopamine, necessary for oviposition (29, 86) and *cyclophorin-like (Cypl)* discovered in a screen (105), is expressed in the oviduct and has been proposed to be required for oviposition in the mated female.

Evidence for genetic divergence between the signals and receptors mediating postmating reproductive behaviors is inferred from the increased size and duration of the insemination reaction mass observed following matings between genetically differentiated populations of *D. mojavensis* (80) and, to an even greater degree, between related species, which is accompanied by increases in the time until females oviposit (110; T.A. Markow, unpublished observations). In extreme cases, those involving interspecific mating, the mass may remain forever, effectively preventing the female from ever remating or laying eggs (111).

CONNECTING THE DOTS

What are the nature and number of the genetic changes underlying the diversification of reproductive behavior in *Drosophila*? To what extent has the evolution of these species differences involved changes in regulatory rather than structural genes (60, 69)? Do the loci or chromosomal regions identified in QTL studies correspond to any of the candidate genes discovered through mutagenesis screens in *D. melanogaster* (81), and if so, do they retain the same functions in other species? If the identical function of a gene is retained across unrelated species, what is the level of sequence divergence observed in that gene? What are the levels of variation in natural populations of any of the species at the loci implicated in the interspecific differences?

One of the goals of mapping characters onto a phylogeny is to learn which states of the characters are ancestral and which are derived. With respect to many of the reproductive behaviors in *Drosophila* discussed above, data are available for large numbers of species. There are clear gaps, however, as not all species have been equally popular or easy to rear and study. Filling in some of these gaps will be important in ultimately understanding the evolu-

tion of the phenotypes and the genetic systems controlling them. Such studies can bridge the often disparate disciplines of systematics and genomics. When there is a change in a character state, for example, is it more commonly attributable to regulatory changes or to changes in the function or number of structural proteins? The switch-points seen on the phylogenetic maps of reproductive behavioral characters, where an entire lineage has undergone a major shift in something like host use or the use of female auditory signals or visual signals, would appear to be a juncture at which some major change in a signaling process has occurred. For example, the use of necrotic cacti might be associated with the appearance of a functionally different sent of olfactory receptors, odorant binding proteins, or arrangement of types of neurons in the sensillae. The same question could be asked of signalizing that occurs inside of the mated female. In several lineages, female remating frequency has shifted between monogamy and frequent remating. Remating of females, in *D. melanogaster* at least, is delayed by the action of one or more seminal fluid proteins. What is the nature or number of changes that have occurred in those lineages where seminal fluid clearly does not produce this effect? Additional and as yet unresolved questions include whether genes mediating intraspecific sexual selection are important to the evolution of sexual isolation between species (13) and the extent to which there is coevolution between characters such as male and female sexual signals (12), or host preference and larval performance during the evolution of host shifts (31). With the availability of genome sequences and the development of expression profiling systems and other tools for 12 *Drosophila* species (101), differing to various degrees in their evolutionary distances, we can begin to address these questions.

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