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The courtship song of *Drosophila santomea* and a comparison to its sister species *D. yakuba* (Diptera: Drosophilidae)

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Abstract. We describe the male courtship song of the newly-discovered species *Drosophila santomea*, endemic to the island of Saõ Tomé and the sister species of the widely distributed *D. yakuba*, which also inhabits the island. The song of *D. santomea* resembles that of *D. yakuba* in lacking a "sine" song, but the interpulse interval (IPI) in the "pulse" song is about 67 ms for the former species and 110 ms for the latter. This is the largest difference in IPI observed between any two sister species in the *D. melanogaster* subgroup, and may play a role in the strong sexual isolation between these two species.

INTRODUCTION

Male courtship in *Drosophila* is often elaborate (Spieth, 1952; Bastock & Manning, 1955), involving wing displays and vibrations, with the latter producing audible courtship "songs" (Sturtevant, 1915; Bennet-Clarke & Ewing, 1967; for a recent review see Tauber & Eberl, 2003). Most species in the well-studied *Drosophila melanogaster* subgroup (Fig. 1) have three types of song in their repertoire: "sine song" "pulse song", and "thud song" (Von Schilcher, 1976; Demetriades et al., 1999).

Pulse and thud songs both consist of sharp pulses separated by an interpulse interval (IPI), although these two types of song differ in how they are produced. Pulse songs are generated by the repetitive vibration of a single wing, usually the one closest to the female during courtship, which is extended horizontally to the body of the fly at an angle of 90° or slightly more. The trailing edge is slightly lowered and vibrated (Bennet-Clarke & Ewing, 1967), making a noise that, when amplified for the human ear, sounds like a purring cat. In *D. melanogaster*, *D. simulans* (Kyriacou & Hall, 1982), and *D. yakuba* (Demetriades et al., 1999), the length of the average IPI in pulse songs also changes slightly and predictably between successive pulse bouts, with this change itself forming a long sinusoidal pattern called the "Kyriacou-Hall" cycle (Kyriacou & Hall, 1982). Evidence for IPI song cycles has also been seen in *D. persimilis* (Noor & Aquadro, 1998).

The thud song of *D. melanogaster*-group species sounds like a motorboat when amplified, and is produced by a rapid scissoring action of both wings held at an angle of about 40° away from the male's abdomen (Demetriades et al., 1999). Thud songs may consist of anywhere from one to several pulsations produced at an irregular frequency. Typically, pulse and thud songs are produced in distinct bursts but, on occasion, a pulse song will begin and then change quickly to a thud song or vice versa (Demetriades et al., 1999).

The sine song, so called because of the continuous waveform that is produced when it is recorded and amplified, is made by the repeated vibration of a single wing. When amplified, the sine song sounds like a buzzing mosquito and, as the name suggests, there are no audibly distinct pulses.

Previous work has indicated that among many closely related species of *Drosophila* the IPIs of pulse songs differ in a species-specific way and therefore might play a central role in courtship and species recognition (Bennet-Clarke & Ewing, 1968; Hoikkala & Lumme, 1984; Ewing & Miyan, 1986; Ritchie & Gleason, 1995; Noor & Aquadro, 1998). If species-specificity of IPIs is essential for intraspecific mate recognition, then IPI differences could also lead to sexual isolation among species. Using a playback experiment, von Schilcher (1976) showed that *D. melanogaster* females confined with a wingless conspecific male copulated more rapidly when subjected to a 34 millisecond IPI (characteristic of *D. melanogaster*) than when subjected to white noise. Furthermore, *D. melanogaster* females paired with wingless conspecific males and subjected to an artificial song with an IPI of 48 ms (mimicking the IPI of the sister species *D. simulans*) were slower to copulate than females subjected to an artificial song with the conspecific IPI of 34 ms.

A ninth species, *D. santomea*, was recently discovered in the *D. melanogaster* subgroup (Lachaise et al., 2000). This species, endemic to the island of São Tomé, a 860 km^2 volcanic island 255 km west of Gabon, is the sister species of the pan-African *D. yakuba* (Lachaise et al., 2000), and probably arose when the ancestor of *D. yakuba* colonized Sao Tome about 300,000 years ago (Llopart et al., 2002, 2005a). *D. yakuba* subsequently reinvaded the island, and now coexists with *D. santomea*. The two sibling species form a classic hybrid zone at middle elevations (the only hybrid zone known in the genus *Drosophila*), where there is a smooth transition between low-altitude populations consisting almost entirely of *D. yakuba* individuals and higher-altitude sites harboring only of *D. santomea* (Llopart et al., 2005a). Such hybrid zones have long been hailed as "windows of the evolutionary process", mainly because they allow one to dissect how different traits can produce barriers to gene flow and thereby contribute to the formation of new species. Previous studies (Llopart et al., 2005a, b) showed that in nature there is almost no gene flow across the *D. yakuba/D. santomea* hybrid zone, probably because of strong mate discrimination between these species as well as postzygotic barriers including hybrid male sterility (Coyne et al., 2002, 2005).

Here we characterize for the first time the male courtship song (pulse song) of *D. santomea* and compare it to that of its sister species *D. yakuba*. Unlike other species in the *D. melanogaster* subgroup, *D. yakuba* has no sine song (Cowling &

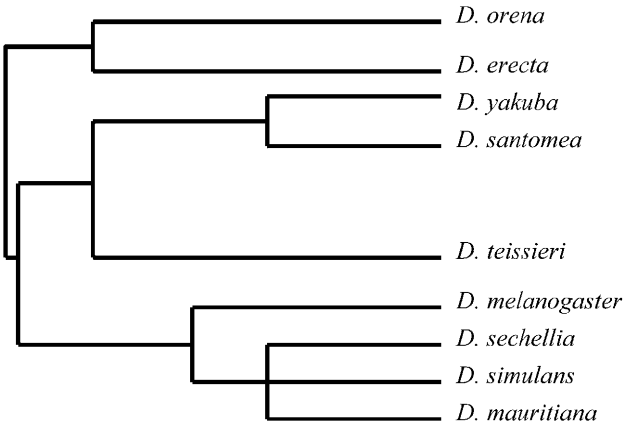

Fig. 1. Phylogeny of *D. melanogaster* subgroup, showing the sister-species relationship of *D. yakuba* and *D. santomea.* See Llopart et al. (2005a) for references on which this phylogeny is based.

Burnet, 1981), and has been reported to produce a pulse song of IPI approximately 120 ms (Demetriades et al., 1999). Since thud songs are usually produced when males are not oriented towards or in close proximity to the female, they do not have a clear role in courtship (Demetriades et al., 1999). Moreover a comparison of pulse and sine songs in *D. melanogaster* shows that only pulse song appears to affect mate choice and interspecific discrimination (Talyn & Dowse 2004). Since the species discussed in this paper lack sine song, we recorded and measured only the parameters of the pulse song.

MATERIAL AND METHODS

***Drosophila* stocks**

Flies were raised on standard cornmeal-yeast-agar medium at 24°C and kept in incubators on a 12-h light-dark cycle. Stocks of *D. yakuba* and *D. santomea* founded from single females captured in the wild were used as well as "synthetic" stocks (mixtures of isofemale lines) described below. In the latter stocks, several isofemale lines from a single population were combined to reconstitute the genetic variation of natural populations and eliminate the effects of any inbreeding that had occurred in the laboratory. (As the Results show, song parameters did not differ significantly between single or mixed isofemale lines.)

Two *D. santomea* stocks, STO.4 and 2005 SYN, were used in the analysis. The STO.4 stock was derived from a single inseminated female collected in the Obo Natural Reserve on São Tomé Island in 1998 within the zone of sympatry with *D. yakuba* (Lachaise et al., 2000). The 2005 SYN stock was derived from a combination of six isofemale lines collected by JAC in the zone of sympatry in January 2005.

We also used *D. yakuba* stocks Taï 18 and 2005 SYN in our analysis. The Taï 18 stock was collected in 1983 by D. Lachaise in the Taï rainforest on the border of Liberia and Ivory Coast. The *D. yakuba* stock 2005 SYN was made by combining six isofemale lines collected by JAC in the area of species overlap in January 2005.

Male hybrids for song analysis were produced by reciprocally crossing the 2005 SYN lines of *D. santomea* and *D. yakuba*.

Mating behaviors of *D. yakuba* and *D. santomea*

While not yet formally analyzed, the visible (non-auditory) courtship behaviors of *D. santomea* are, to the human eye, similar to those of *D. yakuba*. During their initial introduction into a mating chamber, males and females either remain motionless or wander aimlessly. When the two flies come into contact, or close proximity, males approach the female while producing a thud song. If the female stops walking in the chamber, the male will approach her from behind and produce a pulse song. After a train of about 20 pulses, the male will attempt to copulate. If the first copulation attempt is unsuccessful, the male repeats this pattern until copulation is successful or the male breaks off courtship completely.

Table 1. Differences in IPI between *D. santomea* and *D. yakuba*. The species mean IPI was calculated from the individual means within each species. The standard error provided is the standard error of the grand mean for each strain. Hybrids were generated by reciprocally crossing the 2005 SYN lines of *D. santomea* and *D. yakuba*.

Pure species	Line	N	Mean IPI (ms)	SE	Range
D. santomea	2005 SYN	15	65.2	8.5	19.0
	STO.4	10	68.0	6.1	22.2
D. yakuba	2005 SYN	15	105.9	12.7	38.6
	Taï 18	10	113.3	11.1	37.7
Cross	Mother	N	Mean IPI (ms)	SE	Range
F_1 [S X Y]	*santomea*	28	74.3	1.7	39.2
F_1 [Y X S]	*yakuba*	29	80.4	1.8	39.5

Song analysis

Flies used in the analysis were collected under CO_2 anesthesia within 8 h of eclosion. Sexes were isolated and kept in vials of 15 flies. Only flies 4–6 days old were used in the analysis. All songs were performed by aspirating a single male into an "Insectavox" song-recording device (Gorczyca & Hall, 1987) containing a single conspecific female at a temperature of 23 ± 2°C. We amplified the recordings using a Radio Shack amplifier (Catalog no. 277–1008C) connected to a Macintosh Powerbook computer. The analog signals were digitized and basic song parameters analyzed by Canary sound analysis software (version 1.2.4, Cornell Bioacoustics).

For each male, we recorded every bout of pulse song produced, and used only one song for each male. Songs included in the analysis were over 10 pulses in length. Males who did not initiate courtship were discarded after 5 min while courting males were discarded after 10 min. After each successful recording, the ambient temperature inside the Insectavox was measured using a thermocouple (±0.1°C).

To reduce the temperature effects on song parameters, we recorded songs only within a narrow temperature range (4.0°, from 21.2–25.2°C) and also alternated recordings of the two pure species and the two reciprocal F_1 hybrids to reduce the effects systematic changes in temperature. The songs of the two types of F_1 males were assayed by placing them with a *D. santomea* 2005 SYN female. The males of both species readily court heterospecific females (Coyne et al., 2002).

RESULTS

Table 1 shows the differences in interpulse interval between the species and among the two strains tested within each species. The IPI was recorded for each song and a mean was found for each individual. The mean IPI for each strain was determined by averaging the individual means among males; the standard error given is the standard error among the means of different males tested in each strain. The difference in interpulse interval between these species is large and nonoverlapping. The mean IPIs for the two strains of *D. santomea* are 65.2 ms and 68.0 ms, while those for the two strains of *D. yakuba* are 105.9

Table 2. Analysis of variance for interpulse intervals, *D. santomea* vs. *D. yakuba.*

Source	DF	Sum of Squares	F Ratio	Prob >F
Species	1	20604.6	205.87	< 0.0001
Strain (within species)	2	219	1.0942	0.3434
Error	46	4603.9		

ms and 113.3 ms. The mean IPIs of the species thus differ by about 43 ms — a difference far greater than seen between any other sister species in this subgroup. For instance, *D. melanogaster* and *D. simulans* have a difference of 14 ms between their IPI (Bennet-Clarke & Ewing, 1968), while in the *D. obscura* group, the well-studied *D. persimilis* and *D. pseudoobscura* differ in IPI by only 18 ms (Noor & Aquadro, 1998). (The IPIs of the *melanogaster*-group species are roughly 34 ms for *D. melanogaster*, 48 for *D. simulans*, 67 ms for *D. sechellia*, and 39 ms for *D. mauritiana* [Bennett-Clarke & Ewing, 1968; Gleason & Ritchie, 1994; Pugh & Ritchie, 1996]).

Table 2 shows the nested analysis of variance for these data. The differences among strains within a species were not significant ($F_{2,46}$ = 0.34). The differences between the *species*, however, is highly significant ($F_{1,2}$ = 205.8, $p < 0.0001$). The IPI differences thus appear to be species-specific, as they are consistent among different strains of the same species collected in different years and locations.

Looking at the reciprocal F_1 males, one observes a slight (6.1 ms) but significant effect of the X chromosome on mean IPI (t = 2.55, 55 d.f., p = 0.013). This difference is in the direction expected if the X chromosome carries genes contributing to the observed difference between the species, since those hybrids carrying the *D. yakuba* X chromosome have a larger mean IPI. However, the difference contributed by the X chromosome is a relatively small fraction of the total species difference in IPI (6.1/43 or about 14%). This 14% difference is roughly the same as the fraction of genetic material contained on the *D. yakuba* X chromosome — about 18%. Thus the X chromosome does not have a disproportionately large effect on this species difference. In addition, there is some dominance of the *D. santomea* autosomes on the trait since both F_1 hybrids have an IPI closer to that of pure *D. santomea* males than to that of pure *D. yakuba* males. We did not find a significant correlation between temperature and mean IPI (t = 0.36, 49 d.f., p = 0.72). Although such correlations have been seen in other species (Shorey, 1962), we used a much narrower temperature range.

DISCUSSION

Our study shows that *D. santomea* males, like their *D. yakuba* relatives (Cowling & Burnet, 1981), lack a sine song and possess both a thud and a pulse song. These two species are the only ones lacking a sine song in the *D. melanogaster* subgroup, and thus its absence is probably a derived condition that evolved in the common ancestor of these two species (see Fig. 2).

During their courtship, *Drosophila* males communicate with females via visual, acoustic, olfactory, tactile, and gustatory sensory signals. Epicuticular hydrocarbons, fatty-acid derived hydrocarbons present on the *Drosophila* cuticle, have pheromonal activity in some species and are involved in mate recognition in *D. mojavensis, D. arizonae,* and *D. navojoa* (Etges & Jackson, 2001), as well as *D. simulans, D. melanogaster, D. sechellia, D. simulans* (Coyne et al., 1994; Coyne, 1996; Coyne & Charlesworth, 1997). However, recent studies by Llopart et al. (2002) indicate that it is unlikely that these hydrocarbons play a role in sexual isolation between *D. yakuba* and *D. santomea*, as their predominant epicuticular hydrocarbons are nearly identical. Furthermore, Llopart et al. (2002) also measured courtship behaviors under light and dark conditions and showed that the absence of light does not affect sexual isolation in these two species. This suggests that cues other than visual signals are required for the strong sexual isolation shown between *D. yakuba* and *D, santomea.*

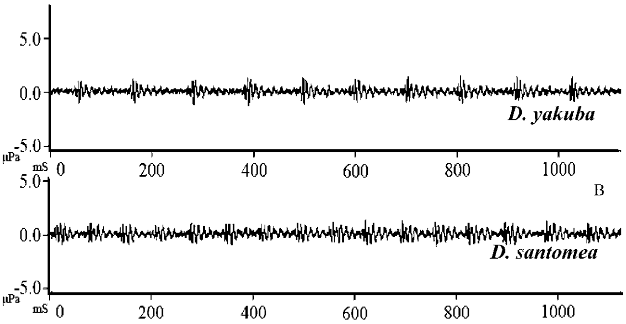

Fig. 2. Sample waveform recordings of *D. yakuba* and *D. santomea* courtship songs.

Sexual isolation between these species derives largely from female *D. santomea* refusing the persistent courtship of *D. yakuba* males (Coyne et al., 2005). The difference in mean IPI between the species reported here — larger than that described in any pair of sibling species in this group — might play a role in this sexual selection. We are studying this possibility using artificial song.

Preliminary genetic analyses indicate that the X chromosome has an effect of 6.1 ms difference between reciprocal F1s on the IPI. This result is approximately that expected based on the relative size of the X chromosome compared to the rest of the genome. This is in contrast to some theories that predict that the X chromosome will carry most of the genes responsible for sexual dimorphisms (Rice, 1984). Future genetic work will focus on quantitative-trait-locus (QTL) analysis of IPI differences, with the goal of finding candidate loci.

Finally, it remains to be seen whether *D. santomea* and *D. yakuba* pulse songs possess Kyriacou-Hall cycles in the length of IPIs. There is doubt, however, whether such cycles could cause sexual isolation, as this would require females to detect and discriminate against a pattern that varies over a long period of time. Finally, although the thud song was not studied in this analysis, it is possible that it differs between the species in a way that may differentially affect intraspecific or interspecific communication.

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