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## Title

**Evolution and diversity of the courtship repertoire in the *Drosophila montium* species group (Diptera: Drosophilidae)**

## Running title

**Courtship in *Drosophila montium* group**

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## Abstract

Changes in elements of courtship behaviour can influence sexual isolation between species. Large-scale analyses of changes, including loss and gain of courtship elements, across a relatively complete phylogenetic group are rare but needed to understand the significance of such changes, for example whether the gain and loss of courtship elements are essentially arbitrary or equally reversible. In most species of *Drosophila*, courtship, including singing, mainly occurs before mounting as pre-mounting courtship. The *Drosophila montium* species group is unusual because loss of pre-mounting courtship and gain of post-mounting one has been detected in this group. Here we provide an extensive analysis on the courtship repertoire and songs of 42 species in this group. Synchronously captured video and audio recordings were analysed to describe courtship patterns and male courtship songs and changes were analysed in a phylogenetic context. Ancestral state reconstruction suggests that a gain of post-mounting courtship singing at the ancestor of this species group has been accompanied with a concurrent decrease in the incidence of pre-mounting courtship singing and has led to subsequent further decrease and eventually complete loss of pre-mounting courtship song in several lineages. Alongside this evolutionary trend towards post-mounting courtship, sine song and a special type of “high pulse repetition song” have become more widely used for courtship during species diversification in the montium group. It is likely that the elaboration of post-mounting courtship behaviours is associated with changes in the relative importance of pre- and post-mounting components of mating systems, such as sperm competition or cryptic female choice.

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## Introduction

Premating isolation is often one of the first barriers to reduce gene flow between diverging species (Coyne & Orr, 1997; Servedio & Burger, 2014). Courtship behaviour and courtship signals are critical components of premating isolation between many animal species, and changes in the structure of courtship traits and preferences are often thought to be a major contribution to speciation (Ritchie, 2007; Kraaijeveld et al., 2011). Less attention has been focussed on changes in the presence or absence of elements used during courtship, their origins or loss. However, some studies suggest that courtship elements can often be lost, sometimes despite the presence of female preferences for them (Wiens, 2001; Bailey et al., 2019). Traits can be lost due to changes in selection from environmental or social factors, or perhaps genetic drift. Also, the importance of different courtship elements can switch, when (for example) chemical communication may become more important than acoustic due to changes in the acoustic environment, or courtship may change from one type of acoustic signal to another. Studies detailing and examining such evolutionary trends require detailed descriptions of courtship behaviour alongside comparative studies, and are needed to understand the extent, causes and consequences of losses and gains in courtship communication. We have remarkably few detailed studies of changes in the utilisation of courtship elements in a strong phylogenetic context.

Courtship of *Drosophila melanogaster* Meigen is one of the best studied behavioural repertoires and is multimodal and complex (Pavlou & Goodwin, 2013; Grillet, Ferveur & Everaerts, 2018). The courtship repertoire within the genus *Drosophila* Fallén is more variable than is widely appreciated. Spieth (1952) produced a pioneering paper describing the courtship behaviour of 101 species within *Drosophila*. There are significant qualitative and quantitative differences among species, and many changes in the predominant mode or method of courtship (Ewing, 1983; Wen & Li, 2011; Jezovit, Levine & Schneider, 2017). Courtship signals produced by courtship elements include acoustic songs by wing vibration, chemical stimuli by tapping and licking, visual stimuli by body circling, wing scissoring, flicking, waving and rowing (Schneider, Dickinson & Levine, 2012; Yeh & True, 2014).

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These courtship elements can be gained and lost repeatedly across the genus (Wen & Li, 2011) but whether changes amongst the elements are random or correlated is unknown.

Wing vibration is probably the most noticeable component of the courtship of *Drosophila* (Sturtevant, 1915) to observers, and in most species, wing vibration produces species-specific pulse songs (Alonso-Pimentel, Spangler & Heed, 1995; Ritchie & Gleason, 1995). Wing vibration may be triggered by the detection of visual or chemical sensory signals by the male during courtship (Greenspan & Ferveur, 2000). In many species, for example *D. melanogaster*, almost all successful males court females with wing vibration before they mount; song reduces female locomotion prior to mounting (Kowalski, Aubin & Martin, 2004). In some species the resulting courtship song is essential for mating success as females will not mate with silenced males or in the presence of heterospecific song, for example, *D. montana* Stone, Griffen & Patterson (Liimatainen et al., 1992), *D. buzzatii* Patterson & Wheeler (Iglesias & Hasson, 2017) and *D. athabasca* Sturtevant & Dobzhansky (Yukilevich et al., 2016). However in others, including *D. melanogaster*, song is only stimulatory because females mate more quickly with homospecific song, but not obligatory in that females will mate in the absence of song or in the presence of the song of closely related species (Ritchie, Halsey & Gleason, 1999; Tomaru, Yamada & Oguma, 2004).

Most species produce song as part of the courtship repertoire before mounting. However, signals such as courtship song can continue to be delivered after mounting and during copulation (Wen & Li, 2011). Wing vibration after mounting has previously been reported in 30 out of 94 species of the *Drosophila montium* species group of the subgenus *Sophophora* Sturtevant (Tomaru & Oguma, 1994; Hoikkala, Crossley & Castillo-Melendez, 2000; Chen et al., 2013), and in a few species of other species groups, such as *D. algonquin* Sturtevant & Dobzhansky in the *affinis* subgroup of the *obscura* group of *Sophophora* (Chang & Miller, 1978) and *D. phalerata* Meigen in the *quinaria* group of the subgenus *Drosophila* (Neems et al., 1997). The function of such songs is poorly understood. In three species of the *D. lini* complex, species-specific copulatory sine songs reduce repelling behaviour of conspecific females and extend the duration of copulation (Wen et al., 2011; Li, Wen & Ritchie,

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2012), so it may favour males in processes such as cryptic female choice or sperm competition after mounting.

The incidence of courtship before mounting is variable in some species of the *montium* group, e.g. in *D. serrata* Malloch, fewer than 30% of males sing before mounting (Chen et al., 2013). Hoikkala & Crossley (2000) suggested that all males of this species still have the ability to sing to a female before mounting, but that they only use this ability when required. There is genetic variation for the incidence of courtship before mounting in *D. serrata* because it can respond to artificial selection in the laboratory (Chen, 2016). Some species in the *montium* group (e.g. *D. lini* Bock & Wheeler) show no courtship before mounting, only producing courtship songs during copulation (Wen et al., 2011; Li, Wen & Ritchie, 2012; Chen et al., 2013).

In addition to changes in the timing of producing song during courtship, the song pattern itself has rapidly differentiated among species. Courtship songs are very diverse in the genus *Drosophila* (Chang & Miller, 1978; Cowling & Burnet, 1981; Hoikkala & Lumme, 1987; Tomaru & Oguma, 1994; Alonso-Pimentel, Spangler & Heed, 1995; Ritchie & Gleason, 1995; Wen et al., 2011). *D. melanogaster* and its relatives produce two types of song, pulse song and sine song (Bennet-Clark & Ewing, 1967; Rybak et al., 2002). In some species there are multiple types of pulse song. A primary song and a secondary song are detected among some species in the *affinis* subgroup of the *obscura* group, the *biplectinata* complex of the *ananassae* group and the *willistoni* group of the subgenus *Sophophora* and the *funebria* group of the subgenus *Drosophila* (Ewing & Bennet-Clark, 1968; Chang & Miller, 1978; Crossley, 1986; Ritchie & Gleason, 1995). In the *repleta* group of the subgenus *Drosophila*, there are two types of pulse song: “A song” with short, regular pulse trains occurs at the beginning of courtship; but “B song” with longer pulse trains of more widely spaced pulses occurs later in courtship (Ewing & Miyan, 1986). Variations in important parameters of courtship songs have been identified between sibling species, and can play important roles in sexual selection and isolation (Alonso-Pimentel, Spangler & Heed, 1995; Ritchie & Gleason, 1995; Wen et al., 2011).

We know little about the evolution of courtship songs, because few studies have revealed how courtship songs evolved in a phylogenetic context (Oliveira et al., 2013). Two types of song in the *repleta* group have undergone loss, elaboration and reduction during evolution, possibly due to physiological and energetic constraints (Ewing & Miyan, 1986). The evolution of courtship songs in the *virilis* group has gone in two different directions. The *virilis* phylad has probably evolved towards longer and denser pulse trains, while the *montana* phylad has evolved towards short interpulse intervals (Hoikkala & Lumme, 1987). In the *willistoni* group, song divergence is not correlated with genetic divergence. Their songs have evolved more quickly than divergence in sexual isolation (Gleason & Ritchie, 1998). In the *buzzatii* species cluster, courtship songs are also uncorrelated with the degree of species divergence, having evolved so rapidly that any signature of evolutionary affinity has been erased between closely related species (Oliveira et al., 2013). In order to shed more light on the evolution of this high variety of courtship songs among *Drosophila* species, it is necessary to compare their songs in a larger phylogenetic context.

The *montium* group includes 94 species and is the largest in the subgenus *Sophophora* (Da Lage et al., 2007; Yassin, 2018; Toda, 2019). Da Lage et al. (2007) upgraded two previous subgroups, i.e. the *ananassae* and *montium* subgroups, of the *melanogaster* group to independent species groups, respectively, and Yassin (2018) recently proposed a comprehensive classification of species subgroups and complexes within the *montium* group based on a well resolved molecular phylogenetic tree inferred from a large (44 species) taxon sampling (Yassin et al., 2016). Here, following their classification system, we present a detailed analysis on the courtship behaviour of 42 species of the *montium* group. We (Chen et al., 2013) already presented some results of a small-scaled study from the same perspective. The present study increased the taxon sampling from 28 species in Chen et al. (2013) to 42 spp. as ingroup and added 14 spp. of *Sophophora* as outgroup, and also increased marker genes for phylogenetic analyses from three (mitochondrial *COI*, *COII* and nuclear *Adh*) to five (by adding two nuclear genes, *Amy1*, and *Amyrel*). This has radically improved the resolution of the resulting tree topology. Based on this phylogeny, we estimate ancestral states for some courtship traits by various analytical methods, and describe the loss or gain of courtship song types before or after

mounting and variation in song pattern in a phylogenetic context. This represents the most comprehensive analysis of the variation of courtship repertoire in any group of *Drosophila*, and we find an apparently systematic loss of courtship singing before mounting and diversification in song pattern through the evolution of this group.

## Materials and Methods

### Flies and recordings of courtship behaviour and songs

The *montium* group is currently divided into seven subgroups: the *parvula* (including 4 spp.), *montium* (28 spp.), *punjabiensis* (5 spp.), *serrata* (19 spp), *kikkawai* (9 spp.), *seguyi* (22 spp.), and *orosa* (3 spp.) subgroups (Yassin, 2018). We obtained culture stocks of 42 species of all the seven subgroups (Table 1, Table S1) from the *Drosophila* Stock Centre of Ehime University (DSEU) to examine their courtship patterns and courtship songs. Flies were maintained on cornmeal-malt medium at 23°C under a 12:12 h light cycle and 50–60% humidity. Virgin flies were collected and separated into sexes without anaesthesia within 12 h of emergence. Male flies were kept individually in vials (9.5 cm height × 1.5 cm diameter) containing culture medium before the experiment, while females were maintained in groups of five in vials. Reproductively mature (5–9 days old) virgin flies were used for all experiments (Wen et al., 2011), and each fly was used once.

Videos were made of courtship behaviour of all species and the behavioural repertoire analysed from these recordings. Detailed song recordings were made using an ‘insectavox’, digitised and analysed using standard methods. Full details are provided in Appendix S1.

### Analysis of courtship incidence

By observing courtship behaviour on video recordings, male performance of courtship before and during/after mounting (henceforth termed pre- and post-mounting courtship, respectively) was scored for each species. Less sexually active pairs, which did not succeed in mounting, were excluded from our data.

Pre-mounting courtship began with ‘orienting’ and ‘following’ in all observed species: the male pointed his head toward the female, approached her, and followed her when she moved. Some other courtship elements such as ‘tapping’, ‘circling’ and ‘licking’ were not observed in the *montium* group. Wing action (including ‘wing vibration’ and ‘wing waving’) was the only observable courtship element before mounting in the *montium* group. The incidence of wing action before mounting largely varied among different species, and even among males of the same species. We define here the pre-mounting courtship as the performance of wing action. Then, we calculated the incidence of pre-mounting courtship for each species as follows:  $I_{\text{pre}} = N_{\text{pre}} / N$ , where  $N_{\text{pre}}$  is the number of males that performed pre-mounting courtship, and  $N$  is the total number of observed pairs.

Post-mounting courtship was performed with only wing actions, *viz.* vibrating wing(s) strongly to produce songs, or slightly vibrating or waving without obvious song production during/after mounting (i.e. even sometimes before copulation; therefore, the conventional term ‘copulatory’ courtship may be inappropriate). In this study, therefore, we judged the incidence of post-mounting courtship by performance of any wing action.

### **Analysis of courtship songs**

Songs were categorized into three types, pulse song, a special type of high pulse repetition song and sine song (Fig. 1). The name of ‘high pulse repetition song’ comes from ‘high pulse repetition sound’ described by Chang & Miller (1978) for *affinis* group species; songs with a similar pattern have been called ‘A’ song in Ewing & Miyan (1986), or ‘pulse song of bursts of pulses’ in Tomaru & Oguma (1994). There were definite differences in oscillogram pattern among the three types of song (Fig. 1, Table S3). A pulse song is a burst of strong sound pulses, each of which comprises polycyclic waves and is interspaced with a pause (Fig. 1A). A high pulse repetition song is a burst of pulses, each of which is composed of a few waves different in amplitude and/or waveform, and such short pulses are repeated continuously without any pause, initially increasing and terminally decreasing gradually in amplitude (Fig. 1B). Hence they lack the clear inter-pulse interval of typical pulse song. A sine song is a burst of humming sound composed of more or less homogeneous sine-like waves with gradual

initial increase and terminal decrease in amplitude (Fig. 1C) as that in a high pulse repetition song. Song parameters were analysed in DataView10.2.1. For the pulse song, three parameters, pulse length (PL), interpulse interval (IPI) and intrapulse frequency (IPF), were measured. PL is the length of sound pulse, IPI is the interval between one pulse to the next pulse, and IPF is the carrier frequency of pulse sound measured by frequency spectrum analysis. Pulses were marked, and parameters were measured and outputted automatically from DataView. For the high pulse repetition song, pulses were marked in each song burst (Fig. 1B), PLs (= IPIs) of the marked pulses were obtained from DataView, and the average PL of each burst was used for calculating the ‘pulse repetition rate (PRR)’ (Chang & Miller, 1978) by the following formula:  $PRR = 1000 \text{ (ms)}/PL \text{ (ms)}$ . For the sine song, song bursts were marked, and sine song frequency (SSF) was calculated with the FFT (Fast Fourier Transform) event spectrum in DataView.

### Phylogenetic analysis

A total of 56 species in the subgenus *Sophophora* of the genus *Drosophila* were used for the phylogenetic analysis, including the 42 species of the *montium* group and 14 outgroup species (Table S1). Two mitochondrial (*COI* and *COII*) and three nuclear (*Adh*, *Amy1*, and *Amyrel*) genes were used. Nucleotide sequences of *COI*, *COII*, and *Adh* genes were determined de novo for 26 species of the *montium* group (Table S1). The methods of DNA extraction, PCR amplification, and sequencing reaction were the same as those in Chen et al. (2013). The *COI*, *COII*, and *Adh* sequences for the remaining species and all the *Amy1* and *Amyrel* sequences were obtained from GenBank (Table S1).

Intron sequences of nuclear genes were removed before the analysis due to a high degree of alignment ambiguity. Nucleotide sequences of individual gene regions were aligned using MUSCLE (Edgar, 2004) implemented in SeaView 4.6.1 (Gouy, Guindon & Gascuel, 2010) or MEGA 7.0.14 (Kumar, Stecher & Tamura, 2016) with default settings. Individual alignments were concatenated by using FASconCAT 1.0 (Kuck & Meusemann, 2010) to generate a dataset of 5862 base pairs. Phylogenetic trees were constructed with concatenated genes, using the maximum likelihood (ML) and Bayesian methods, in which sequences were partitioned by codon position (1st, 2nd, and 3rd). ML analyses



were conducted in RAxML 8.2.9 (Stamatakis, 2014) with raxmlGUI 1.3.1 (Silvestro & Michalak, 2012), in which the “GTRGAMMA” model was applied for all data partitions. A bootstrap analysis of 1000 pseudoreplicates was performed by using the “ML + rapid bootstrap” search. Bayesian analyses were conducted by using MrBayes 3.2.6 (Ronquist et al., 2012). The best fit substitution model for each data partition was determined with the Akaike information criterion (AIC) implemented in MrModeltest 2.3 (Nylander, 2004), together with PAUP\* 4.0b10 (Swofford, 2003). A Markov-Chain Monte-Carlo (MCMC) search was performed with four chains, each of which was run for 10 million generations. Trees were sampled every 100 generations, and the first 25% of the samples were discarded as burn-in. The trace file generated by the Bayesian MCMC runs was inspected in TRACER 1.6.0 (Rambaut et al., 2014) to check whether the number of sampling generations and effective sample sizes (ESS) were large enough for reliable parameter estimates. A consensus of sampled trees was computed by using the “sumt” command with the “contype=allcompat” option, and the posterior probability for each interior branch was obtained to assess the robustness of inferred relationships.

### **Ancestral state reconstruction**

**Courtship incidence.** Based on the rooted molecular phylogenetic tree resulting from the Bayesian analysis, ancestral states for the incidence ( $I_{pre}$  as a continuous variable) of pre-mounting courtship were reconstructed by the method of PCMs (Phylogenetic Comparative Methods) using the R *Rphylopars* package (Goolsby, Bruggeman & Ané, 2017). In our dataset, there were no data of  $I_{pre}$  for *D. subauraria* Kimura and the outgroup species (Table 1). For *D. subauraria*, *Rphylopars* imputed its  $I_{pre}$  value. For the outgroup species, we assumed, based on the literature shown in Table 1, that sexually active males of these species always perform pre-mounting courtships, i.e.  $I_{pre} = 1.00$ . It is known, for most of the outgroup species, that almost all successful males court females with wing vibration producing songs before they mount. However, wings are used differently during pre-mounting courtship in some species. For example, males of *D. subobscura* Collin wave wings silently in front of females (Wen & Li, 2011), and circling and silent wing scissoring are widely used by species with spotted wings, such as *D. biarmipes* Malloch, *D. elegans* Bock & Wheeler and *D.*

*prolongata* Singh & Gupta (Ewing & Bennet-Clark, 1968; Yeh, Liou & True, 2006; Lai, Gleason & True, 2009; Tomaru & Yamada, 2011; Kudo et al., 2015). Males of *D. nebulosa* Sturtevant perform wing-lifting movements without song and fan an anal droplet towards the female (Gleason et al., 2012).

The incidence of post-mating courtship was all-or-nothing: C+ (the incidence  $I_{\text{post}} = 1.00$ ) or C- ( $I_{\text{post}} = 0.00$ ). Males of all the studied species of the *montium* group always performed any wing action during/after mating, but those of *D. melanogaster* never did it. Thus, they were assigned to C+ and C-, respectively. Chang & Miller (1978) reported that *D. algonquin* produced courtship songs during copulation (thus to be classified as C+), but that its relative *D. affinis* Sturtevant only occasionally produced a loud high pulse repetition rate “buzzing burst” just before copulation (thus C-). For the other outgroup species, no explicit description about their post-mating courtship is available in the literature. Therefore, their states for post-mating courtship were set as unknown (“?”) in the following analysis. It is highly likely that these species do not produce post-mating courtship song (personal observations), but a lack of an unusual courtship step is not usually explicitly included in courtship descriptions. Evolutionary gains/losses of post-mating courtship were estimated parsimoniously by mapping the character states on the tree in ACCTRAN (accelerated transformation) and DELTRAN (delayed transformation) analyses, using PAUP\* 4.0a164 (<https://paup.phylosolutions.com/>).

**Song type.** Ancestral states for song type were reconstructed in two ways. In one way (“A”), pre- and post-mating courtship songs were treated as independent characters, and song types as states of each character. In addition to the three song-types, i.e. pulse song (P), high pulse repetition song (HPR) and sine song (S), songs composed of two song types, i.e. P+HPR and P+S, and no song (-) and unknown (?) states were incorporated as distinct song-type states into the character evolution analyses. The song-type states of the outgroup species were determined from the literature (see Fig. 2); especially, the ‘high pulse repetition sounds’ produced by *D. affinis* and *D. algonquin* were judged to

be HPRs based on oscillograms reported by Chang & Miller (1978: Fig. 2A, D). In the other (“B”) analysis, three song types, P, HPR and S, were treated as independent characters with states of presence and absence, where any combinations of them were allowed in the process of estimating the most parsimonious character evolution. In both “A” and “B” analyses, evolutionary changes, gains or losses of song-types were estimated, separately for pre- and post-mounting courtship, by ACCTRAN and DELTRAN of PAUP\*.

## Results

### Courtship incidence

By carefully observing 1594 video recordings of courtship behaviour in 42 species of the *montium* group, we confirmed that post-mounting courtship was constantly performed (i.e. C+) by males of every species, while the incidence of pre-mounting courtship varied even within and among species (Table 1). The incidence of pre-mounting courtship was very high in four species of three subgroups: *D. asahinai* Okada ( $I_{\text{pre}} = 1.00$ ) and *D. biauvaria* Bock & Wheeler (0.92) in the *montium* subgroup, *D. nikananu* Burla (1.00) in the *seguyi* subgroup, and *D. parvula* Bock & Wheeler (0.89) in the *parvula* subgroup. In contrast, pre-mounting courtship was completely lacking ( $I_{\text{pre}} = 0.00$ ) in 12 species of three subgroups: 5 spp. out of 14 spp. in the *montium* subgroup, 5 spp. out of 6 spp. in the *kikkawai* subgroup, and 2 spp. out of 8 spp. in the *serrata* subgroup. Among the remaining species, except for *D. subauraria* with no available data in the present study, the incidence ranged from 0.03 to 0.75.

### Courtship songs

**Song types.** We recorded analysable songs of pre-mounting courtship from 116 pairs, and of post-mounting courtship from 426 pairs (Tables S2, S3). Among the 42 species of the *montium* group, five species produced no analysable songs in pre-mounting or post-mounting courtship. Of the remaining species, 24 species sang only one type of song through the entire courtship repertoire (Fig. 2, Table S3). 13 species produced two types of song (Fig. 2, Table S3): seven species produced different types (P/S, P/HPR or S/P) in pre-/post-mounting courtships; three species (*D. barbarae* Bock & Wheeler, *D. burlai* Tsacas & Lachaise and *D. jambulina* Parshad & Paika) produced two types (P

and S) in pre-mounting courtship; but three species (*D. lini*, *D. ogumai* Zannat & Toda and *D. ohnishii*) of the *lini* complex produced two types (P and S) in post-mounting courtship. Frequencies (i.e. the number of species that produced each type of song in pre- or post-mounting courtship) of the three song-types among the studied species of the *montium* group were not significantly different between pre- and post-mounting courtships (Chi-square test:  $\chi^2 = 4.005$ ,  $df = 2$ ,  $P = 0.135$ ; Table 2). Of the 42 species, 25 species produced both pre- and post-mounting courtship songs (Fig. 2, Table 3). Among them, 16.5 species produced songs of the same type in pre- and post-mounting courtships (Table 3), but the remaining 8.5 species produced different types between pre- and post-mounting courtships. These frequencies were compared to those expected from random coincidence (see a footnote of Table 3), and were significantly different from the latter, showing a much higher frequency of coincidence (Chi-square test:  $\chi^2 = 11.408$ ,  $P = 0.0007$ ; Table 3).

**Song parameters.** We analysed the following parameters of courtship song for each species: pulse length (PL), interpulse interval (IPI) and intrapulse frequency (IPF) for pulse song, pulse repetition rate (PRR) for high pulse repetition song, and sine song frequency (SSF) for sine song (Table S2). For species that produced the same type songs before and during/after mounting, parameter values were compared between pre- and post-mounting songs by the independent sample *t*-test using IBMSPSS statistics version 19.0.0. The following species produced songs with identical parameter values before and during/after mounting: *D. biauraria*, *D. auraria* Peng, *D. rufa* Kikkawa & Peng and *D. bunnanda* Schiffer & McEvey sang high pulse repetition songs; *D. barbarae* and *D. diplacantha* Tsacas & David sine songs; and *D. mayri* Mather & Dobzhansky, *D. seguyi* Smart and *D. parvula* pulse songs. On the other hand, parameter values were significantly different between pre- and post-mounting songs in the following species: pulse songs of *D. punjabiensis* Parshad & Paika, *D. orosa* Bock & Wheeler and *D. malagassya* Tsacas & Rafael; high pulse repetition songs of *D. cauverii* Muniyappa, Reddy & Prakash and *D. truncata* Okada; and sine songs of *D. burlai*. In *D. jambulina*, furthermore, pulse songs recorded before mounting from one pair were significantly longer in PL and IPI than those from the other pairs. Moreover, its post-mounting pulse songs were significantly different in PL, IPI and IPF from both or either type(s) of pre-mounting pulse song (Tables S2, S3).

## Phylogeny

Our phylogenetic analysis was almost comprehensive in taxon sampling from the *montium* group, including all the subgroups and complexes, except the *megapyga* complex (Yassin, 2018; Table S1). The ML and Bayesian analyses generated the same tree topology (Fig. S1, Fig. 2), which was largely congruent with those of previous studies (Schawaroch, 2002; Yassin et al., 2016; but see Zhang et al., 2003). Of the seven subgroups, five were recovered as monophyletic groups. However, the monophyly of the *serrata* subgroup was not supported in the present study, and *D. orosa* representing the *orosa* subgroup, which was not included in Yassin et al. (2016), was placed as the sister to the *punjabiensis* subgroup.

## Ancestral state reconstruction

**Courtship incidence.** The ancestral state reconstruction for the incidence of pre-mounting courtship revealed that the incidence ( $I_{pre}$ ) largely decreased on the root branch (i.e. MRCA: the most recent common ancestor) of the *montium* group (Fig. 3). This trend of decreasing incidence continued in the subsequent process of diversification in this species group, resulting in nearly complete losses of pre-mounting courtship at the MRCA of the *kikkawai* subgroup, in some lineages of the *montium* subgroup and at several terminal species. On the other hand, secondary increase of the incidence occurred mostly on and near terminal branches, except for a slight increase at the MRCA of the *seguyi* subgroup (Fig. 3).

The results of ACCTRAN and DELTRAN analyses to estimate evolutionary gains/losses of post-mounting courtship in the subgenus *Sophophora* were the same and are shown in Fig. 3. It was estimated that the MRCA for all the species was a typical *Drosophila* species performing courtship only before mounting but never during/after mounting (C-). Then, post-mounting courtship was estimated to have been gained (C+) independently at least twice in the subgenus *Sophophora*: first at the MRCA of the *montium* group and then never lost in its descendants, and second at a terminal taxon, *D. algonquin*, of the outgroup. Thus, it is inferred that post-mounting courtship would have

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newly arisen at the MRCA and then became prevalent throughout the *montium* group.

**Song type.** The results of ancestral state reconstruction were quite different between the “A” and “B” analyses, in which song types were treated as character states or independent characters, respectively. Evolutionary song-type changes (including gains/losses) estimated by the character optimization analyses using PAUP\* are mapped onto corresponding branches of the Bayesian tree (Fig. 2) resulting from the molecular phylogenetic analysis, separately for pre- and post-mounting courtship, in Fig. 4 (the “A” analysis) and Fig. S2 (the “B” analysis). The results of ACCTRAN and DELTRAN analyses were partly inconsistent with each other. The “A” analysis inferred that a few events of song gain had occurred in earlier ancestors with subsequent changes of song types in descendants (Fig. 4), while the “B” analysis inferred that most of song gains had independently occurred on or near terminal branches of extant species (Fig. S2). This may be due to the difference in the number of evolutionary steps necessary for song-type changes between the two analyses. When the song type changes from P to S, for example, such a change needs only 1 step in the “A” analysis, but 2 steps (loss of P and gain of S) in the “B” analysis. Thus, song-type changes would be more parsimonious in the “A” analysis, while independent song gains would be so in the “B” analysis. In terms of the tree length and goodness-of-fit parsimony statistics, the “A” analysis was better than the “B” analysis (Table 4). In addition, the inference from the “B” analysis for the post-mounting courtship song was inconsistent with the results of ancestral state reconstruction for the incidence of post-mounting courtship: the latter analysis inferred that post-mounting courtship had been gained at the MRCA of the *montium* group (Fig. 3), while the “B” analysis inferred that the post-mounting courtship song had been lacking in ancestors at deep nodes of the *montium* group (Fig. S2). Therefore, we adopted the results of “A” analysis as our preferred hypothesis of courtship song evolution in the *montium* group.

The song-type state of the MRCA at the root of the phylogenetic tree was estimated to be P (pulse song) for pre-mounting courtship but – (no song) for post-mounting courtship. Pulse song is predominant in pre-mounting courtship among the outgroup species. This state is also kept in the

*parvula* subgroup that first branched off in the *montium* group, but was estimated to have once been lost in early branch(es): at the MRCA of the *melanogaster* + *montium* groups (ACCTTRAN) or MRCAs of the *montium* subgroup and of the *kikkawai* subgroup (DELTRAN). In post-mounting courtship, HPR (high pulse repetition song) was estimated to have been gained by an early ancestor of the *montium* group: the MRCA of the *montium* group excepting the *parvula* subgroup (ACCTTRAN) or the MRCA of the *kikkawai* + *punjabiensis* + *orosa* + *serrata* + *seguyi* subgroups (DELTRAN).

From all the estimated changes, information about the frequency of each song-type change (e.g. P to S), gain (e.g. – to P) or loss (e.g. P to –) was extracted by ignoring taxon and phylogenetic information and summarized in Fig. 5, separately for pre- and post-mounting courtship and for ACCTTRAN and DELTRAN. The evolution of pre-mounting courtship song in the *montium* group was characterized primarily by frequent losses and regains of pulse (P) song and secondarily by gains and changes (from pulse song) of high pulse repetition (HPR) and sine (S) songs (Fig. 5A). Most of these song-type state changes occurred on terminal branches representing extant species, except for the early loss of P song, gain of HPR song by the MRCA of the *auraria* complex, and change from P to S on a deeper branch in the *seguyi* subgroup (Fig. 4). It is noteworthy that S and HPR songs were gained in two lineages of outgroup as well, *D. melanogaster* and the *affinis* subgroup, respectively (Figs 4, 5A). On the other hand, the evolution of post-mounting courtship song was characterized by frequent changes between P, HPR and S songs; after the early gain of HPR song, main transformation routes were HPR to S, or HPR to P to S (Figs 4, 5B).

## Discussion

### Loss of pre-mounting courtship and gain of post-mounting courtship

Our results clearly suggest that the loss of pre-mounting courtship song and gain of post-mounting courtship singing is an evolutionary feature of the *montium* group. The ancestral state reconstruction analyses indicate that post-mounting courtship singing behaviour has been gained and pre-mounting courtship has decreased in incidence concurrently at the MRCA of the *montium* group (Fig. 3). After the gain of post-mounting courtship by the MRCA, this behaviour has never been lost in any of its

descendants in the *montium* group. Wing vibration is a typical element of post-mounting courtship in the *montium* group, and the resulting song is likely to lead to longer copulation. Three species of the *lini* complex produce species-specific sine songs which suppress female repelling behaviour during copulation (Wen et al., 2011). Courting males of all the 42 species in the present study always displayed wing vibration during/after mounting, so once adopted this seems to have become an important if not obligatory component of courtship behaviour of these species.

The increased importance of copulatory courtship behaviour has alongside decreased the incidence of pre-mounting courtship in the *montium* group, eventually resulting in complete loss of the latter courtship in 12 out of the 42 studied species (Table 1, Fig. 3). However, pre-mounting courtship has secondarily increased in incidence or been regained on or near several terminal branches, recovering very high incidences ( $I_{\text{pre}} = 0.89\text{--}1.00$ ) in four species, *D. biauraria*, *D. asahinai*, *D. nikananu* and *D. parvula*. In the other species that performed pre-mounting courtship at intermediate frequencies, some males courted before mounting but others did not even in cases of successful mating, suggesting that the pre-mounting courtship is not necessary or essential for successful mating. In *D. serrata*, the incidence of pre-mounting courtship varied among stocks from different geographic populations, and experimental selection for high and low incidence has been successful in some strains (Chen, 2016). It seems likely that the gain of post-mounting courtship has been associated with relaxed selection on pre-mounting courtship and allowed its incidence to decline during the diversification of the *montium* group.

Why has post-mounting courtship evolved and, once appeared, been favoured? Most species of *Drosophila* produce signals prior to mounting but in the *montium* group they have switched to a post-mounting mode of courtship, especially singing. Little is known about the mating system or natural ecology of these species. Increased intensity of sperm competition might select for male tactics which favour sperm competition. Playback experiments with *D. lini* suggest that copulation is extended due to post-mounting song (Li, Wen & Ritchie, 2012), which might allow the delivery of more sperm or other ejaculate components. In *D. serrata*, there is a high level of polyandry in the field,



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with wild caught females having mated with as many as 8 or 9 males with a skewed paternity success (Frentiu & Chenoweth, 2008), so the incidence of sperm competition is high. Interestingly, *D. prolongata* of the *rhopaloa* subgroup of the *melanogaster* group shows a prominent leg sexual dimorphism, and pre-mounting courtship includes leg vibration, increasing female receptivity, but with a risk of interception of the female by rival males. Therefore, males shift their courtship behaviour from leg vibration to ‘rubbing’ in the presence of rivals (Setoguchi et al., 2015). So the risk of mate interception might also lead to the evolutionary loss of some pre-mounting courtship elements alongside increasing post-mounting courtship. However, the incidence of sperm competition and male competition is widespread in *Drosophila* (Holman, Freckleton & Snook, 2008; Rouse, Watkinson & Bretman, 2018) and we do not know if the incidence covaries with the likelihood of post-mounting courtship, which would be very interesting to study further. It would also be interesting to correlate post-mounting courtship with reproductive features such as testes and accessory gland size or genital morphology. Cryptic female choice is often thought to be based on copulatory components of courtship (Eberhard, 1994) but often this is only presumed. Manipulation studies are required to decisively demonstrate that copulatory courtship allows this component of female choice (e.g. Edvarsson & Arnqvist, 2000) but once present, it may be more persistent than more arbitrary female preferences.

A feature of the change to post-mounting courtship is the associated loss of pre-mounting courtship singing. At one level it is surprising that a major courtship trait can be lost, but sexually selected traits have often been lost during evolution, perhaps as they change in importance during cycles of trait-preference coevolution or under negative selection from eavesdroppers (Pascoal et al., 2014). We know that pre-mounting song in flies can vary from being obligatory in some species to only stimulatory in others, and in at least two species, *D. heteroneura* and *D. venezolana*, no stimulatory effect of pre-mounting song was found, even though males still commonly produce this as part of courtship (Boake & Poulsen, 1997; Iglesias & Hasson, 2017). That pre-copulatory courtship loss is reversible implies that preferences for pre-mating courtship have not been lost. It is extremely interesting that pre- versus post-mounting courtship do not seem to be equally “disposable” in an

evolutionary sense, so there may be switching of the importance of courtship elements rather than outright loss (c/f Wiens, 2001). It would be very interesting to assess if female preference for copulatory courtship song relies on the same neurogenetic substrates, perhaps facilitating the reversibility of loss of preference for pre-mating courtship song.

It is, however, impossible to identify which possible mechanisms are responsible for the gain of post-mounting courtship and reduction/loss of pre-mating courtship in the MRCA of the *montium* group, from the current data. Candidate species for comparison of biological features relevant to the hypothetical mechanisms should be extant species that show different modes of courtship from close relatives. For the gain of post-mounting courtship, *D. algonquin* and *D. phalerata* can be compared with closely related species such as *D. affinis* and *D. innubila* Spencer, respectively, which produce no post-mounting courtship song (Wen & Li, 2011). For pre-mounting courtship, *D. leontia* Tsacas & David is the only species that regained the pre-mounting courtship ( $I_{\text{pre}} = 0.41$ ) within the *kikkawai* subgroup, and is to be compared with its sibling species, *D. bocki* Baimai ( $I_{\text{pre}} = 0.00$ ), which is morphologically indistinguishable but reproductively isolated from *D. leontia* (Baimai, 1979). Another candidate pair is *D. birchii* Dobzhansky & Mather, having completely lost the pre-mounting courtship ( $I_{\text{pre}} = 0.00$ ), and *D. mayri* Mather & Dobzhansky ( $I_{\text{pre}} = 0.55$ ) of the *birchii* complex.

### **Evolutionary changes in song type**

We recognised three different types of song, *viz.* pulse, high pulse repetition and sine songs, produced during pre- and post-mounting courtships in the *montium* group. Alongside the gain of post-mounting courtship and changes (loss, decrease, increase or regain) in the incidence of pre-mounting courtship, the song type used for pre- and post-mounting courtships can also change during diversification in the *montium* group. However, the same type of song tended to be used for both pre- and post-mounting courtships in individual species (Table 3). The parsimonious estimation (by ACCTRAN and DELTRAN) of ancestral courtship song type inferred that pulse song sung before mounting by most of outgroup species and some early ancestors of the *montium* group was also employed for the post-mounting courtship in a number of their descendant species (Fig. 4), sometimes with

modification of song parameters as seen in *D. jambulina*, *D. seguyi*, *D. malagassya*, *D. mayri*, *D. orosa* and *D. punjabiensis* (Fig. 2, Tables S2, S3). On the other hand, pulse song changed to high pulse repetition or sine song in some lineages. Pre-mounting pulse song changed to sine song on a deeper branch in the *seguyi* subgroup, and the sine song has been applied to post-mounting courtship as well in *D. diplacantha* and *D. burlai*, with parameter modification in the latter (Figs 2, 4, Tables S2, S3). In the post-mounting courtship, high pulse repetition song was gained by an early ancestor of the *montium* group, and changed to sine song at the MRCA of the *montium* subgroup and then back to high pulse repetition song at the MRCA of the *auraria* + *rufa* complexes (Fig. 4). Therefore, high pulse repetition song sung before mounting by some species, such as those of the *auraria* complex, *D. rufa*, *D. bunnanda*, *D. truncata* and *D. cauverii*, would have derived from the same type of post-mounting courtship song evolved in their ancestors, with some modification of the song parameter PRR in the last two species (Figs 2, 4, Tables S2, S3). Thus, it is likely that the timing of singing the same type of song has shifted between before- and after-mounting phases of courtship throughout the evolution of the *montium* subgroup. In addition to these song-timing changes somewhat reflecting phylogenetic patterns, a number of song-type changes independently occurred on terminal branches, i.e. in extant individual species.. The types of pre- and post-mounting courtship songs were different between *D. leontia* (pre-mounting song: P, post-mounting song: S) and its siblings *D. bocki*/*D. kikkawai* (pre: –, post: HPR) of the *kikkawai* complex, and significant differences of song parameters were detected among four species of the *auraria* complex (Tomaru & Oguma, 1994) and three species of the *lini* complex (Wen et al., 2011). Whether these differences all influence female preference is unknown, except in the *lini* complex, where female copulation is extended when species-specific song is heard (Li, Wen & Ritchie, 2011). It seems likely that song divergence is an important component of sexual isolation amongst these species.

The frequency distribution of song-type changes (including gains and losses) extracted from the phylogenetic song-type transformation analyses (Fig. 4) did not show any prominent trend of directional changes in pre-mounting courtship; the three types (P, HPR, and S) of song were repeatedly lost and gained, and transformed to and from each other (Fig. 5A). In post-mounting

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courtship, however, the song-type transformation was predominated by changes from HPR to S and from HPR to P to S (Fig. 5B). Thus, HPR seems to be at a key position in the evolution of post-mating courtship songs. In terms of sound oscillogram pattern, HPR can be changed to S by homogenising pulse-composing waves in amplitude and/or form and to P by inserting interpulse pauses, and P can be changed to S by prolonging a pulse (Fig. 1, Table S3).

This study provides a detailed examination of losses and gains in elements of courtship behaviour. We demonstrate that the diversity of courtship singing behaviour across the largest species group of the subgenus *Sophophora* includes changes in the relative timing, elements and structure of song, and infer these evolutionary changes in a phylogenetic context. The ecological and mating system forces selecting for these changes are largely unknown. It has been concluded that copulatory courtship is a very labile element of courtship behaviour in insects (Eberhard, 1994) but it is extremely interesting that, within this group, copulatory courtship seems to become an essential, irreversible, component of courtship once adopted.

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## Figure legends:

**Figure 1.** Three types of courtship song in the *Drosophila montium* species group. (A) pulse song of *D. leontia*; (B) high pulse repetition song of *D. kikkawai*; (C) sine song of *D. leontia*.

**Figure 2.** A Bayesian phylogenetic tree and courtship song types for 42 species of the *Drosophila montium* group and 14 outgroup species of the subgenus *Sophophora*. The Bayesian tree was constructed, based on two mitochondrial (*COI* and *COII*) and three nuclear (*Adh*, *Amy1*, and *Amyrel*) genes, using MrBayes 3.2.6. Branch support is indicated by the Bayesian posterior probability ( $\geq 0.9$ ; \* = 1.0). Song types and/or other wing displays are indicated, separately for pre- and post-mounting courtship, in parentheses following species name: P, pulse song; HPR, high pulse repetition song; S, sine song; –, no courtship; ?, unknown. When a species produces songs of the same type but with different parameter values (see Table S2), those songs are discriminated with subscripts 1–3.

Information from literature or personal communication is indicated with superscript showing its reference: [1] Tomaru & Oguma (1994); [2] Wen *et al.* (2011); [3] Lai, Gleason & True (2009); [4] Geng *et al.* (1989); [5] Wheeler, Field & Hall (1988); [6] Yeh, Liou & True (2006); [7] Yamada *et al.* (2002); [8] Crossley (1986); [9] Chang & Miller (1978); [10] Noor & Aquadro (1998); [11] Immonen *et al.* (2009); [12] Gleason *et al.* (2012); [13] Ritchie & Gleason (1995); [14] Gleason (personal communication).

**Figure 3.** Ancestral state reconstruction for the incidence of pre-mounting courtship using the R *Rphylopars* package and for the occurrence of post-mounting courtship by parsimonious character optimization (ACCTRAN and DELTRAN) analyses of PAUP\* 4.0a164, shown with superimposed mapping onto the Bayesian tree. The incidence ( $I_{pre}$ ) values are shown with black (observed values for the ingroup species except *Drosophila subauraria*), gray (assumed values for the outgroup species) and blue (estimated values for ancestors at nodes and *D. subauraria*), and changes of more than 0.25 between successive ancestors or terminal species are indicated on corresponding branches with bars

(light blue: decrease; dark blue: increase). In the character optimization analyses, post-mounting courtships were categorized into three classes: presence (C+), absence (C-) and unknown (?). The results (completely consistent between ACCTAN and DELTRAN) are shown with red bars representing the gain on corresponding branches.

**Figure 4.** Evolutionary song-type changes mapped onto the Bayesian tree (Fig. 2). The ancestral state reconstruction was performed separately for pre- and post-mounting courtship; pre- and post-mounting courtship songs were treated as independent characters, and song types as states of each character. Song-type states were coded as P (pulse song), HPR (high pulse repetition song), S (sine song), - (no song), and ? (unknown); and songs composed of two song types, i.e. P+HPR and P+S, were regarded as distinct song-type states. Song-type changes were estimated parsimoniously by ACCTAN (accelerated transformation) and DELTRAN (delayed transformation), using PAUP\* 4.0a164, and are shown on corresponding branches with bars; inconsistent results of ACCTAN and DELTRAN are shown with short bars and letter 'A' (ACCTAN) or 'D' (DELTRAN). Gain of another type of song, which has caused a dual state of song-type, is indicated with '+'. Subgroups of the *Drosophila montium* species group are shown with Roman numerals corresponding to those of Yassin (2018): I, *parvula*; II, *montium*; III, *punjabiensis*; IV, *serrata*; V, *kikkawai*; VI, *seguyi*; and VII, *orosa*.

**Figure 5.** Evolutionary changes of song-types in pre- and post-mounting courtships of the *Drosophila montium* species group (black arrows) and outgroup species (gray arrows), extracted from the song-type evolution (Fig. 4) estimated by ACCTAN (accelerated transformation) and DELTRAN (delayed transformation). The number of changes from one type to another, gains or losses is shown beside each arrow; gain of another type of song, which has caused a dual state of song-type, was counted as 0.5. Song-types: P, pulse song; HPR, high pulse repetition song; S, sine song; -, no song. The song-type state estimated for MRCA (the most recent common ancestor) at the root of the phylogenetic tree (Fig. 4) is shown with \*.

**Table 1** Incidence of pre-mounting courtship in the studied species of the *Drosophila montium* group and the outgroup species

In-/Out-group Subgroup/(Group)	Species	Strain code	Incidence of pre-mounting courtship ( $I_{pre}^*$ )		
			<i>N</i>	Observed/(Assumed)	Reference <sup>†</sup>
<b>Ingroup</b>					
<i>montium</i>	<i>D. auraria</i>	A662	20	0.15	
	<i>D. triauraria</i>	T544	36	0.08	
	<i>D. biauraria</i>	B16	25	0.92	
	<i>D. subauraria</i>	ONM29	1	NA	
	<i>D. asahinai</i>	AM2K-1	17	1.00	
	<i>D. rufa</i>	rufa-OGM	24	0.75	
	<i>D. lacteicornis</i>	IR96-1	40	0.00	
	<i>D. neoasahinai</i>	OKNH2K	34	0.00	
	<i>D. tani</i>	tani-YF	83	0.00	
	<i>D. fengkainensis</i>	XT33	26	0.00	
	<i>D. trapezifrons</i>	Bavi31	11	0.27	
	<i>D. pectinifera</i>	OGS98m	34	0.00	
	<i>D. baimaii</i>	14028-0481.00	30	0.27	
	<i>D. pseudobaimaii</i>	K41	22	0.23	
	<i>kikkawai</i>	<i>D. bocki</i>	IR2-37	29	0.00
<i>D. leontia</i>		AO-2	39	0.41	
<i>D. kikkawai</i>		NAHA1	80	0.00	
<i>D. lini</i>		BGS3146.1	32	0.00	
<i>D. ohnishii</i>		MMY326	23	0.00	
<i>D. ogumai</i>		RGN3	30	0.00	
<i>punjabiensis</i>	<i>D. punjabiensis</i>	CJB212	44	0.11	
	<i>D. watanabei</i>	14028-0531.02	22	0.14	
<i>orosa</i>	<i>D. orosa</i>	14028-0611.00	23	0.09	
<i>serrata</i>	<i>D. barbarae</i>	14028-0491.01	31	0.42	
	<i>D. cauverii</i>	cauv-CNRS	31	0.68	
	<i>D. birchi</i>	14028-0521.00	64	0.00	
	<i>D. mayri</i>	14028-0591.00	42	0.55	
	<i>D. bicornuta</i>	BOG1	26	0.00	
	<i>D. truncata</i>	RGN179	25	0.24	
	<i>D. bunnanda</i>	14028-0721.00	50	0.10	
	<i>D. serrata</i>	Q122	226	0.27	
	<i>seguyi</i>	<i>D. burlai</i>	L6	20	0.20
<i>D. diplacantha</i>		14028-0586.00	73	0.44	
<i>D. nikananu</i>		14028-0601.00	23	1.00	
<i>D. greeni</i>		14028-0712.00	27	0.44	
<i>D. malagassya</i>		J6	27	0.41	
<i>D. seguyi</i>		K59	34	0.24	
<i>D. vulcana</i>		14028-0711.00	70	0.03	
<i>D. tsacasi</i>		14028-0701.00	9	0.22	
<i>D. jambulina</i>		F76	32	0.69	
<i>parvula</i>	<i>D. kanapiae</i>	14028-0541.00	23	0.04	
	<i>D. parvula</i>	SHL17	36	0.89	
<b>Outgroup</b>					
<i>(melanogaster)</i>	<i>D. biarmipes</i>	-	-	(1.00)	[1]
	<i>D. takahashii</i>	-	-	(1.00)	[2]
	<i>D. melanogaster</i>	-	-	(1.00)	[3]
	<i>D. elegans</i>	-	-	(1.00)	[4]
<i>(ananassae)</i>	<i>D. ananassae</i>	-	-	(1.00)	[5]

	<i>D. bipectinata</i>	-	-	(1.00)	[6]
( <i>obscura</i> )	<i>D. affinis</i>	-	-	(1.00)	[7]
	<i>D. algonquin</i>	-	-	(1.00)	[7]
	<i>D. pseudoobscura</i>	-	-	(1.00)	[8]
	<i>D. subobscura</i>	-	-	(1.00)	[9]
( <i>willistoni</i> )	<i>D. nebulosa</i>	-	-	(1.00)	[10]
	<i>D. tropicalis</i>	-	-	(1.00)	[11]
	<i>D. willistoni</i>	-	-	(1.00)	[11]
( <i>saltans</i> )	<i>D. saltans</i>	-	-	(1.00)	[12]

\*The incidence of pre-mounting courtship:  $I_{\text{pre}} = N_{\text{pre}}/N$ , where  $N_{\text{pre}}$  is the number of males that showed pre-mounting courtship, and  $N$  is the total number of observed pairs.

†References: [1] Lai, Gleason & True (2009); [2] Geng et al. (1989); [3] Wheeler, Field & Hall (1988); [4] Yeh, Liou & True (2006); [5] Yamada et al. (2002); [6] Crossley (1986); [7] Chang & Miller (1978); [8] Noor & Aquadro (1998); [9] Immonen et al. (2009); [10] Gleason et al. (2012); [11] Ritchie & Gleason (1995); [12] Gleason (personal communication).

**Table 2** Number of species having produced songs of each type in pre- and post-mounting courtships among studied species of the *Drosophila montium* group

Song type	$N_{\text{total}}$ *	P		HPR		S		Chi-square test†
		$N_{\text{P}}$	%	$N_{\text{HPR}}$	%	$N_{\text{S}}$	%	
Pre-mounting	31	16	51.6	8	25.8	7	22.6	$\chi^2 = 4.005$
Post-mounting	42	12	28.6	16	38.1	14	33.3	$P = 0.135$

P, pulse song; HPR, high pulse repetition song; S, sine song.

\* Species that produced two types of song were counted twice.

† By using SAS 9.21.



**Table 3** Song-type coincidence between pre- and post-mounting courtships in species having performed both courtships

Song-type	No. of species having produced songs of each type <sup>†</sup>		No. of species having produced songs of the same type in pre- and post-mounting courtships		Chi-square test			
	Pre-mountin	Post-mountin	Observe	Expected	Song-types in pre- and post-mountin g courtships	No. of species		$\chi^2$
	g	g	d	‡		Observe	Expecte	
P	13.5	8	6.5	4.3	Same	16.5	8.5	7.529
HPR	8	10	8	3.2	Different	8.5	16.5	3.879
S	3.5	7	2	1.0	Total	25	25	11.408
Total	25	25	16.5	8.5				$P = 0.0007^{\S}$

\* Song-type: P, pulse song; HPR, high pulse repetition song; S, sine song.

† Species having produced two types of song in pre- or post-mounting courtship is counted half for each song-type.

‡ The expected value was calculated by the following formula:

$$E_i = S \times (Pr_i \times Po_i),$$

where  $E_i$  is the expected number of species that produce songs of type  $i$  in both pre- and post-mounting courtships,  $S$  is the total number of species (in the above case,  $S = 25$ ),  $Pr_i$  is the probability that song of type  $i$  is sung in pre-mounting courtship (in the above case of P song,  $Pr_P = 13.5/25$ ), and  $Po_i$  is the probability that song of type  $i$  is sung in post-mounting courtship (in the above case of P song,  $Po_P = 8/25$ ).

§ Calculated by the function of Excel.

**Table 4** Comparison of tree lengths and goodness-of-fit parsimony statistics between two ("A" and "B") analyses of ancestral state reconstruction for pre- and post-mounting courtship songs

Analysis*	Pre-mounting courtship song		Post-mounting courtship song	
	"A"	"B"	"A"	"B"
Tree length	23	25	19	29
Consistency index (CI)	0.2174	0.1200	0.2105	0.1034
Homoplasy index (HI)	0.7826	0.8800	0.7895	0.8966
Retention index (RI)	0.4194	0.4634	0.3750	0.3500
Rescaled consistency index (RC)	0.0912	0.0556	0.0789	0.0362

\* "A": song types were treated as states of a character (pre- or post-mounting courtship song); "B": song types (P, HPR, and S) were treated as independent characters.

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## Supporting Information

**Appendix S1** Detailed methods for recording courtship behaviour and songs

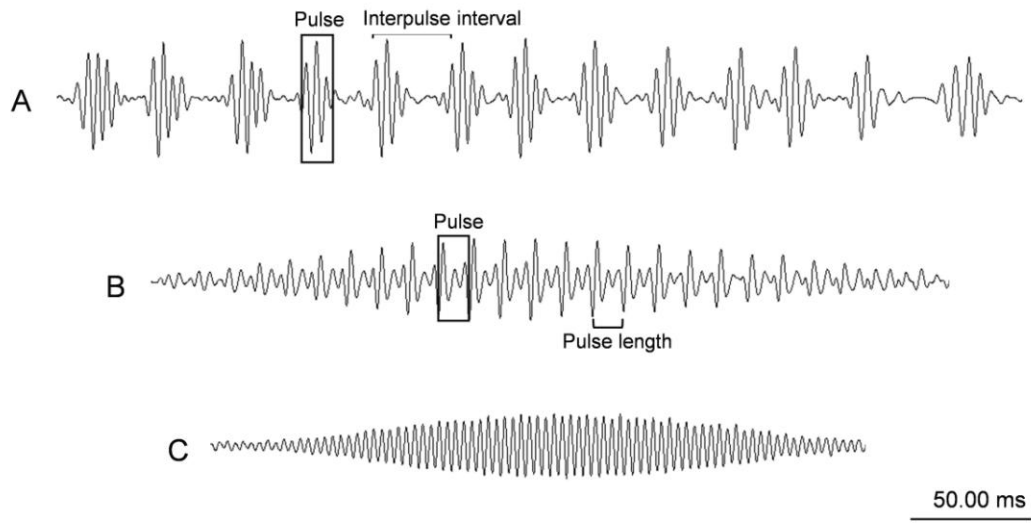
**Table S1** Species of the subgenus *Sophophora* of *Drosophila* subjected to the phylogenetic analysis in this study

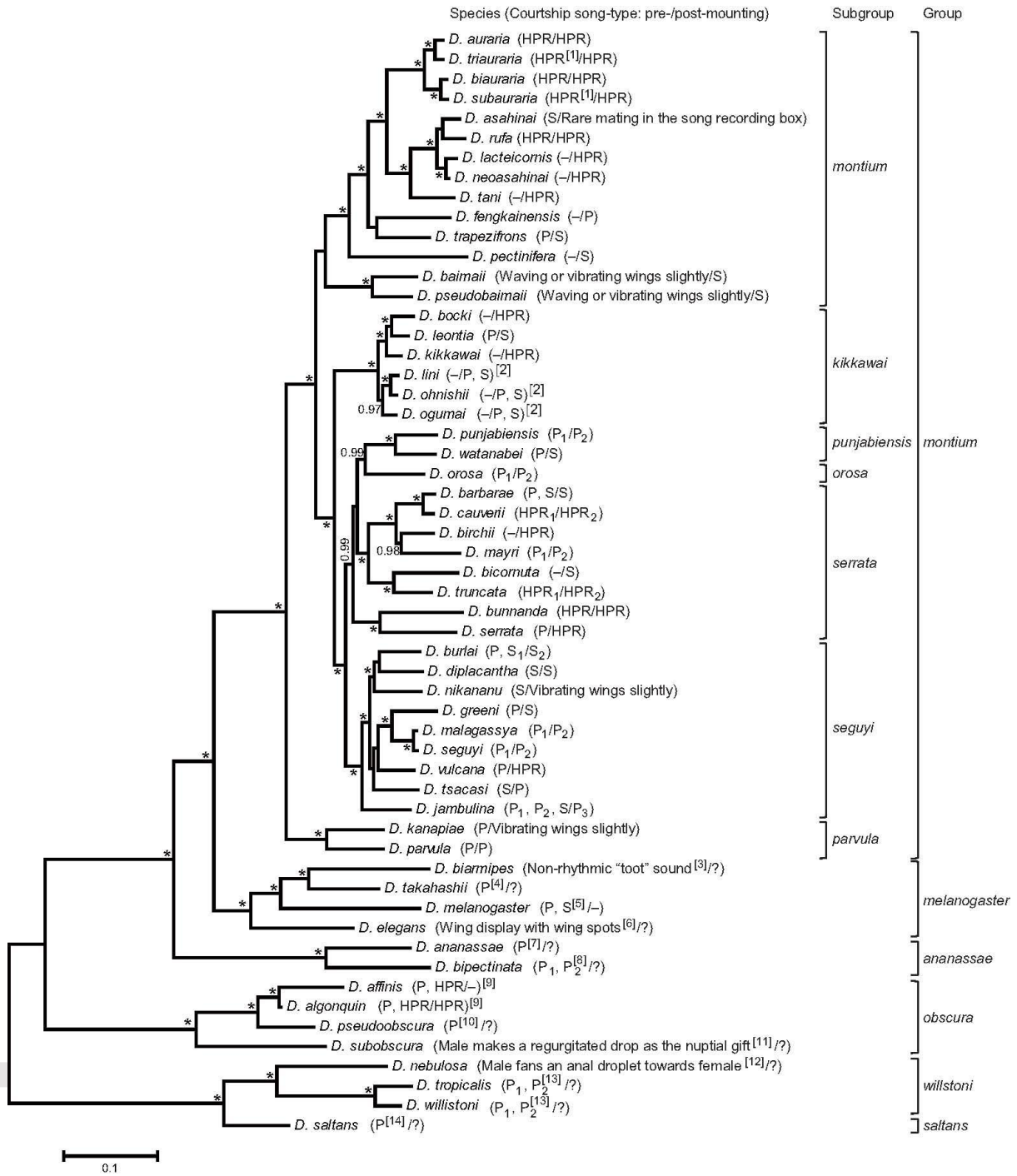
**Table S2** Parameters of courtship songs in the *Drosophila montium* species group

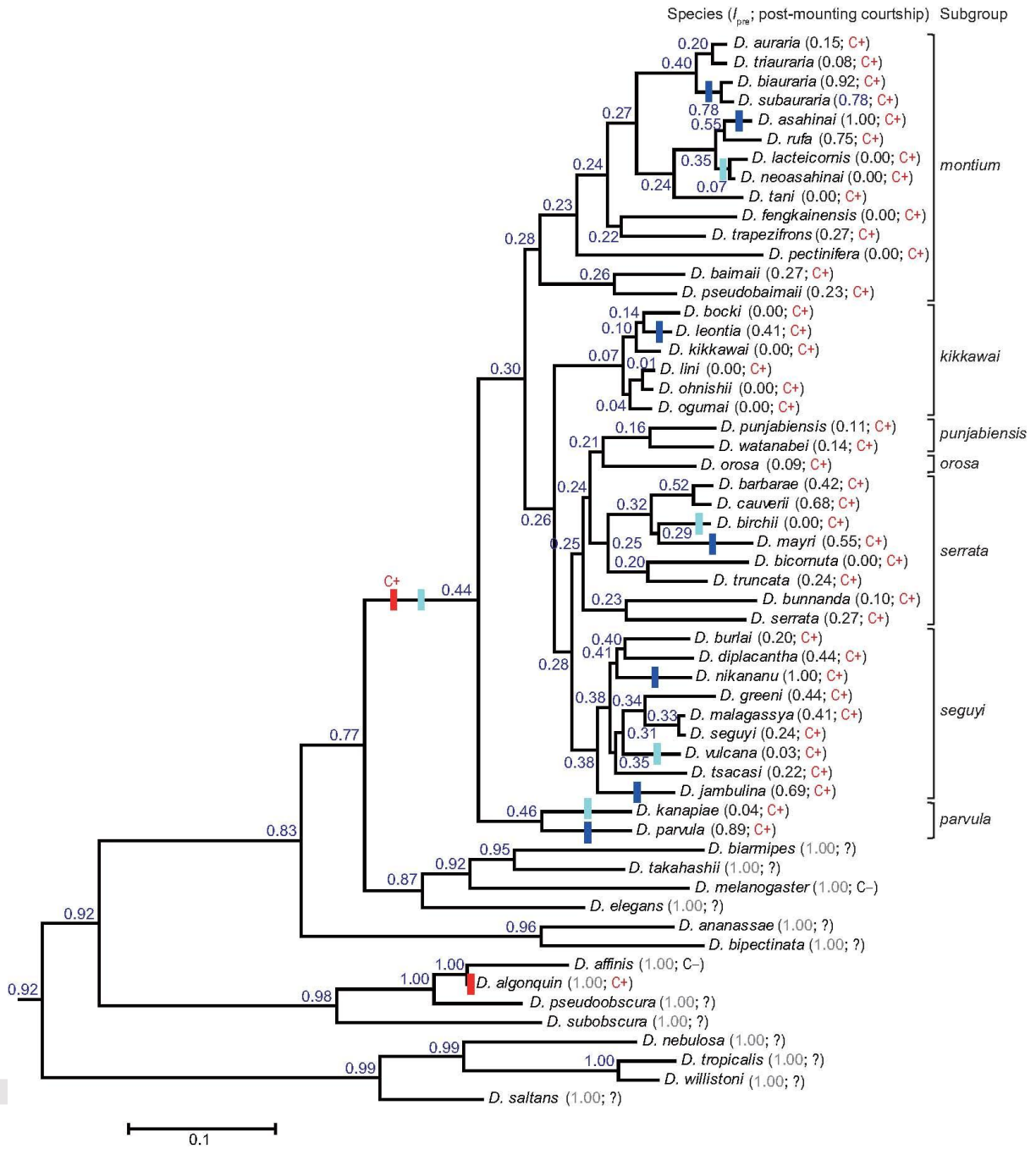
**Table S3** Songs produced during pre- and post-mounting courtships in the *Drosophila montium* species group

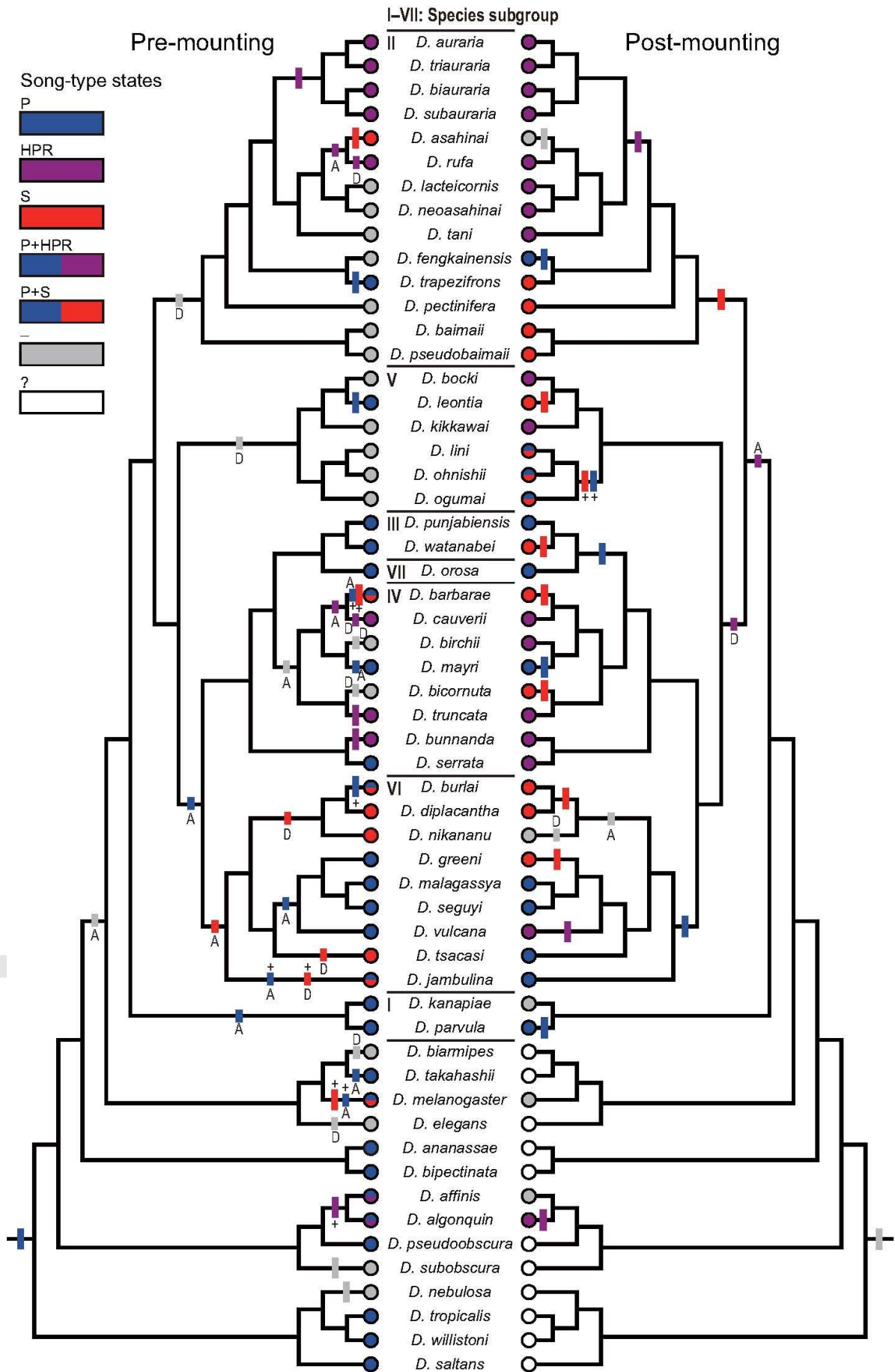
**Figure S1** Phylogenetic tree constructed by a maximum likelihood (ML) analysis of the concatenated dataset. Branch support is indicated by bootstrap value ( $\geq 50\%$ ).

**Figure S2** Evolutionary song-type changes mapped onto the Bayesian tree (Fig. 2). The ancestral state reconstruction was performed separately for pre- and post-mounting courtship; three song types, P (pulse song), HPR (high pulse repetition song) and S (sine song), were treated as independent characters.

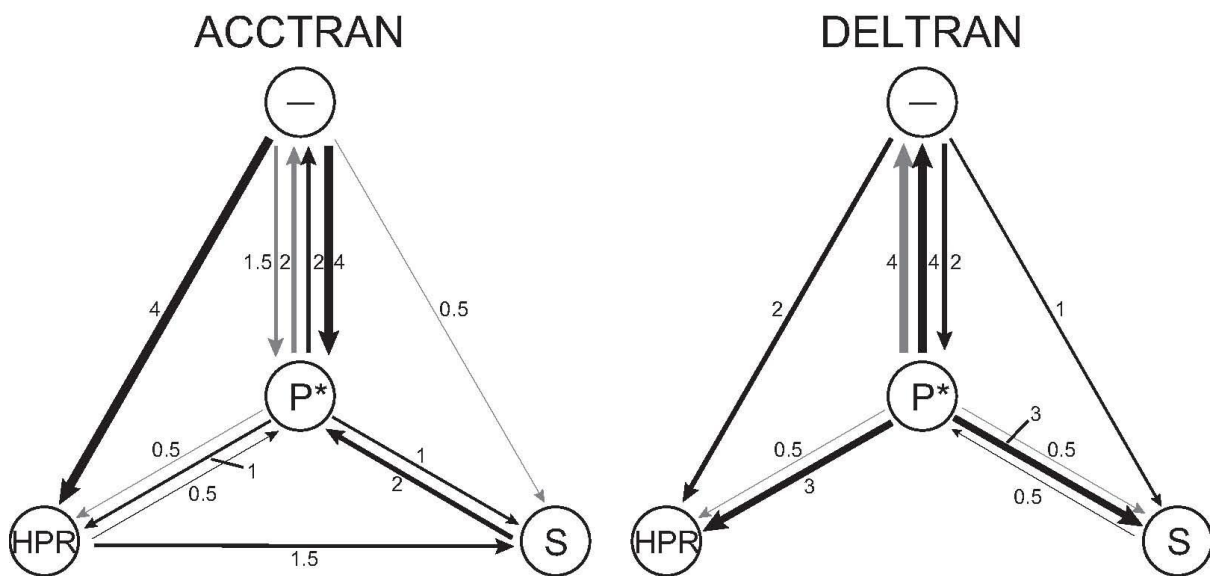








(A) Pre-mounting



(B) Post-mounting

