Correspondence: Masanori J. Toda, The Hokkaido University Museum, Kita 10-jo, Nishi 8-chome, Kita-ku, Sapporo, Hokkaido Prefecture 060-0810, Japan. email: hutian@lemon.plala.or.jp

Shuo-Yang Wen, Department of Entomology, College of Plant Protection, South China Agricultural University, 483 Wushan Road, Guangzhou 510642, China. email: shywen@scau.edu.cn

ORIGINAL ARTICLE

Divergence and correlated evolution of male wing spot and courtship display between *Drosophila nepalensis* and *D. trilutea*

Wen-Zhou Mo[1], Zhuo-Miao Li[1], Xiang-Mei Deng[1], Ai-Li Chen[1], Michael G. Ritchie[2], De-Jun Yang[3], Zhuo-Bin He[3], Masanori J. Toda[4] and Shuo-Yang Wen[1]

[1]*Department of Entomology, College of Plant Protection, South China Agricultural University, 483 Wushan Road, Guangzhou, 510642, China;*

[2]*School of Biology, University of St Andrews, St Andrews, Fife KY16 9TH, UK;*

[3]*Acoustics Lab, Guangdong Institute of Metrology, South China National Centre of Metrology, 30 Songbai East Street, Guangzhou, 510405, China;*

[4]*The Hokkaido University Museum, Kita 10-jo, Nishi 8-chome, Kita-ku, Sapporo, Hokkaido Prefecture 060-0810, Japan*

Abstract Male-specific wing spots are usually associated with wing displays in the courtship behavior of *Drosophila* and may play important roles in sexual selection. Two closely related species, *D. nepalensis* and *D. trilutea*, differ in wing spots and scissoring behavior. Here we compare male morphological characters, pigmentation intensity of male wing spots, wing-scissoring behavior, courtship songs and reproductive isolation between two species. F1 fertile females and sterile males result from the cross between females of *D. nepalensis* and

2

males of *D. trilutea*. The pigmentation of wing spots is significantly weaker in *D. trilutea* than in *D. nepalensis* and the F1 hybrid. Males scissor both wings in front of the female during courtship, with a posture spreading wings more widely, and at a faster frequency in *D. nepalensis* than in *D. trilutea* and the F1s. Males of *D. trilutea* vibrate wings to produce two types (A and B) of pulse songs, whereas *D. nepalensis* and the F1s sing only type B songs. The incidence of wing vibration and scissoring during courtship suggests that wing vibration is essential but scissoring is a facultative courtship element for successful mating in both species. The association between the darker wing spots with more elaborate scissoring might be the consequence of correlated evolution of these traits in *D. nepalensis*, however *D. trilutea* retains wing scissoring during courtship despite having weaker pigmentation of wing spots. The genetic architecture of two traits differs in the F1s, consistent with maternal or sex-linked effects for spots but non-additive effects for scissoring.

Key words correlated evolution; courtship song; *Drosophila nepalensis*; *D. trilutea*; wing display; wing spot

3

Introduction

Melanic wing pigmentation is seen in a number of drosophilid species. In most cases, such as the Hawaiian picture-wing group, wing pigmentation occurs in both female and male flies (True *et al.*, 1999). In monomorphic species, wing pattern may not influence mate choice, for example the polka-dotted wing pattern of the sexually monomorphic *Drosophila guttifera* (Niida & Koshikawa, 2021). In contrast, sexually dimorphic wing pigmentation is relatively unusual in *Drosophila* and is always associated with male wing displays during courtship in the *Drosophila melanogaster* species group (Mcrobert & Jackson, 1989; True *et al.*, 1999; Prud'homme *et al.*, 2006). The association between such sexually dimorphic morphology and display behavior seems likely to have coevolved through strong sexual selection (Svensson & Waller, 2013; Yeh & True, 2014; Santos & Machado, 2016). Based on a molecular phylogenetic analysis, Prud'Homme *et al.* (2006) inferred that wing-spot pigmentation was gained once and lost independently at least five times within the *melanogaster* group. Visual cues are often important elements in the *Drosophila* courtship repertoire (Greenspan & Ferveur, 2000). Male flies show some wing movements during courtship, such as wing vibration, wing scissoring, and wing waving. Males produce courtship songs as an acoustic signal by wing vibration, extending one or both wings from the resting position and then moving the wing(s) rapidly up and down. Wing scissoring or wing waving are displays of

4

visual signals by opening and closing of both wings in a scissoring-like movement usually in between bouts of wing vibration or by slowly spreading one wing 90 degrees outward from the body and holding this position without vibration (Spieth, 1952). Males with wing spots always perform wing displays to show wing spots in front of a female during courtship, whereas related spotless species generally lack these displays (Yeh *et al.*, 2006; Kopp & True, 2002; Yeh & True, 2014).

Wing pigmentation often varies in pattern and intensity among related species. Little is known about the variation in the intensity of wing pigmentation and its association with wing display during courtship. However, correlated divergence between these morphological and behavioral traits has been investigated between a pair of sibling species of the *elegans* subgroup, wing-spotted *D. elegans* and non-spotted *D. gunungcola* (Yeh *et al.*, 2006; Yeh & True, 2014; Massey *et al.*, 2020). Another possible model group of species for studies on coevolution between wing spot and its courtship display is the *takahashii* subgroup. Most species of the *takahashii* subgroup are morphologically uniform to a large extent, though Bock and Wheeler (1972) suggested that they can be identified by a combination of "coloration of male wings; number of sex-comb rows and bristles on the metatarsus and second tarsal segment; and structure of the basal branch of the posterior paramere". The male wing pigmentation varies in intensity among species of this subgroup: *D. nepalensis* males

5

possess wings with a black spot on the antero-distal portion (Okada, 1955); male wings of *D. prostipennis* and *D. pseudotakahashii* are diffusely darkened antero-distally (Bock & Wheeler, 1972; Kopp & True, 2002); but males of the remaining species have clear wings (Bock & Wheeler, 1972). However, some species classified in the last group, such as *D. takahashii*, *D. lutescens* and *D. trilutea*, actually have very light pigmentation on the apical region of male wings, leading to their variable categorization into non-spotted (Kopp & True, 2002) or spotted groups (Prud'homme *et al.*, 2006). We focus here on two sibling species, *Drosophila nepalensis* Okada, 1955 and *D. trilutea* Bock & Wheeler, 1972. Males of these two species distinctly differ in the intensity of wing pigmentation from each other. According to their original descriptions (Okada, 1955; Bock & Wheeler, 1972), however, there are few diagnostic morphological characters other than the wing pigmentation to distinguish these two species. In this study, therefore, we first test the species status for "*D. nepalensis*" and "*D. trilutea*" by examining their detailed morphological characters and the degree of sexual and reproductive isolation between them. Then, to address evolutionary associations between the male wing spot and courtship behaviors, we compare the intensity of wing pigmentation, the degree of wing scissoring behavior and the courtship songs between the two "sibling species" and their F1 hybrid, to infer if these are heritable and show similar patterns of inheritance.

6

Materials and methods

Flies

Strain BHL066 of *D. nepalensis* was established from a female collected from Baihualing (Yunnan, China) in the summer of 2014, and VT05-05 of *D. trilutea* (from northern Vietnam) was provided by Prof. Masayoshi Watada (Ehime-Fly, Drosophila Stocks of Ehime University). Flies were maintained on standard cornmeal-malt medium at 23°C under a 12 h : 12 h light cycle. Virgin flies were collected and separated into sexes without anesthesia within 12 h of emergence. Male flies were kept individually in vials (9.5 cm height × 1.5 cm diameter) containing culture medium before experiments, whereas females were maintained in groups of five in vials. Reproductively mature (5–7 days old) virgin flies were used for all experiments, and each fly was used only once.

Morphological comparison

Since it is almost impossible to identify female flies of the *takahashii* subgroup to species by morphological characters (Toda, 1991), only male morphology was compared between *D. trilutea* and *D. nepalensis*. Ten male specimens were examined for each species. The

7

specimens examined were collected from the field or obtained from culture strains (Table 1). If the culture strains were isofemale ones, i.e. originated from a single female, only one male specimen was selected from each strain. To observe detailed structures, the male terminalia and fore leg were detached from the body, cleared by warming in 10% KOH solution at approximately 100°C for several minutes, mounted in a droplet of glycerin on a cavity slide, and examined under a light microscope. Some samples were microphotographed using a DinoLite® Digital Eyepiece Camera. Metric characters were measured with an ocular micrometer installed on a stereomicroscope. The numbers of dorsal and ventral branches of aristae and the number of thick setae forming each TBR (Transverse Bristle Rows) (Baumina & Kopp, 2007) of sex combs on the fore tarsomeres I and II were counted. Statistical differences were examined using the Wilcoxon test with the software JMP®9.0.2 (SAS Institute Inc, Cary, NC, USA). To describe morphological characteristics of the two species in this study, we applied the most recent, morphological terminology of Rice *et al.* (2019) for the male terminalia, Cumming and Wood (2017) for the body parts other than the male terminalia, and Zhang and Toda (1992) for the definitions of measurements and indices (Table S1). All voucher specimens are deposited in Systematic Entomology, the Hokkaido University Museum, Hokkaido University, Sapporo, Japan (SEHU).

Mate-choice tests

Five to seven-day old virgin flies were used in mate-choice tests. In a "female-choice" test, one virgin female and two virgin males of each species were introduced into a glass vial (10.5 cm × 3 cm) containing food medium using an aspirator without anesthesia. A space 30 mm in diameter and 30 mm in height was left as a behavior-observation chamber above the food medium by inserting a cotton plug into the vial (Wen *et al.*, 2011). Similarly, "male-choice" tests used one virgin male and two virgin females of each species. Forty replicates were set up for each of four combinations: *trilutea♀×trilutea+nepalensis♂*, *nepalensis♀×trilutea+nepalensis♂*, *trilutea+nepalensis♀×trilutea♂*, and *trilutea+nepalensis♀×nepalensis♂*. In order to distinguish the species, females of one species in male-choice tests were marked by feeding them on a medium colored with Methylene Blue for one day before the experiment. Of the 40 replicates, 20 were color-marked for one species, and the other 20 were color-marked for the other species (Wen *et al.*, 2011). Males in female-choice tests were distinguished by their wing spots. Each replicate was observed from the introduction of experimental flies into the vial to the end of successful copulation, which was judged when genital intromission continued for more than 5 min. During the observation, all mounting events were recorded: which female was mounted by the male in a male-choice test, or which male mounted the female in a female-choice test. It was also recorded with

9

which female the male eventually achieved successful copulation in a male-choice test, or which male achieved successful copulation with the female in a female-choice test. The extent of sexual isolation was evaluated separately both before and after mounting. To quantify sexual isolation, we used the frequency in which the first mounting was homo- or heterogamic, excluding the data for any second and later mounting(s) to eliminate effects of previous mounting and/or rejected experience(s). For evaluation of sexual isolation after mounting, we used the frequency of homo- or heterogamic matings (successful copulation). The "pair sexual isolation coefficient" (*PSI*) and the "joint isolation index" (I_{PSI}) were obtained from the software JMATING 1.0.8 (Carvajal-Rodriguez & Rolan-Alvarez, 2006) (http://webs.uvigo.es/acraaj/JMsoft.htm). *PSI* is the ratio of the observed frequency to the expected one under random mounting/mating for each species pair, values of *PSI* above/below 1 thus indicating excess/deficit of observed mounting/mating frequency relative to the expected one from random mounting/mating, and I_{PSI} ranges from –1 to 1, with –1 representing disassortative mounting/mating, 0 representing random mounting/mating, and 1 representing complete sexual isolation (Rolán-Álvarez & Caballero, 2000; Pérez-Figueroa *et al.*, 2005; Wen *et al.*, 2011).

Crossing experiments

10

Six-day old virgin flies were used in the two reciprocal combinations: *D. nepalensis*♀×*D. trilutea*♂ and *D. trilutea*♀×*D. nepalensis*♂. Any virgin F1 progeny were collected and backcrossed to both parental species. Ten and five replicates were made for each combination of cross and backcross experiments, respectively. For each replicate, five females and ten males were introduced into a vial (10.5 cm × 3 cm) containing food medium with an aspirator without anesthesia. After five days, flies were transferred into a new vial to allow them to lay eggs for five more days, and then discarded from the vials. When adults eclosed, the number of progeny were counted, and the sex ratio calculated. An additional experiment under a condition of increased number of parental flies (20 females and 40 males) was performed with ten replicates for the cross of *D. trilutea*♀×*D. nepalensis*♂.

Pigmentation intensity of wing spots

Six to seven-day old virgin male flies of *D. nepalensis*, *D. trilutea*, and their F1 hybrid of *D. nepalensis*♀×*D. trilutea*♂ (the reciprocal cross *D. trilutea*♀×*D. nepalensis*♂ produced no offspring; see Results) were dissected in ethanol: both wings were detached under an anatomical microscope. The wings were transferred onto a cavity slide and mounted in a droplet of glycerin. Digital images were taken using an EVOS FL automated fluorescence

11

microscope (Thermo Fisher Scientific, USA). The wing-spot area was defined as a rectangle with four landmarks as shown in Fig. 1. The original images were transferred into target 8-bit images in the grey mode with a threshold of 130 using Image J 1.45v (https://imagej.nih.gov/ij/). The target images were re-opened and the 'Grey Value' (GV) of wing-spot area was measured for each of left and right wings, and the bilateral asymmetry was evaluated as the measure of directional asymmetry: $DA = GV_{left} - GV_{right}$ (Graham et $al.$, 2010). First, the mean GVs, separately for each of left and right wings, and the mean DAs were compared among $D.$ $nepalensis$, $D.$ $trilutea$, and the F1 hybrid by Welch's F test one-way ANOVA, along with the Bartlett test for the homogeneity of variance. If a significant difference was detected, the $post$ hoc multiple comparisons were made by the Tukey-Kramer method in cases satisfying the prerequisite of homoscedasticity but by the Steel-Dwass method in cases of heteroscedasticity. For the mean DAs, the 95% confidence intervals were calculated to examine the presence or absence of directional asymmetry (i.e. deviation from 0). And the variance of DA as an index of fluctuating asymmetry (Graham et $al.$, 2010) was compared among/between $D.$ $nepalensis$, $D.$ $trilutea$, and the F1 hybrid by the Bartlett tests. The statistical analyses were made using the software JMP®9.0.2 (SAS Institute Inc, Cary, NC, USA).

12

Video recording of courtship behavior and comparison of wing scissoring behavior

Six to seven-day old virgin flies of *D. nepalensis*, *D. trilutea*, and their F1 hybrid (*D. nepalensis*♀×*D. trilutea*♂) were used for observation and/or video recording of courtship behavior. Digital video was made using a multiple well plate and macro lens digital camera linked to a laptop under the operation of the AMCap-DirectShow 8.12 (Microsoft Corp.). Fly pairs were introduced into mating chambers (15 mm diameter × 6 mm height) containing culture medium, and covered with transparent coverslips, under LED light. Temperature was maintained at 24±2°C. The observation/recording was continued until copulation finished for each pair, but interrupted at 4 h where the copulation was still continuing. Sequences of courtship behavioral elements (orientation, circling, tapping, wing vibration and scissoring, and mounting attempt) were recorded for each pair by observation and/or repeated examination of the videos. From the behavioral records of 40, 44 and 74 successful mating pairs of *D. nepalensis*, *D. trilutea* and the F1 hybrid, respectively, the incidence of each courtship element was calculated as the number of males who performed the element. Male flies often performed bursts of wing scissoring in front of the female. To compare this behavior among the three groups, we examined video recordings in more detail for the following pairs: seven pairs of *D. nepalensis*, ten of *D. trilutea*, and 14 of the F1 hybrid. The wing-scissoring posture was defined by the maximum and minimum angles of spread wing from the body axis. We measured the following attributes for each pair: the total duration (s) of scissoring bursts during courtship (Σt_{burst}, where t_{burst} = the duration of each burst); and the total number of scissorings during courtship (Σn_{sci}, where n_{sci} = the number of scissorings per

13

burst). Then, we calculated the frequency of scissoring per second ($f_{sci} = \Sigma n_{sci}/\Sigma t_{burst}$ s^{-1}).

Statistical comparisons for these attributes were made using the same methods as for

wing-spot pigmentation intensity.

Recording and analysis of courtship songs

   Courtship behavior and songs were recorded using a custom-made *Drosophila*

Acoustic-Behavioral Experimental Soundproof System (Guangzhou, China; Fig. S1). This

consists of a mating chamber (with 12 mm inside diameter, 2.5 mm internal height and

acoustically transparent nylon net floor) placed above a pre-polarized microphone (Type

4176, B&K, Denmark) and a CCLD microphone preamplifier (Type 2671, B&K, Denmark)

connected to a computer and operated by Audacity 2.1.0 (Audacity Team). Above the mating

chamber is a macro lens operated by ArcSoft ShowBiz 3.5.15.68 (ArcSoft Inc.) to

synchronously capture videos and audio from the B&K microphone. Courtship songs were

digitized at a sampling frequency of 8 kHz using Audacity 2.1.0 (Audacity Team) and saved

in the .wav format. The following song parameters were subsequently analyzed using

DataView 10.2.1 (https://www.st-andrews.ac.uk/~wjh/dataview/), while watching the

synchronous videos: pulse length (PL), interpulse interval (IPI), intrapulse frequency (IPF),

and cycle number per pulse (CN). Statistical comparisons for these parameters were made

using the same methods as for wing-spot pigmentation intensity.

14

Results

Morphology

Although males of the two species, *D. trilutea* and *D. nepalensis*, can easily be distinguished from each other by the wing color pattern, they are otherwise very similar (Fig. 2A,E). However, significant interspecific differences were detected in the following characters (Table 1): WL (distance from humeral cross vein to wing apex), WW (maximum wing width), sctlp (distance between ipsilateral scutellar setae / cross distance between apical scutellar setae), C3F (relative length of heavily setigerous region in 3rd costal section of wing), and the numbers of thick setae forming the apical (TBR_{I-i}) and subapical (TBR_{I-ii}) sex combs on the fore tarsomere I and TBR_{II-i} on the tarsomere II, indicating that the wing is larger, the apical scutellar setae are separated from each other more, the relative proportion of heavily setigerous part in 3rd costal section is larger, and the sex combs comprise more thick setae in *D. trilutea* than in *D. nepalensis* (Table 1; Fig. 2B,F). In addition, the numbers of primary and secondary teeth on the surstylus tended to be larger in *D. trilutea* (10–14 and 5–8, respectively, *n* = 4) than in *D. nepalensis* (9–11 and 5, respectively, *n* = 3) (Fig. 2C,G). However, these quantitative differences were not large or diagnostic (i.e. non-overlapping) and would not be significant if we applied a Bonferroni correction. On the other hand, another distinct interspecific difference was found in the postgonite, which is the term unified

15

by Rice *et al.* (2019) for "basal branch of the posterior paramere" of Bock and Wheeler (1972) or "basal process of aedeagus" of Hu and Toda (2001). Bock and Wheeler (1972) recognized this organ as being the most useful to distinguish some species of the *takahashii* subgroup. In *D. trilutea*, the postgonite bears coarse serrations basally to subapically along the outer margin and a few small serrations on the distal surface (Fig. 2D), while in *D. nepalensis*, the postgonite is serrated only on the basal to submedial margin, but is smooth in the distal portion (Fig. 2H).

Premating isolation

The frequencies of first homo- and hetero-gamic mountings and of homo- and hetero-gamic matings are shown in Table 2 with the values of *PSI*, I_{PSI}, and the bootstrap tests for *PSI* and I_{PSI}. In female-choice tests, females of *D. trilutea* and *D. nepalensis* were first mounted more often by conspecific males giving $I_{PSI} = 0.409$ ($P = 0.0002$), i.e. the species show significant pre-mounting sexual isolation. In male-choice tests, almost half-half females were mounted by both homo- and hetero-gamic males. The I_{PSI} of 0.1774 ($P = 0.1156$) indicates that males did not differentiate between females of these two species before mounting, i.e. they mounted at random. Hence the assortative first mounting is probably due

16

to female choice. After being mounted, females showed strong repelling behavior against heterospecific males. Hence most females were mated by conspecific males with I_{PSI} of 0.9520 and 0.8836 in the male- and female- choice tests, respectively, resulting in almost complete sexual isolation between the two species, except four females of *D. nepalensis* who mated with *D. trilutea* males in the female-choice test (Table 2).

Postmating isolation

We performed two reciprocal crosses between *D. nepalensis* and *D. trilutea* and four combinations of backcross (their F1 hybrid × parental species). Results are shown in Table 3. F1 progeny were produced only from the combination of *D. nepalensis*♀×*D. trilutea*♂, as expected from the results of mate-choice tests described above. The crosses between *D. trilutea* ♀×*D. nepalensis* ♂ were infertile even under the condition of increased number of parental flies (20 females and 40 males per replicate). Backcross progeny were obtained from the F1 females with parental males, but none were produced from the F1 males with parental females, indicating that F1 females are fertile but males sterile. The fertility (the number of offspring produced) per female from the combination of *D. nepalensis*♀×*D. trilutea*♂ was 40.7, and the sex ratio was normal at 1.04. The backcrosses of F1 females with males of *D.*

17

nepalensis and of *D. trilutea* showed a reduced fertility to 12.7 and 8.0, respectively, and again the sex ratio did not deviate from 1.

Wing-spot pigmentation

We investigated the pigmentation intensity of male wing spots of *D. nepalensis*, *D. trilutea* and the F1 hybrid of *D. nepalensis*♀×*D. trilutea*♂ by measuring the grey value (*GV*) of wing-spot area (Fig. 1). The mean *GV*s were significantly different among the three groups (Table 4), being significantly smaller in *D. trilutea* than in *D. nepalensis* and the F1 hybrid, but the difference between the last two was not significant (Fig. 3A,B). Indeed, the F1s were almost identical to *D. nepalensis*. The mean *DA*s (directional asymmetry) were neither significantly different among the three groups nor deviated from 0 (Table 4, Fig. 3C), indicating no directional asymmetry in any of the three groups. On the other hand, the variance of *DA* (fluctuating asymmetry) was the largest in the F1 hybrid, significantly different from *D. trilutea* but not from *D. nepalensis* (Table 4).

18

Courtship and wing-scissoring behavior

There are several behavioral elements of the courtship of *D. nepalensis* and *D. trilutea*: orientation, tapping, circling, and vibration, waving/scissoring of wing(s), and attempting to mount. The sequence of courtship behavior is very similar in the two species and their F1 hybrid (Fig. 4, Supplementary Movies S1, S2 and S3): (1) a male orients himself to a female by running to her rear, while spreading either his left or right wing when he approaches her from the left or right side, respectively; (2) he taps her, vibrates the spread wing to produce courtship songs, and attempts to mount her; and (3) if she rejects him by flapping her wings, he spreads both of his wings, circles to her front, and performs wing display by waving or scissoring his both wings. If she keeps steady, he turns back to her rear to tap and sing, and repeats the sequence from (2) to (3) until she accepts his mounting. The incidence of each element in successfully mated males was compared among the three groups, i.e., *D. nepalensis*, *D. trilutea* and their F1 hybrid (Table 5). Although the courtship elements of orientation, wing vibration and mounting attempt were always performed in both species and the F1 hybrid, tapping was absent in the courtship of a few males of *D. trilutea* (Fisher's exact test, $P = 0.0359$; Table 5), and a few males did not perform circling or scissoring in either parental species or the F1 hybrid. However, the proportion of males having performed (+) or not performed (–) these elements was not significantly different among the two species

19

and the F1 hybrid (Fisher's exact test, $P > 0.05$; Table 5). These results imply that tapping or scissoring is not a necessary element for successful courtship.

However, as with most wing-spotted *Drosophila* species, males of *D. nepalensis*, *D. trilutea* and their F1 hybrid often perform bursts of wing-scissoring during courtship. In a burst both wings are repeatedly scissored, but in noticeably different ways between *D. nepalensis*, *D. trilutea* and their F1 hybrid. *D. nepalensis* males scissor both wings between the angles of 90° (at the maximum) and ≥45° (at the minimum) against the body axis, while *D. trilutea* and the F1 hybrid males do so between 90° and <45° (Fig. 4, Supplementary Movies S1, S2 and S3). We measured the total duration of scissoring bursts (Σt_{burst}) and the total number of scissorings (Σn_{sci}) during the courtship of each male from the video recordings, and calculated the frequency of scissoring ($f_{sci} = \Sigma n_{sci}/\Sigma t_{burst}$). Although the first two measures were not significantly different among the three groups, males of *D. nepalensis* scissored the wings significantly more quickly at an average frequency of 1.773 s^{-1} than those of *D. trilutea* (1.437 s^{-1}) and the F1 hybrid (1.399 s^{-1}) (Fig. 3, Table 6, SM1, 2 and 3).

20

Courtship songs

The patterns of courtship songs produced by *D. nepalensis*, *D. trilutea* and their F1 hybrid

are shown in Fig. 5. Songs were categorized into two types of pulse song based on the cycle

number per pulse (CN): a type A pulse consists of only one cycle, which is specific to *D.*

trilutea, but a type B pulse contains multiple (2 to 5) cycles, which is produced by the three

groups of males (Fig. 5, Table 7, Movies S4–S7). Four parameters, CN, PL, IPI and IPF, of

the type B song were significantly different among the three groups ($P < 0.0001$, Welch's F

test one-way ANOVA). *Post hoc* multiple comparison tests detected significant differences in

PL and IPF between all pairs of the three groups, but CN and IPI were not significantly

different between *D. nepalensis* and the F1 hybrid (Table 7). Thus, the F1 hybrid is more

similar to the maternal parent, *D. nepalensis*, in CN and IPI of the type B song and lacking

the type A song, suggesting a similar inheritance to wing-spot intensity.

Discussion

Male-specific wing pigmentation and associated courtship behavior would likely have

coevolved through strong sexual selection (Svensson & Waller, 2013; Yeh & True, 2014;

Santos & Machado, 2016). Behavior and morphology often coevolve but can sometimes

21

become disassociated in *Drosophila*. Massey *et al.* (2020) found a variation in male courtship behavior between local populations of *D. gunungcola*, which is a wing-spotless species very closely related to the wing-spotted *D. elegans.* Males of a wild population in East Java perform wing displays in front of a female during courtship despite completely lacking wing spots, while males of another strain, SK from Sukarami, West Sumatra, have neither of these traits. Furthermore, they confirmed that these two traits are genetically distinct: the presence or absence of wing spots is controlled by a ∼440 kb region including a candidate wing patterning gene optomotor-blind (*omb*) on the X chromosome, whereas at least two loci on the X chromosome and two loci on autosomes govern the evolution of wing display. The morphological change involves fewer candidate genes and may have evolved prior to the more complex behavioral change (Massey *et al.*, 2020). Here we present another instance of dissociated changes of morphological and behavioral displays between a pair of sibling species in the *takahashii* subgroup.

D. nepalensis and *D. trilutea* are very similar to each other in the general morphology. However, we found that males of these two species can be distinguished by a slight but distinct difference in serration of the postgonite (Fig. 2D,H) in addition to the difference in the intensity of wing-spot pigmentation (Fig. 1). Furthermore, a number of quantitative

22

characters are significantly different between them (Table 1). Our mate-choice tests detected the presence of premating isolation between *D. nepalensis* and *D. trilutea* (Table 2). Before mounting, females of both species recognized conspecific mates to some degree through courtship interactions. Upon mounting, most females refused to copulate with heterospecific males. Consequently, successful copulations were almost always achieved by homospecific mates. However, *D. nepalensis* females rarely copulated with *D. trilutea* males, and produced F1 hybrid offspring; but the reciprocal cross (*D. trilutea*♀×*D. nepalensis*♂) was unsuccessful in reproduction. The backcross experiments detected partial postmating isolation (Table 3): the F1 hybrid females were fertile but males sterile, consistent with Haldane's Rule (Orr, 1997). We therefore conclude that *D. nepalensis* and *D. trilutea* are closely related "good" species. This is further supported by their occurrence in sympatry across a wide geographic range: *D. nepalensis* is distributed in Ryukyu Is., Taiwan Is., southern and southwestern China, Thailand, Singapore, Myanmar, Nepal and northern India, and *D. trilutea* in Taiwan Is., central, southern and southwestern China and northeastern India (Toda, 2021). However, they can hybridize and produce partially fertile offspring, through which introgression might be possible to some extent between them.

23

Through courtship behaviors *Drosophila* females and males exchange various signals to recognize potential mates (Aranha & Vasconcelos, 2018, Hall, 1994, Ishimoto & Kamikouchi, 2020, Spieth, 1974). In the courtship of *D. nepalensis* and *D. trilutea*, males sing songs by vibrating either wing from behind a female before trying to mount her. If the female rejects the male's attempt by flicking her wings, the male circles to her front to perform a display by scissoring his wings, then turns back to her rear again to sing and try to mount, and repeats these behaviors (Fig. 4, Supplementary Movies S1, S2) until she finally accepts or he gives up. In their courtship, wing vibration (producing song) and scissoring are noticeable behavioral elements. In our observation of >150 successful mating interactions, all males performed wing vibration, but about 30% males did not perform scissoring (Table 5). This suggests that wing vibration is essential but scissoring not always necessary for successful mating. Courtship songs are different in composition and parameters between *D. trilutea* and *D. nepalensis* (Fig. 4, Table 7) such that songs could serve as effective cues for mate recognition. Which components (song type and/or parameters) act as essential acoustic signals could be examined by song playback experiments (Klappert *et al.*, 2007; Li *et al.*, 2012).

Even though the wing-scissoring display is not essential for mating success, this wing display would provide some visual signals for mate recognition. The pigmentation intensity of male wing spots is significantly stronger in *D. nepalensis* than in *D. trilutea* (Figs. 1 and 3, Table 4); Kopp and True (2002) have previously regarded *D. trilutea* as lacking male wing spots. Despite the weak pigmentation on male wings, *D. trilutea* still shows pronounced scissoring behavior. However, it is not the same as in the dark spotted *D. nepalensis*. Males of *D. nepalensis* scissor both wings in a more widespread posture (between the angles of 90° and ≥45° against the body axis) at a faster frequency (1.773 scissorings per second) than *D. trilutea* males do between 90° and <45° and at 1.437 s^{-1} (Figs. 3 and 4, Table 6, Supplementary Movies S1 and S2).

Sexually dimorphic characters can diverge very rapidly among closely related species (Kopp & True, 2002) and even within a species (Hegde *et al.*, 2005; Roy & Gleason, 2019). As color phenotypes are easy to study, their evolutionary changes or diversification can be traced (Koshikawa, 2020). For example, Moest *et al.* (2020) identified genes controlling wing patterns, such as *aristaless* of chromosome 1, *cortex* of chromosome 15, *optix* of chromosome 18 and *WntA* of chromosome 10, in *Heliconius* butterflies, and demonstrated that these genes are under very strong natural and sexual selection and influence mimicry and

25

speciation. In *Drosophila*, Yeh *et al.* (2006), Yeh and True (2014) and Massey *et al.* (2020) investigated the genetic basis of correlated divergence in wing pigmentation and associated courtship display between the sibling species *D. elegans* and *D. gunungcola*: the wing-spotted *D. elegans* males display the spot to females by extending their wings laterally during courtship, while the non-spotted *D. gunungcola* (SK strain) males perform no wing display. They made hybridization experiments and found partial maternal effects on the wing pigmentation and display of F1 hybrid males: F1 males from *D. elegans*♀×*D. gunungcola*♂ possessed the wing spots (but small) and showed wide wing display angles like the maternal species *D. elegans*, while F1 males from *D. gunungcola*♀× *D. elegans*♂ lacked the wing spots similar to the maternal species *D. gunungcola* but showed narrower wing display angles. Furthermore, using recombinant backcross progeny generated via fertile F1 hybrid females produced by both reciprocal crosses between these species*,* Massey *et al.* (2020) found that introgression of the X-linked region affecting wing spot development from *D. gunungcola* into *D. elegans* reduced pigmentation in the wing spots but did not affect the wing display, indicating that these are genetically separable traits.

The genetic information available from F1 crosses is limited but can show interesting differences between morphology and behavior. Our study species allow similar genetic

26

analyses as in *D. elegans* and *D. gunungcola*. The production of F1 hybrids from *D. nepalensis*♀×*D. trilutea*♂ allows us to compare the wing pigmentation and courtship behavior among parent species and the F1 hybrid. The F1 hybrid males have dark wing spots not significantly different in the intensity from those of maternal species, but with a larger variance of *DA* (i.e. bilateral fluctuating asymmetry) than the parental species as generally expected due to developmental abnormality or weakness of F1 hybrid (Figs 1 and 3, Table 4), suggesting that the pigmentation intensity is controlled either by genes on the X chromosome (as in *D. elegans* and *D. gunungcola*) or by dominant autosomal alleles of *D. nepalensis*. A similar inheritance is seen in courtship song: the F1 hybrid and maternal species produce only the type B pulse song with CN and IPI not significantly different between them (Fig. 5, Table 7). On the other hand, the wing scissoring behavior of F1 hybrid males is nearly the same in the scissoring posture (angles) and frequency as that of paternal species (Fig. 3, Table 6, Supplementary Movie S3). Thus, dominant genetic effects were the major component for scissoring, implying that the underlying genetic architecture is different for this trait. This could affect their potential to respond to selection, which will differ according to the degree of linkage and dominance (Charlesworth *et al.*, 1987).

Conclusions

In the present study, we described divergence of genitalia, reproductive isolation, wing spots, wing scissoring behavior and courtship songs between the two sibling species *D. nepalensis* and *D. trilutea*. Males of *D. nepalensis* display their darker wing spots in front of a female by scissoring both wings in a more widespread posture and at a faster frequency during courtship, probably as a consequence of correlated evolution of these two traits. On the other hand, despite weak pigmentation on male wings, *D. trilutea* still retains pronounced wing-scissoring behavior in courtship. Different inheritance of these traits (wing pigmentation and display) might reflect their response to selection. While courtship song produced by wing vibration is an essential courtship element, wing-scissoring display may be a facultative element for successful mating. This, along with the potentially different genetic architectures for the morphological and some behavioral traits, could have influenced the divergence of these traits between the two species. However, more detailed elucidation of the genetic basis of these morphological and behavioral traits is needed to better understand their potential for independent evolution.

Acknowledgments

28

Disclosure

All authors have seen and agree with the contents of the manuscript and there is no conflict of interest, including specific financial interest and relationships and affiliations relevant to the subject of manuscript.

29

Reference

Aranha, M.M. and Vasconcelos, M.L. (2018) Deciphering *Drosophila* female innate behaviors. *Current Opinion in Neurobiology*, 52, 139–148.

Baumina, O. and Kopp, A. (2007) Sex-specific expression of a HOX gene associated with rapid morphological evolution. *Developmental Biology*, 311, 277–286.

Bock, I.R. and Wheeler, M.R. (1972) *The Drosophila melanogaster species group*. The University of Texas Publications, USA. 102 pp.

Carvajal-Rodriguez, A. and Rolan-Alvarez, E. (2006) JMATING: a software for the analysis of sexual selection and sexual isolation effects from mating frequency data. *BMC Evolutionary Biology*, 6, 40.

Charlesworth, B., Coyne, J.A. and Barton, N.H. (1987) The relative rates of evolution of sex chromosomes and autosomes. *American Naturalist*, 130, 113–146.

Cumming, J.M. and Wood, D.M. (2017) *Adult morphology and terminology. Manual of Afrotropical Diptera*. eds. A.H. Kirk-Spriggs & B.J. Sinclairs, South African National Biodiversity Institute, Pretoria. pp. 89–133.

Graham, J.H., Raz, S., Hel-Or, H. and Nevo, E. (2010) Fluctuating asymmetry: methods, theory, and applications. *Symmetry*, 2, 466–540.

Greenspan, R.J. and Ferveur, J.F. (2000) Courtship in *Drosophila*. *Annual Review of Genetics*, 34, 205–232.

Hall, J.C. (1994) The mating of a fly. *Science*, 264, 1702–1714.

Hegde, S.N., Chethan, B.K. and Krishna, M.S. (2005) Mating success of males with and without wing patch in *Drosophila biarmipes*. *Indian Journal of Experimental Biology*, 43, 902–909.

Hu, Y. and Toda, M.J. (2001) Polyphyly of Lordiphosa and its relationships in Drosophilinae (Diptera: Drosophilidae). *Systematic Entomology*, 26, 15–31.

Ishimoto, H. and Kamikouchi, A. (2020) A feedforward circuit regulates actionselection of pre-mating courtship behavior in female *Drosophila*. *Currrent Biology*, 30, 396–407 e4.

Klappert, K., Mazzi, D., Hoikkala, A. and Ritchie, M.G. (2007) Male courtship song and female preference variation between phylogeographically distinct populations of *Drosophila montana*. *Evolution*, 61, 1481–1488.

31

Kopp, A. and True, J.R. (2002) Evolution of male sexual characters in the Oriental *Drosophila melanogaster* species group. *Evolution Development*, 4, 278–291.

Koshikawa, S. (2020) Evolution of wing pigmentation in *Drosophila*: Diversity, physiological regulation, and cis-regulatory evolution. *Development Growth Differentiation*, 62, 269–278.

Lande, R. (1980) Sexual dimorphism, sexual selection, and adaptation in polygenic characters. *Evolution*, 34, 292–305.

Li, Y.-F., Wen, S.-Y. and Ritchie, M.G. (2012) Copulatory song in three species of the *Drosophila montium* subgroup extends copulation and shows unusual genetic control. *Animal Behaviour*, 83, 233–238.

Massey, J.H., Rice, G.R., Firdaus, A.S., Chen, C.Y., Yeh, S.D., Stern, D.L., *et al.* (2020) Co-evolving wing spots and mating displays are genetically separable traits in *Drosophila*. *Evolution*, 74, 1098–1111.

Mcrobert, S.P. and Jackson, L.L. (1989) Description of sexual behaviors of *Drosophila rajasekari*, the role of (*Z, Z*)-7,11-Heptacosadiene. *Journal of Chemical Ecology*, 15, 1423–1432.

Moest, M., Van Belleghem, S.M., James, J.E., Salazar, C., Martin, S.H., Barker, S.L., *et al.* (2020) Selective sweeps on novel and introgressed variation shape mimicry loci in a butterfly adaptive radiation. *PLOS Biology*, 18, e3000597.

Niida, T. and Koshikawa, S. (2021) No evidence for contribution of sexually monomorphic wing pigmentation pattern to mate choice in *Drosophila guttifera*. *Ethology*, 127, 527-536.

Okada, T. (1955) *Drosophila. Fauna and flora of Nepal Himalaya. Scientific results of the Japanese expeditions to Nepal Himalaya.* (ed. H. Kiharas), Fauna and Flora Research Society, Kyoto University. pp. 387–390.

Orr, H.A. (1997) Haldane's rule. *Annual Review of Ecology and Systematics*, 28, 195–218.

Pérez-Figueroa, A., Caballero, A. and Rolán-Alvarez, E. (2005) Comparing the estimation properties of different statistics for measuring sexual isolation from mating frequencies. *Biological Journal of the Linnean Society*, 85, 307–318.

Prud'homme, B., Gompel, N., Rokas, A., Kassner, V.A., Williams, T.M., Yeh, S.D., *et al.* (2006) Repeated morphological evolution through cis-regulatory changes in a pleiotropic gene. *Nature*, 440, 1050–1053.

33

Rice, G., David, J.R., Kamimura, Y., Masly, J.P., Mcgregor, A.P., Nagy, O., *et al.* (2019) A standardized nomenclature and atlas of the male terminalia of *Drosophila melanogaster*. *Fly* (Austin), 13, 51–64.

Rolan-Alvarez, E. and Caballero, A. (2000) Estimating sexual selection and sexual isolation effects from mating frequencies. *Evolution*, 54, 30–36.

Roy, P.R. and Gleason, J.M. (2019) Assessing the use of wing ornamentation and visual display in female choice sexual selection. *Behavioural Processes*, 158, 89–96.

Santos, E.S. and Machado, G. (2016) Sexual dichromatism in wing pigmentation of New World dragonflies follows Rensch's rule. *Journal of Evolutionary Biology*, 29, 1447–1454.

Spieth, H.T. (1952) Mating behavior within the genus *Drosophila* (Diptera). *Bulletin of the American Museum of Nature History*, 99, 395–474.

Spieth, H.T. (1974) Courtship behavior in *Drosophila*. *Annual Review of Entomology*, 19, 385–405.

Svensson, E.I. and Waller, J.T. (2013) Ecology and sexual selection: evolution of wing pigmentation in calopterygid damselflies in relation to latitude, sexual dimorphism, and speciation. *American Naturalist*, 182, E174–195.

Toda, M.J. (1991) Drosophilidae (Diptera) in Myanmar (Burma) VII. The *Drosophila melanogaster* species-group, excepting the *D. montium* species-subgroup. *Oriental Insects*, 25, 69–94.

Toda, M.J. (2021) DrosWLD a database on taxonomy of Drosophilidae. https://bioinfo.museum.hokudai.ac.jp/db/modules/stdb/index.php?action=tbl&tbl_xml :id=21.

True, J.R., Edwards, K.A., Yamamoto, D. and Carroll, S.B. (1999) *Drosophila* wing melanin patterns form by vein-dependent elaboration of enzymatic prepatterns. *Current Biology*, 9, 1382–1391.

Wen, S.Y., Yamada, H., Li, Y.F., Kimura, M.T., Oguma, Y., Sawamura, K., *et al.* (2011) Copulatory courtship behavior and sine song as a mate recognition cue in *Drosophila lini* and its sibling species. *Zoological Science*, 28, 469–475.

Yeh, S.D., Liou, S.R. and True, J.R. (2006) Genetics of divergence in male wing pigmentation and courtship behavior between *Drosophila elegans* and *D. gunungcola*. *Heredity* (Edinb), 96, 383–395.

Yeh, S.D. and True, J.R. (2014) The genetic architecture of coordinately evolving male wing

pigmentation and courtship behavior in *Drosophila elegans* and *Drosophila*

gunungcola. *G3* (Bethesda), 4, 2079–2093.

Zhang, W.X. and Toda, M.J. (1992) A new species-subgroup of the *Drosophila immigrans*

species-group (Diptera, Drosophilidae), with description of two new species from

China and revision of taxonomic terminology. *Japanese Journal of Genetics*, 60,

839–850.

Supporting Information

Fig. S1 A custom-made *Drosophila* Acoustic-Behavioral Experimental Soundproof System.

The system consists of a mating chamber (1) placed above a pre-polarized microphone (Type

4176, B&K, Denmark) and a CCLD microphone preamplifier (Type 2671, B&K, Denmark)

connected to a computer operated by Audacity 2.1.0 (Audacity Team). Above the mating

chamber is a macro lens (2) operated by ArcSoft ShowBiz 3.5.15.68 (ArcSoft Inc.) to

synchronously capture videos and audio from the B&K microphone.

Table S1. Definitions of metric characters of adult Drosophilidae.

Movie S1. Scissoring behavior of *D. nepalensis*.

Movie S2. Scissoring behavior of *D. trilutea*.

Movie S3. Scissoring behavior of F1 hybrid of *D. nepalensis*♀×*D. trilutea*♂.

Movie S4. Type A of courtship song of *D. trilutea*.

Movie S5. Type B of courtship song of *D. trilutea*.

Movie S6. Type B of courtship song of *D. nepalensis*.

Movie S7. Type B of courtship song of F1 hybrid of *D. nepalensis*♀×*D. trilutea*♂.

Table 1 Comparison of quantitative characters between males of *Drosophila trilutea* and *D. nepalensis*

Character†	D. trilutea					D. nepalensis					Wilcoxon test
	n‡	Mean	SD	Minimum	Maximum	*n*‡	Mean	SD	Minimum	Maximum	*P*
BL (mm)	10	2.092	0.238	1.808	2.489	10	2.109	0.146	1.827	2.306	0.4958
ThL (mm)	10	1.057	0.062	0.932	1.128	10	0.996	0.074	0.882	1.103	0.0689
WL (mm)	10	2.183	0.095	1.985	2.281	10	2.030	0.165	1.783	2.274	0.0278 *
WW (mm)	10	0.997	0.040	0.926	1.058	10	0.921	0.067	0.825	1.021	0.0187 *
FW/HW	10	0.447	0.009	0.427	0.462	10	0.457	0.018	0.427	0.478	0.2162
ch/o	10	0.116	0.021	0.088	0.146	10	0.133	0.021	0.094	0.154	0.0710
prorb	10	0.873	0.090	0.732	1.000	7	0.924	0.036	0.851	0.955	0.1532
rcorb	10	0.420	0.047	0.362	0.519	8	0.391	0.049	0.293	0.444	0.3718
vb	9	0.791	0.102	0.625	0.921	9	0.756	0.087	0.641	0.882	0.5071
dcl	10	0.600	0.030	0.559	0.645	8	0.626	0.036	0.558	0.667	0.1179

	N	Mean	SE			N	Mean	SE			p	
sctl	8	0.898	0.053	0.787	0.951	9	0.847	0.068	0.706	0.944	0.0823	
sterno	10	0.643	0.056	0.538	0.730	5	0.620	0.078	0.522	0.731	0.4990	
sterno2	10	0.385	0.049	0.302	0.460	6	0.375	0.057	0.311	0.451	0.6613	
orbito	10	0.726	0.068	0.625	0.833	10	0.729	0.087	0.625	0.880	0.8198	
dcp	10	0.409	0.036	0.348	0.460	10	0.443	0.040	0.386	0.513	0.0743	
sctlp	10	1.065	0.064	1.000	1.136	10	1.142	0.094	1.000	1.270	0.0394	*
C	10	2.811	0.139	2.577	3.042	10	2.755	0.170	2.456	2.958	0.5197	
4c	10	0.990	0.034	0.920	1.024	10	1.052	0.126	0.873	1.219	0.3611	
4v	10	2.081	0.105	1.938	2.250	10	2.203	0.302	1.881	2.895	0.4265	
5x	10	2.181	0.206	1.884	2.500	10	2.125	0.179	1.848	2.394	0.5702	
ac	10	2.340	0.178	2.063	2.627	10	2.336	0.202	2.000	2.651	0.7912	
M	10	0.713	0.046	0.638	0.794	10	0.747	0.085	0.615	0.886	0.4046	
C3F	1	0.3	0.0	0.339	0.434	1	0.3	0.0	0.240	0.375	0.0109	*

	0	71	28			0	21	43			

Aristal branches

	n	Mean	SD	Min	Max	n	Mean	SD	Min	Max	P	
dorsal	10	4.1	0.3	4	5	10	4.3	0.5	4	5	0.2758	
ventral	10	3	0	3	3	10	3.2	0.4	3	4	0.1462	

Sex combs on tarsomere I

	n	Mean	SD	Min	Max	n	Mean	SD	Min	Max	P	
TBR_{I-i}	10	4.4	0.5	4	5	10	3.8	0.6	3	5	0.0383	*
TBR_{I-ii}	10	1.2	0.6	0	2	10	0.5	0.5	0	1	0.0205	*

Sex combs on tarsomere II

	n	Mean	SD	Min	Max	n	Mean	SD	Min	Max	P	
TBR_{II-i}	10	3.1	0.3	3	4	10	2.5	0.5	2	3	0.0095	**
TBR_{II-ii}	10	1.2	0.4	1	2	10	1.1	0.3	1	2	0.5416	

* $P < 0.05$, ** $P < 0.01$.

†Explained in Table S1.

‡Examined specimens: *D. trilutea*: 1♂ from NDSSC (National Drosophila Species Stock Center) Stock No. 14022-0321 (Texas Stock No. 3066.9; the type isofemale strain), 1♂ from isofemale strain VT05-ST (Vietnam), 1♂ collected from Dinghushan, Guandong, China, 21‑28.xii.1986, 1♂ collected from Hehuanshan, Taiwan, China 29,30.viii.2017, 6♂ F1 offspring of females collected from Hehuanshan, Taiwan, China, 13–15.vii.2017; *D. nepalensis*: 1♂ from isofemale strain BHL066 (Baihualing, Yunnan, China), 1♂ from isofemale strain BHL109 (Baihualing, Yunnan, China), 1♂ from isofemale strain BHL112 (Baihualing,

Yunnan, China), $1\male$ collected from isofemale strain SWB172 (Shwebo, Myanmar), $6\male$ collected from Pyinoolwin, Myanmar, 30.xii.1981‑6.i.1982.

Table 2 Sexual isolation before and after mounting in mate-choice tests.

Combination	N	Frequency of first mounting (*PSI*)		$I_{\mathrm{PSI}} \pm$ SD	Frequency of mating (*PSI*)		$I_{\mathrm{PSI}} \pm$ SD
Female : Male		Homogamic	Heterogamic	Bootstrap test	Homogamic	Heterogamic	Bootstrap test
Female-choice tests							
trilutea : trilutea/nepalensis	40	30 (1.3636*)	10 (0.5556*)	0.4090 ± 0.1034	40 (2.0000*)	0 (0.0000*)	0.8836 ± 0.0495
nepalensis : trilutea/nepalensis	40	26 (1.4444*)	14 (0.6364*)	$P = 0.0002$	36 (2.0000*)	4 (0.1818*)	$P < 0.0001$
Male-choice tests							
trilutea/nepalensis : trilutea	40	25 (1.1628)	15 (0.8108)	0.1774 ± 0.1121	40 (2.0000*)	0 (0.0000*)	0.9520 ± 0.0335
trilutea/nepalensis : nepalensis	40	22 (0.8372)	18 (1.1892)	$P = 0.1156$	40 (2.0000*)	0 (0.0000*)	$P < 0.0001$

PSI: pair sexual isolation coefficient. I_{PSI}: joint isolation index for *PSI* coefficient. *PSI* and I_{PSI} were calculated by the JMATING 1.0.8 (Carvajal-Rodriguez & Rolán-Álvarez, 2006).

41

*Significant (after Bonferroni correction at α = 0.05) deviation from 1 expected under random mating.

Table 3 Numbers of progeny emerging in the *Drosophila nepalensis × D. trilutea* cross and backcross (their F1 hybrid × parental species) experiments.

Cross/ Backcross	Combination	Number of replicates (*N*)	Parents /replicate ♀	Parents /replicate ♂	Offspring: Mean (/replicate) ± SD ♀	Offspring: Mean (/replicate) ± SD ♂	Fertility /female	Sex ratio (♂/♀)
Cross	*nepalensis*♀ × *trilutea*♂	10	5	10	99.6 ± 11.4	103.7 ± 18.0	40.7 ± 5.3	1.04 ± 0.15
	trilutea♀ × *nepalensis*♂	10	5	10	0	0	–	–
		10	20	40	0	0	–	–
Backcross	F1♀ × *nepalensis*♂	5	5	10	33.4 ± 7.7	30.2 ± 2.4	12.7 ± 1.6	0.95 ± 0.25
	F1♀ × *trilutea*♂	5	5	10	19.4 ± 3.4	20.8 ± 7.5	8.0 ± 2.0	1.10 ± 0.33
	nepalensis♀ × F1♂	5	5	10	0	0	–	–
	trilutea♀ × F1♂	5	5	10	0	0	–	–

42

Table 4 Comparison of wing-spot pigmentation intensity (*GV*: Grey Value) and bilateral asymmetry of male left and right wings among *Drosophila nepalensis*, *D. trilutea* and their F1 hybrid (*D. nepalensis*♀ × *D. trilutea*♂).

	D. nepalensis	F1 hybrid	*D. trilutea*
N	28	455	33
Left wing *GV*			
Mean	137.051	137.485	48.667
Welch's F test ANOVA		$P < 0.0001$***	
Variance	245.514	209.520	290.477
Bartlett test		$P = 0.3735$	
Right wing *GV*			
Mean	137.726	136.987	48.031
Welch's F test ANOVA		$P < 0.0001$***	
Variance	150.185	188.840	302.499
Bartlett test		$P = 0.0999$	
Bilateral asymmetry (*DA*)			
Mean (directional asymmetry)	-0.675	0.497	0.636
Welch's F test ANOVA		$P = 0.8736$	
95% confidence interval	-5.254 – 3.905	-0.667 – 1.661	-2.496 – 3.769
Variance (fluctuating asymmetry)	139.495	159.628	78.045

43

		Bartlett test			$P = 0.0425*$			
		Bartlett test (pairwise)[†]		ab		b		a

* $0.01 < P < 0.05$, *** $P < 0.001$.

[†] The same letters indicate non-significant difference at $\alpha = 0.05$.

Table 5 Numbers of males having performed six courtship elements in successful mating pairs of *Drosophila nepalensis*, *D. trilutea* and their F1 hybrid (*D. nepalensis*♀×*D. trilutea*♂).

Species	N	Orientation		Wing vibration		Tapping		Circling		Scissoring		Mounting attempt	
		+	−	+	−	+	−	+	−	+	−	+	−
D. nepalensis	40	40	0	40	0	40	0	38	2	29	11	40	0
F1 hybrid	74	74	0	74	0	74	0	68	6	55	19	74	0
D. trilutea	44	44	0	44	0	41	3	37	7	24	20	44	0
Fisher's exact test[†]		n/a		n/a		$P = 0.0359*$		$P = 0.2442$		$P = 0.0762$		n/a	

*$P < 0.05$.

[†]Using R version 3.5.1 (R Core Development Team, 2018).

Table 6 Comparison of 'scissoring' behavior during courtship among *Drosophila nepalensis*, *D. trilutea* and their F1 hybrid (*D. nepalensis*♀×*D. trilutea*♂).

44

	D. nepalensis	F1 hybrid	*D. trilutea*
N	7	14	10
Total duration of scissoring during courtship: Σt_{burst}[†] (s)			
Mean	43.99	45.20	41.12
Welch's F test ANOVA		$P = 0.8414$	
Variance	584.79	380.81	163.08
Bartlett test		$P = 0.2152$	
Total number of scissorings during courtship: Σn_{sci}[†]			
Mean	75.1	64.6	57.5
Welch's F test ANOVA		$P = 0.4812$	
Variance	1412.7	1055.1	134.7
Bartlett test		$P = 0.0073**$	
Scissoring frequency per second (f_{sci}): $\Sigma n_{\text{sci}}/\Sigma t_{\text{burst}}$ (1/s)			
Mean	1.773	1.398	1.437
Welch's F test ANOVA		$P = 0.0009***$	
Variance	0.025	0.027	0.028
Bartlett test		$P = 0.9963$	

** $0.001 < P < 0.01$, *** $P < 0.001$.

[†]t_{burst} = the duration of each burst; n_{sci} = the number of scissorings per burst.

45

Table 7 Pulse song parameters of *Drosophila trilutea*, *D. nepalensis* and their F1 hybrid (*D. nepalensis*♀×*D. trilutea*♂)

Species	N_{pair}	Song type	N_{song}	CN Mean ± SE	CN Range	CN Test[†]	PL (ms) Mean ± SE	PL Test[‡]	IPI (ms) Mean ± SE	IPI Test[‡]	IPF (Hz) Mean ± SE	IPF Test[†]
D. trilutea	6	A	27	1.0 ± 0.0	1	(n/a)	3.51 ± 0.17	(n/a)	45.33 ± 0.31	(n/a)	465.07 ± 15.49	(n/a)
		B	28	2.2 ± 0.1	2–3	a	10.26 ± 0.62	a	45.80 ± 1.29	a	298.25 ± 9.29	c
F1 hybrid	13	B	36	2.8 ± 0.1	2–4	b	20.53 ± 0.55	c	59.09 ± 1.14	b	202.07 ± 8.20	a
D. nepalensis	5	B	24	3.1 ± 0.1	2–5	b	15.22 ± 0.67	b	63.20 ± 1.40	b	240.12 ± 10.04	b

N_{pair}, the number of pairs examined; N_{song}, the number of songs examined; CN, the number of cycles per pulse; PL, pulse length; IPI, interpulse interval; IPF, intrapulse frequency.

[†]Tukey-Kramer test with the homoscedasticity (P = 0.1132 in Bartlett test), and [‡]Steel-Dwass test with the heteroscedasticity (P < 0.05 in Bartlett test): the same letters indicate non-significant difference at α = 0.05

Figure legends

Fig. 1 Male right wings of *Drosophila nepalensis*, *D. trilutea*, and the F1 hybrid of *D. nepalensis♀×D. trilutea♂*, with definition of wing-spot area (rectangle) for which the Grey Value is measured: landmark 1 is at the intersection point of dm-m (posterior crossvein) and M_1 (4th longitudinal vein); landmark 2 at the end point of M_1; landmark 3 at the end point of R_{4+5} (3rd longitudinal vein); and landmark 4 at the contact point of C (costal vein) and the upper side of the rectangle.
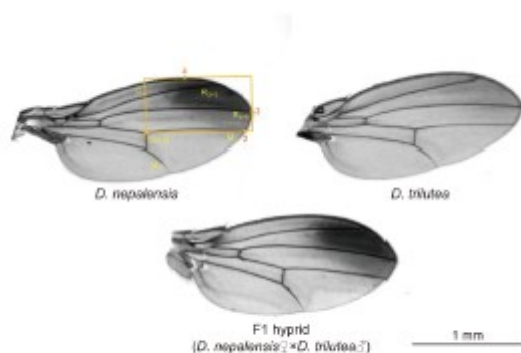

D. nepalensis

D. trilutea

F1 hyprid
(D. nepalensis♀×D. trilutea♂)

1 mm

Fig. 2 Male morphology of *Drosophila* (*Sophophora*) *trilutea* (A–D) and *D.* (*S.*) *nepalensis* (E–H). (A, E) Habitus; (B, F) sex combs on tarsomeres I and II of fore leg; (C, G) periphallic organs (pr teeth, primary teeth; sc teeth, secondary teeth; sur, surstylus) in posterior view; (D, H) phallic organs (aed, aedeagus; aed s, aedeagal sheeth; pgt, postgonite; preg, pregonite) in posterior view. Scale bars: 1 mm in A and E; 0.1 mm in the others.

47

Fig. 3 Comparison of male wing-spot pigmentation (A–C) and 'scissoring' behavior in courtship (D–F) among *Drosophila nepalensis* (nep), *D. trilutea* (tri) and their F1 hybrid (F1). The results of *post hoc* multiple comparisons by the Tukey-Kramer method are shown with letters near violin-plots (A, B, F): the same letters indicate non-significant difference at $\alpha = 0.05$.
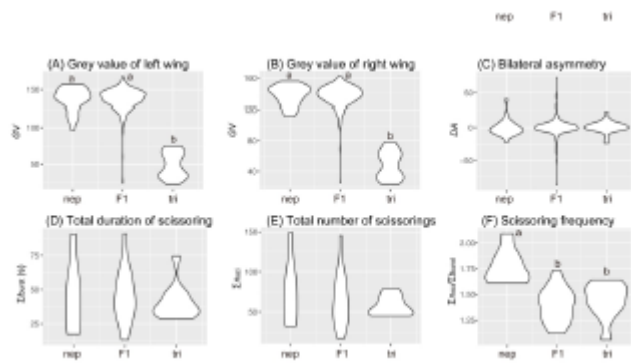
Fig. 4 The sequence of courtship behavior and scissoring postures of *Drosophila nepalensis* and *D. trilutea*. α: the maximum angle of wing spread from the body axis; β: the minimum angle wing spread from the body axis.

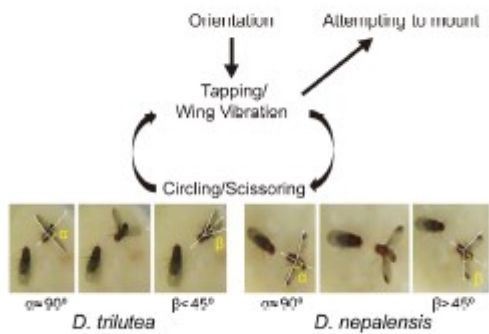49

Fig. 5 Male courtship songs of *Drosophila nepalensis*, *D. trilutea*, and the F1 hybrid of *D. nepalensis*♀×*D. trilutea*♂.

50