ARTICLE

Sexual dimorphism and courtship behavior in *Drosophila prolongata*

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Abstract Sexual dimorphism is often derived from sexual selection. In sexually dimorphic *Drosophila* species, exaggerated male structures are used for specific behaviors in male-to-male competition or courtship toward females. In *Drosophila prolongata*, a member of the *melanogaster* species group, males have enlarged forelegs whereas females do not. However, the adaptive role of the enlarged forelegs is unclear because little is known about the behavior of *D. prolongata*. In this study, the courtship behavior of *D. prolongata* was investigated in comparison with closely related species. Males of *D. prolongata* use their forelegs in a specific behavior, "leg vibration", in which the male vigorously vibrates the female's abdomen by extending his forelegs from in front of her. Leg vibration was observed immediately before "attempting copulation", indicating that it has an adaptive role in the mating process. In contrast, leg vibration was not observed in closely related species. Because the large forelegs are necessary to accomplish leg vibration, it was suggested that the sexual dimorphism of *D. prolongata* forelegs is currently under the influence of sexual selection in courtship behavior.

Introduction

Sexual dimorphism, which is frequently expressed in the form of exaggerated structures in males, is often derived from sexual selection (Andersson 1994). It is also presumed that sexually dimorphic structures tend to be accompanied by evolution of specific behavior, in which the exaggerated structures play important roles.

In *Drosophila* fruit flies, several species have evolved sexually dimorphic structures that are used in specific behaviors. For example, males of *Drosophila heteroneura* have a broadened head, which is used in male-to-male competition (Spieth 1981; Boake et al. 1997). When males compete for territory, they take up a head-to-head position and push their opponent. The male with a wider head tends to win, and consequently he has a higher probability of mating success (Boake et al. 1997). Another example is seen in male-specific wing pigmentation and courtship behavior among the species that belong to the *melanogaster* group. In addition to the standard courtship elements in *Drosophila*, such as orientation, following, wing vibration, and licking (Spieth 1952; Cobb et al. 1986;

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Yamamoto and Koganezawa 2013), males of species that have wing spots perform "wing display" in front of the female (Fuyama 1979; Yeh et al. 2006), whereas species lacking wing spots do not. Wing spots are associated with wing display in at least seven species that are phylogenetically independent from each other (Yeh et al. 2006). As indicated by these examples, the adaptive roles of sexually dimorphic structures can be better understood in the context of related behavior. In other words, understanding how sexually dimorphic structures are used in the behavioral context gives important insights into the mechanisms by which the morphology evolved through sexual selection.

Drosophila prolongata, a member of the *rhopaloa* subgroup of the *melanogaster* species group, is endemic to southwestern China, northeastern India, Myanmar, and Vietnam (Singh and Gupta 1977; Toda 1991; H. Takamori, unpublished observation). The forelegs of *D. prolongata* are extraordinarily thick and elongated in males. Like other cases of sexual dimorphism, it is presumed that the enlarged forelegs have evolved under sexual selection. However, the adaptive role of the enlarged forelegs is unknown, mostly because of the lack of information on the behavior of *D. prolongata*. Because such enlarged forelegs have not been observed in any other *Drosophila* species, it is difficult to infer their function from the known behavior of other species.

In this study, the role of the enlarged forelegs of *D. prolongata* males in courtship behavior was investigated, with reference to closely related species. We found that the enlarged legs were used in a specific behavior that was observed immediately before attempting copulation, indicating that sexual dimorphism in *D. prolongata* is under the influence of sexual selection in mating process.

Materials and methods

Fly strains

Drosophila prolongata (BaVi044), *D. rhopaloa* (BaVi5327), and *D. kurseongensis* (SaPa058) were collected in Vietnam by H. Takamori in March 2005, September 2004, and March 2009, respectively. Isofemale lines were established by H. Takamori and T. Aotsuka, and maintained at Tokyo Metropolitan University on ordinary cornmeal medium for *Drosophila* culture. An undescribed species, KB866, was kindly provided by Dr. Artyom Kopp (Barmina and Kopp 2007).

Phylogenetic analysis

The phylogenetic relationships between the four species used in this study were inferred using *D. elegans*, *D.*

takahashii, *D. melanogaster*, and *D. kikkawai* as an outgroup. Four nuclear genes were selected from loci that have been demonstrated to be applicable for phylogenetic analysis of the *melanogaster* group (Kopp 2006; Yang et al. 2012), comprising *extra sexcombs* (*esc*), *hunchback* (*hb*), *kinase suppressor of ras* (*ksr*), and *Phosphoglucose isomerase* (*Pgi*). For each gene, the sequence of the longest exon containing the coding DNA sequence (CDS) was obtained from the Flybase *D. melanogaster* gene annotation (R5.52). These were FBgn0000588:1, FBgn0001180:2, FBgn0015402:1, and FBgn0003074:4, for *esc*, *hb*, *ksr*, and *Pgi*, respectively. The corresponding sequences were obtained from the draft genome assembly via the Flybase BLAST web interface for *D. takahashii*, *D. elegans*, *D. kikkawai*, and *D. rhopaloa*. For the other species, the corresponding sequences were obtained from contigs that were assembled from brain RNA-seq data (accession numbers AB849898–AB849909). Non-protein coding regions and gaps were eliminated. In total, 6,323 sites were included in the analysis. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model with a discrete Gamma distribution model of evolutionary rate differences among sites. The molecular clock was calibrated by the deduced divergence time between *D. melanogaster* and *D. takahashii* at 35 million years ago (MYA) (Tamura et al. 2004). All the evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

Video recording of courtship behavior

All the species were reared on cornmeal medium at 20 °C in a 12:12 h light:dark cycle. Newly eclosed males and females were maintained separately for 7 days before recording. By this stage, the ovary was fully developed in females of all four species. Courtship behavior was recorded during the period from 1 h after the start of light phase to the end of light phase. A male and a female were introduced into a mating chamber (25 mm in diameter, 15 mm in height) in which a disc of wet filter paper was placed on the bottom. A piece of yeast paste was placed at the center of the chamber. Behavior was recorded using a SONY HDR-CX560V digital camera installed 40 cm above the chamber. Seven chambers were recorded at the same time. Recorded movies were played on PC and inspected visually. The slow-replay function was used occasionally as necessary. Behavioral elements were identified and scored manually.

Transition analysis

For selected pairs, the sequence of behavioral elements was scored for the 15 min preceding successful copulation. At least 30 pairs were scored for each species. Transition
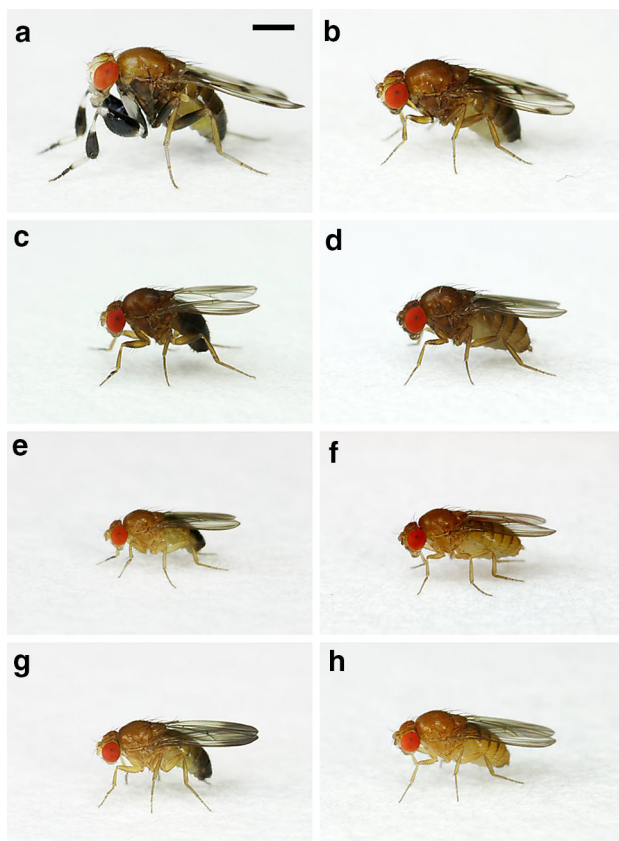
matrices are shown in Supplementary Tables S1–S4. Deviation of the frequency of each transition from the expected value was examined by χ^2 test (Hoikkala and Kaneshiro 1993; Chen et al. 2002; Lasbleiz et al. 2006; Jonsson et al. 2011). The expected frequency of transitions was obtained by the method described by Goodman (1968).

For inter-species comparisons, subsequences of behavior that consist of three contiguous behavioral elements were extracted from the video data. In total, 432 patterns of subsequences were identified from the four species. Differences between a target species and the others in the frequency of pairs that exhibited each pattern at least once were examined by Fisher's exact test with p value adjustment for multiple comparisons by the Bonferroni method.

Results

Morphology and phylogenetic relationship of the species used in this study

Morphology of the forelegs is sexually dimorphic in *D. prolongata* (Singh and Gupta 1977; Toda 1991; Figs. 1a, b,
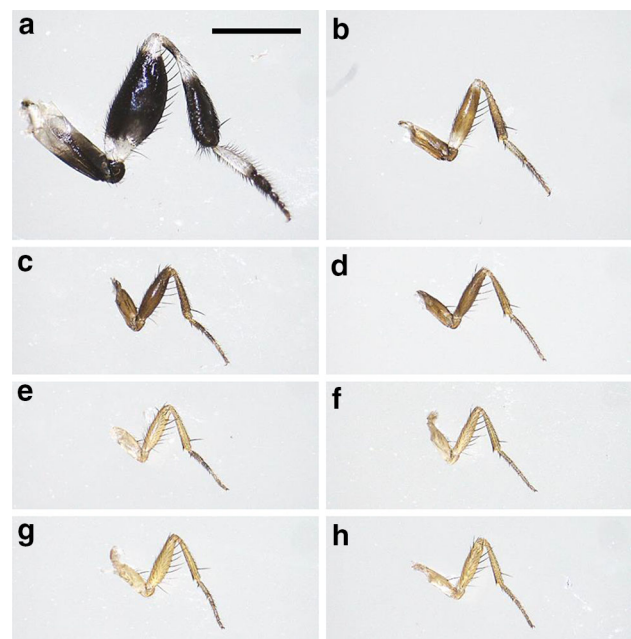
2a, b). Each segment is longer in males, and the femur is thickened along the dorso-ventral axis. The pigmentation pattern is also dimorphic; forelegs have black and white stripes in males. On the other hand, in closely related species, KB866, *D. rhopaloa*, and *D. kurseongensis*, the forelegs are monomorphic, as observed in other *Drosophila* species (Figs. 1c–h, 2c–h).

In contrast to the foreleg morphology, the pigmentation pattern on wings is sexually dimorphic in all four species. *D. prolongata* has five spots on each wing, which are larger in males (Fig. 3a, b). In KB866, the wings of males are shaded at the front edge, while those of females are occasionally pigmented faintly (Fig. 3c, d). In *D. rhopaloa*, only males have pale pigmentation on the wings (Fig. 3e, f), and males of *D. kurseongensis* have a spot at the tip of each wing, whereas this spot is absent in females (Fig. 3g, h).

In spite of these morphological differences, the four species are phylogenetically close to each other. *D. kurseongensis* was estimated to have diverged from the other three species about 5 MYA, and *D. prolongata* diverged from KB866 and *D. rhopaloa* about 4 MYA (Fig. 4), suggesting that the enlarged forelegs in *D. prolongata* evolved rapidly.

Copulation rate, duration of courtship and copulation

For each of the four species, at least 30 independent courtship episodes ending with successful copulation were

Fig. 1 Appearance of the four species used in this study. **a**, **b** *Drosophila prolongata*, **c**, **d** KB866, **e**, **f** *D. rhopaloa*, and **g**, **h** *D. kurseongensis*. **a**, **c**, **e**, **g** Males and **b**, **d**, **f**, **h** females. *Scale bar* 1.0 mm
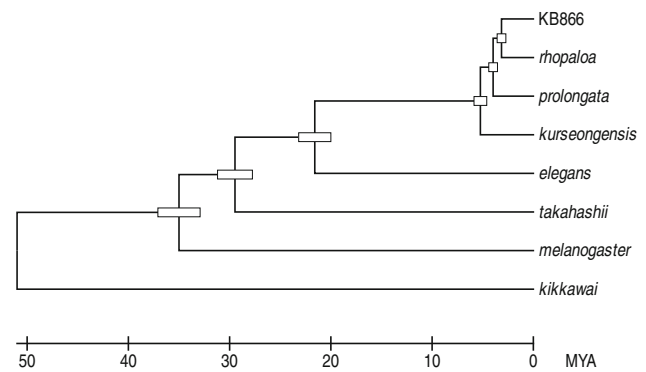

Fig. 2 Forelegs of the four species used in this study. The lateral side of the right foreleg is shown. **a**, **b** *Drosophila prolongata*, **c**, **d** KB866, **e**, **f** *D. rhopaloa*, and **g**, **h** *D. kurseongensis*. **a**, **c**, **e**, **g** Males and **b**, **d**, **f**, **h** females. *Scale bar* 1.0 mm
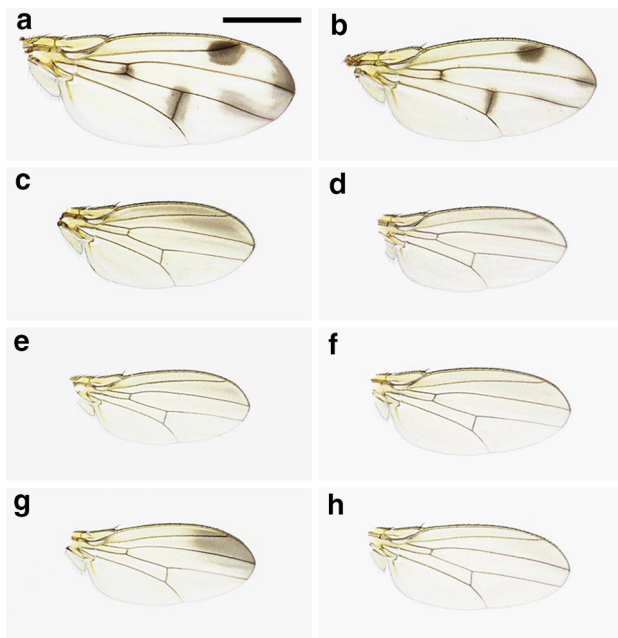
Fig. 3 Wings of the four species used in this study. The dorsal side of the right wing is shown. **a**, **b** *Drosophila prolongata*, **c**, **d** KB866, **e**, **f** *D. rhopaloa*, and **g**, **h** *D. kurseongensis*. **a**, **c**, **e**, **g** Males and **b**, **d**, **f**, **h** females. *Scale bar* 1.0 mm

Fig. 4 Phylogenetic relationship of the four species used in this study. *Drosophila elegans*, *D. takahashii*, *D. melanogaster*, and *D. kikkawai* were included as an outgroup. The Maximum Likelihood tree was deduced from the CDS sequences of the four nuclear genes, *esc*, *hb*, *ksr*, and *Pgi*. The molecular clock was calibrated from the divergence time between *D. melanogaster* and *D. takahashii* at 35 MYA (Tamura et al. 2004). *Boxes* at the internal nodes indicate the standard error

recorded. The total number of observed pairs, however, was quite different between species (Table 1). Because of the low copulation rate, a large number of *D. prolongata* pairs had to be observed to record sufficient number of successful copulations, even though the recording period was set to three times longer (3 h) than that for the other species. Copulation rate was also low in *D. kurseongensis* (Table 1). Duration of courtship until successful copulation was also different between the species. Most pairs of KB866 and *D. rhopaloa* copulated within 5 min, but *D. prolongata* and *D. kurseongensis* spent much longer in courtship, with larger variations between pairs (significantly different by Bartlett's test at $p = 0.05$ level; Table 1; Fig. 5). Although sexual maturation of some *Drosophila* species, such as *D. virilis*, is known to require longer period after eclosion (Huttunen et al. 2008), it might not be the reason of low copulation rate in *D. prolongata* and *D. kurseongensis*, because the ovary was fully developed by the time of analysis in females of all four species (data not shown).

It is known that the longer copulation delays a female from remating, increasing the likelihood of the male being successful in fathering the offspring under conditions involving sperm competition in *D. melanogaster* and *D. montana* (Gilchrist and Partridge 2000; Mazzi et al. 2009). In contrast, the shorter copulation allows females to remate immediately, which benefits females by hedging the risk of mating with a genetically inferior male (Jennions and

Petrie 2000). Thus, duration of copulation is thought to be an important parameter resulting from sexual conflict. In many species of the *melanogaster* group, copulation lasts over 10 min (Hirai et al. 1999; Singh and Singh 2004). Duration of copulation in KB866, *D. rhopaloa*, and *D. kurseongensis* was around 15 min, whereas it was about half of that in *D. prolongata* (Table 1). This result may suggest that the intra- and inter-sexual relationship with regard to sperm competition has changed in *D. prolongata*. For example, the tendency for remating in females might be different from the other species, although this remains to be confirmed.

Elements of courtship behavior

Behavioral elements were extracted from the recorded courtships. In total, 13 elements were identified (Table 2; Fig. 6; Supplementary Movies S1–S4). Ten elements were commonly observed in the all species. Consistent with their wing pigmentation pattern, they exhibited bi-lateral wing vibration, as reported in other wing-spotted species such as *D. suzukii* and *D. elegans* (Fuyama 1979; Yeh et al. 2006).

Leg vibration was observed only in *D. prolongata*, whereas leg shaking was specific to the other three species (Table 2). Leg vibration is a dynamic movement involving (1) quick positioning in front of the female, (2) extension of both wings, facing their surface towards the female, with wing vibration, and (3) extending both forelegs along the body of the female and vibrating the female's abdomen violently (Fig. 6c, d; Supplementary Movie S1). This kind of behavior has not been reported in the other *Drosophila* species. In contrast, leg shaking is different from leg

Table 1 Courtship and copulation characteristics of different *Drosophila* species

	Observation period (h)	Observed pairs	Copulated pairs	Copulation rate (%)	Courtship duration[a] (min) (mean ± SE)	Copulation duration[a] (min) (mean ± SE)
D. prolongata	3	236	30	12.7	25.95 ± 4.47 A	7.62 ± 0.32 A
KB866	1	57	37	64.9	2.12 ± 0.43 B	15.73 ± 0.67 B
D. rhopaloa	1	70	47	67.1	0.93 ± 0.28 C	15.47 ± 0.47 B
D. kurseongensis	1	112	32	28.6	20.13 ± 2.55 A	13.32 ± 0.47 C

For these traits, phylogenetic signal (Pagel's λ) was not significantly different from 0 by likelihood ratio test. Values followed by the same letter are not significantly different by the pairwise Wilcoxon rank sum test at $p = 0.05$ level (adjusted for multiple comparisons by the Bonferroni method) following the Kruskal–Wallis rank sum test

[a] Subset of copulated pairs was used for analysis of courtship duration and copulation duration (*D. prolongata*: $n = 30$; KB866: $n = 33$; *D. rhopaloa*: $n = 41$; *D. kurseongensis*: $n = 32$)

vibration: (1) leg shaking occurs when the male is apart from the female by more than the body length of the fly, (2) in most cases, the male raises and vibrates one foreleg at a time, and (3) the male never touches the female (Supplementary Movies S2–S4).

The frequency of occurrence of each behavioral element is shown in Fig. 7. It should be noted that the elements that appear at earlier stages of courtship may be underrepresented in species that showed longer courtship duration (*D. prolongata* and *D. kurseongensis*) because of the limit of the scored period. As in other *Drosophila* species, unilateral wing vibration was the most frequent element in all four species (Fig. 7). Leg display was frequently observed in *D. prolongata*.

Transition analysis

Next, transitions between two behavioral elements were analyzed. The standard courtship sequence in *Drosophila* starts with orientation, followed by tapping, following, unilateral wing vibration, and licking, ending with attempting copulation and copulation (Spieth 1952; Cobb et al. 1986; Yamamoto and Koganezawa 2013). Consistent with this, the early stage of courtship in the observed four species seemed to comprise orientation, tapping, and following (Fig. 8). However, the other part of courtship was unique to the observed species, and major differences between *D. prolongata* and the other three species were seen in this part.

In KB866, *D. rhopaloa*, and *D. kurseongensis*, transitions between wing waving, leg shaking, and bi-lateral wing vibration were directionally linked in this order (Fig. 8). Attempting copulation was preceded by uni-lateral wing vibration, consistent with the standard *Drosophila* courtship. On the other hand, in *D. prolongata* leg vibration did not form a transition link with wing waving and bi-lateral wing vibration, but it was inserted between uni-lateral wing vibration and attempting copulation.

Transition from licking to attempting copulation was also significantly more frequent.

Inter-species comparison of behavioral sequences

Transition diagrams are widely used for the analysis of behavior structures. However, this method has several problems: (1) the analysis is based on single-step transitions between two elements, and transitions that consist of two or more steps cannot be analyzed; (2) transitions between high-frequency elements tend to be underrepresented because the analysis detects the deviation from the proportionally expected frequency of transitions; and (3) the analysis considers the significance among the transitions within a species, and does not support any statistical comparisons between species. To address these problems, we applied a novel method that is able to compare the frequency of two-step-sequences of behavioral elements between species. Briefly, the proportion of the insect pairs that showed a particular two-step-sequence was compared between a species of interest and the others. Behavioral sequences that appeared more or less frequently than in the other species were listed in the order of statistical significance (Table 3). In this analysis, behavioral sequences that contain a species-specific element cannot be compared with other species, because they are always significantly different. For this reason, leg shaking and leg vibration were treated as an equivalent behavioral element, in order to directly compare the behavioral context in which these elements appeared. Thus, the letter "s" represents leg vibration in *D. prolongata*, whereas it represents leg shaking in the other species.

In *D. prolongata*, "attempting copulation to copulation (a–c)" was more frequently preceded by leg vibration (s) and less by uni-lateral wing vibration (v) than the other species (Table 3). Conversely, attempting copulation to copulation (a–c) was preceded by uni-lateral wing

Fig. 5 Total courtship duration until successful copulation. The distribution of courtship duration is shown in the histogram. The cumulative proportion of copulating pairs is shown as *lines*. *Drosophila prolongata*: *n* = 30; KB866: *n* = 33; *D. rhopaloa*: *n* = 41; *D. kurseongensis*: *n* = 32
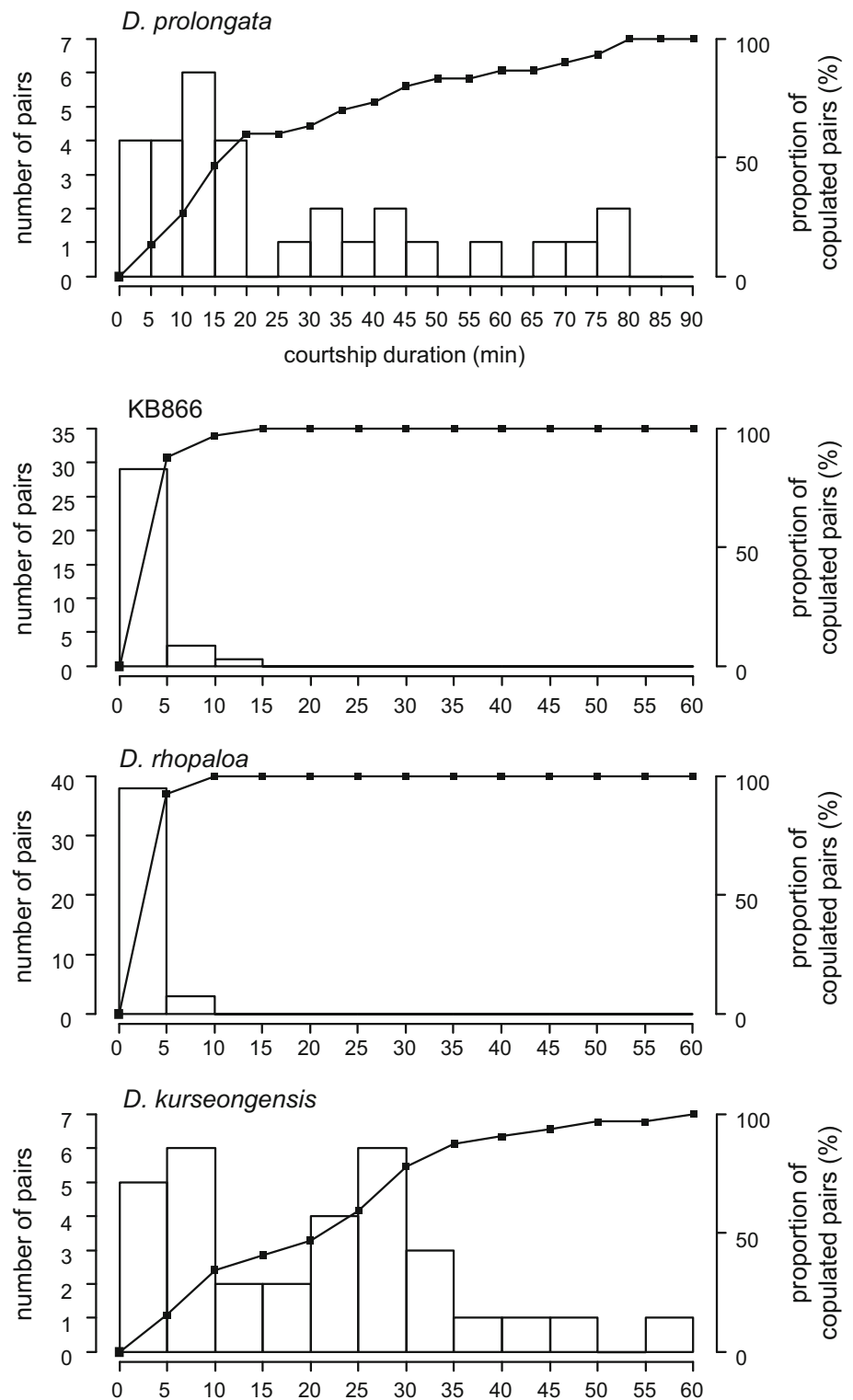
vibration (v) in KB866 and *D. kurseongensis*. These results were consistent with the analysis by transition diagrams, and it was confirmed that these elements were actually followed by successful copulations, not by failed attempts. In *D. rhopaloa*, the frequency of b–a–c (bi-lateral wing

vibration to copulation) was significantly higher than in the other species (Table 3).

Because the occurrence of leg display (d) and uni-lateral wing vibration (v) were most frequent in *D. prolongata* (Fig. 7), transitions between these two elements were

Table 2 Ethogram

Behavioral element[a]	Description	Appearance in courtship[b]			
		pro	KB	rho	kur
Orientation (o)	The male orientates toward the female	+	+	+	+
Tapping (t)	The male taps the female's body with his forelegs	+	+	+	+
Following (f)	The male follows a walking female or approaches a standing female	+	+	+	+
Licking (l)	The male licks the female's abdomen	+	+	+	+
Elbow rubbing (e)	The male rubs the one of his forelegs with another one	+	+	+	+
Uni-lateral wing vibration (v)	The male extends one of his wings and vibrates it. Often accompanied by circling	+	+	+	+
Bi-lateral wing vibration (b)	The male extends both of his wings and vibrates them. Often accompanied by circling	+	+	+	+
Wing waving (w)	The male slowly lifts and extends both of his wings and then closes them	+	+	+	+
Leg shaking (s)	The male lifts one or both of his forelegs and shakes them rapidly. Both wings are spread	−	+	+	+
Leg display (d)	The male lifts his forelegs forward and moves them repeatedly up and down	+	+	−	+
Leg vibration (s)	The male positions anterior to the female and beats her abdomen with his forelegs. Both wings are spread	+	−	−	−
Attempting copulation (a)	The male attempts to mount the female	+	+	+	+
Copulation (c)	The flies successfully copulate	+	+	+	+

pro, *Drosophila prolongata*; KB, KB866; *rho*, *D. rhopaloa*; *kur*, *D. kurseongensis*

[a] The one letter designation of each element is shown in parentheses. The same letter was assigned to leg vibration (s) and leg shaking (s) (see text for explanation)

[b] + the element was observed in courtship, − the element was not observed in courtship
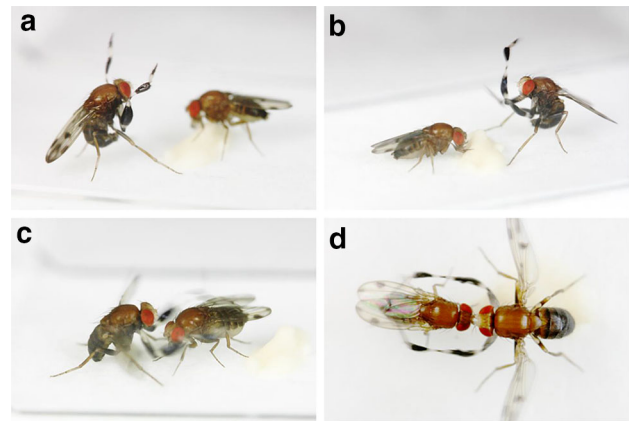
Fig. 6 Selected behavioral elements in the *Drosophila prolongata* courtship. **a** Leg display. **b** Elbow rubbing. **c, d** Leg vibration
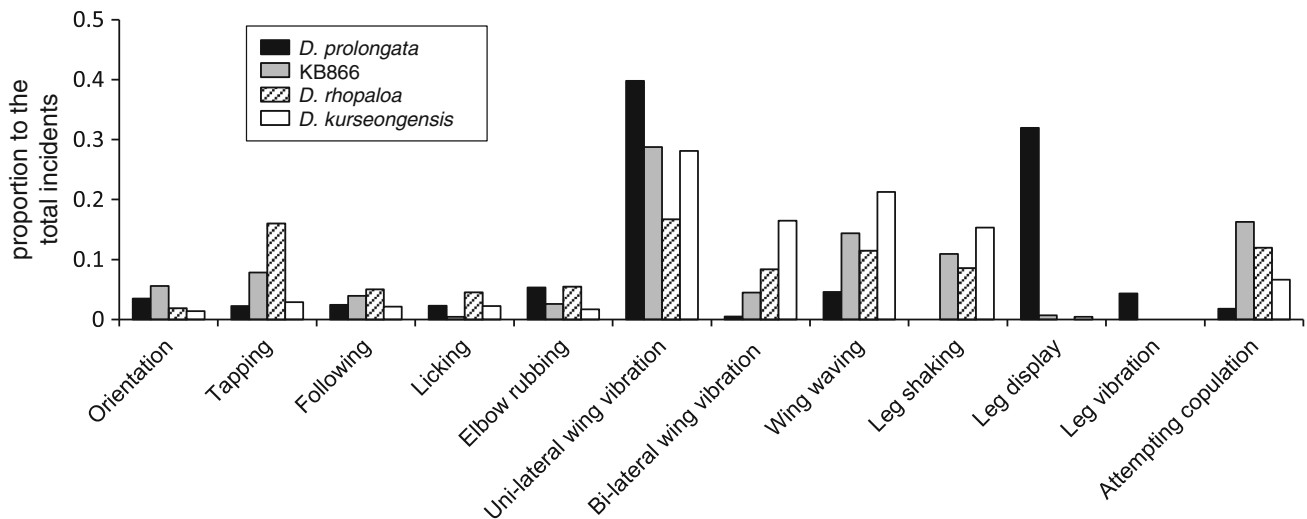
D. prolongata, showing that elbow rubbing (e) is inserted into uni-lateral wing vibration (v).

In *D. kurseongensis*, the transition diagram showed that uni-lateral wing vibration (v), wing waving (w), leg shaking (s), and bi-lateral wing vibration (b) were directionally linked in this order (Fig. 8). In Table 3, v–w–s, w–s–b, s–b–v, and b–v–w were significantly more frequent than in the other species, demonstrating that these four elements form a loop of behavioral elements that is characteristic of *D. kurseongensis*.

The lists of specific sequences for KB866 and *D. rhopaloa* were shorter than those of the other two species (Table 3). One reason was that their courtship was shorter and consisted of a few courtship elements (on average 25 elements in KB866 and 10 elements in *D. rhopaloa*, compared with 81 in *D. prolongata* and 78 in *D. kurseongensis*), resulting in a smaller repertoire of two-step sequences. At the same time, particularly in *D. rhopaloa*, behavior was quite variable between individual pairs, making the frequency of each two-step sequence moderate (not extremely high or low) and not significantly different from other species in statistical examination.

Behavioral elements linked to successful copulation

Because the success of copulation is the ultimate objective of the courtship behavior, we counted the all behavioral elements that appeared within the three steps before successful copulation. In KB866 and *D. kurseongensis*, uni-lateral wing vibration (v) comprised 97.0 and 93.8 %, respectively, of the elements preceding successful copulation (Fig. 9). In *D. rhopaloa*, uni-lateral wing vibration (v), bi-lateral wing vibration (b), tapping (t), and licking (l) were observed before successful copulation. In *D. prolongata*, leg vibration (s) was most frequently observed

underrepresented in the transition diagram (Fig. 8). On the other hand, d–v–d and v–d–v were highly significant in this analysis (Table 3), showing that these transitions are characteristic of *D. prolongata* courtship. In addition, d–v–e, e–v–d, and v–e–v were significantly more frequent in

Fig. 7 Frequency of occurrence of each behavioral element. Frequency is shown as a proportion of the total number of incidents for each species. The occurrence of each behavioral element was scored for 15 min preceding successful copulation. The number of pairs used in this analysis was the same as that in Fig. 5. The total number of the incidents were; *Drosophila prolongata*: 2,420; KB866: 841; *D. rhopaloa*: 418; and *D. kurseongensis*: 2,497

before successful copulation, followed by licking (l), unilateral wing vibration (v), and leg display (d) (Fig. 9).

Discussion

Functional link between foreleg morphology and leg vibration

In this study, it was revealed that the *D. prolongata* males use their forelegs in leg vibration during courtship toward females. Such behavior has not been reported in any other *Drosophila* species. We confirmed that leg vibration was not observed even in the most closely related species, namely, leg vibration is specific to *D. prolongata*. To accomplish leg vibration, the forelegs need to be long enough to reach the female's abdomen from in front of her. In this regard, leg vibration appears to be tightly linked with foreleg morphology. In other words, the functional link might underlie the coincidence of long forelegs and leg vibration in *D. prolongata*.

### Effect of leg vibration on copulation success

Although leg vibration was frequently followed by attempting copulation, and thus it seemed to be a kind of signaling behavior from males to females, its effect on copulation success is unknown. Because half of successful copulations were not preceded by leg vibration (Fig. 9), it is clearly dispensable for a sequence of courtship behavior. Among several possibilities, surrounding facts indicate its function in physically stimulating females to increase the rate of copulation success. First, physical stimulation of the female's abdomen by a male during courtship was reported in several other *Drosophila* species. In *D. silvestris* and closely related species, "leg rubbing" behavior was described as an element of courtship (Spieth 1978). In *D. virilis*, "touching" the female's abdomen from behind was observed immediately before copulation (Vedenina et al. 2013). These behaviors were thought to stimulate females to accept copulation. Second, a recent study revealed that a vibratory signal was used to immobilize the female during courtship in the species of the *melanogaster* subgroup (Fabre et al. 2012). Males of these species showed "quivering" of abdomen, by which they produce substrate-borne vibrations that prevent females escaping from courting males. Considering the low copulation rate in *D. prolongata* (Table 1), males of this species may use leg vibration to make the female more receptive. To understand the function of leg vibration and the effect of leg vibration on female receptivity, as well as the reasons why copulation was not always preceded by leg vibration, remains to be elucidated by further experiments.

Evolution of leg vibration

Evolution of sexual dimorphism is often explained by sexual selection. Because the long forelegs of *D. prolongata* are used in leg vibration during courtship, it is natural to assume that these characters (morphology and behavior) also evolved under sexual selection. However, it is difficult to infer an evolutionary intermediate state of these characters from the current functional link between them; neither the morphology nor the behavior alone would
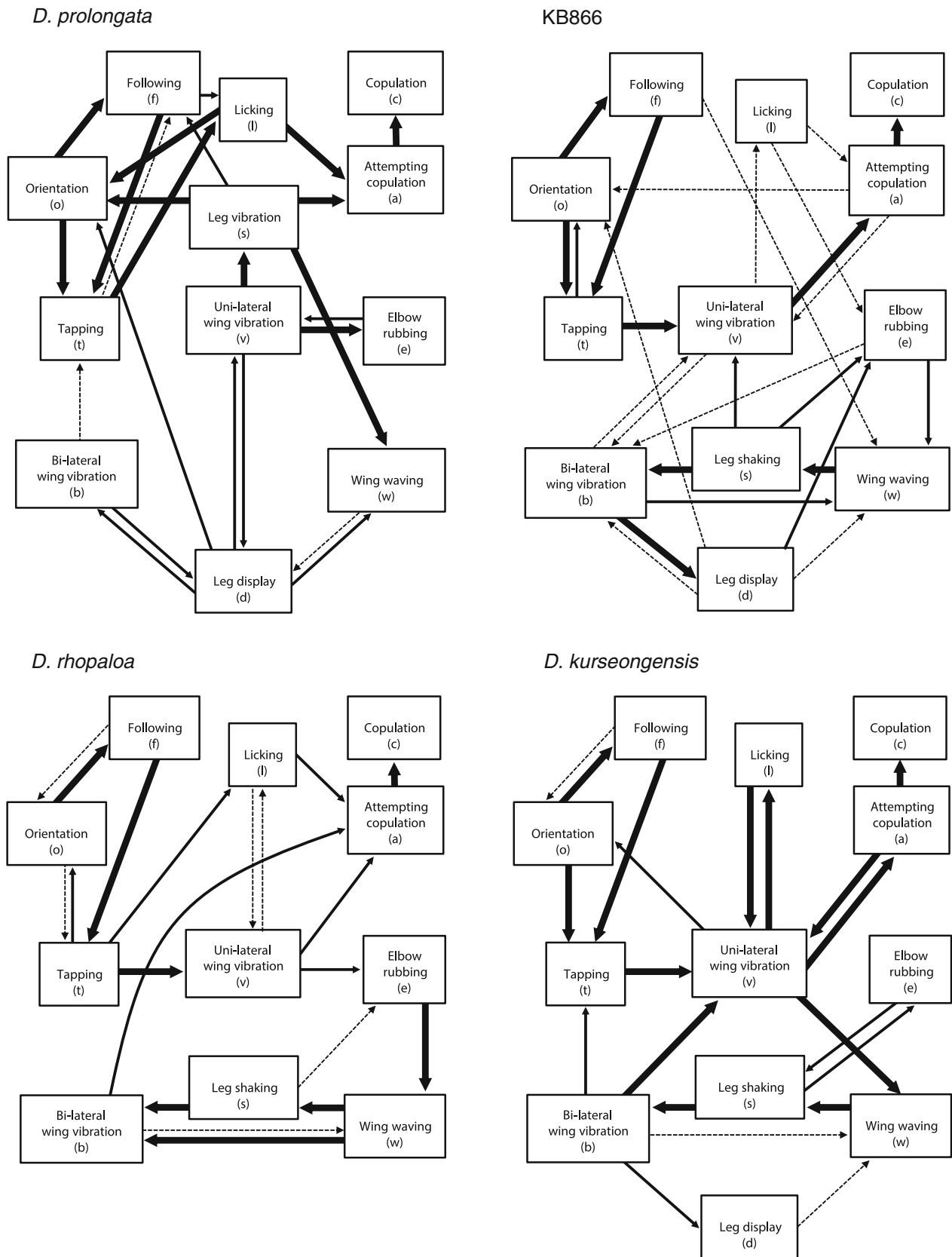
Fig. 8 Transition diagram. Transitions that occurred more frequently than the expected rate are indicated by *arrows*. *Thick arrow* $p < 0.001$; *thin arrow* $0.001 < p < 0.05$; *dotted arrow* $0.05 < p < 0.3$

Table 3 Inter-species comparison of behavioral sequences

Sequence	Frequency of appearance[a]		p[b]	Sequence	Frequency of appearance[a]		p[b]
	Within species	Other species			Within species	Other species	
Drosophila prolongata				KB866			
d–v–d	0.87	0.01	<0.001	s–v–a	0.61	0.13	<0.001
v–d–v	0.87	0.02	<0.001	v–a–c	0.97	0.52	<0.001
d–v–e	0.80	0.00	<0.001	t–v–a	0.76	0.26	0.001
e–v–d	0.77	0.00	<0.001	a–v–a	0.55	0.16	0.005
d–v–s	0.70	0.00	<0.001	v–a–v	0.64	0.27	0.047
v–e–v	0.83	0.08	<0.001				
w–d–v	0.60	0.00	<0.001	*D. rhopaloa*			
w–v–d	0.57	0.00	<0.001	v–w–v[c]	0.00	0.44	<0.001
v–s–a	0.53	0.00	<0.001	v–a–v[c]	0.02	0.47	<0.001
s–a–c	0.50	0.00	<0.001	w–v–w[c]	0.00	0.35	<0.001
v–a–c[c]	0.10	0.81	<0.001	a–v–a[c]	0.00	0.34	<0.001
v–e–d	0.47	0.00	<0.001	b–v–a[c]	0.00	0.31	0.003
d–w–v	0.47	0.00	<0.001	b–a–c	0.22	0.00	0.005
v–d–w	0.50	0.01	<0.001	v–d–v[c]	0.00	0.31	0.006
d–o–f	0.40	0.00	<0.001	d–v–d[c]	0.00	0.30	0.012
v–d–o	0.40	0.00	<0.001	v–l–v[c]	0.02	0.33	0.019
t–v–d	0.40	0.00	<0.001	o–t–v[c]	0.05	0.37	0.021
e–d–v	0.40	0.00	<0.001	a–v–w[c]	0.02	0.31	0.038
f–v–d	0.40	0.00	<0.001	d–v–e[c]	0.00	0.27	0.049
v–w–d	0.37	0.00	<0.001				
v–w–s[c]	0.00	0.54	<0.001	*D. kurseongensis*			
t–v–a[c]	0.00	0.51	<0.001	b–v–a	0.78	0.05	<0.001
w–s–v[c]	0.00	0.49	<0.001	b–v–w	0.66	0.03	<0.001
v–s–d	0.30	0.00	<0.001	s–b–v	0.72	0.07	<0.001
e–v–e	0.40	0.03	<0.001	l–v–w	0.56	0.01	<0.001
v–s–v	0.43	0.05	<0.001	b–w–s	0.72	0.07	<0.001
d–e–v	0.27	0.00	0.001	v–b–v	0.59	0.03	<0.001
d–w–d	0.27	0.00	0.001	v–w–b	0.50	0.01	<0.001
o–t–l	0.27	0.00	0.001	w–s–b	0.78	0.13	<0.001
v–s–o	0.27	0.00	0.001	v–l–v	0.69	0.11	<0.001
l–v–d	0.27	0.00	0.001	b–v–b	0.47	0.01	<0.001
d–v–w	0.33	0.02	0.001	w–v–w	0.69	0.12	<0.001
e–v–s	0.30	0.01	0.002	s–b–w	0.50	0.03	<0.001
o–f–v	0.40	0.05	0.002	v–w–s	0.84	0.25	<0.001
w–s–b[c]	0.00	0.39	0.003	b–v–l	0.44	0.03	<0.001
v-d–l	0.23	0.00	0.006	v–a–v	0.75	0.23	<0.001
l–d–v	0.23	0.00	0.006	t–v–w	0.56	0.10	<0.001
d–v–o	0.23	0.00	0.006	w–v–a	0.47	0.07	<0.001
s–v–d	0.23	0.00	0.006	v–b–w	0.38	0.03	<0.001
d–l–v	0.23	0.00	0.006	a–v–w	0.56	0.13	<0.001
v–a–v[c]	0.03	0.47	0.007	w–s–v	0.72	0.25	0.002
d–o–t	0.27	0.01	0.008	v–w–v	0.66	0.23	0.002
v–s–w	0.27	0.01	0.008	w–b–v	0.34	0.02	0.003
v–d–e	0.20	0.00	0.033	v–a–w	0.41	0.06	0.003
t–d–v	0.20	0.00	0.033	s–v–b	0.25	0.01	0.014
a–v–d	0.20	0.00	0.033	v–a–c	0.94	0.54	0.015

Table 3 continued

Sequence	Frequency of appearance[a]		p[b]	Sequence	Frequency of appearance[a]		p[b]
	Within species	Other species			Within species	Other species	
v–s–f	0.20	0.00	0.033	w–s–w	0.44	0.11	0.039
				s–v–w	0.53	0.17	0.041
				b–w–v	0.19	0.00	0.050

[a] Proportion of pairs that showed the corresponding sequence pattern

[b] Significance of difference examined by Fisher's exact test adjusted by the Bonferroni method for multiple comparisons (number of comparisons = 432)

[c] Behavioral sequences observed less frequently than in the other species

Fig. 9 Behavioral elements preceding successful copulation. Behavioral elements that appeared within three steps before successful copulation (c) are shown. Letters in boxes indicate behavioral elements (see Table 2; Fig. 8). The sizes of the boxes represent the proportion of the frequency of each behavioral element. Lines indicate the transition between elements in behavioral sequences. Drosophila prolongata: $n = 30$; KB866: $n = 33$; D. rhopaloa: $n = 41$; D. kurseongensis: $n = 32$. *In one case in KB866, successful copulation was preceded by only two steps, thus the first element was designated as "−"

D. prolongata

KB866

D. rhopaloa

D. kurseongensis
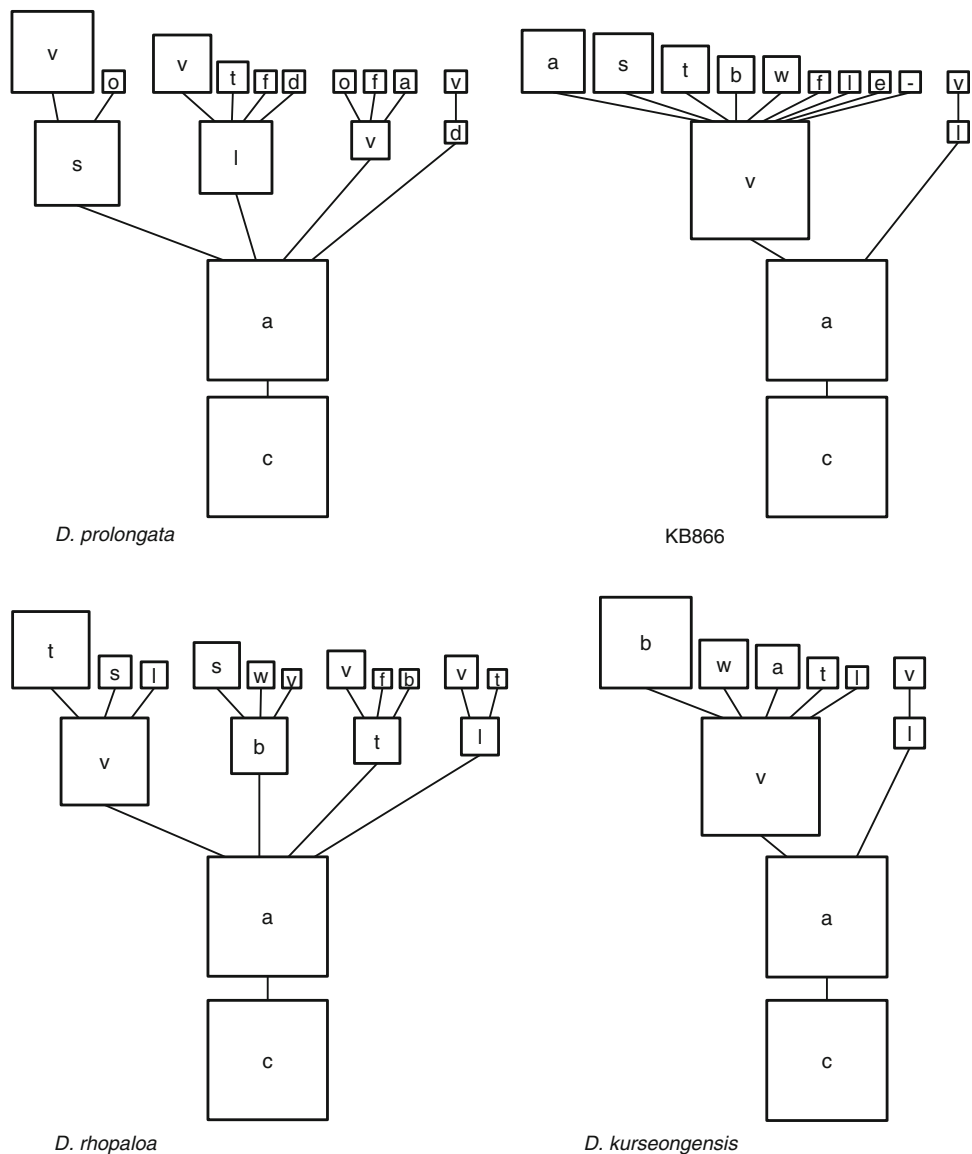
have been adaptive. For example, leg vibration with short forelegs may not be effective if they do not reach the female's abdomen. Likewise, long forelegs may not have enough adaptive advantage to balance with their developmental cost without leg vibration. Unfortunately, our observation of closely related species did not provide any

insights into this issue, because none of them showed leg vibration. At present, we cannot exclude the possibility that the long forelegs in *D. prolongata* originally evolved for other reasons, such as a male-to-male aggressive behavior. It is also noteworthy that leg display was a characteristic element in *D. prolongata* (Fig. 7). Together with the high-contrasting color pattern, the size of the forelegs might have evolved initially as a visual signal. Because leg vibration was dispensable for copulation, it could have evolved after the acquisition of long forelegs. Analysis of the variation among natural populations in foreleg size, as well as in courtship behavior, may provide insights into the evolutionary history of sexual dimorphism and behavior in *D. prolongata*.

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