



# The *Diachasmimorpha longicaudata* complex: Reproductive isolation and geometric patterns of the wing

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## ARTICLE INFO

### Article history:

Received 23 March 2009

Accepted 29 June 2009

Available online 2 July 2009

### Keywords:

*Diachasmimorpha longicaudata*

Thailand

Cryptic species

Crossing experiments

Morphometrics

## ABSTRACT

*Diachasmimorpha longicaudata* is an endoparasitoid of Tephritid fruit fly larvae and is regarded as an important biocontrol agent. However, it is likely that under this specific name several biological species may be contained, the correct identification of which is essential for effective use in control programs. In this paper, three populations (DLA, DLB and DLBB) of *D. longicaudata* designated according to geography and/or natural hosts were reared in the same laboratory. They were tested for reproductive compatibility and characterized by morphometric analyzes. Forced-contact mating technique showed either complete lack of inter-population reproductive compatibility or the production of rare, sterile female offspring. The three populations, indistinguishable on the basis of morphological characters alone, were readily identified by the geometry of the wing. Results strongly suggest that the DLA, DLB and DLBB are distinct biological species, and highlight the usefulness of wing geometry to distinguish them.

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## 1. Introduction

Braconid fruit fly parasitoids (Opiinae) are typically solitary endoparasitoids. Current interest in their systematics comes from their use as biological control agents (Ovruski et al., 2000; Wharton and Gilstrap, 1983). Although there has been some controversy, most opiine genera have now been revised and redescribed (Wharton, 1997). However, this revision did not include the species level, especially for *Diachasmimorpha longicaudata* (Wharton and Gilstrap, 1983). There is suspicion that recognized species may in fact be cryptic species complexes (Kitthawee, 2008; Wharton and Gilstrap, 1983). The biological control user, lacking a reliable means of identification, may find it difficult to determine exactly which species has been released.

The parasitoid, *D. longicaudata*, is a common fruit fly parasitoid and is native to many countries of Southeast Asia where it has been reported infesting a wide variety of host flies in the genus *Bactrocera* (Bess et al., 1961; Clausen et al., 1965; Wharton and Gilstrap, 1983). It has been introduced and established in several other countries for biological control (Clausen, 1978; Ovruski et al., 2000; Sivinski and Webb, 1989; Vargas et al., 1993; Wong and Ramadan, 1987). However, its taxonomic status remains unclear. Wharton and Marsh (1978) observed morphological variations in specimens from different geographical localities, and Wharton

and Gilstrap (1983) listed a number of subspecies (*compensan*, *formosanus*) and varieties (*chocki*, *malaiaensis*, *novocaledonicus* and *taiensis*). In these earlier descriptions and keys, color was used as a major character but different populations of *D. longicaudata* are often proved indistinguishable on the basis of morphology alone (Kitthawee, 2008). Because of this, *D. longicaudata* has been treated as a single taxon in most research and biological control programs. Recently, Kitthawee (2008) reported that the subspecific subdivision was actually more than local differentiation, suggesting that in Thailand *D. longicaudata* is a complex of cryptic species.

Correct taxonomic identification of biological control agents such as *D. longicaudata* is essential to a successful biological control program (DeBach and Rosen, 1991). Therefore, we examined this group of fruit fly parasitoids in Thailand by using reproductive compatibility tests and morphometric techniques for tentative group identification. Reciprocal cross-breeding experiments allowed for the detection of reproductive barriers among three populations. The venation of the wings was then tested as an alternative and low-cost identification technique to accurately differentiate the populations.

## 2. Materials and methods

### 2.1. Parasitoid cultures

Parasitoid populations of *D. longicaudata* were obtained from ripe fruits collected from different types of trees and from various

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**Table 1**

Number specimens of *D. longicaudata* complex (DLA, DLB and DLBB), males (M) and females (F) used for the morphometric study. DL, *D. longicaudata*; N, number of measured specimens; F<sub>1</sub>, offspring of DLB females × DLA males; Host fly, genus *Bactrocera*; Host plant, plant on which *Bactrocera* was reared; M. s., *Musa sapientum* (banana); lab, laboratory; nat, nature.

DL Code	N		Host fly		Host plant	
	F	M	Lab	Nat	Lab	Nat
DLA	53	51	<i>correcta</i>	<i>correcta</i>	M. s.	<i>Psidium guajava</i>
DLB	49	49	<i>dorsalis</i>	<i>dorsalis</i>	M. s.	<i>Terminalia catappa</i>
DLBB	52	49	<i>dorsalis</i>	<i>carambolae</i>	M. s.	<i>Averrhoa carambola</i>
F <sub>1</sub>	11	0	<i>dorsalis</i>	–	M. s.	

geographic locations. They were identified as *D. longicaudata* using the key of Wharton and Gilstrap (1983) and confirmed by Dr. Robert Wharton, Texas A&M University, USA, in 1997. They were tentatively labeled as DLA, DLB and DLBB<sup>1</sup> according to the time and location of collection, the host plant and the host fly (Table 1).

The DLA and DLB samples were collected in Thailand from Nakhon Pathom Province in the central part of the country (Fig. 1). These colonies were initiated with approximately 15–20 pairs in 1997 and 2000 respectively. The DLBB sample came from Phatthalung Province in the more southern area (Fig. 1) and the colony started from approximately 10 pairs in 2001. All these colonies were maintained at the Department of Biology (Mahidol University) for more than 100 consecutive generations before the present experiments were conducted.

## 2.2. Cross-breeding

Combinations of reciprocal pair-matings (Table 2) among the different populations (DLA, DLB and DLBB) were performed by the forced-contact mating technique (Kitthawee, 2008). Each cross pair-mating consisted of reciprocal crosses and controls. In preparation for the cross pair-mating, parasitized pupal hosts from colonies to be crossed were isolated and kept individually in vials in order to obtain virgin males and females. Emerging parasitoids were grouped by population and sex.

The crossing process was conducted as in Kitthawee (2008): in short, an immobilized, virgin female was placed in a plastic vial and arranged in the flight position; an active winged male was then released into the same vial and the vial was slowly moved until the male touched the immobilized female. After successful copulation, females were transferred in groups of 5–10 mated females to a new cage provided with 10% honey in distilled water. They were allowed to lay eggs for 10 days into the same fruit fly species used in parasitoid cultures (Table 1). In each cross pair-mating 3–4 replicates (a total of 25–30) were prepared.

Parasitoid progeny were counted and sexed to determine successful fertilization (genetic compatibility). Due to the haplodiploid mode of reproduction in *D. longicaudata*, incompatibility was attested by the absence of female offspring. Only the presence of female progeny indicated that mating and egg fertilization were both successful. Genetic compatibility was estimated by the percentage of F<sub>1</sub> progeny relative to mating within-population controls, and these frequencies were compared using the  $\chi^2$  test with Yates' correction (Sokal and Rohlf, 1995).

## 2.3. Sample processing for morphometric analyzes

A total of 104 DLA (53 females, 51 males), 98 DLB (49 females, 49 males) and 101 DLBB (52 females, 49 males) were studied (Table 1). Specimens from each colony of DLA, DLB and DLBB were



**Fig. 1.** Geographic origin (see black stars) of the parasitoids: DLA and DLB from central Thailand, province of Nakhon-Pathom, and DLBB from southern Thailand, province of Phatthalung.

dissected. Both left and right fore wings of female and male parasitoids were mounted on the glass slides. Right fore wings only were photographed using a digital camera connected to a stereo microscope at 40× magnification.

## 2.4. Data collection and analyzes

Wings were digitized at 10 landmarks (Fig. 2), all of them of “type I” (venation intersections) (Bookstein, 1991). To avoid

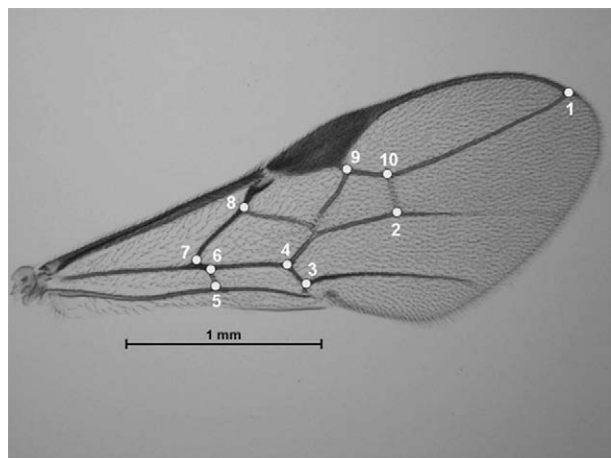
**Table 2**

Crossing combinations among the DLA, DLB and DLBB populations of *D. longicaudata*. Between brackets, the number of F<sub>1</sub> females.

Crosses Female × Male	Pairs tested	Total progeny	% (F <sub>1</sub> female)
DLA × DLA*	30	548	51 (280)
DLA × DLB	30	380	None
DLB × DLA	30	522	3 (17)
DLB × DLB*	30	614	53 (330)
DLB × DLBB	25	470	None
DLBB × DLB	30	411	None
DLBB × DLBB*	30	408	35 (144)
DLBB × DLA	25	285	None
DLA × DLBB	25	320	None

\* Control crosses.

<sup>1</sup> The voucher specimens of each population were kept at Mahidol University (Bangkok, Thailand).



**Fig. 2.** Fore wing of *Diachasmimorpha longicaudata* showing 10 landmarks used in geometric morphometric analysis. All landmarks are the junction of two different veins, and are Type I landmarks (Bookstein, 1991).

possible optical distortion at the periphery of the optical lens, each wing was located at the center of the visual field.

### 2.5. Repeatability (*R*)

In order to reduce the impact of digitizing error, all the landmarks were taken by the same person. The measurement error was estimated comparing two sets of measurements. It was computed as “1–*R*”, with “*R*” the “repeatability” index as described by Arnqvist and Mårtensson (1998), i.e. a Model II oneway ANOVA on repeated measures, where “*R*” is provided by the ratio of the between individual variance and the total variance.

### 2.6. Size comparison

For comparing overall wing size among different populations, we used the isometric estimator known as “centroid size” (CS) derived from coordinates data. It is defined as the square root of the sum of the squared distances between the center of the configuration of landmarks and each individual landmark (Bookstein, 1991). The centroid sizes of parasitoids (DLA, DLB and DLBB) were compared by non-parametric analyzes based on permutations (1000 runs) allowing to compare both means and variances of size (Caro-Riaño et al., 2009).

### 2.7. Shape variation and allometry

Shape variables were obtained through the Generalized Procrustes Analysis (GPA) superimposition algorithm. Thin-plate spline equation produced the “partial warps” (Rohlf, 1990). Both non-uniform (“partial warps”, strictly speaking) and uniform components were used as shape variables. “Relative warps” (RW) are the principal components derived from the shape variables.

Shape variation was illustrated for males and females, separately, by the factorial map of the two first principal components, RW1 and RW2. Allometry was tested by linear regression of RW1 on centroid size. The thin-plate spline interpolation function was used to apply the shape changes (both uniform and non-uniform components) among groups to a virtual squared grid providing a direct and quantitative implementation of the D’Arcy-Thompson transformation “grids” (Bookstein, 1991). Thus, a total of six different PCA were performed to compare, in each sex, DLA to DLB, DLA to DLBB, and DLB to DLBB. To visualize the anatomical differences between species, arrows are indicating the direction of change at each landmark relative to the first principal component, RW1.

## 2.8. Classification based on metric properties

Wings were reclassified according to their similarity to the average shape of each group. This was performed using Mahalanobis distances as an estimation of metric distance. As a validating procedure, distances were computed on discriminant axes estimated without the individual to be classified. Each individual to be classified was then introduced as supplementary data (cross-validated classification).

## 2.9. Software

Data collection, analyzes and graphical output were performed using the various modules (COO, TET, MOG, VAR, PAD and COV) of specialized software developed by Dujardin J.P. and freely available at <http://www.mpl.ird.fr/morphometrics>. The deformation grids were produced using TPSregr program (Rohlf, 1993).

## 3. Results

### 3.1. Hybridization tests

The results of cross-breeding experiments among the different populations of the *D. longicaudata* complex are summarized in Table 2. Due to the haplodiploid mode of reproduction in *D. longicaudata*, reproductive incompatibility was assessed by the absence of female offspring. Homogamic (within-population) control crosses produced the diploid females at varying percentages relative to the total offspring. The percentage of female offspring was 51%, 53% and 35% for DLA, DLB and DLBB populations, respectively. The female offspring from homogamic crosses in DLBB population was significantly lower than in DLA and DLB populations ( $\chi^2$  test,  $P < 0.05$ ) while no significant difference was detected between DLA and DLB ( $\chi^2$  test,  $P > 0.05$ ).

Heterogamic crosses did not produce progeny, or produced only a few sterile female offspring (Table 2). Crosses between DLBB  $\times$  DLB or DLA populations of *D. longicaudata* produced no  $F_1$  female hybrids at all. Crosses between the DLA and DLB populations produced either 0% (female DLA  $\times$  male DLB) or 3% (female DLB  $\times$  male DLA). Female progeny from the heterogamic crosses was significantly lower than progeny of homogamic crosses, in DLA and/or DLB ( $\chi^2$  test,  $P < 0.001$ ). In addition, all adult  $F_1$  female hybrids were sterile.

### 3.2. Morphometric analyzes

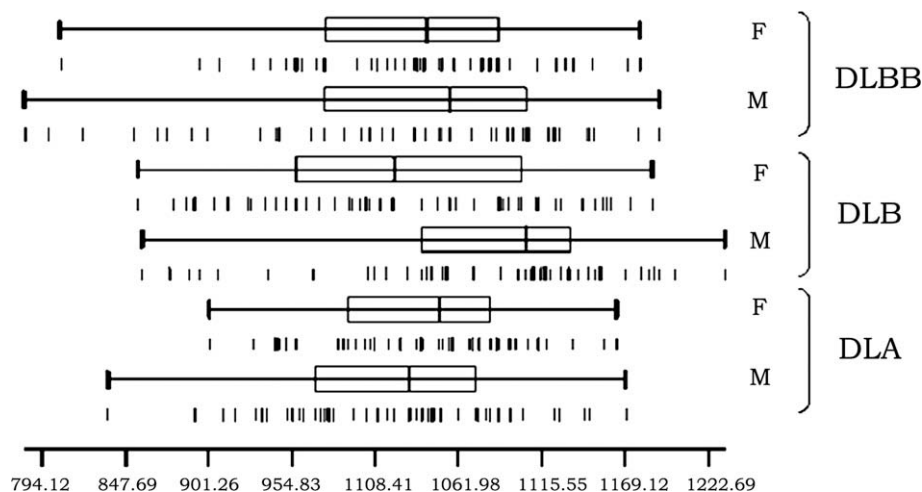
A total of 303 specimens were studied (154 females and 149 males, see Table 1 for details). Also, a set of 11 “DLA  $\times$  DLB” hybrid female specimens was examined and compared to the parental groups.

### 3.3. Repeatability (*R*)

The comparison of two repeated sets of measurements of the same set of wings showed very good agreement for the centroid size ( $R = 0.99$ ), as well as for the four first relative warps ( $R = 0.97$  on average) representing most (81%) of the shape variation. As observed by Arnqvist and Mårtensson (1998) and by Caro-Riaño et al. (2009), the repeatability was then decreasing on the remaining RW.

### 3.4. Size variation

The size variation was graphically presented as quantile plots (Figs. 3 and 4). Only male DLA were significantly smaller than



**Fig. 3.** Centroid size variation (in pixels) presented as quantile plots for DLA males (M), females (F); DLB males (M), females (F) and DLBB males (M), females (F). The box shows the median as a line across the middle and the quartiles (25th and 75th percentiles) as its ends.

the male DLB ( $P < 0.01$ ). The sterile female DLA  $\times$  DLB progeny were significantly larger than their parents ( $P < 0.01$ ). Size ranges among the three populations were overlapping and did not show significant differences except for the DLA females having significantly lower variance ( $P < 0.05$ ) than DLB females. Hybrids also showed a significantly lower variation of size than both parents ( $P < 0.01$ ).

### 3.5. Shape comparison

In accordance with the poor size variation among groups, there was no significant contribution of size to the first relative warp variation (detailed results not shown). Contrary to size variation, shape was similar between sexes, but it strongly differed among groups. In both males and females, the first relative warp (RW1, horizontal axis of Figs. 5 and 6) separated DLA from the remaining groups, i.e. DLB and DLBB, themselves almost completely separated on RW2 (the vertical axis of Figs. 5 and 6).

The principal component analysis (PCA) was also used to explore the shape variation in the  $F_1$  offspring of DLB females  $\times$  DLA males. According to the factorial map (Fig. 7), the wings showed an “intermediate” shape between parents, with more similarity to the DLB group.

### 3.6. D’Arcy-Thompson “grids”

Although each species comparison showed a different set of landmark displacements, variations among males and females were visually very similar within each pair comparison (Fig. 8).

### 3.7. Classification

The classification and the cross-checked classification of the specimens based on the Mahalanobis distances allowed a perfect assignment of all individuals. The cross-checked classification provided complete (100%) discrimination among the three populations (DLA, DLB and DLBB) in males as well as in females. When performing such classification on both males and females considered together, there was no confusion among species, but only between sexes within species (detailed results not shown).

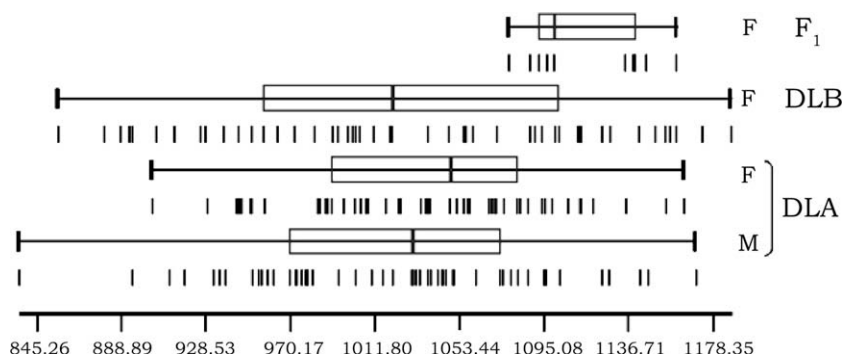
Furthermore, the cross-validated classification of the  $F_1$  offspring was performed relatively to the parents, i.e. female DLB and male DLA (Fig. 7). Hybrids were classified as follows: 64% ( $n = 7$ ) assigned to DLB and 36% ( $n = 4$ ) to DLA.

## 4. Discussion

The use of different approaches such as cross-breeding experiments and geometric morphometric analyzes was able to validate three biological species of the *D. longicaudata* complex in Thailand. In this report, the use of geometric morphometrics appears as a promising alternative or as a useful complement to morphological characterization.

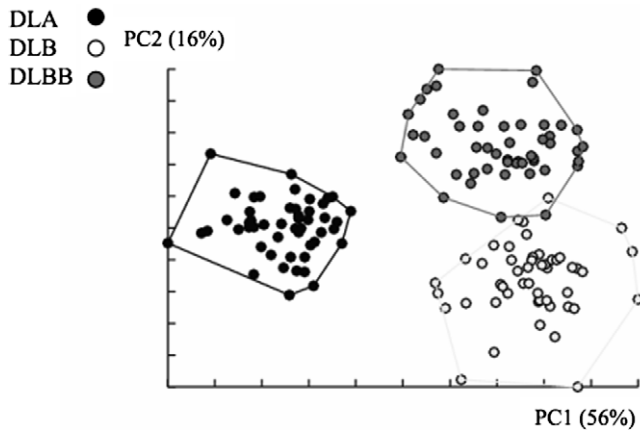
### 4.1. *D. longicaudata* is a complex of species

Data from cross-breeding experiments may serve as evidence for the need to reevaluate current classification (Pinto and Stouthamer, 1994). Lack of progeny, as was the case for the crosses with



**Fig. 4.** Centroid size variation (in pixels) presented as quantile plots for female and male DLA, female DLB and female  $F_1$ . The box shows the median as a line across the middle and the quartiles (25th and 75th percentiles) as its ends.  $F_1$ , the female progeny of female DLB and male DLA; F, females; M, males.





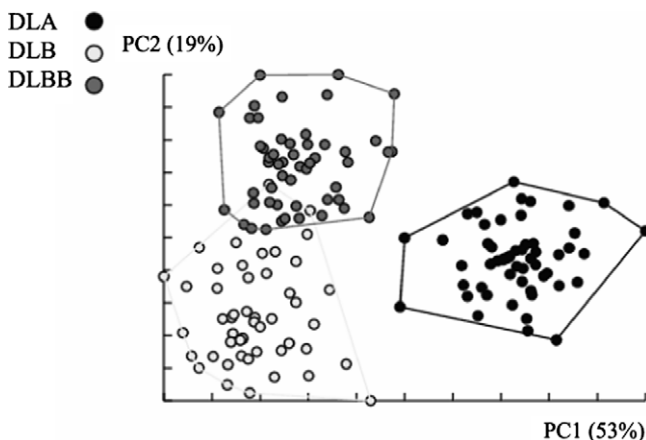
**Fig. 5.** First two components of a principal component analysis (PCA) performed on partial warps from male *D. longicaudata*, plotted by populations (solid black = DLA, opened circle = DLB and solid gray = DLBB). PC1, first principal component; PC2, second principal component; between brackets, the proportion of total variance. Almost complete separation is observed among different populations.

DLBB, and the production of sterile progeny, as observed between DLA and DLB, was valid arguments to consider them as three biological species. Additional arguments include differences in host specificity and clear-cut morphometric divergence.

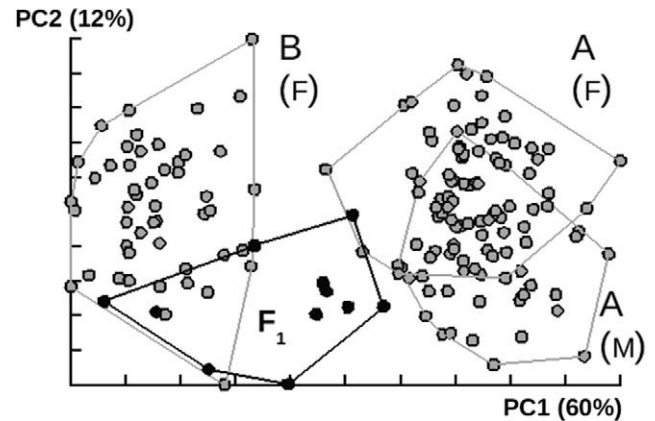
The mechanism of this speciation could be related to ecogeographical differences. The DLBB species is a geographically isolated population, found in southern Thailand, while DLA and DLB are sympatric populations, found together in the central part of Thailand (Fig. 1). As a very first hypothesis, we thus could suggest an allopatric speciation mechanism explaining the reproductive isolation observed for DLBB, and a sympatric mechanism for the separation of DLA and DLB. The DLA species prefers the host fly *B. correcta*, collected from *Psidium guajava* (guava), while the DLB species prefers *B. dorsalis*, collected from *Terminalia catappa* (Indian almond) (Table 1) (Kitthawee, 2000, 2008). These different hosts and habitats could represent a significant factor of speciation by separating the sympatric DLA and DLB.

#### 4.2. The geometry of the wings as a species specific character

Geometric morphometric analysis has proved to be useful in distinguishing close species (De la Riva et al., 2001; Villegas



**Fig. 6.** First two components of a principal component analysis (PCA) performed on partial warps ("relative warps") from female *D. longicaudata*, plotted by populations (solid black = DLA, opened circle = DLB and solid gray = DLBB). PC1, first principal component; PC2, second principal component; between brackets, the proportion of total variance. Almost complete separation is observed among different populations.



**Fig. 7.** Principal component analysis (PCA) is used to investigate F<sub>1</sub> offspring of DLB females × DLA males. The first component (PC1) is the horizontal axis and presents here 60% of the total variance, the second component (PC2) presents 12%. F<sub>1</sub> offspring (black dots) lie between DLB (gray dots, labeled B) and DLA (gray dots, labeled A), either males (M) or females (F).

et al., 2002), geographic or ecotopic conspecific populations (Dujardin et al., 2003; Camara et al., 2006; Dujardin and Le Pont, 2004), laboratory lines (Jirakanjanakit and Dujardin, 2005), and it has been shown powerful enough to allow the identification at the individual level (Dujardin et al., 2007). The present study demonstrates that the geometric pattern of the wing venation contains taxonomically relevant information when properly quantified and oriented to the description of shape (Dujardin and Le Pont, 2004; Dujardin and Slice, 2007).

#### 4.3. Size

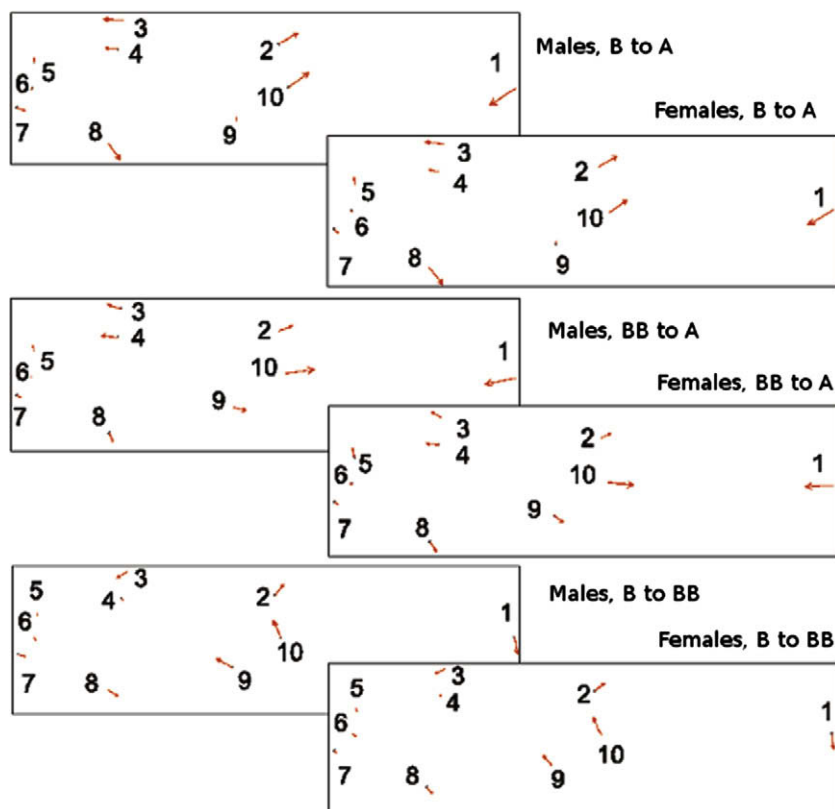
Size is often very contributive to species distinction, but in the case of *D. longicaudata* complex, we could not detect any significant difference except for the males of DLA and DLB. The lack of size differences among species could be a consequence of the common laboratory conditions to which specimens were submitted during many generations.

#### 4.4. Shape

Contrary to general size similarity, there was a clear-cut shape divergence among groups. According to the analysis of allometry, this was not a passive consequence of size variation. Furthermore, it could not be related to different hosts, at least for DLB and DLBB since they were reared on the same fruit fly *B. dorsalis* (Table 1).

The landmark positions differed among groups significantly enough to recognize them as completely separate clouds when plotted onto the two first principal components of shape (Figs. 5 and 6). The principal component analysis (PCA) does not use the group information, so that the disclosing of separate clouds is always a significant signal. Another signal of consistent geometric differences was the parallelism of shape changes in males and females. Since the Procrustes superimposition uses the least square criterion of optimality, landmark displacements should not be examined one by one, but it is worth noting a general similarity among the deformation grids (Fig. 8): males and females (right wings) showed similar landmark displacements from one species to another.

In sum, shape differences were able to produce separate clusters with simple principal component analysis, they were allometry-free and they affected the same landmarks in the same way for each sex. Since the three populations were reared under the



**Fig. 8.** Relative displacements of landmarks in wing of males (left) and females (right) when going from one species to another along the first relative warps (RW1). Top: from DLB to DLA (RW1 representing 69% of the total variance in males, and 67% in females), Mid: from DLBB to DLA (RW1 representing 66% of the total variance in males, and 63% in females) and Bottom: from DLB to DLBB (RW1 representing 39% of the total variance in males, and 46% in females).

same laboratory conditions, except for the host fly of DLA (see Table 1), these differences are suggestive of evolutionary divergence.

#### 4.5. Classification

The cross-checked classification scores produced 100% correct assignments. Our data, together with previous studies on parasitoids (Baylac et al., 2003), suggest that detection and identification of sibling species of parasitoids could be readily achieved by geometric morphometrics. The discriminating power of geometric analyzes on pictures allows to imagine a future database of reference pictures belonging to distinct cryptic species as a systematic tool for classification of unknown specimens. This tool is to be developed in the near future as described in Dujardin (2008).

These results should stimulate more interest in geometric techniques to improve recognition of sibling species by non-molecular tools.

#### 5. Conclusions

Reproductive isolation and shape divergence indicated that *D. longicaudata* in Thailand is a species complex comprised of at least three distinct species designated here as: *longicaudata* A, B and BB. These cryptic species are only partially morphologically distinguishable, but can be separated and confirmed on the basis of wing morphometrics and cross-breeding experiments. Speciation mechanisms are unknown, but among possible factors we could suggest are geographic separation (allopatric speciation for species BB), host plant and/or host fly specificity (for the sympatric species A and B).

For successful control programs targeting fruit flies, accurate tools are necessary to discriminate within the *Diachasmimorpha*

complex in Thailand. We recommend geometric morphometrics as a low-cost, fast and promising approach.

#### Acknowledgments

To R.A. Wharton for helping in the morphological identification of the biological material used in this study. To A. Henry for critical reading of the paper. This work was supported by the Thailand Research Fund and the Commission on Higher Education, RMU5080060, and by the TRF/BIOTEC Special Program for Biodiversity Research and Training, BRT R\_251010.

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