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Wing shape as a potential discriminator of morphologically similar pest taxa within the *Bactrocera dorsalis* species complex (Diptera: Tephritidae)

M.K. Schutze[1,2]*, A. Jessup[3] and A.R. Clarke[1,2]

[1]Discipline of Biogeosciences, Queensland University of Technology, GPO Box 2434, Brisbane 4000, Queensland, Australia: [2]Cooperative Research Centre for National Plant Biosecurity, LPO Box 5012, Bruce, A.C.T. 2617, Australia: [3]Insect Pest Control Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna, Austria

Abstract

Four morphologically cryptic species of the *Bactrocera dorsalis* fruit fly complex (*B. dorsalis s.s.*, *B. papayae*, *B. carambolae* and *B. philippinensis*) are serious agricultural pests. As they are difficult to diagnose using traditional taxonomic techniques, we examined the potential for geometric morphometric analysis of wing size and shape to discriminate between them. Fifteen wing landmarks generated size and shape data for 245 specimens for subsequent comparisons among three geographically distinct samples of each species. Intraspecific wing size was significantly different within samples of *B. carambolae* and *B. dorsalis s.s.* but not within samples of *B. papayae* or *B. philippinensis*. Although *B. papayae* had the smallest wings (average centroid size = 6.002 mm ± 0.061 SE) and *B. dorsalis s.s.* the largest (6.349 mm ± 0.066 SE), interspecific wing size comparisons were generally non-informative and incapable of discriminating species. Contrary to the wing size data, canonical variate analysis based on wing shape data discriminated all species with a relatively high degree of accuracy; individuals were correctly reassigned to their respective species on average 93.27% of the time. A single sample group of *B. carambolae* from locality 'TN Malaysia' was the only sample to be considerably different from its conspecific groups with regards to both wing size and wing shape. This sample was subsequently deemed to have been originally misidentified and likely represents an undescribed species. We demonstrate that geometric morphometric techniques analysing wing shape represent a promising approach for discriminating between morphologically cryptic taxa of the *B. dorsalis* species complex.

Keywords: canonical variate analysis, cryptic species, fruit flies, generalised Procrustes analysis, interspecific variation, intraspecific variation

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Introduction

The Tephritidae, or true fruit flies, represent a highly diverse dipteran family of over 4000 globally distributed species (White & Elson-Harris, 1992). The majority attack the seed-bearing organs of plants, with many species inflicting

*Author for correspondence
Fax: +61 7 3138 1535
E-mail: m.schutze@qut.edu.au

significant economic losses to commercial horticulture through fruit destruction (White, 1996).

As with any pest insect, management of tephritids relies on accurate identification of the target species (Walter, 2003); however, several important tephritid pest species remain difficult to identify as they belong to cryptic, sibling complexes. Sibling species can potentially vary in important traits, such as host use and pest status, geographic distribution and seasonal phenology, yet their study and management remains confounded due to high levels of morphological similarity (Clarke *et al.*, 2001; Garros *et al.*, 2006; Barik *et al.*, 2009). Importantly, mating behaviour will also vary among sibling species, which has implications for the sterile insect management technique for which the mating success of treated insects is critical (Dyck *et al.*, 2005).

One tephritid species complex, the *Bactrocera dorsalis* complex, contains over 70 described species (Drew & Hancock, 1994), some of which are regarded as the most destructive horticultural pests within their native south-east Asian range (Clarke *et al.*, 2005). Moreover, the invasive movement of certain species within the complex, including the spread of *B. carambolae* Drew & Hancock into South America during the mid 1970s (van Sauers-Muller, 1991) and the recent expansion of *B. invadens* Drew, Tsuruta & White into Africa (Drew *et al.*, 2005; Khamis *et al.*, 2009) renders this group a truly global problem. Despite their economic importance, identification of some species of the complex is difficult and is of ongoing concern (Clarke *et al.*, 2005). This is a particular challenge as some of the most destructive pest species in the complex are also the most morphologically similar (= cryptic), especially *B. papayae* Drew & Hancock, *B. carambolae*, *B. philippinensis* Drew & Hancock and *B. dorsalis s.s.* (Hendel). Difficulties chiefly arise due to broad intraspecific morphological variation between members of the *B. dorsalis* complex, which can seriously confound their identification (Drew *et al.*, 2008). Without supporting information such as geographical origin (itself not a true 'taxonomic character'), unambiguous species identification of these four species is often impossible. Further, diagnostic characters used in taxonomic keys are sometimes described using subjective or relational terminology, such as 'narrow', 'slightly expanded' and 'occasionally' (Lawson *et al.*, 2003; Drew & Hancock, 1994), making definitive identifications more difficult. Traditional morphometric approaches based on the length of the aedeagus have achieved some success in delineating species within the *B. dorsalis* complex, particularly between the sympatric species of *B. occipitalis* and *B. philippinensis* and, with some success, *B. papayae* and *B. carambolae* (Iwahashi, 1999a,b; Drew *et al.*, 2008). Unfortunately, however, as aedeagus length is a continuous character state, even this measure is not always definitive. Traditional wing morphometric studies assessing vein length can effectively separate some members of the complex but not all of the four critical pest taxa listed above (Adsavakulchai *et al.*, 1998).

Whereas some other morphologically cryptic insect complexes have been adequately resolved using alternative approaches to morphological taxonomy, such as molecular data and ecological information (e.g. Scheffer & Lewis, 2001), efforts to discriminate between certain populations or species in the *B. dorsalis* complex have failed to reach a similar consensus. For instance, while molecular approaches have achieved some headway in discriminating certain species of the *B. dorsalis* complex (Muraji & Nakahara, 2002; Naeole & Haymer, 2003), there is yet to be found a consistent marker to adequately resolve species boundaries between the four abovementioned species, with the exception of *B. carambolae* for which molecular markers have been identified (Yong, 1995; Armstrong & Cameron, 1998; Armstrong & Ball, 2005). Similarly, studies of the constituents of the male pheromone gland reveal a high degree of similarity between most pest species of the complex, again with the exception of *B. carambolae* which possesses a distinct suite of pheromones compared to chemically similar *B. dorsalis s.s.*, *B. papayae* and *B. philippinensis* (Fletcher & Kitching, 1995; Wee & Tan, 2005).

As the biological species status of the four taxonomically described species in question (i.e. *B. dorsalis s.s.*, *B. papayae*, *B. carambolae* and *B. philippinensis*) is not universally supported by alternate lines of evidence, there remain two possibilities, either (i) that the species as described are biologically valid, but appropriate diagnostic markers remain to be found, or (ii) diagnostic markers do not exist as these species are at such an early stage in their evolutionary divergence that sufficient 'species criteria' (*sensu* de Queiroz, 1998) are yet to accrue. Indeed, what we observe for these four species in question, i.e. a lack of consistent evidence of species boundaries, is precisely what we might expect given a case of a recent and rapid evolutionary radiation (Shaffer & Thomson, 2007), as is the case hypothesised for this tropical species complex (Clarke *et al.*, 2005). Given the need to seek resolution on the specific status of these flies for management and trade reasons, we are currently pursuing new methods for their discrimination by seeking to use tools suited to detecting fine-scale differences which may be expected following recent radiations (Shaffer & Thomson, 2007).

One such approach is shape analysis, the quantification of the relative positions of homologous structures, e.g. landmarks or outlines, and then comparing variation in shape data between individuals or groups, a technique known as geometric morphometrics (Rohlf & Marcus, 1993; Rohlf, 1999). This technique has been applied across a range of taxonomic levels, from revealing cryptic species to discriminating intraspecific populations and to resolving relationships at the generic level (Gilchrist & Crisafulli, 2006; Bouyer *et al.*, 2007; Marsteller *et al.*, 2009; Michez *et al.*, 2009). While traditional morphometric approaches (i.e. linear measurements and their ratios) can be applied to shape analysis (Daly, 1985), they are often used only to compare variation in size and proportion rather than to quantify shape itself (Drew *et al.*, 2008). Geometric morphometric techniques, however, focus directly on shape variation and provide potentially greater taxonomic utility over traditional techniques, as shape is regarded as a more heritable character than size (Bitner-Mathé & Klaczko, 1999; Birdsall *et al.*, 2000; Dujardin *et al.*, 2003). Further, continuous characters, such as shape, are hypothesised to be among the first to show differences following isolation events (Bouyer *et al.*, 2007). Therefore, sensitivity to small shape changes between populations may be particularly useful for detecting differences between groups of organisms following recent evolutionary radiations. As the *B. dorsalis* species complex is considered to contain taxa that have undergone a recent radiation (Clarke *et al.*, 2005), geometric morphometric analysis, therefore, may be sensitive enough to resolve differences should they exist.

The aim of this study, therefore, was to use geometric morphometric techniques to measure wing size and shape for previously identified specimens of *B. dorsalis s.s.*, *B. papayae*, *B. carambolae* and *B. philippinensis* to determine: (i) whether

Table 1. Collection localities of *B. dorsalis s.l.* flies used in the current study.

Species	Code label	Locality	Province	Country	*N*	Total
B. dorsalis s.s	DOR Taiwan	Chiayi	Taiwan	Rep of China	20	
	DOR India	Bangalore	Karnataka	India	20	59
	DOR Thailand	Chiang Mai	Chiang Mai	Thailand	19	
B. carambolae	CAR Kuala Kangsar	Kuala Kangsar	Perak	Malaysia	18	
	CAR TN Malaysia	TN 6, 12, 14		Malaysia	19	55
	CAR Suriname	Paramaribo	Paramaribo	Suriname	18	
B. papayae	PAP Malaysia	Cameron Highlands		Malaysia	20	
	PAP Borong	Borong	Flores	Indonesia	19	57
	PAP Mataram	Mataram	Lombok	Indonesia	18	
B. philippinensis	PHI Batangas	Batangas City	Batangas	Philippines	21	
	PHI Trece	Trece Martires City	Cavite	Philippines	18	54
	PHI Cebu	Cebu City	Cebu	Philippines	15	
B. tryoni	TRY	Brisbane	Queensland	Australia	20	20

N, number of individuals.

wing size or shape is an effective discriminator between the currently defined species; and, if so, (ii) to what degree the species' wings differ from each other; but, if not, (iii) should any of these species be suspected as conspecific based on morphometric shape data? Importantly, we specifically wanted to test the strength of this approach towards resolving specimens which had been *a priori* identified and labelled in the laboratory of R.A.I. Drew and colleagues (i.e. the original author/s of three of the four cryptic species) without further species identification undertaken by us. We believe that if wing shape data accurately reflected the species groupings as previously determined by the species' original authors, this technique could also show promise as an effective technique to apply to new, unidentified specimens.

Materials and methods

Sample collection and curation

Individuals for four species from the *B. dorsalis* complex were chosen for analysis. These were: *B. papayae*, *B. carambolae*, *B. philippinensis* and *B. dorsalis s.s.* Additionally, one congeneric species from outside the *B. dorsalis* complex, *B. tryoni* (Froggatt), was included as an out-group to provide a relative measure of unambiguous interspecific variation.

Individuals were sourced primarily from the Queensland Department of Employment, Economic Development and Innovation (DEEDI) insect collection, located in Brisbane, Australia. Specimens from DEEDI were used for the following reasons: (i) in most cases the specific identities of individuals from the collection were determined by the original species' authors (i.e. R.A.I. Drew and D. Hancock); and (ii) the specimens within the collection have been collected from across a broad spatial distribution, allowing us to account for that source of variation in our analysis. The only flies included in the study that were not sourced from the DEEDI insect collection were *B. carambolae* from Suriname, South America and *B. tryoni* from Brisbane, Australia. The Suriname *B. carambolae* were collected by A. van Sauers-Muller as pupae from infested carambola fruit (*Averrhoa carambola*) placed in the field at Paramaribo, Suriname during August 2009; and wild, cue-lure trap-caught males of *B. tryoni* were collected by MKS during November 2009 in Brisbane, Australia. Details of collection records for all flies used are given in table 1.

Identification of material from the DEEDI collection was based on determinations carried out by R.A.I. Drew when revising the *B. dorsalis* complex in the 1990s (Drew & Hancock, 1994). Material from Suriname is considered to be *B. carambolae*, based on identifications made at the time of the first invasion (van Sauers-Muller, 1991). The out-group, *B. tryoni*, was identified by MKS. Only males were examined for all populations, as most available specimens had been collected using the male specific attractant, methyl eugenol. Where females were available (for example in the Suriname material), we chose to ignore them to avoid potentially sex biased results. We recognise the limitation of using one sex and, therefore, recommend that any expansion on this work should include both sexes where possible.

Between 54 and 59 individuals per species from at least three geographically distinct collection sites were randomly selected from available specimens for each in-group species (table 1). In some instances, flies from more than one trap and from more than one collection date were used; each group of flies from a collection site is termed a 'sample'. Within the limits of the specimens available, samples were chosen to maximise the geographic distance between collection sites so as to increase the amount of potential intraspecific variation measured. Generally, one sample came from one site, the only major exception being *B. carambolae* from 'TN' (table 1). These flies were collected along 'Transect North', Peninsular Malaysia (Drew & Hancock, 1994). There are no precise site details for the collection sites along the transect; however, each TN site is described as being approximately 20 km apart along a 200–250 km distance between the extreme localities of Kuala Kangsar (TN1) and Kota Bharu (TN17), Peninsular Malaysia (Drew & Hancock, 1994). Samples in the present study were from TN6, 12 and 14 (table 1).

All specimens were assigned a cross-referenced code number between QUT 001 and QUT 284, which was affixed to both (i) the slide-mounted wing and (ii) the pinned or alcohol-preserved voucher specimen (n.b. not all slide-mounted wings were included in the analysis due to damage or obscured landmarks). Only the following DEEDI insect collection material had DEEDI database accession numbers: *B. carambolae* from Kuala Kangsar Malaysia (selected specimens between QDPC 0-141684 and QDPC 0-141777) and *B. carambolae* from the TN Malaysia transect (selected specimens between QDPC 0-141663 and QDPC 0-141742). All loaned material is due to be redeposited in the original

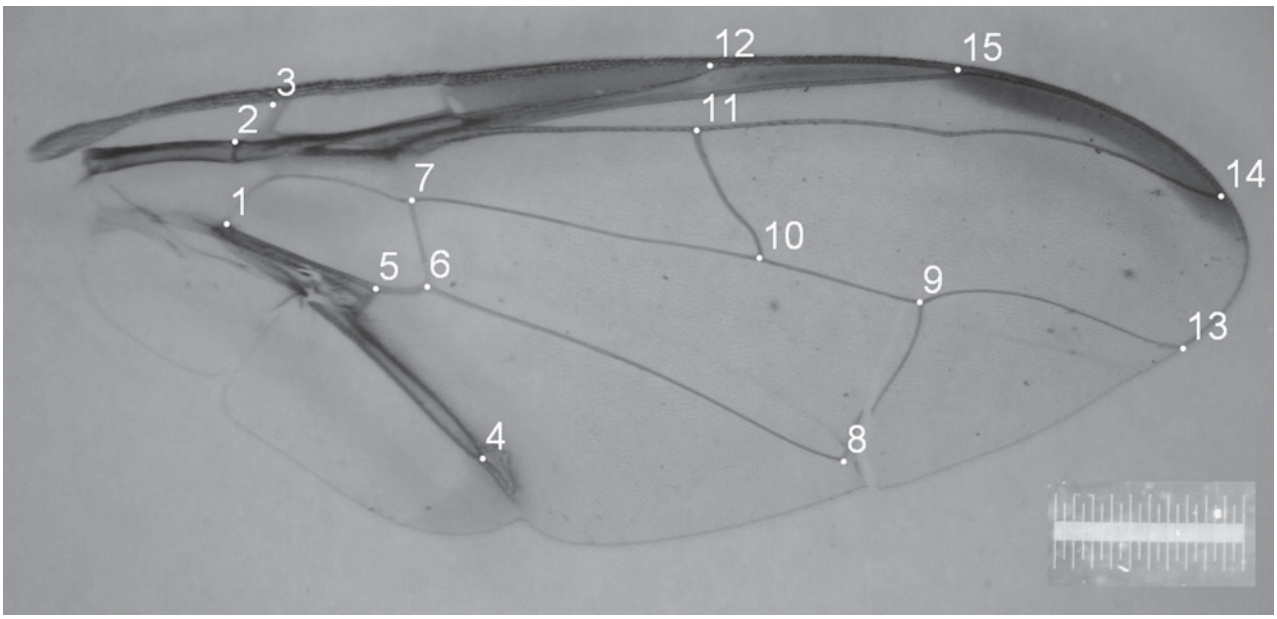

Fig. 1. Right-hand wing of a *Bactrocera dorsalis s.s.* individual showing each of the 15 landmarks used in the geometric morphometric analysis. Scale bar = 1 mm. 1, basal junction of veins of cell bm; 2, anterior-most point of the suture located towards the base of vein sc; 3, inner antero-distal corner of cell bc; 4, junction of veins A_1 and CuA_2; 5, junction of CuA_1 and CuA_2; 6, junction of vein CuA_1 and dm-bm cross vein; 7, junction of vein M and dm-bm cross-vein; 8, junction of vein CuA_1 and dm-cu; 9, junction of vein M and dm-cu; 10, junction of vein M and r-m cross-vein; 11, junction of vein R_{4+5} and r-m cross-vein; 12, junction of vein R_1 and costal vein; 13, termination of vein M; 14, termination of vein R_{4+5}; 15, termination of vein R_{2+3}.

collection at the expiry of the loan (2012), while remaining voucher specimens are maintained at the Queensland University of Technology, Brisbane, Australia.

Morphometric analysis

In most specimens, the right wing of each fly was removed and slide-mounted using Canada balsam. In some instances (19% of flies sampled), the right wing was either damaged or missing and so the left wing was used instead. As fluctuating asymmetry (differences between the left and right wing) does not occur in other *Bactrocera* species (Gilchrist & Crisafulli, 2006), we believe that the occasional (and non-systematic across-samples) use of the left wing would not bias the analysis. Wings were photographed at 10 × magnification using an AnMo Dino-Eye microscope eye-piece camera (model AM423B) mounted into a Leica MZ6 stereo-microscope and saved in bitmap format using the computer program DinoCapture v3.2.0.5 (produced by AnMo). Fifteen homologous Type 1 landmarks (Bookstein, 1991) were selected for comparison, these being the following vein junctions, vein terminations or vein suture: (1) basal junction of veins of cell bm; (2) anterior-most point of the suture located towards the base of vein sc; (3) inner antero-distal corner of cell bc; (4) junction of veins A_1 and CuA_2; (5) junction of CuA_1 and CuA_2; (6) junction of vein CuA_1 and dm-bm cross vein; (7) junction of vein M and dm-bm cross-vein; (8) junction of vein CuA_1 and dm-cu; (9) junction of vein M and dm-cu; (10) junction of vein M and r-m cross-vein; (11) junction of vein R_{4+5} and r-m cross-vein; (12) junction of vein R_1 and costal vein; (13) termination of vein M; (14) termination of vein R_{4+5}; and (15) termination of vein R_{2+3} (fig. 1). Each landmark was digitised using the computer program tpsDig2 v2.12 (Rohlf, 2008) for which x, y coordinates were generated and saved as a text file.

Centroid size was used as the measure of size for each fly wing, calculated using the computer program morphologika2 v2.5 (O'Higgins & Jones, 2006). Centroid size is an isometric estimator of size, calculated as the square root of the summed squared distances of each landmark from the centre of the landmark configuration (i.e. the mean position of all co-ordinates). Tests for significant differences among samples for each *a priori* defined species (based on their original classification) were undertaken using a one-way ANOVA with a Tukey *post-hoc* test. Further, comparisons among species (with intra-specific groups combined) were also tested using a one-way ANOVA with a Tukey *post-hoc* test; however, the individuals of the *B. carambolae* sample from TN Malaysia were removed from this analysis (see reasons below).

Raw landmark co-ordinate data were imported into the program CoordGen6f (part of the IMP software series (Sheets, 2006)) and aligned using a generalised Procrustes analysis procedure, which is a process for removing non-shape variation (i.e. rotation, translation and scale) from the data (Rohlf, 1999).

As wing size can also significantly influence the shape of the wing (allometry), we undertook a multivariate regression of the dependant variable (wing shape) on centroid size (independent variable) (e.g. Drake & Klingenberg, 2008) using the software package MorphoJ v 1.02E (Klingenberg, 2011). This approach uses the shape of each specimen as represented by a vector of landmark coordinates following Procrustes superimposition and then relates this to size. The statistical significance of the regression was tested by permutation tests (10,000 replicates) against the null hypothesis of independence.

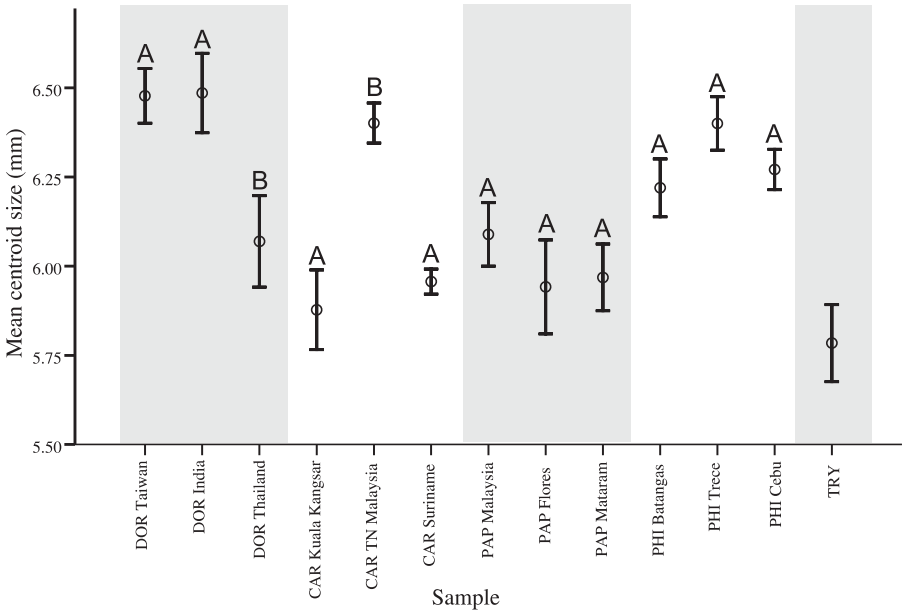

Fig. 2. Wing centroid size (mean ± 1 SE) for males from each sampled location of *Bactrocera dorsalis s.l.* and the out-group *B. tryoni* (TRY). DOR, *B. dorsalis s.s.*; CAR, *B. carambolae*; PAP, *B. papayae*; PHI, *B. philippinensis*. See table 1 for locality details. Shading was added to delineate different species. Samples sharing the same letter within a species are not statistically different from each other based on intra-specific one-way ANOVA with Tukey *post hoc* test ($P > 0.05$).

Procrustes transformed co-ordinate data was then imported into the statistical analysis package SPSS v 17.0 for subsequent canonical variates analysis (CVA), and also the computer program CVAGEN6J (Sheets, 2006) for group assignment tests. The latter test reassigns individuals to an *a priori* defined group following CVA (based on Mahalanobis distances between the individual and group centroids).

For CVA, individuals were *a priori* defined as members of one of 13 samples based on collection locality (i.e. three sites for each of the four in-group species, one site for the out-group *B. tryoni*). Following CVA, intraspecific groups (except for TN Malaysia *B. carambolae*, see Discussion) were combined according to species to form five groups, i.e. *B. dorsalis s.s.*, *B. papayae*, *B. carambolae*, *B. philippinensis* and *B. tryoni*, for which the assignment test was undertaken to determine the number of individuals from any one sample being reassigned to their *a priori* taxonomically defined species.

Results

Wing size

Intraspecific wing size variation among sampled populations was significantly different for *B. dorsalis s.s.* ($F_{(2, 56)} = 4.876$, $P < 0.05$) and *B. carambolae* ($F_{(2, 52)} = 14.628$, $P < 0.001$) but not so for either *B. papayae* ($F_{(2, 54)} = 0.559$, $P > 0.05$) or *B. philippinensis* ($F_{(2, 51)} = 1.610$, $P > 0.05$) (fig. 2). The Tukey *post hoc* test for *B. dorsalis s.s.* revealed that the Thailand sample was significantly smaller than either of those from Taiwan or India, with the latter two samples not significantly different from each other. The sample of *B. carambolae* from 'TN Malaysia' was significantly larger than the *B. carambolae* samples from Kuala Kangsar and Suriname, which again were not significantly different from each other. The out-group *B. tryoni* possessed the smallest wings (fig. 2).

Table 2. Wing centroid size (mean ± SE) for each of the five *Bactrocera* species used in this study.

Species	*N*	1	2	3
B. tryoni	20	5.79 (± 0.11)		
B. papayae	57	6.00 (± 0.06)	6.00	
B. carambolae	37		6.15 (± 0.05)	6.15
B. philippinensis	54			6.29 (± 0.04)
B. dorsalis s.s	59			6.35 (± 0.07)

Species which are not significantly different ($P > 0.05$; based on the Tukey *post hoc* test following one-way ANOVA) occur within the same column. *N*, number of individuals.

Table 3. Details of the ten significant canonical variates produced following CVA on 15 Procrustes transformed landmark data for all *B. dorsalis s.l.* and *B. tryoni* specimens used in the current study.

Function	Eigenvalue	% of variance	Cumulative %	Canonical correlation
1	3.675	28.8	28.8	0.887
2	3.262	25.6	54.4	0.875
3	2.129	16.7	71.1	0.825
4	1.452	11.4	82.5	0.770
5	0.584	4.6	87.1	0.607
6	0.407	3.2	90.3	0.538
7	0.370	2.9	93.2	0.520
8	0.294	2.3	95.5	0.477
9	0.223	1.8	97.3	0.427
10	0.191	1.5	98.7	0.400

For the combined samples of each in-group plus the out-group, wing size varied significantly across species ($F_{(4, 222)} = 9.239$ $P < 0.05$). The Tukey *post hoc* test showed,

Table 4. Functions for group centroids for all significant canonical variates produced from CVA on 15 Procrustes transformed landmark data for all *B. dorsalis s.l.* and *B. tryoni* specimens used in the current study.

Sample group	Function									
	1	2	3	4	5	6	7	8	9	10
B. dorsalis Taiwan	2.762	0.443	–0.683	–1.504	0.155	–0.413	–1.480	–0.073	0.008	–0.243
B. dorsalis India	2.789	0.391	0.288	–0.135	0.267	–0.126	0.658	0.632	–0.966	0.260
B. dorsalis Thailand	2.011	0.300	–0.078	–0.314	0.431	0.632	0.558	–0.672	0.159	0.499
B. carambolae Kuala Kangsar	0.048	–0.026	–1.816	0.449	–0.061	–1.552	0.533	–0.012	0.742	0.366
B. carambolae TN Malaysia	–3.920	–1.732	–2.272	–1.432	0.754	0.309	–0.106	0.050	–0.436	0.199
B. carambolae Suriname	0.152	0.582	–2.082	3.218	–0.083	0.422	–0.347	–0.359	–0.310	–0.414
B. papayae Malaysia	0.710	1.011	–0.249	–0.360	0.391	1.084	0.189	0.096	0.764	0.034
B. papayae Flores Indo	–0.375	–0.304	0.005	–1.036	–2.031	0.003	0.367	–0.757	–0.292	–0.155
B. papayae Mataram Indo	–0.433	0.195	–0.360	–0.135	–0.685	0.164	0.311	1.334	0.267	–0.571
B. philippinensis Batangas	–1.737	1.464	1.644	1.015	0.021	–0.371	–0.522	0.145	–0.209	0.629
B. philipinensis Trece	–1.800	1.425	1.902	–0.023	–0.492	0.349	–0.429	0.126	0.244	0.221
B. philippinensis Cebu City	–1.407	1.913	1.822	–0.477	1.261	–0.616	0.611	–0.594	–0.108	–0.998
B. tryoni	0.618	–5.127	1.900	0.776	0.211	–0.036	–0.116	–0.032	0.185	–0.121

however, that no species, including the out-group *B. tryoni*, was able to be discriminated from at least one other species (table 2). It is thus concluded that wing size is not a good discriminator of these taxa.

Wing shape

Generalised Procrustes superimposition produced a new set of co-ordinate data for each of the individuals used in the study. Multiple regression of wing shape on centroid size revealed a significant association ($P<0.0001$), which is perhaps not unexpected considering the degree of variation in wing sizes among the specimens sampled (see above). However, despite the statistically significant association observed, wing size was predicted to account for only 5.8% of total shape variation. Therefore, despite the significance of the association, its influence is considered relatively minor.

Canonical variates analysis on the Procrustes transformed data-set subsequently resulted in ten significant ($\alpha=0.05$) canonical variates (CVs) (table 3), of which the first three explained 71.1% of the variation (CV1 Wilks' $\lambda=0.001$, $\chi^2=1552.726$, $P<0.001$; CV2 Wilks' $\lambda=0.005$, $\chi^2=1207.279$, $P<0.001$; CV3 Wilks' $\lambda=0.019$, $\chi^2=882.518$, $P<0.001$). For each of the ten canonical variates, functions at group centroids were also generated (table 4), the first three of which are graphically represented for each of the 13 sample groups (fig. 3). Based on the graphical presentation of the first three canonical variates (i.e. fig. 3), samples cluster into species groups with the exception of *B. carambolae* 'TN Malaysia', which falls distant from the remaining *B. carambolae* samples.

Success rate for the reassignment of individuals from any one sample to their *a priori* defined species ranged from 81% (*B. papayae*) to 100% for *B. tryoni* (table 5). Note that the number of *B. carambolae* used for reassignment was reduced to 36 due to the exclusion of *B. carambolae* samples from 'TN Malaysia'. This sample group was removed from analysis after the initial canonical variates as *post hoc* morphological examination of the specimens by this paper's authors and R.A.I. Drew (one of the authors of the species *B. carambolae*) confirmed that this group of specimens likely represents a new species other than *B. carambolae*, therefore indicating a misclassification when the samples were originally identified.

Discussion

Variation in wing size

Wing centroid size for each of the *B. dorsalis s.l.* populations studied revealed that some species (i.e. *B. dorsalis s.s.* and *B. carambolae*) contained one group significantly different in size from the remaining sample groups (fig. 2). Importantly, wing centroid size does not effectively discriminate one species from the other as there is no consistent significant difference in wing size between the species studied here (table 2).

Body size in tephritids is considered to be heavily influenced by environmental variables such as temperature and larval food source (Hooper, 1978; Krainacker *et al.*, 1987). As a result, it is not surprising to record variation among samples within species. Such variation in intraspecific sizes between populations may explain the difference in wing sizes observed between groups of *B. dorsalis s.s.* seen in this study, particularly as each of the groups have been collected from geographically isolated, and presumably environmentally different, locations (i.e. Thailand, Taiwan and India). In contrast, wing centroid size among sample groups of both *B. papayae* and *B. philippinensis* was not significantly different. The collection localities for each of these species were, relative to those of *B. dorsalis s.s.*, geographically close, especially for the latter species (table 1). Each sample was, therefore, likely to have been exposed to similar environmental conditions, possibly resulting in adults of similar size.

The situation of the sample of *B. carambolae* from 'TN Malaysia' is different. This group of flies has wings significantly larger than either of the other two samples of *B. carambolae* studied (fig. 2), including the group from Kuala Kangsar, which is, similarly to 'TN Malaysia', located in Peninsular Malaysia. Consequently, unlike the situation of *B. dorsalis s.s.*, for which each sample group was collected from geographically isolated locations, it is less likely that such variation in wing size between two sample groups from Peninsular Malaysia can be explained due to different environmental conditions experienced during immature development. As stated previously, the difference in size between the 'TN Malaysia' sample and the remaining *B. carambolae* is likely due to this group of specimens representing a different species to *B. carambolae*, as determined following *post hoc* morphological examination (R.A.I. Drew, Griffith University, personal communications). This

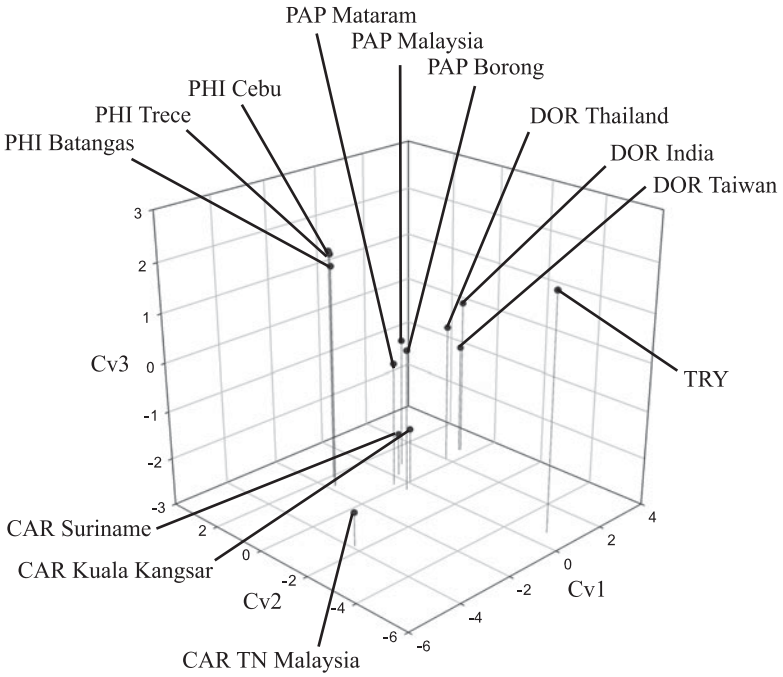

Fig. 3. Plot of function centroids of the first three canonical variates produced from CVA on Procrustes coordinate data based on 15 wing landmarks from all individuals sampled for *B. philippinensis*, *B. papayae*, *B. dorsalis s.s.*, *B. carambolae* and *B. tryoni*.

conclusion is further supported by our results based on wing shape data (fig. 3). The reason for the original misidentification remains unclear; however, individuals from this sample superficially resemble *B. carambolae*, particularly with regard to the expanded costal band on the wing. However, the 'TN Malaysia' specimens possess a costal band that is much broader and extends well beyond vein R_{2+3} and almost to vein R_{4+5} (not characteristic of *B. carambolae* for which the band only slightly overlaps vein R_{2+3} (Drew & Hancock, 1994)).

We conclude that while wing centroid size may reveal differences between populations of any given species (or indeed reveal a misdiagnosed population), it lacks utility as a diagnostic tool for effectively discriminating between the four morphologically cryptic species studied here.

Variation in wing shape

While wing size data may have limited utility in separating the four pest species of the complex, wing shape data appears to have potential for discriminating between *B. dorsalis s.s.*, *B. papayae*, *B. carambolae* and *B. philippinensis*. This is demonstrated by the close proximity of group function centroids between conspecific populations (fig. 3) and the relatively high success rate for the correct reassignment of individuals to their *a priori* species (table 5). Importantly, this intraspecific conservation in wing-shape remains despite large geographic distances between some of the populations sampled, e.g. *B. dorsalis s.s.* is represented by samples from Taiwan, northern Thailand and India (a distance of over 4500 km between the extremes). Additionally, the high similarity in wing shape between the Malaysian and South American populations of *B. carambolae* (fig. 3 and table 5) further supports a view that wing shape is highly conserved within species regardless of the geographic proximity of regional populations. An important aspect to bear in mind is, however, the allometric effect of wing size on shape. As our results reveal the relatively minor overall influence of wing size on shape (5.8%), this association is statistically significant and should be considered in future studies.

Table 5. Reassignment of *Bactrocera* individuals to their a *priori* defined species, based on Mahalanobis distances to group centroids following canonical variates analysis on Procrustes transformed wing landmark data.

Species		**1**	**2**	**3**	**4**	**5**	*N*	% correct
B. dorsalis	**1**	**54**	1	3	0	1	59	**92**
B. carambolae	**2**	1	**33**	2	0	0	36	**92**
B. papayae	**3**	6	3	**46**	2	0	57	**81**
B. philippinensis	**4**	0	0	3	**51**	0	54	**94**
B. tryoni	**5**	0	0	0	0	**20**	20	**100**

Rows, *a priori* groupings; columns, number of individuals reassigned to *a priori* group. % correct calculated based on the number of individuals reassigned to their *a priori* species group. *N*, number of individuals.

While emerging as a potentially effective species level diagnostic tool, shape analysis also reinforces the very close morphological similarity between *B. papayae* to both *B. dorsalis* and *B. carambolae*. Of the 11 *B. papayae* individuals assigned to another species, six were reassigned as *B. dorsalis* and three to *B. carambolae* (the remaining two specimens placed with *B. philippinensis*) (see table 5). Such close affinity among these species with respect to shape data may reflect the close biological relationship between these taxa which may support them being considered conspecific; this is impossible to confirm, however, without further supportive biological information. Nevertheless, observations have been made previously regarding various levels of successful interspecific mating among these species under field-cage conditions

(McInnis *et al.*, 1999); and, therefore, their biological species limits remain unresolved.

Mechanisms driving wing shape variation for *B. dorsalis* species are unknown. Wing shape may play a functional role in the mating systems of *B. dorsalis* complex flies, as it does for species of the neotropical tephritid genus *Blepharoneura*, for which wing shape may influence audible signals produced during courtship (Marsteller *et al.*, 2009). Further studies into the mating systems of *B. dorsalis* complex flies are required to determine if wing shape plays a role in producing different sounds among species and, more importantly, if sound is used as a key component in their specific mate recognition systems (*sensu* Paterson, 1985).

The use of wing shape information is not, in isolation, an argument to confirm or refute species limits but rather one line of evidence to be used with other data sets (e.g. mating data and genetic studies). Shape variation has been documented for intraspecific systems, such as for *Glossina palpalis gambiensis* Vanderplank (Diptera: Glossinidae) populations occurring over geographical gradients in Africa (Bouyer *et al.*, 2007), conspecific strains of *B. tryoni* experiencing different environmental conditions during development (e.g. laboratory versus wild) (Gilchrist & Crisafulli, 2006) and for other cryptic complexes within the Tephritidae (Kitthawee & Dujardin, 2010). Therefore, fine-scale shape variation between populations of *B. dorsalis s.l.* may simply be evidence of divergences occurring during the early stages of evolutionary radiation, at a stage which is concomitant with inter-population, rather than interspecific, differences. Such differences may be unresolvable using techniques such as molecular analysis, hence the current lack of molecular markers to discriminate between these species (with the exception of *B. carambolae*). Indeed, the high degree of intraspecific and overlapping morphological variation among the species studied here, combined with pheromone and cross-mating data suggesting hybridisation between some of these species (McInnis *et al.*, 1999; Wee & Tan, 2005), further emphasises the possibility that these taxa represent species in the early stages of divergence rather than distinct and reproductively isolated biological species.

In conclusion, geometric morphometric analyses demonstrate a capacity to resolve fine-scale differences in the *B. dorsalis* complex. Shape analysis using these techniques provides a promising opportunity to quickly and relatively easily identify morphologically cryptic pest species of the complex and has the potential for use as a rapid identification tool given a broader, more comprehensive dataset. More importantly, however, further behavioural and ecological research is required to confirm or refute whether the currently defined taxa studied here represent sound biological species, following which more reliable identification tools can be developed.

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