Bridging the morphological and biological species concepts: studies on the *Bactrocera dorsalis* (Hendel) complex (Diptera: Tephritidae: Dacinae) in South-east Asia

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Defining species accurately is a critical need in fundamental disciplines such as ecology and evolutionary biology and in applied arenas such as pest management. The validity of species designations depends on agreement of different methods of species diagnosis for unique biological species. The *Bactrocera dorsalis* complex of fruit flies provide an excellent opportunity for such a test of the congruence of different techniques (e.g. morphological, molecular, host-plant based, chemotaxonomy) used for species diagnosis. The complex contains a large number of closely-related species, is distributed over a wide geographical range in South-east Asia and considerable information has been compiled on some species. In the present study, the morphological and biological species boundaries were compared using new data from morphometric analyses of reproductive and body parts, together with a review of data on morphology, chemistry of male pheromones that are important in courtship and mating, molecular analyses, and endemic rainforest host plants. For the populations studied (*Bactrocera carambolae*, *Bactrocera dorsalis*, *Bactrocera occipitalis*, *Bactrocera papayae*, *Bactrocera philippinensis*, *Bactrocera kandiensis* and *Bactrocera invadens*) there appears to be significant congruence between the morphological and biological species boundaries. © 2008 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2008, 93, 217–226.

ADDITIONAL KEYWORDS: biology – genetic boundaries – molecular – morphology.

INTRODUCTION

The concept that species are the basic unit of evolution, each with its own unique genetic makeup, is widely accepted amongst evolutionary biologists (Carson, 1957; Paterson, 1993; Drew, 2004; Balakrishnan, 2005). The accurate identification and description of biological species is vital and morphological taxonomists must continue to use diagnostic systems that elucidate, or are in agreement with, genetic boundaries (Balakrishnan, 2005). Although molecular methods such as DNA barcoding may assist in species resolution (Tautz *et al*., 2003), their value needs to be assessed in light of what constitutes a species (Fitzhugh, 2006). Furthermore, for practical purposes of ongoing species identification, particularly for fauna in the tropics, identification keys using nonmolecular data are still vital (Balakrishnan, 2005). The challenge therefore is to base these identification systems on morphological criteria that, in turn, are based on or reflect the true genetic boundaries of species populations.

The tropical fruit flies (Tephritidae: Dacinae) are an example of a group of insects that have speciated prolifically throughout the tropics and subtropics.
(Clarke et al., 2005), especially in South-east Asia, where groups of sibling species have been identified (Drew, 1989; Drew & Hancock, 1994). The detailed study of the Bactrocera dorsalis complex by Drew & Hancock (1994) has led to considerable debate over species and a number of published works aimed at defining the limits of some species populations (Armstrong & Cameron, 2000; Nakahara et al., 2000; Muraji & Nakahara, 2001, 2002; Clarke et al., 2005).

To date, the morphological techniques used by Drew & Hancock (1994), when combined with male pheromone chemistry and endemic rainforest host plant records, has provided sound evidence for the status of species within the dorsalis complex (Clarke et al., 2005). Likewise, several molecular techniques have also confirmed the species status of some taxa in this complex (Clarke et al., 2005). There is, however, a need to continue research on this complex to provide validity or otherwise for all species in the complex, for both economic reasons and for refining the systematics of the Subfamily Dacinae. In the present study, we analyse morphological and biological species boundaries for some species in the dorsalis complex to assess concordance between them. We review molecular, male pheromone chemistry, and endemic host plant data and present new data on the morphometrics of male and female characters, to improve the resolution of species boundaries.

### Material and Methods

**Morphometric Measurements**

Comprehensive morphological comparisons were made by Drew & Hancock (1994) for all known species in the dorsalis complex. In the present study, we focus on the seven species that are most difficult to distinguish from each other on external adult morphology, Bactrocera carambolae Drew & Hancock, Bactrocera dorsalis (Hendel), Bactrocera kandiensis Drew & Hancock, Bactrocera occipitalis (Bezzi), Bactrocera papayae Drew & Hancock, Bactrocera philippinensis Drew & Hancock, and Bactrocera invadens Drew, Tsuruta & White. Bactrocera carambolae and B. papayae are sympatric throughout most of their geographical range as are B. occipitalis and B. philippinensis. Where possible, specimens were collected at the type localities of species, reared from known major host fruits and obtained from male lure traps (Table 1). Specimens of B. carambolae, B. papayae, and B. philippinensis are distributed more widely than the other four species and thus were collected from different allopatric populations to measure intraspecific geographical variation.

The following characters were measured: length of thorax including scutellum (in dorsal view), lengths of fore and hind femora and fore and hind tibiae, lengths of wing and vein CuA1, width of medial longitudinal...
dark band on abdominal tergum IV and lengths of male aedeagus and female aculeus. The nonreproductive characters were chosen on the basis that they do not change after death of the insect and thus can be used in ratio analyses to counter any possible changes in the reproductive parts with size of the individual flies. The reproductive characters, male aedeagus and female ovipositor, were chosen for the morphometric analyses because they are related to biological species boundaries. The lengths of the female aculeus and male aedeagus were used previously to separate some species (Iwaizumi, Kaneda & Iwahashi, 1997; White & Hancock, 1997; Iwahashi, 1999a, b; Iwahashi, 2000) within the dorsalis complex. However, some of these studies suffered from methodological limitations of low sample sizes, primary use of material from laboratory-reared colonies and exclusive use of male lure based sampling.

Aedeagus measurements were taken on species 1–7 in Table 1 and aculeus measurements for species 1, 2, 4 and 5. The aedeagus measurements are the length of the long coiled stem, not including the glans. All specimens were assigned a unique label, softened in 10% KOH, washed, dissected under water, and preserved individually in 70% ethyl alcohol for future reference, in either the Griffith University or Queensland Department of Primary Industries insect collections.

We sought answers to the following questions: (1) Can aedeagus length be used to differentiate species, based on measurements of the seven species? (2) Can measurements of certain external body parts be used to differentiate species, based on data from seven species? (3) Can species be differentiated on ratios of aedeagus length to certain external body part measurements, based on data from seven species? (4) Does intraspecific geographical variation in aedeagus length occur, based on a study of allopatric populations of four species? (5) Does individual fly size influence aedeagus length, based on a study of three ratios of aedeagus length to certain body part measurements, for four species? (6) Does sexual dimorphism occur in external body part measurements, based on data on seven characters from four species? (7) Is there a correlation between aedeagus length and aculeus length within species, based on data from four species?

**RESULTS**

**Morphometric measurements**

External morphological characters on the Costal band, femora and abdominal terga III–V that separate all seven species, have been identified (Table 2). However, intraspecific variation in these characters in B. carambolae, B. dorsalis, B. papayae, and B. philippinensis causes difficulties in successful diagnosis, particularly in males.

The four most difficult species to separate morphologically, B. carambolae, B. dorsalis, B. papayae, and B. philippinensis are all significantly different in aedeagus length (Fig. 1A). Likewise, B. invadens, recently described from Sri Lanka and Africa, is significantly different from these four species. This interpretation does not change appreciably when aedeagus length is controlled for fly size, by analysing the ratios of aedeagus length to length of thorax, hind tibiae, and wing vein CuA1 (Fig. 1B, C, D). Given that the results based on aedeagus length and the ratios of aedeagus length to body parts, are similar, it appears that the aedeagus length does not change with fly size intraspecifically.

In the comparison of species on body size, there was no significant difference between them in thorax length, with B. carambolae (mean = 2.91 mm), B. dorsalis (2.93 mm), B. papayae (2.95 mm), B. occipitalis (3.06 mm), B. kandiensis (3.10 mm), B. invadens (3.10 mm), and B. philippinensis (3.14 mm). Similarly, there were no significant differences in wing length with B. carambolae (mean = 5.75 mm), B. papayae (5.86 mm), B. dorsalis (6.00 mm), B. occipitalis (6.05 mm), B. philippinensis (5.93 mm), B. kandiensis (6.18 mm), and B. invadens (6.21 mm); wing vein length with B. carambolae (mean = 2.09 mm), B. papayae (2.12 mm), B. dorsalis (2.18 mm), B. occipitalis (2.21 mm), B. invadens (2.25 mm), B. kandiensis (2.25 mm), and B. philippinensis (2.18 mm); and hind tibia length with B. carambolae (mean = 1.74 mm), B. papayae (1.77 mm), B. dorsalis (1.76 mm), B. occipitalis (1.83 mm), B. invadens (1.85 mm), B. kandiensis (1.86 mm), and B. philippinensis (1.84 mm).

With respect to fore femur length, B. carambolae (mean = 1.51 mm) and B. philippinensis (1.61 mm) were significantly different. However, B. carambolae and B. philippinensis, considered separately, were not significantly different from B. dorsalis (1.52 mm), B. papayae (1.53 mm), B. occipitalis (1.57 mm), B. invadens (1.6 mm), and B. kandiensis (1.63 mm). With respect to hind femur length, B. carambolae (mean = 2.03 mm) and B. kandiensis (2.26 mm) were significantly different. However, similar to the fore femora measurements, B. carambolae and B. kandiensis, individually, were not significantly different.

**Statistical analyses**

Data were analysed using analyses of variance with species or location as factors and the various morphometric measures and ratios as dependent variables. All analyses were performed using SAS, version 9.0 (SAS Institute) or SPSS, version 14.0 (SPS Inc.). Data are presented as means within the text (+95% confidence intervals in the figures).
from B. papayae (2.06 mm), B. dorsalis (2.07 mm), B. occipitalis (2.11 mm), B. philippinensis (2.18 mm) and B. invadens (2.24 mm) and, with respect to fore tibia length, B. kandiensis (mean = 1.39 mm) and B. philippinensis (1.42 mm) were significantly different from B. carambolae (1.29 mm), B. papayae (1.30 mm), B. dorsalis (1.30 mm), B. invadens (1.37 mm), and B. occipitalis (1.37 mm), which were not significantly different from each other.

**Table 2.** Morphological character differences between seven sibling species of the dorsalis complex

| Species                                    | Costal band                          | Legs (femora)                           | Abdominal terga III-V |
|--------------------------------------------|--------------------------------------|----------------------------------------|-----------------------|
| Bactrocera carambolae (Drew and Hancock)   | Overlapping R_2,3 especially before apex of this vein, and widening across apex of R_{4,5} | Femora entirely fulvous, with a subapical dark spot on outer surfaces of fore femora, usually in females | Medial longitudinal black band of medium width; anterolateral corners on tergum IV large and rectangular in shape |
| Bactrocera dorsalis (Hendel)               | Confluent with R_{2,3} and remaining narrow and of uniform width to apex of wing (occasionally with a slight swelling around apex of R_{4,5}) | Femora entirely fulvous | Medial longitudinal dark band narrow; anterolateral corners of tergum IV small and triangular |
| Bactrocera invadens (Drew, Tsuruta and White) | Confluent with R_{2,3} and remaining narrow to apex of wing | Femora entirely fulvous | Medial longitudinal dark band narrow to medium width; lateral dark margins on terga IV and V narrow; tergum III may be entirely dark fuscous to black |
| Bactrocera kandiensis (Drew and Hancock)   | Confluent with R_{2,3} and remaining narrow and of uniform width to apex of wing | Femora fulvous with large dark fuscous spots preapically on fore- and mid-femora and around apices of hind femora. | Medial longitudinal dark band very narrow; anterolateral corners of tergum IV very small and triangular |
| Bactrocera occipitalis (Bezzi)             | Overlapping R_{2,3} and widening markedly across apex of wing | Femora entirely fulvous | Medial longitudinal dark band broad; lateral dark margins of all three terga broad but usually paler on posterolateral areas of tergum IV |
| Bactrocera papayae (Drew and Hancock)     | Confluent with R_{2,3} and remaining narrow and of uniform width to apex of wing (occasionally with a slight swelling around apex of R_{4,5}) | Femora entirely fulvous | Medial longitudinal dark band narrow; anterolateral corners of tergum IV small and triangular |
| Bactrocera philippinensis (Drew and Hancock) | Confluent with or just overlapping R_{2,3} and usually expanding into a fish-hook barb pattern around apex of R_{4,5} (occasionally of uniform width to apex of wing) | Femora entirely fulvous | Medial longitudinal dark band narrow to medium width; anterolateral corners of tergum IV small and triangular |

**ASSESSMENT OF INTRASPECIFIC GEOGRAPHICAL VARIATION IN AEDEAGUS LENGTH**

All populations of B. papayae were within the limits expected for that species (2.54–3.35 mm) (Iwahashi, 1999b; R. A. I. Drew unpubl. data). However, the Malaysian population of this species was significantly different from those from Australia, Bali, and Papua New Guinea (Fig. 2). Populations from Australia,
Bali, Papua New Guinea, and Christmas Island were not significantly different from each other and, likewise, the populations from Malaysia and Christmas Island were not significantly different. For *B. carambolae* from Bali and Malaysia, there was no statistical difference in aedeagus length between the populations whereas, for *B. philippinensis*, the mean value of aedeagus length for the Philippines (3.135 mm) was significantly different from that for the specimens from Palau (3.197 mm), although both values were within the known range of measurements for this species in the Philippines (3.12–3.39 mm) (Iwahashi, 1999a; R. A. I. Drew unpubl. data).

**ASSESSMENT OF SEXUAL DIMORPHISM IN MORPHOMETRIC MEASUREMENTS**

Females of *B. dorsalis* were significantly larger than males in the lengths of the wing vein CuA₁, whereas there were no such differences between the sexes in the other measurements (Fig. 3). For *B. philippinensis*, females were significantly smaller than males in the lengths of the fore femur and fore tibia and significantly larger than the males in the length of the wing vein CuA₁, whereas there were no differences in the other measurements. *Bactrocera carambolae* females were significantly larger than the males in all seven measurements, and *B. papayae* females were significantly larger than the males in the hind femur and wing vein CuA₁.

Across all four species studied, the female was significantly larger than the male in wing vein CuA₁. Other differences were erratic in occurrence except for *B. carambolae*, in which the females were larger than the males in all traits. The mean aedeagus length and mean aculeus length for the four species, where both sexes were available for study, were strongly correlated (*r* = 0.967; *P* = 0.033; Fig. 3).

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DISCUSSION

A view or concept of species has direct influence on our understanding of the genetics of species which, in turn, influences our understanding of species diversity (Paterson, 1989). Although, in taxonomy, we define species primarily on morphological characters, it is becoming increasingly evident that we must understand and elucidate the genetic and behavioural boundaries of species. This is particularly important in groups of economically important species such as those in the *B. dorsalis* complex.

In the review of the *dorsalis* complex (Drew & Hancock, 1994), some species were defined on minor morphological differences in external body characters, combined with some supporting biological evidence. This has led to considerable debate regarding the validity of some species, particularly ones of economic significance. Consequently, in the present study, we have reviewed these difficult-to-define species of Drew & Hancock (1994) in order to test their species status. A summary of the comparisons made on new and existing data on morphology, molecular analyses, chemistry of male pheromones, endemic rainforest host plants, and morphometric analyses of the male aedeagus and female aculeus lengths, is provided in Table 3 for the five species that cause most confusion.

Within the Dacinae, the morphological characters used to define species are almost entirely based on colour patterns. Within the *dorsalis* complex, *B. carambolae, B. dorsalis, B. occipitalis, B. papayae,* and *B. philippinensis* are separated from each other on a combination of shape and size of the lateral postural yellow vittae, width and shape of the costal band on the wing, colour of the femora (legs), and shape and size of the dark colour patterns on abdominal terga III–V (Table 2). *Bactrocera invadens* is more distinct in possessing a mostly red–brown scutum and *B. kandiensis* in having large dark fuscous to black markings on the apices of all femora. On external morphological characters, *B. carambolae* and *B. occipitalis* are clearly different from each other and the other three species (*i.e. B. dorsalis, B. papayae,* and *B. philippinensis;* Table 3). There are some small but consistent differences between the latter three species but it is difficult to attribute specific status based on these characters alone. In a laboratory and field cage study of *B. dorsalis* (introduced from Hawaii) and *B. papayae* field collected in Malaysia, Tan (2003) recorded hybridization resulting in the production of morphological intermediate forms. However, such hybridization between *Bactrocera* species is easy to achieve in laboratory cages, even with species in different subgenera (Cruickshank, Jessup & Cruickshank, 2001). In eastern Australia, apparent field hybrids of *Bactrocera tryoni* (Froggatt) and *Bactrocera neohumeralis* (Hardy) based on the occurrence of morphological intermediates within sympatric populations have been shown, through microsatellite analyses, to be confined primarily to one species, *B. tryoni,* and that hybridization between these species is a rare event (Gilchrist & Ling, 2006). As shown by our results, natural geographical variations in the external morphological characters occur in these species but these variations cannot be presumed to be the result of hybridization as Tan (2003) concluded.

![Figure 2. Comparison of aedeagus length of *Bactrocera papayae* between Australia (North Queensland), Christmas Island, Bali, Malaysia, and Papua New Guinea; *Bactrocera carambolae* between Bali and Malaysia and *Bactrocera philippinensis* between Palau and the Philippines. Bars (means + 95% confidence intervals (CI)) with the same letter within species are not statistically different, as indicated by a Tukey’s HSD test.](image-url)
Molecular evidence supports the specific status of five species (Clarke et al., 2005) and diagnostic markers are available to distinguish between them (Armstrong, Cameron & Frampton, 1997). Naeole & Haymer (2003) developed molecular markers based on oligonucleotide arrays for *B. dorsalis*, *B. carambolae*, and *B. papayae* whereas Muraji & Nakahara (2001) found nucleotide sequence differences be-

Table 3. Summary of differences, between five *dorsalis* complex species, based on external morphology, DNA, male pheromones, endemic host plants and morphometric analyses of male aedeagus length

|                      | *Bactrocera carambolae* | *Bactrocera dorsalis* | *Bactrocera occipitalis* | *Bactrocera papayae* | *Bactrocera philippinensis* |
|----------------------|-------------------------|-----------------------|--------------------------|----------------------|-----------------------------|
| *Bactrocera carambolae* | X                       | A, B, C, E            | A, B, C, D, E            | A, B, C, E           |                             |
| *Bactrocera dorsalis*  | X                       | A, B                  | B, E                     | B, E                 |                             |
| *Bactrocera occipitalis* | X                       | A, B, E               | A, B, E                  | A, B, E              |                             |
| *Bactrocera papayae*   | X                       | X                     | X                        | B, E                 |                             |
| *Bactrocera philippinensis* | X                      | X                     | X                        | X                    |                             |

A, morphology (external); B, molecular (mitochondrial DNA d-loop + 12S & 16S); C, male pheromones; D, host plants (data for *carambolae* and *papayae* only); E, morphometric (aedeagus length).

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between B. carambolae, B. dorsalis, and B. philippinensis. Armstrong et al. (1997) provided strong evidence for the application of molecular data for the determination of Bactrocera species. Isozyme studies by Yong (1994b, 1995) on B. dorsalis (from Hawaii), and B. carambolae and B. papayae from Malaysia were able to distinguish B. carambolae on the hydroxybutyrate dehydrogenase enzyme and indicated that B. dorsalis and B. papayae had a close genetic affinity. Although genetic similarities between allopatric populations of B. dorsalis and B. papayae have been documented (Tan, 2003), this does not cast doubt on their species status. The validity of species can only be tested using molecular data by evaluating similarities and differences between species in allopatry and sympatry. In particular, only fixed differences in sympatry can provide sound confirmation of species.

Male pheromones are extremely important mate recognition systems in courtship and mating behaviour within Bactrocera species (Drew, 2004). The release of a volatile pheromone by males at mating time (generally dusk) is used to attract conspecific females. Consequently, we believe that differences in the chemical composition of the male pheromones can be used to define species. In a comprehensive review of pheromone chemistry studies on the dorsalis complex, Fletcher & Kitching (1995) showed that B. carambolae was markedly different from B. dorsalis, B. papayae, and B. philippinensis (specimens of B. occipitalis were not available in their study) and that ‘B. papayae, B. philippinensis and B. dorsalis possess minor consistent differences’ and concluded that ‘the nature of the rectal glandular components may be a powerful taxonomic criterion’. Four of the species could be separated (Table 3).

Host plants in the endemic rainforest habitat of tropical Dacinae have long been recognized as important to the maintenance of the fruit fly species gene pool (Drew, 2004). Many Bactrocera species utilize one or a few specific plant species within only one plant family. Where B. carambolae and B. papayae occur in sympatry, significant differences have been found in their utilization of rainforest host plants in this study and by Yong (1994a) (similar extensive rainforest fruit records for B. dorsalis, B. invadens, B. kandiensis, B. occipitalis, and B. philippinensis have never been obtained). Basically, B. papayae utilizes 21 host plant families that are not used by B. carambolae whereas B. carambolae utilizes only four families not used by B. papayae (Allwood et al., 1999). In addition, B. papayae breeds more prolifically in the rainforest host fruits that have been recorded than does B. carambolae resulting in a greater abundance of the former in rainforest ecosystems whereas the latter is more prevalent in disturbed habitats (Clarke et al., 2001). Given that the host plants are utilized by many Bactrocera species for courtship and mating and specific larval food sites, differences in their host plant records can be used to, at least, infer specific status. On this basis, B. carambolae and B. papayae appear to be valid species (Table 3).

The morphometric studies undertaken in the present study were conducted on large sample numbers collected in the type localities of the species, attracted to male lure (methyl eugenol) and, where possible, reared from host fruits. The length of the male aedeagus was significantly different for all five of the most difficult-to-identify species (i.e. B. carambolae, B. dorsalis, B. occipitalis, B. papayae, and B. philippinensis), except for B. dorsalis compared with B. occipitalis. There is a strong correlation between the length of the male aedeagus and female aculeus but no significant intraspecific variation with fly size or geographical distribution (except minor intraspecific variation with geographical distribution in B. papayae and B. philippinensis). If we were to accept Paterson’s (1985) definition of a species being ‘that most inclusive population of . . . organisms which share a common fertilization system’, these characters appear to be sound ones to define species. This is in agreement with Iwahashi (1999a) on studies of B. occipitalis and B. philippinensis and Iwahashi (1999b) on B. carambolae and B. papayae.

With sibling species complexes, there appears to be no substitute, at present, for defining species boundaries based on tests for congruence between morphological, molecular and biological data sets. A recent example of this approach is the work of Schiffer, Carew & Hoffman (2004) on cryptic species of Drosophila. The Bactrocera dorsalis complex of species appear to be evolving rapidly (Clarke et al., 2005) and this may partly explain variations in their morphological characters. In the present study, we compared five species of this complex based on morphological and biological criteria, particularly those that have significance in maintaining the ‘field for gene recombination’ (sensu Carson, 1957) in the natural environment. The agreement between external morphology, morphometrics, molecular analyses, pheromone chemistry, and endemic host range indicates that it is reasonable to assume that the current specific status of the B. dorsalis complex species investigated is valid.

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