Mating Compatibility Among Four Pest Members of the *Bactrocera dorsalis* Fruit Fly Species Complex (Diptera: Tephritidae)

M. K. SCHUTZE,[1,2,3] A. JESSUP,[4,5] I. UL-HAQ,[4] M.J.B. VREYSEN,[4] V. WORNOAYPORN,[4] M. T. VERA,[6,7] AND A. R. CLARKE[1,2]

J. Econ. Entomol. 106(2): 695–707 (2013); DOI: http://dx.doi.org/10.1603/EC12409

ABSTRACT *Bactrocera dorsalis* (Hendel), *Bactrocera papayae* Drew & Hancock, *Bactrocera philippinensis* Drew & Hancock, and *Bactrocera carambolae* Drew & Hancock are pest members within the *B. dorsalis* species complex of tropical fruit flies. The species status of these taxa is unclear and this confounds quarantine, pest management, and general research. Mating studies carried out under uniform experimental conditions are required as part of resolving their species limits. These four taxa were collected from the wild and established as laboratory cultures for which we subsequently determined levels of prezygotic compatibility, assessed by field cage mating trials for all pair-wise combinations. We demonstrate random mating among all pair-wise combinations involving *B. dorsalis*, *B. papayae*, and *B. philippinensis*. *B. carambolae* was relatively incompatible with each of these species as evidenced by nonrandom mating for all crosses. Reasons for incompatibility involving *B. carambolae* remain unclear; however, we observed differences in the location of couples in the field cage for some comparisons. Alongside other factors such as pheromone composition or other courtship signals, this may lead to reduced interspecific mating compatibility with *B. carambolae*. These data add to evidence that *B. dorsalis*, *B. papayae*, and *B. philippinensis* represent the same biological species, while *B. carambolae* remains sufficiently different to maintain its current taxonomic identity. This poses significant implications for this group's systematics, impacting on pest management, and international trade.

KEY WORDS Oriental fruit fly, prezygotic compatibility, sexual isolation

Tropical fruit flies of the genus *Bactrocera* Macquart (Diptera: Tephritidae) are distributed principally in Asia and the Pacific (Drew and Hancock 2000). The taxonomy of this large genus is complex, including ≈500 species placed in different subgenera, within which a number of species complexes exist (Drew 1989). Some of these complexes, such as the *B. dorsalis* complex (Drew and Hancock 1994), the *Bactrocera tau* complex (Drew and Romig 1997), the *Bactrocera musae* complex (Drew et al. 2011), and the *Bactrocera tryoni* complex (Clarke et al. 2011) contain important pest species. Of these, the *Bactrocera dorsalis* complex is regarded as the most economically important and arguably the most taxonomically challenging (Clarke et al. 2005). Members of this complex include the Oriental fruit fly *Bactrocera dorsalis* sensu stricto (s.s.) (Hendel) (hereafter referred to as *B. dorsalis*), the Asian papaya fruit fly *Bactrocera papayae* Drew & Hancock, the Philippine fruit fly *Bactrocera philippinensis* Drew & Hancock, the carambola fruit fly *Bactrocera carambolae* Drew & Hancock, and the invasive fruit fly *Bactrocera invadens* Drew, Tsuruta & White. These species are widely acknowledged as either serious agricultural pests where they occur, or as high-level quarantine threats in countries where they are absent but capable of invasion and establishment (White and Elson–Harris 1992, Clarke et al. 2005). Indeed *B. dorsalis*, *B. carambolae*, and *B. invadens* have invaded and become established in the Pacific (Vargas et al. 2007), South America (Sauers–Muller 1991), and Africa (Drew et al. 2005), respectively; and predictive modeling of the geographic distribution of both *B. dorsalis* and *B. invadens* based on climatic tolerance suggests that these pests have the potential to invade Mediterranean Europe and south–eastern North America (Stephens et al. 2007, Meyer et al. 2010).

The *B. dorsalis* species complex was formally revised in the late 1960s to consist of 16 species (Hardy 1969), however, subsequent revisions have expanded the complex to now include over 70 taxa (Drew 1989, Drew and Hancock 1994, Clarke et al. 2005). These taxonomic revisions were based predominantly on

[1] CRC for National Plant Biosecurity, LPO Box 5012, Bruce, A.C.T. 2617, Australia.
[2] School of Earth, Environmental and Biological Sciences, Queensland University of Technology, G.P.O. Box 2434, Brisbane 4000, Queensland, Australia.
[3] Corresponding author, e-mail: m.schutze@qut.edu.au.
[4] Insect Pest Control Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna, Austria.
[5] NSW Department of Primary Industries, Locked Bag 26, Gosford NSW 2250, Australia.
[6] Cátedra de Terapéutica Vegetal, Departamento de Sanidad Vegetal de la Facultad de Agronomía y Zootecnia de la UNT, Tucumán, Argentina.
[7] CONICET, Crisostomo Alvarez 722, S. M. de Tucumán 4000, Tucumán, Argentina.

0022-0493/13/0695–0707$04.00/0

morphological characters; however, the characterization and analysis of allozyme data (Yong 1995), DNA information (Muraji and Nakahara 2002, Naeole and Haymer 2003, Armstrong and Ball 2005, Yu et al. 2005), morphometric characters (Iwaizumi et al. 1997; Adsavakulchai et al. 1998; Iwahashi 1999a,b,; Mahmood 1999; Iwahashi 2001; Drew et al. 2008), pheromone composition (Fletcher and Kitching 1995, Wee and Tan 2005, Tan et al. 2011), and behavior (McInnis et al. 1999; Wee and Tan 2000a,b; Tan 2003), have since been applied to either support or challenge the existing classification, particularly of *B. dorsalis*, *B. papayae*, *B. philippinensis*, *B. invadens*, and *B. carambolae.* The primary aims of such studies have been to resolve species boundaries and improve identification accuracy for quarantine and insect pest management of these most damaging and taxonomically challenging members of the complex.

Despite the large amount of work on these taxa, there remains ongoing debate regarding the true species status of these members of the *B. dorsalis* complex; that is, do they all represent real biological species, or are some of them taxonomic synonyms of a single biological entity (Clarke et al. 2005, Drew et al. 2008, Tan et al. 2011)? This debate has arisen because of problems in identifying consistent and unambiguous discriminatory taxonomic characters (morphological or otherwise) that are universally accepted by relevant authorities and workers in tephritid taxonomy, ecology, and management (see Clarke et al. (2005) for a review). The fundamental question therefore remains whether: 1) these are real biological species for which current diagnostic techniques are inadequate; or 2) the diagnostic tools are adequate and the complex has been 'split' too far, with two or more 'taxonomic species' representing a single biological species.

Notwithstanding the work of previous studies into delimiting species boundaries for members of the *B. dorsalis* complex, there has been a noticeable lack of comparative behavioral information; particularly with respect to work on pre- and postzygotic compatibility (e.g., mating compatibility and offspring viability, respectively). While the importance of such data to help resolve biological species boundaries is widely recognized (Walter 2003), the limited research in this field is principally because of logistical and regulatory difficulties with respect to establishing live colonies of all *B. dorsalis* complex pest species in a single location. Such difficulties arise because these taxa are generally allopatric to each other (only *B. papayae* and *B. carambolae* are sympatric) (Drew and Hancock 1994) and quarantine concerns limit opportunities to culture all species together for behavioral research. Consequently, what is known of interspecific mating compatibility among pest members of the complex comes from independent pair-wise studies undertaken by research groups using nonstandardized experimental conditions in different countries, such as for *B. dorsalis* versus *B. carambolae* in Suriname (McInnis et al. 1999), *B. papayae* versus *B. carambolae* (Wee and Tan 2000b) and *B. dorsalis* versus *B. papayae* in Malaysia (Tan 2000), and *B. dorsalis* versus *B. philippinensis* in the Philippines (Medina et al. 1998). These studies have demonstrated varying degrees of interspecific mating compatibility, from relative incompatibility for comparisons involving *B. carambolae*, to high levels of compatibility among *B. dorsalis*, *B. papayae*, and *B. philippinensis;* however, as comprehensive and accessible published data are available only from McInnis et al. (1999) and Wee and Tan (2000b), direct comparisons between all studies remains difficult. Therefore, a need remains for a cohesive quantification of interspecific mating comparisons among these economically important members of the complex.

The aim of the current study was to determine mating compatibility among each of the following four members of the *B. dorsalis* complex: *B. dorsalis*, *B. papayae*, *B. carambolae*, and *B. philippinensis.* While the taxonomic resolution of *B. invadens* is of equal importance, we have not included it in this study as a follow-up project is underway to specifically examine compatibility between African *B. invadens* and Asian *B. dorsalis* s.s. For each possible cross among the four taxa, prezygotic compatibility was assessed using standardized field cage mating tests for all species combinations. Flies were used as close to 'wild' as possible, which in practice was within five generations from the field.

Materials and Methods

Source Material. We evaluated four members of the *B. dorsalis* species complex: *B. dorsalis*, *B. papayae*, *B. carambolae*, and *B. philippinensis.* All except *B. philippinensis* were directly obtained as larvae or pupae from the wild and established as fresh laboratory cultures at the FAO-IAEA Insect Pest Control Laboratory, Seibersdorf Austria. *B. philippinensis* was collected from the field and maintained for one generation at the National Mango Research and Development Centre (Guimaras Island, Philippines) before F1 pupae were sent to Seibersdorf. No specific permits were required for the described field studies or for the importation of live material into the Seibersdorf laboratories. Collection locations were not privately owned and no endangered or protected species were involved in the study.

Because of the recognized difficulties in discriminating between these species, every effort was made to obtain each from an area where it is considered allopatric from the remaining three. The native distribution of *B. dorsalis* is from Pakistan to eastern China (including Taiwan) extending southwards to central/southern Thailand; *B. papayae* and *B. carambolae* are largely sympatric with each other in their native range in south–east Asia, occurring from southern Thailand through the Indo-Malay Archipelago (the species are allopatric in their invasive ranges, *B. papayae* in Papua New Guinea and *B. carambolae* in South America); and *B. philippinensis* is restricted to the Philippines. Therefore, we obtained *B. carambolae* from its allopatric population in Paramaribo, Suriname (ex *Averrhoa carambola* L.), arriving at Seibersdorf March 2010 as ≈1,000 pupae; *B. dorsalis* was collected from within its native range in Saraburi, Central Thai-

land (ex *Mangifera indica* L.), arriving March 2010 as ≈500 pupae; and *B. philippinensis* was obtained from Guimaras Island, Philippines (ex *Carica papaya* L.), arriving August 2010 as ≈500 pupae. *B. papayae* were collected in November 2010 from Serdang, Peninsular Malaysia, where this species occurs in sympatry with *B. carambolae;* however, to ensure we collected *B. papayae* and not *B. carambolae*, ≈400 wild flies were obtained from banana (*Musa acuminata x balbisiana* hybrids, varieties Mas, Berangan, and Lemak) that is a recorded host of *B. papayae* but not for *B. carambolae* (Clarke et al. 2001, Sauers–Muller 2005). Flies were morphologically examined for external and internal (e.g., genitalia) characters to confirm their identity in accordance with their taxonomic descriptions (Drew and Hancock 1994). In addition, Professor R.A.I. Drew, the co-authority who described *B. papayae*, *B. carambolae*, and *B. philippinensis*, also confirmed the identity of the cultures based on pinned material, diagnostic micrographs, and genitalia measurements. Representative voucher samples were preserved as dried (pinned) and wet (>95% alcohol) material at both the Seibersdorf facility and Queensland University of Technology, Brisbane Australia.

General Rearing Protocol. Emerged adult flies were provided a standard diet of enzymatic yeast hydrolysate and sugar (1:3) with water supplied ad libitum. Sexually mature flies were exposed to egg-cups dosed with commercial guava juice (Rubricon, Rubricon Products, Middlesex, United Kingdom) as an oviposition stimulant, or in some cases they were provided fruit (*M. indica*) for oviposition in cases where egg cups were inadequate at early stages of colony establishment (as was the case for *B. carambolae*). Collected eggs were incubated overnight (25 ± 2°C, 65% relative humidity [RH]) on moist filter paper placed on wet sponge in petri dishes and then transferred to carrot diet (Tanaka et al. 1970) for larval development (27–28°C, 55% RH). Pupae were collected into and sifted from moistened teak sawdust, and transferred to either experimental (20 cm diameter × 27 cm height) or colony cages (50 × 50 × 50 cm). The maximum amount of material possible was used to initiate each generation to minimize the loss of genetic variability.

Mating Compatibility Tests. Adult flies were sexed within 4 d of emergence that is well before sexual maturation (15–20 d for wild cultures) as based on unpublished data (M.K.S.; data not shown) and information from previous studies (McInnis et al. 1999, Wee and Tan 2000a). Flies were then maintained under low-stress conditions of 100–200 flies per cylindrical cage (20 cm diameter × 27 cm height). General procedures followed those outlined in the FAO/IAEA/USDA (2003) Manual for Product and Quality Control. For fly species identification, a small dot of colored water-based paint was applied to the scutum of each fly's thorax using a soft paint-brush at least 48 h before each field cage test (colors were randomized among tests). The mating tests were conducted using flies aged between 20–30 d to ensure 100% maturity (particularly necessary for later-maturing *B. carambolae*).

Field cage tests were conducted inside a glasshouse (maintained at ≈25°C and ≈50% RH, and exposed to natural light) that contained four partitioned flight cages (2.0 × 1.6 × 1.9 m), each housing a single, nonfruiting potted *Citrus sinensis* Osbeck (Rutaceae) tree of 2 m in height with a canopy of ≈ 1.1 m in diameter. Field cage tests were conducted between September 2010 and March 2011, with most compatibility tests undertaken between December and February (however, *B. dorsalis* vs. *B. carambolae* tests were undertaken in September and October 2010; and *B. carambolae* vs. *B. philippinensis* tests were undertaken in October and December 2010). We recognize that undertaking experiments over different months while under natural light conditions may influence the outcome of experiments because of seasonal fluctuation in ambient light conditions differentially affecting fly behavior; however, as most comparisons were conducted over a 2–3 mo period we do not believe this effect was significant. We feel confident in this as while two halves of some trials were conducted at two times of the year and 2 mo apart (e.g., *B. carambolae* vs. *B. philippinensis;* first four replicates conducted in October, the second four conducted in December), the results across all replicates in this instance were statistically homogeneous (see Results).

Flies were released into the experimental field cage at a 1:2 male:female sex ratio. The biased sex ratio sought to ameliorate the effect of differences in temporal variation at the onset of sexual activity from biasing measures of prezygotic compatibility. Preliminary trials (data not presented) showed that 'early starting males' monopolised 'early starting females' in cases of slight temporal differences between species at onset of mating, thereby depriving 'late-starting males' from accessing early starting females given a 1:1 sex ratio; this over-inflated isolation indices. With a female biased population there was always an excess of females for mating, and should a temporal difference exist late-starting males would still have access to at least as many early starting females. This temporal difference did not exist for all comparisons (see Results); however, we decided to undertake this protocol for all pair-wise tests to maintain consistency across tests. We considered an alternative approach to ameliorate the problem of early mating monopolization (i.e., replacing flies as couples were removed), yet this solution was decided against for the following reasons: the continual introduction of new flies into the arena may disturb existing flies; standard protocols state that males be introduced into the mating arena for a period of time before female release (therefore, newly introduced males are 'unequal' to established males and may behave differently); and it was not possible to identify a suitable release point for new flies. Importantly, temporal variation in sexual activity is recorded using this method and this information can be examined as a potential factor in cases of incompatibility.

For each replicate, 20 males of each of the two species under study were released into a field cage 1 to 2 hr before sunset (as the study species mate at dusk), followed by the release of 40 females of each of

the same two species ≈30 min afterwards (i.e., 40 males and 80 females per cage); at which point the experimental observations began. As each pair formed, the couples were gently coaxed into plastic vials (3.7 cm diameter × 4.0 cm) that were capped and sequentially numbered. The following data were recorded for each pair: male species; female species; time of mating; position (cage or tree) and elevation in the cage or canopy (low [from within the canopy or on the field cage wall] or high [the very top of the canopy or on the field cage ceiling]). Light readings (in lux) were also made at time of mating for some replicates (measurement made at the location of the mating couple), along with periodic measurements of temperature (°C) and relative humidity in the cage. Experiments concluded when flies became inactive, which was after sunset when light intensity dropped below 10 lux. Six or eight replicates were completed for each of the possible pair-wise comparisons and they were generally conducted over two evenings per comparison (i.e., four replicates per evening).

Data Analysis. Relative percentages of each of the four possible couples (i.e., Sp 1 ♀ x Sp1 ♂, Sp 1 ♀ x Sp2 ♂, Sp 2 ♀ x Sp1 ♂, Sp 2 ♀ x Sp2 ♂) for each of the compatibility tests were calculated for each replicate. Comparisons of means among couples were undertaken using one-way analysis of variance (ANOVA) with post hoc Tukey test after arcsine transformation of raw percentage data following Rull et al. (2012).

Sexual compatibility was measured using the Index of Sexual Isolation (ISI) in conjunction with the Male Relative Performance Index (MRPI) and the Female Relative Performance Index (FRPI) (Cayol et al. 1999). Values of ISI range from +1 (complete positive assortative mating, i.e., males and females only mating with their respective species) to 0 (complete random mating) to −1 (complete negative assortative mating; i.e., all males of one species mating with all females of the opposite species and vice versa). Values of MRPI range from 1 (only males of one species mated; i.e., the first listed in the test) to 0 (males of both species participated equally in mating) to −1 (only males of the reciprocal species mated; i.e., the second listed in the test). The FRPI is the equivalent of the MRPI but as applied to females. The combined application of ISI, MRPI, and FRPI provide a comprehensive measure of mating compatibility as it demonstrates the degree of isolation between species and the relative participation of the sexes of each species (and whether asynchronous participation by the sexes is the cause of elevated isolation indices).

Ninety-five percent confidence intervals of isolation indices (ISI, MRPI, and FRPI) for each of the six treatments were calculated to determine deviations from random mating (ISI = 0) or equal participation by the respective sexes (MRPI and FRPI = 0). Confidence intervals that included zero were considered to represent cases of random mating and equal participation between the species (our H_0). Heterogeneity χ^2 analyses across replicates for each treatment were undertaken to determine if data could be combined before further analysis. After heterogeneity tests, chi-squared tests of independence were applied to determine if males mated predominantly with females of one species over the other.

The mean time to begin mating (mating latency) was estimated by calculating how many minutes had elapsed between the time each couple initiated mating and the time of the first observed mating couple (=time zero) within each particular cage replicate. Although we could have analyzed mating time against time of official sunset on the day of each replicate, light intensity is known to heavily influence mating behavior in *Bactrocera* species (Roan et al. 1954, Barton–Browne 1957) and differences in cloud cover and the physical location of each field cage resulted in altered light intensity for different cages during each experiment. Our assumption was, therefore, that ambient localized light conditions were satisfactory for mating when the first couple mated and this biological light requirement would be approximately uniform across replicates. Statistical analyses were conducted for each of the six pair-wise tests, with latency data for each of the four possible mating combinations combined before one-way ANOVA (with Tukey post hoc test where appropriate) to determine significant differences (α = 0.05) in latency among mating combinations.

We conducted a one-way ANOVA (with Tukey post hoc test where appropriate) on arcsine transformed percentage data of couples collected on the tree for each of the six pair-wise mating tests to determine if there was a significant difference in couple location (cage or tree). Similarly, one-way ANOVA on arcsine transformed proportion elevation data were undertaken to determine if there was a significant difference among the four possible couples with respect to their location in the field cage (low or high), with an associated *t*-test for each couple to determine significant differences between proportion of couples collected from high versus low elevation.

Results

Eight replicates of mating compatibility tests were completed for all pair-wise comparisons except between *B. papayae* and *B. philippinensis*, for which there were six replicates. The overall mean percent of mated couples for each pair-wise cross ranged from an average of 57.5 to 85.4% (Table 1).

Total numbers and mean percentages of each of the four possible mating-pair combinations (i.e., Sp 1 ♀ x Sp1 ♂, Sp 1 ♀ x Sp2 ♂, Sp 2 ♀ x Sp1 ♂, Sp 2 ♀ x Sp2 ♂) for the six compatibility tests are presented in Fig. 1. One-way ANOVA on arcsine-transformed percentage-mated data for each of the possible mating comparisons revealed significant differences between the numbers of each mating-pair combination across replicates for all crosses (Table 1). *Post hoc* Tukey tests for combinations involving *B. dorsalis*, *B. papayae*, and *B. philippinensis* revealed no consistent patterns of differences between the proportions of homotypic versus heterotypic couples (Fig. 1). In contrast, treatments involving *B. carambolae* showed a much greater dichotomy between the relative numbers of homo-

Table 1. Mean overall percentage of possible couples mated for each species combination and results of ANOVA testing for significant differences in the relative proportions of each of the four possible mating pairs for each combination (i.e., Sp 1 ♀ x Sp1 ♂, Sp 1 ♀ x Sp2 ♂, Sp 2 ♀ x Sp1 ♂, Sp 2 ♀ x Sp2 ♂)

Combination	Overall % mated (mean ± SE)	ANOVA		
		F	df	*P*
B. dorsalis$^{(F5)}$ vs *B. papayae*$^{(F1)}$	74.1 ± 3.6%	5.74	3,28	0.003
B. dorsalis$^{(F4,F5)}$ vs *B. philippinensis*$^{(F3,F4)}$	63.8 ± 4.0%	3.48	3,28	0.029
B. papayae$^{(F1)}$ vs *B. philippinensis*$^{(F4)}$	85.4 ± 4.2%	3.61	3,20	0.031
B. dorsalis$^{(F2,F3)}$ vs *B. carambolae*$^{(F2,F3)}$	67.8 ± 3.7%	29.94	3,28	<0.001
B. carambolae$^{(F3)}$ vs *B. philippinensis*$^{(F2)}$	66.3 ± 3.4%	26.19	3,28	<0.001
B. carambolae$^{(F5)}$ vs *B. papayae*$^{(F1)}$	57.5 ± 4.5%	12.58	3,28	<0.001

Fn = no. of laboratory-bred generations from wild.

versus heterotypic couples, especially that of *B. dorsalis* versus *B. carambolae* for which homotypic couples significantly outnumbered heterotypic couples; and in particular those pairs involving *B. carambolae* males and *B. dorsalis* females for which there were only seven couples (Fig. 1).

Ninety-five percent confidence intervals included isolation indices corresponding to random mating (i.e., ISI = 0) for all treatments among *B. dorsalis*, *B. papayae*, and *B. philippinensis* (Fig. 2). By contrast all confidence intervals for isolation indices calculated for treatments involving *B. carambolae* did not include 0, but tended toward values indicative of positive assortative mating (i.e., mating incompatibility) (Fig. 2).

Males of both species participated equally in copulations for all tests (based on 95% C.I. about the mean

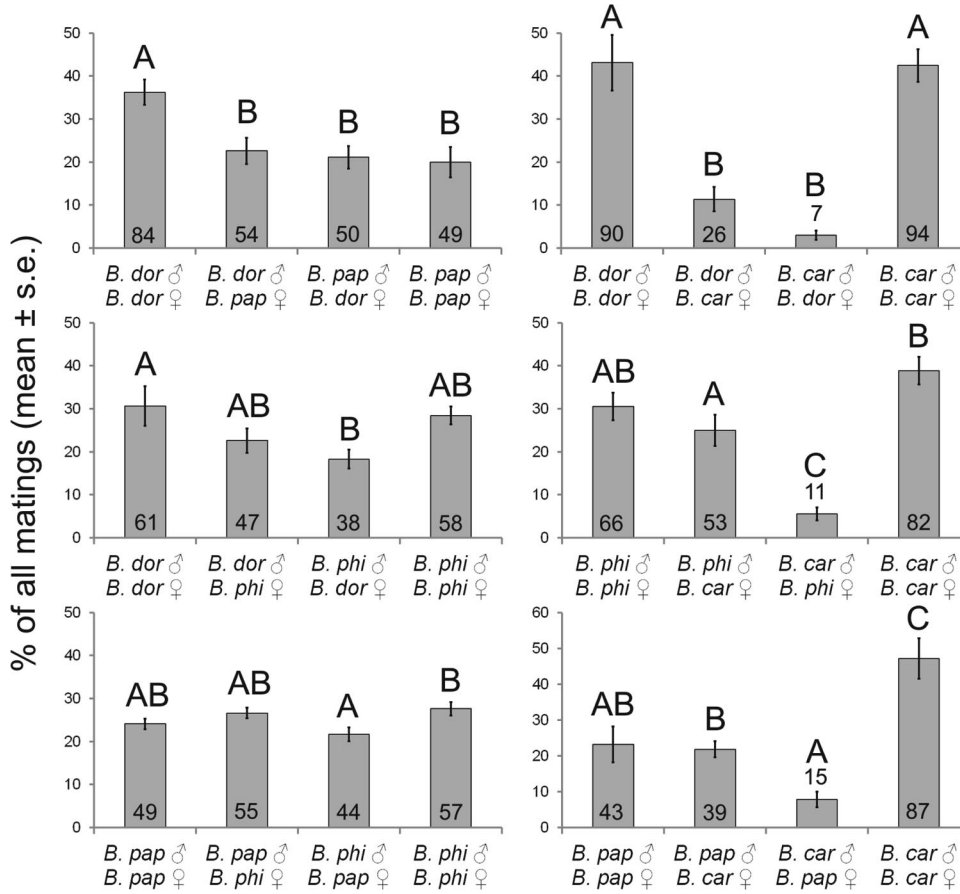

Fig. 1. Relative percentages and total numbers of each possible couple formed for each of the six mating comparisons among *B. dorsalis* (B. dor), *B. papayae* (B. pap), *B. philippinensis* (B. phi), and *B. carambolae* (B. car). Numbers in bars are total numbers of each couple formed summed across replicates. Different letters denote statistically significant differences ($\alpha = 0.05$) between relative numbers of couples formed (one-way ANOVA with Tukey post hoc test using arcsine-transformed percentage data) within each comparison.

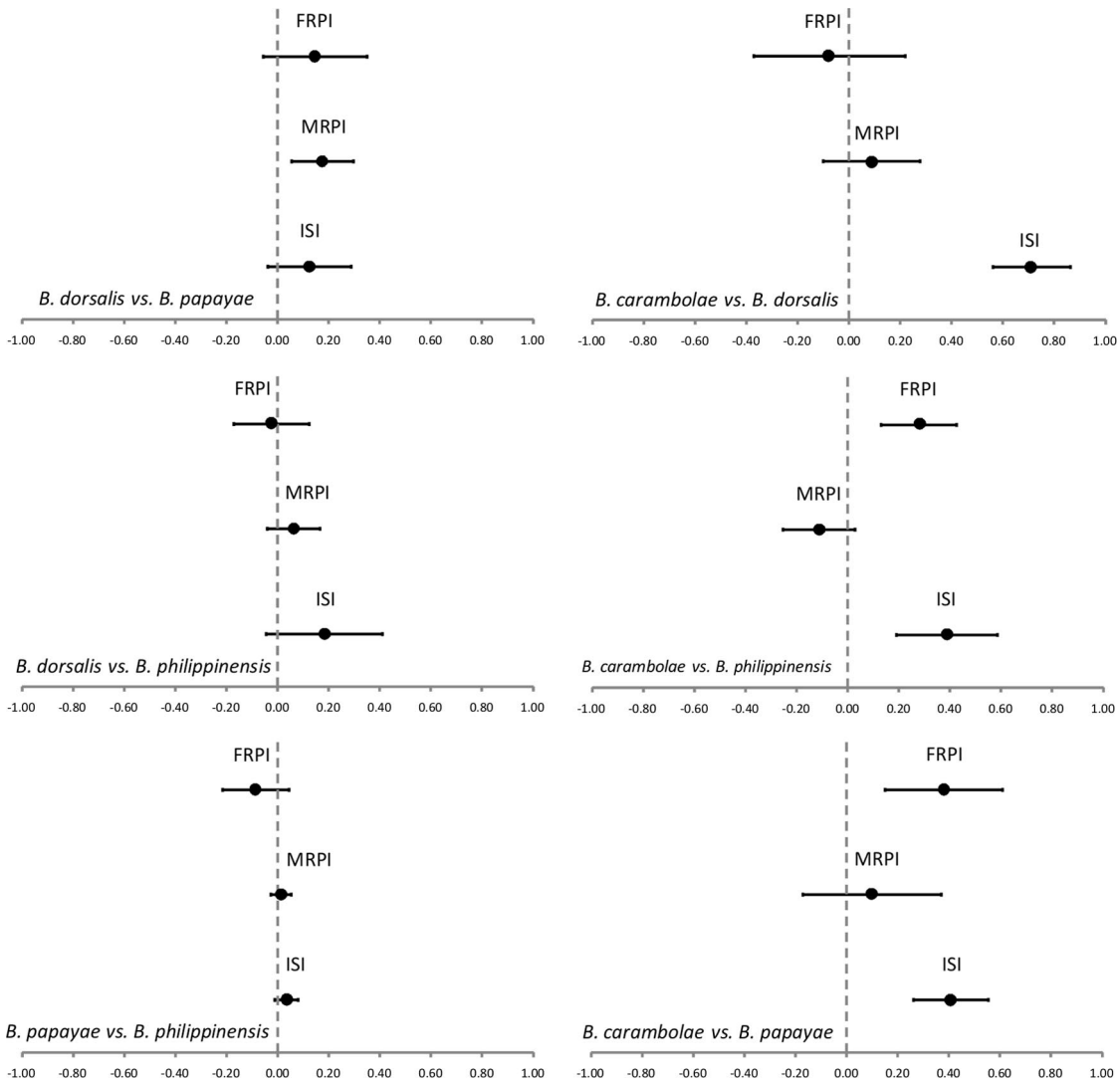

Fig. 2. Index of Sexual Isolation (ISI) and relative performance indices for males (MRPI) and females (FRPI) with associated 95% CIs calculated for each of the six mating compatibility comparisons among *B. dorsalis*, *B. papayae*, *B. philippinensis*, and *B. carambolae*. Dotted line (0.00) represents random mating.

of MRPI) except that of *B. dorsalis* versus *B. papayae* (more *B. dorsalis* males mated; $P\ (0.06 \leq \mu \leq 0.30)$). Females of both species participated equally in copulations for all tests except for *B. carambolae* versus *B. papayae* ($P\ (0.15 \leq \mu \leq 0.61)$) and *B. carambolae* versus *B. philippinensis* ($P\ (0.13 \leq \mu \leq 0.43)$), for which more *B. carambolae* females mated in both tests (Fig. 2).

Chi-squared tests of independence demonstrated statistical homogeneity for all replicates for all comparisons (*B. dorsalis* vs. *B. papayae* $\chi^2 = 8.76, P = 0.270$; *B. dorsalis* vs. *B. philippinensis* $\chi^2 = 11.43, P = 0.121$; *B. dorsalis* vs. *B. carambolae* $\chi^2 = 2.06, P = 0.956$; *B. papayae* vs. *B. philippinensis* $\chi^2 = 0.41, P = 0.955$; *B. papayae* vs. *B. carambolae* $\chi^2 = 4.61, P = 0.708$; *B. philippinensis* vs. *B. carambolae* $\chi^2 = 8.45, P = 0.295$). Consequently chi-squared tests of independence were conducted on summed replicates within comparisons.

Two crosses involving *B. dorsalis*, *B. papayae*, and *B. philippinensis* displayed nonsignificant results based on analysis of summed replicates, implying there was no bias by males to mate with females from either species. These crosses were *B. dorsalis* versus *B. papayae* ($\chi^2 = 2.52$; $P = 0.112$) and *B. papayae* versus *B. philippinensis* ($\chi^2 = 0.26$; $P = 0.610$). In contrast the mating comparison of *B. dorsalis* versus *B. philippinensis* was statistically significant, implying that males mated significantly more often with homotypic females than with heterotypic females ($\chi^2 = 5.81$; $P = 0.016$). This result was driven chiefly by a single outlier replicate for which there were a disproportionately high number of homotypic *B. dorsalis* couples (57% of all couples obtained) as compared with the remaining seven replicates. This outlier replicate was conducted simultaneously as one other replicate that used flies

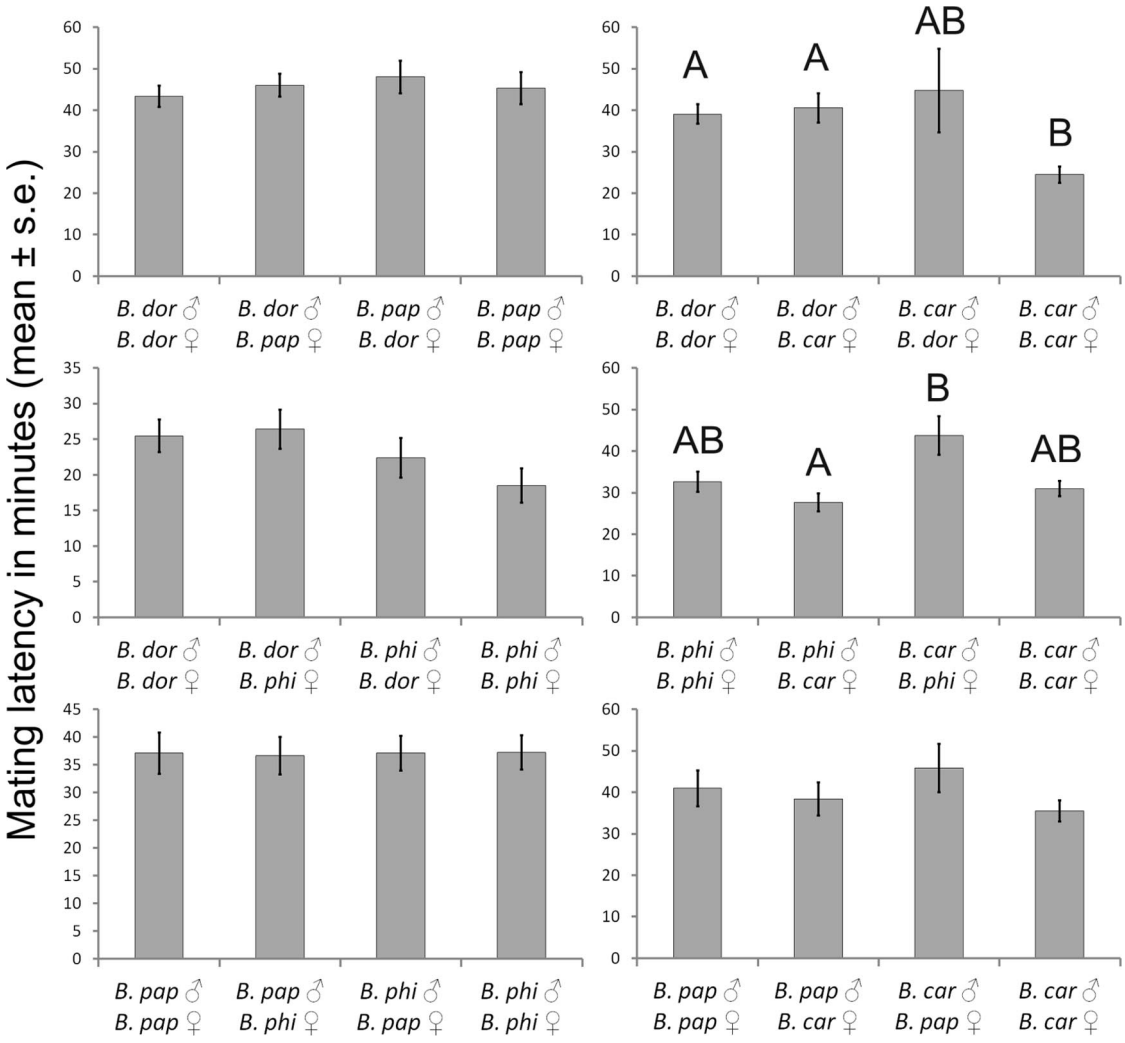

Fig. 3. Mating latency as average time since first couple observed for couples formed for each of the six mating compatibility comparisons among *B. dorsalis* (B. dor), *B. papayae* (B. pap), *B. philippinensis* (B. phi), and *B. carambolae* (B. car). Couple combinations with different letters are significantly different from each other ($\alpha = 0.05$) based on one-way ANOVA followed by Tukey post hoc test (no letters = no significant difference among couples).

prepared at the same time and that had been sourced from identical stock material; however, this parallel replicate did not have the same high number of homotypic *B. dorsalis* couples (20% in this instance). Closer analysis of the data revealed no information as to why one outlier replicate resulted in twice as many homotypic *B. dorsalis* couples and we are hence unable to explain why this occurred. However, if this replicate is removed and the remaining seven replicates reanalyzed, the result subsequently becomes nonsignificant ($\chi^2 = 2.08$; $P = 0.149$).

For crosses involving *B. carambolae*, the chi-squared test of independence for each cross showed that males mated significantly more with homotypic females than with heterotypic females (*B. dorsalis* vs. *B. carambolae* $\chi^2 = 109.04$, $P < 0.001$; *B. papayae* vs. *B. carambolae* $\chi^2 = 29.98$, $P < 0.001$; *B. philippinensis* vs. *B. carambolae* $\chi^2 = 42.97$, $P < 0.001$). Males of *B. carambolae* mated with far fewer heterotypic females (ranging from a contribution of 3.2–8.2% of couples within a cross) relative to reciprocal males mating with *B. carambolae* females for each of the three crosses (*B. dorsalis* 12.0%, *B. papayae* 21.2%, and *B. philippinensis* 25.0%) (Fig. 1).

Mating latency data of copulation initiation for couples from across combinations are presented in Fig. 3. Where measurements were taken, couples were found to start mating anywhere between 320 lux to over 2,000 lux. Averaged across all combinations and comparing only for homotypic couples, *B. philippinensis* mated first with a latency of 29.5 ± 1.6 min ($n = 181$) at mean light intensity of 409.7 ± 70.6 lux ($n = 40$), followed by *B. carambolae* with a latency of 30.2 ± 1.3 min ($n = 263$) at an average of 202.7 ± 25.4 lux ($n = 35$), then

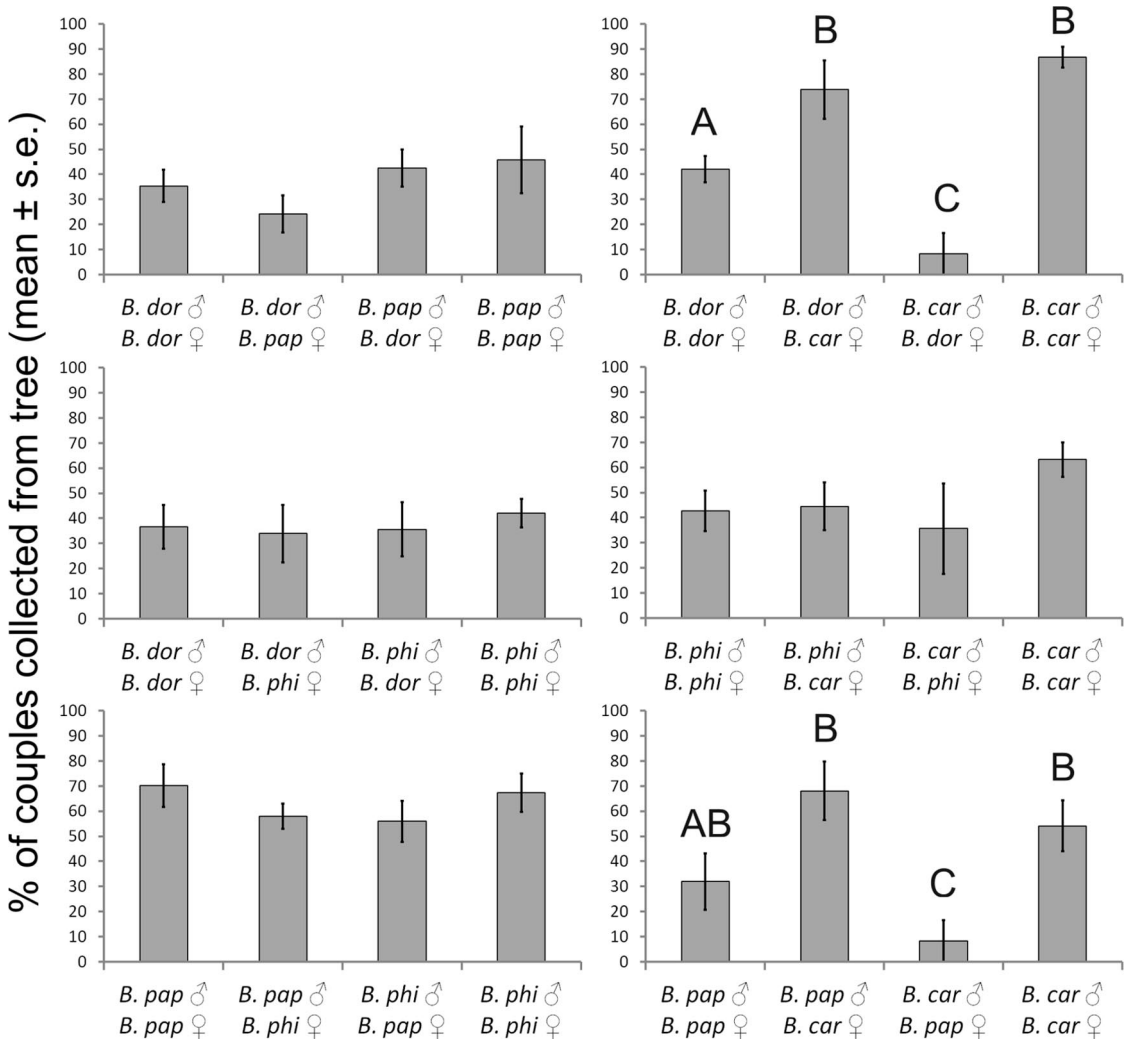

Fig. 4. Average percentage of respective couples collected from the tree for of each of the six mating compatibility comparisons among *B. dorsalis* (B. dor), *B. papayae* (B. pap), *B. philippinensis* (B. phi), and *B. carambolae* (B. car). Couple combinations with different letters are significantly different from each other ($\alpha = 0.05$) based on one-way ANOVA of arcsine transformed percentage data followed by Tukey post hoc test (no letters = no significant difference among couples).

B. dorsalis with 37.1 ± 1.5 min ($n = 235$) and 172.4 ± 25.9 lux ($n = 25$), and finally *B. papayae* at 41.1 ± 2.3 min ($n = 141$) and 318.3 ± 68.0 lux ($n = 33$). With respect to each cross, there was no significant difference in latency among couple combinations for *B. dorsalis* versus *B. papayae* ($F_{(3,233)} = 0.387, P = 0.762$), *B. dorsalis* versus *B. philippinensis* ($F_{(3,200)} = 2.139, P = 0.097$), *B. papayae* versus *B. philippinensis* ($F_{(3,201)} = 0.006, P = 0.999$), and *B. papayae* versus *B. carambolae* ($F_{(3,180)} = 0.954, P = 0.416$) (Fig. 3). A significant difference in mating latency was observed, however, in 1) the cross of *B. dorsalis* versus *B. carambolae* ($F_{(3,213)} = 9.755, P < 0.001$) for which homotypic *B. carambolae* couples had a significantly reduced latency as compared with the remaining three combinations; and 2) *B. carambolae* versus *B. philippinensis* ($F_{(3,208)} = 2.801, P < 0.05$) for which there was a significant difference in latency between the heterotypic combinations (see Fig. 3).

There was no significant difference in the proportion of the four possible couples occurring on the tree relative to the cage for any crosses involving *B. dorsalis*, *B. papayae*, and *B. philippinensis* (*B. dorsalis* vs. *B. papayae:* $F_{(3,28)} = 1.238, P = 0.315$; *B. dorsalis* vs. *B. philippinensis:* $F_{(3,28)} = 0.195, P = 0.899$; *B. papayae* vs. *B. philippinensis:* $F_{(3,20)} = 0.969, P = 0.427$). For the comparison of *B. dorsalis* versus *B. papayae* and *B. dorsalis* versus *B. philippinensis* most couples were collected from the cage wall (i.e., <50% from the tree) whereas for *B. papayae* versus *B. philippinensis* most couples were collected from the tree (Fig. 4). Further, while more homotypic *B. carambolae* couples occurred on the tree relative to the other three combinations for the *B. carambolae* versus *B. philippinensis*

Table 2. Mean (±SE) percentages of all mating combinations collected from one of two elevations within the field cage for all six mating compatibility tests among four *B. dorsalis* complex species

Cross		Elevation		*t*-test
Male	Female	High	Low	
B. dorsalis	*B. dorsalis*	90.59% ± 2.92	9.41% ± 2.92	$t = 10.301$; df = 14; $P < 0.0001$
B. dorsalis	*B. papayae*	93.86% ± 3.02	6.14% ± 3.02	$t = 11.661$; df = 14; $P < 0.0001$
B. papayae	*B. dorsalis*	90.92% ± 4.78	9.08% ± 4.78	$t = 8.795$; df = 14; $P < 0.0001$
B. papayae	*B. papayae*	81.55% ± 8.01	18.45% ± 8.01	$t = 5.076$; df = 14; $P < 0.0001$
	One-way ANOVA	$F = 0.565$; df = 3, 28; $P = 0.643$		
B. dorsalis	*B. dorsalis*	96.13% ± 2.54	3.87% ± 2.54	$t = 14.620$; df = 14; $P < 0.0001$
B. dorsalis	*B. philippinensis*	87.53% ± 5.40	12.47% ± 5.40	$t = 7.311$; df = 14; $P < 0.0001$
B. philippinensis	*B. dorsalis*	92.01% ± 3.98	7.99% ± 3.98	$t = 9.729$; df = 14; $P < 0.0001$
B. philippinensis	*B. philippinensis*	81.53% ± 5.19	18.47% ± 5.19	$t = 9.925$; df = 14; $P < 0.0001$
	One-way ANOVA	$F = 2.388$; df = 3, 28; $P = 0.090$		
B. papayae	*B. papayae*	83.07% ± 8.75	16.93% ± 8.75	$t = 4.674$; df = 10; $P < 0.001$
B. papayae	*B. philippinensis*	89.40% ± 2.37	10.61% ± 2.37	$t = 10.907$; df = 10; $P < 0.0001$
B. philippinensis	*B. papayae*	79.40% ± 5.76	20.60% ± 5.76	$t = 5.175$; df = 10; $P < 0.0001$
B. philippinensis	*B. philippinensis*	84.32% ± 5.59	15.68% ± 5.59	$t = 5.780$; df = 10; $P < 0.0001$
	One-way ANOVA	$F = 0.287$; df = 3, 20; $P = 0.834$		
B. carambolae	*B. carambolae*	42.31% ± 12.42A	57.69% ± 12.42[a]	$t = -1.097$; df = 12; $P = 0.294$
B. carambolae	*B. dorsalis*	80.00% ± 20.00AB	20.00% ± 20.00[a]	$t = 2.121$; df = 8; $P = 0.067$
B. dorsalis	*B. carambolae*	47.55% ± 14.23A	52.45% ± 14.23[a]	$t = -0.408$; df = 12; $P = 0.690$
B. dorsalis	*B. dorsalis*	97.14% ± 2.86B	2.86% ± 2.86	$t = 15.355$; df = 12; $P < 0.0001$
	One-way ANOVA	$F = 4.906$; df = 3, 22; $P < 0.01$		
B. carambolae	*B. carambolae*	76.41% ± 4.17	23.59% ± 4.17	$t = 5.986$; df = 14; $P < 0.0001$
B. carambolae	*B. philippinensis*	71.43% ± 14.87	28.57% ± 7.37[a]	$t = 2.038$; df = 12; $P = 0.064$
B. philippinensis	*B. carambolae*	95.31% ± 4.69	4.69% ± 7.19	$t = 12.068$; df = 14; $P < 0.0001$
B. philippinensis	*B. philippinensis*	91.76% ± 4.25	8.24% ± 3.87	$t = 9.467$; df = 14; $P < 0.0001$
	One-way ANOVA	$F = 2.333$; df = 3, 27; $P = 0.096$		
B. carambolae	*B. carambolae*	86.52% ± 5.47	13.48% ± 5.47	$t = 7.192$; df = 14; $P < 0.0001$
B. carambolae	*B. papayae*	100.00% ± 0.00	0.00% ± 0.00*	
B. papayae	*B. carambolae*	88.13% ± 4.53	11.88% ± 4.53	$t = 7.794$; df = 14; $P < 0.0001$
B. papayae	*B. papayae*	92.38% ± 5.13	7.62% ± 5.13	$t = 8.849$; df = 12; $P < 0.0001$
	One-way ANOVA	$F = 1.923$; df = 3, 25; $P = 0.152$		

High elevation is defined as the top of the canopy of the test tree or the ceiling of the cage; low elevation is defined as the mid-lower canopy or the side-lower cage wall. Letters within a column presented instances of significant differences in elevation among couples within a test (following one-way ANOVA on arcsine transformed percentage data with Tukey post hoc test).

[a] Cases where there is no significant difference ($\alpha = 0.05$) between high and low elevation as determined via pair-wise *t*-test on arcsine-transformed data for each male/female couple (*, denotes the single case where a statistical test was not possible because of no variation among replicates for *B. carambolae* ♂/*B. papayae* ♀).

cross (i.e., as compared with *B. philippinensis* homotypic and both heterotypic combinations), this difference was not statistically significant ($F_{(3,27)} = 0.946$; $P = 0.432$) (Fig. 4). There was, however, a significant difference in couple location for the *B. dorsalis* versus *B. carambolae* and *B. papayae* versus *B. carambolae* crosses ($F_{(3,24)} = 16.865$, $P < 0.001$ and $F_{(3,25)} = 5.430$, $P < 0.01$, respectively). The majority of homotypic *B. carambolae* couples and those heterotypic couples involving *B. carambolae* females for the *B. dorsalis* versus *B. carambolae* cross were collected from the tree as compared with couples involving *B. dorsalis* females (that were predominantly collected from the cage wall) (Fig. 4). This was mirrored in the cross between *B. papayae* and *B. carambolae* for which those couples involving *B. carambolae* females were mostly collected from the tree as compared with predominantly cage-collected couples involving *B. dorsalis* females (Fig. 4).

Significantly more couples involving *B. dorsalis*, *B. papayae*, and *B. philippinensis* were collected from high in the field cage (at either the very top of the canopy or from the ceiling of the cage) relative to lower in the canopy or the side of the cage (Table 2). However, some mating tests involving *B. carambolae* resulted in couples being collected significantly more frequently from lower within the mating arena (Table 2). This was particularly the case for the *B. dorsalis* versus *B. carambolae* mating test for which couples involving *B. carambolae* females were collected significantly more often from lower in the canopy/cage relative to those couples involving *B. dorsalis* females (Table 2). This trend was also evident for the *B. carambolae* versus *B. philippinensis* (except for the *B. philippinensis* male x *B. carambolae* female combination for which 95% of couples were collected from a high elevation), however, while this difference among couples was not statistically significant, there was difference in the proportion of high and low elevation couples of *B. carambolae* male x *B. philippinensis* female (Table 2). Similarly, there was no significant difference in elevation among couples for the *B. carambolae* versus *B. papayae* test, with all couples of all four crosses being collected from the high elevation in the field cage (Table 2).

Discussion

While considerable effort and resources have been applied toward assessing the genetic, morphological, and physiological relationships among *B. dorsalis*, *B.*

papayae, *B. philippinensis*, and *B. carambolae*, this study represents the first instance where all four species have been simultaneously examined for their prezygotic mating compatibility. We demonstrate high compatibility among all comparisons of *B. dorsalis*, *B. papayae*, and *B. philippinensis* as evidenced by random mating; but relative incompatibility between any of these three members of the *B. dorsalis* complex and *B. carambolae* as demonstrated by significant deviation from random mating (toward assortative mating).

Previous research has found varying levels of sexual compatibility among these four members of the *B. dorsalis* complex which generally agrees with our findings. In separate pair-wise studies, Tan (2003) and Medina et al. (1998) reported high levels of mating compatibility between *B. dorsalis* versus *B. papayae* and *B. dorsalis* versus *B. philippinensis;* and subsequently argued that they represent the same biological entity or are at least "not distinct genetic species" (Tan 2003). Hybrid progeny of *B. dorsalis* and *B. papayae* were also successfully reared up to the third generation with no evidence of hybrid sterility (Tan 2003). An affinity among these three species is further exemplified by the body of work on male pheromone constituents for these three taxa. *Bactrocera dorsalis*, *B. papayae* (originally called 'Mal B'), and *B. philippinensis* ('Philippine B') possess similar male rectal gland components, albeit with minor yet consistent differences (Perkins et al. 1990); and subsequent male pheromone studies have recorded similar results (Tan 2000). According to the recognition concept of species, in that a species is 'that most inclusive populations of individual biparental organisms which share a common fertilization system' (sensu Paterson (1985)), all levels of analysis into the mating systems of these species so far imply that *B. dorsalis*, *B. papayae*, and *B. philippinensis* represent the same species for which the only mechanism preventing reproduction is their allopatric distributions. Importantly, we recognize the next step toward fully resolving sexual compatibility among these species is to complete postzygotic studies through to the F2 stage as species may demonstrate little to no discrimination at the prezygotic level (i.e., randomly mate); however, there may be breakdown in subsequent generations resulting from parental hybrids.

The situation regarding *B. carambolae* is different. As in the study of McInnis et al. (1999), we found a relatively high degree of mating incompatibility between *B. dorsalis* and *B. carambolae* because of the disproportionately high number of homotypic couples formed relative to heterotypic ones (92% homotypic couples for both species in McInnis et al. (1999); 86% in the current study). Similarly, and even though experiments were conducted under a no choice scenario, Wee and Tan (2000b) showed that *B. papayae* females exposed to *B. carambolae* and *B. papayae* males formed 95 ± 9.5 homotypic couples compared with only 8.0 ± 4.6 couples with *B. carambolae* males. Importantly, however, when *B. carambolae* females were exposed to males of both species under the same no choice scenario there was no preference (16.0 ± 2.1 homotypic *B. carambolae* and 16.7 ± 5.2 heterotypic) (Wee and Tan 2000b). This observation is partially supported by our results, in that *B. dorsalis*, *B. papayae*, and *B. philippinensis* males were more successful at mating with *B. carambolae* females as compared with *B. carambolae* males mating with heterotypic females. This is mirrored for *B. philippinensis* and to a lesser degree *B. dorsalis* (possibly because this comparison was conducted with earlier-generation closer-to-wild flies). In this case at least, it seems males of *B. papayae* and *B. philippinensis* did not actively discriminate between homotypic females or those of *B. carambolae* and if we had conducted our experiments under a no choice scenario as did Wee and Tan (2000b) we may have obtained similar results.

A number of possible explanations have been proposed to explain differences in mating behavior between *B. carambolae* and other members of the complex. Wee and Tan (2000b), for instance, propose that male *B. papayae* flies are more aggressive and promiscuous maters that their *B. carambolae* counterparts. Furthermore, differences in pheromone constituents between *B. carambolae* and the other three species are well documented (Perkins et al. 1990). As stated above, *B. dorsalis*, *B. papayae*, and *B. philippinensis* males have very similar rectal gland compounds (Perkins et al. 1990, Fletcher and Kitching 1995) that are considerably different from those of *B. carambolae.* Male pheromones are emitted at dusk and are accompanied by vigorous wing fanning to produce a 'pheromone cloud' (Ohinata et al. 1982). These pheromones are generally regarded as important for attracting females to males (Shelly and Kaneshiro 1991), however, a number of other physiological roles of these male-emitted pheromone clouds are possible such as female excitation (Bellas and Fletcher 1979) or as an aggregation cue to attract other males (Tan 2000). While we did not record specific data, our cage observations closely mirrored those documented for Hawaiian field *B. dorsalis* flies (Shelly and Kaneshiro 1991) in that the clear majority of males within the mating arena did not engage in wing fanning, yet many 'nonfanners' still successfully mated with females without obvious courtship behavior. It is clear that further studies into proximal mate recognition cues among these species are necessary.

Other factors may also contribute to reduced compatibility between *B. carambolae* and the other three members of the *B. dorsalis* complex studied here. As for other species of fruit fly for which differences in mate location has been observed (Vera et al. 2006), we demonstrate variation in the location of couples within the mating arena for some comparisons. For both *B. carambolae* versus *B. papayae* and *B. carambolae* versus *B. dorsalis* the location of couples varied considerably among combinations and seemed to be driven by the location of females whether they occur on the cage or tree. For both comparisons the majority of couples involving *B. carambolae* females occurred on the tree, whereas most of the couples involving *B. dorsalis* or *B. papayae* females occurred on the field cage (usually on the ceiling) (Fig. 4). Additionally, there was a considerable difference in the elevation

within the field cage for which couples were collected from depending on the male/female combination; particularly for the comparison involving *B. carambolae* and *B. dorsalis.* For this comparison a greater proportion of couples involving *B. carambolae* were collected from lower in the mating arena (28–33%) as compared with the homotypic *B. dorsalis* couples that were almost exclusively collected from high in the field cage (Table 2). Unexpectedly, however, this was not evident for the test between *B. carambolae* and *B. papayae* for which >80% of all four couple combinations were collected from high in the mating arena. Furthermore, the reverse situation occurred for the test between *B. carambolae* and *B. philippinensis* for which while there was no difference in the location of couples with respect to cage or tree, there was a relative increase in the number of couples involving *B. carambolae* occurring lower in the field cage as compared with the homotypic *B. philippinensis* mating pairs (and one heterotypic combination; Fig. 4, Table 2). Similar differences in couple location have been observed for populations of the tephritid *Anastrepha fraterculus* (Wiedemann) for which an 'ecological isolation' hypothesis (sensu Dobzhansky (1937)) has been proposed (Vera et al. 2006). 'Temporal isolation' may also result in reduced compatibility, as is the case of many closely related taxa of fruit fly, such as *B. tryoni* and *B. neohumeralis*, for which the latter mates at dusk and the former in the middle of the day; and despite their close genetic and biological affinity these two species rarely encounter each other for the purpose of mating (Clarke et al. 2011). Our results do not, however, show any strong differences between species in terms of time of mating, with all species generally mating an average of 20–30 min before sunset and with no particular variation in mating latency that correlates with measures of incompatibility. Therefore, our data does not support the hypothesis that time of mating between *B. carambolae* and the other species is a causal agent for reduced mating compatibility, but rather that other subtle behavioral differences, including preferred mating location (alongside other factors such as pheromone composition), may play more important roles that deserves further attention.

We do, however, recognize that *B. carambolae* used in these trials was sourced from its invasive range of South America. These flies likely represent a population that has experienced a bottleneck and subsequently has reduced genetic variation. This may result in South American *B. carambolae* behaving differently from *B. carambolae* from its native range of Asia. Recent phylogenetic studies by the authors (under review) do, however, reveal both Asian and South American *B. carambolae* to belong to the same monophyletic clade, and our results closely parallel behavioral studies undertaken by others using Asian material (listed above). We endorse further investigation using *B. carambolae* from its home range in future compatibility assessments; especially between Asian and South American members of this species to determine the degree of sexual compatibility among *B. carambolae* between its native versus invasive range.

Mating compatibility experiments conducted in this study closely followed guidelines as detailed in the quality control manual used for assessing insect quality in relation to the sterile insect technique (SIT) (FAO/IAEA/USDA 2003). The differences between the manual and our study are the inclusion of data from couples collected from the cage wall and the doubling of female to male ratios in the field cage. We agree with Vera et al. (2006) in that couples caught on the field cage wall should be included in mating compatibility studies among closely related cryptic species, as different species may exhibit diverse preferences in their choice of mating location, or be influenced by the other species present in the mating arena. As such the exclusion of cage wall-caught couples from analysis may result in these observations being overlooked and the identification of possible causes of incompatibility (e.g., ecological isolation) missed. Second, we doubled the female to male ratio to ameliorate the potential effects of slight differences in time from adversely inflating isolation indices. We consider this to avoid the potential of all females of one species from being monopolized by a species (or population) of males that may begin mating vigorously yet slightly earlier than males of the other group. Considering the objective of this study was mating compatibility and not strictly competition among males (for which equal ratios should be used), we consider future work assessing mating compatibility among cryptic species for the purposes of identifying species limits use females at double ratio where slight temporal differences may occur.

In conclusion, we demonstrate for the first time within a single study that *B. dorsalis, B. papayae*, and *B. philippinensis* mate randomly with each other under seminatural field cage conditions, yet all these members of the *B. dorsalis* complex are equally relatively incompatible with *B. carambolae.* We believe that in addition to previously reported differences between these species (e.g., pheromone composition), especially for *B. carambolae*, that behavioral variation (e.g., differences in mating location) contribute to the mating incompatibility observed here between *B. carambolae* and the remaining three species. While these data alone cannot be used to redefine species limits or to assert full sexual compatibility (because of the absence of full postzygotic testing; not completed in this study yet underway) and the inherent caveats of artificial cage studies (Walter 2003), we consider our results closely parallel previous research suggesting that *B. dorsalis, B. papayae*, and *B. philippinensis* represent the same biological species (Medina et al. 1998, Wee and Tan 2000b, Tan 2003, Krosch et al. 2013, Schutze et al. 2012). Such an outcome has significant implications not only for their taxonomic identities, but has consequences for pest management (especially for the SIT for which knowledge of species limits and mating compatibility are critical) and international trade.

Acknowledgments

We are deeply thankful to our colleagues who assisted us in obtaining live material for this project, namely Weerawan Amornsak, Wigunda Rattanapun, Helen Bignayan, Vijay Shanmugam, M. Hanifah Yahaya, and Alies van Sauers Muller. Particular appreciation goes to R.A.I. Drew for his assistance with species identifications. We also thank Thilakasiri Dammalage, Ulysses St. Tomas, Amirul Islam, Sohel Ahmad, Nwe Nwe Yin, Mokhtar F. El-Wakkad, Adalecio Kovaleski, Todd Shelly, Guy Hallman, and Scott Myers for their tireless assistance in the field cages. We also acknowledge Jorge Hendrichs for his support of this work; and Magali Evrard, Adrene Despars, Anne Lorenz, and Tamara Wimberger for administrative assistance. Funding for this work was provided by the Australian Cooperative Research Centre for National Plant Biosecurity project 20115. M.K.S. and A.R.C. would like to acknowledge the support of the Australian Government's Cooperative Research Centres Program.

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Received 28 September 2012; accepted 13 December 2012.