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EVIDENCE OF NATURAL HYBRIDIZATION BETWEEN TWO SYMPATRIC SIBLING SPECIES OF *Bactrocera dorsalis* COMPLEX BASED ON PHEROMONE ANALYSIS

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Abstract—*Bactrocera carambolae* and *B. papayae* are major fruit fly pests and sympatric sibling species of the *B. dorsalis* complex. They possess distinct differences in male pheromonal components. In the 1990's, wild *Bactrocera* fruit flies with morphological traits intermediate between those of *B. carambolae* and *B. papayae* were often captured in traps baited with methyl eugenol (ME). Chemical analyses of rectal glands of ME-fed males revealed that the laboratory F_1, F_2, and backcross hybrids possessed ME-derived sex pheromonal components ranging from that typical of *B. papayae* to that of *B. carambolae* without any specific trend, which included a combination of pheromonal components from both parental species within an individual hybrid. ME-fed hybrids without any ME-derived pheromonal components were also detected. Further chemical analysis of rectal glands from wild *Bactrocera* males, after ME feeding in the laboratory, showed a combination of pheromonal components similar to that found in the ME-fed, laboratory-bred hybrids. These findings present circumstantial evidence for the occurrence of a natural hybrid of the two *Bactrocera* species.

Key Words—*Bactrocera carambolae*, *B. papayae* (=*B. dorsalis*), hybrid, methyl eugenol, rectal gland, male pheromone, 2-allyl-4,5-dimethoxyphenol, (*E*)-coniferyl alcohol, 6-oxo-1-nonanol.

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INTRODUCTION

Two sibling species of the *Bactrocera dorsalis* complex (Diptera: Tephritidae), *B. carambolae* Drew and Hancock and *B. papayae* Drew and Hancock, are major fruit pests in Southeast Asia, particularly Malaysia, Thailand, and Indonesia. These sympatric species are found readily throughout the peninsular Malaysia, with *B. carambolae* and *B. papayae* being more abundant in the southern and central regions, respectively (Tan and Nishida, 1996).

Following a taxonomic revision by Drew and Hancock (1994), *B. carambolae* can be differentiated morphologically from *B. papayae* by three key traits: (1) wing costal band slightly enlarged at apex of R_{4+5}, (2) the presence of a dark spot on foreleg femora, and (3) the appearance of a bar-shaped pattern of the lateral abdominal band on terga III–V. Subsequently, Iwaizumi et al. (1997) proposed the use of genital characters to differentiate sibling species within the *B. dorsalis* complex. However, identification of wild fruit flies based on these morphological and genitalia traits has been difficult and unreliable because they often show considerable variation (Iwaizumi et al., 1997; Iwahashi, 1999). Nevertheless, distinctive differences were found in volatile components of the male rectal gland, genetic polymorphism in intron sequences, and ribosomal DNA analyses (Perkins et al., 1990; He and Haymer, 1997; Armstrong and Cameron, 2000).

Both species are strongly attracted to and compulsively feed on methyl eugenol (ME), a potent male attractant frequently used in area-wide fruit fly control programs. Males of *B. papayae*, after consuming ME, produced 2-allyl-4,5-dimethoxyphenol (DMP) and (*E*)-coniferyl alcohol (CF) (Nishida et al., 1988; Tan and Nishida, 1996), whereas males of *B. carambolae* produced only CF (Tan and Nishida, 1996; Wee, 2000) in addition to their major endogenous rectal gland component, 6-oxo-1-nonanol (OXO), and other minor components (Perkins et al., 1990). These pheromonal compounds are sequestered and stored in the rectal gland prior to release during courtship at dusk.

Since the early 1990's, wild *Bactrocera* fruit flies with intermediate morphological traits between *B. carambolae* and *B. papayae* (hereafter referred to as intermediates) often have been recovered from traps baited with ME in Malaysian ecosystems where these species are known to coexist (Wee, 2000). This has raised speculation that natural hybridization might have occurred. Nevertheless, the intermediates have been regarded as morphological variants of their respective species (Iwahashi, 1999). Attempts to mate the two sibling species in the laboratory yielded viable hybrids with similar morphological traits to those of the field intermediates (Wee and Tan, 2000a; Wee, 2000). However, to date, there is no conclusive evidence as to whether natural hybridization between the sibling species occurs in the wild.

Here, we report evidence of natural hybrids between *B. carambolae* and *B. papayae* using gas chromatographic analyses of pheromonal components

accumulated in the male rectal glands after ME consumption, involving laboratory-bred hybrids and wild flies, respectively.

METHODS AND MATERIALS

Chemicals. 1,2-Dimethoxy-4-[2-propenyl]benzene (methyl eugenol; >99.8% purity) was obtained from Agrisense-BCS Ltd. (UK). Authentic samples (>96% purity) of CF (96% *trans*), DMP, and OXO were synthesized and supplied by R. Nishida (Kyoto University, Japan).

Insects. Laboratory strains of *B. carambolae* and *B. papayae* were pure-bred lines for more than 30 generations. These fruit fly species possessed the phenotypic traits typical of *B. carambolae* or *B. papayae*, respectively, according to Drew and Hancock (1994). Fruit fly colonies were cultured as described in Wee and Tan (2000b). Male and female flies were segregated by the fourth day after adult eclosion. Virgin males and females of each species were kept in separate cages with food and water in an insectary that had 12 D:12 L regimes with room temperature of 25–29°C and 83–90% RH.

Laboratory Crosses. All crosses were conducted in a screen cage ($40 \times 40 \times 40$ cm^3) placed indoors. Equal numbers (50–100) of sexually mature males of one species and females of the other species were transferred into the experimental cage in the morning and were acclimatized for at least 8 hr before courtship began. At 21:00 hr, under red light, each mating pair was carefully collected in a specimen vial, labeled, and allowed to continue mating until dawn.

The pure-bred lines of *B. carambolae* (BC) and *B. papayae* (BP) were crossed reciprocally to produce F_1 hybrids (BC ♀ × BP ♂ and BP ♀ × BC ♂). Six additional sources of flies, comprising a variety of the three key morphological traits in combination (that is, pattern of wing costal band at R_{4+5} and abdominal band at terga III–V, and absence/presence of a femoral spot on prothoracic legs) between BC and BP, were obtained from the following crosses of flies: (1) reciprocal crosses of F_1 hybrids were self-crossed to produce F_2 generations; (2) F_1 hybrids (from BP ♀ × BC ♂) were backcrossed with the parental species (BP ♀ × hybrid ♂ and hybrid ♀ × BC ♂); and (3) F_1 hybrids (from BC ♀ × BP ♂) were backcrossed with the parental species.

The emerged flies were maintained on artificial diet until sexual maturity (Wee and Tan, 2000b; Wee, 2000) and then allowed to feed on ME. This was performed on 30–74 male flies for each of the above crosses.

Wild Fruit Fly Collections. Wild fruit flies were obtained either by field trapping using ME-baited trap or collecting field-infested fruits from various locations in peninsular Malaysia (northern region: Penang; central region: Perak; and southern region: Johor), which were subsequently reared in the laboratory.

For field trapping, improved and modified clear traps from Tan (1985) (Figure 1) baited with ME were set up at sites located near hillside forests. The trap was designed in such a way as to prevent the trapped flies from feeding on the ME source, thus avoiding regurgitation and anal feeding behavior (Tan, 2000). These traps were used only once (trapping conducted between 08:00 and 14:00 hr) and discarded after use. Trapping was conducted on different dates in July 2000. Occasionally, flies were caught using specimen vials before they could enter the trap. In contrast, male flies raised from rotten fruits were maintained until sexual maturity (20–28 d), and allowed to feed on ME for

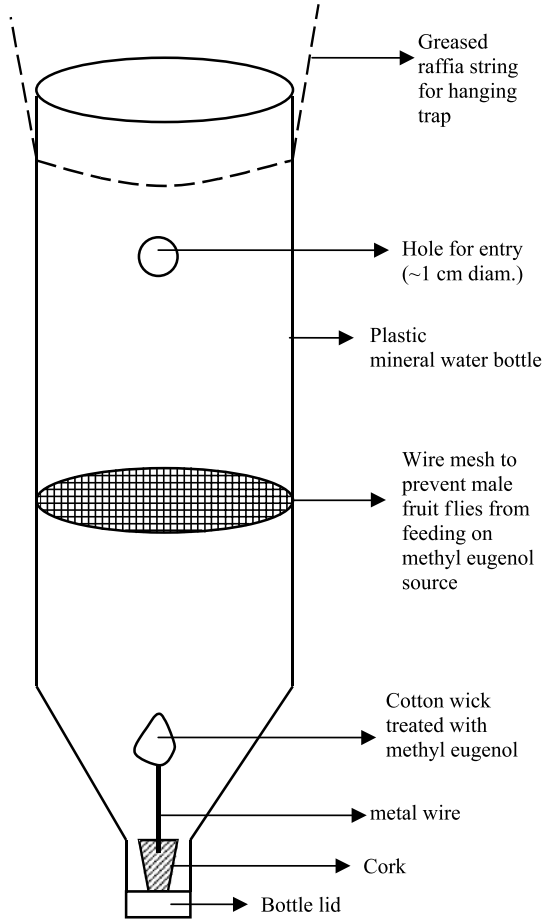

FIG. 1. Methyl eugenol-baited trap used to trap wild *Bactrocera* fruit fly males.

subsequent pheromonal analysis. A total of 167 wild males (Penang, 106; Perak, 11; and Johor, 50) were analyzed for pheromonal components.

Consumption of ME. In the laboratory, each male fruit fly was allowed to feed on 0.1 μl ME dispensed on a small piece of filter paper (1×1 cm^2; Whatman® No. 1) for 10 min during their peak response to ME (10:00–11:00 hr) (Tan, 1985). After 24 hr, male rectal glands were excised and soaked individually in 20–50 μl of redistilled ethanol spiked with an internal standard (1-dodecanol to monitor evaporation of sample solvent and not for quantification) placed in a screw-cap glass vial, and stored at −20°C for further analysis.

Rectal gland samples were obtained from (1) parental males of *B. carambolae* and *B. papayae* ($N = 30$ each), (2) males of laboratory hybrids from F_1 and F_2 generations plus backcrosses, and (3) wild males from both field trapping and male flies raised from field-infested fruits.

Pheromone Analyses. Rectal glands in ethanol (see above) were carefully homogenized with a fine glass rod followed by 5 min sonication. After centrifugation at ~1,000 *g* for 2–3 min, 1 μl aliquots of the supernatant were subjected to gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses, respectively.

Analysis of rectal gland volatiles was conducted using splitless injections on a Shimadzu GC-14A gas chromatograph, with an HP Ultra-1 column (Agilent, 25 m × 0.25 mm × 0.33 μm) programmed from 80°C/1 min to 240°C at 10°C/min. The peak areas were calibrated with those of the authentic standard samples using a C-R6A integrator (Shimadzu). Chemical identification was performed by comparison with the retention time, use of authentic internal standards, and mass spectral fragmentation patterns of authentic standards.

GC-MS analyses were performed with an HP 5989B mass spectrometer (electron impact at 70 eV) connected to an HP-5MS column (30 m × 0.25 mm, 0.33 μm film thickness) using the same running conditions as above.

RESULTS

The rectal gland of laboratory-reared *B. papayae* males following ME consumption contained CF and DMP, whereas CF was detected in the rectal gland of *B. carambolae* male along with its endogenously produced rectal gland component, OXO (Figure 2A and B). Identification of the rectal gland components was confirmed by comparison with the mass spectra of authentic standards (Figure 3A, B, and C).

Rectal gland analysis of ME-fed laboratory hybrids revealed the presence of various combinations of components between the two parental species (Table 1). A majority of the ME-fed F_1 hybrid males (68–84%) contained only CF in their

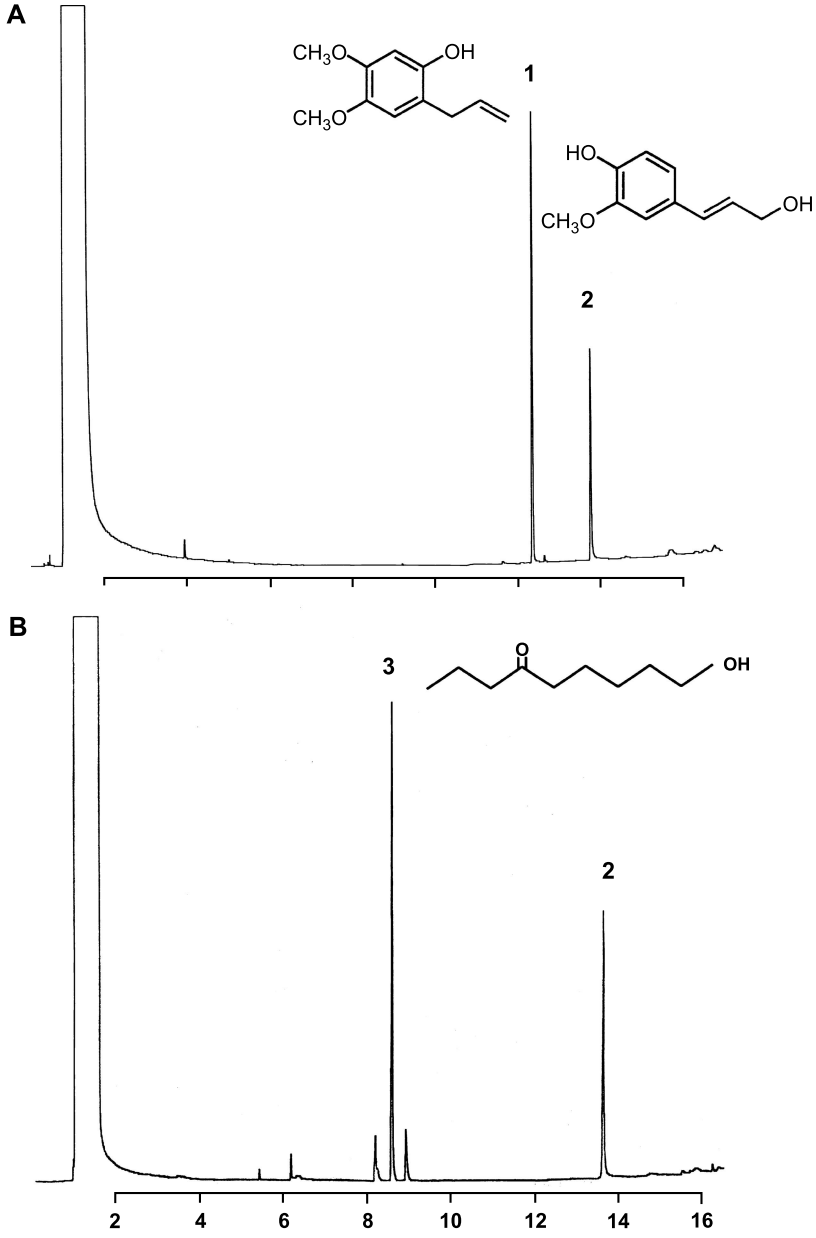

FIG. 2. Gas chromatograms of laboratory male rectal gland of (**A**) *Bactrocera papayae* and (**B**) *B. carambolae*, 24 hr after methyl eugenol consumption. (**1**) 2-Allyl-4,5-dimethoxyphenol, (**2**) (*E*)-coniferyl alcohol, and (**3**) 6-oxo-1-nonanol.

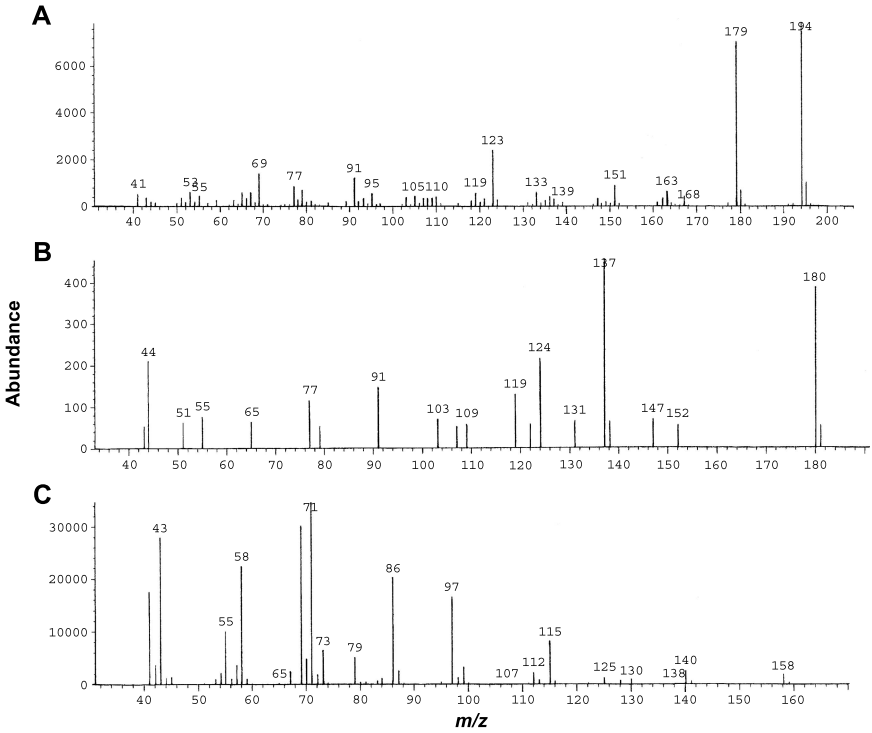

FIG. 3. Mass spectra of (**A**) 2-allyl-4,5-dimethoxyphenol and (**B**) (*E*)-coniferyl alcohol, the methyl eugenol-derived sex pheromonal components in male rectal gland of *B. papayae* and *B. carambolae* (with absence of **A**); and (**C**) 6-oxo-1-nonanol, the major endogenous component of sexually mature male *B. carambolae*.

rectal glands, whereas these percentages showed gradual decrease to below 30% when the respective F_1's were self-crossed (i.e., F_2) or backcrossed to the parental species (Table 1).

Between 14 and 18% of F_1 males possessed both CF and DMP in their rectal gland, which is typical for *B. papayae* males which have consumed ME. Percentages of hybrids that possessed this combination increased when they were subjected to self-crossing (Table 1). Backcrossing F_1 hybrids with parental *B. papayae* further increased the percentages of individuals bearing the *B. papayae* rectal gland blend (Table 1).

Similarly, the occurrence of *B. carambolae* blend (OXO + CF) was low among the F_1 hybrids but showed an increase in the F_2 generation. Backcrossing to the parental *B. carambolae* increased the percentage of hybrids having the *B. carambolae* rectal gland blend (Table 1).

TABLE 1. RECTAL GLAND CONTENTS PROFILE (%) OF HYBRIDS F_1, F_2 AND BACKCROSSED PROGENIES RESULTING FROM INTERBREEDING BETWEEN SIBLING SPECIES OF THE *dorsalis* COMPLEX, *B. carambolae* (BC) AND *B. papayae* (BP), AFTER METHYL EUGENOL CONSUMPTION

Progeny	F_1		F_2		Backcrosses			
Crosses: Female × Male (progeny designation)	BC × BP (R)	BP × BC (S)	R × R	S × S	BC × R	R × BP	BP × S	S × BC
Chemical combination	(N = 74)	(N = 50)	(N = 30)	(N = 50)	(N = 53)	(N = 50)	(N = 50)	(N = 49)
CF	68.9	84.0	33.3	36.9	17.0	28.0	26.0	10.2
DMP + CF[a]	17.6	14.0	43.4	43.5	5.7	66.0	64.0	–
OXO + CF[b]	8.0	2.0	23.3	15.2	75.4	–	–	73.4
OXO + DMP + CF	4.1	–	–	4.4	1.9	2.0	–	–
DMP	1.4	–	–	–	–	4.0	–	–
OXO	–	–	–	–	–	–	–	8.2
None	–	–	–	–	–	–	10.0	8.2

Notes: CF = (*E*)-coniferyl alcohol; DMP = 2-allyl-4,5-dimethoxyphenol; OXO = 6-oxo-1-nonanol; R = hybrid of cross between female BC and male BP; S = hybrid of cross between female BP and male BC.

[a]Combination of rectal gland components typical of a *B. papayae* male after methyl eugenol consumption.

[b]Combination of rectal gland components typical of a *B. carambolae* male after methyl eugenol consumption.

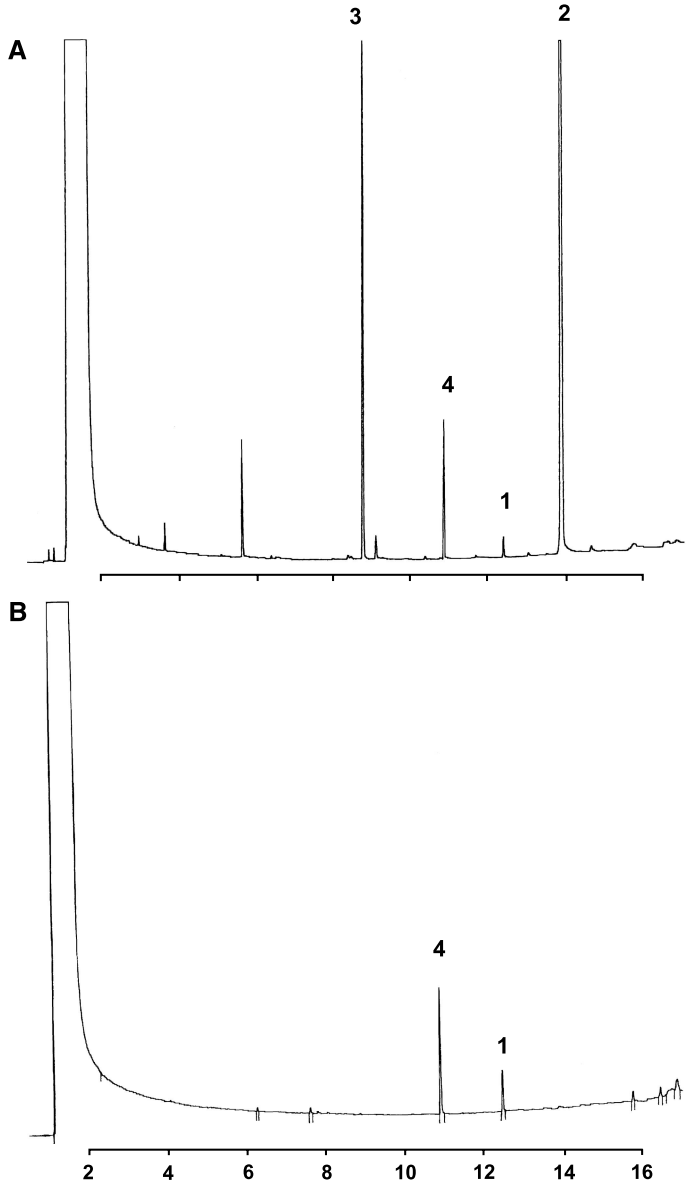

FIG. 4. Gas chromatograms of wild *Bactrocera* male rectal gland, 24 hr after methyl eugenol feeding in the laboratory: (**A**) pheromone profile featuring a combination of both *B. carambolae* and *B. papayae* pheromonal components and (**B**) pheromone profile containing DMP only. (**1**) 2-Allyl-4,5-dimethoxyphenol, (**2**) (*E*)-coniferyl alcohol, (**3**) 6-oxo-1-nonanol, and (**4**) 1-dodecanol as internal standard.

A unique rectal gland blend that featured the combination of both parental species, i.e., OXO + CF + DMP, was detected among the F_1 and F_2 generations as well as when hybrids of *B. carambolae* female and *B. papayae* male were backcrossed with their parental species (Table 1). A low percentage (1–4%) of hybrids possessed DMP only in their rectal glands when *B. carambolae* females interbred with *B. papayae* males, and when their F_1 hybrids were backcrossed to the male parental species (Table 1). Hybrids with OXO component only or without any rectal gland production were not found in F_1 or F_2 generations, but appeared in the backcrossed hybrids of *B. papayae* females and *B. carambolae* males (Table 1).

Rectal gland analyses revealed that several wild males with intermediate morphological traits possessed the unique rectal gland blend from the combination of both *B. carambolae* and *B. papayae* parents, i.e., OXO + CF + DMP (one male each from Penang and Johor; Figure 4A). Apart from this, there were also males detected with DMP only (one male from Perak and two males from Johor; Figure 4B) and without any pheromonal components (three males from Johor).

Interestingly, two wild males collected from Johor of the typical *B. papayae* phenotype were found to possess OXO + CF, which are the typical rectal gland blend of a *B. carambolae* male. In addition, there was a male with the *B. carambolae*-like morphology that contained CF and DMP (typical rectal gland blend of *B. papayae*) in its rectal gland.

DISCUSSION

Among the 52 sibling species in the *B. dorsalis* complex, four (*B. carambolae*, *B. dorsalis*, *B. papayae*, and *B. philippinensis*) have been observed to interbreed in the laboratory. *B. carambolae* interbreeds with *B. dorsalis* (McInnis et al., 1999) and with *B. papayae* (Wee and Tan, 2000a), and *B. dorsalis* interbreeds with *B. papayae* (Tan, 2000). Sexual compatibility was also observed between wild *B. dorsalis* and wild *B. philippinensis* (Medina et al., 1998). However, to date, there is no documented evidence of interspecific mating of these species in the field.

Whereas pheromonal analyses were suggested to be of use in chemotaxonomy of *Bactrocera* flies (Krohn et al., 1992), this paper describes for the first time the use of pheromonal analyses to detect evidence of natural hybrids of the ME-attracted *Bactrocera* species. The distinctive pheromonal components, together with the common phenylpropanoid CF, detected in *B. carambolae* and *B. papayae* were consistent with previous reports (Nishida et al., 1988; Perkins et al., 1990; Tan and Nishida, 1996; Wee, 2000). Therefore, the detection of pheromonal combinations between that of *B. papayae* and *B. carambolae* in a

wild *Bactrocera* fruit fly similar to that of the laboratory hybrids suggests that natural hybridization between *B. carambolae* and *B. papayae* had occurred in the wild.

Wild *Bactrocera* species with intermediate morphological characteristics have been observed regularly since the 1990's (Wee, 2000). Therefore, if natural hybridization happened in the past, some degree of self-crossing among the F_1 or backcrossing to the parental species must have occurred throughout many generations. Laboratory studies have shown that intermediate pheromone combination, such as OXO + CF + DMP, even though at low percentages, persist in F_1, F_2, and backcross generations. Thus, the detection of two wild males with OXO + CF + DMP rectal gland combination in the northern (Penang) and southern region (Johor) of peninsular Malaysia is consistent with the results of our laboratory studies.

The fact that low percentages of captured wild males possessed combinations of pheromonal components may reflect the rarity of the interbreeding that occurs in the wild. However, based on the sample size of trapped wild males, a low capture of those that do interbreed in the field cannot be discounted. Fewer hybrids would suggest higher chances of the hybrid individuals backcrossing with the parental species than among the hybrid themselves. In this study, a higher number of wild males was detected containing DMP only or without any pheromonal components compared with intermediates detected that possess OXO, DMP, and CF. This is also consistent with the laboratory interbreeding results that showed a higher percentage for such occurrence when the F_1 hybrids were backcrossed with the parental species.

The results showed that more hybrids were detected in Johor. Both *B. carambolae* and *B. papayae* are dusk-mating species and exhibit similar mating behavior (Wee, 2000). Although *B. papayae* preceded *B. carambolae* in mating at a slightly higher light intensity (1,000 *versus* 600 lx), their courtship and mating period overlapped (Wee, 2000). A wind tunnel study has shown that females of both species respond by upwind anemotactic flight to conspecific and heterospecific males during courtship period (Wee and Tan, 2000a). In field cages that simulated heterospecific sexual encounters of females, *B. papayae* females showed a much higher prereproductive isolation than *B. carambolae* females (Wee and Tan, 2000a; unpublished data). This suggests that females are able to distinguish between conspecific and heterospecific males at close range/contact during courtship encounters. However, *B. carambolae* females were less discriminating than *B. papayae* females in interspecific encounters (Wee and Tan, 2000a). The fact that *B. carambolae* is more abundant in Johor, and that *B. carambolae* females are nonselective in mate preference may account for natural interbreeding in the field. Moreover, *B. papayae* males are more aggressive in mating behavior than *B. carambolae* males, which could lead to

higher chances of securing a female, be it a conspecific or a heterospecific female (Wee, 2000; Wee and Tan, 2000a). This may explain the higher incidence of wild hybrids captured in Johor.

In contrast, in areas where *B. carambolae* population density is low, high prereproductive isolation in the case of *B. papayae* females may also contribute to the low recovery of hybrids in the wild. However, in the absence of conspecific males, females of both *B. papayae* and *B. carambolae* mate with heterospecific males (Wee, 2000; Wee and Tan, 2000a). In addition, the production of a common ME-derived sex pheromone, in both *B. papayae* and *B. carambolae* males, would further promote and facilitate interspecific encounters and mating in the wild because natural sources of ME are plentiful, especially in the tropics where there are many plant species that contain ME (Tan and Nishida, 2000; Tan et al., 2002; Nishida et al., 2004). ME, as a pheromone precursor (Tan and Nishida, 1996, 1998), enhances male mating competitiveness in *B. dorsalis*, *B. papayae*, and *B. philippinenses* after ME consumption (Shelly and Dewire, 1994; Shelly et al., 1996; Tan and Nishida, 1996, 1998; Hee and Tan, 1998; Shelly, 2000). This may account for the occurrence of the two hybrid flies in the Perak and Penang regions.

Pheromonal analysis also showed that even a wild *Bactrocera* species with the typical phenotype of *B. carambolae* can possess a typical rectal gland blend of a *B. papayae* male and vice versa. Thus, species identification based solely on morphological traits may not be totally reliable, at least in the *B. dorsalis* complex, and especially for the two sympatric species. The discovery of this phenomenon certainly further complicates the existing problem in the taxonomic status of *B. carambolae* and *B. papayae*, the latter of which was recently shown to be neither a distinct biological nor genetic species from *B. dorsalis* (Hendel) (Naeole and Haymer, 2003; Tan, 2003).

Interbreeding between a *B. carambolae* female and *B. papayae* male produced F_1 males with rectal gland blends closer to a *B. papayae* male. However, backcrossing this F_1 male to *B. carambolae* female parent yielded a high percentage (75%) of hybrids having *B. carambolae* rectal gland blend. Therefore, pheromone production in the *Bactrocera* species appears to be a sex-limited trait whereby genes responsible for pheromone production show a 100% penetrance in males only, and although females inherited the same gene, the gene was not expressed in the female progeny.

In contrast, based on these laboratory interbreeding results, backcrossing of the hybrids to the parental species over many generations may produce not only higher percentages of hybrids that resemble the parental species in terms of rectal gland blend, but also, in terms of morphological traits. Therefore, at the time of this investigation, a true hybrid may look like the parental species in all aspects, and, thus, escape any morphological/pheromonal means of screening. Only those minority categories with unique intermediate rectal gland blends

may be detected by pheromone analysis. A more sensitive molecular technique may help in the determination of the hybrids' polymorphism/genetic make-up.

In *B. papayae*, the *in vitro* conversion of ME into its metabolites involves the cytochrome P-450 mixed-function monooxygenases (Lim et al., 1998). Hybridization has resulted in a lower penetrance observed in more than 80% of the F_1 hybrids of the reciprocal crosses, and in some cases, a low percentage of the backcross offspring has been found not to produce either CF or DMP after ME consumption. This lowered and "zero" penetrance may have resulted from the complex interaction between segregated maternal and paternal genes during hybridization of sibling species, causing alteration/deletion or inhibition of the gene responsible for ME conversion, thus disabling the respective gene(s). However, this does not discount other possibilities that may contribute to this lowered penetrance for the sex-limited trait.

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