ORIGINAL ARTICLE

Allozyme electrophoretic evidence for a complex of species within the *Bactrocera tau* group (Diptera: Tephritidae) in Thailand

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Abstract

Saelee, A., Tigvattananont, S. and Baimai, V.
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Songklanakarin J. Sci. Technol., 2006, 28(2) : 249-259

Electrophoretic analysis of the *Bactrocera* (*Zeugodacus*) *tau*-like flies collected from wild populations coupled with morphological observation and cytological evidence has revealed seven species within this taxon, temporarily designated as species A (= *B. tau s.s*), C, D, E, F, G and I. These enzyme electrophoretic characteristics distinguishing these species (including four sympatric and two allopatric species) are described in this study. The value of Wright's fixation index, F_{ST}, among populations was found to be +0.769. Partitioning of each species reduced the mean F_{ST} to +0.053. This suggests strong species-specific mating. UPGMA clustering of Nei's unbiased genetic distance was estimated by analysis of allele frequencies at 12 enzyme loci. The resulting dendrogram shows that two lineages exist in the *B. tau* complex, one consisting of species C and I and the other comprising four species including *B. tau* s.s. (=A), species D, E, F and G. Of these, species F and G are truly sibling species because of their morphological similarity.

Key words : allele frequencies, *Bactrocera tau*, genetic distance, sibling species, phylogenetic tree

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Received, 9 June 2005 Accepted, 30 August 2005

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หลักฐานด้านแอลโลไซม์อิเล็กโตรฟอริซิสสำหรับสปีชีส์ซับซ้อนภายในกลุ่ม *Bactrocera tau* (Diptera: Tephritidae) ในประเทศไทย

ว. สงขลานครินทร์ วทท. 2549 28(2) :

การวิเคราะห์ตัวอย่างแมลงวันผลไม้กลุ่มคล้าย *Bactrocera* (*Zeugodacus*) *tau* จากประชากรธรรมชาติโดยใช้เทคนิคอิเล็กโตรฟอริซีสควบคู่กับหลักฐานด้านสัณฐานวิทยาและเซลล์วิทยา พบว่า มี 7 ชนิดที่เรียกชื่ออย่างไม่เป็นทางการว่า ชนิด A (= *B. tau*), C, D, E, F, G, I แต่ละชนิดแสดงลักษณะเฉพาะของเอ็นไซม์ดังที่ได้แสดงไว้ในการศึกษานี้ ซึ่งสามารถแบ่งออกเป็น 2 กลุ่ม คือ กลุ่มชิมแพตริกกับกลุ่มแอลโลแพตริก และพบว่าค่า F_{ST} (Wright's fixation index) ระหว่างประชากรเท่ากับ +0.769 และค่าเฉลี่ย F_{ST} ระหว่างสปีชีส์ลดลงถึง +0.053 ข้อมูลนี้แสดงให้เห็นว่าการผสมพันธุ์จำกัดอยู่ภายในสปีชีส์เท่านั้น การจัดกลุ่ม UPGMA ตามหลักความห่างทางพันธุกรรมของ Nei โดยอาศัยความถี่แอลลีลของเอ็นไซม์ 12 ตำแหน่ง ผลการวิเคราะห์เป็น dendrogram แสดงให้เห็นว่าชนิดซับซ้อนของ *B. tau* นี้แบ่งออกเป็น 2 กลุ่ม คือ กลุ่มหนึ่งประกอบด้วยชนิด C และ I ส่วนอีกกลุ่มหนึ่งประกอบด้วยชนิด *B. tau* s.s (= A), D, E, F, และ G และยังพบว่าชนิด F และ G มีรูปร่างทางสัณฐานวิทยาคล้ายคลึงกันมากจึงนับว่าเป็น sibling species ที่แท้จริง

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The tephritid fruit fly, *Bactrocera* (*Zeugodacus*) *tau* (Walker), is an economically important pest of many fruits in South and South-east Asia. The fly preferentially attacks fruit of the family Cucurbitaceae, including species of *Cucumis, Luffa,* and *Trichosanthes*. However, *B. tau* has been reared from the fruits of several other plant families such as Anacardiaceae (*Dracontomelon* and *Mangifera* spp.), Moraceae (*Artocarpus* sp.), Oxalidaceae (*Averrhoa* sp.) and Sapotaceae (*Manilkara* sp.) (White and Elson-Harris, 1992). *Bactrocera tau* has been previously known under several different names including *Chaetodacus tau* (Walker), *Dacus hageni* (De Meijere), *D. nubilus* Hendel, and *D. tau* (Walker). This is evidence for the taxonomic confusion regarding this pest. It has been suggested that *B. tau* is made up of large complex of sibling species (Drew and Romig, 1997) and so presents a taxonomic challenge. Cytological analysis of heterochromatin in mitotic chromosomes supports this view, suggesting that *B. tau* actually consists of at least seven cryptic species tentatively named as *B. tau* sp. A, B, C, D, E, F and G (Baimai *et al*., 2000). The existence of these species is also supported by molecular analysis of COI gene (Jamnongbek *et al*., 2003). These taxonomic problems require intensive systematic and ecological studies so that pests of edible fruit crops can be diagnosed and their phylogenetic relationships and evolution can be understood.

Allozyme electrophoresis has been used to quantify the amount of genetic variation and resolve taxonomic relationships in several tephritid species complexes. Berlocher *et al*. (1993) used allozymes to revise the phylogeny of seven taxa in the *Rhagoletis pomonella* species complex from North America. A new sibling species of the *B. dorsalis* complex, *B. opiliae* from Australia, was described based on electrophoretic characters (Drew and Hardy, 1981). In addition, Satayalai (1995) reported electrophoretic evidence for several new species in the *B. dorsalis* complex in Thailand.

We present electrophoretic evidence supporting the existence of seven sibling species within the *B. tau* complex. Genetic markers for

species identification and phylogenetic relationships of these species are also described.

Materials and Methods

1. Sample collection

Larval samples of the *B. tau* complex were obtained from infested fruit collected from 22 populations distributed in 11 provinces of Thailand from April 1996, to February 1998 (Table 1). Larvae in the infested fruit were brought back to the laboratory at Mahidol University where they were reared to adults. Morphological characters (Tigvattananont, unpublished descriptions) were used to distinguish *B. tau* sp. A through to sp. I. The adults were stored in liquid nitrogen (-70ºC) for electrophoretic study. Voucher pin specimens are kept at King Mongkut Institute of Technology, Lat Krabang, Bangkok, Thailand, by S. Tigvattananont.

2. Enzyme electrophoresis

Sample preparations and polyacrylamide horizontal slab gel electrophoresis followed the methods of Green *et al*. (1990). Staining methods for enzymes were modified from Berlocher (1980), Harris and Hopkinson (1977) and Steiner and Joslyn (1979).

The following enzymes were examined using two kinds of gel and buffer system: (1) aspartate aminotransferase (AAT), alcohol dehydrogenase (ADH) and superoxide dismutase (SOD) run on

Table 1. Collection details for samples of seven species of the *Bactrocera tau* complex collected for electrophoretic study in Thailand [North: Nan (NA), Uttaradit (UD); Northeast: Mukda-Han (MH); West: Kanchanaburi (KB); Central: Phetchaburi (PH), Ratchaburi (RB); South: Ranong (RN), Phatthalung (PL), Pattani (PN), Yala (YL), Songkhla (SO)].

Population	Species	Collection code	Host-plant species	Sample size
1	A	NA(D)7	*Trichosanthes tricuspidata* [a]	111
2	A	UD(D)1	*Luffa cylindrica* [a]	60
3	A	MH(D)2	*Luffa cylindrica* [a]	40
4	A	PH(B)3	*Trichosanthes tricuspidata* [a]	63
5	A	RB(B)4	*Momordica cochinchinensis* [a]	34
6	A	YL(C)5	*Trichosanthes tricuspidata* [a]	84
7	A	PL(C)1	*Trichosanthes tricuspidata* [a]	38
8	A	PN(B)2	*Trichosanthes tricuspidata* [a]	85
9	A	SO(D)2	*Trichosanthes cordata* [a]	50
10	C	KB(S)50	*Momordica cochinchinensis* [a]	48
11	C	PH(B)1	*Momordica cochinchinensis* [a]	71
12	C	RB(B)4	*Momordica cochinchinensis* [a]	70
13	D	RN(H)22	*Trichosanthes tricuspidata* [a]	18
14	D	PL(C)1	*Trichosanthes tricuspidata* [a]	34
15	D	YL(C)5	*Trichosanthes tricuspidata* [a]	57
16	E	RN(M/2)492	*Strychnos thorelii* [b]	32
17	E	RN(M/2)499	*Strychnos thorelii* [b]	60
18	F	RN(M/2)223	*Hydnocarpus anthelminthicus* [c]	51
19	G	KB(S)3	*Hydnocarpus anthelminthicus* [c]	33
20	I	YL(C)5	*Trichosanthes tricuspidata* [a]	58
21	I	PN(B)2	*Trichosanthes tricuspidata* [a]	60
22	I	SO(D)2	*Trichosanthes cordata* [a]	47

[a] **Family Cucurbitaceae,** [b] **Family Strychnaceae,** [c] **Family Flacourtiaceae**

a 6.0% acrylamide matrix with a TEB buffer system, and (2) glucose phosphate isomerase (GPI), glyceral-3-phosphate dehydrogenase (G3PDH), isocitate dehydrogenase (IDH), malate dehydrogenase (MDH), malic enzyme (ME), and phosphogluconate dehydrogenase (PGD) run on a 5.5% acrylamide matrix with a TC buffer system.

To facilitate standardization among gels, reference samples of *B. dorsalis* and *B. tau* from laboratory colonies with known monomorphic electromorphs were run on each gel. The most common allele in *B. dorsalis* was taken as the "100" reference allele, and the mobilities of all other alleles were calculated in relation to this standard allele (Green *et al*., 1990).

3. Data analysis

Genetic differentiation between populations was analyzed using Wright's F-statistics. The total genetic variability (F_{IT}) was partitioned into within (F_{IS}) and between (F_{ST}) population variation (Wright, 1943; Wright, 1951; Weir and Cockerham, 1984). The unweighted pair-group method with arithmetic averages (UPGMA) was used to construct a phylogenetic tree based on Nei's unbiased genetic distance (Nei, 1978). Data analysis was performed using the software POPGENE version 1.1 (Yeh and Boyle, 1996). *Bactrocera cucurbitae*, which was used as an outgroup, is a member of the subgenus *Zeugodacus*. but morphologically distinct from the *B. tau* complex.

Results

A total of 1,204 individuals of the *B. tau* complex were obtained from different populations as shown in Table 1. Species A-G were the same as those described by Baimai *et al*. (2000) and species B and H were not included as the sample size was too small. Six host-plant species from three families were found to be infested with larvae of the *B. tau* complex. *Bactrocera tau* sp. A that emerged from fruit of four host-plant species were studied. Species C occurred in *Momordica cochinchinensis* fruit while species D and E were found in *Trichosanthes tricuspidata* and *Strychnos thorelii* fruit, respectively. The closely related sibling species F and G were found in fruit of the same species of host-plant, *Hydnocarpus anthelminthicus*. They occurred in allopatric populations, i.e., species F in Ranong, southern Thailand and species G in Kanchanaburi, western Thailand. Species I was found infesting *T. tricuspidata* and *T. cordata* fruit in southern Thailand.

Twelve loci of nine enzyme systems were interpretable and scorable (Appendix 1). Examples of gels are shown in Figure 1. All enzymes migrated anodally, except for *Adh-3*, which migrated cathodally in most cases (except for some individuals of species D in which it migrated anodally). Identification of all loci was unambiguous in all species except for loci *Adh-1* and *Adh-2* in species C, E and I. No bands were expressed for these two loci in these three species. This may be because the enzymes migrated too rapidly or they were silenced, either by a mutation at the active site or by regulation.

With the exception of *G3pdh* all loci were polymorphic based on the criterion that the frequency of the most abundant allele was less than 0.95. Alleles with frequencies less than 0.05 in each species were pooled into a "rare alleles" category.

The seven species can be divided into four groups based on electrophoretic data (Figure 2): (*i*) species D distinguished from other species by a single diagnostic allele for each of five loci, (*ii*) species E distinguished from other species by the presence of the Mdh^{100} and Sod^{110} alleles, (*iii*) species C and I which were di stinguishable from the other species by the possession of two unique alleles (Idh^{125} and $Pgd\text{-}2^{122}$), and (*iv*) species A (=*B. tau*), F and G distinguished from other species by possessing unique alleles at two loci ($Adh\text{-}1^{66,69}$ and $Adh\text{-}2^{71,74}$). Further details of these groups are given in what follows.

Of the seven investigated species in the *B. tau* complex, only species D showed unique fixed alleles (absolutely monomorphic). These alleles ($Adh\text{-}1^{100}$, $Adh\text{-}2^{100}$, Gpi^{100}, Me^{100} and $Pgd\text{-}2^{113}$) can be used as genetic markers for distinguishing this

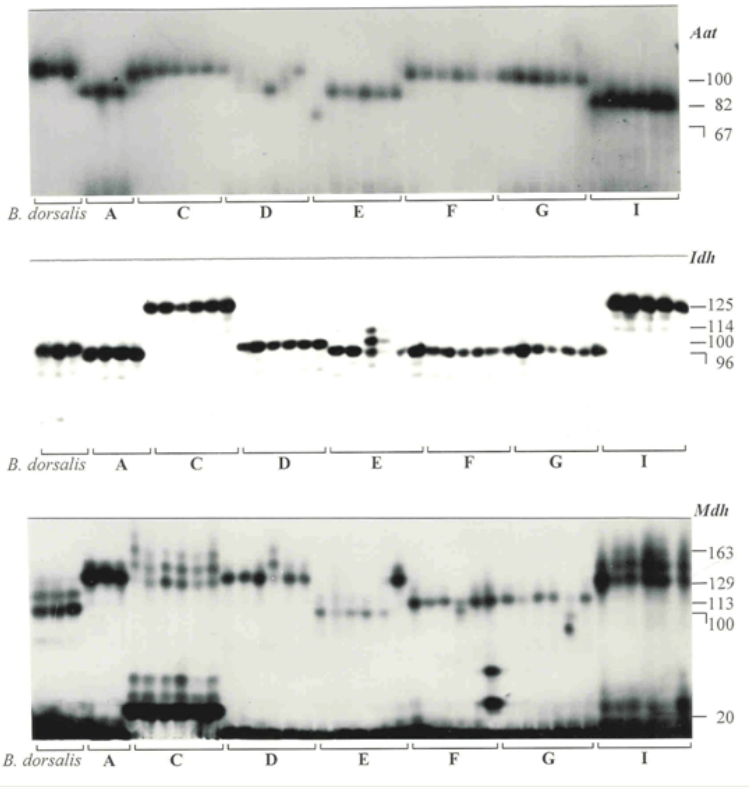

Figure 1. Gels showing common electromorphs at three loci of seven species of the *Bactrocera tau* complex. (species A [= *B. tau*], C, D, E, F, G and I).

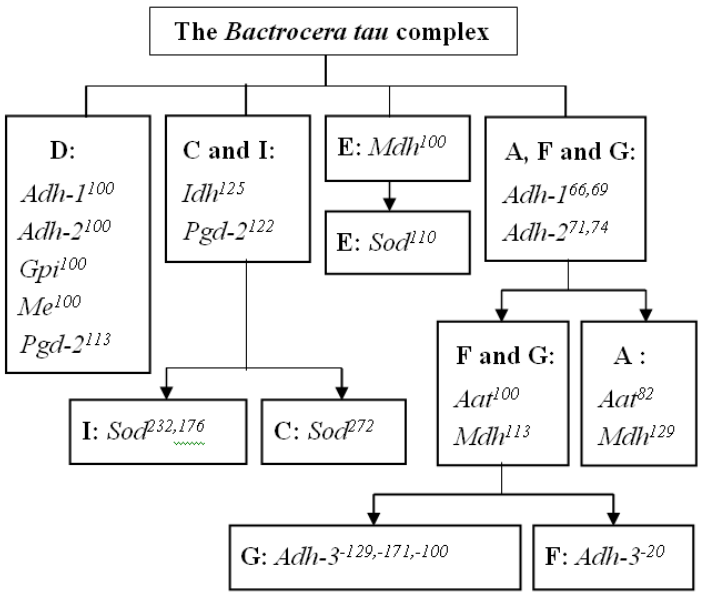

Figure 2. Electromorphic alleles used as genetic markers for classification of the seven species (A, C, D, E, F, G and I) of the *Bactrocera tau* complex (see Appendix 1).

Table 2. Summary of F-statistics for seven species of the *Bactrocera tau* complex (species and populations as in Table 1, standard error in parentheses). I, all populations; II, mean value per species (n = 5 species including A, C, D, E and I); III, mean value per pair of sympatric species (n = 6 pairs); IV, the two most closely related species that occur in allopatry, i.e., species F and G.

Criterion	F_{IS} [a]	F_{ST} [a]	F_{IT} [b]
I	0.352	0.769	0.850
II	0.158	0.053	0.203
	(0.086)	(0.029)	(0.083)
III	0.094	0.590	0.629
	(0.041)	(0.094)	(0.079)
IV	0.334	0.131	0.421

[a] **F_{IS} and F_{ST} are measures of the genetic variability within and among populations, respectively;**
[b] **F_{IT} measures the total genetic variability.**

species from the other six.

Although no single diagnostic allele was detected for distinguishing unambiguously between species A, C, E, F, G and I, allele frequency differences over multiple loci were sufficient to distinguish individuals of each species with a high degree of probability. Two enzyme loci, *Mdh* and *Sod*, are useful in diagnosing species E. Mdh^{100} appears to be the only specific electromorph, with a typical frequency of 0.958. If rare alleles appear at the *Mdh* locus, then Sod^{110} can be used to distinguish species E.

The fixation of Idh^{125} and $Pgd\text{-}2^{122}$ distinguished species C and I from all other species of the *B. tau* complex. Sod^{272}, which is fixed in species C, can then be used to separate species C from species I, in which Sod^{272} does not occur.

Species A, F and G formed a group in which each species shared two or more of the following alleles: $Adh\text{-}1^{66}$, $Adh\text{-}1^{69}$, $Adh\text{-}2^{71}$ and $Adh\text{-}2^{74}$. No other species had any of these alleles. Species A was separated from species F and G by using Aat^{82} or Mdh^{129} (Figure 1). The most difficult separation in the *B. tau* complex is species F from G, since they share at least one allele at all loci, although at different frequencies. However, *Adh-3* may be of use in distinguishing these species. From Figure 2 and Appendix 1, $Adh\text{-}3^{-20}$ is possessed by species F (91.1%) but not by species G.

Table 2 presents F-statistics of the *B. tau* complex. The F_{ST} value for all populations was +0.769. Partitioning the populations by species resulted in a mean of F_{ST} value of +0.053. Values of F-statistics were calculated for each pair of sympatric host-plant species: species A and C from *M. cochinchinensis* collected in Ratchaburi; species A and D from *T. tricuspidata* in Phatthalung; species A and D, species A and I, species D and I from *T. tricuspidata* in Yala and species A and I from *T. tricuspidata* in Pattani. There was considerable genetic variation among populations in sympatry (F_{ST} = +0.590), indicating that more than half of the genetic diversity occurred between species. In contrast, the pair of most closely related allopatric species (F and G) which infest the same host-plant species showed little variation (F_{ST} = +0.131).

Table 3 shows the average genetic distances (*D*) among these species of the *B. tau* complex. The average value of *D* between populations within species was 0.023, and ranged from 0.002 (species I) to 0.050 (species E). The greatest genetic distance between species was between C and E (1.811), while the least was between species F and G (0.136). If genetic distance between species reflects evolutionary divergence (Marinkovic *et al.*,

1978), then the phylogeny of the *B. tau* complex could be represented by Figure 3. The *B. tau* complex can be divided into 2 major groups: group 1 consisting of species C and I, and group 2 comprising species A, D, E, F and G.

Discussion

Morphological (Tigvattananont, unpubl.) and cytological observations (Baimai *et al*., 2000) as well as biogeographical differentiation of host-plant species (Tigvattananont, unpubl.) have revealed at least seven closely related species within the *B. tau* complex. Our electrophoretic studies confirm the existence of these seven species. These species were temporarily designated as species A (= *B. tau*), C, D, E, F and G by Baimai *et al*. (2000) and species I (Baimai *et al*., unpubl.) in their cytological investigations.

We compared allozyme polymorphisms of these seven species of the *B. tau* complex and have established diagnostic electromorphs for each of them. Thus, species D can be distinguished on the basis of a fixed allele in one enzyme. Multiple loci can be used as genetic markers in all other species (Figure 2).

The most closely related allopatric species (F and G) infest *H. anthelminthicus*, but in different localities. Species F was found in Ranong province while species G occurred in Kanchanaburi province, about 500 kilometers north of Ranong. No single fixed allelic difference was observed between these two sibling species but there were a number of gene frequency differences (Appendix 1). The average Nei's unbiased genetic distance between species F and G was estimated as 0.136 (Table 3) and this combined with genetic variation of 13.1% (Table 2), supports the separation of these

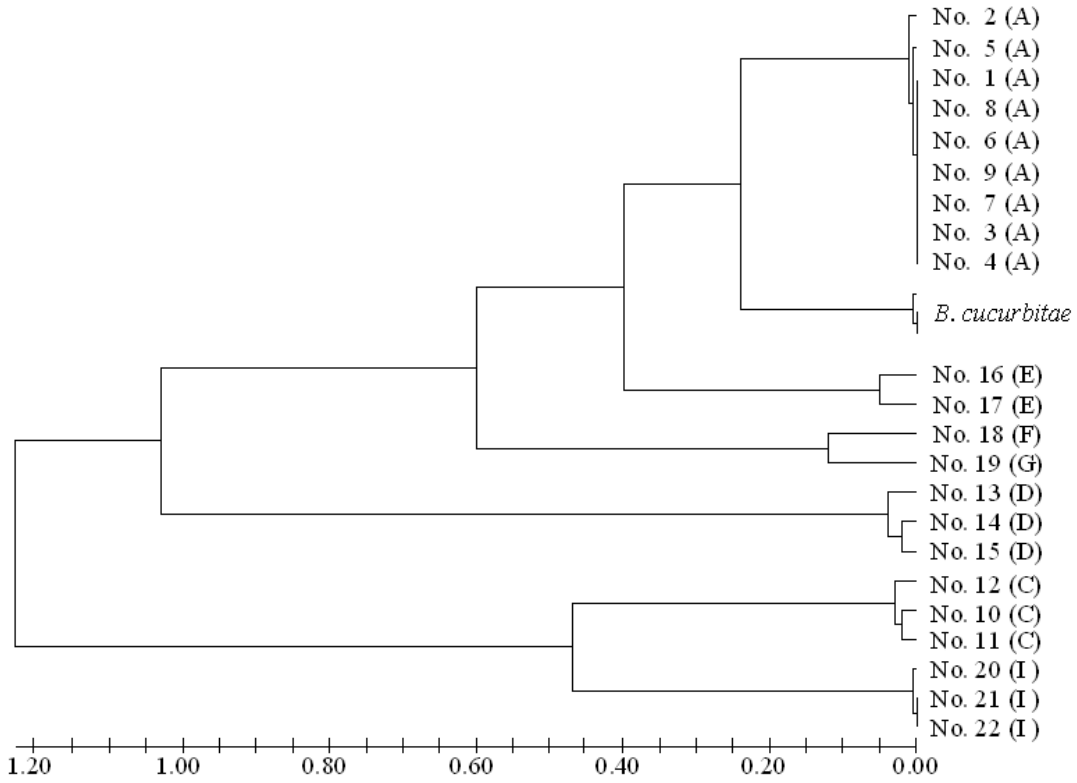

Figure 3. A phylogram of the *Bactrocera tau* complex. *Bactrocera cucurbitae* was used as an outgroup species. (scale = values of genetic distance between populations).

Table 3. Mean Nei's (1978) unbiased genetic distances (D) between species of the *Bactrocera tau* complex and *B. cucurbitae* (=Y) ((indicates mean genetic distances between populations within species).

Species	No. of populations	A	C	D	E	F	G	I	Y
A	9	0.004*							
C	3	1.544	0.025*						
D	3	0.937	1.804	0.032*					
E	2	0.384	1.811	1.158	0.050*				
F	1	0.792	1.567	1.548	0.700	-			
G	1	0.379	1.193	1.495	0.531	0.136	-		
I	3	0.822	0.780	0.878	0.916	1.522	1.399	0.002*	
Y	1	0.289	2.293	1.475	0.603	1.144	0.731	1.249	0.001*

two species as shown by cytological evidence (Baimai *et al*., 2000).

The two main groups of the *B. tau* complex, evident in the phylogenetic tree (Figure 3), are also supported by morphological evidence. The apex of the aculei of both species C and I which comprise one group has a unique trilobed shape, when examined by scanning electron microscopy (Phinchongsakuldit, personal communication). This morphological characteristic is similar to that of *B. nubilus* as described by Hardy (1973). Hence, species C and I could belong to the same group as *B. nubilus*. This group may represent another interesting complex of species.

Species of the *B. tau* complex, especially species A, E, F and G, are morphologically similar and they may be considered as cryptic species. The genetic distances between these cryptic species (Table 3) were greater than that between sibling species of *Rhagoletis* for which the average genetic distance is smaller than 0.05 for 47% of the loci (Berlocher and Bush, 1982).

Multiple lines of evidence from alternative analyses (electrophoresis, morphology and cytogenetics) are useful for species identification in cases of species complexes. For example, *B. tau* (species A) and species E show very little difference in sex chromosomes (Baimai *et al*., 2000) and the adult males are morphologically nearly indistinguishable (Tigvattananont, unpubl.). However, electrophoretic data show several alleles with a high probability of occurrence that can be used to separate species, e.g., Mdh^{129} for species A and Mdh^{100} for species E (Figure 1), which are easy to recognize. The two siblings, F and G, can hardly be separated morphologically (Tigvattananont, unpubl.) and electrophoretically (this study), but they can be easily distinguished by differences in appearance of autosome no. 4 and of the Y chromosome in their mitotic karyotypes (Baimai *et al*., 2000). Species C and D form a final example. These two species have been found to exhibit similar mitotic karyotypes based on the amount and distribution of pericentric heterochromatin in the autosomes and sex chromoso}es (Baimai *et al*., 2000), but they can be easily distinguished by external morphology (Tigvattananont, unpubl.) and electromorphs (this study). Thus, all these approaches are necessary for the study of sibling species of closely related species as exemplified by the *B. tau* complex.

Acknowledgements

We wish to thank R. Sharpe, P.J. Grote and J. Milne for valuable comments on the manuscript. We also thank S. Tiengtrong and C. Piyottip for technical assistance. This work was supported by the Thailand Research Fund (RTA 3880008 and RTA 4280001) and the TRF/BIOTEC Special Program for Biodiversity Research and Training (BRT 542009).

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Appendix 1. Allele frequencies at 12 allozyme loci for seven species (A, C, D, E, F, G and I) of the *Bactrocera tau* complex. (N = sample size; * indicates undetectable electromorph).

Locus	A	C	D	E	F	G	I
N	565	189	109	92	51	33	165
Aat							
100	0	0.751	0.297	0	0.961	0.985	0
90	0	0.213	0	0	0	0	0
82	0.981	0	0.683	0.973	0	0	0.982
Rare alleles	0.019	0.036	0.020	0.027	0.039	0.015	0.018
Adh-1							
100	0	*	1.000	*	0	0	*
69	0	*	0	*	1.000	0.515	*
66	1.000	*	0	*	0	0.485	*
Adh-2							
100	0	*	1.000	*	0	0	*
74	0	*	0	*	1.000	0.515	*
71	1.000	*	0	*	0	0.485	*
Adh-3							
50	0	0	0.421	0	0	0	0
-20	0	0	0.138	0.120	0.911	0	0
-100	0	0	0.441	0	0	0.062	0
-129	0.871	0.889	0	0.087	0	0.750	0
-171	0	0	0	0.761	0	0.094	0.964
Rare alleles	0.129	0.111	0	0.032	0.089	0.094	0.036
G3pdh							
87	0.993	0.997	1.000	0.978	1.000	1.000	0.973
Rare alleles	0.007	0.003	0	0.022	0	0	0.027
Gpi							
122	0	1.000	0	0	0	0	0.988
108	0	0	0	0	1.000	0.515	0
100	0	0	1.000	0	0	0	0
95	0.992	0	0	0.940	0	0.485	0
Rare alleles	0.008	0	0	0.060	0	0	0.012
Idh							
125	0	1.000	0	0	0	0	1.000
100	0	0	0.973	0	0	0	0
96	0.989	0	0	0.907	1.000	0.970	0
Rare alleles	0.011	0	0.027	0.093	0	0.030	0
Mdh							
129	0.981	0	0.986	0	0	0	0.992
113	0	0	0	0	0.971	0.849	0
100	0	0	0	0.958	0	0	0
20	0	1.000	0	0	0	0	0
Rare alleles	0.019	0	0.014	0.042	0.029	0.151	0.008
Me							
122	0	1.000	0	0	0	0	0.991
100	0	0	1.000	0	0	0	0
97	0.993	0	0	0.949	1.000	1.000	0

Appendix 1. (continued)

Locus	A	C	D	E	F	G	I
Rare alleles	0.007	0	0	0.051	0	0	0.009
Pgd-1							
136	0	0.060	0	0	0	0	0
128	0	0.156	0	0	0	0	0
122	0.964	0.550	0.923	0.978	1.000	1.000	0.973
107	0	0.161	0.064	0	0	0	0
Rare alleles	0.036	0.073	0.013	0.022	0	0	0.027
Pgd-2							
122	0	1.000	0	0	0	0	1.000
113	0	0	1.000	0.058	0	0	0
97	0.984	0	0	0.942	1.000	1.000	0
Rare alleles	0.016	0	0	0	0	0	0
Sod							
272	0	1.000	0	0	0	0	0
232	0.833	0	0.959	0	0	0	0.685
176	0.148	0	0	0	0.980	0.970	0.267
110	0	0	0	0.804	0	0	0
Rare alleles	0.019	0	0.041	0.196	0.020	0.030	0.048