**DNA barcode variability and host plant usage of fruit flies
(Diptera: Tephritidae) in Thailand**

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DNA barcode variability and host plant usage of fruit flies (Diptera: Tephritidae) in Thailand

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Running title: DNA barcoding of fruit flies in Thailand

Abstract

The objectives of this study were to examine the genetic variation in fruit flies (Diptera: Tephritidae) in Thailand and to test the efficiency of the mitochondrial cytochrome *c* oxidase subunit I (COI) barcoding region for species-level identification. Twelve fruit fly species were collected from 24 host plant species of 13 families. The number of host plant species for each fruit fly species ranged between one and 11, with *Bactrocera correcta* found in the most diverse host plants. A total of 123 COI sequences were obtained from these fruit fly species. Sequences from the NCBI database were also included, for a total of 17 species analyzed. DNA barcoding identification analysis based on the best-close match method revealed a good performance, with 94.4% of specimens correctly identified. However, many specimens (3.6%) had ambiguous identification, mostly due to intraspecific and interspecific overlap between members of the *B. dorsalis* complex. A phylogenetic tree based on the mitochondrial barcode sequences indicated that all species, except for the members of the *B. dorsalis* complex, were monophyletic with strong support. Our work supports recent calls for synonymization of these species. Divergent lineages were observed within *B. correcta* and *B. tuberculata*, and this suggested that these species need further taxonomic reexamination.

Key words: *Bactrocera*, DNA barcode, fruit fly, host specificity, Tephritidae

Introduction

Fruit flies (Diptera: Tephritidae) are among the most important insect pests of fruits and vegetables. Globally, there are approximately 5 000 described species of fruit flies assigned into six subfamilies (Uchôa 2012). Most pest species of Tephritidae belong to the genera *Anastrepha* Schiner, *Bactrocera* Macquart, *Ceratitis* Macleay, *Dacus* Fabricius, and *Rhagoletis* Loew (White and Elson-Harris 1992; Van Houdt et al. 2010). The genus *Bactrocera* is one of the largest genera of Tephritidae, with more than 600 described species arranged in 30 subgenera (Drew 1989, 2004; Drew and Hancock 1994). Several species of the genus *Bactrocera* are capable of infesting a wide variety of economically important fruits and vegetables (White and Elson-Harris 1992; Allwood et al. 1999; Clarke et al. 2005). For example, the *Bactrocera dorsalis* (Hendel) species complex, which includes more than 75 sibling species, can infest nearly 200 host plant species from 50 plant families (Drew 1989; Drew and Hancock 1994; Hollingsworth et al. 2003; Clarke et al. 2005; Kumar et al. 2011).

Rapid and accurate identification of specimens at the species level is a primary requirement for all aspects of fruit fly biology and management (Armstrong and Ball 2005; Jinbo et al. 2011). However, morphological identification of fruit flies has some limitations. The taxonomic identification of fruit flies based on morphology for the immature stages (i.e. egg, larva, and pupa) is extremely difficult because of a lack of diagnostic characters to distinguish species (Armstrong et al. 1997). Morphological identification is reliable only in the adult stage, but faces difficulties in the case of high within-species variation or morphological similarity of closely-related species, such as found within species complexes. The classic example is the case of the *B. dorsalis* species complex. Three species, namely *B. papayae* Drew and Hancock, *B. philippinensis* Drew and Handcock, and *B. invadens* Drew, Tsuruta and White, were recognized as different species based on morphological differences (e.g. abdominal color patterns, aedeagus length, wing shape, postsutural lateral vittae and

thoracic color patterns). However, multidisciplinary studies including molecular genetics (using the nuclear ribosomal internal transcribed spacer (ITS), mitochondrial cytochrome *c* oxidase subunit I (COI) and NADH dehydrogenase 4 (ND4) genes), cytogenetics, mating compatibility, and chemoecology suggested that these species should be merged into a single biological species with *B. dorsalis* (Schutze et al. 2015a). The cytogenetic technique has also contributed to fruit fly taxonomy (Zacharopoulou et al. 2011), which can be used to differentiate the members of some species complexes (Baimai et al. 2000). However, cytological taxonomy requires high levels of personal expertise for interpretation and is workable only for some species because of poor quality in visualization of the chromosomes.

Previous studies have shown that DNA barcodes could effectively be used to identify fruit fly species (Armstrong and Ball 2005). The barcode region (Hebert et al. 2003; Ratnasingham and Hebert 2007) of the COI sequences have been used successfully for specimen identification to the species level (Armstrong and Ball 2005; Blacket et al. 2012), although discriminating members of species complexes was less successful (Barr et al. 2012; Blacket et al. 2012; Jiang et al. 2014). In this study, we investigated the efficiency of DNA barcoding for identification of morphospecies of fruit flies of the genus *Bactrocera* in Thailand. Although several studies have now used COI barcode sequences in *Bactrocera*, the success of this method still relies mainly on the availability of the database (Virgilio et al. 2010; Jinbo et al. 2011). In addition, it is also preferable for genetic variation across the geographic range of the species to be included in the database. Thus, specimens from a wide geographical range should be included to acknowledge intraspecific genetic variation (Meyer and Paulay 2005). In Thailand, fruit flies are highly diverse: there are at least 103 species from 15 subgenera of four genera (*Bactrocera*, *Dacus*, *Ichneumonopsis*, *Monacrostichus*) recorded in the country (Drew and Romig 2013). DNA barcode sequences have only been reported from seven species (*Bactrocera correcta* (Bezzi), *B. latifrons* (Hendel), *B. umbrosa*

(Fabricius), *B. zonata* (Saunders), *B. caudata* (Fabricius), *B. isolata* (Hardy), *B. rubella* (Hardy)) (Meeyen et al. 2014; Jiang et al. 2014; Kunprom et al. 2015). Therefore, our results would increase the sequence library for fruit fly species in Thailand, and this will encourage rapid and accurate specimen identification. We also report the host plant species of the fruit flies included in our study, which is generally lacking in previous DNA barcode studies of the Tephritidae. This information will be useful for further control and management as well as fruit fly biology studies.

Materials and Methods

Sample collection and morphological identification

Fruit fly specimens were collected from 25 sites in Thailand between March 2012 and February 2015 (Table 1 and Fig.1). The infested fruits from 24 host plant species belonging to 13 families were obtained from both natural forests and fruit orchards (Table 2). The infested fruit was placed in plastic boxes containing sawdust in the bottom, covered by calico, and kept at room temperature until adults emerged. Adult flies were collected and stored in 80% ethanol at -20°C . Voucher specimens were kept at Department of Biology, Faculty of Science, Mahasarakham University, Thailand. Species were identified morphologically using the keys and descriptions of White and Elson-Harris (1992), Plant Health Australia (2011), and Drew and Romig (2013).

DNA extraction, amplification and sequencing

Genomic DNA was extracted from each adult fly, randomly chosen from each host plant species from each collecting locality using the GF-1 Tissue DNA Extraction Kit (Vivantis, Selangor Darul Ehsan, Malaysia) following the manufacture's protocol, with a final elution volume of 100 μl . A portion of the mitochondrial cytochrome *c* oxidase I (COI)

gene was amplified using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). Polymerase chain reactions (PCR) were carried out in a final volume of 50 µl containing 2 µl of DNA template, 2 µl of each primer (10 µM), 3 µl of 50 mM MgCl₂, 5 µl of 10x PCR buffer, 1.6 µl of 10 µM dNTPs, and 0.4 µl of *Taq* DNA polymerase (5 U/ µl). The temperature profile of the PCR included an initial denaturation at 94°C for 2 min followed by 36 cycles of 94°C for 30 sec, 45°C for 45 sec, and 72°C for 45 sec with the final extension at 72°C for 5 min. PCR products were checked with a 1% agarose gel and purified using the HiYield Gel/PCR DNA Extraction Kit (RBC Bioscience). Sequencing was performed in both directions at Macrogen (Seoul, Korea) using the same primers as in the PCR. DNA sequences were aligned using CLUSTAL X (Thompson et al. 1997), with a final visual inspection. Sequences were translated into amino acids to verify that they were correct using MEGA 6 (Tamura et al. 2013).

Data analysis

A total of 1146 COI sequences were included in the analyses. One hundred and twenty three sequences (GenBank accession numbers: KT588319-KT588441) were obtained in this study, and the remainder were from GenBank. Sequences from GenBank were obtained using the species name as the key word to search for the data. We included all of the available sequences in GenBank of the species that we detected during our study plus sequences of the species that know to occur in Thailand. In total, 17 species were included in the data analysis; 12 were obtained in the present study, and five were from GenBank. According to a previous suggestion (Schutze et al. 2015a), members of the *B. dorsalis* complex (*B. dorsalis*, *B. invadens*, *B. papayae*, *B. philippinensis*) were treated as a single morphological species for phylogenetic analysis. However, the original morphological

identifications were retained for the detailed presentation of the tree of this species complex. Intraspecific and interspecific genetic divergences were calculated based on the Kimura 2-parameter (K2P) model using MEGA 6 (Tamura et al. 2013). The frequency of successful identification was tested using the best match and best close match methods in the program TaxonDNA (Meier et al. 2006). The best match method assigns specimens to species according to the best-matching barcode sequence (Meier et al. 2006). The best close match method assigns specimens to species based on the level of sequence similarity below the threshold value, which is 95% of all intraspecific distances (Meier et al. 2006). The published sequences of all species were used as the reference data set for the best close match analysis. All unique haplotypes (n = 461) were used to infer phylogenetic relationships using the maximum-likelihood (ML) method implemented in PhyML 3.0 (Guindon et al. 2010). Node support was calculated using approximate likelihood ratio tests (Anisimova and Gascuel 2006). The program jModeltest v.2.1.9 (Darriba et al. 2012) was used to select the best-fit DNA substitution model for the ML analysis based on the Akaike information criterion (AIC). The best-fit model was the general time-reversible (GTR) model with a proportion of invariable sites of 0.382 and with a gamma distribution of 1.006. *Ceratitis capitata* (Wiedemann) and *Rhagoletis pomonella* (Walsh) (GenBank accession numbers DQ116370 and DQ116382, respectively) were used as the outgroups (Krosch et al. 2012). To present the relationship between the host plant and the fruit fly phylogeny, information about the host plant families from Table 2 were mapped onto the ML tree.

Results

Fruit fly species and host plant diversity

A total of 12 fruit fly species was collected in this study (Table 2). Among these, there were 11 known, and one was an unknown species (*Bactrocera* sp. 1) of the subgenus

Javadacus. These species were collected from 24 host plant species of 13 families (Table 2). *B. correcta* is the fruit fly species that was found from the most diverse range of host plants in this study. This species was collected from 11 plant species distributed in 10 plant families. *Bactrocera* (*Zeugodacus*) *scutellaris* (Bezzi), *B. (Bactrocera) thailandica* Drew and Hancock, *B. (Bactrocera) tuberculata* (Bezzi), *B. (Gymnodacus) symplocos* Drew and Romig, *B. (Hemigymnodacus) diversa* (Coquillett), and *Bactrocera* sp. 1 were collected from only a single host plant species each (Table 2).

COI barcode sequence variation and DNA barcode statistics

A total of 1 146 sequences from 17 fruit fly species (12 species collected in the present study and 5 species obtained from GenBank) of the genus *Bactrocera* were included in the data analysis. Intraspecific genetic divergence based on K2P genetic distances ranged from 0% to 10.47%, with a mean of 0.64% (Table 3). The maximum intraspecific genetic divergence was found in *B. dorsalis* complex (10.47%). The K2P genetic distance to the closest species ranged from 0% to 15.88%. Low interspecific genetic divergences (0%) were found among members of the *B. dorsalis* complex (i.e. *B. carambolae* and *B. dorsalis*). Specimens of *B. tau* and *B. cucurbitae* also showed very low genetic divergence, with a minimum K2P value of 0.88%. Levels of intraspecific and interspecific genetic divergence values showed overlap for four species (Fig. 2). To compare our results based only on species that occur in Thailand with all available sequences of genus *Bactrocera* in the public database, we performed a barcode gap analysis in BOLD (Ratnasingham and Hebert 2007; accessed 18 February 2016). In total, 1 669 sequences represent 68 species were included in the analysis. The levels of intraspecific (0% to 12.49%, mean 1.63%) and interspecific genetic divergence (smallest pairwise distance to the nearest neighbor species, 0% to 12.63%, mean 3.5%) were similar to our data. However, some COI sequences in this database could

be from misidentified specimens. For example, the same COI haplotype was found for morphologically highly different species, such as *B. carambolae* and *B. cucurbitae*. This could be a limitation of using all sequences from public databases for conduct barcode-based identifications.

The best match and best close match methods revealed similar levels of correct specimen identification to species (Table 4). The overall percent correct specimen identification for the best close match method was 94.42% (1 105 of 1 146 sequences), 2.87% was for ambiguous identification (33 sequences), and 0.52% was for misidentification (six sequences). Two sequences (0.17%) were without any match closer than the threshold value (2.05%), which is a genetic distance at 95% of all intraspecific genetic distances. With only two exceptions for *B. cucurbitae* and one for *B. tau* that show low (<0.88%) interspecific genetic divergence, all sequences that were ambiguous or displayed misidentifications were from members of the *B. dorsalis* complex. One haplotype was shared by different species (*B. dorsalis* and *B. carambolae*) of the members of the *B. dorsalis* complex.

Phylogenetic relationships

A maximum likelihood (ML) tree (Fig. 3) revealed two main clades (clades I and II). Clade I was comprised of eight species of the subgenus *Bactrocera*, including three species of the *B. dorsalis* complex (*B. carambolae*, *B. dorsalis*, *B. thailandica*), *B. correcta*, *B. latifrons*, *B. umbrosa*, *B. zonata*, and *B. tuberculata*, and one species of the subgenus *Gymnodacus* (*B. (Gymnodacus) symplocos*). Clade II was comprised of six species of the subgenus *Zeugodacus* (*B. caudata*, *B. cucurbitae*, *B. isolata*, *B. rubella*, *B. scutellaris*, *B. tau* complex), one species of the subgenus *Hemigymnodacus* (*B. diversa*), and one unknown species (*Bactrocera* sp. 1). Clade II was further divided into two subclades. *B. caudata*, *B. isolata*, *B. rubella*, *B. scutellaris* and *B. diversa* comprised clade II-1. *B. cucurbitae*, *B. tau*

complex, and one unknown species (*Bactrocera* sp. 1) formed clade II-2. At the species level, all species were monophyletic with strong bootstrap support, except for members of the *B. dorsalis* complex (Fig. 4). Three species (*B. invadens*, *B. papayae*, *B. philippinense*) were recently treated as the synonyms of *B. dorsalis* (Schutze et al. 2015a) and formed a single clade (Fig. 4). Thus, our results supported this synonymization. Another member of the *B. dorsalis* complex, *B. carambolae*, was treated as a valid species based on multidisciplinary evidence (Schutze et al. 2015). The ML tree partially supported this as most of the haplotypes of this species formed a single clade, although two haplotypes of *B. dorsalis* were also included in this clade (Fig. 4). Specimens of an unknown species, *Bactrocera* sp. 1, formed a monophyletic clade with strong support (posterior probability = 1).

Members of the fruit fly species in clade I were found in diverse host plant families, notably, *B. dorsalis* and *B. correcta* (Fig. 3). All 13 families (20 species) of the host plants recorded in this study were exploited by the fruit fly species in clade I. In contrast, only two plant families (five species) were recorded for the fruit fly species in clade II. The family Cucurbitaceae is the major host plant for the fruit fly species in this clade (Fig. 3).

Discussion

The primary objective of the DNA barcode method is to identify unknown specimens to known species using a short DNA sequence (Hebert et al. 2003). Previous studies indicated that the DNA barcoding technique based on COI sequences was effective for specimen identification of fruit flies (Armstrong and Ball 2005; Barr et al. 2012; Blacket et al. 2012) as well as other insects (e.g., Pramual and Adler 2014). Our results revealed a good performance (96.42% correct identification) of the DNA barcode for identification of morphospecies of fruit flies in Thailand. However, a considerable proportion (3.58%, 41 sequences) of unsuccessful identification (ambiguous and incorrect identification) was also revealed. Nearly

all of the ambiguous or incorrect identifications were due to overlapping intraspecific and interspecific genetic divergences among members of the *B. dorsalis* complex (i.e., between *B. dorsalis* and *B. carambolae*). A previous study also found that the existence of a species complex reduced the efficiency of the DNA barcode for species differentiation. Jiang et al. (2014) found that when species complexes (*B. dorsalis* and *B. tryoni* complexes) were included in the analysis, the DNA barcode only had successful identification for 74% of species.

Unsuccessful identification of the *B. dorsalis* complex was correlated to the phylogenetic analysis based on COI barcoding sequences that found the members of the *B. dorsalis* complex included in this study (*B. carambolae*, *B. dorsalis*) were not recovered as reciprocally monophyletic. Several factors could explain this phenomenon including (i) incomplete lineage sorting, (ii) interspecific introgression, (iii) inadequate phylogenetic signal for the DNA sequences being used, and (iv) imperfect taxonomy (Funk and Omland 2003).

High intraspecific genetic divergences were found among the members of *B. dorsalis*, and the successful identification of other species suggested that the COI sequence used in this study possessed enough variation. Extensive taxonomic studies of the *B. dorsalis* species complex based on a multidisciplinary approach using morphology, molecular genetics, cytogenetics, sexual compatibility, and chemoecology (Schutze et al. 2012, 2015a, b; Boykin et al. 2014) provided consistent evidence to support *B. papayae*, *B. philippinensis*, and *B. invadens* as junior synonyms of *B. dorsalis*, while *B. carambolae* was retained as a valid species (Schutze et al. 2015a). According to these studies, imperfect taxonomy is unlikely to explain the species non-monophyly of *B. dorsalis* and *B. carambolae*. Therefore, the most likely explanations are incomplete lineage sorting and interspecific introgression. Although *B. carambolae* was treated as the valid biological species, this species is closely related to *B.*

dorsalis (Schutze et al. 2015a). Phylogenetic analysis based on multiple gene sequences also found that *B. carambolae* is nested within the paraphyletic *B. dorsalis* clade (San Jose et al. 2013). As these species are closely related, incomplete lineage sorting could explain the species non-monophyly, which led to unsuccessful barcode-based identifications. Interspecific introgression could also explain this situation. Although the two species are valid biological species, and differentiation in mating behavior resulting in a low rate of interbreeding was observed (Schutze et al. 2013), a field cage study revealed that interspecific hybridization could occur between these species (McInnis et al. 1999).

In addition to providing a rapid and accurate method for specimen identification, DNA barcoding can also reveal hidden diversity. Our results revealed that five species (*B. correcta*, *B. cucurbitae*, *B. dorsalis*, *B. carambolae*, *B. tuberculata*) possessed high (>3%) intraspecific genetic divergence. Previous studies in other insects, such as black fly (Simuliidae) (Pramual and Adler 2014), freshwater midge (Chironomidae) (Proulx et al. 2013), and fruit fly (Jiang et al. 2014), suggested that species possessing intraspecific genetic variation higher than 3% were likely to be a species complex. Two genetically divergent lineages were revealed in the phylogenetic analysis of *B. correcta*. The results were consistent with previous studies. Population genetics and molecular phylogenetic studies of *B. correcta* in Thailand found two genetically divergence lineages of this species (Jamnongluk et al. 2003a; Kunprom et al. 2015). These lineages were geographically sympatric and found on the same range of host plant species. Examination of the relationship between host plant families and genetic lineages on the ML tree of this species revealed no apparent association (Fig. 5). Therefore, it is likely that they are two genetically divergent species, but morphologically similar within the *B. correcta* morphospecies. Further investigation is required to determine its taxonomic status.

Specimens of *B. tuberculata* included in this study also showed two divergent lineages. These lineages were associated with geographic origin, i.e., Thailand and China. Genetic differentiation between these lineages was high (3.87%). Thus, it is likely that they could be distinct species. Morphological comparisons with the photographs of this species in the DNA barcode database (www.boldsystems.org) revealed that they were slightly different on the abdomen. *B. tuberculata* from Thailand collected in this study had a pale fulvous on the tergum II at the position adjoining the tergum III, with a narrow transverse black band across the anterior edge reaching the narrow black lateral edge. These characters agree well with the description of this species by Drew and Roming (2013). In contrast, the specimen of *B. tuberculata* from China reported in BOLD had a fulvous tergum II adhering to the tergum III, but the narrow transverse black band did not reach the lateral edges. Therefore, the taxonomic status of these species needs reexamination.

Bactrocera sp.1 was collected from *Coccinia grandis* (L.) Voigt. This species was morphologically classified into the subgenus *Javadacus* as it had a pair of scutellar setae and prescutellar setae, anterior supra-alar and postpronotal setae absent. All male specimens had a pecten of cilia on the abdominal tergum III and a posterior lobe of the male surstylus long. These characteristics are considered to be the dominant features of the subgenus *Javadacus* (Drew and Roming 2013). *Bactrocera* sp.1 is similar to *B. trilineata*. They are also found on the same major host plant, *C. grandis* of the family Cucurbitaceae. However, *Bactrocera* sp. 1 could be differentiated from *B. trilineata* by the color of the abdominal tergum III. *Bactrocera* sp.1 had a broad transverse black band across the tergum III covering two-thirds of the tergum, but *B. trilineata* had a broad transverse black band across the tergum III covering almost the entire tergum. The scapular setae on the thorax was the most distinctive character differentiating *Bactrocera* sp. 1 from *B. trilineata*. The former species had two scapular setae, while the latter had four. There are no reports for the COI barcoding sequence

of *B. trilineata*; thus, we were unable to compare these species. Therefore, further taxonomic work is needed to clarify the species status of *Bactrocera* sp. 1.

Bactrocera tau in Thailand was proposed to be a species complex comprised of nine (A – I) cryptic species (Baimai et al. 2000; Sumrandee et al. 2011). Molecular genetics and morphometric analysis of the wing shape supported the distinctions of some species within the complex (Jamnongluk et al. 2003b; Thanaphum and Thaenkhram 2003; Kitthawee and Dujardin 2010; Dujardin and Kitthawee 2013). Our results, however, revealed a single monophyletic lineage with low genetic diversity for *B. tau*. The most likely explanation for this situation is that our specimens represent a single species, *B. tau* A. Although we were not able to identify our specimens of *B. tau* into cryptic species because this would have required a close examination of the microscopic morphology of the shape of the ovipositor (Sumrandee et al. 2011), Baimai et al. (2000) suggested that geographic origin and host plant could be used for cryptic species recognition in this species. Our specimens were collected from the north and northeast of Thailand from three host plant species that only *B. tau* A was recorded from. Therefore, it is likely that our specimens included in this study represent only a single species (i.e. *B. tau* A) of the *B. tau* species complex. The addition of other species within the *B. tau* complex is needed for fully understanding the genetic diversity and efficiency of the DNA barcode for cryptic species delimitation in this group.

In conclusion, our results indicated that COI barcode sequences were successful for identifying fruit fly species in Thailand. However, the efficiency of this barcoding sequence was limited when members of a species complex were included in the analysis. A further limitation was found when sequences from a public database were used where species identification is uncertain, such as morphologically highly different species having the same COI sequence or the same species name being applied for highly divergence COI sequences. A similar problem has been reported in other barcoding studies of insects such as in

Chironomidae (Pramual et al. 2016). This indicates that care must be taken to examination the identification source and method when using full public databases to obtain species-level identifications. Levels of host plant specificity vary across fruit fly species. Members of the subgenus *Bactrocera* were found in diverse host plants, while those of the subgenus *Zeugodacus* were confined to host plants of the family Cucurbitaceae. There was no evidence of association between the intraspecific genetic lineages and the host plant species. However, the limitation of the host plant information for the sequence data from public database prevented us from confirming this, and further examination will be required.

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Table 1. Details of specimen collection sites of fruit flies in this study.

Location	Code	Geographic region	Latitude / Longitude	Elevation (m)
Fang, Chaing Mai Province	CMI	North	18° 32' 36" N/ 98° 31' 03" E	1 330
Mueang Chaing Rai, Chiang Rai Province	CRI1	North	19° 51' 17" N/ 99° 53' 21" E	122
Mae Chan, Chiang Rai Province	CRI2	North	20° 11' 28" N/ 99° 56' 47" E	409
Mueang Lampang, Lampang Province	LPG1	North	18° 17' 01" N/ 99° 30' 09" E	229
Mae Mo, Lampang Province	LPG2	North	18° 24' 29" N/ 99° 51' 25" E	598
Wang Nuea, Lampang Province	LPG3	North	19° 04' 11" N/ 99° 43' 45" E	607
Mae La Noi, Mae Hong Son Province	MSN1	North	18° 22' 39" N/ 97° 56' 30" E	315
Pai, Mae Hong Son Province	MSN2	North	19° 26' 45" N/ 98° 19' 09" E	1430
Mueang Phayao, Phayao Province	PYO	North	19° 13' 03" N/ 99° 44' 16" E	572
Long, Phrae Province	PRE	North	18° 00' 53" N/ 99° 42' 55" E	156
Kaset Sombun, Chaiyaphum Province	CPM	Northeast	16° 22' 45" N/ 101° 58' 22" E	226
Mueang Khon Kaen, Khon Kaen Province	KKN	Northeast	16° 32' 41" N/ 102° 50' 37" E	173
Na Haeo, Loei Province	LEI	Northeast	17° 21' 17" N/ 101° 03' 42" E	794
Muang Maha Sarakham, Maha Sarakham Province	MKM1	Northeast	16° 08' 13" N/ 103° 18' 33" E	159
Kantharawichai, Maha Sarakham Province	MKM2	Northeast	16° 15' 17" N/ 103° 13' 50" E	150
Nong Sung Mukdahan, Mukdahan Province	MDH	Northeast	16° 27' 38" N/ 104° 26' 26" E	204
Mueang Nong Khai, Nong Khai Province	NKI1	Northeast	17° 48' 59" N/ 102° 42' 58" E	181
Si Chiang Mai, Nong Khai Province	NKI2	Northeast	17° 56' 28" N/ 102° 35' 43" E	252

Location	Code	Geographic region	Latitude / Longitude	Elevation (m)
Nong Phok, Roi-Et Province	RET	Northeast	16° 18' 35 N/ 104° 12' 11" E	179
Prang Ku, Si Sa Ket Province	SSK	Northeast	14° 49' 43 N/ 104° 03' 32" E	136
Mueang Ubon Ratchathani, Ubon Ratchathani Province	UBN	Northeast	15° 13' 44 N/ 104° 51' 15" E	121
Mueang Chanthaburi, Chanthaburi Province	CTI1	East	12° 36' 25" N/ 102° 04' 49" E	10
Laem Sing, Chanthaburi Province	CTI2	East	12° 28' 26" N/ 102° 05' 32" E	11
Mueang Kamphaeng Phet, Kamphaeng Phet Province	KPT	Central	16° 26' 04" N/ 99° 22' 06" E	98
Mueang Nakhon Pathom, Nakhon Pathom Province	NPT	Central	13° 50' 32 N/ 100° 01' 24" E	10

Table 2. Fruit fly species and host plants collected in this study.

Fruit fly species	Host plants		Source	Number of fruit flies	Sampling Location ^b
	Family	Species			
<i>B. (Bactrocera) carambolae</i> Drew & Hancock	Myrtaceae	<i>Psidium guajava</i> L.	Fruit orchards	1	CRI1
		<i>Syzygium samarangense</i> (Blume) Merr & L.M. Perry	Fruit orchards	4	MDH
<i>B. (Bactrocera) correcta</i> (Bezzi)	Anacardiaceae	<i>Mangifera indica</i> L.	Fruit orchards	1	RET
	Anacardiaceae	<i>Mangifera indica</i> L.	Fruit orchards	13 ^a	
	Annonaceae	<i>Artabotrys siamensis</i> Mig.	Fruit orchards	1 ^a	
	Cucurbitaceae	<i>Coccinia grandis</i> (L.) Voigt	Fruit orchards	1 ^a	
	Irvingiaceae	<i>Irvingia malayana</i> Oliv. ex A.W. Benn.	Wild fruits	2 ^a	
	Malpighiaceae	<i>Malpighia glabra</i> L.	Fruit orchards	13 ^a	
	Myrtaceae	<i>Syzygium samarangense</i> (Blume) Merr & L.M. Perry	Fruit orchards	61 ^a	
		<i>Psidium guajava</i> L.	Fruit orchards	50 ^a	
	Oxalidaceae	<i>Averrhoa carambola</i> L.	Fruit orchards	7 ^a	
	Solanaceae	<i>Solanum melongena</i> L.	Fruit orchards	8 ^a	
	Tiliaceae	<i>Muntingia calabura</i> L.	Fruit orchards	1 ^a	
	Verbenaceae	<i>Ziziphus mauritiana</i> Lam.	Fruit orchards	14 ^a	
<i>B. (Zeugodacus) cucurbitae</i> (Coquillett)	Cucurbitaceae	<i>Coccinia grandis</i> (L.) Voigt	Wild fruits	7	CMI, KPT
		<i>Cucurbita moschata</i> Duchesne	Fruit orchards	2	MKM2
		<i>Luffa cylindrica</i> (L.) M. Roem.	Fruit orchards	1	PYO, PRE
		<i>Gymnopetalum integrifolium</i> (Roxb.) Kurz	Fruit orchards	4	SSK
<i>B. (Hemigymnodacus) diversa</i> (Coquillett)	Cucurbitaceae	<i>Cucurbita moschata</i> Duchesne	Fruit orchards	1	NKI2
<i>B. (Bactrocera) dorsalis</i> (Hendel)	Anacardiaceae	<i>Mangifera indica</i> L.	Fruit orchards	1	CPM, CTI1
	Annonaceae	<i>Annona squamosa</i> L.	Fruit orchards	3	MKM1
	Myrtaceae	<i>Psidium guajava</i> L.	Fruit orchards	4	MKM2
		<i>Syzygium samarangense</i> (Blume) Merr & L.M. Perry	Fruit orchards	12	NPT, NKI1

Fruit fly species	Host plants		Source	Number of fruit flies	Sampling Location ^b
	Family	Species			
	Verbenaceae	<i>Ziziphus mauritiana</i> Lam.	Fruit orchards	1	SSK, UBN
<i>B. (Bactrocera) invadens</i>	Anacardiaceae	<i>Mangifera indica</i> L.	Fruit orchards	1	KKN, SSK
Drew, Tsuruta & White	Annonaceae	<i>Annona squamosa</i> L.	Fruit orchards	1	MKM1
(= <i>B. (Bactrocera) dorsalis</i>	Myrtaceae	<i>Psidium guajava</i> L.	Fruit orchards	2	MKM2
(Hendel))		<i>Syzygium samarangense</i> (Blume) Merr & L.M. Perry	Fruit orchards	1	
	Oxalidaceae	<i>Averrhoa carambola</i> L.	Fruit orchards	1	
	Sapindaceae	<i>Dimocarpus longan</i> Lour.	Fruit orchards	1	
	Verbenaceae	<i>Ziziphus mauritiana</i> Lam.	Fruit orchards	1	
<i>B. (Bactrocera) papayae</i>	Anacardiaceae	<i>Mangifera indica</i> L.	Fruit orchards	4	CPM,
Drew & Hancock	Ebenaceae	<i>Diospyros decandra</i> Lour.	Fruit orchards	1	MKM2
(= <i>B. (Bactrocera) dorsalis</i>	Myrtaceae	<i>Syzygium samarangense</i> (Blume) Merr & L.M. Perry	Fruit orchards	1	SSK
(Hendel))		<i>Averrhoa carambola</i> L.	Fruit orchards	2	
<i>B. (Bactrocera) philippinensis</i>	Annonaceae	<i>Annona squamosa</i> L.	Fruit orchards	2	MKM2
Drew & Hancock	Irvingiaceae	<i>Irvingia malayana</i> Oliv. ex A.W. Benn.	Fruit orchards	1	NK11
(= <i>B. (Bactrocera) dorsalis</i>	Myrtaceae	<i>Psidium guajava</i> L.	Fruit orchards	1	SSK
(Hendel))		<i>Syzygium samarangense</i> (Blume) Merr & L.M. Perry	Fruit orchards	1	
<i>B. (Bactrocera) latifrons</i>	Solanaceae	<i>Capsicum frutescens</i> L.	Fruit orchards	1	CTI2, CRI1 LPG1, RET
(Hendel)		<i>Solanum melongena</i> L.	Fruit orchards	3	
		<i>Lycopersicon esculentum</i> Mill.	Fruit orchards	1	
		<i>Capsicum frutescens</i> L.	Fruit orchards	55 ^a	
		<i>Solanum melongena</i> L.	Fruit orchards	7 ^a	
		<i>Solanum torvum</i> Sw.	Fruit orchards	14 ^a	
		<i>Solanum trilobatum</i> L.	Fruit orchards	21 ^a	
<i>B. (Zeugodacus) scutellaris</i>	Cucurbitaceae	<i>Cucurbita moschata</i> Duchesne	Wild fruits	4	MSN2
(Bezzi)					
<i>B. (Zeugodacus) tau</i> (Walker)	Cucurbitaceae	<i>Coccinia grandis</i> (L.) Voigt	Wild fruits	3	CPM, CRI2

Fruit fly species	Host plants		Source	Number of fruit flies	Sampling Location ^b
	Family	Species			
		<i>Momordica cochinchinensis</i> (Lour.)	Fruit orchards	5	LPG2, LEI
		<i>Gynopetalum integrifolium</i> (Roxb.) Kurz	Wild fruits	16	PYO
<i>B. (Bactrocera) thailandica</i> Drew & Hancock	Myrtaceae	<i>Psidium guajava</i> L.	Fruit orchards	9	CMI
<i>B. (Bactrocera) tuberculata</i> (Bezzi)	Barringtoniaceae	<i>Careya sphaerica</i> Roxb.	Wild fruits	7	MKM1
<i>B. (Gymnodacus) symplocos</i> Drew & Romig	Anacardiaceae	<i>Spondias pinnata</i> (L.f.) Kurz	Wild fruits	7	LPG3
<i>Bactrocera</i> sp.1	Cucurbitaceae	<i>Coccinia grandis</i> (L.) Voigt	Wild fruits	4	MSN1
Total				391	

^aSequences obtained from GenBank. ^bDetails of the locations are given in Table 1.

Table 3. Intraspecific Kimura 2-parameter (K2P) genetic divergence and distance to nearest neighbor of 17 fruit fly species based on mitochondrial *cytochrome c oxidase I* sequences.

Species	Number of COI sequences				Percent intraspecific genetic divergence (mean)	Percent pairwise K2P distance to the closest species (mean)
	This study	GenBank		Total (number of haplotypes)		
		Thailand	Other countries			
<i>B. (Zeugodacus) caudata</i>	-	3	-	3 (2)	0.15-0.61 (0.40)	7.14-7.79 (7.32)
<i>B. (Bactrocera) correcta</i>	1	185	24	210 (99)	0-3.80 (1.23)	6.69-10.00 (7.72)
<i>B. (Zeugodacus) cucurbitae</i>	14	-	119	133 (23)	0-4.43 (0.17)	0.88-5.88 (5.06)
<i>B. (Hemigymnodacus) diversa</i>	1	-	10	11 (9)	0-1.70 (0.86)	7.14-7.79 (7.32)
<i>B. (Zeugodacus) isolata</i>	-	2	-	2 (1)	0	12.17-12.83 (12.56)
<i>B. (Bactrocera) latifrons</i>	5	97	33	135 (47)	0-1.70 (0.30)	11.21-13.92 (11.95)
<i>B. (Zeugodacus) rubella</i>	-	7	-	7 (3)	0-1.33 (0.38)	11.00-13.33 (11.75)
<i>B. (Zeugodacus) scutellaris</i>	4	-	18	22 (16)	0-1.10 (0.15)	11.00-13.33 (11.75)
<i>B. (Zeugodacus) tau</i>	24	-	79	103 (51)	0-1.10 (0.32)	0.88-5.88 (5.06)
<i>B. (Bactrocera) tuberculata</i>	7	-	5	12 (7)	0-5.60 (2.77)	5.90-10.59 (7.65)
<i>B. (Bactrocera) umbrosa</i>	-	10	-	10 (2)	0-0.30 (0.09)	10.71-11.60 (11.06)
<i>B. (Bactrocera) zonata</i>	-	3	-	3 (2)	0-0.61 (0.41)	6.69-10.00 (7.72)
<i>B. (Gymnodacus) symplocos</i>	7	-	-	7 (6)	0-1.40 (0.65)	10.71-11.60 (11.06)
<i>Bactrocera</i> sp.1	4	-	-	4 (3)	0-2.80 (1.40)	12.91-15.88 (14.19)
<i>B. (Bactrocera) carambolae</i>	5	-	82	87 (40)	0-3.70 (0.61)	0-9.80 (1.35)
<i>B. (Bactrocera) dorsalis</i>	21	7	199	227 (115)	0-10.47 (0.93)	0-9.80 (1.35)
<i>B. (Bactrocera) thailandica</i>	9	-	13	22 (4)	0-0.80 (0.14)	1.83-9.14 (2.87)
Total	123	314	709	1 146 (461)	0-10.47 (0.64)	0-15.88 (8.10)

Table 4. DNA barcode statistics for 17 fruit fly species based on best match and best-close match methods in TaxonDNA (Meier et al. 2006).

	Best match	Best close match
Correct identification (n)	96.50% (1 106 sequences)	96.42% (1 105 sequences)
Misidentification (n)	0.61% (7 sequences)	0.52% (6 sequences)
Ambiguous identification (n)	2.87% (33 sequences)	2.87% (33 sequences)
Sequences without any match closer than threshold value	0	0.17% (2 sequences)
Threshold ^a	-	2.05%

^aThreshold value is genetic distance at 95% of all intraspecific genetic distances (Meier et al. 2006).

Figure legends

Fig. 1. Collection sites of fruit flies in Thailand. Details of sampling sites are given in Table 1.

Fig. 2. Range of intraspecific Kimura 2-parameter genetic distances and pairwise divergences to the closest species for 17 species of fruit flies of the genus *Bactrocera* based on the barcode region of the mitochondrial *cytochrome c oxidase I* sequences.

Fig. 3. Maximum likelihood tree for *cytochrome c oxidase I* (COI) sequences of 17 species of the fruit fly genus *Bactrocera*. Members of *Bactrocera dorsalis* complex were treated as one species. Variability within each morphospecies was collapsed into triangle for visualization. Node support based on likelihood ratio test is shown above or near branch. Scale bar represents 0.04 substitutions per nucleotide position. Numbers in parentheses represent the total number of sequences followed by number of haplotypes of each species. Host plant families of each fruit fly species are shown next to the taxon name, with the number of fruit fly specimens for each plant family shown inside the legend. Asterisks indicate that the host plant family for sequences from GenBank was unknown, and thus, only the numbers of specimens from present study were shown. Question marks indicate that the host plant is unknown.

Fig. 4. Maximum likelihood tree for *cytochrome c oxidase I* (COI) barcode sequences of 190 unique sequences of members of the *Bactrocera dorsalis* complex. Node support based on likelihood ratio test is shown above or near branch. Scale bar represents 0.04 substitutions per nucleotide position. Numbers in parentheses represent the number of haplotypes of each species.

Fig. 5. Maximum likelihood tree for *cytochrome c oxidase I* (COI) barcode sequences of 99 unique sequences of *Bactrocera correcta*. Node support based on likelihood ratio test is shown above or near branch. Scale bar represents 0.04 substitutions per nucleotide position.

Host plant families of each haplotype are shown. Question marks indicate that the host plant is unknown.

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Fig. 1. Collection sites of fruit flies in Thailand. Details of sampling sites are given in Table 1.
205x293mm (300 x 300 DPI)

Fig. 2. Range of intraspecific Kimura 2-parameter genetic distances and pairwise divergences to the closest species for 17 species of fruit flies of the genus *Bactrocera* based on the barcode region of the mitochondrial cytochrome c oxidase I sequences.
303x197mm (300 x 300 DPI)

Fig. 3. Maximum likelihood tree for cytochrome c oxidase I (COI) sequences of 17 species of the fruit fly genus *Bactrocera*. Members of *Bactrocera dorsalis* complex were treated as one species. Variability within each morphospecies was collapsed into triangle for visualization. Node support based on likelihood ratio test is shown above or near branch. Scale bar represents 0.04 substitutions per nucleotide position. Numbers in parentheses represent the total number of sequences followed by number of haplotypes of each species. Host plant families of each fruit fly species are shown next to the taxon name, with the number of fruit fly specimens for each plant family shown inside the legend. Asterisks indicate that the host plant family for sequences from GenBank was unknown, and thus, only the numbers of specimens from present study were shown. Question marks indicate that the host plant is unknown.

Fig. 4. Maximum likelihood tree for cytochrome c oxidase I (COI) barcode sequences of 190 unique sequences of members of the *Bactrocera dorsalis* complex. Node support based on likelihood ratio test is shown above or near branch. Scale bar represents 0.04 substitutions per nucleotide position. Numbers in parentheses represent the number of haplotypes of each species.

172x207mm (600 x 600 DPI)

Fig. 5. Maximum likelihood tree for cytochrome c oxidase I (COI) barcode sequences of 99 unique sequences of *Bactrocera correcta*. Node support based on likelihood ratio test is shown above or near branch. Scale bar represents 0.04 substitutions per nucleotide position. Host plant families of each haplotype are shown. Question marks indicate that the host plant is unknown.