# NATURAL VARIATION AND BIOGEOGRAPHY OF THE MELON FRUIT FLY, *ZEUGODACUS CUCURBITAE* (DIPTERA: TEPHRITIDAE), IN SOUTHEAST-ASIA AND THE WEST-PACIFIC


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# Keywords

Natural variation, biogeography, melon fly, *Zeugodacus cucurbitae*, Southeast-Asia, The West-Pacific, integrative taxonomy, geometric morphometrics, aedeagus analysis, *cox1*, microsatellites, population structure, Thai biogeography, Thai-Malay peninsular, Isthmus of Kra, Kangker-Pattani line, host race, cryptic species, native range, invasive range, invasive pathway

# Abstract

The melon fly, *Zeugodacus cucurbitae*, is a widely distributed horticultural fruit fly pest that infests fruit of over 125 plant species, predominantly cucurbits. With a native range including the Indian Subcontinent and Southeast-Asia, the melon fly has since invaded Africa, the West-Pacific, and parts of Oceania. While much is known about some aspects of this fly, host-related and geographic variation across Southeast-Asia and the West-Pacific is poorly understood. This study assesses variation in *Z. cucurbitae* populations from Southeast-Asia and the West-Pacific within an integrative taxonomic framework, with a particular focus on biogeographic and host-associated affects in Thailand.

Multidisciplinary approaches greatly enhance our understanding of phenotypic and genotypic variation, particularly if tools that can resolve population structure and variance over different geographic and temporal scales are applied. Hence, an integrative taxonomic approach incorporating morphological attributes of wing size, wing shape and aedeagus length, molecular data (*cox1* and microsatellite) and host-use data was applied to resolve natural variation in *Z. cucurbitae*. The aim was to examine research questions addressing: i) fine scale biogeographic hypotheses in Thailand and along a transect of the Thai-Malay peninsula (Chapter 3); ii) potential for host-associated populations (Chapter 4); and, iii) a broad scale examination of potential invasion signatures throughout Southeast-Asia and the West-Pacific (Chapter 5).

Two hypotheses were tested regarding *Z. cucurbitae* population structuring within Thailand in the first research chapter. The first posited that natural variation and genetic diversity were correlated with biogeographic and regional environmental conditions across Thailand. Analyses, based on population samples from six mainland regions and two islands in Thailand, revealed that population structure of *Z. cucurbitae* within Thailand is relatively homogeneous, except for populations from the Northeast. This region is separated from the rest of Thailand by a mountain range which represents a potential natural barrier to fly dispersal. The second hypothesis addressed whether the Isthmus of Kra barrier correlates

with natural variation in *Z. cucurbitae*, thus constituting a biogeographic barrier, or contact zone, separating populations from northern and southern regions of the Thai-Malay Peninsula. Data were obtained from 17 sites along a north-south Thai-Malay transect (incorporating the Isthmus of Kra). Overall, wing shape was similar across most sites, but there were significant wing shape differences between flies from Nan (northernmost site) and Selangor (southernmost site). Flies from Yala and Narathiwat (the southernmost Thai sites) were more similar to flies from Malaysia than to other flies from Thailand.

In Chapter 4, I examined *Z. cucurbitae* diversity in Thailand by focusing on host-plant relationships to determine whether there is evidence for the existence of host races or cryptic species in this highly polyphagous species. Melon fly were collected from ten different host plant species with a focus on two plant families (Cucurbitaceae and Fabaceae) within the one Thai biogeographic region. Morphological and molecular results from this study were inconsistent; yet, revealed a low level of differentiation among flies reared from different host plants. There was, however, very subtle structure evident for some host-related comparisons. Nevertheless, results do not support the existence of host races, thus confirming that *Z. cucurbitae* in Thailand represents a single biological species utilising a range of host plants across different families.

Specimens were obtained from ten sites from Southeast-Asia (native range) and four sites from the West-Pacific (invasive range) for the broad-scale study undertaken in Chapter 5. Analysis of morphological and molecular data revealed greatest diversity in Southeast-Asia commensurate with this being the native range of melon fly, with subsequent dispersal into and across the West-Pacific where genetic and morphological variability was much reduced. However, mitochondrial and microsatellite data supports the notion of multiple introductions to Hawaii from Southeast-Asia due to relatively greater variability in comparison to other Pacific sites. High levels of similarity were found between some Southeast-Asia sites and those into the invasive range, thereby informing of potential invasion pathways into the West-Pacific region.

Overall, this integrative study of *Z. cucurbitae* variation across its native and invasive range of Southeast-Asia and the West-Pacific provides strong support for a single-species hypothesis for a taxon that was *a priori* suspected of potentially representing a cryptic complex of either different species or host races. Sub-structuring was nevertheless revealed at finer scales; particularly relating to geographic boundaries and, to a much lesser extent, host use. Given this understanding, data obtained during this study may be used to develop a practical identification guide and diagnostic protocol for *Z. cucurbitae* and other cucurbit fruit fly pests. Further resolution of population structure across the region, particularly with respect to better understanding subtle inter-regional biological variability (e.g., sexual behaviour in a natural context) will provide further comprehensive insights into this destructive pest and inform future management programmes. Finally, the results of this study emphasise the value of integrating multiple data sets to develop a better understanding of dispersal pathways in highly variable insect species with the potential to become globally important invasive pests.

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# Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

QUT Verified Signature

Signature:

Date: 23 February 2016

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# CHAPTER 1

## GENERAL INTRODUCTION

## 1.1 BACKGROUND

The insect family Tephritidae (Insecta: Diptera) consists of over 5000 species of "true fruit flies" (Fletcher, 1987). Approximately 10% of tephritids are pests of fruits, vegetables and flowers (Christenson & Foote, 1960; Weems et al., 1999; Diamantidis et al., 2008) and cause annual economic losses greater than US$1 billion worldwide (STDF, 2010). The melon fly, *Zeugodacus cucurbitae* (Coquillet), is one of several tephritid species that are of particular economic importance to Southeast-Asia and the West-Pacific. *Zeugodacus cucurbitae* is distributed across a range of climatic regions, including the Indian subcontinent, Southeast-Asia, Africa, the Hawaiian Islands and other parts of Oceania (Dhillon et al., 2005). This fly infests the fruit of more than 125 plant species, predominantly cucurbits (i.e., melons) (Piñero et al., 2006).

Despite the economic importance of *Z. cucurbitae*, only limited research has been undertaken on the genetic and morphological variation of this species within its native range of Southeast-Asia. Additionally, the reasons for the very broad geographical distribution of *Z. cucurbitae*, particularly with respect to the relative importance of natural and human factors in aiding spread, are unclear. To better understand factors explaining this fly's distribution and natural variation, this thesis applies an integrated approach using host-use, morphological information, and molecular data to quantify and resolve the natural variation of *Z. cucurbitae* at different geographic scales.

The melon fly has dispersed from Asia westwards to Africa and eastwards far into the Pacific region (Weems et al., 2001; Dhillon et al., 2005). Thailand represents part of its native distribution and is located at the approximate middle of the global range of the species; we may therefore expect to see maximum diversity here as reflected in genetics and morphology. Throughout Thailand, there are abundant and diverse host plants, and a range of forest and agricultural habitats; thus, any differentiation into host races is likely to be detectable should they exist. Moreover, Thailand is located in a biotic transition zone between Indochinese and Sundaic floral and faunal elements, suggesting that there could be enhanced diversity here. Thailand is also logistically convenient for this kind of study. Most of the country

and a great variety of host plants are easily accessible by road, which enables intensive sampling by trapping and rearing from host fruit. In addition, infested fruit can be safely transferred from collection sites to rearing facilities, without creating unacceptable, phytosanitary risk. All of these factors suggest that it is an optimal model system for investigating variation.

This thesis, therefore, investigates if host plant races exist in *Z. cucurbitae*, and what, if any, population structuring occurs within the species based on geographic distribution, known biogeographic barriers, and recent invasions over the last 50 to 100 years. The thesis begins with a literature review to set the experimental background for the thesis and identify gaps in knowledge. This introductory chapter is grouped into two broad sections: (i) background to inter and intra-species variation in tephritid fruit flies, with particular reference to cryptic taxa of agricultural importance and how they can be studied, and (ii) an introduction to the subject animal, *Z. cucurbitae,* including what is known of the melon fly in Thailand. The chapter concludes with an overview of the thesis structure and a description of the flow of the experimental chapters.

## 1.2 THE DIVERSITY OF TEPHRITID FRUIT FLIES

There are myriad forms of insects on earth (Erwin, 1982, 1991; Stork, 1993) and the true fruit flies (Diptera: Tephritidae) are a dramatic example of the diversity that can exist within a single insect family. Nearly 5000 tephritid species have been described (Snustad et al., 2000; Aluja & Norrbom, 1999), and they are widespread across every continent except Antarctica. Several are pest species found throughout Africa, Asia, the Americas, Europe, and Australia; thus making them a group of truly global significance (Stonehouse et al., 1998; Siebert, 1999; Hendrichs, 2000). In addition to their pest status, the remarkable diversity of the tephritids renders them an ideal group for biodiversity and evolutionary studies (Aluja & Norrbom, 1999). This diversity encompasses not only the familiar fruit-feeding groups, but also saprophagous (i.e., feeding on decaying organic matter) species of 'Antlered flies' (genus *Phytalmia*) and the flower-head daisy feeding tephritines (McAlpine & Schneider, 1978; Schutze et al., 2007).

Natural variation in tephritids has attracted particular attention from applied biologists because of their economic importance (Feder et al., 2003; Linn et al., 2003). The following section introduces our current understanding of variation in tephritids, particularly with respect to applied biology. Because of their importance, the section focuses on sibling species complexes within the pest tephritids, including why we need to resolve such complexes, and how morphological and genetic variation within and between species can be studied using an integrative taxonomic approach.

This section does not cover possible drivers of diversity in fruit flies, such as host plant specialisation (Novotny et al., 2005), local adaptation (Templeton, 1986), biogeographic barriers (De Meyer et al., 2008, 2010) and large scale isolation and invasion (Busch-Petersen et al., 1988); these are the foci of individual research chapters, and relevant literature is covered in the introductions of those chapters.

### 1.2.1 Morphological variation and taxonomy in tephritids

Tephritid morphological variation can manifest itself in many ways, such as in simple characteristics relating to variation in body size or appendage length; in complex characters such as the shape of the wing; number and arrangement of setae; leg or abdomen colour patterns; or in attributes of developing stages (Mousseau & Dinglt, 1991). As a result, tephritid taxonomy, like that of many other insect groups, has been almost entirely based on morphological characters: features such as general shape (e.g., outline shape of the abdomen or relative size and shape of the sides of the head), colour patterns (e.g., shape of dark markings on the face, dorsal thorax, wings, legs or abdominal tergites) and chaetotaxy (e.g., arrangement of bristles of the head or dorsal thorax, and microtrichial patterns of the wing) have been of particular use (Drew, 1989; White & Elson-Harris 1992; Drew & Hancock, 2000). In fact, the bulk of tephritid taxonomy is still based on these serviceable and readily observable features. Further, there are differences between the sexes and this dimorphism represents an additional complication; for example, a posteriodorsal spot is more common on the fore femur in female Carambola fruit fly, *Bactrocera carambolae* Drew and Hancock, than in males (Kapoor, 2006).

Morphological features that we can observe do not always reflect actual biological species, and it has become increasingly apparent that the relatively simple external characters, such as those mentioned above, may be insufficient to adequately resolve species, especially within problematic tephritid complexes (Stone, 1942; White & Elson-Harris, 1992; Drew & Hancock, 1994; Vera et al., 2006; Cameron et al., 2010; Asokan et al., 2011; Kitthawee & Rungsri, 2011; Hernández-Ortiz et al., 2012; Schutze et al., 2012a, 2012b; 2015a, 2015b). Part of the problem stems from the fact that intraspecific variation may overlap interspecific differences. Intraspecific variation may be subtle, such as in the size or shape of shape of dark spots on a fly's thorax; or considerable, such as bold, well-defined marking across the abdomen (Steck, 1999; Kapoor, 2006); however, crucial and conspicuous characteristics (e.g., abdominal colour, wing venation, or chaetotaxy) have been shown to vary among individuals of the same species almost as much as among species (Iwahashi & Routhier, 2001) which may render them less than reliable characters for defining species. Their use in diagnostic keys may, as a consequence, be impractical.

Traditional morphological studies alone are not always adequate for taxonomic studies. This can be illustrated by two extremes scenarios. In the first, subtle morphological variation is taken to indicate the existence of different species, when in fact the different forms constitute the same biological species. This is well demonstrated in the *B. dorsalis* complex: a group that comprises over 100 taxa that share a defined set of morphological characters: abdominal terga III-V with a medial longitudinal dark (T-band), a mostly black scutum and with a band and variable dark patterns on the lateral margins (Drew & Hancock, 1994; Drew & Romig, 2013). In Southeast-Asia, many species have been described in this complex, including major pest species *B. philippinensis* Drew and Hancock, *B. papayae* Drew and Hancock, and *B. carambolae.* These taxa had been differentiated from *B. dorsalis* on the basis of subtle morphological characters pertaining to colour patterns of the wing and length of the aculeus (Drew & Hancock, 1994; Drew & Romig, 2013). Moreover, an invasive fruit fly in Africa that is morphologically very similar to *B. dorsalis* (Lux et al., 2003; Khamis et al., 2012) was

described as yet another species, namely *B. invadens* Drew, Tsuruta & White (Drew et al., 2005; Drew & Romig, 2013). The African fly was distinguished from *B. dorsalis* based primarily on the colour of the scutum (the dorsal surface of the thorax), aedeagus length, postsutural vittae width, a dark transverse band on the abdomen, and a dark anterolateral marking on abdominal tergites (Drew et al., 2005; Drew & Romig, 2013).

Research on these "species" and their management has been confounded by their supposed, close morphological (e.g., shape) (White & Elson, 1992; Drew & Hanhook, 1994; Iwahashi, 2001), geometric morphometric, molecular (Armstrong & Ball, 2005; Chua et al., 2010; Schutze et al., 2012a, 2012b, 2015a, 2015b; Krosch et al., 2013), physiological and behavioural similarities (Fletcher & Kitching, 1995; Tan, 2003; Medina et al., 1998). Moreover, the identification of these species has been largely based on their respective geographical distributions (Drew & Hancock, 1994), despite known problems in using geography as a taxonomic character (Sites-Jnr & Marshall, 2003; Fitzpatrick et al., 2009). As more samples across the entire geographic range have been subsequently examined, it became apparent that all of these species reflect variation at the population level rather than that at the species level. Consequently, Drew & Romig (2013) synonymised *B. philippinensis* with *B. papayae*; and Schutze et al., (2015a) took the further step of incorporating a range of multidisciplinary tools to synonymize *B. papayae* and *B. invadens* with *B. dorsalis*. While very closely related, the integrated comparative datasets which included chemoecological, molecular, and morphological information consistently supported *B. carambolae* as being a distinct biological species.

In the second scenario, a lack of reliable diagnostic characters has led taxonomists to underestimate the number of actual biological species in a single taxonomic species: the South American fruit fly, *Anastrepha fraterculus*, is a classic example. For a long time, each was considered to be a single biological species; however, subtle yet consistent evidence from multiple disciplines has revealed that each comprises numerous species, albeit having broadly similar morphologies. A great quantity of information has been recently published that illuminates the taxonomic status of the problematic *A*. *fraterculus* group in particular. It has long

been suspected that individuals and populations of this species may represent a complex of undescribed cryptic species (Stone, 1942; Hernández-Ortiz et al., 2004). The populations are very heterogeneous, with variability encompassing pest status, behaviour, pheromones, karyotype, Wolbachia strains, isozymes and molecular sequence data (Smith-Caldas et al., 2001; Vera et al., 2006; Cáceres et al., 2009; Hernández-Ortiz et al., 2012); *cox1* data was especially revealing, in that it yielded a surprisingly large number of lineages suggestive of different species (Smith-Caldas et al., 2001). Furthermore, recent morphometric work (Hernández-Ortiz et al., 2004) has encouragingly shown that a morphological approach may be used to identify and define entities (in this case a Mexican morphotype of *A. fraterculus*). Mating compatibility work has been decisive in recognising which entities are most likely to be reproductively isolated (Vera et al., 2006), and recent sequence data and karyotypes, in combination with evidence of reproductive isolation, have revealed that such morphotypes indeed represent natural and distinct entities for which seven distinct morphotypes (and likely biological species) within currently defined *A. fraterculus* (Hernández-Ortiz et al., 2012).

Closer to my study organism, *Z. cucurbitae*, there are also cases where minor morphological differences previously overlooked or dismissed as intraspecific variation, have proved consistent and indicative of closely related, very similar-looking, valid species (i.e., cryptic species). For example, differences in the colour bands of the thorax and abdomen, in the shape of the wing, in the aculeus, ovipositor shape, wing morphometrics, chromosomes and molecular data have been used with spectacular success to distinguish at least eight distinct species within *Zeugodacus tau* Walker in Thailand (Saelee et al., 2006; Kitthawee & Dujardin, 2010; Sumrandee, et al., 2011; Kitthawee & Rungsri, 2011). In the '*tau* group', there has been more variation than could be accommodated within the traditional definition of the species. None of the now-recognised '*tau* lineages' currently have formal names and they continue to be referred to as species A, B, C, D, E, F, G and I, with species 'A' representing *Z. tau* sensu stricto (Baimai et al., 2000). Thus, readily observable features are largely adequate for the differentiation of species in the *tau* group.

*Zeugodacus cucurbitae* has been defined using a combination of gross morphological features, morphometrics and colour characters of the adults (White & Hancock, 1997; Lawson et al., 2003; De Meyer et al., 2015). The range of variation encompassed by the definition is substantial, but the prevailing view of *Z. cucurbitae* has been that it is a single highly variable species. The view that *Z. cucurbitae* comprises a single, variable species rests on the simple fact that it has not been possible to recognize correlations among patterns of variation. It has been a default view rather than the outcome of a comprehensive, robust analysis of variation. Nevertheless, evidence for multiple lineages within *Z. cucurbitae* has been building and there has been a growing suspicion that this species might prove to be similar to *A. fraterculus* and *Z. tau* in that multiple independent lineages may exist under a single named species. Perhaps the clearest lesson from the literature on *A. fraterculus* and *Z. tau* is that data from multiple sources (molecular, behavioural, morphology) are required to enable an understanding of these taxonomically challenging complexes. These data are required, firstly to provide resolution of the entities within the complex and, secondly, to enable analysis for potentially meaningful correlations in variation. It is also clear that while simple 'DNA barcode' molecular data provide some of the most potent evidence of relationships among populations, systematic analysis of alternative molecular approaches-combined with analysis of subtle morphological variation-represents a promising avenue for determining the presence of cryptic races or species; particularly when paired with additional biological (e.g., host use) data. Notably, while mating compatibility studies provide another strong line of evidence for resolving species boundaries, as evidenced by work on those taxa mentioned above, undertaking such studies is a highly challenging approach particularly for pest species of quarantine concern. The present investigation of *Z. cucurbitae* draws upon these salutary lessons.

## 1.3 WHY CRYPTIC TAXA ARE IMPORTANT FOR PLANT BIOSECURITY

There is a broad consensus that biological security (= biosecurity) problems are worsening due to globalization, specifically due to the ever increasing volume, speed and diversity of trade, travel, transportation and tourism (Zeng et al., 2008; Waage & Mumford, 2008). Within this larger issue, human-assisted movement of

pests appears to be the major cause of plant biosecurity problems (Defra, 2002; Gordh & McKirdy, 2013). The great diversity of crops and their rich insect and pathogen complexes guarantee a continuing and high level of new pest and disease introductions.

Different species, or even different populations of pests, may have different host use patterns (Wilson et al., 1994; Clarke et al., 2001), climatic tolerances (van Herrwege & David, 1997), abundance and distribution patterns (Bale et al., 2002), and patterns of pesticide resistance (Crow, 1957). Understanding and responding to this diversity may be the difference between applying an effective phytosanitary treatment and applying a treatment that allows a pest to survive and invade a new area (Walter, 2003). Given this, (cryptic) variation between and within species can greatly confuse and negatively impact biosecurity risk assessment and quarantine operations (Boykin et al., 2012). Cryptic species also create practical problems for quarantine inspectors and diagnosticians. Regulators in exporting countries need their officers to be able to identify organisms reliably so they can certify the absence of particular pests; while importing countries need to reliably identify organisms to maintain effective and consistent quarantine controls. Cryptic taxa within quarantine relevant species can, therefore, create significant policy and management problems for the national plant protection organisations in both exporting and importing countries (Bigler et al., 2005; Dyck et al., 2005; Murray at el., 2012).

### 1.3.1 The challenges of sibling/cryptic species complexes in tephritids

Additional to over-lapping character states, fruit fly taxonomy is further confounded by the existence of cryptic species complexes, i.e., groups of species which are morphologically very similar or identical, and which may or may not also be genetically very similar (Clarke & Schutze, 2014). Biological species within complexes may be indistinguishable from each other, yet may remain reproductively isolated and unable to mate even though they may be sympatric (Steyskal, 1972; Lincoln et al., 1982). Widely cited examples of species complexes within the tephritids include the Australian *Bactrocera tryoni* species complex (*Bactrocera tryoni* (Froggatt), *Bactrocera neohumeralis* (Hardy), *Bactrocera aquilonis*

(May) and *Bactrocera melas* (Perkins & May)) (Drew & Lambert, 1986; Morrow et al., 2000; Wang et al.,2003; Cameron et al., 2010); the *A. fraterculus* complex from South and Central America (Stone, 1942; Hernández-Ortiz et al., 2004; Smith-Caldas et al., 2001; Vera et al., 2006; Cáceres et al., 2009; Hernández-Ortiz et al., 2012); and from Asia the *B. dorsalis* complex consisting of almost 100 species (Drew & Hancock, 1994; Clarke et al., 2005; Drew et al., 2008; Schutze et al., 2012a, 2012b, 2013, 2015a, 2015b), and the *Z. tau* complex of at least eight species (Jamnongluk et al., 2003; Saelee et al., 2006; Kitthawee & Dujardin, 2010; Sumrandee, et al., 2011; Kitthawee & Rungsri, 2011; Drew & Romig, 2013).

In the case of the *B. dorsalis* and *B. tryoni* complexes, the number of formally described species almost certainly exceeds the number of biological species (i.e., the biological species have been taxonomically 'split' too far) (Morrow et al., 2000; Wang et al., 2003; Cameron et al., 2010). In contrast, for the *Z. tau* and the *A. fraterculus* complexes, there is strong evidence for numerous, taxonomically undescribed biological species (Hernández-Ortiz et al., 2004); that is, there are more biological species than taxonomic names. The lessons of these species complexes is that whenever one is working with a tephritid "species", particularly one with a diverse host range and distributed over a large geographic range, the question of whether one is dealing with a complex of sibling species needs to be considered. Given that *Z. cucurbitae* is both polyphagous and geographically widespread, it falls into the category of a species for which the potential of it being a complex exists.

The existence of numerous species complexes may indicate that taxa are rapidly diversifying (Clarke et al., 2005). If undergoing rapid evolutionary change, with new variants emerging, selection will still be shaping variation and reproductive isolation between lineages may be incomplete (de Queiroz, 1998, 1999). Such lineages are often difficult to accommodate in traditional taxonomic frameworks (Clarke & Schutze, 2014). However, while they may be difficult to allocate into morphologically-defined species units, these complexes and sibling species provide intriguing insights into fundamental evolutionary processes at the same time as posing major challenges to applied workers.

### 1.3.2 The study of variation and cryptic taxa

Several disciplines, including behaviour and genetics, are available to support the study of cryptic species and species-level variation, and these are detailed below. However, information from these individual disciplines are increasingly being used together within an integrative taxonomic framework, where the ultimate conclusions reached are strengthened by the use of multiple, independent datasets analysed using the tools of a variety of disciplines (Dayrat, 2005; Schlick-Steiner et al., 2010 Clarke & Schutze, 2014). The simultaneous application of multiple disciplines to address a specific taxonomic question (i.e., integrative taxonomy), represents a powerful approach towards resolving species and population-level relationships in complex species (Dayrat, 2005; Will et al., 2005; Padial et al., 2010; Yeates et al., 2010). The parallel use of molecular, morphological and morphometric (particularly geometric morphometric shape analysis), and comparative biological data (e.g., host use) can provide deeper insights into groups for which only one or two traditional taxonomic measures have been applied (Lefébure et al., 2011). The approach has become very popular in recent years (Krosch et al., 2013; Schutze et al., 2012b, 2015a, 2015b), having been used to demonstrate synonymy between species previously regarded as distinct, such as species of predatory mites (Tixier et al., 2011) and whiteflies parasitoids (Polaszek et al., 2004), and to refine species-boundary hypotheses such as in the mealy bug genus *Ferrisia* (Gullan et al., 2010). This thesis takes an integrative taxonomic approach to determine if variation between *Z. cucurbitae* populations is best explained as intra-or inter-specific variation, with that variation potentially driven by both geography and host use. Specifically, integrative taxonomy may be characterised as an approach that:

(1) makes use of evidence from multiple disciplines and data sets;
(2) draws on the literature of classical taxonomy but is not driven by existing taxonomic constructs;
(3) takes its theoretical rationale from the literature of speciation and population genetics;

(4) explicitly tests hypotheses regarding the definition of species using the tools of multiple disciplines; and
(5) uses increasingly sophisticated or detailed studies of taxa to confirm or refute previously defined species boundaries (Padial et al., 2010; Clarke & Schutze, 2014).

In essence, the integrative taxonomic approach uses morphological, morphometric, genetic, behavioural and other biological data sets of individuals or populations to test species boundary hypotheses. The outcome is that individuals or populations are assigned to one or more species (Dayrat, 2005; Padial et al., 2010). An important theoretical basis of integrative taxonomy is that many of the observable attributes of biological lineages, such as differences in courtship behaviour, niche specialisation, or morphological differences, may be the outcome of independently evolving biological lineages rather than the drivers of speciation (de Queiroz, 2007) (Figure 1.1). Where independently-evolving lineages (e.g., different species) occur, a pair of populations may exhibit differences in attribute 'A' but not in attribute 'B', while another pair of populations may be identical with respect to attribute 'A' but be quite different with respect to attribute 'B'. Clearly, it is unlikely that an analysis of species delimitation based solely on attribute 'A' would yield a robust conclusion. It follows that species delimitation should use as many species attributes as possible. The integrative taxonomic approach uses this theoretical insight by applying multiple species delimitation tools to a single question (e.g., integrative taxonomy will use attribute 'A' and 'B', not attribute 'A' or 'B'; Clarke & Schutze, 2014).

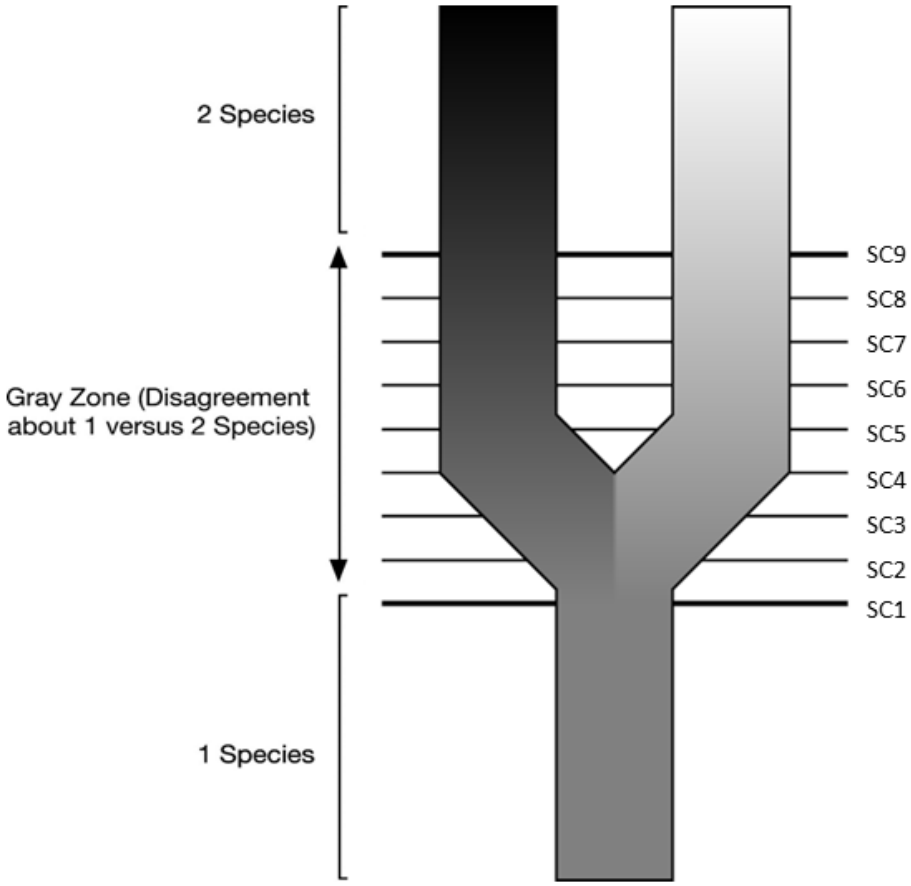



**Figure 1.1** Lineage separation and divergence (speciation) and species concepts. This highly simplified diagram represents a single lineage/species splitting to form two lineages/species (Modified from de Queiroz, 2007).

The integrative taxonomic approach does not depend on a single, novel, analytical technique. For example, many of the techniques used in this thesis to analyse molecular data are tried-and-tested instruments from the population geneticist's tool box. The procedures used for analysing the shapes of structures such as wings are relatively recent, but alone they are not enough to make the integrative taxonomic approach work. The integrative taxonomic approach advocates neither analytical techniques nor essential data sets (Schlick-Steiner et al., 2010). Indeed, it is to be expected that the most informative data sets would vary from one group of organisms to the next. For example, courtship data would be more likely to be useful in an analysis of species of crickets which have diverse stridulatory calls (Fitzpatrick & Gray, 2001; Gray, 2005) than in some cockroach species where males present tergal glands to the female and feeds with glandular

secretions (Nojima et al., 1999). The integrative taxonomic approach does not prescribe how many data sets are sufficient, yet a recommendation of at least three independent criteria be used has been made and seems workable (Schlick-Steiner et al., 2010). As yet, there is no consensus as to whether the approach should be predicated on a particular "species theory" (de Queiroz, 2007a, b; Schlick-Steiner et al., 2010; Clarke & Schutze, 2014). However, the unified species concept *sensu* de Queiroz is widely accepted.

The application of integrative taxonomy to the delimitation of fruit fly species is still in its infancy, but a set of species delimitation criteria that should be used has been proposed by Clarke & Schutze (2014):

(1) morphological and geometric morphometric analysis;
(2) multi-locus comparisons;
(3) pre- and post-zygotic mate compatibility tests. Mate choice trials in large cages can provide evidence on mate recognition and reproductive isolation, but such trials are not always logistically feasible; and
(4) pheromone analysis. The role of pheromones in sexual communication requires elucidation, especially to determine how this criterion interacts with pre-zygotic mate compatibility (criterion 1).

These and other potential criteria ("tools") are reviewed in the following pages.

## 1.4 TOOLS FOR FRUIT FLY INTEGRATIVE TAXONOMY

Fruit flies are well suited to integrative taxonomic studies as they possess numerous morphological and molecular characters that can be recorded from preserved specimens, while they can also be reared under controlled conditions for analysis of behavioural and chemoecological information (Aluja & Norrbom, 1999). This section describes the most commonly used characters for fruit fly integrative taxonomy (Clarke & Schutze, 2014).

### Morphological approaches

Because of the perceived unreliability of some external characters of fruit flies, researchers have often focussed on the length of the male aedeagus in classifying taxa in challenging groups such as the *B. dorsalis* complex (Iwaizumi et

al., 1997; Schutze et al., 2012a, 2012b, 2015a, 2015b; Krosch et al., 2013) and the *Z. tau* complex (Sumrandee et al., 2011). The shape of the ovipositor and the form of minute denticles embellishing the eversible ovipositor membrane have also been used to distinguish highly similar species of *Bactrocera*, again including those of the *B. dorsalis* complex (Hardy, 1973; Drew, 1989; Drew & Hancock, 1994). However, internal features are also subject to intra-specific variation, and some characters have been shown to be of little taxonomic value in some cases (Mahmood, 2004; Kapoor, 2006).

Traditional morphometric analysis remains appropriate for some characters, such as measurements of the aedeagus (Iwahashi, 2001; Drew et al., 2008) or ovipositor (Hernández-Ortizetal et al., 2004; Selivon et al., 2005; Sumrandee et al., 2011). However, measurements that collectively define shapes, such as that of the wing, can also be analysed using the methods of geometric morphometrics: a technique that examines variation in shape, where shape is defined as the remaining geometric information after the effects of location, scale (i.e., size) and rotation have been removed, via a technique known as generalised Procrustes superimposition, prior to multivariate analysis (Rohlf & Marcus, 1993; Dryden & Mardia, 1998). Wing shape has been found to be a particularly useful tool for resolving population-level variation in *B. dorsalis* (Iwahashi, 2001; Schutze et al., 2012a, 2012b, 2015a, 2015b; Krosch et al., 2013) through to resolving interspecific differences among species from the *Z. tau* complex (Kitthawee & Rungsri, 2011). Such variation in wing shape may have significant biological consequences: recognition of wing patterns by opposite sexes plays a role in courtship in some tephritids; and male flies often produce sounds and distribute pheromones by wing-fanning as part of pre-copulatory behaviour, from which females may discriminate among mates (Sivinski & Pereira, 2005). Thus, variation in wing shape may justifiably be considered a likely contender as a species attribute (or "species criterion" *sensu* de Quieroz, 2007) within an integrative taxonomic context.

Genetic approaches

Allozymes, random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFLPs), microsatellites and cytochrome *c* oxidase I (*cox1*)

haplotype analysis are all molecular/genetic approaches available for use to study tephritid variation (Tajima, 1989; Clark et al., 1998; Rozas et al., 2003; Xu, 2012). However, the preferred tools for recent studies of genetic variation in insects have been haplotype variation in the *cox1* gene and microsatellites (Nabholz et al., 2009).

DNA barcoding (Hebert et al., 2003) has been promoted as a valuable tool for species identification and discovery and has been proposed as a powerful methodology in biosecurity and the identification of invasive species (Armstrong & Ball, 2005). The *cox1* gene region has become the standard DNA barcode region (≈ 650 base pair fragment) for a global identification system for animals (Hebert et al., 2003; Pauls et al., 2010). *Cox1* is particularly suitable for analytical and diagnostic purposes because of ease of isolation, high copy number, conservation of sequence and structure, absence of recombination, maternal inheritance and variable mutation rates across different regions of the gene (Moritz et al., 1987; Simon, 1991; Hebert, et al., 2003). Efficient PCR primers are available, and there is abundant comparative data for evolutionary studies (Kerr et al., 2009). The DNA barcoding approach has not been without its detractors, noting the apparent lack of correlation between *cox1* and nuclear DNA data or the absence of a practicable barcode gap (Hurst & Jiggins, 2005; Whitworth et al., 2007; Wiemers & Fiedler, 2007). Even some of the advantages promoted for *cox1*, such as the absence of recombination and mutation rates, do not always hold (Rubinoff et al., 2006). Perhaps the most trenchant criticism of the barcoding approach relates to its simplistic application to species discovery, in which *cox1* sequences would be the sole discriminator of new taxa (Rubinoff et al., 2006). In fact, in the past decade, barcoding has tended to be used in species discovery to indicate where there might be previously undetected lineages and to direct the search for additional discriminating characters, rather than to justify the recognition of new species. Thus, for example, Pauls et al. (2010) were inclined to accept that several lineages of Chilean hydropsychid caddisflies, which could not be discriminated reliably using morphological or other characters, were in fact cryptic species; nevertheless, they hesitated to formally describe these lineages as new species based solely on barcodes. Huemer et al. (2014) were even more cautious and used “discordant”

barcodes among European moths (which are relatively well known taxonomically) to suggest where incorrect identifications, hybridisation, introgression, or even *Wolbachia* infections might be involved, rather than to recommend recognising cryptic species based on barcode differences.

Barcoding in the sense of Armstrong & Ball (2005), i.e., based on the approximately 650 base pair fragment of the *cox1* gene region, has limited utility in the identification of some tephritid species complexes (e.g., *B. dorsalis, B. tryoni, A. fraterculus, Ceratitis* spp.) (Barr et al., 2006; Liu et al., 2013). In these instances, the existence of cryptic species, inadequate sampling of all genetic subgroups, and high levels of geographic differentiation may combine to complicate identification (Armstrong & Ball, 2005). In some complexes, it may be that there is insufficient variation in *cox1* to provide evidence of recent lineage divergences, but this does not appear to be the case in other taxa, such as *Z. cucurbitae* (Armstrong & Ball, 2005).

*Mitochondrial cytochrome c oxidase I (cox1)*

Haplotype variation in the *cox1* gene can be used as a measure to differentiate sibling species, or to measure variation within a species. For example, Jamnongluk et al. (2003) used *cox1* sequences to study the molecular phylogeny of eight species from the *Z. tau* complex and found sequence divergence between species ranged from 0.06 to 28%. While Muraji (2002) used 1.6 kb sequences of mitochondrial DNA for a study of the population genetics of *Bactrocera* sp., Jamnongluk et al. (2003) analysed the molecular phylogenetic relationships among *Bactrocera* subgenera by using the shorter *cox1* sequences (nucleotide sequence 636 bp of *cox1*). The role of *cox1* in species-level phylogenetic reconstruction has been well demonstrated (Smith-Caldas et al., 2001), particularly when used in combination with other loci, including nuclear data, in multi-locus analyses (Krosch et al., 2012; Virgilio et al., 2015). *Cox1* and 16S ribosomal RNA together defined distinct clades within *B. caudata* Doleschall, with one clade for flies from Malaysia-Thailand-China and a second from Bali-Lombok (i.e., straddling Wallace's Line) (Lim et al., 2012).

*Microsatellites*

Microsatellites have been widely used in studies of population structure and speciation (Goldstein & Schlötterer, 1999). Microsatellites are tandem repeats of a short nucleotide motif repeated up to 60 times (Tautz & Schlötterer, 1994) which are widespread throughout the genome (Hancock, 1999; Sunnucks, 2000; Zhang & Hewitt, 2003), easy and reliable to score, and are highly polymorphic (Bruford & Wayne, 1993).

Microsatellites are commonly employed in tephritid population genetic studies. For example, the genetic relationships among three Australian sibling species, *B. tryoni*, *B. neohumeralis* and *B. aquilonis* were investigated using microsatellites. Microsatellite data indicated that *B. tryoni* and *B. neohumeralis* are sympatric, while *B. aquilonis* is allopatric to both. This data also indicated that even though *B. tryoni* and *B. neohumeralis* are genetically distinct and there are differences in mating time between the two species, there was some gene flow between them (Wang et al., 2003). Microsatellites also reveal the dynamic nature of populations of *B. tryoni* in eastern Australia (Gilchrist et al., 2006). In ancestral areas, where there are large, long-established populations, differentiation is low, presumably reflecting extensive gene flow. However, in more recently invaded, temperate areas, *B. tryoni* exists as either small metapopulations derived from the ancestral areas or very small metapopulations that rapidly decline due to pest control measures. These metapopulations in the recently invaded areas are far more genetically diverse, presumably because of genetic drift. The microsatellites suggest recurring migration of flies both from the ancestral areas to the many scattered populations in the invaded area and from these metapopulations to more distant, ephemeral populations in the far west, which is one of the country's most important quarantine areas (Gilchrist et al., 2006).

Data from 11 polymorphic microsatellite loci revealed high levels of genetic diversity among populations of medfly, *C. capitata* in South Africa (Karsten et al., 2013; Karsten et al., 2015). However, although individual medfly adults rarely fly more than 10 km (Meats & Smallridge, 2007), there was little population structure at any studied scale within South Africa leading the authors to deduce that gene

flow among the populations (some of which were separated by more than 1600 km) likely resulted from human-assisted dispersal.

Microsatellite data for *B. dorsalis* in China and Southeast-Asia show that genetic diversity is lower in southeast China than in southwest China, and that Southeast-Asian *B. dorsalis* exhibit relatively rich genetic diversity (Shi et al., 2010). This is consistent with *B. dorsalis* being introduced into South-western China and with an invasion route between China and Southeast-Asia. Microsatellite data revealed no significant population structuring in *B. dorsalis* in the Thai-Malay Peninsula (Krosch et al., 2013) and provided a strong argument that *B. dorsalis* and *B. papayae* are conspecific, resulting in their synonymy (Schutze, 2015a).

Behavioural approaches (pre-and post-zygotic mate compatibility)

Species identification based only on morphological and molecular tools may not always be totally reliable, especially in cryptic species (Nadler & De Leon, 2011; Jörger & Schrödl, 2013). It is becoming increasingly evident that an understanding of the behavioural boundaries of species can clarify such morphological and molecular ambiguity. Comparative behavioural information relevant to pre-zygotic compatibility, especially mating compatibility, is widely recognised as being important in resolving biological species (Walter, 2003). Pre-zygotic behavioural mechanisms may prevent wasteful mating and post-zygotic incompatibility which can be detected through reduced viability of offspring. Tephritids vary in their preferred time of mating, location of mating, response to waving of wings, and auditory calling (AliNiazee, 1974; Sivinski et al., 1984; Miyatake, 1997). Variation in these characteristics may be the basis for isolation of lineages and thus further differentiation. This kind of variation can be revealed by mating experiments and field observation. Many behavioural studies have provided valuable information for discriminating between the members of species complexes in fruit flies. Schutze et al. (2013) studied mating behaviour in the *B. dorsalis* complex and found consistent behavioural differences, particularly with respect to mating location, between *B. carambolae* on one hand, and *B. philippinensis*, *B. papayae* and *B. dorsalis*, on the other. These data add to evidence that *B. dorsalis*, *B. papayae* and *B. philippinensis* represent the same biological species and are distinct from *B. carambolae*. In

another example, differences in mating behaviour indicated that among several populations of *A. fraterculus* from South America are reproductively isolated. Most of the populations were non-compatible with each other and thus sexually isolated. Using results from mating compatibility combined with morphometrics, genetic confirmed the cryptic species complex in *A. fraterculus* (Vera et al., 2006). Likewise, a study of mating behaviour in two strains of uncertain status in the *A. fraterculus* complex from Argentina and Peru indicated that they belong to different, biological species (Cáceres et al., 2009).

Further, some populations differ in the time of day they mate: the *Bactrocera tryoni* complex contains *B. tryoni* and two closely related species*, B. neohumeralis* and *B. neohumeralis.* There are slight differences in morphology, particularly the colour of the humeral callus, and mating time in that *B. tryoni* mates only at dusk while, *B*. *neohumeralis* mates during the day (Drew & Lambert, 1986; Morrow et al., 2000); however, these species cannot be resolved on genetic grounds. While others fruit flies may prefer to mate on different parts of the host tree, in another tephritid genus, *Blepharoneura*, diversification may involve shifts in use of both host taxa and host parts (Condon & Steck, 1997). Condon et al. (2008) observed mating behaviour among species of *Blepharoneura* and found that courtship behaviours may play a role in maintaining reproductive isolation among sympatric species. *Blepharoneura* spp. avoid mis-matings by courting and mating on different parts of the host plant (Condon & Norrbom, 1999).

Pheromone differences

Pheromones, in general, are important in the courtship and mate recognition system of many insects (Ayasse et al., 2001; Wyatt, 2003; Wicker-Thomas, 2007); fruit flies are no exception. Tephritid males produce volatile compounds that have been implicated in attracting and securing mates (Nation, 1990; Drew, 2004; Cáceres et al., 2009). Because of this, pheromones are species specific (Drew & Hancock, 1994; Drew et al., 2008); hence, they are robust species delimitation criteria and the application of pheromone analyses is of increasing importance in the identification of cryptic species (Aluja & Martin, 1999).

In the *B. dorsalis* complex, there are distinct differences in the volatile components of the male rectal gland between *B. carambolae* and *B. dorsalis* (Perkins et al., 1990; Wee & Tan, 2005), but very few or no differences between *B. dorsalis* and those species previously referred to as *B. papayae* and *B. philippinensis* (Fletcher & Kitching, 1995). Males of *B. papayae*, *B. philippinensis* and *B. dorsalis* consume the potent male attractant methyl eugenol (ME) and biotransform it to two oxidized analogues (2-allyl-4, 5-dimethoxyphenol and (E)-coniferyl alcohol) for storage in the rectal gland and subsequent use in courtship interactions (Tan & Nishida, 1996, 2012; Tan et al., 2013). This results in a mating advantage for males that consume ME (Shelly & Dewire, 1994; Shelly et al., 1996; Tan & Nishida, 1996). Similarly, male "*B. invadens"* also respond to and consume ME and biosynthesize the same rectal gland pheromone constituents (2-allyl-4, 5-dimethoxyphenol and (E)-coniferyl alcohol) as *B. dorsalis* (Tan et al., 2011; Tan & Nishida, 2012). All of these species are now regarded to be conspecific (Schutze et al., 2015a).

As the case for *B. papayae* and *B. philippinensis*, the pheromones released by sexually mature males of the South American fruit fly, *A. fraterculus* from different geographical populations differ quantitatively and qualitatively in the composition of volatiles (Cáceres et al., 2009). It has been suggested that the differences among the pheromone mixtures might be regulated by multiple genes and that variations in pheromone would be indicative of incipient speciation (Brízová et al., 2013).

Cuticular hydrocarbons

Cuticular hydrocarbons (CHCs) are one of several components of insect epicuticle. In addition to playing an important physiological role in water conservation and desiccation resistance (Gibbs, 2011), they are important for inter-individual recognition for some insect groups, particularly among the social groups including termites, ants and bees (d'Ettorre & Lenoir, 2010; Rottler et al., 2013; Curtis et al., 2013; Jennings et al., 2014). Particular CHC profiles are characteristic of numerous insect lineages and are often distinct and stable over large geographical areas (Martin et al., 2008; Martin & Drijfhout, 2009). Food source and other

environmental factors may influence CHC profiles in insects (Fedina et al., 2012), and they may also differ between sexes (Jennings et al., 2014). While the precise constitution of CHCs in the epicuticle may vary depending on environmental influences, CHC composition is also determined by the underlying genotype; hence, specific CHC composition may reflect differences at the species and population (intraspecific) level. Thus, they are particularly useful as chemotaxonomic tools to resolve cryptic lineages. They are important in mate recognition and as indicators of mate quality (Blomquist & Bagnères, 2010; Guillem et al., 2012; Kather & Martin, 2012; Vaníčková et al., 2014). Similar to pheromones, different species have different constituent compounds and these can be used for identification. Consequently, they are very effective tools for species delimitation.

CHCs can be obtained from specimens that have been damaged, dried or otherwise rendered unusable for morphometric or DNA analysis. CHC analysis is particularly useful for the study of cryptic species that are difficult to recognize based on classical morphological or molecular characteristics (Sutton, 1994; Vaníčková et al., 2014). CHCs have been used to differentiate species in tephritids, such as among of larvae and adults of *A. ludens*, *A. suspensa*, *C. capitata*, *C. rosa*, *Z. cucurbitae*, and *B. dorsalis.* CHC profiles for these species exhibit statistical differences between species, similarity in profiles of conspecific life forms (e.g., pupae of *A. suspensa* from different locations), and the same four major alkanes in all *Anastrepha* and *Ceratitis* larvae. The alkane patterns of adults of *Z. cucurbitae* and *B. dorsalis* were distinctly different to the alkane profiles of *Ceratitis* and *Anastreph*a. Although the two *Anastrepha* species showed the same major peaks as *Ceratitis*, the patterns were different (Carlson & Yocom, 1986). CHCs have also been used to distinguish between larvae of *A. suspensa* and *C. capitata* (Sutton & Steck, 1994) and between two species (*Bactrocera* Malaysia A and *Bactrocera* Malaysia B) of the *Bactrocera dorsalis* complex from Malaysia. Cuticular hydrocarbons suggested that '*B.* Malaysia A' and '*B.* Malaysia B' are closely related species which are almost indistinguishable morphologically except by comparison of the female ovipositors (Goh et al., 1993). These species were subsequently described as *B. carambolae* (Malaysian A) and *B. papayae* (Malaysian B; now *B. dorsalis*) (Drew &

Hancock, 1994). Recently, gas chromatography and pattern recognition techniques were developed to classify larvae and adults of both sexes in the *Anastrepha* complex (Lavine et al., 1992; Vaníčková et al., 2014) and cryptic species of the *Ceratitis* FAR complex of African tephritids (Vaníčková et al., 2014).

Comparative host use

The fruit fly literature is replete with host records suggesting comparative host use may be useful for recognising lineages, races and species (cryptic or otherwise). This is consistent with the generalisation that different biological species have different host requirements and are correspondingly attracted to different hosts (Bernays, 1998). Indeed, the previous sections have noted how diet and affect pheromone composition and CHC profiles, which in turn can influence mate recognition. However, in practice, host data has not proved definitive in the recognition of tephritid lineages (Mitter & Futuyma, 1983). This is largely because, in reality, some species have genuinely wide host ranges, because some supposed polyphagous "species" have proved to be composites of several species, and because host shifts undeniably occur. Hence, host associations, without the support of molecular evidence indicating reduced gene flow or other data indicating reproductive isolation, are unreliable indicators of host races or biological species (cryptic or otherwise). Cryptic host lineages may be especially predicted in polyphagous species (Brunner et al., 2004).

A host race can be said to exist in a plant-feeding insect when different lineages exhibit a strong preference for different species or cultivars of a host plant or for a range of closely related host plants (Drès & Mallet, 2002). The lineage may be totally restricted to one or a few plant species or varieties, yet may or may not be recognisable using morphological, morphometric, or other traditional characters. One of the most widely known cases of host race formation is that of the apple maggot fly *Rhagoletus pomonella* (Walsh). *Rhagoletus pomonella* originally utilised native hawthorn fruits, but secondarily came into association with new hosts, such as apple and cherry, which ripened at different periods of the year (Bush, 1974; Feder, 1998; Linn et al., 2005). Selection of allele frequencies to adapt to these hosts was accompanied by changes in habitat-specific mating and oviposition

behaviours (Feder et al., 1997; Linn et al., 2004). This provoked rapid, sympatric, evolutionary divergence of the species into distinct races (Whitman & Agrawal, 2009).

Host data can be used combined with other characters as evidence for species delimitation and sometimes can be highly practicable for fruit fly identification. For example, fruit flies in the *B. dorsalis* complex usually have many kinds of the same host plants. However, extensive rearing data for *B. carambolae* and *B. dorsalis* in native and introduced ranges (Thai-Malay Peninsula and Suriname) found that *B. dorsalis* infested banana (*Musa* sp.) but *B. carambolae* does not, either in Southeast-Asia or in its invasive northern South American range of Suriname (Clarke et al., 2001; van Sauers-Muller, 2005). Similarly, *Z. tau* has been subdivided into eight forms which can be differentiated on cytological, morphological and molecular characteristics and which differ substantially in host-plant preferences. For example, one form is polyphagous, one form occurs only in fruit of *Strychnos thorelii* Pierre ex Dop, one form only in fruit of *Siphonodon celastrineus* Griff, while two other forms attack the same host plant species, *Momordica cochinchinensis* (Lour.) Spreng*.,* and yet another two forms both utilise the same host, *Hydnocarpus anthelminthicus* Pierre ex Laness*,* but in different localities (Tigavattananont, 1986; Meksongsee et al., 1991; Baimai et al., 2000).

Clearly there are an abundance of disciplines available in the application of an integrative taxonomic study of tephritid fruit flies, as for any insect species. This does not mean that all lines of evidence be pursued; particularly considering time and logistical constraints associated with the study of a widespread pest species that occurs in a different country to that where it is being investigated. Therefore, for this thesis, an integrative study of *Z. cucurbitae* was undertaken focussing on multiple morphological (wing morphometric, aedeagus analysis) and genetic approaches (*cox1* and microsatellite) which were examined in light of biogeographic and host association hypotheses. Live insect studies, as would be required for behavioural, mating compatibility, or chemoecological studies, would have posed too great a biosecurity risk and were considered beyond the scope of this thesis.

## 1.5 THE MELON FLY, *ZEUGODACUS CUCURBITAE*

### 1.5.1 Economic impact, taxonomic position and geographic distribution

The melon fly, *Z. cucurbitae* (Figure 1.2 and Chapter 1 cover-page) is one of the major pest fruit fly species of the Oriental region. It infests the fruit of more than 125 plant species, predominantly from the Cucurbitaceae (Piñero et al., 2006) (Table 1.1), and inflicts heavy commercial losses in its native and invasive ranges (Stonehouse et al*.,* 1998; Siebert, 1999; Koyama et al., 2004).

The adult melon fly is 6 to 8 mm in length and has a reddish-coloured body, with three bright yellow longitudinal vittae (stripes) on the scutum (dorsum of thorax) (Figure 1.2D). A coloured mark across the dm-cu crossvein is one of the distinctive characteristics of the melon fly wing pattern (Ibrahim, 1990) (Figure 1.2C). Until early 2015, melon fly was placed in the subgenus *Zeugodacus* Hendel of the genus *Bactrocera* Macquart, i.e., its taxonomic name was *Bactrocera* (*Zeugodacus*) *cucurbitae* (Drew & Romig, 2013). However, subsequent molecular phylogenetic analysis suggested that *Zeugodacus* should be regarded as a genus distinct from *Bactrocera* (Sugura et al., 2006; Krosch et al*.,* 2012)*,* and this was supported by a more recent study that formally erected *Zeugodacu*s to generic rank (Virgilio et al*.*, 2015). Hence, in this thesis, I refer to the species as *Z. cucurbitae*, even though in most literature it is referred to as *B. cucurbitae*.

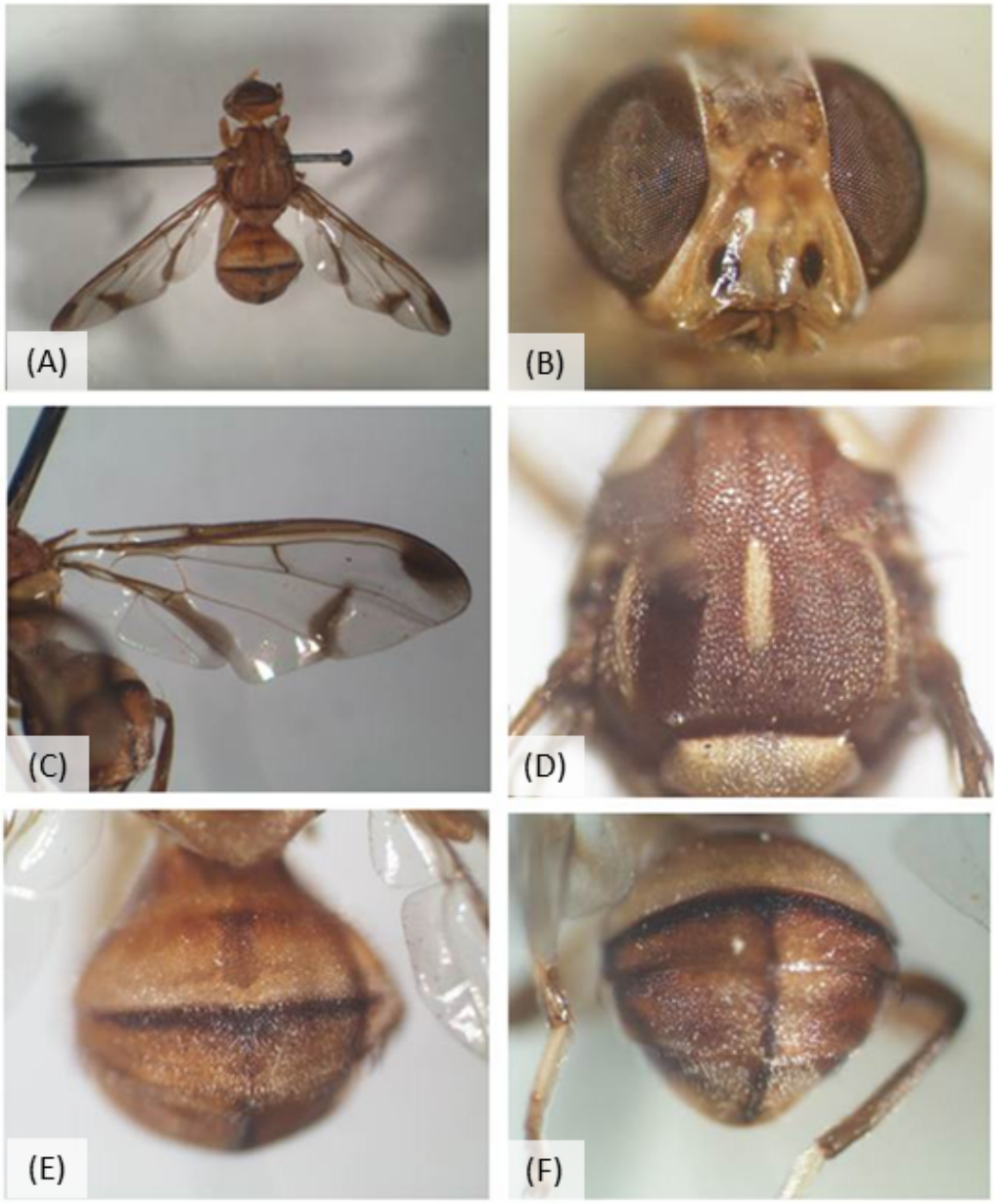



**Figure 1.2** *Zeugodacus cucurbitae,* habitus and body details. (A) melon fly (B) head with compound eyes (C) wing (D) scutum with a medial postsutural vitta (E) abdomen (F) abdomen and hind legs.

**Table 1.1** Host plants of *Zeugodacus cucurbitae* (Modified from Dhillon, 2005)

| Scientific Name | Family | Common Name | References |
|---|---|---|---|
| *Benincasa hispida* (Thunb.) Cogn. | Cucurbitaceae | Chinese melon | Narayanan & Batra, 1960 |
| *Citrullus lanatus var. lanatus* (Thunb.) Matsum. & Nakai | Cucurbitaceae | Water melon | Narayanan, 1953; Narayanan & Batra, 1960; Pareek & Kavadia, 1994; Allwood et al., 1999; Weems & Heppner, 2001; Vayssieres et al., 2007 |
| *Cucumis sativus* L. | Cucurbitaceae | Cucumber | Narayanan, 1953; Narayanan & Batra, 1960; Pareek & Kavadia, 1994; Allwood et al., 1999; Weems & Heppner, 2001; Vayssieres et al., 2007 |
| *Cucurbita moschata* Duchesne ex Poir. | Cucurbitaceae | Pumpkin | Back & Pemberton, 1917; Narayanan, 1953; Narayanan & Batra, 1960; Wen, 1985; Pareek & Kavadia, 1994; Hollingsworth et al., 1997; Allwood et al., 1999; Weems & Heppner, 2001; Vayssieres et al., 2007 |
| *Lagenaria siceraria* (Mol.) Standl. | Cucurbitaceae | Calabash (water-bottle) | Narayanan & Batra, 1960; Allwood et al., 1999; Wen, 1985; Weems & Heppner, 2001; Vayssieres et al., 2007 |
| *Luffa acutangula* (L.) Roxb. | Cucurbitaceae | Ribbed gourd | Narayanan, 1953; Narayanan & Batra, 1960; Pareek & Kavadia, 1994; Allwood et al., 1999; Weems & Heppner, 2001 |
| *Luffa cylindrica* M.Roem | Cucurbitaceae | Sponge gourd | Narayanan, 1953; Narayanan & Batra, 1960; Pareek & Kavadia, 1994; Allwood et al., 1999; Weems & Heppner, 2001; Vayssieres et al., 2007 |
| *Momordica charantia* Duchesne ex Poir. | Cucurbitaceae | Bitter gourd | Narayanan, 1953; Narayanan & Batra, 1960; Wen, 1985; Wong et al., 1989; Uehida et al., 1990; Pareek & Kavadia, 1994; Hollingsworth et al., 1997; Allwood et al., 1999; Weems & Heppner, 2001; Vayssieres et al., 2007 |

**Table 1.1** Continued

| Scientific Name | Family | Common Name | References |
|---|---|---|---|
| *Trichosanthes cucumerina* L. | Cucurbitaceae | Wild snake gourd | Narayanan, 1953; Narayanan & Batra, 1960 |
| *Vignia unguiculata* L. | Fabaceae | Long bean or cowpea | Narayanan and Batra, 1960 |
| *Phaseolus vulgaris* L. | Fabaceae | String/French bean | Narayanan &Batra, 1960 |
| *Phaseolus limensis* L. | Fabaceae | Lime bean | Narayanan & Batra, 1960 |
| *Phaseolus radiculatus* | Fabaceae | Green gram | Narayanan & Batra, 1960 |
| *Dolichos lablad* (L.) Sweet | Fabaceae | Hyacinth bean | Narayanan & Batra, 1960 |
| *Cajanus cajan* (L.) Millsp. | Fabaceae | Pigeon pea | Narayanan & Batra, 1960 |
| *Carica papaya* L. | Caricaceae | Papaya | Narayanan 1953, Narayanan & Batra, 1960, Wong et al. 1989, Vargas et al. 1990, Weems & Heppner, 2001 |
| *Abelmoschus* esculentus (L.) Moench | Malvaceae | Okra | Narayanan & Batra, 1960; Ranganath & Veenakumari, 1997 |
| *Anacardium occidentale* L. | Anacardiacea | Cashew nut | Vayssieres et al., 2007 |
| *Annona reticulate* L. | Annonaceae, | Custard apple | Narayanan & Batra, 1960 |
| *Averrhoa carambola* L. | Oxalidaceae | Starfruit/carambolas | Wen, 1985; Armstrong et al., 1995; Vayssieres et al., 2007 |
| *Persea Americana* Mill. | Lauraceae | Avocado | Narayanan, 1953, Narayanan & Batra, 1960 |
| *Brassica oleracea* L. | Brassicaceae | Cauliflower | Dhillon et al., 2005 |
| *Mangifera indica* | | Mango | Narayanan & Batra 1960; Weems & Heppner, 2001 |
| *Citrus sinensis* L. | Rutaceae | Orange | Narayanan & Batra, 1960; Weems & Heppner, 2001; Vayssieres et al., 2007 |

*Zeugodacus cucurbitae* is widely distributed throughout the warmer regions of the world and has a near global distribution including the Indian subcontinent, Southeast-Asia, Africa, the Hawaiian Islands and other parts of Oceania (Weems et al., 1999; Dhillon et al., 2005) (Figure 1.3, Table 1.2). The species' origin was postulated to be the Indo-Oriental region (Drew & Hancock, 2000), with molecular evidence (microsatellite and *cox1* data) supporting the geographic origin of *Z. cucurbitae* as sub-continental Asia (Pakistan-India-Bangladesh) (Virgilio et al., 2010). From this origin, *Z. cucurbitae* is postulated to have dispersed into Indochina and through the Southeast-Asian archipelago. The fly was detected in Hawaii in 1895, and invaded other Pacific islands during the 20$^{th}$ century, including Guam (1936), Papua New Guinea (1940), and the Solomon Islands (1984) (Back & Pemberton, 1917; Drew et al.,1982; Eta, 1985; Waterhouse, 1993; Dhillon et al., 2005; Wu et al., 2009; Virgilio et al., 2010; Putulan, 2014; Vargas et al., 2015). Some of this dispersal may have been 'natural' (e.g., it is conceivable that melon fly naturally dispersed across the Indonesian archipelago to Papua New Guinea and Solomon Islands), but some is almost certainly human assisted (e.g., from Japan to Hawaii, Weems et al., 2011). *Zeugodacus cucurbitae* has also spread into North and East Africa, but it is not clear how it found its way to Africa; and African populations are genetically well-differentiated compared to those from Asia (Virgilio et al., 2010). The lack of detectable bottlenecks suggested that recent human-assisted African invasions might have originated from elsewhere in the African continent (Khamis et al., 2009; Virgilio et al. 2010).

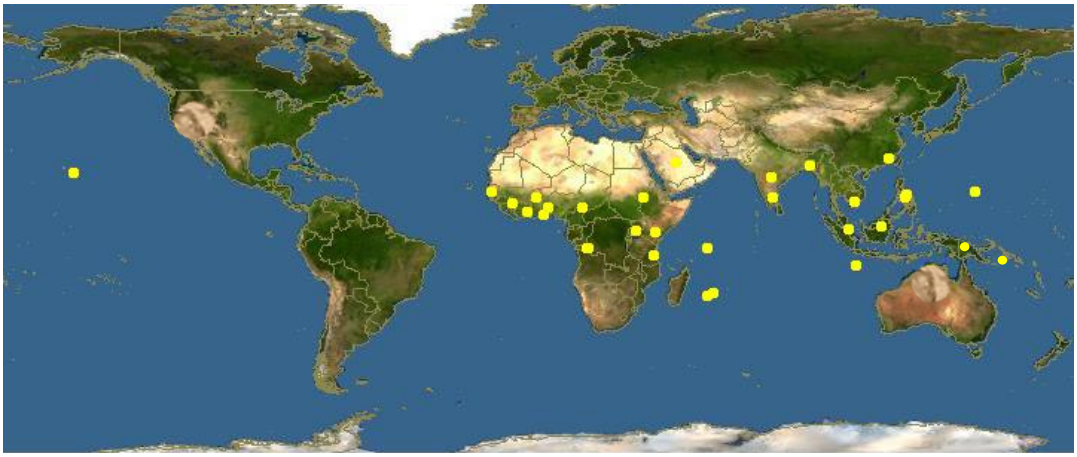


**Figure 1.3** Map of the worldwide distribution of *Zeugodacus cucurbitae* (Modified from Anonymous, 2015a)

**Table 1.2** Geographic distribution of melon fruit fly, *Zeugodacus cucurbitae* (Modified from Dhillon et al., 2005)

| Continents | Type of distribution | Countries |
|---|---|---|
| Asia | Native | India and Bangladesh |
| | Invasive (over historical time) | Afghanistan, Brunei, Cambodia, China (numerous provinces), Christmas Island, East Timor, Indonesia (numerous islands),Iran, Laos, Malaysia, Burma, Nepal, Oman, Pakistan, Philippines, Singapore, Sri Lanka, Taiwan, Thailand, United Arab Emirates, Vietnam |
| North America | Invasive (known human assisted) | United States: established in Hawaii, periodic interceptions in other states |
| Oceania | Invasive (assumed human assisted) | Guam, Kiribati, Nauru, Northern Mariana Islands, Papua New Guinea, Solomon Islands, Hawaiian Islands |
| Africa | Invasive (possibly over historical time and human assisted) | Cote d'Ivoire, Cameroon, Egypt, Gambia, Kenya, Mali, Mauritius, La Reunion, Seychelles, Somalia, Tanzania |

### 1.5.2 Morphological and genetic variation in *Z. cucurbitae*

*Cox1 and intra-specific genetic variation*

Wu et al. (2009) inferred relationships among populations of *Z. cucurbitae* from mitochondrial DNA. Their results were consistent with an Indian origin hypothesis and the subsequent spread of populations into other hot-humid regions of Asia and Southeast-Asia. They found the western Asian region showed the highest level of genetic diversity and so assumed that *Z. cucurbitae* had expanded its range from west to east. Other studies have failed to detect this regional variation in genetic diversity. Seven populations of *Z. cucurbitae* from China and two populations from Southeast-Asia exhibited exceedingly low diversity of *cox1* across all populations (Hu et al., 2008). *Cox1* data for *Z. cucurbitae* from India, China, Japan, Sri Lanka, Thailand and Hawaii, albeit based on a small number of geographic samples, suggested that the species comprises one widespread population that is relatively homogenous and which may have originated only a relatively recent 400 thousand years ago (Prabhakar et al*.,* 2012). This theory of a single, panmictic population of *Z. cucurbitae* is based largely on a small number of samples from Southeast-Asia and these from just one country (Thailand) (Prabhakar et al., 2012). While existing studies suggest that *Z. cucurbitae* is a single phylogenetic lineage (Hu et al., 2008; Wu et al., 2011; Jacquard et al., 2013), all studies are based on very small sample site numbers and more sequences from a wider range of localities and host plants in Southeast-Asia are required to fully resolve this species in the region.

*Microsatellite studies*

Population structure and gene flow in *Z. cucurbitae* in Southeast-Asia and China has been studied using 12 polymorphic microsatellite markers (Wu et al., 2011). Genetic diversity was found to be low in China but high in Southeast-Asia. However, these studies sampled only three sites from Southeast-Asia (Indonesia, Burma, Thailand) and, critically, incorporated no sequence data from *Z. cucurbitae* populations in peninsular Southeast-Asia or from the Indo-Malay archipelago (Wu et al., 2011). A study of macrogeographic population structure and phylogeography in *Z. cucurbitae* using 13 microsatellite markers and material from 25 sites across

the world (except the Pacific) found that genetic diversity was highest in Pakistan (Virgilio et al. 2010). The Southeast-Asian region, which is the focus of my thesis, was conspicuously under-sampled by Virgilio et al., (2010) with only five sites represented. In summary, sequence data currently available provides only the most preliminary view of the population structure of *Z. cucurbitae* over the bulk of its endemic Southeast-Asian distribution.

*Host races and melon fly*

*Zeugodacus cucurbitae* predominantly attacks cucurbit fruits (family Cucurbitaceae) (Bezzi, 1913; Shah et al., 1948; Allwood et al., 1999; Clarke et al., 2001; Jacquard et al., 2013) and is known from over 100 plant species, attacking over 70 host plants in India alone (Doharey, 1983). Many of these host records may, however, be based on erroneous records following observations of adults resting on plants or caught in traps set in non-host species, rather than by demonstrated infestation (White & Elson-Harris, 1992). Indeed, only nine species of cucurbit and a total of 13 host species have been recorded for *Z. cucurbitae* in surveys in Papua New Guinea, the Solomon Islands, Nauru and Northern Marianas Islands (Wong et al., 1989; Allwood & Drew, 1996).

With such a wide range of host plants, some may be available more or less continuously, while others may be available only intermittently, seasonally, or only in particular habitats. Further, *Z. cucurbitae* is also regularly reared from some non-cucurbit hosts, such a beans (Narayanan & Batra, 1960; Wong et al., 1989; Allwood & Drew, 1996; Weems & Heppner, 2001). In such a situation, it is important to determine whether there is any evidence for host races since their existence would add an extra layer of complexity to interpretations of the species' population structure and dispersal history. Alternatively, it may be that *Z. cucurbitae* simply exhibits extreme phenotypic plasticity (Whitman & Agrawal, 2009), which would help explain the success of *Z. cucurbitae* as an invasive pest (Papadopoulos, 2014).

### 1.5.3 Melon fly and plant biosecurity in Thailand

Thailand is an agricultural country; approximately 21 million ha, or 40.9%, of the total area is used for agricultural production (OAE, 2008). The climate and soils of Thailand are particularly conducive to the production of fruit and vegetables (Long, 1973), and these commodities generate important income for Thai farmers (Krongkaew, 1985). Nevertheless, a constantly growing Thai population, rising incomes, and increasing levels of urbanization are all increasing the demand for fruit and vegetables (Cheng & Lee, 1991). In addition to domestic use, Thailand is a major exporter of agricultural produce, especially fresh tropical fruit and vegetables (Zhou & Thomson, 2009). Some export markets have minimal phytosanitary requirements, but many of the most profitable destinations for Thai produce are increasingly sensitive to phytosanitary risk (Anonymous, 2015b). Consequently, the pressure on Thailand to manage plant pest threats increases as global markets become more regulated (U. Unahawutti, personal communication, April 22, 2014). Unfortunately the climatic and geographical characteristics of Thailand, and the sheer diversity of Thai horticulture, promote a diverse and flourishing array of pests, including thrips, whiteflies, aphids, mealybugs, lepidopteran larvae and leaf mining flies. In the past, Thai farmers have relied heavily on pesticides, either in the field or post-harvest, to deal with pests such as fruit flies (Allwood & Drew, 1996; Chinajariyawong et al*.,* 2003); however, chemical control options are diminishing because of the increasing sensitivity of markets to pesticide residues (Panuwet et al., 2012).

Thailand and its neighbours are now members of the Association of Southeast Asian Nations (ASEAN) Economic Community (Plummer & Yue, 2009). Commercial integration of the ASEAN Member Countries creates rich market opportunities for Thai agricultural products, but it is accompanied by expectations among neighbours of easy access to the Thai market for their agricultural exports. There is a risk that the political drive towards liberalised trade will overwhelm the capacity of biosecurity agencies to assess and manage the risk of moving plant pests. Thailand shares porous borders with several countries whose biosecurity capabilities remain

a work in progress. These neighbours are yet to document their pest status in any systematic way and certainly have minimal capacity to manage pest threats.

Tephritid fruit, flies are important threats to Thailand agriculture and cause significant crop losses. While there are many tephritid species in Thailand, two species stand out as being of the greatest economic importance, *Z. cucurbitae* and *B. dorsalis* (Guamán, 2009). Both are considered to be key insect pests of fruit production in Thailand, causing loss of yield and degradation of quality (Department of Agriculture, 2012); cucumber and bitter gourd particularly suffer from fruit infestation by *Z. cucurbitae* (Ramadan & Messing 2003).

Even though fruit flies are one of the groups of insect pests that cause most problems in Thai agriculture, there have been relatively few research activities that have focused on fruit flies, in particular melon fly. Kittayapong et al*.* (2000) and Jamnongluk et al*.* (2002) studied the distribution of the reproduction-modifying bacteria, *Wolbachia*, in natural populations of tephritid fruit flies (including *Z. cucurbitae*) in Thailand. Clarke et al*.* (2001), studying the seasonal abundance and host use patterns of seven species of fruit fly in Thailand and Peninsular Malaysia, reported that *Z. cucurbitae* showed no clear pattern in population modality between regions and confirmed that the fly is predominantly a cucurbit pest. Ramadan and Messing (2003) studied parasitism of *Z. cucurbitae* in Thailand and found that melon fly sometimes were parasitised by *Aceratoneuromyia* species (Hymenoptera: Eulophidae) and Guamán (2009) monitored fruit files in Thailand and concluded that *Z. cucurbitae* and *B. dorsalis* are most active in the morning. Even though fruit flies cause massive problems in Thai agriculture (Sutantawong et al., 2004; Orankanok et al., 2007), it can only be concluded that research on fruit flies in Thailand has been inadequate for most applied purposes.

Few Thai researchers have applied molecular tools for fruit fly identification or ecological studies. However, these research projects have all been conducted from university laboratories and have thus far tended to be quite narrow in scope, focusing on chromosome and phylogenetic analysis (Baimai et al., 1995, Jamnongluk et al., 2003; Saelee et al., 2006). For example, Baimai et al. (1995) studied chromosomal variation in the *B. dorsalis* complex and five closely related

species designated *B. dorsalis* s.s. species A, B, C, D, and E. In addition, *B. dorsalis* complex including flies from Thailand using *cox1* and microsatellites were conducted (Krosch et al., 2013; Schutze et al., 2012b). These work have contributed to the resolution of the species of the *B. dorsalis* complex in Thailand and demonstrated that *B. dorsalis* is the same species as *B. papayae*, resulting in the synonymization of these taxa (Schutze et al., 2015a).

Another pest group, the *Z. tau* complex, has been found via molecular allozyme analysis to consist of at least eight species in Thailand (Baimai et al., 2000; Saelee et al., 2006); while *cox1* phylogenetic analyses have identified four clades within the complex (Jamnongluk et al., 2003b). Furthermore, subgeneric-level *cox1* phylogenetic studies within *Bactrocera* have shown that subgenera containing cucurbit-attacking species (i.e., *Asiadacus*, *Hemigymnodacus* and *Zeugodacus*) are more closely related to each other than to other *Bactrocera* subgenera containing highly polyphagous species that attack a wider range of plant families (Jamnongluck et al., 2003a).

There is evidence that at least some fruit flies move freely across Thai biogeographic regions. *Cox1* data for *B. latifrons* from 11 locations in northern and northeastern Thailand revealed no major divergent lineages (Meeyen et al., 2014), suggesting high rates of gene flow among populations. Two divergent lineages were detected among 15 populations of the guava fruit fly, *B. correcta,* from locations in Central, East and Northeast Thailand (Kunprom et al., 2015), but overall genetic differentiation among populations was low, again suggesting extensive exchange of flies among the populations.

Molecular data have been used to investigate fruit fly invasion pathways and colonisation involving Thailand. Microsatellite data for the *B. dorsalis* complex in seven countries, including Thailand and Hawaii, showed no recent, genetic bottlenecks and suggested a migration route from China to the west (Aketarawong et al., 2006, 2007). Microsatellite data for *B. dorsalis* in six countries in East-Asia (including Thailand) has suggested that human assistance has played a strong role in dispersal and colonisation in this species (Aketarawong et al., 2014b).

Microsatellites have also been used to demonstrate the potential value of sterile insect release technologies (SIT) for control of *B. dorsalis* in Thailand. In trials

conducted in central of Thailand, there was no genetic admixture between released, mass-reared flies and wild flies within the release (i.e., potential control) area (Aketarawong et al., 2011).

There have been no molecular studies on *Z. cucurbitae* in Thailand. A much more comprehensive understanding of *Z. cucurbitae* in Thailand is necessary to enable the Thai biosecurity system to respond to regional trade pressures, make sense of the inevitable discovery of previously undocumented variation in the species, and to cope with the likely ongoing effects of biological invasions.

## 1.6 STRUCTURE OF THE THESIS AND PH.D. OBJECTIVE

In Thailand, the traditional taxonomic approach using morphological characteristics will remain central to fruit fly identification (Department of Agriculture, 2010). However, identifying species will increasingly depend on additional methods, and in the not too distant future the necessities of screening large numbers of samples for particular species will demand molecular screening methods. There are many new approaches that can be applied to species resolution and diagnosis such as geometric morphometrics, DNA barcoding, population genetics, or genomic approaches. Acquiring these tools can be costly for plant biosecurity agencies in Thailand but they are modest compared to the potential biosecurity-related consequences to Thai agriculture (Kong et al., 2012). As a member of the Royal Thai Department of Agriculture, I am particularly concerned about how little is known of *Z. cucurbitae* in Thailand and neighbouring regions. To address this knowledge gap, this thesis studies the population variation (as assessed through morphometrics, geometric morphometrics and population genetics) of native and introduced melon fly populations to examine natural variation, population structure, distribution, host plant use and putative invasive pathways.

The overall aim of this thesis is to explore population level variation of *Z. cucurbitae* with the supplementary goal of supporting recommendations for melon fly management in Thailand. Following this introductory chapter, chapter 2 provides a description of the general materials and methods, followed by three research chapters and a final discussion chapter.

In chapter 3 focuses on *Z. cucurbitae* population structuring at what I refer to in this thesis as the intermediate spatial scale encompassing the whole of Thailand, with a few sampling sites in peninsular Malaysia. Within Thailand, historical biogeographic effects and human mediated movement may be contributing to contemporary population structuring. To test this, I present results of population analyses based on samples from six mainland regions and two islands in Thailand, with a focus on 17 sites along the Thai-Malay transect (Isthmus of Kra). Two hypotheses are tested regarding *Z. cucurbitae* population structuring within Thailand. The first hypothesis posits that natural variation and genetic diversity are correlated with biogeographic and regional environmental conditions in different parts of Thailand. The second hypothesis addresses whether the Isthmus of Kra barrier has influenced natural variation in *Z. cucurbitae* and constitutes a geographic barrier or contact zone separating populations from the northern and southern parts of the Malay Peninsula. In summary, this chapter reveals that flies within Thailand are relatively homogeneous, except for flies from the Northeast. Moreover, there was no association strictly with Isthmus of Kra but the results were consistent with a different biogeographic barrier at Kangar-Pattani line. The question of whether these differences imply a phytosanitary risk to the rest of Thailand or to international trading partners are explored in chapter 5, which examines these differences (and others) in the context of past invasive pathways.

Accordingly, chapter 4 data are assembled based on host plant associations for Thailand. These data enable investigation of morphological and genetic variation among the flies which have been collected from different host plants. This chapter focuses on host-plant relationships to test the hypothesis that host races of *Z. cucurbitae* exist in Thailand, as would be suggested by genetic or morphological differentiation associated with host-reared material from different hosts. To obtain maximum geographical and host coverage, samples were reared from ten different fruit and vegetable species collected from ten sites within central Thailand. However, host plant use may not be the only factor explaining variation in *Z. cucurbitae*, with sub structuring related to geography equally important.

The population structure of *Z. cucurbitae* over a broad geographic range scale, covering Southeast-Asia and the West-Pacific, is analysed in chapter 5. In contrast to sympatric host effects, this chapter looks at the other extreme of population structuring where historical divergence, historical bottlenecks and genetic drift are likely to be the most important factors in driving genetic structure. Specifically, chapter 5 investigates morphological and genetic data under a hypothesis of multiple and recent introductions from Southeast-Asia into the West Pacific. Greatest diversity amongst the populations studied is in Southeast-Asia, and this suggests this region as the origin of melon fly with dispersal into and across the other regions (e.g., Africa, India and China). Increased diversity in part of the Pacific supports the notion of multiple introductions to some Islands.

The final chapter (Chapter 6) provides a general discussion of the morphological and genetic variation in *Z. cucurbitae* in fine and broad scales and also includes consideration of host plant associations. Together, chapters 3, 4 and 5 contribute to an understanding of the three most likely reasons for morphological variation and population structuring in *Z. cucurbitae*. This understanding supports recommendations for melon fly management in Thailand and also provides guidance to phytosanitary regulators. Current assessments of phytosanitary risk are based on the belief that *Z. cucurbitae* is a single entity but the present study examines this assumption. This study also sheds light on the question of whether populations in newly invaded parts of the melon fly's range are the result of one introduction, multiple introductions or potentially ongoing invasions. This information is valuable when reviewing quarantine arrangements. Finally, the investigation of the evidence for host races provides guidance to the search for fresh avenues for pest management. For example, the new data are relevant to the question of whether crop rotation and removal of alternate hosts can be integrated into new pest management practices.

# CHAPTER 2

## MATERIALS AND METHODS

## 2.1 INTRODUCTION

This thesis takes an integrative taxonomic approach to study variation in *Z. cucurbitae* at different scales, from host use through to local and regional variation. While the questions vary from chapter to chapter, the methodologies to collect data are often common across chapters. To avoid repetition, this chapter describes the general methods applied in these investigations. Specifically, I describe in this chapter the specimen collection process, procedures used in morphological studies (traditional morphological and geometric morphometric), and molecular studies (*cox1* and microsatellite markers). General statistical methods are also described.

## 2.2 SPECIMEN COLLECTION

Adult fruit fly specimens were collected from 20 sites in Thailand and other Southeast-Asian and West-Pacific countries by adult lure trapping or by rearing from infested fruits. Trapping and fruit rearing in Thailand were performed either by myself or colleagues from the Thai Department of Agriculture under my instruction. Trapping in other countries was performed by colleagues who were sent a package of equipment and consumables that included detailed instructions for trapping, handling of specimens, and shipment back to Queensland University of Technology (QUT) Brisbane, Australia.

### 2.2.1 Host fruit rearing

Infested ripe fruit were collected from the field and returned to the laboratory where larvae completed development. Targeted fruit depended on availability, but the nominated hosts were species that are typically attacked by *Z. cucurbitae* and are members of the family Cucurbitaceae, such as *Momordica charantia* L., *Coccinia grandis* (L.) Voigt, *Cucumis sativus*, *Luffa acutangula* or *L. aegyptiaca* Mill. *Z. cucurbitae* was also reared from non-cucurbit hosts such as *Vigna unguiculata* (Fabaceae); *Mangifera indica* (Anacardiaceae); *Averrhoa carambola* (Averrhoaceae); *Syzygium jambos* L. (Alston) (Myrtaceae); and *Persea americana* Mill. (Lauraceae) (Figure 2.1). This range of hosts was sampled to maximize the chances of detecting host races should they exist. Fruit species and variety, fruit weight, fruit number,

date and collection locations were recorded. Over 1500 individual fruit pieces, weighing a total of approximately 366 kg and covering 22 fruit species and varieties were sampled (See Chapter 4 for full details).

Fruit were brought back to the laboratory where they were held over a >1 cm-thick layer of moistened, sterilized sawdust. Drainage was provided to prevent larvae from drowning. Mature larvae emerged and jumped out of the fruit when ready to pupate in the substrate. Resultant pupae were placed in a small emergence cage containing water and sugar for subsequent adults to consume *ad libitum*. Emergent adult flies were permitted to live for one week to allow cuticle sclerotization to ensure all morphological characters developed prior to being killed and preserved for subsequent analysis (Figure 2.2).

In some cases, emergent flies were held for culturing, for which recently emerged adults were identified as *Z. cucurbitae* based on external morphology and transferred to a separate 60 x 60 x 30 cm culturing cage. The sorting process eliminated other species that may have emerged from the fruit, including parasitoids and other tephritids. Cultures were maintained at 23 ± 2 °C and 60 - 80% R.H., and adults were provided a food mixture made from hydrolyzed enzymatic yeast (10 g); corn protein (10 g); sugar (40 g); and water (50 ml). Additional water was supplied *ad libitum*.


**Figure 2.1** Examples of fruit from which *Zeugodacus cucurbitae* were collected in Thailand. (A) *Luffa acutangula* (B) *Momordiaca charantia* (C) *Citrullus lanatus* (D) *Cucumis melo* L. (E) *Coccinia grandis* (F) *Cucumis sativus* (G) *Cucurbita pepo* L. *var. cylindrica* Paris (H) *Luffa cylindrica* (I) *Momordica cochinchinensis* (Lour.) Spreng. (J) *Cucurbita moschata* Duchesne ex Poir. (K) *Sechium edule* (Jacq.) Sw. (L) *Vigna unguiculata* (M) *Mangifera indica* (N) *Averrhoa carambola* (O) *Syzygium jambos* L. (Alston) (P) *Persea americana.*

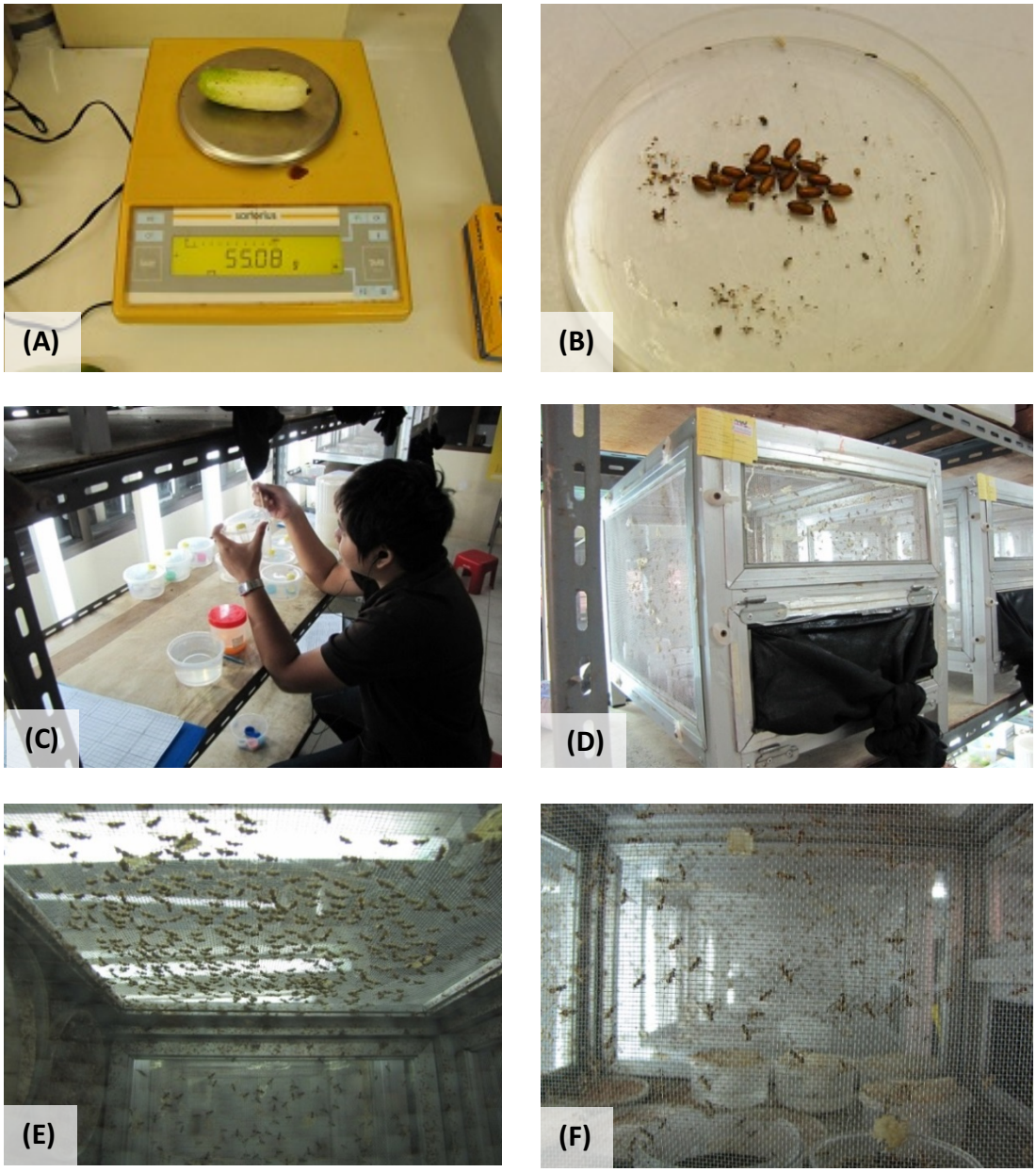



**Figure 2.2** Mass-rearing of *Zeugodacus cucurbitae* from host plants in the insect laboratory located at the Insect Taxonomy Group, Department of Agriculture, Bangkok, Thailand. (A) Weighing host plants that collected from plant plantations; (B-C) collecting pupae from host plants; (D-F) *Z. cucurbitae* emerging from pupae and feeding on water and sugar in holding cages.

### 2.2.2 Adult lure trapping

Fruit flies were collected into cue lure (CL) and methyl eugenol (ME) insecticide-baited hanging traps containing propylene glycol as a preserving agent (Schutze et al., 2012b; Krosch et al., 2013). Most research states that ME is not attractive to males of *Z. cucurbitae*, and that this species is only attracted to cue lure (Shelly & Villalobos, 1995; Shelly et al., 2004). However, ME was also used because local researchers in Thailand and the Philippines have found *Z. cucurbitae* to be attracted to this lure (R. Orqui personal communication, March 9, 2012 and S. Srikachar, personal communication, April 16, 2012). Additionally, many gardeners in Thailand use ME intensively in *Z. cucurbitae* management. Cue lure is difficult to find in Thailand and researchers have to import this lure from abroad. It is impractical and too expensive for gardeners to do this, so they have resorted to using the more readily available ME to control melon fly instead of Cue lure (pers. obs. and from work in progress; 5% from dragon fruit orchard, Chantaburi Province and 3% from Jujube apple plantation, Samutsakorn Province), fruit fly surveillance in Thailand (Plant Protection Research and Development Office, Department of Agriculture, Bangkok, Thailand).

Standard bucket-type fruit fly traps were obtained from Bugs for Bugs Pty Ltd, Australia (Figure 2.3B). Each trap contained propylene glycol preservation fluid and a cotton wick that was impregnated with Malathion insecticide (1ml/wick) and one of two male lure compounds: either ME or cue lure (3 ml/wick). Three of each trap type (ME or Cue) were placed in trees at each sampling location (Figure 2.3A-C). Traps were separated by 3 m on different trees. One trap was hung in each of trees spaced approximately 3 m apart, and remained in place until a sufficient sample (20-50 specimens) had been trapped or for up to a maximum of one week. Flies were identified based on external morphology (Ibrahim, 1990). All samples were collected between 2012 and 2013 and returned to QUT where specimens were transferred into and stored in 100% ethanol at -20˚C until removed for morphological analysis and genetic studies. Voucher samples will be deposited at QUT and the Department of Agriculture (DOA), Bangkok, Thailand.

Sampling outside Thailand was undertaken by regional colleagues. For this, pre-packed trapping kits containing all necessary collecting equipment (traps, preservation fluids, vials, quarantine paperwork) were prepared and shipped for regional collections. As for Thailand collections, fruit flies were collected into ME and cue lure/insecticide-baited traps containing propylene glycol as a preserving agent. Traps were left suspended in place for one week or until they had attracted sufficient specimens (roughly 20-50 individuals of *Z. cucurbitae*). All samples were collected between 2012 and 2014 and returned to QUT where specimens were transferred into and stored in > 95% ethanol at -20˚C.

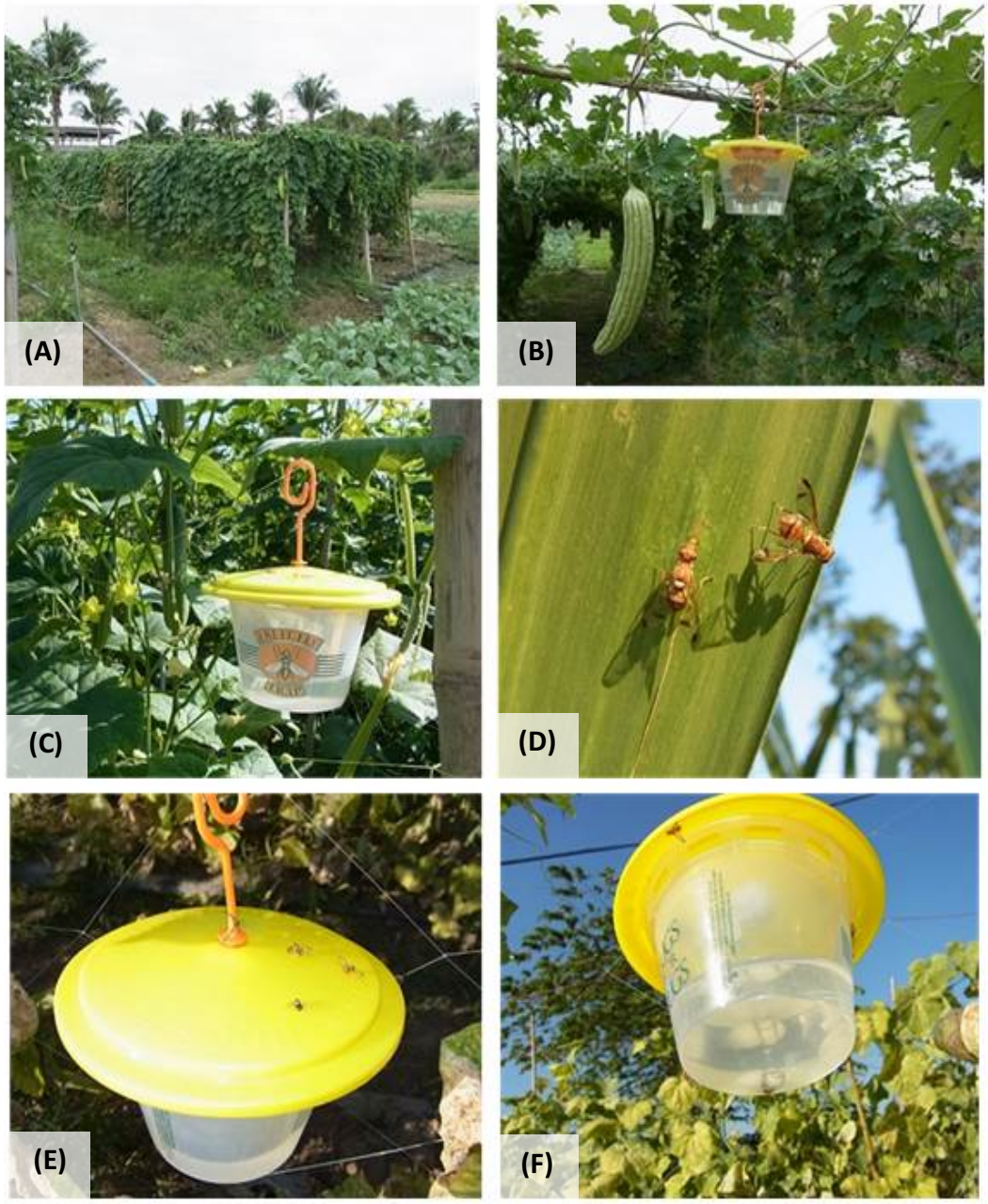



**Figure 2.3** (A) Cucurbit plantation (B-C); setting fruit fly trap in plantations; (D) *Zeugodacus cucurbitae* found at trapped location; (E-F) tephritids were collected into lures and insecticide-baited traps that contained propylene glycol as a preserving agent.

## 2.3 MORPHOMETRIC ANALYSES

### 2.3.1 Geometric morphometrics analyses

#### 2.3.1.1 Specimen preparation

Only males were examined for all populations as most available specimens had been collected using the male-specific attractants cue lure and ME. Twenty male flies were randomly selected from each collection location for analysis. Total number of specimens depended on host plant (Chapter 3) or location (Chapters 4 & 5). Right-hand wings were slide mounted in Canada balsam and air-dried prior to image capture at 10X magnification using an ANMO Dino-Eye microscope eye-piece camera (model no. AM423B) mounted on a Leica (Wild MZ3) stereo-microscope. Image capture was achieved using the program 'DinoCapture 2.0 version 1.4.4' (produced by AnMo); images were saved as BMP files (1280*1024 pixels). Only one wing from each specimen was used because tephritid wings are symmetrical (Gilchrist & Crisafulli, 2006). In rare cases where the right wing was damaged, the left wing was used instead. DNA analysis was performed on the same speciments used for morphological analysis.

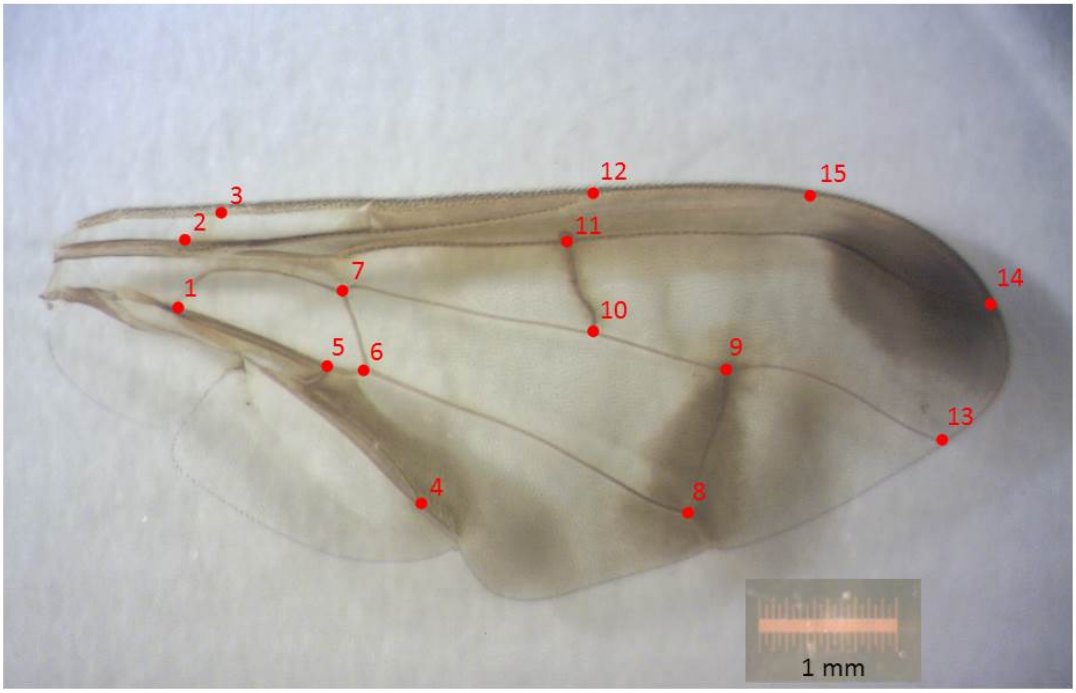
#### 2.3.1.2 Landmark determination and acquisition

Geometric morphometric shape analysis began by taking records of biologically relevant landmarks from all wing images. Landmarks represent Cartesian coordinates that are homologous locations on all specimens included for analysis (Zelditch et al., 2012). Fifteen Type I landmarks (Bookstein et al., 1991, Schutze et al., 2012a) located at junctions and terminations of wing veins were recorded from all specimens (Table 2.1 and Figure 2.4). Landmarks were digitised for each wing using the 'TPSDIG2 Version 2.17' software (Rohlf, 2013) for which landmark x, y coordinates were generated and saved as a text file.

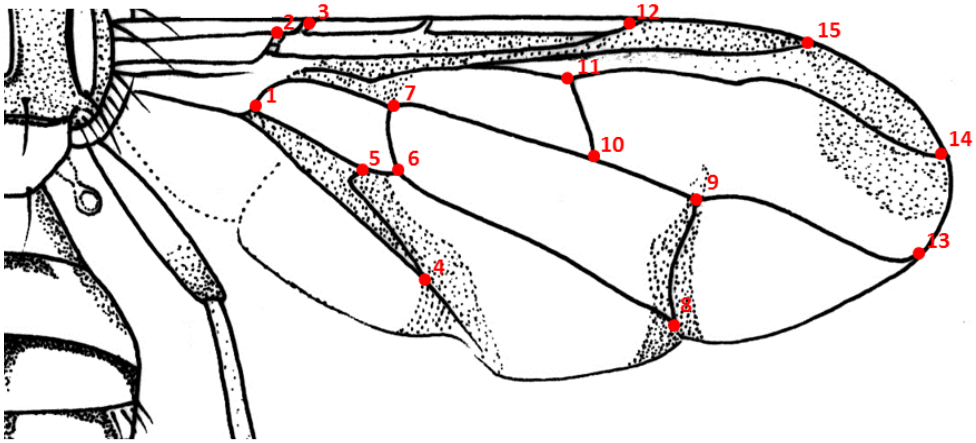
**Table 2.1** Fifteen landmarks located at the junctions and terminations of wing veins chosen for this study.

| Landmarks | Position on wing |
|---|---|
| 1 | Basal junction of veins of cell bm |
| 2 | Anterior-most point of the suture located towards the base of vein sc |
| 3 | Inner antero-distal corner of cell bc |
| 4 | Junction of veins A1 and CuA2 |
| 5 | Junction of CuA1 and CuA2 |
| 6 | Junction of vein CuA1 and dm-bm cross vein |
| 7 | Junction of vein M and dm-bm cross-vein |
| 8 | Junction of vein CuA1 and dm-cu |
| 9 | Junction of vein M and dm-cu |
| 10 | Junction of vein M and r-m cross-vein |
| 11 | Junction of vein R4+5 and r-m cross-vein |
| 12 | Junction of vein R1 and costal vein |
| 13 | Termination of vein M |
| 14 | Termination of vein R4+5 |
| 15 | Termination of vein R2+3 |

Landmark co-ordinate data (the .tps file) were transferred to the analytical software 'MORPHOJ version 1.06B' (Klingenberg, 2011). This program undertakes geometric morphometric analyses such as Procrustes superimposition (Rohlf, 1999), centroid size calculation, multivariate statistical analysis such as PCA and CVA (Zelditch et al., 2012).




(A)




(B)

**Figure 2.4** Right-hand wing of a *Zeugodacus cucurbitae* individual showing each of the 15 land marks used in the geometric morphometric analysis: (A) wing with 15 landmarks using TPS Program (B) wing drawing with 15 landmarks. Landmark information can be found in Table 2.1.

#### 2.3.1.3 Centroid size calculation

A value known as "centroid size" is a measure of wing size that is more useful for shape analysis than raw measurements. It is largely uncorrelated with shape (Mitterroecker et al., 2013) and enables standardisation of measurements for rigorous comparison of shapes (Debat et al., 2003). The centroid size of each wing was calculated by summing the distance between each landmark and the centre of the configuration of landmarks (i.e., the mean position in two dimensional space of all of the coordinates) and then obtaining the square root of this total (Figure 2.5). A one-way ANOVA with a *post-hoc* Tukey test was (where appropriate) was used to test if there were any significant differences in centroid sizes among samples sites; this was done using the statistical data analysis package SPSS version 22 (IBM SPSS Statistics, IBM Corporations, NY). A t-test was used to compare between two groups (e.g., Upper and lower Isthmus of Kra barrier, the West Pacific and South-east Asia) were performed. All data were tested to confirm that they were consistent with assumptions of normality and homogeneity of variance before further analysis was undertaken carried out (by appropriate log transformation). If assumptions were not met, the data were appropriately transformed. If the data still did not meet assumptions after these transformations were still not met, equivalent non-parametric tests (Kruskal-wallis or Mann Whitney U tests) were performed.

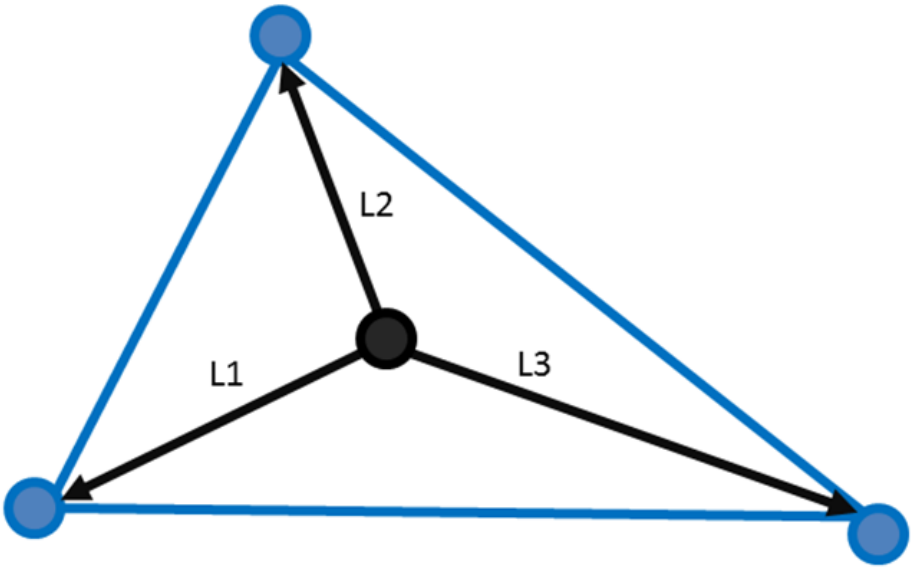


**Figure 2.5** A geometric depiction of the calculation of centroid size, which equals the square root of the summed squared lengths of lines segments L1, L2, L3 (redrawn from Zelditch et al., 2012).

#### 2.3.1.4 Procrustes superimposition

In addition to information relating to shape, landmark co-ordinate data from TPSDIG2 Version 2.17 includes variation which derives from rotation, translation and scale (i.e., size) of the wings. This largely artifactual variation was removed by importing the raw landmark coordinate data into the program MORPHOJ (Klingenberg, 2011; Schutze, et al., 2012a) where it was subjected to a generalised Procrustes analysis, which is a method of statistical analysis that can be used to compare the shapes of objects (Rohlf, 1999).

#### 2.3.1.5 Determination of allometric effect

Effectively, the shape of each specimen was represented as a vector of landmark coordinates following Procrustes superimposition and then this vector was related to size. The shape of the wing can be influenced by wing size; i.e., through allometry, the relation between the size of an organism and the shape of any of its body parts (Klingenberg, 1998; Debat et al., 2003). This enabled meaningful, morphological comparison of samples free of the influence of the size of the specimen (Klingenberg, 2009; Sidlauskas et al., 2011). To detect allometric effects, multivariate regression analyses of the dependent variable (shape) against the independent centroid size variable were performed (e.g., Drake & Klingenberg, 2008; Schutze et al., 2012a). The null hypothesis was that the shape of the wing was independent of the size of the wing. Permutation tests (with 10000 replicates) were used to test the significance of the regression. Wherever a significant association was detected (i.e., indicating changes in shape that were significantly correlated with wing size), data were corrected before being used in subsequent analyses. To correct for allometric contribution towards shape variation, subsequent analyses were undertaken using the residual components as determined from the regression of shape on centroids size.

#### 2.3.1.6 Wing shape analyses

- Principal Component Analysis (PCA)

Principal Component Analysis was conducted to assess differences in wing shape between populations under a non-hypothesis (i.e., exploratory) scenario. PCA is the most widely used method in morphometric studies; it provides a way to display variation within a sample and to sketch out the main features of this variation in this case, shape variation. Commonly, PCA is utilised for the first exploration of large data sets from several samples. PCA can provide an informative, visual impression of overall variation in the data. However, it does not take into account any group structure (such as if the observations can be regarded as falling into a number of classes, such as several different, putative species, males and females, or patients and controls). PCA can be used to ascertain whether there are any obvious subdivisions, but it does not always reveal subdivisions present in the data. Importantly for the present assessment of wing shapes, PCA can be used to see which shape changes are associated with the greatest variation or with the least variation, to identify which shape features are particularly variable or constant. Various types of graphs can be used to visualise shape changes as indicated by landmark displacements after superimposition. Deformation in overall shape can be depicted by wireframes based on each of the principal axes. Wireframe graphs depict the morphological context of the landmarks; pairs of wireframes graphically depict the starting and target shape and represent the structure under study by the software MORPHOJ (Klingenberg, 2011).

- Canonical variate analysis (CVA)

The individuals were assigned to groups based on the hypotheses being tested. Canonical variate analysis (CVA) is another analytical method which can be used to assess differences in wing shape between populations. In contrast to PCA, this method can be used to find the shape features that offer the best prospects for distinguishing among multiple groups of specimens. For CVA, group membership of individual samples is assumed to be known *a priori*. To assess wing shape in the present study, the inputs for CVA were the differences between populations (i.e., the residual components as determined from the regression of shape). As was done

in the centroid size analysis, samples were *a priori* assigned to one or other of the sample locations. The subsequent CVA determined relative differences in wing shape among the groups-groups defined on the basis of host fruit (Chapter 3) or location (Chapters 4 & 5) prior to CVA. Significant differences were determined via permutation tests (1000 permutation rounds) for Mahalanobis distances among groups. It was also necessary to correct for multiple comparisons by performing Bonferroni corrections (Rice, 1989) throughout the analysis. Canonical variate shape change transformations were produced for the first two canonical variates and wireframes used to depict relative changes in shape among the datasets.

#### 2.3.1.7 Isolation-By-Distance (IBD) for wing shape

Correlation among geographic and Mahalanobis distance was assessed via linear regression analysis. A linear regression of pairwise Mahalanobis distance (calculated from CVA) against geographic distance (km) to test for isolation-by-distance effects (Wright, 1943).

### 2.3.2 Aedeagus morphometrics analysis

#### 2.3.2.1 Specimen preparation

The same groups of 20 males as used for wing shape analysis were also examined for variation in aedeagus length. Total number of specimens depended on host fruit (Chapter 3) or location (Chapters 4 & 5). The complete abdomen of each individual was removed and placed in 5 ml of 10% potassium hydroxide (KOH) solution and left overnight at room temperature; this process softened and cleared the structures for ease of dissection. Following softening, abdomens were dissected under water. Each aedeagus was removed from the remaining genitalic structures and straightened on a microscope slide. Aedeagi were measured from the base of the aedeagus to the base of the distiphallus according to Iwaizumi et al. (1997) (Figure 2.6). Measurements were made by eye-piece micrometer and converted into millimeters. Measurements were taken to the nearest 0.01 mm.

### 2.3.2.2 Data (aedeagus) analysis

ANOVA with a *post hoc* Tukey test (where appropriate) was used to assess for significant differences among sites using SPSS. As for wing measurements, all data were verified for conformity with assumptions of normality and homogeneity of variance before being analysed. Again, if assumptions were not met, the data were appropriately log-transformed. If the assumptions still were not met, equivalent non-parametric tests; Kruskal-wallis to compare all locations or Mann-Whitney to compare between two groups (e.g., Upper and lower Isthmus of Kra barrier, the West pacific and South-east Asia) were undertaken.

### 2.3.2.3 Isolation- By -Distance for aedeagus length

Isolation-by-Distance was assessed based on linear regression analysis of geographic (km) distance against aedeagus length for flies collected throughout sampled locations using the program SPSS.

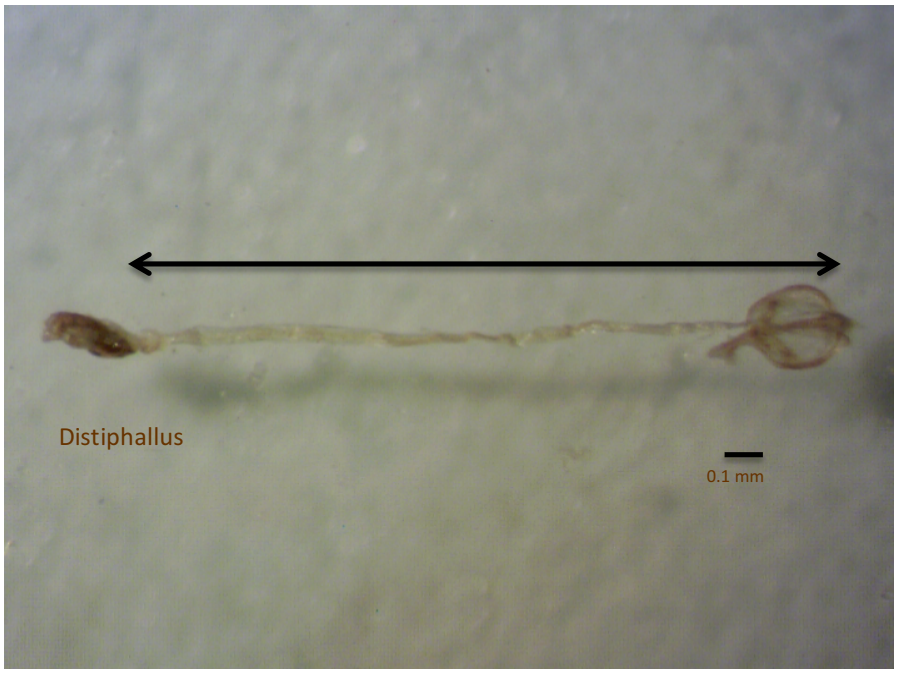



**Figure2.6** Dissected aedeagus of *Zeugodacus cucurbitae* representing measurement taken between base of the aedeagus (on far right) and base of the distiphallus (on left).

## 2.4 MOLECULAR PROCEDURES AND ANALYSES

### 2.4.1 Mitochondrial DNA procedure and analyses

#### 2.4.1.1 Mitochondrial DNA extraction, polymerase chain reaction (PCR) amplification sequencing and Sanger sequencing

The same groups of 20 males as used for morphometric analysis were also examined in Mitochondrial DNA. Three legs (fore, mid and hind) were removed from each individual for genomic DNA extraction using the ISOLATE II Genomic DNA Kit (Bioline, Australia) following the manufacturer's protocol. A 650 bp fragment of mitochondrial cytochrome c oxidase subunit I (*cox1*) was amplified using the universal invertebrate *cox1* primers;

LCO1490 (forward: GGTCAACAAATCATAAAGATATTGG) and

HCO2198 (reverse: TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al., 1994; Wilson, 2012).

PCR amplification was carried out with 2 µl of template DNA, 0.5 µl of each primer (10 pmol/µl) (Integrated DNA Technologies (IDT), USA), 2 µl of 5X polymerase buffer (Bioline), 3.0 µl of 25 mM $MgCl_2$ (Bioline), 0.1 µl of 5 U/µl MyTaq HS Red DNA Taq polymerase (Bioline, Australia), and made up to a final volume of 25 µl with distilled deionized water ($ddH_2O$) Amplifications were performed in an Eppendorf Mastercycler® Pro S thermal cycler with an initial denaturing step at 94˚C for 3 minutes followed by 28 cycles at 94˚C for 30 seconds, 50˚C for 30 seconds, 72˚C for 30 seconds, and a final elongation step at 72˚C for 5 minutes. One µl of PCR product was separated in 1.5% (w/v) agarose gel using TBE buffer (40 mM Tris-acetate, 1 mM EDTA) to confirm the quality of PCR product.

Total PCR product was purified using the commercial ISOLATE PCR and Gel Kit (Bioline) following the manufacturer's guidelines. Purified PCR product was amplified in a sequencing reaction containing 1.0 µl of PCR product, 1.0 µl of forward primer (3.2pmol/µl), 0.5 µl of version 3.1 ABI Prism® Big Dye Terminators (Applied Biosystems, California, USA), 3.5 µL of 5x sequencing dilution buffer (400mM Tris pH9, 10mM $MgCl_2$), adjusted to a total reaction volume of 20µL with $ddH_2O$. The sequencing cycle protocol involved initial denaturing at 96°C for 5

minutes, followed by 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes, before a final hold at 15°C for 10 minutes.

Sequencing fragments were cleaned using a standard ethanol precipitation protocol prior to sequencing at the Molecular Genetics Research Facility of the Faculty of Science and Technology (MGRF), QUT. All sequences will be deposited in GenBank (Accession Numbers will be supplied upon acceptance of manuscripts currently in preparation).

**2.4.1.2 Mitochondrial DNA analyses**

- Mitochondrial DNA Gene diversity

Sequences were aligned by eye and checked for internal stop codons and double peaks (indicative of pseudogenes) using BioEdit Sequence Alignment Editor Version 7.2.5 (Hall, 1999). Analysis of genetic diversity (gene diversity, equivalent to expected heterozygosity; $\Theta_\pi$, a diversity estimate based on the mean number of pairwise differences among populations; number of haplotypes; Tajima's *D*; and Fu's $F_S$) were performed using MEGA version 4.0 (Tamura et al., 2007) and ARLEQUIN Version 3.5.1.2 (Excoffier et al., 2005). Tajima's *D* (Tajima, 1993) and Fu's $F_S$ (Fu, 1997) were used to detect deviations in gene diversity from what would be expected under neutrality and to search for demographic changes or the effects of selection on gene diversity (Fu & Li, 1993). Both tests included coalescent simulations in DnaSP Version 5.0 (Librado & Rozas, 2009). Tajima's *D* tests of neutrality were performed for each site and for the total dataset. A significant negative Tajima's *D* suggests that there are more low frequency polymorphisms than would be expected, whereas a significant positive Tajima's *D* suggests there are low levels of low and high frequency polymorphisms (Tajima, 1989, 1989, 1993). Fu's $F_S$ is based on the probability of observing an expected number of haplotypes (k) or more in a sample of a given size and is particularly useful for detecting whether populations have departed from equilibrium (e.g., following population expansion). A significant negative value indicates that there are more haplotypes than expected, which may occur if the population has recently expanded. A positive value of $F_S$ indicates fewer haplotypes than would be expected, which may occur if the population had recently been through a bottleneck (Fu, 1997).

Genetic differentiation among each geographical population or host plant was estimated using pairwise $F_{ST}$ incorporating the Tamura and Nei model evolution ($\Phi_{ST}$) using ARLEQUIN. Median joining networks among *Z. cucurbitae* haplotypes were constructed and post-processed under maximum parsimony in Network Version 4.6.1.1 (Bandelt et al., 1999). Median joining networks were considered the most appropriated method in this case over other alternatives (e.g., minimum spanning and maximum parsimony). Minimum spanning networks perform best when sampling of haplotypes across the population is relatively complete; they do not perform well if there are significant gaps in sampling across the distribution of a species and where some internal node haplotypes are not sampled. On the other hand, median joining networks incorporate the maximum parsimony criterion and infer internal node haplotypes that may have been missed by incomplete sampling (Cassens et al., 2005). This gives a better estimate of the true genealogy (Woolley et al., 2008).

- Genetic differentiation

An analysis of molecular variance (AMOVA) was conducted in ARLEQUIN to assess partitioning of variation within and among sites. AMOVA is a robust method for testing hypotheses about hierarchical differentiation directly from molecular data. Genetic structure (within individuals, within populations, within groups of populations, among groups) was tested using non-parametric permutation procedures (Excoffier et al., 1992). Samples were constrained according to geographical location in relation to different host plants (Chapter 4) and biogeographic barrier (the six Thai biogeographical regions and two islands, Isthmus of Kra [Chapter 3], Southeast-Asia and West-Pacific [Chapter 5]) to assess the partitioning of variation under different hypotheses of structure.

#### 2.4.1.3 Isolation-By-Distance for genetic distance ($\Phi_{ST}$).

Isolation-By-Distance was assessed based on linear regression analysis of geographic distance against genetic distance among populations ($\Phi_{ST}$).

### 2.4.2 Microsatellite genotyping

#### 2.4.2.1 Polymerase chain reaction (PCR) amplification sequencing and Sequence cleaning

The same groups of 20 males as used for morphometric analysis, mitochondrial DNA were also examined in microsatellite. Specimens were genotyped for 14 microsatellite markers developed in previous studies (Delatte et al., 2010; Wu et al., 2009) (Table 2.2). Initially, these fourteen microsatellite loci were amplified in a 12.5 µl reaction using a standard PCR protocol of 1 µl of 1:10 diluted gDNA, 6.25 µl of Multiplex PCR Master Mix (Qiagen, USA), 4 µl of H2O (RNA free) and 1.25 µl of primer mix. Primer mixes were divided into two multiplex reactions over seven loci each to avoid allele length overlap: 'Multiplex 1' (BcCIRH9, BcCIRG1, BcuA2.5, BcCIRH10, BcCIRD3, BcCIRD11, BcCIRF3) and 'Multiplex 2' (BcCIRC3, BcuG3.4, BcCIRH7, BcCIRE8, BcuB4.3, BcCIRE7, BcuB5.2). Each stock primer mix comprised 8 µl of each primer (50uM) and 88 µl of TE buffer. Unlabelled reverse primers were manufactured by Integrated DNA Technologies (California, USA); dye-labelled forward primers were manufactured by Applied Biosystems (Carlsbad, USA). Thermal cycling involved initial denaturing at 95°C for 2 min, followed by 30 cycles of 95°C for 15 m, 90 s at 57°C for annealing, and 72°C for 90 s. Final extension was carried out at 60°C for 30 s. Fragments were analysed on an ABI 3500 sequencing platform in a sequencing reaction of 10 µl of Hi-Di™ formamide (ABI), 1 µl of GSLIZ600 sequencing size standard (ABI) and 1 µl of each PCR product (diluted 3 times with ddH2O); two loci (BcCIRH10 and BcCIRH11) failed to amplify for sufficient numbers of samples and were excluded from further study.

### 2.4.1.2 Microsatellite analyses

- Microsatellite diversity

Allele sizes were checked and scored in GeneMapper Version 5 (ABI) and the number of alleles and allelic richness per locus was calculated using FSTAT Version 2.9.3 (Goudet, 2001). Microsatellite Toolkit 3.1 was used to measure observed and expected heterozygosity (Park, 2001). GENEPOP was used to screen for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD). Genetic diversity estimates were calculated using ARLEQUIN. Bonferroni corrections (Rice, 1989) were performed throughout the analysis to correct for multiple comparisons. One locus (BcCIRH9) exhibited highly significant heterozygote deficiency ($P < 0.0001$) in several populations (possibly because of the presence of null alleles) and this locus was excluded from the study.

- Genetic differentiation, clustering methods and spatial patterns

Heterozygote deficit/excess for each population was estimated using the multilocus f estimator, $F_{IS}$ (Weir & Cockerham, 1984) in FSTAT. Differentiation among populations was tested using the sum of squares, allele size difference method (pairwise $R_{ST}$) implemented in ARLEQUIN. $R_{ST}$ is a stepwise, mutation-based measurement of genetic differentiation taking into account the variance of microsatellite allele size, and is more appropriate than other measures of population differentiation, like $F_{ST}$, when population structure is expected to be more pronounced and migration rates are lower (Balloux & Goudet, 2002). To estimate the amount of molecular variation associated with different sets of population groupings, hierarchical analysis of molecular variance (AMOVA) based on among-site $R_{ST}$ was undertaken using ARLEQUIN, based on among-site $R_{ST}$.

### 2.4.1.3 Isolation-By-Distance for genetic distance ($R_{ST}$)

Isolation-By-Distance was assessed based on linear regression analysis of geographic distance against genetic distance among populations ($R_{ST}$).

- Population structure using Bayesian clustering

Input files for Bayesian cluster analysis were created by using CONVERT software (Glaubitz, 2004). STRUCTURE Version 2.3 (Pritchard et al., 2000) was used

to assess admixture among individuals and infer population structure in the absence of *a priori* population origin information. This program uses multilocus microsatellite allele frequencies and a Bayesian Markov Chain Monte Carlo (MCMC) approach to assign individuals to *K* number of clusters (Falush et al., 2003, 2007; Pritchard et al., 2000). The program was run for 10 iterations of 1000000 generations, with the initial 100,000 generations removed as burn-in from each iteration. The method described by Evanno et al. (2005) was used to determine the greatest number of *K* in the dataset as implemented by the online resource STRUCTUREHARVESTER (Earl & vonHoldt, 2011). Generally, if log-probabilities for several values of *K* are very similar, the smallest *K* that captures the major structure in the data should be chosen. However, in some situations where hierarchical structure is present, the *ad hoc* statistic Δ*K* should be used to determine the uppermost level of structure (Evanno et al., 2005; Meirmans, 2015). In this case, the highest Δ*K* is considered to be the best estimator of the number of clusters. The program CLUMPP Version 1.1.2 (Jakobsson & Rosenberg, 2007) was used to align outputs across replicates and digest cluster membership coefficient matrices for each value of *K*. The admixture proportions of samples and individuals were produced by DISTRUCT software Version 11 (Rosenberg, 2004) to display the bar plot under the most likely *K* value.

The program POPULATIONS Version 1.2.31 (Langella, 1999) was used to construct Neighbour-Joining (NJ) trees for the microsatellite data using Cavalli-Sforza and Edwards chord distance, $D_C$ (Cavalli-Sforza & Edwards, 1967). This analytical method was used for the nuclear dataset rather than methods based on other distance models because it was considered to be most appropriate where differentiation is largely driven by drift (Ensing et al., 2011). This metric is particularly useful for deriving meaningful trees based on microsatellite sequences (Takezaki & Nei, 1996).

Genetic relatedness among populations was examined in two dimensional spaces by using Principal Component Analysis (PCA), based on allele frequencies, using PCAGEN 1.2 (Goudet, 2005).

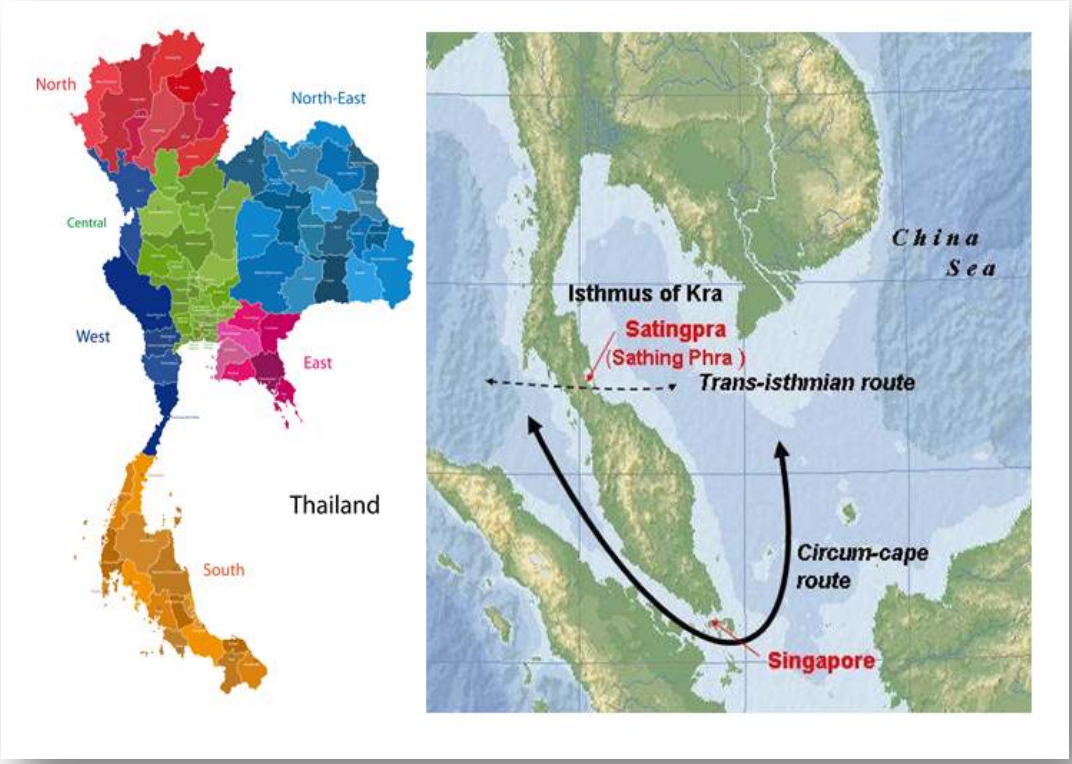
**Table 2.2** Primer details and indices of genetic variation for 14 microsatellite loci used in this study. Tm: locus-specific annealing temperature, He, expected heterozygosity and Ho: observed heterozygosity.

| | Locus name | Primer sequences | Repeat motif | Fluorescent label | Allele range size (bp) | Tm | Number of alleles | He | Ho |
|---|---|---|---|---|---|---|---|---|---|
| 1 | BcCIRD3* | F: CTGATGAGTCCAATAAAATGC<br>R: CTGCCATCATATCCTTTGTT | (ca)7 | PET | 150-170 | 55°C | 6 | 0.54 | 0.71 |
| 2 | BcCIRE8* | F: CGACTTTGGAGTGCTTTG<br>R: ACACGAGCGCATAACAAC | (ca)7 | VIC | 179-200 | 56°C | 7 | 0.63 | 0.69 |
| 3 | BcuG3.4** | F:HEX-GGATGAAGTCGCAAAGGAAG<br>R:TCAGCTTGTTTGTCCTGCTG | (gca)7 | FAM | 164-177 | 73°C | 13 | 0.78 | 0.67 |
| 4 | BcCIRG1* | F: AAAATGTTAGATAAACAAAATACTG<br>R: CCATACAACACAATGCTCT | (ca)8 | FAM | 179-197 | 52°C | 7 | 0.57 | 0.58 |
| 5 | BcuB5.2** | F:FAM-CCAAAACCAATCACGACG<br>R:AAACATACGCACGCAACT | (tg)7 | NED | 125-135 | 80°C | 7 | 0.57 | 0.52 |
| 6 | BcuA2.5** | F:FAM-TTTCGTTTTCCTGCCAGAGTT<br>R:CAATGCAGCAAATTCACCTAC | (gt)8 | FAM | 101-110 | 78°C | 6 | 0.54 | 0.46 |
| 7 | BcuB4.3** | F:TAMRA-CTCGCCGTAATAGCCTGT<br>R:GGGTCGTAAATTCCGTTG | (tg)7 | VIC | 122-134 | 80°C | 7 | 0.47 | 0.37 |
| 8 | BcCIRF3* | F: CCGGATAGACGTAAGCACT<br>R: AACCGTAGGTGACGTGTG | (ac)9 | NED | 161-194 | 55°C | 7 | 0.42 | 0.38 |
| 9 | BcCIRH9* | F: CAACAACCTAACTTCAATCACA<br>R: AACCTTCTCTTAAACCGTTAGAC | (ac)9 | FAM | 251-281 | 55°C | 8 | 0.53 | 0.32 |
| 10 | BcCIRD11* | F: ATCTCTGCGGTGCATTTA<br>R: CAATAATAACAGCAATAACAACG | (ac)7 | VIC | 160-177 | 55°C | 8 | 0.49 | 0.32 |
| 11 | BcCIRC3* | F: AAGCGTCAATGAGACAGC<br>R: CTGCTTGAGGGCAAGTAA | (tg)7 | FAM | 201-217 | 55°C | 6 | 0.15 | 0.11 |
| 12 | BcCIRE7* | F: CTGCCACTATCCCTCTTG<br>R: CCAACGAGAAAAGCAATAA | (ttg)5 | NED | 179-200 | 54°C | 7 | 0.10 | 0.11 |
| 13 | BcCIRH10* | F: TCAGCTCTGCACCTACTCA<br>R: TGCTGTAATGCACGATTG | (ca)9 | PET | 214-256 | 55°C | 9 | 0.09 | 0.10 |
| 14 | BcCIRH7* | F: GTGCAGCTAGGCAGGTAG<br>R: GATTCGTTGCGAAGGTAG | (tg)8 | PET | 140-164 | 54°C | 5 | 0.41 | 0.05 |

* Delatte et al., 2010 and **Wu et al., 2009

# CHAPTER 3

**Resolving population structure of *Zeugodacus cucurbitae* in Thailand: natural barriers to a great disperser**



## 3.1 INTRODUCTION

### 3.1.1 General introduction

Biological variability results from both genetic and environmental influences, and their interactions (Beebee & Rowe, 2008). Thus, although the genome of a species is a powerful determinant of phenotype, not all phenotypic variation is solely the result of genetic variation. Environmental influences on plant-feeding species are diverse and include the effects of inorganic and organic substrates or food (e.g., soil type or host plant species) (Via, 1984), the effects of prevailing climatic conditions (e.g., temperature, rain, wind, and relative humidity) (Post et al*.,* 1997), competition from other organisms (Travis, 1980), or the impact of parasites and predators (Poulin & Thomas, 1999). Such environmental influences may play a significant role in shaping variation, particularly in morphology, physiological performance, and behaviour (Awmack & Leather; 2002, Beebee & Rowe, 2008; Freeland et al*.,* 2011). Changes stimulated by the environment can occur within a short period of time for a single individual, within the lifespan of an organism, or over multiple generations and therefore can be of evolutionary significance (Miner et al., 2005).

The joint impact of historical and contemporary environmental influences on the distribution and biological variation of a species can be investigated using the methods of historical biogeography, taking into account the possible effects of dispersal and vicariance (Parenti & Humphries, 2004; Crisci, 2006; Posadas et al., 2006). Commonly, across a large geographical scale, suitable habitat is fragmented and the capacity for dispersal by an individual will depend on both the geographical pattern of suitable habitat and biological traits of the organism (Kodandaramaiah, 2009). Historical changes driven by climate and geomorphology can modify and shape the distribution of suitable habitat over time and, in turn, profoundly influence patterns of dispersal and vicariance, and ultimately the distribution of species (Wiens & Donoghue, 2004). Although external factors, such as historical changes in climate and geological events tend to influence population structure at the large scale, both external factors (e.g., heterogeneity in habitat) and factors innate to a species, such as dispersal ability, can also shape population structure

(Bossart & Prowell, 1998). Population structure in highly dispersive species with wide geographical distributions may be expected to associate with broad scale biogeographical factors, while species with low dispersive characteristics might be expected to show population differentiation at much finer spatial scales (Bezault et al., 2011). *Zeugodacus cucurbitae* currently has a broad global distribution. However, significant parts of this distribution are known to have resulted from human assistance (Vargas et al., 2015) and it is unclear how much population structuring may occur within its presumed native range (i.e., Southeast-Asia). This chapter investigates this question, asking whether the present-day structuring of genetic and morphological variation of *Z. cucurbitae* in Thailand reflects, in any way, the different environmental regions of the country, or current theories concerning the biogeography of the country and neighboring region.

### 3.1.2 Biogeography of Thailand

It is generally accepted that Thailand, and at least parts of the Southeast-Asian countries which adjoin Thailand along with parts of South-Asia, comprise the native range of the melon fly, and that this species has dispersed from this native range west to Africa, east into the Pacific, and north to China (Hu et al., 2005; Wu et al., 2009; Virgilio et al., 2010; Prabhakar et al., 2012). Thailand approximates the mid-point of this species' modern day distribution and, most significantly, the species is considered to have existed in this region for a substantial proportion of its evolutionary history. Based on general patterns of high within-species biological diversity observed in endemic regions (Muminhanov, 2008), it is reasonable to expect that substantial morphological, biological and genetic diversity will occur in *Z. cucurbitae* in this region. Accordingly, this chapter pays particular attention to morphometric variation and variation in key genetic markers in melon fly from Thailand. Thailand is also of exceptional biogeographic interest because it is here that Asian, Indochinese and Sundaic biota overlap to create a notably rich flora and fauna (Penny, 2001; Hughes et al., 2003; Woodruff, 2003; Hepburn & Radloff, 2011; Woodruff & Turner, 2009).

*Biogeographic regions of Thailand*

Thailand lies at the heart of mainland Southeast-Asia. Its 513115 square kilometres are surrounded by Burma (Myanmar) to the west, Laos to the north and east, Cambodia to the southeast, and the Gulf of Thailand to the south. The border with the northern Malaysian states is relatively short. Peninsular Thailand is part of the Malay Peninsula; Burma extends along the western portion of the peninsula as far as the Isthmus of Kra (Kislenko, 2004) (Figure 3.1).


**Figure 3.1** Map of Thailand and surrounding countries (redrawn from Anonymous, 2015c).

Thailand can be divided into six biogeographical units, which to some extent reflect the restricted ranges of many local and endemic species (Collins et al., 1991; Lekagul et al., 1991). A six-region system is commonly used within Thailand for geographical and scientific purposes (Gullette, 2013) (Figure 3.2) and these regions are as follows:

1) The Northern Highlands are surrounded by mountain ridges and wide valleys that extend southward from the borders with Burma and Laos. Prior to anthropogenic disturbance, the areas above 1000 metres supported extensive, evergreen,

montane forests, and the lower slopes were covered with dense, mixed deciduous and dry dipterocarp forests.

2) The Khorat Plateau includes the northeastern area of Thailand between the Petchabun Range in the west and the Donglak (Dang Raek) Range in the south along the Cambodian border. The plateau was once densely wooded with abundant wild animals, but is now widely deforested with some tropical rainforests and dry evergreen forests persisting in the hills.

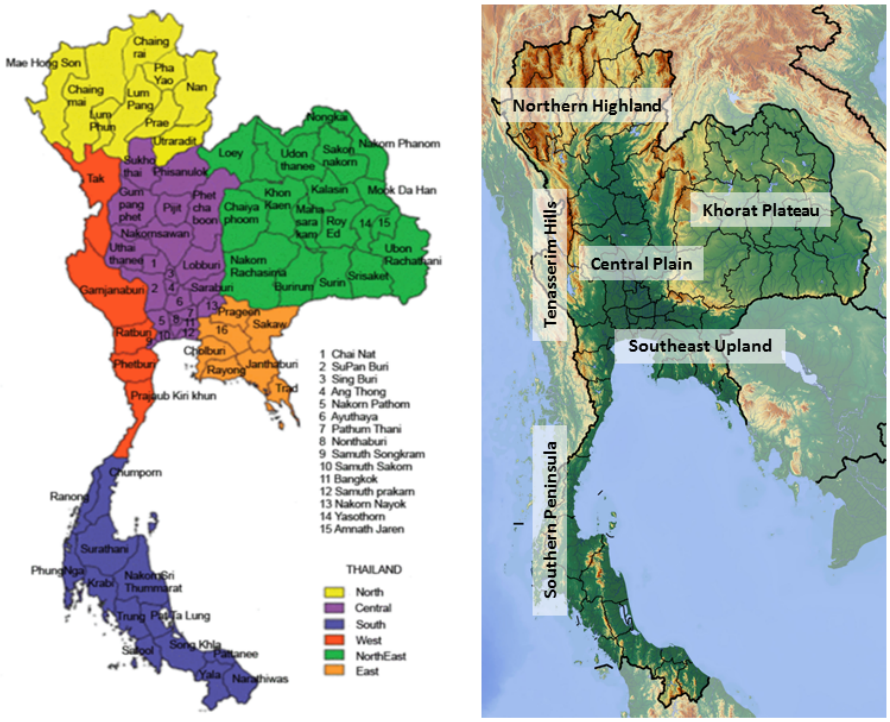



**Figure 3.2** Maps of Thailand showing (left) its six geographic regions (North, Northeast, East, Central, West and South) and (right) the biogeographic regions (redrawn from Anonymous, 2015d).

3) The Central Plain of the Chao Phraya River is an immense, fertile, alluvial plain that rises in altitude from south to north. It is now almost entirely cultivated as rice paddy fields which have completely replaced the previously existing freshwater swamps and monsoon forests.

4) The Southeast Uplands is the smallest division and extends from the Cardamom Mountains in Cambodia to the Gulf of Thailand. Semi-evergreen forests mostly

cover the upland areas, and the lowlands are also increasingly given over to agriculture.

5) The Tenasserim Hills extend southward from the Northern Highlands and run south along the border with Myanmar, rising steeply from the Central Plains to about 1000 metres above sea level. Even though the hills are situated in the rain shadow of the higher mountains on the Myanmar side of the range, the hills have long supported semi-evergreen forests on the higher elevations.

6) The Southern Peninsula includes the region south of the Kra Isthmus and extends to the Malaysian border. High precipitation in the peninsula supports dense rain forests.

Across these regions, there are important differences. The Northern Highlands, for example, have a more temperate climate and are notably cooler than the more southern parts of Thailand. For example, in January the overnight minimum temperatures at high elevations in the north can be near to freezing, while the overnight minima at Suratthani in the south rarely fall below 22°C. The Khorat Plateau is markedly drier than other regions, whereas the Southern Peninsula receives rain from both the southern and northern Monsoon. These climatic differences, driven by the distinct altitudinal and latitudinal characteristics of each of the six Thai biogeographical units affect the distribution of local plant species (Muminhanov, 2008). It is possible that, in turn, these environmental differences may also influence the population structure of associated herbivores, including fruit flies.

Coupled with the distinct environmental characteristics of the biogeographic regions, local adaptation may be another factor influencing the population structure of fruit flies in Thailand and neighbouring regions. *Cox1* data for *B. latifrons* from 11 sites in northern and north-eastern Thailand revealed no major divergent lineages associated with biogeographic region, however, flies from one site in northern Thailand were significantly different genetically from all other local populations and possibly were derived from adjacent natural forest (Meeyen et al., 2014). Similarly, *B. correcta* were found to be relatively homogeneous across 12 population sites in

north-eastern Thailand except for divergent lineages at two sites (Kunprom et al., 2015).

*Islands of Thailand*

In addition to the main biogeographic regions in the mainland of Thailand, the country also includes many islands. Most of these islands have been isolated geographically since sea levels rose at the end of the Pleistocene, some 8,000-10,000 years ago (Ruttner, 1988) and it would not be unexpected to find that populations of at least a few of the plants and animals on these islands were in some way distinctive from conspecific mainland populations. Two of the largest Thai islands are Samui Island and Chang Island, of which Samui Island is much further from the mainland (40 km) than Chang Island (10 km). There is significant evidence that populations of a species of bee, *Apis cerana* Fabricius, from Samui Island are considerably morphologically and genetically distinct from *A. cerana* from other parts of Thailand (Sylvester et al., 1998; Hepburn et al., 2001; Sittipraneed et al., 2001a, 2001b; Warit et al., 2006). However, there are no comparable studies of other species from Samui Island, and there has been no population-level research at all on the flora or fauna of Chang Island.

*Kra Isthmus*

The area of Thailand south of the Kra Isthmus, in Peninsular Thailand, is perhaps the most distinctive of the country's biogeographical units. The Isthmus of Kra (Figure 3.1) represents the principal contact zone and biogeographic barrier between historical Sundaland (now mostly part of the Southeast-Asian archipelago) and the major part of the Asian mainland (Woodruff, 2010). The regions separated by the modern-day Kra Isthmus are climatically distinct and possess divergent flora and fauna. The Kra Isthmus is a well-documented biogeographic barrier historically limiting the distributions of numerous animal species, including amphibians (Inger, 1999), honey bees (Hepburn et al., 2001; Sittipraneed et al., 2001a, 2001b), forest bird species (Hughes et al., 2003; Round et al., 2003), freshwater crustaceans (de Bruyn et al., 2005), starfish (Benzie, 1999) and marine gastropods (Crandall et al., 2007). Paradoxically, plant species show a strong divide in their biogeographical

distribution approximately 500 km further south of the Kra Isthmus at the Kangar-Pattani Line (Van Steenis, 1950; Good, 1964; Keng, 1970; Whitmore, 1984, 1998; Ashton, 1992; Baker et al., 1998; Wikramanayake et al., 2002; Lohman et al., 2011) (Figure 3.1). The most recent hypothesis to explain the cause of changes in the distribution of biota at the Isthmus of Kra is that a rise in sea level narrowed the isthmus, without actually cutting it, caused compression of faunal populations along the Thai peninsula. This compression is thought responsible for the zoogeographic transition now observed (Woodruff & Turner, 2009).

### 3.1.3 Chapter structure

Taken together, numerous studies across vertebrates, invertebrates and vascular plants have tested, and in some cases demonstrated, population structuring associated with the biogeographic regions of Thailand and disjunctions centered around the Isthmus of Kra. However, the underlying processes to explain current faunal and floral distributions have been more implicit than explicit. For example, the change in animal species distributions appears to correspond to a change in forest types, rather than because of historical biogeographic processes acting directly on the animals (Hughes et al., 2003). Similarly, there have been few comparative studies across closely related species, especially for insect groups. Morphometric and microsatellite data for Oriental fruit fly (*B. dorsalis*) populations across the Kra Isthmus did not indicate any genetic barrier or genetic heterogeneity across this zone (Krosch et al., 2013; Aketarawong et al., 2014a), but in the absence of any other comparable fruit fly study if cannot be determined how generally this finding might hold.

All of this heightens interest in the study of the population structure of *Z. cucurbitae* in Thailand. Both population-genetic and biogeographic theories predict that significant population of *Z. cucurbitae* might be expected in Thailand, as a region of endemism for the species with a complex biogeographic history and diverse contemporary climate. Not just for academic sake, but also because of the implications for internal quarantine and international trade, it is worth asking whether population structuring of *Z. cucurbitae* within Thailand does occur, or whether the populations are largely panmictic.

This chapter examines evidence for structured biological variation for *Z. cucurbitae* among the Thai biogeographical regions, whether there is evidence for differentiation across the Isthmus of Kra, and if populations have differentiated on the two largest Thai islands. Using samples from the six mainland regions, two Islands, and 17 sites along the Thai-Malay Peninsula, morphometric (wing and aedeagus), *cox1* gene and microsatellite data were used to infer population structure, diversity and demographic history of *Z. cucurbitae* in Thailand. In addition to increasing the fundamental understanding of the natural variation of *Z. cucurbitae* in Thailand, this study also supports current efforts to better understand the biogeography of the Thai-Malay Peninsula.

This chapter is lengthy and addresses two major biogeographic topics (i.e., the six Thai biogeographic regions and the Kra Isthmus). It is retained as a single chapter because both topics draw upon interpretation of data from a common set of sampling sites. However, recognizing the results are extensive, the chapter is split into two parts: the first deals with Thai biogeography in general, while the second on the specific question of the Thai-Malay north-south transect and the Isthmus of Kra.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Sampling strategies

*Biogeographic region coverage*

Sampling of *Z. cucurbitae* between 2012-2014 was undertaken using cue-lure and Methyl eugenol baited fruit fly traps at 20 sites distributed over each of the six biogeographical regions (North, West, Central, Upper South, Lower South and Eastern), as well as Samui and Chang Islands (Figure 3.3). Specimens were collected at a minimum of two sites in each region (Table 3.1), and combined to create a single sample for each region. Because of this combining of samples, regression tests of genetic or morphological variation against latitude and geographic distances were not undertaken.

*Thai-Malay peninsula*

Field expeditions were undertaken along a north-south transect of the Thai-Malay peninsula, crossing the Isthmus of Kra. This transect comprised 17 sample sites (Chiangmai, Nan, Tak, Suphanburi, Nonthaburi, Ratchaburi, Petchaburi, Prachupkririkran, Bangsaphan, Chumphon, Thachana, Suratthani (Muang), Samui Island, Phang-nga, Yala, Narathiwat and Selangor (Malaysia) located approximately 80-100 km apart between the northern extremity of Thailand and Thai-Malay Peninsula (Figure 3.4 and Table 3.1).

*Trap catches*

The performance of the fruit fly traps baited with three traps of each kind (cue-lure and methyl eugenol) were set at each site indicated the different density of fruit flies at the sites at the time of trapping. A total of 3300 *Z. cucurbitae* individuals was captured in cue-lure traps during the collecting period (Table 3.2). Traps baited with methyl eugenol captured only ten *Z. cucurbitae.*

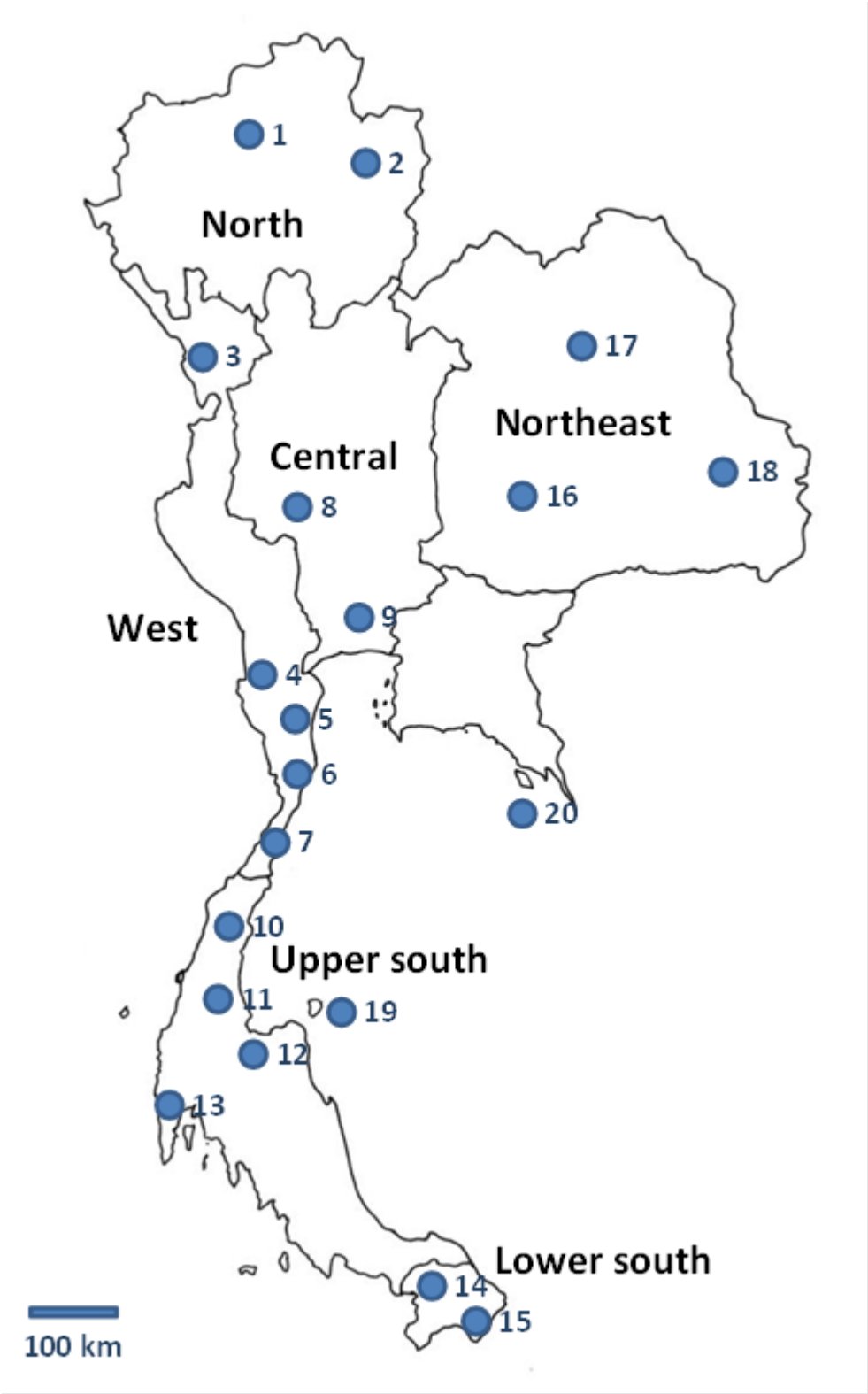



**Figure 3.3** Locations of sampling sites in the six Thai biogeographical regions (North, West, Central, Upper South, Lower South and Eastern) and two islands (Samui Island and Chang Island) at which *Zeugodacus cucurbitae* were collected. Specific collection data is presented in Table 3.1

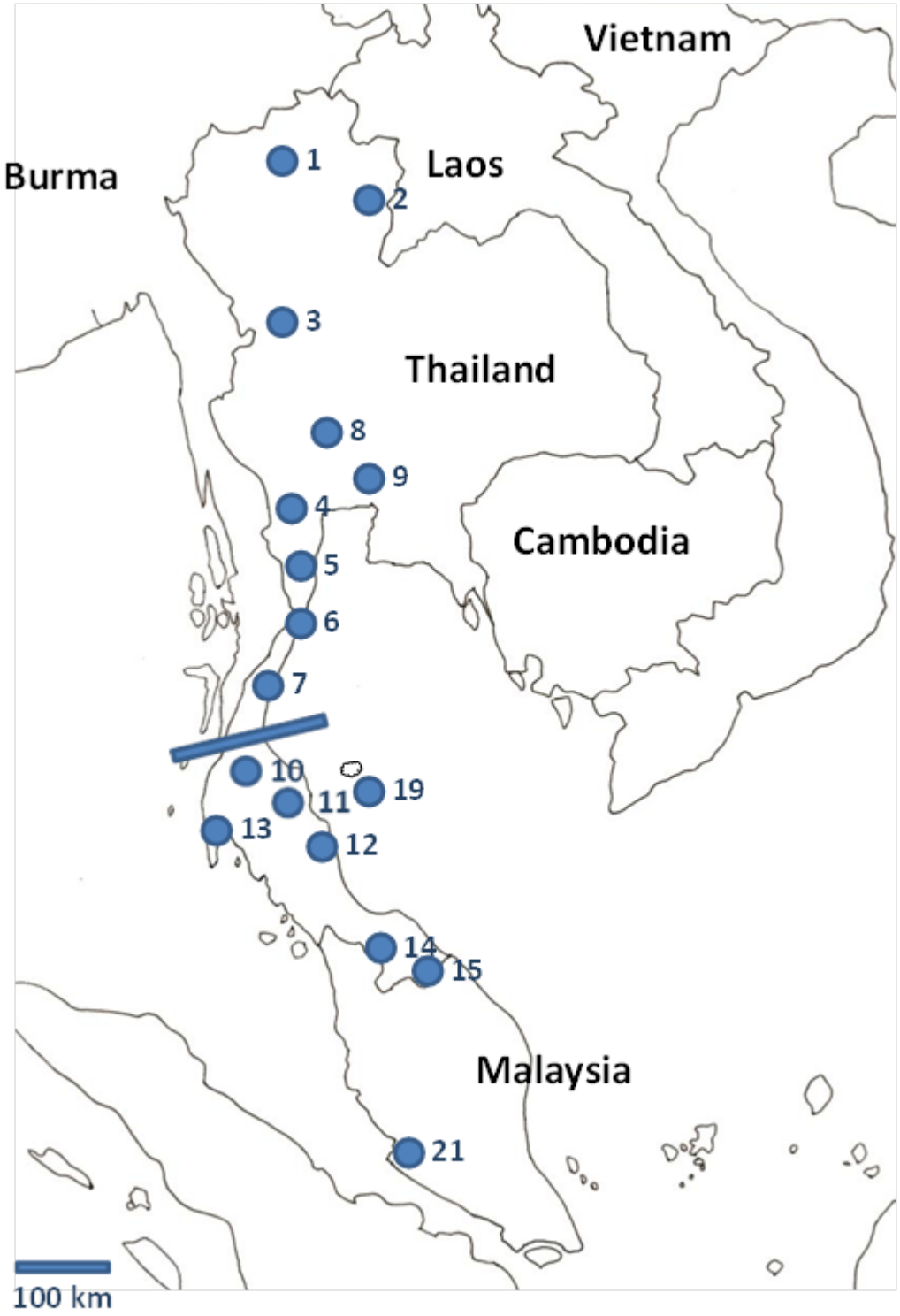



**Figure 3.4** Geographical location of sample sites along the Thai-Malay Peninsula from which *Zeugodacus cucurbitae* were collected. Specific collection data is presented in Table 3.1. The putative location of the Isthmus of Kra seaway is given as a solid blue line bisecting the Peninsula after Woodruff (2010) and Lohman et al. (2011).

**Table 3.1** Collection locations and numbers of individuals used for wing shape, aedeagus length, *cox1* and microsatellite analyses for samples of male *Zeugodacus cucurbitae* collected from six regions and two islands of Thailand, and one sites from Malaysia.

| Locat-ion | Region | Location | Date | Latitude | Longitude | Wing shape | Aedeagus | *cox1* | Microsatellite |
|---|---|---|---|---|---|---|---|---|---|
| 1 | North | Chiangmai* | 7 - 12 Feb - 13 | 18.942 | 99.021 | 20 | 20 | 20 | 20 |
| 2 | North | Nan* | 1 - 5 March - 13 | 18.764 | 100.773 | 20 | 20 | 20 | 20 |
| 3 | West | Tak* | 1 - 5 April - 13 | 17.225 | 99.157 | 20 | 20 | 20 | 19 |
| 4 | West | Ratchaburi* | 10 - 14 Sep - 12 | 13.221 | 99.434 | 20 | 20 | 20 | 20 |
| 5 | West | Petchaburi* | 5 - 7 Jan - 13 | 12.421 | 99.542 | 20 | 20 | 20 | 20 |
| 6 | West | Prachupkririkran* | 10 - 14 Sep - 12 | 11.541 | 99.464 | 20 | 20 | 20 | 20 |
| 7 | West | Bangsaphan* | 10 - 14 Sep - 12 | 11.135 | 99.255 | 20 | 20 | 20 | 20 |
| 8 | Central | Suphanburi* | 10 - 14 Sep - 12 | 14.478 | 100.050 | 20 | 20 | 20 | 20 |
| 9 | Central | Nonthaaburi* | 10 - 14 Sep - 12 | 14.030 | 100.181 | 20 | 20 | 20 | 20 |
| 10 | Upper South | Chumphon* | 10 - 14 Sep - 12 | 10.225 | 99.055 | 20 | 20 | 20 | 18 |
| 11 | Upper South | Tachana* | 10 - 14 Sep - 12 | 9.361 | 99.085 | 20 | 20 | 20 | 20 |
| 12 | Upper South | Suratthani* | 10 - 14 Sep - 12 | 9.110 | 99.281 | 20 | 20 | 20 | 20 |
| 13 | Upper South | Phang-nga* | 5 - 7 March - 13 | 8.852 | 98.350 | 20 | 20 | 20 | 20 |
| 14 | Lower South | Yala* | 4 - 15 Feb - 13 | 6.305 | 101.162 | 20 | 20 | 20 | 20 |
| 15 | Lower South | Narathiwat* | 4 - 15 Feb - 13 | 6.069 | 101.875 | 20 | 20 | 20 | 20 |
| 16 | Northeast | Nakhonratchasima | 21 - 24 March - 13 | 14.848 | 101.226 | 20 | 20 | 20 | 20 |
| 17 | Northeast | Udonthani | 3 - 9 April - 13 | 17.218 | 102.762 | 20 | 20 | 20 | 20 |
| 18 | Northeast | Ubonratchathani | 9 - 13 Jan - 13 | 15.233 | 105.029 | 20 | 20 | 20 | 20 |
| 19 | Samui Island | Suratthani* | 9 - 25 March - 13 | 9.285 | 99.562 | 20 | 20 | 20 | 20 |
| 20 | Chang Island | Trad | 7 - 9 Jan - 13 | 12.074 | 102.165 | 20 | 20 | 20 | 20 |
| 21 | Selangor | Malaysia* | 9 - 17 Feb - 14 | 3.005 | 101.421 | 20 | 20 | 20 | 20 |

*Location from which melon fly were collected for the Thai-Malay

**Table 3.2** Locations and numbers of individuals of male melon fly collected from 20 sites (six regions and two islands) from Thailand by fruit fly trap with two kind of lure (Cue lure and methyl eugenol, insecticide-baited) and number of melon fly trapped (per trap per day). Three traps of each kind were set at each location.

| | **Location** | **No. of individual *Z. cucurbitae*** | ***Z. cucurbitae* /trap/day** |
|---|---|---|---|
| 1 | Chiangmai | 170 | 9.44 |
| 2 | Nan | 31 | 2.07 |
| 3 | Tak | 56 | 3.73 |
| 4 | Ratchaburi | 171 | 11.4 |
| 5 | Petchaburi | 99 | 6.6 |
| 6 | Prachupkririkran | 65 | 4.3 |
| 7 | Bangsaphan | 21 | 1.4 |
| 8 | Suphanburi | 220 | 14.67 |
| 9 | Nonthaburi | 59 | 6.55 |
| 10 | Chumphon | 86 | 5.70 |
| 11 | Thachana | 134 | 8.90 |
| 12 | Suratthani | 138 | 9.20 |
| 13 | Phang-nga | 82 | 9.15 |
| 14 | Yala | 299 | 8.30 |
| 15 | Narathiwat | 335 | 9.30 |
| 16 | Nakhonratchasima | 85 | 7.08 |
| 17 | Udonthani | 275 | 13.09 |
| 18 | Ubonratchathani | 429 | 13.00 |
| 19 | Samui Island | 514 | 28.54 |
| 20 | Chang Island | 31 | 3.44 |
| 21 | Selangor, Malaysia | 30 | N.A. |
| | **Total** | **3300** | |

### 3.2.2 Morphometric analyses

#### 3.2.2.1 Geometric morphometric analyses

Wings from 20 individuals from each of the 20 sites from six regions and two islands from Thailand were mounted, measured and the data used to test the first hypothesis regarding Thai biogeography (North, West, Central, Upper South, Lower South and Eastern; Samui Island and Chang Island). Additionally, flies from 17 sites, 16 from Thailand and one from Malaysia, were mounted, measured and the data analysed to test hypotheses regarding the Isthmus of Kra barrier. All details regarding specimen preparation, landmarking, Procrustes superimposition, and calculation of wing size, are presented in Chapter 2.

*Wing size* (*centroid size*): The individuals were assigned to groups based on the hypotheses being tested. These were:

(i) To test whether *Z. cucurbitae* wing data reflected the currently recognized Thai biogeographical regions, i.e., the data were used to compare among the six regions and two islands to test the "Thai biogeography" hypothesis. Individuals/sampling localities from within a region were thus clustered within that region.

(ii) To test the Isthmus of Kra barrier hypothesis, samples were compared across the 17 sites (see Figure 3.4 & Table 3.1) along the Thai-Malay transect.

All data were first tested to confirm that they were consistent with assumptions of normality and homogeneity of variance before further analysis was undertaken. The data met assumptions and one-way ANOVAs were performed to test for differences in centroid size between *a priori* defined groups for Thai biogeography (six regions and two islands) and for testing for an Isthmus of Kra barrier from 17 sampled sites. T-test was performed to compare between the group of sites above the Isthmus of Kra barrier (the 9 "Upper" sites) and the group of sites below the Isthmus of Kra biogeographic barrier (the 8 "Lower" sites) (Figure 3.4).

*Linear regression analysis*: Linear regressions were performed to test whether aspects of wing size in melon fly varied with latitude and, therefore, showed

evidence for clinal variation. The test of regression was used to determine the relation of wing size against latitude for flies collected from along Thai-Malay transect using the program SPSS. IBD was tested on Mahalanobis distance against geographic distance (km) to assess variation of wing shape and geographic distance for the 17 sites along the Thai-Malay Peninsula.

*Wing shape analyses*:

- Principal Component Analysis (PCA) was conducted to assess differences in wing shape between populations under a non-hypothesis scenario. PCA can be used to ascertain whether there are any obvious subdivisions, but it does not always reveal subdivisions present in the data. Importantly for the present assessment of wing shapes, PCA can be used to see which shape changes are associated with the greatest variation or with the least variation, to identify which shape features are particularly variable or particularly constant.

- Canonical variate analysis (CVA) was performed on Procrustes transformed data. The individuals were assigned to groups based on the hypotheses being tested. Individuals were retained in their *a priori* defined groups for CVA, and separate analyses were carried out based on the hypotheses being tested (same as wing size). These analyses (and sampling) were as above for wing size:

(i) To test whether *Z. cucurbitae* wing shape reflected the currently recognized Thai biogeographical regions. Individuals/sampling localities from within a region were thus clustered within that region.

(ii) To test the Isthmus of Kra barrier hypothesis (see Figure 3.4 & Table 3.1).

Significant differences were determined via permutation tests (10000 permutations) for Mahalanobis distance among the *a priori* defined groups. It was also necessary to correct for multiple comparisons by performing Bonferroni corrections (Rice, 1989) throughout the analysis.

Canonical variate shape change transformation wireframes were produced for the first three canonical variates to depict relative changes in shape among the datasets.

*Linear regression analysis*: Regression analysis was conducted for Mahalanobis distances against geographic distance (km) to determine if there was an isolation by distance effect regarding wing shape across the Thai-Malay transect.

**3.2.2.2 Aedeagus morphometrics**

Males used for wing shape analysis were also examined for variation in aedeagus length. All details regarding specimen preparation of aedeagi are presented in Chapter 2.

*Statistical analysis of aedeagus length*: Data were tested to confirm that they were consistent with assumptions of normality and homogeneity of variance before further analysis was undertaken. The data met assumptions, and ANOVA was used to compare and test for differences in aedeagus length between *a priori* defined groups for Thai biogeography (six regions and two islands) and for testing variation among 17 sites along the Isthmus of Kra. The T-test was used to compare aedeagus length between those sites from the Upper and Lower Isthmus of Kra barrier.

*Linear regression analysis*: To assess if aedeagus length varied on a north-south gradient, aedeagus length was regressed against latitude for specimens collected from the 17 sites along Thai-Malay transect.

**3.2.3 Molecular procedure and analyses**

**3.2.3.1 Mitochondrial DNA procedure and analyses**

**- Mitochondrial DNA extraction, polymerase chain reaction (PCR), Sanger sequencing, amplification sequencing and sequence clean up**

Males used for geometric morphometric analyses and aedeagal morphometrics were also subjected to molecular analyses. Specimens of *Z. cucurbitae* were sequenced for *cox1* - 20 individuals from each of the 20 sites from six regions and two islands from Thailand and a total of 340 specimens of *Z. cucurbitae* from 17 sites on a transect commencing in the far north of Thailand and encompassing the entire length of peninsular Thailand, including sites on either side of the Isthmus of Kra and one site in peninsular Malaysia. All details regarding

*cox1* amplification, PCR protocol, reactions and sequencing are presented in Chapter two.

**- Mitochondrial DNA analyses**

Statistical analysis was undertaken to measure mitochondrial DNA gene diversity of *Z. cucurbitae* and also to investigate genetic differentiation. All details of basic statistical analyses employed are presented in Chapter 2.

*Statistical analysis of Molecular Variance*: An Analysis of Molecular Variance (AMOVA) was conducted in ARLEQUIN to assess partitioning of variation within and among sites. Samples were constrained according to geographical region within which the samples were collected to evaluate the Thai biogeography hypothesis (i.e., samples were grouped according to the six regions and two islands) and to test the Isthmus of Kra biogeographical barriers to assess the partitioning of variation.

*Isolation-by-distance*: IBD was evaluated by linear regression analysis between geographic distance (km) and genetic distance among groups ($\Phi_{ST}$) to determine variation of genetic distance and geographic distance from the North of Thailand to Selangor, Malaysia.

**3.2.3.2 Microsatellite genotyping**

**- Polymerase chain reaction (PCR) amplification sequencing and Sequence cleaning**

Eleven microsatellite loci that had been screened for variation in *Z. cucurbitae* were used for genotyping. All details regarding microsatellite loci, PCR protocol, reactions are presented in Chapter 2.

**- Microsatellite analyses**

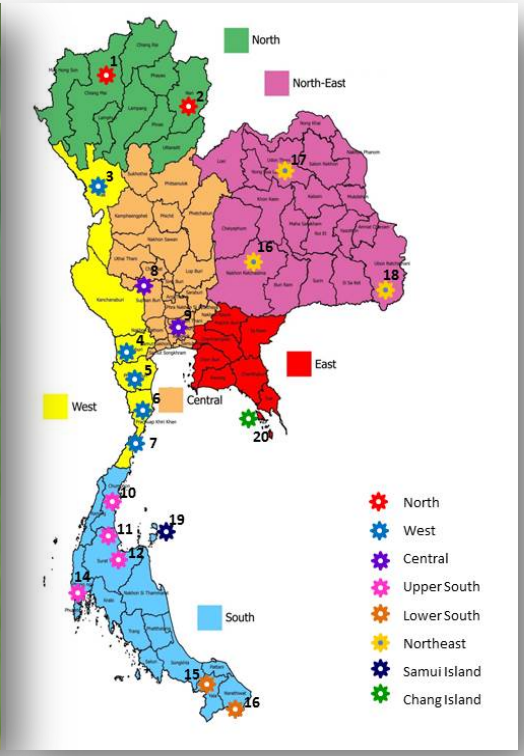
Statistical analysis was undertaken to measure microsatellite diversity of *Z. cucurbitae* and also to investigate genetic differentiation and population structure. All details of basic statistical analyses employed are presented in Chapter 2.

*Statistical analysis of Molecular Variance*: An analysis of molecular variance was conducted in ARLEQUIN to confirm population clusters and to differentiate the

variation component among the populations from six regions and two islands; and the 17 sites along Thai-Malay transect. The analysis was based on among-site $R_{ST}$ estimates.

*Isolation-by-distance*: The hypothesis of IBD was assessed between geographic distance and genetic distance ($R_{ST}$) among populations by linear regression analysis. Thus, the pairwise values of genetic distance ($R_{ST}$) among the 17 sites along Thai-Malay transect were regressed against geographic distance from the North of Thailand to Selangor, Malaysia.

# RESULTS PART 1: THAI BIOGEOGRAPHY



## 3.3 RESULTS PART 1: THAI BIOGEOGRAPHY

### 3.3.1 Morphometric results

#### 3.3.1.1 Geometric morphometric wing shape analysis

*Centroid size calculation*

Four-hundred males from 20 sites across six regions and two islands were examined. *Zeugodacus cucurbitae* wing size among sampled populations was significantly different ($F_{7, 392} = 3.684$; $P < 0.05$). Wings of flies from the North were largest with an average wing centroid size of 6.365 ± 0.398 mm, which was significantly larger than wings of flies sampled from Lower South, Samui and Chang Island. There was considerable overlap in wing size among North, West, Central, Upper South and Northeast regions, and those wing centroid sizes were larger than those from two islands and the Lower south (Figure 3.5).

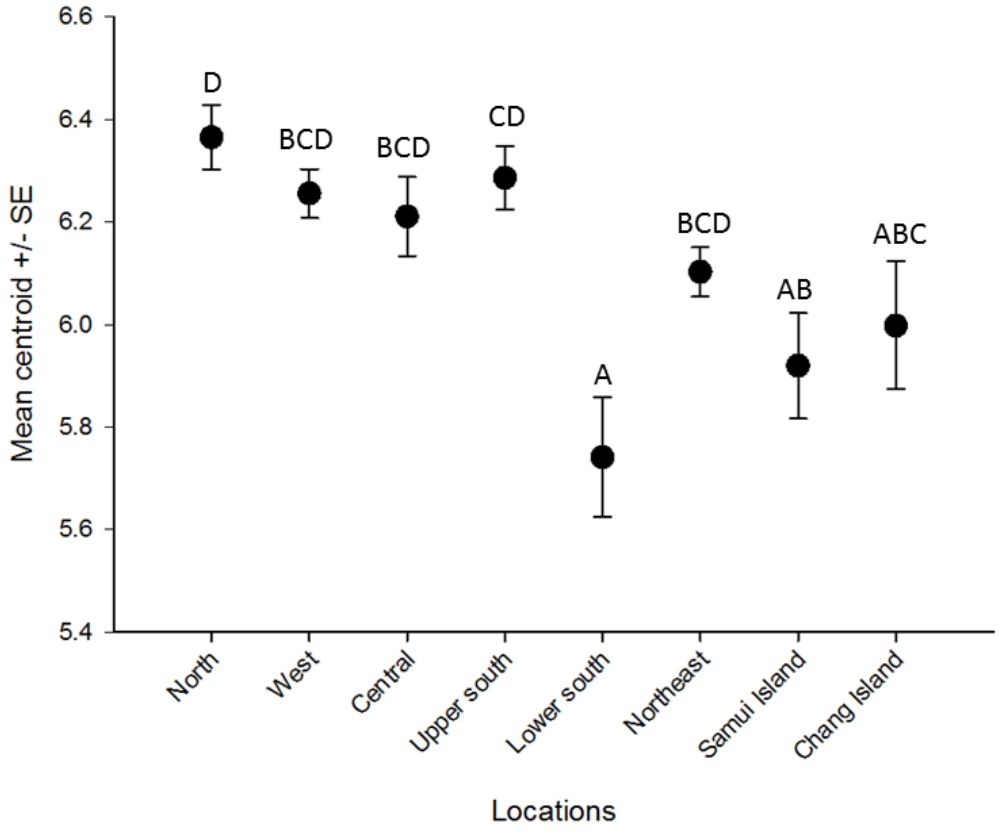



**Figure 3.5** Mean (± SE) wing centroid size of *Zeugodacus cucurbitae* from six biogeographic regions and two islands of Thailand. Samples sharing the same letter are not statistically different from each other based on one-way ANOVA with a Tukey *post hoc* test ($F_{7, 392} = 3.684$; $P > 0.05$).

*Procrustes Superimposition*

Initially, generalized Procrustes superimposition was used to generate new co-ordinate data for each individual fly used in the study. Fifteen landmarks in two dimensions were investigated and the average shape was rendered in two axes (Table 3.3). The Procrustes sum of squares value was 0.2440, and the Tangent sums of squares value 0.24388. This Procrustes superposition indicated that landmarks 12 and 15 were more variable than other landmarks; no outliers were detected (Figure 3.6).

**Table 3.3** Average position on two axis of fifteen landmarks in two dimensions of *Zeugodacus cucurbitae* wings from individuals collected from 17 sites across six regions and two islands of Thailand. The position of the landmarks in relation to a real wing is shown in Figure2.4.

| Landmark | Axis 1 (X) | Axis 2 (Y) |
|---|---|---|
| 1 | -0.330412 | -0.007908 |
| 2 | -0.328323 | 0.050554 |
| 3 | -0.300418 | 0.077501 |
| 4 | -0.090629 | -0.167076 |
| 5 | -0.185563 | -0.052438 |
| 6 | -0.152966 | -0.052325 |
| 7 | -0.175681 | 0.016825 |
| 8 | 0.152352 | -0.159668 |
| 9 | 0.187706 | -0.029137 |
| 10 | 0.050160 | -0.002352 |
| 11 | 0.027479 | 0.078547 |
| 12 | 0.064723 | 0.135314 |
| 13 | 0.392129 | -0.079863 |
| 14 | 0.434283 | 0.048364 |
| 15 | 0.255161 | 0.143661 |

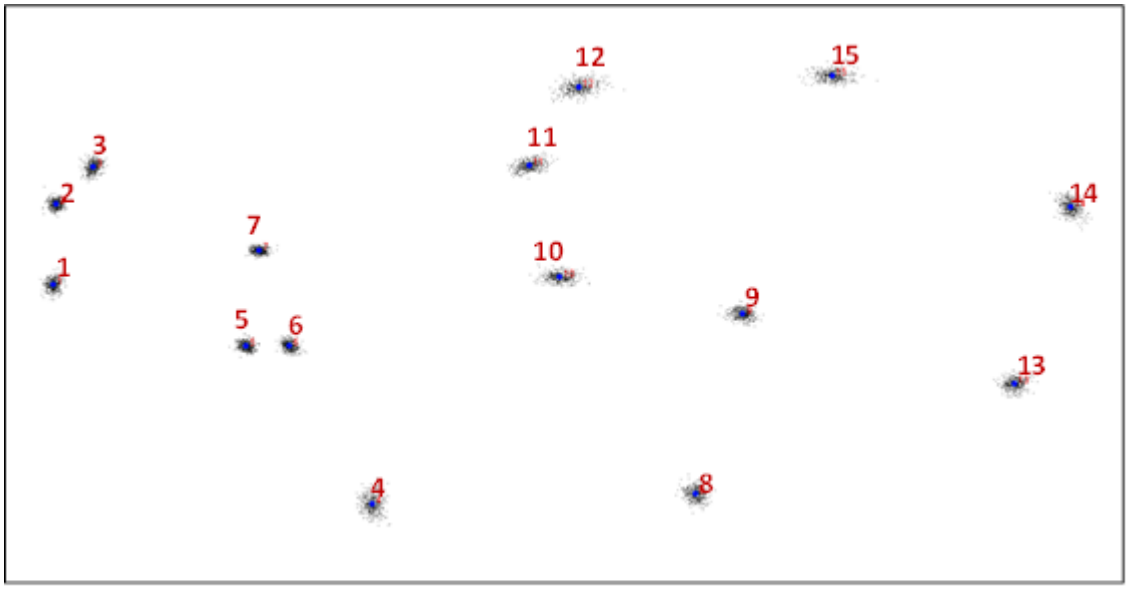



**Figure 3.6** Procrustes superposition showing the variation of 15 landmarks of *Zeugodacus cucurbitae* wings from individuals collected in six biogeographic regions and two islands of Thailand. The figure shows the configurations of landmarks for which differences in position, scale and orientation have been removed. The position of the landmarks in relation to a real wing is shown in Figure2.4.

*Determination of allometric effect*

Multiple regression of wing shape on centroid size showed a significant allometric effect ($P < 0.0001$) and accounted for 5.44 % of shape variation (Figure 3.7). Accordingly, the subsequent CVA was conducted on data corrected to take allometric effect into account.

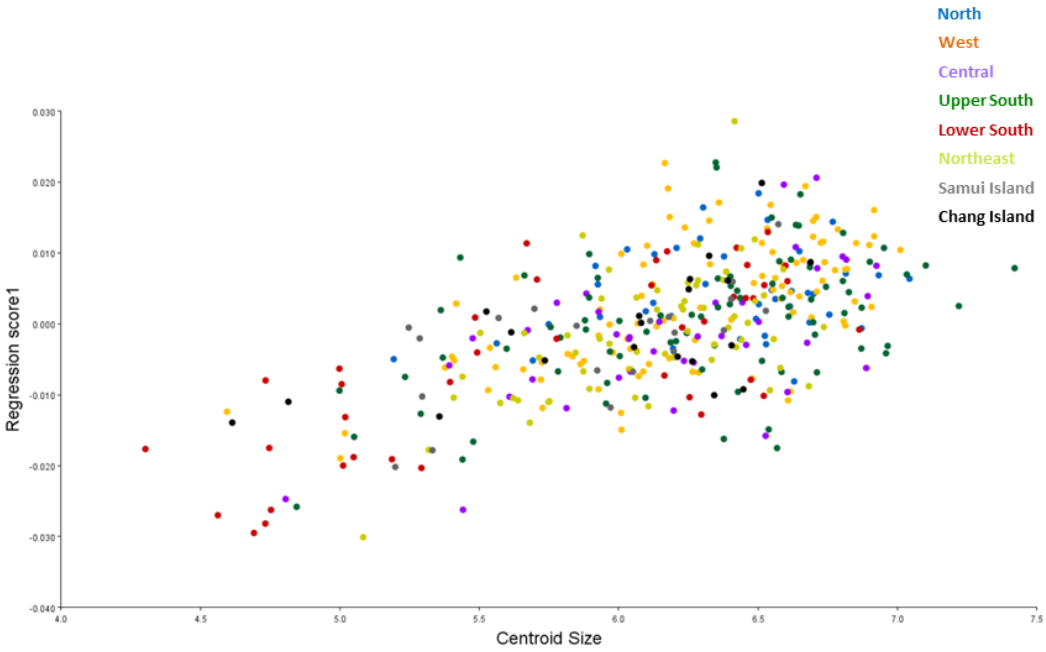



**Figure 3.7** Multiple regression of wing shape (regression score one) on centroid size (a measure of wing size) from six biogeographic regions and two islands of Thailand. Each coloured dot represents the wing of a fly from one of the listed locations.

*Wing shape analyses*

- Principal component analysis

The first two principal components accounted for 31.8% of the total shape variation and therefore, they accounted for a relatively low proportion of the total shape variation (Table 3.4 and Figure 3.8). Plots on the first two principal axes do not show distinct or even partial separation of *Z. cucurbitae* populations from different Thai biogeographic regions (Figure 3.9). The principal component analysis demonstrated little variation in wing shape and discrete groups are not evident in the scatter plots.

**Table 3.4** Summary statistics for principal component analysis of groups of *Zeugodacus cucurbitae* collected from six biogeographic regions and two islands of Thailand. The table shows eigenvalues, percent variation and cumulative percentage of variance explained by each principal component axis (PC) for the entire dataset.

| PC | Eigenvalues | % Variance | Cumulative % |
|---|---|---|---|
| 1 | 0.000110 | 18.007 | 18.007 |
| 2 | 0.000084 | 13.784 | 31.791 |
| 3 | 0.000082 | 13.424 | 45.215 |
| 4 | 0.000062 | 10.195 | 55.410 |
| 5 | 0.000044 | 7.265 | 62.675 |
| 6 | 0.000035 | 5.771 | 68.446 |
| 7 | 0.000029 | 4.778 | 73.224 |
| 8 | 0.000024 | 3.909 | 77.133 |
| 9 | 0.000019 | 3.127 | 80.260 |
| 10 | 0.000017 | 2.732 | 82.993 |
| 11 | 0.000014 | 2.301 | 85.293 |
| 12 | 0.000012 | 1.925 | 87.218 |
| 13 | 0.000010 | 1.704 | 88.923 |
| 14 | 0.000009 | 1.534 | 90.456 |
| 15 | 0.000009 | 1.501 | 91.957 |
| 16 | 0.000008 | 1.253 | 93.210 |
| 17 | 0.000007 | 1.089 | 94.298 |
| 18 | 0.000006 | 0.960 | 95.259 |
| 19 | 0.000006 | 0.918 | 96.177 |
| 20 | 0.000005 | 0.854 | 97.031 |
| 21 | 0.000004 | 0.735 | 97.766 |
| 22 | 0.000004 | 0.604 | 98.371 |
| 23 | 0.000003 | 0.546 | 98.917 |
| 24 | 0.000003 | 0.431 | 99.347 |
| 25 | 0.000002 | 0.344 | 99.691 |
| 26 | 0.000002 | 0.309 | 100.000 |

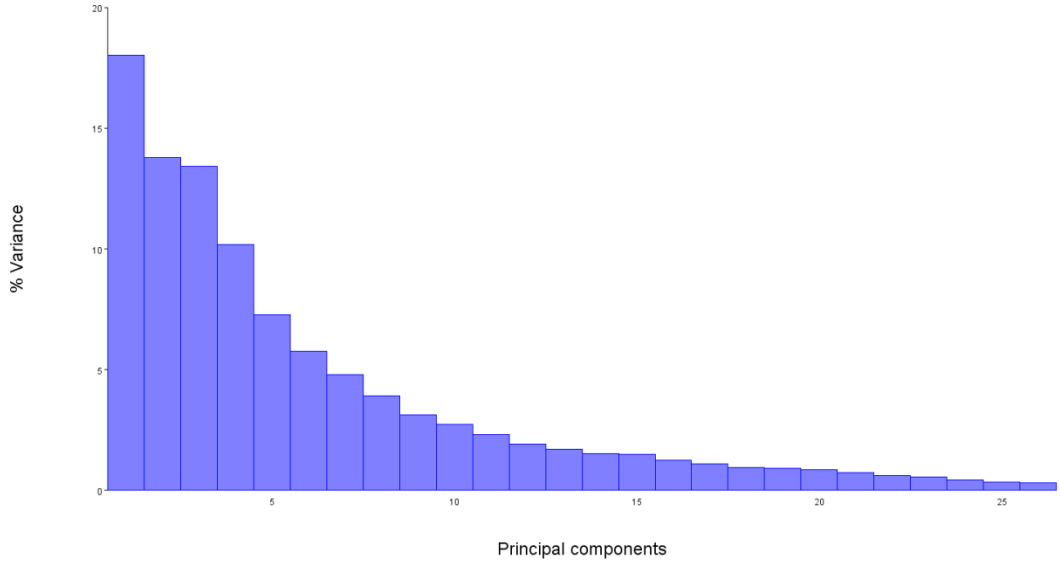



**Figure 3.8** Histogram of variances of the principle components of all the variables of groups of *Zeugodacus cucurbitae* collected from six biogeographic regions and two islands of Thailand.

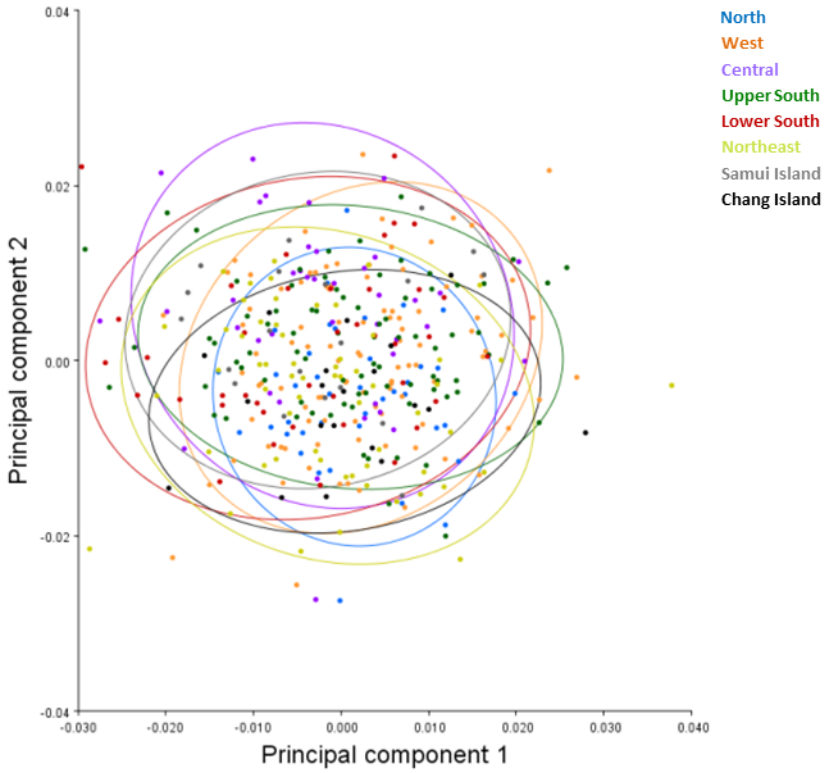



**Figure 3.9** Plots of principle component 1 vs principle component 2 based on wing shape data from wings of *Zeugodacus cucurbitae* sampled from six biogeographic regions and two islands of Thailand; 95% confidence ellipses are shown for each group. Each coloured dot represents the wing of a fly from one of the listed locations.

- Canonical variate analysis

Canonical variate analysis of the entire dataset based on the eight *a priori* defined groups resulted in seven canonical variates obtained for wing shape data, with the first two accounting for 70.42% of the variation (Table 3.5 and Figure 3.10). Distinction among the groups was particularly evident along the first canonical axis, which explained over 50% of the variation. However, the first two variates did not strongly resolve any particular groups. The proportion of significant differences in Mahalanobis distances between sites revealed some structure among wing shape of flies based on Thai biogeography. Flies from Samui Island were not significantly different to West, Upper South and Lower South, indicating the shape of *Z. cucurbitae* wings from Samui Island were similar to the shape of wings from adjacent, mainland populations in the Upper and Lower south and West populations. There were also similarities between Lower and Upper south populations and between the West and Upper south populations (i.e., between sites separated by the isthmus of Kra). Moreover, wings from the northeast were similar with wing shape of flies from the North and Chang Island, while northeast was similar to the wing shape of files from North (Table 3.6).

**Table 3.5** Summary statistics for canonical variates analysis of groups of *Zeugodacus cucurbitae* collected from six biogeographic regions and two islands of Thailand. The table shows eigenvalues, percent variation and cumulative percent variation explained by each canonical variate axis for the entire dataset.

| CV | Eigenvalues | % Variance | Cumulative % |
|---|---|---|---|
| 1 | 0.695 | 50.08 | 50.08 |
| 2 | 0.282 | 20.34 | 70.42 |
| 3 | 0.134 | 9.62 | 80.04 |
| 4 | 0.131 | 9.41 | 89.45 |
| 5 | 0.074 | 5.35 | 94.81 |
| 6 | 0.042 | 3.01 | 97.82 |
| 7 | 0.030 | 2.18 | 100.00 |

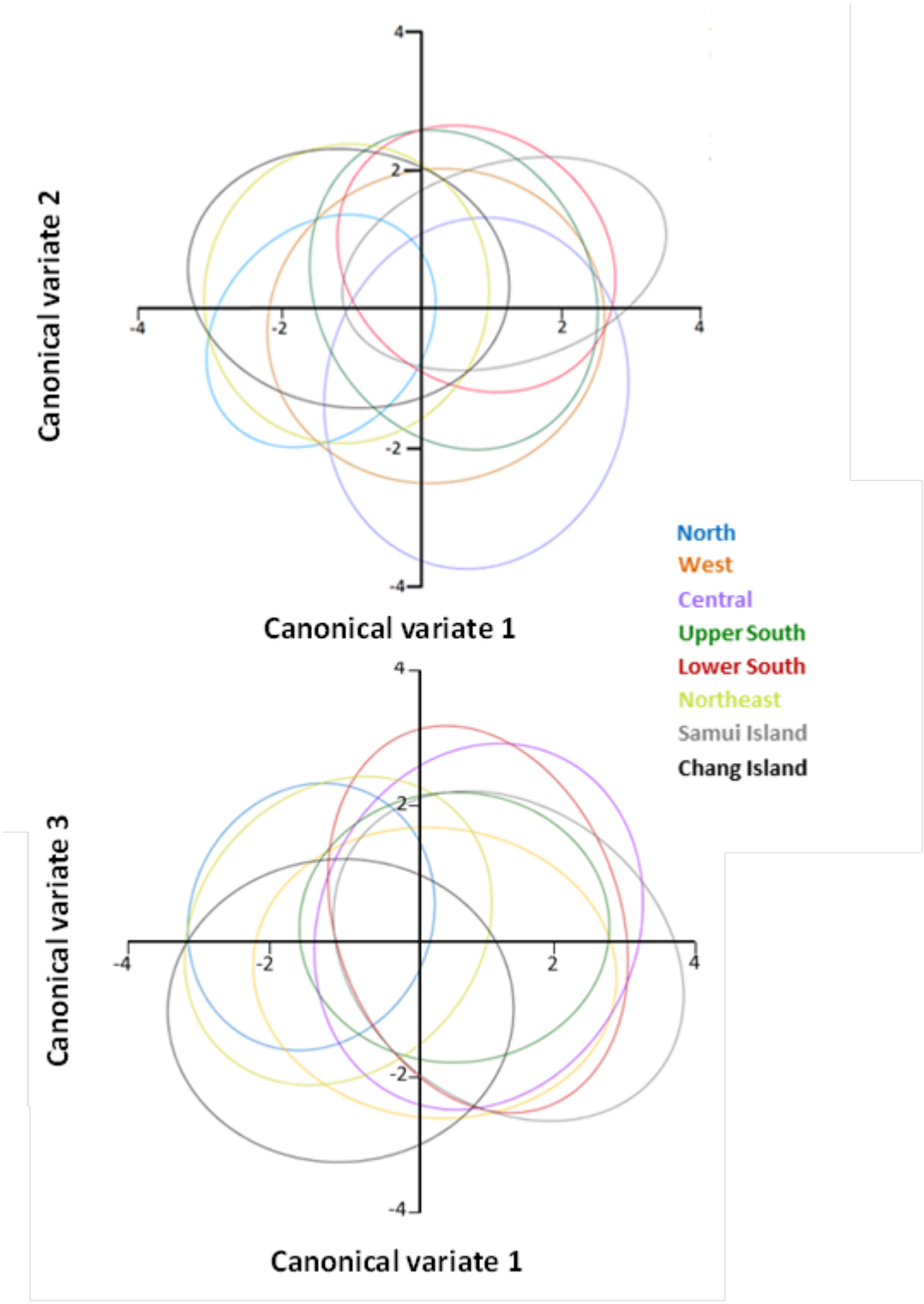



**Figure 3.10** Plot of the first three variates following canonical variate analysis of geometric morphometric wing shape data for *Zeugodacus cucurbitae* sampled from six biogeographic regions and two islands of Thailand. Twenty wings were analysed per site; 95% group confidence ellipses are represented; individual data points have been removed for clarity.

**Table 3.6** Mahalanobis distances between *Zeugodacus cucurbitae* wing shape for eight populations collected across Thai biogeographical regions. Values represent Mahalanobis distances as calculated from canonical variates analysis across six biogeographic regions and two islands sites. Values in bold are significant after Bonferroni correction for multiple tests, with the initial $P = 0.05$ and the corrected $P < 0.001$.

| Regions | North | West | Central | Upper South | Lower South | Northeast | Samui Island |
|---|---|---|---|---|---|---|---|
| North | - | | | | | | |
| West | **1.925** | - | | | | | |
| Central | **2.624** | **1.482** | - | | | | |
| Upper south | **2.189** | 1.039 | **1.604** | - | | | |
| Lower south | **2.596** | **1.457** | **2.079** | 1.081 | - | | |
| Northeast | 1.373 | **1.634** | **2.383** | **1.649** | **2.118** | - | |
| Samui Island | **2.967** | 1.693 | **2.250** | 1.380 | 1.504 | **2.617** | - |
| Chang Island | **1.997** | **1.855** | **2.764** | **2.039** | **2.449** | 1.409 | **2.725** |

### 3.3.1.2 Aedeagus morphometric analysis

Significant differences in mean *Z. cucurbitae* aedeagus length occurred among sites ($F_{7, 392} = 3.684$; $P < 0.05$). The mean aedeagus length of the North population (2.710 ± 0.108 mm) was significantly longer than that of populations from the Northeast (2.592 ± 0.108mm) and Chang Island (2.608 ± 0.139 mm). Aedeagal lengths of males from other populations were intermediate between these two groups (Figure 3.11).

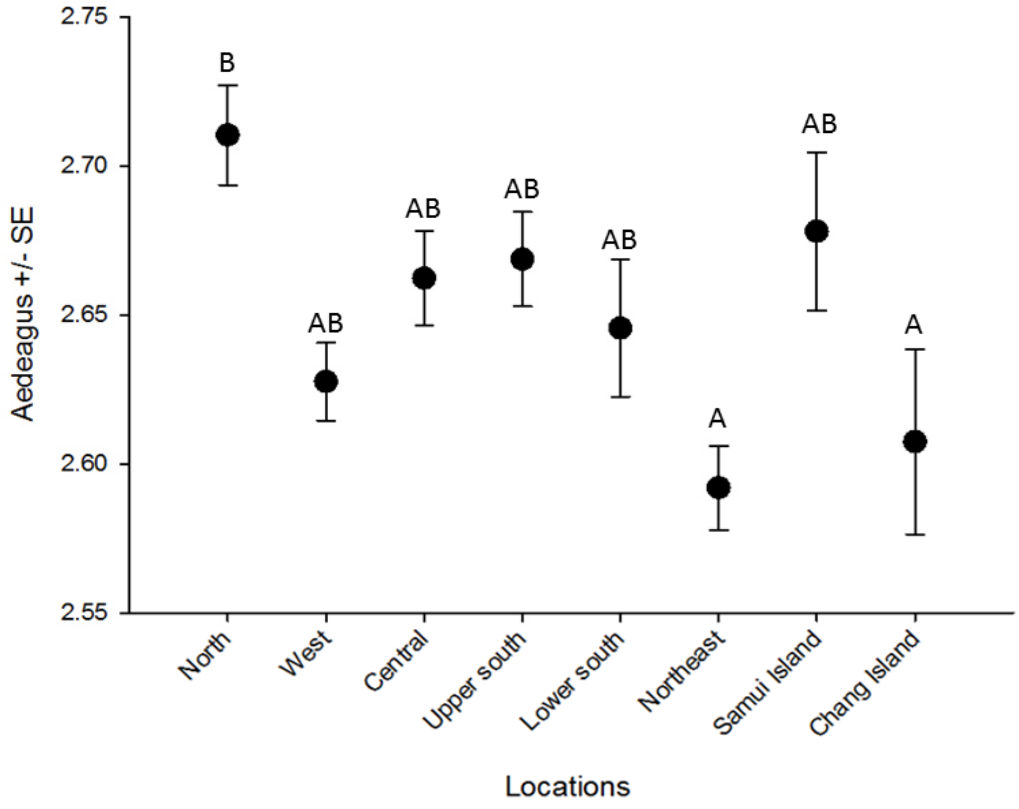



**Figure 3.11** Aedeagus length (mean ± SE) of *Zeugodacus cucurbitae* from six biogeographic regions and two islands of Thailand. Samples sharing the same letter are not statistically different from each other based on one-way ANOVA with Tukey *post hoc* test ($F_{7, 392} = 3.684$; $P < 0.05$).

### 3.3.2 Molecular results

#### 3.3.2.1 Mitochondrial DNA (*cox1*) analysis

*Mitochondrial DNA gene diversity*

A 652-base pair sequence of the *cox1* gene was amplified for 400 specimens of *Z. cucurbitae* collected from six regions and two islands locations across Thailand (Table 3.1). A total of 40 polymorphic positions (defining unique haplotypes) was detected in the aligned sequences. The two most common haplotypes, Haplotype 1 (H1) and Haplotype 2 (H2), were present at all sampling sites. Samples from mainland locations exhibited higher diversity than the samples from the two islands. The samples from Upper South showed the most diversity with 16 haplotypes, followed by samples from the Northeast (14 haplotypes), West (11 haplotypes), Central (9 haplotypes), Lower South and North (5 haplotypes), Chang Island (4 haplotypes) and Samui Island (4 haplotypes). Only ten of the 40 haplotypes (haplotypes 4, 5, 11, 12, 14, 15, 16 and 34) were found in more than one site, with two of them (haplotypes 1 and 2) found across most sites; 30 haplotypes were restricted to a single site (Table 3.7).

Figure 3.12 depicts the median-joining (MJ) network for *cox1*. It shows the relationships among *cox1* haplotypes and reveals no major divergent lineage(s). The network is a double starburst-like shape centred on the two common haplotypes (H1 and H2), with numerous singletons linked directly to these haplotypes. Most haplotypes were connected by short branch lengths. The network did not show any distinct pattern among haplotypes and their geographical distribution; the central haplotypes occur most frequently and were shared by several geographically widespread populations (Table 3.7, Figure 3.12).

**Table 3.7** Distribution of *cox1* haplotypes of *Zeugodacus cucurbitae* from six biogeographic regions and two islands of Thailand, highlighted in grey haplotypes observed in more than one region.

| Sample site | North | West | Central | Upper South | Lower South | Northeast | Samui Island | Chang Island |
|---|---|---|---|---|---|---|---|---|
| No. samples | 40 | 100 | 40 | 80 | 40 | 60 | 20 | 20 |
| Hap_1 | 13 | 24 | 11 | 9 | 5 | 11 | 3 | 4 |
| Hap_2 | 24 | 63 | 22 | 53 | 31 | 33 | 16 | 14 |
| Hap_3 | - | - | 1 | - | - | - | - | - |
| Hap_4 | - | 1 | 1 | - | - | 1 | - | - |
| Hap_5 | - | - | 1 | 1 | - | - | - | - |
| Hap_6 | - | - | 1 | - | - | - | - | - |
| Hap_7 | - | - | 1 | - | - | - | - | - |
| Hap_8 | - | - | 1 | - | - | - | - | - |
| Hap_9 | - | - | 1 | - | - | - | - | - |
| Hap_10 | - | 1 | - | - | - | - | - | - |
| Hap_11 | - | 3 | - | 2 | - | 3 | - | - |
| Hap_12 | - | 1 | - | 2 | - | 3 | - | - |
| Hap_13 | - | - | - | 1 | - | - | - | - |
| Hap_14 | 1 | - | - | 1 | - | 1 | - | - |
| Hap_15 | - | 1 | - | 2 | - | - | - | - |
| Hap_16 | - | - | - | 1 | 1 | - | 1 | - |
| Hap_17 | - | - | - | 1 | - | - | - | - |
| Hap_18 | - | - | - | - | 1 | - | - | - |
| Hap_19 | - | - | - | - | 1 | - | - | - |
| Hap_20 | - | - | - | - | - | - | - | 1 |
| Hap_21 | - | - | - | - | - | - | - | 1 |
| Hap_22 | 1 | - | - | - | - | - | - | - |
| Hap_23 | 1 | - | - | - | - | - | - | - |
| Hap_24 | - | - | - | 1 | - | - | - | - |
| Hap_25 | - | - | - | 1 | - | - | - | - |
| Hap_26 | - | - | - | 1 | - | - | - | - |
| Hap_27 | - | 1 | - | - | - | - | - | - |
| Hap_28 | - | - | - | - | - | 2 | - | - |
| Hap_29 | - | - | - | - | - | 1 | - | - |
| Hap_30 | - | - | - | - | - | 1 | - | - |
| Hap_31 | - | - | - | - | - | 2 | - | - |
| Hap_32 | - | - | - | - | - | 1 | - | - |
| Hap_33 | - | - | - | - | - | 1 | - | - |
| Hap_34 | - | 1 | - | - | - | 1 | - | - |
| Hap_35 | - | - | - | - | - | 1 | - | - |
| Hap_36 | - | 1 | - | - | - | - | - | - |
| Hap_37 | - | 1 | - | - | - | - | - | - |
| Hap_38 | - | - | - | 1 | - | - | - | - |
| Hap_39 | - | - | - | 1 | - | - | - | - |
| Hap_40 | - | - | - | 1 | - | - | - | - |
| **Total** | **5** | **11** | **9** | **16** | **5** | **14** | **3** | **4** |

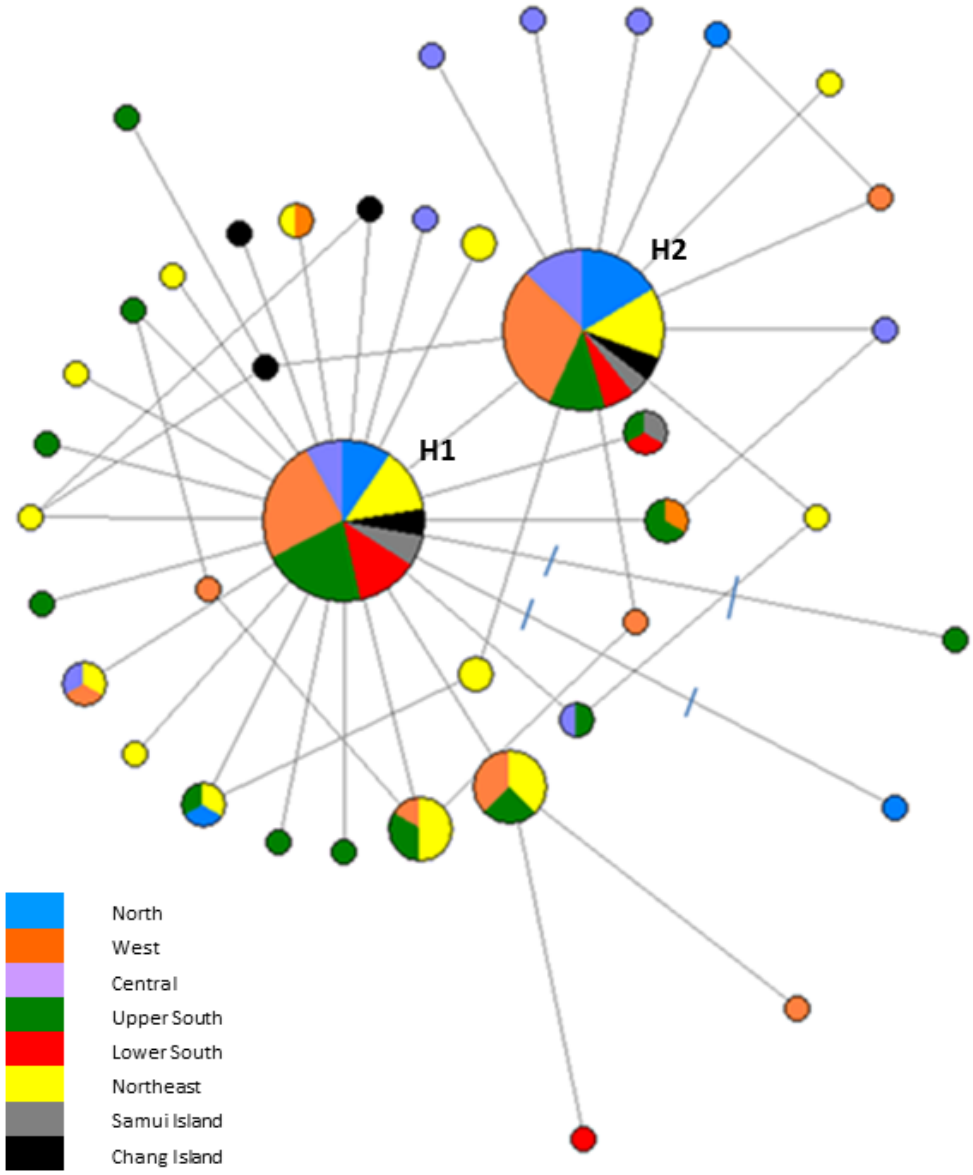



**Figure 3.12** Median-joining haplotype network generated from *Zeugodacus cucurbitae* collected in six biogeographic regions and two islands of Thailand. The 40 *cox1* haplotypes are colour coded by collecting location. Sizes of nodes and pie segments are proportional to haplotype frequency. Length of branches is proportional to number of mutational changes between haplotypes.

Samui Island showed the lowest values in genetic diversity (0.353 ± 0.123) and genetic diversity parameter (θπ) (0.368). Both genetic diversity and θπ were greatest in the northeast region (0.687 ± 0.080 and 0.943) (Table 3.8). There were highly significant negative values of Tajima's *D* tests of neutrality for the total dataset (*D* = -1.474, *P* < 0.0001).

**Table 3.8** Population-genetics summary-statistics from *cox1* analysis of *Zeugodacus cucurbitae* from six biogeographic regions and two islands of Thailand. Values in bold are statistically significant at α = 0.05.

| Sample site | Number of individuals | Gene Diversity | Θ$\pi$ | Tajima's D | Tajima's *D P*-value | Fu'*F*s | Fu'*F*s *P*-value |
|---|---|---|---|---|---|---|---|
| North | 40 | 0.546 ± 0.058 | 0.667 | -1.101 | 0.147 | -1.415 | 0.189 |
| West | 100 | 0.530 ± 0.048 | 0.676 | -1.670 | **0.021** | -7.824 | **0.000** |
| Central | 40 | 0.633 ± 0.065 | 0.831 | -1.592 | **0.044** | -5.602 | **0.000** |
| Upper South | 80 | 0.540 ± 0.066 | 0.728 | -2.299 | **0.001** | 17.053 | **0.000** |
| Lower South | 40 | 0.359 ± 0.092 | 0.472 | -1.537 | **0.034** | -2.392 | **0.033** |
| Northeast | 60 | 0.687 ± 0.080 | 0.943 | -1.800 | **0.012** | 11.437 | **0.201** |
| Samui Island | 20 | 0.353 ± 0.123 | 0.368 | -0.821 | 0.189 | -0.775 | 0.199 |
| Chang Island | 20 | 0.489 ± 0.117 | 0.537 | -0.975 | 0.204 | -1.406 | 0.058 |

*Genetic differentiation (cox1)*

Population pairwise $F_{ST}$ analysis indicated that 96.43% of the pairwise populations were not significantly different genetically (Table 3.9). Hierarchical Analysis of Molecular Variance of *cox1* data was conducted to test three levels of variation (within population, population within region, between regions). AMOVA analysis of populations grouped according to biogeographic region revealed no significant genetic differentiation between those regions (0.40%) ($F_{CT}$ = 0.039). The variation was highest within populations (97.60%), followed by populations within regions (1.98%) (Table 3.10).

**Table 3.9** Pairwise $F_{ST}$ distances between populations of *Zeugodacus cucurbitae* collected from six biogeographic regions and two islands of Thailand. The value in bold was significant after Bonferroni correction for multiple tests, with the initial $P$ = 0.05 and the corrected $P < 0.001$.

| Sample site | North | West | Central | Upper South | Lower South | Northeast | Samui Island |
|---|---|---|---|---|---|---|---|
| North | - | | | | | | |
| West | 0.001 | - | | | | | |
| Central | -0.015 | 0.008 | - | | | | |
| Upper South | 0.057 | 0.021 | **0.067** | - | | | |
| Lower South | 0.049 | 0.008 | 0.055 | -0.011 | - | | |
| Northeast | 0.005 | -0.005 | 0.014 | 0.011 | 0.002 | - | |
| Samui Island | 0.040 | 0.000 | 0.045 | -0.022 | -0.033 | -0.008 | - |
| Chang Island | 0.010 | -0.011 | 0.015 | -0.012 | -0.018 | -0.015 | -0.029 |

**Table 3.10** Results of the Analysis of Molecular Variance for the *cox1* dataset generated for Thai *Zeugodacus cucurbitae* popluations. Samples were constrained according to geographical region in relation to six biogeographic regions and two islands of Thailand. For locations of sites and biogeographic zones see Figures 3.3 and Table 3.1.

| Source of variation | d.f. | Sum of square | Variance of components | % total variance | Fixation indices |
|---|---|---|---|---|---|
| Among regions | 7 | 3.897 | 0.00141 | 0.40 | $F_{CT}$ = 0.039 |
| Among populations within region | 12 | 5.864 | 0.10071 | 1.98 | $F_{ST}$ = .0240* |
| Within populations | 378 | 131.667 | 0.34833 | 97.63 | $F_{SC}$ = 0.020 |
| Total | 397 | 103.337 | 0.40548 | | |

* $P > 0.005$

### 3.3.2.2 Microsatellite analysis

*Microsatellite diversity*

Microsatellite markers (11 loci) were used to assess contemporary population structure in Thai *Z. cucurbitae*, with a total of 400 individuals screened from six regions and two islands across Thailand. The number of alleles per locus varied from 3.64 (Samui Island and Chang Island) to 5.18 (West).

Average allelic richness ranged from 1.47 to 3.65. Curiously, the lowest allelic richness value was found on Samui Island and the highest value on Chang Island (Table 3.11). Average gene diversity over all loci per sample site varied from 0.432 ± 0.239 (in the Lower south) to 0.552 ± 0.304 (on Chang Island). Observed heterozygosity varied from 0.326 (Samui Island) to 0.4206 (West), which indicated moderate levels of intra-population diversity (Table 3.11).

**Table 3.11** Locations, sample sizes, microsatellite diversity estimates and Hardy-Weinberg (H-W) equilibrium test values for *Zeugodacus cucurbitae* populations from Thailand. No values were significant after Bonferroni correction for multiple tests, with the initial $P = 0.05$ and the corrected $P < 0.001$.

| Sample site | Number of individuals | Expected heterozygosity | Observed heterozygosity | No Alleles | Allelic Richness | Fis | H-W | Gene Diversity |
|---|---|---|---|---|---|---|---|---|
| **North** | 40 | 0.4499 | 0.4035 | 4.00 | 3.527 | 0.104 | 0.0097 | 0.491 ± 0.268 |
| **West** | 100 | 0.4888 | 0.4206 | 5.18 | 2.466 | 0.14 | < 0.0001 | 0.526 ± 0.286 |
| **Central** | 40 | 0.4862 | 0.4138 | 4.36 | 2.500 | 0.151 | < 0.0001 | 0.492 ± 0.268 |
| **Upper South** | 80 | 0.4790 | 0.4005 | 5.18 | 2.165 | 0.165 | < 0.0001 | 0.455 ± 0.249 |
| **Lower South** | 40 | 0.4365 | 0.3540 | 4.09 | 3.120 | 0.191 | < 0.0001 | 0.432 ± 0.239 |
| **Northeast** | 60 | 0.5136 | 0.4059 | 4.82 | 3.499 | 0.211 | < 0.0001 | 0.498 ± 0.270 |
| **Samui Island** | 20 | 0.4532 | 0.3260 | 3.64 | 1.475 | 0.287 | < 0.0001 | 0.437 ± 0.248 |
| **Chang Island** | 20 | 0.4635 | 0.4419 | 3.64 | 3.649 | 0.048 | 0.7600 | 0.552 ± 0.304 |

*Differentiation and genetic relationships among populations*

Population pairwise $R_{ST}$ analysis revealed that 89.29% of pairwise populations were not significantly different (Table 3.12). Analysis of molecular variance (AMOVA) was conducted based on eight groups (6 regions: north, west, central, upper south, lower south, northeast; and two islands: Samui Island and Chang Island). There was non-significant variation ($F_{CT}$ = 0.0084; $P$ > 0.05) between populations from six regions and two islands. The results indicated that 0.84% of the genetic variation was partitioned among the eight groups, 1.68% among sampling sites within groups, and 97.47% within sampling sites (Table 3.13).

**Table 3.12** Pairwise $R_{ST}$ distances between populations of *Zeugodacus cucurbitae* collected from six biogeographic regions and two islands of Thailand. Values in bold were significant after Bonferroni correction for multiple tests, with the initial $P$ = 0.05 and the corrected $P$ < 0.001.

| Sample site | North | West | Central | Upper South | Lower South | Northeast | Samui Island |
|---|---|---|---|---|---|---|---|
| North | - | | | | | | |
| West | 0.008 | - | | | | | |
| Central | 0.007 | -0.003 | - | | | | |
| Upper South | 0.008 | -0.003 | 0.004 | - | | | |
| Lower South | 0.046 | 0.028 | 0.044 | 0.023 | - | | |
| Northeast | -0.010 | 0.008 | 0.006 | 0.007 | **0.041** | - | |
| Samui Island | 0.004 | -0.012 | -0.017 | -0.006 | 0.013 | 0.003 | - |
| Chang Island | 0.060 | 0.012 | 0.004 | 0.027 | **0.075** | **0.059** | -0.001 |

**Table 3.13** Results of Analysis of Molecular Variance (AMOVA) for microsatellite data for populations of *Zeugodacus cucurbitae* from Thailand. Samples were constrained according to six biogeographic regions and two islands in Thailand. For geographical locations of sites and Thai biogeography see Figures 3.3 and Table 3.1.

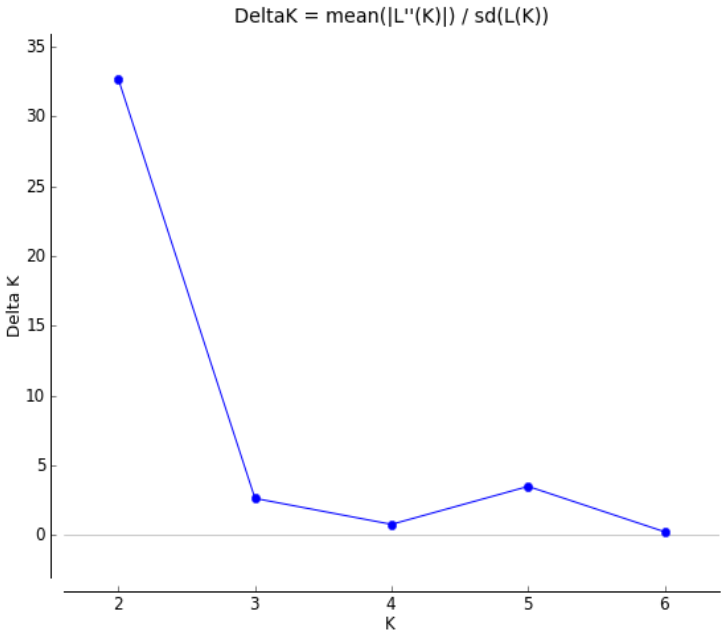
| Source of variation | Sum of square | Variance of components | % total variance | Fixation indices |
|---|---|---|---|---|
| Among regions | 10372.883 | 5.73315 | 0.84436 | $F_{CT}$ = 0.0084 |
| Among populations within region | 12786.009 | 11.40853 | 1.68021 | $F_{ST}$ = 0.0252* |
| Within populations | 469134.392 | 661.85368 | 97.47544 | $F_{SC}$ = 0.0169* |
| Total | 492293.284 | 678.99536 | | |

* $P < 0.05$

*Population structure using Bayesian clustering*

Genetic structuring among the six biogeographic regions and two islands was tested by Bayesian cluster analysis (using STRUCTURE) on the individual genotypes. The Evanno et al. (2005) method indicated the highest (delta *K*) value of 2 as an optimal *K* value (Figure 3.13A), while results of Bayesian clustering of the dataset similarly supported two (Δ*K*) distinct groups (Figure 3.13B). The two population clusters identified were Northeast (Orange) and others (Blue) (Figure 3.14). This result was congruent with results from PCA and the neighbour-joining population tree (Figure 3.15 and Figure 3.16).

(A)




(B)

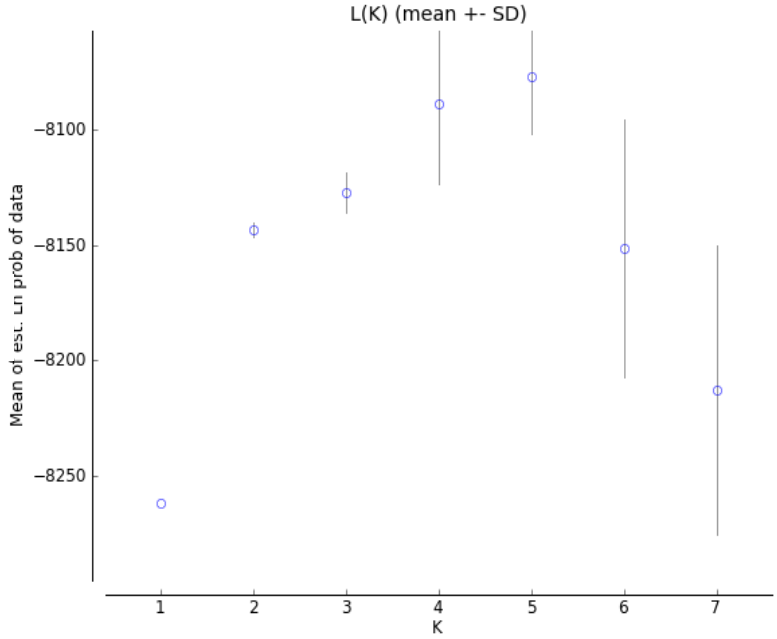



**Figure 3.13** Bayesian clustering results for microsatellite data of *Zeugodacus cucurbitae* in Thailand; (A) plot of Δ*K* with the median value is that which is most highly supported as the optimum value of *K* for the analysed sample, (B) Mean of log probability of data (LnP (D)).

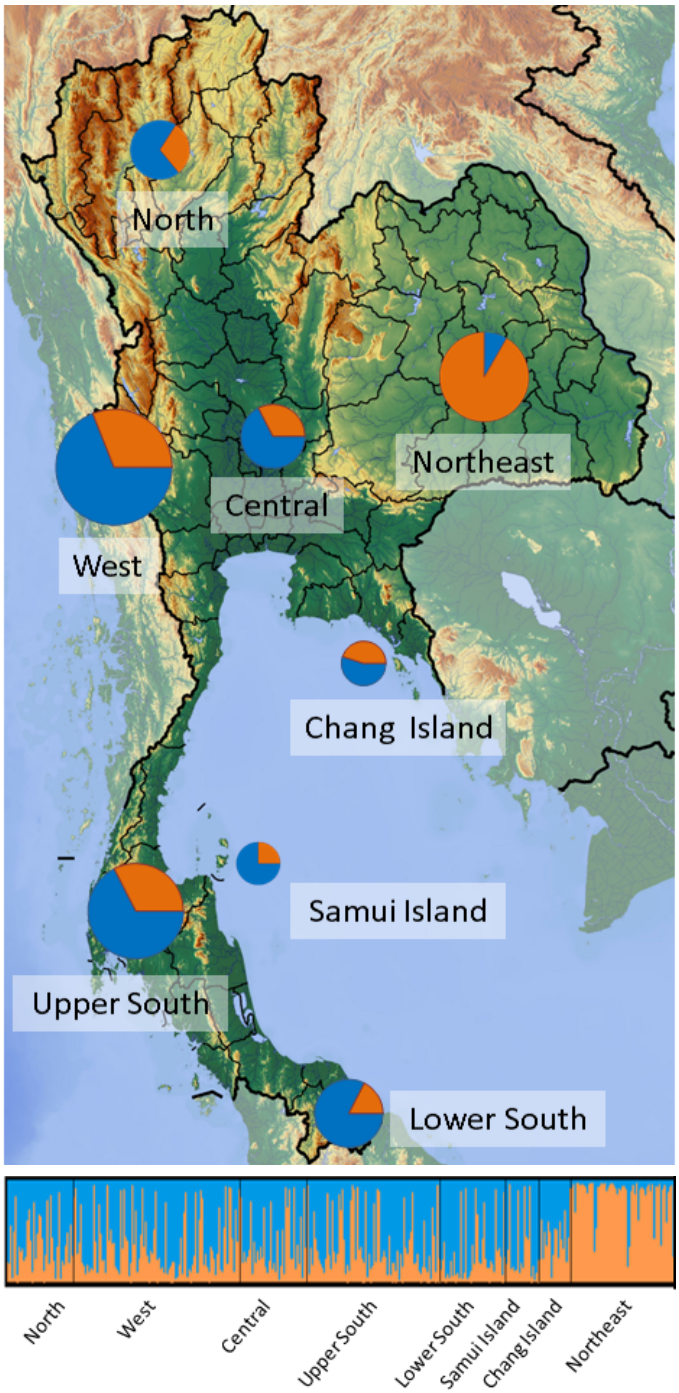



**Figure 3.14** Structure bar plot of Bayesian cluster analysis based on 11 microsatellite loci obtained from *Zeugodacus cucurbitae* collected from six biogeographic regions and two islands of Thailand. The plot shows population assignment results for different values of *K* = 2. Each horizontal line represents a single individual and its cluster assignment to a particular cluster is given as a particular colour. Solid black lines separate individuals from each of the eight sites. Pie charts represent assignment probability of belonging to each of *K* = 2 clusters identified by structure based on microsatellite allele frequencies, with probability values normalized using CLUMPP. Pie chart sizes are relative to sample size at each sampling location.

The neighbour-joining phylogenetic tree based on Cavalli-Sforza and Edwards (1967) distances also supported the Structure cluster assignment. Although the Structure result showed that Northeast population as almost completely separate from other regions, the unrooted dendrogram supported this population as a sister group to the Central, West and Upper South populations. The Northeast population was separated by a long branch relative to the rest of the internode branch lengths in the tree, which suggested the Northeast population was quite divergent from other populations. Furthermore, the tree supported a close relationship between the North and Chang Island populations and between Samui Island and the Lower south (Figure 3.15).

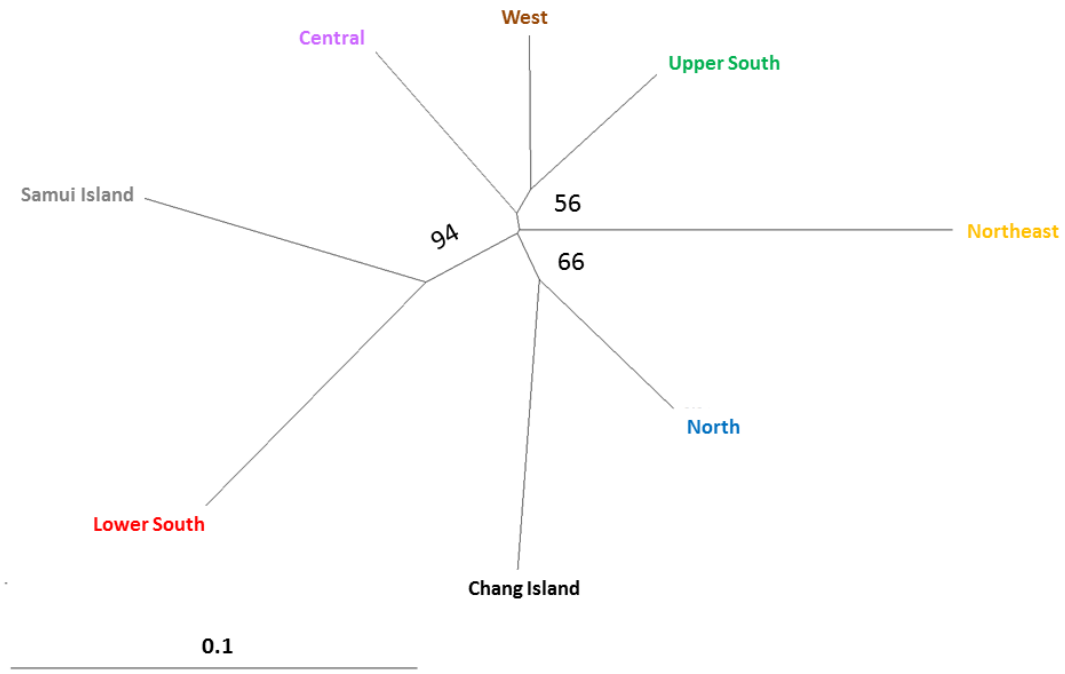



**Figure 3.15** Neighbour-joining unrooted tree of population relationships from the microsatellite dataset of *Zeugodacus cucurbitae* from six biogeographic regions and two islands of Thailand, based on Carvalli-Sforza and Edwards (1967) distances matrix. Bootstrap values were calculated using 1000 replications and are given as percentage, with only values greater than 50% showed.

Principle component analysis (PCA) of the 11 loci revealed population clustering similar to that obtained from Bayesian cluster analysis and neighbour-joining. The first two axes in the microsatellite PCA explained 85.87% of the variation in the data. Over 70.72% of the variation in the data was explained by the PC1 axis, which effectively separated the Northeast region from all other sampled regions. A secondary and more subtle pattern of structure was explained by the second PC axis (15.15%), which appeared to separate the North, West, Central, Upper South and Chang Island from the Lower South and Samui Island populations (Figure 3.16).

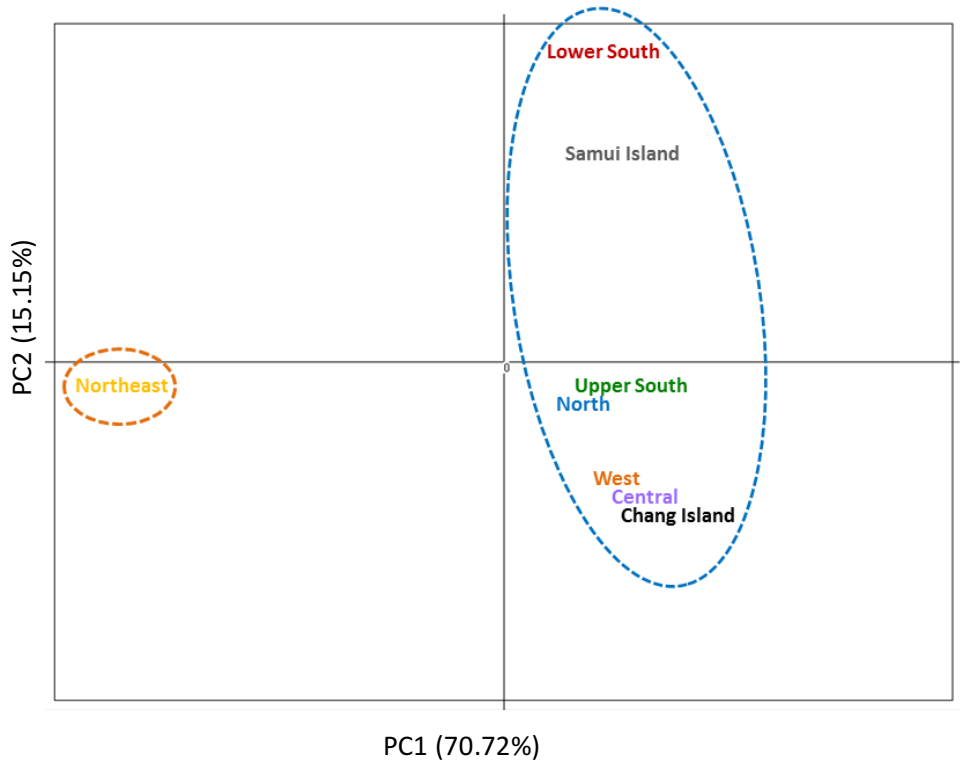
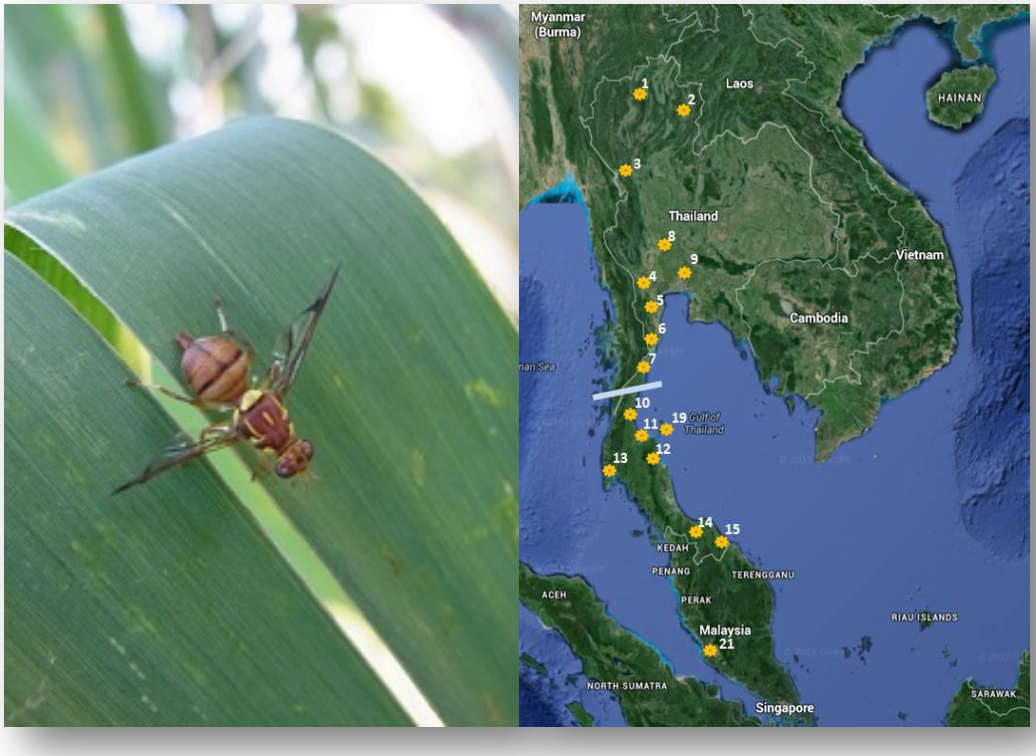



**Figure 3.16** Principal components analysis (PCA), based on allele frequencies of 11 microsatellites loci, for *Zeugodacus cucurbitae* collected from six biogeographic regions and two islands of Thailand. The blue and tan dashed lines correspond to population clusters identified using STRUCTURE.

*The first part of the results have provided a picture of the population structure of melon fly across Thailand, based on the generally accepted biogeographical regions within the country. These results show relatively minor structuring of Z. cucurbitae populations according to region, with the marked exception of flies from the northeast which have a notably different microsatellite structure. I will now turn to the chapter's second major topic, the population structure of melon fly along the Thai-Malay Peninsula, in order to address the question of a possible biogeographical barrier at the Isthmus of Kra.*

# RESULT PART 2: THAI-MALAY BIOGEOGRAPHY



## 3.4 RESULTS PART 2: THAI-MALAY BIOGEOGRAPHY

### 3.4.1 Morphometric results

#### 3.4.1.1 Geometric morphometric wing shape analysis

*Centroid size calculation*

Wing size and shape variation were examined using geometric morphometric analysis in a data set for 340 males from 17 sample sites (20 samples/sites) along the Thai-Malay transect. Wing centroid sizes were significantly different among sites ($F_{16, 332} = 3.437$; $P < 0.05$) for flies sampled along Thai-Malay transect. There was a significant difference between populations below (=south of) and above (= north of) the Isthmus of ($t_{1, 338} = 3.316$; $P < 0.05$). Wing size differed significantly among sites below the Kra Isthmus ($F_{7, 159} = 4.187$; $P < 0.05$), but did not significantly differ among sites above the Isthmus of Kra ($F_{8, 179} = 9.71$; $P > 0.05$). The wing centroid size of flies from below the Isthmus of Kra were on average smaller (6.070 ± 0.641 mm) and more variable than those from above the Isthmus of Kra (6.270 ± 0.465 mm). Wings of flies from Narathiwat were the smallest in size (5.634 ± 0.821 mm), while wings of flies from Thachana were the biggest (6.437 ± 0.348 mm) (Figure 3.17).

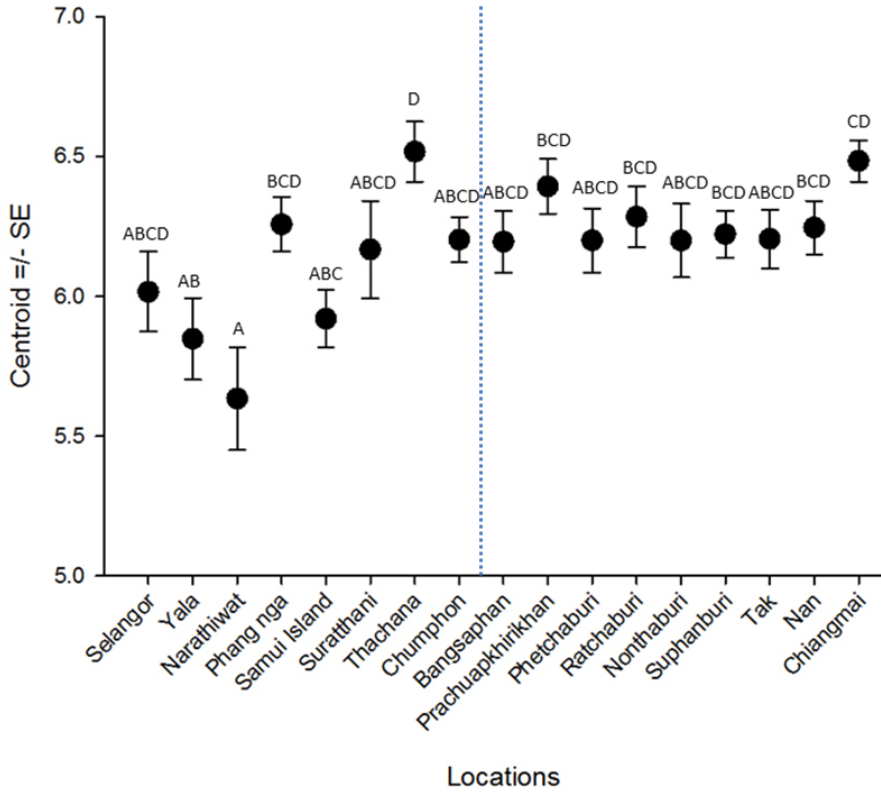


**Figure 3.17** Wing centroid sizes (mean ± SE) of *Zeugodacus cucurbitae* from sites along the Thai-Malay transect. Samples sharing the same letter are not statistically different from each other based on one-way ANOVA with Tukey *post hoc* test ($F_{16, 323} = 3.437$, $P > 0.05$). The dotted blue line represents the Isthmus of Kra barrier that divides Lower (southern) and Upper (northern) Isthmus of Kra locations.

*Isolation by distance*

A weak, but statistically significant and positive relationship was found between melon fly wing centroid size and increasing latitude ($R^2 = 0.049$; $P < 0.05$) (Figure 3.18).

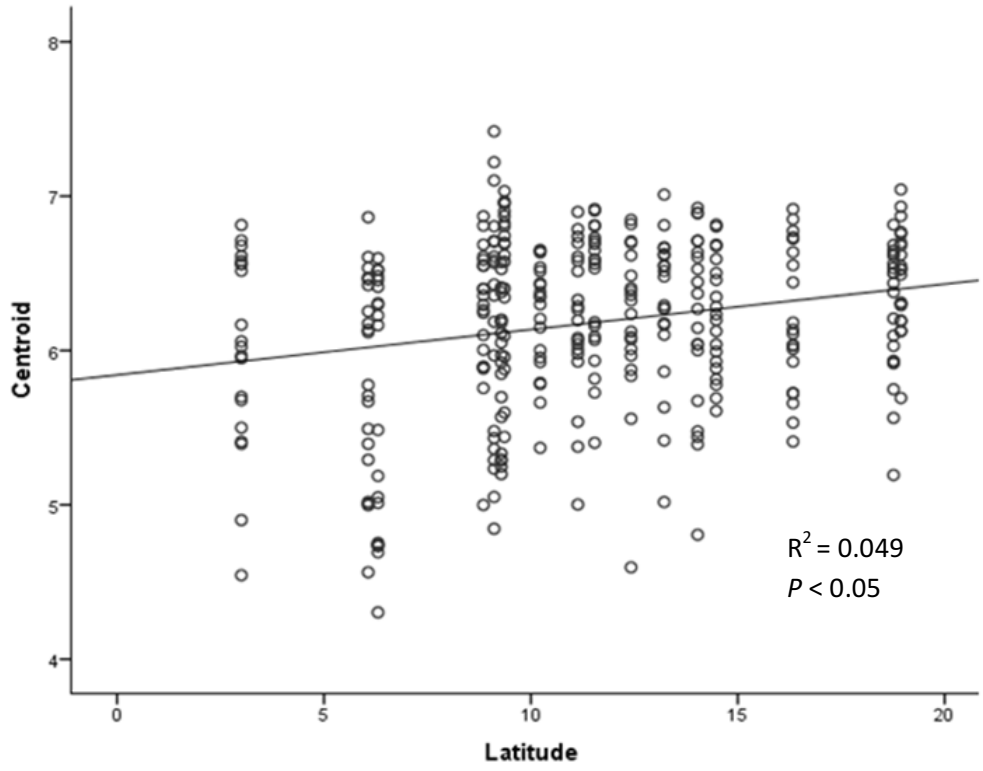



**Figure 3.18** Regression of *Zeugodacus cucurbitae* wing centroid size (mm) against latitude for flies collected from 17 sites along the Thai-Malay transect. The vertical column of values on the far left of the graph (3°N) comprises centroid values for flies from Selangor; the closely adjacent columns on the far right are values for flies from Nan in far northern Thailand.

*Procrustes superimposition*

Once again, generalized Procrustes superimposition produced a fresh set of co-ordinate data for each fly. Fifteen landmarks in two dimensions were investigated, and average shape rendered in two axes (Table 3.14). The Procrustes sum of squares was 0.2146, and Tangent sums of squares were 0.2145. The Procrustes superposition highlighted landmarks 12 and 15 as more variable than the other landmarks; once again, no outliers were evident in the data set (Figure 3.19).

**Table 3.14** Average position on two axes of fifteen landmarks in two dimensions of *Zeugodacus cucurbitae* wings from 17 sites along the Thai-Malay transect. The position of the landmarks in relation to a real wing is shown in Figure 2.4.

| Landmark | Axis 1 (X) | Axis 2 (Y) |
|---|---|---|
| 1 | -0.330489 | -0.008668 |
| 2 | -0.328490 | 0.050126 |
| 3 | -0.300666 | 0.076961 |
| 4 | -0.090254 | -0.166427 |
| 5 | -0.185585 | -0.052230 |
| 6 | -0.152848 | -0.051843 |
| 7 | -0.176078 | 0.016997 |
| 8 | 0.152841 | -0.159261 |
| 9 | 0.187922 | -0.028882 |
| 10 | 0.050139 | -0.001906 |
| 11 | 0.027583 | 0.078796 |
| 12 | 0.063893 | 0.134686 |
| 13 | 0.392313 | -0.079643 |
| 14 | 0.434517 | 0.048006 |
| 15 | -0.330489 | -0.008668 |

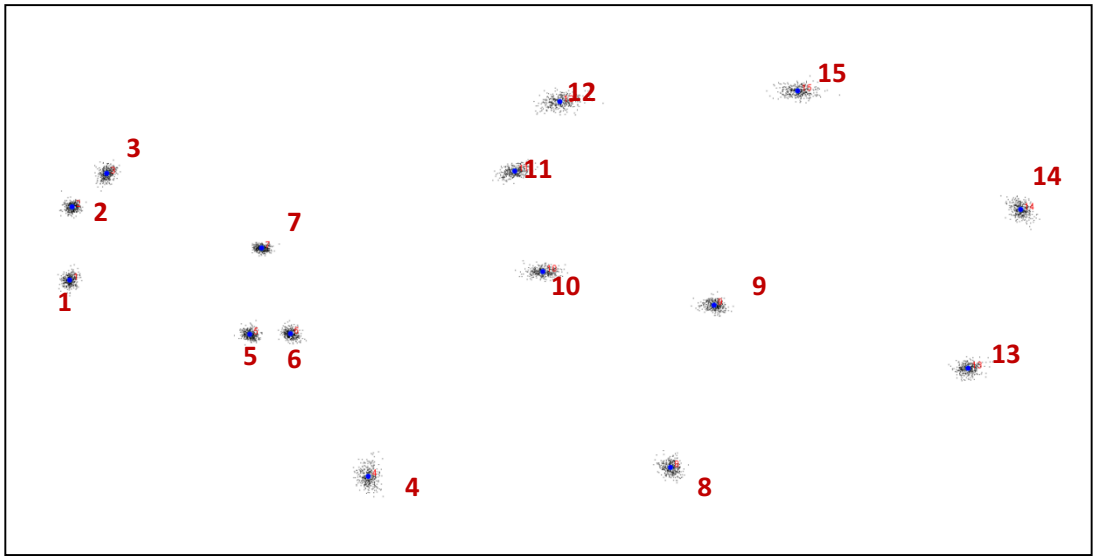



**Figure 3.19** Procrustes superpositions showing the variation of 15 landmarks of Zeugodacus cucurbitae wings from individuals collected from 17 sites along the Thai-Malay transect. The figure shows the configurations of landmarks for which differences in position, scale and orientation have been removed. The position of the landmarks in relation to a real wing is shown in Figure 2.4.

*Determination of allometric effect*

Multiple regression of wing shape on centroid size showed a significant allometric effect ($P < 0.0001$) and accounted for 4.37 % of shape variation (Figure 3.20). Accordingly, the subsequent CVA was conducted on data corrected to take allometric effect into account.

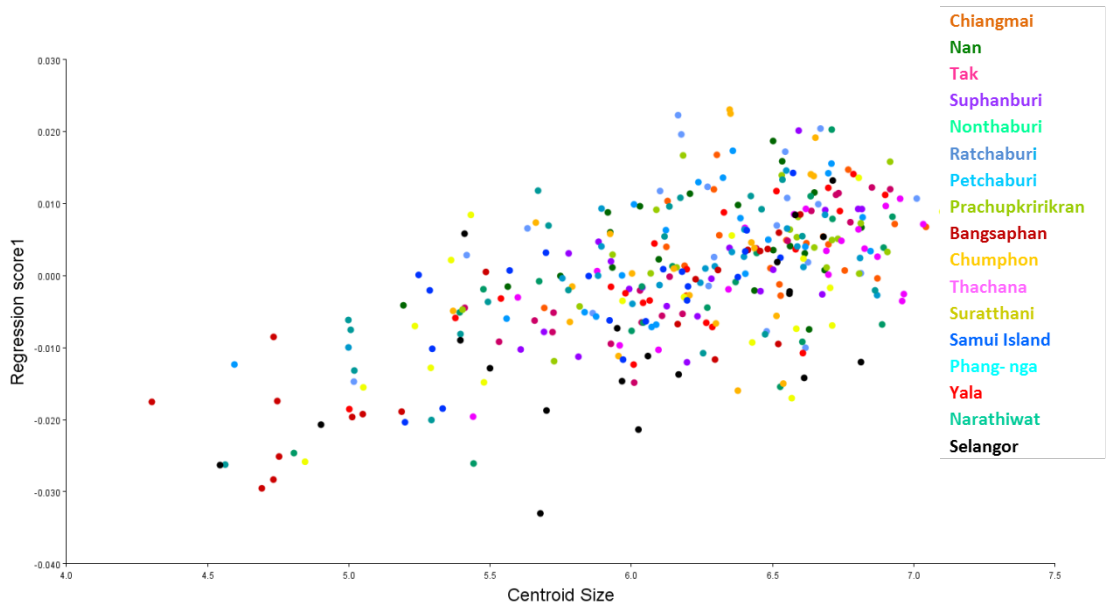



**Figure 3.20** Multiple regression of wing shape (regression score one) on centroid size (wing size) for *Zeugodacus cucurbitae* individuals collected from 17 sites along the Thai-Malay transect.

*Wing shape analyses*

- Principal component analysis

Principal component analysis was conducted on 15 wing landmarks, of which the first two principal components explained 18.19% and 14.78% of the variation, respectively (Table 3.15 and Figure 3.21). The first two principal axes did not show distinct separation of the populations (Figure 3.22). While the principal component analysis detected variations in the wing shape of melon flies along the Thai-Malay transect, scatter plots of the data did not reveal discrete groups.

**Table 3.15** Summary statistics for Principal component (PC) analysis of groups of *Zeugodacus cucurbitae* collected from 17 sites along the Thai-Malay transect. Eigenvalues, percent variation and cumulative percent explained by each Principal component axis for the entire dataset.

| PC | Eigenvalues | % Variance | Cumulative % |
|---|---|---|---|
| 1 | 0.00011507 | 18.186 | 18.186 |
| 2 | 0.00008716 | 13.776 | 31.962 |
| 3 | 0.00008683 | 13.724 | 45.686 |
| 4 | 0.00006856 | 10.835 | 56.521 |
| 5 | 0.00004462 | 7.051 | 63.572 |
| 6 | 0.00003483 | 5.505 | 69.078 |
| 7 | 0.00002975 | 4.702 | 73.779 |
| 8 | 0.00002501 | 3.953 | 77.732 |
| 9 | 0.00001843 | 2.913 | 80.645 |
| 10 | 0.00001662 | 2.626 | 83.271 |
| 11 | 0.00001434 | 2.267 | 85.538 |
| 12 | 0.00001165 | 1.842 | 87.379 |
| 13 | 0.00001120 | 1.77 | 89.149 |
| 14 | 0.00000987 | 1.559 | 90.708 |
| 15 | 0.00000877 | 1.385 | 92.094 |
| 16 | 0.00000764 | 1.207 | 93.301 |
| 17 | 0.00000679 | 1.073 | 94.374 |
| 18 | 0.00000614 | 0.97 | 95.344 |
| 19 | 0.00000601 | 0.951 | 96.294 |
| 20 | 0.00000507 | 0.801 | 97.095 |
| 21 | 0.00000443 | 0.701 | 97.796 |
| 22 | 0.00000378 | 0.597 | 98.393 |
| 23 | 0.00000347 | 0.548 | 98.942 |
| 24 | 0.00000270 | 0.427 | 99.368 |
| 25 | 0.00000206 | 0.326 | 99.695 |
| 26 | 0.00000193 | 0.305 | 100.000 |

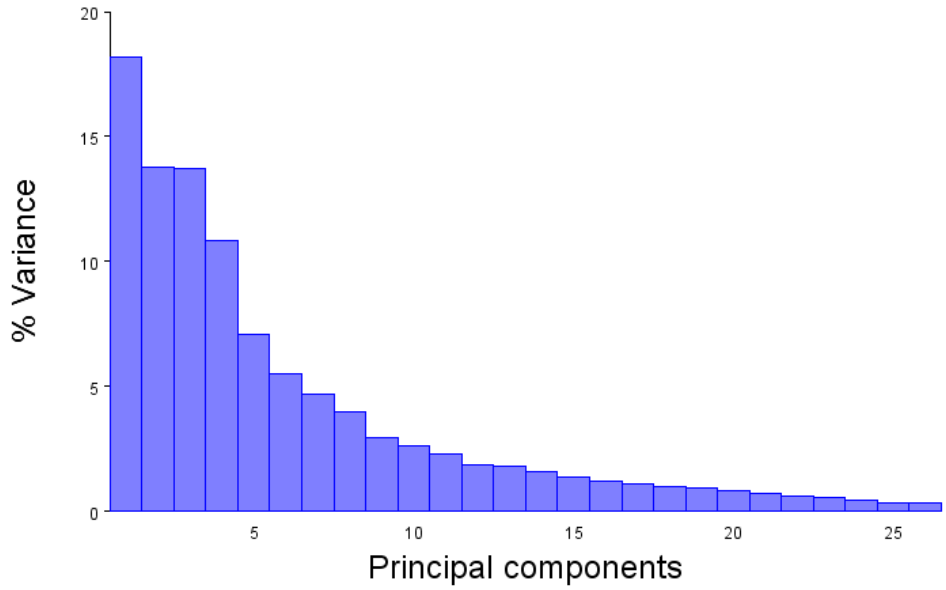



**Figure 3.21** Histogram of variances of the principal components following principal component analysis of wing shape of *Zeugodacus cucurbitae* collected from 17 sites along the Thai-Malay transect.

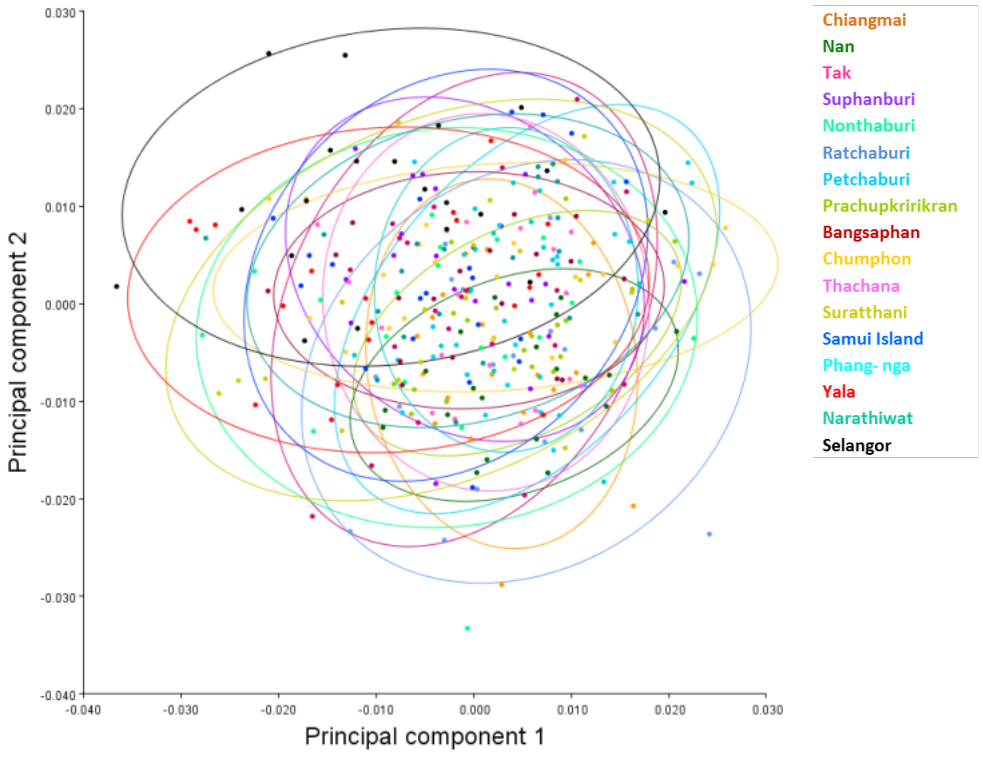



**Figure 3.22** Plots of principal component 1 vs principal component 2 based on wing shape data of *Zeugodacus cucurbitae* sampled from 17 sites along the Thai-Malay transect; 95% confidence ellipses are shown for each group. Each coloured dot represents the wing of a fly from one of the listed locations.

- Canonical variate analysis

Canonical variate analysis yielded 16 canonical variates, for which the first three accounted for 58.13% of the variation (Table 3.16). Based on the first three canonical variates, the wing shape of flies from Selangor (Malaysia) was clearly delineated from flies from Nan and Chiangmai (Thailand), with the greatest difference occurring along the first canonical axis (Figure 3.23A). Variation in wing shape was depicted as a landmark movement from a consensus configuration using wireframe; greatest deformation along the first and the second canonical variate occurred at landmark 4 (Figure 3.23B). This pattern was reflected in the significant pairwise Mahalanobis distances estimated between these populations (Table 3.17). The two most northern locations, Chiangmai and Nan, were significantly different from all other sites, but were similar to each other (Table 3.17). Selangor, the southernmost site along Thai-Malay transect, was significantly different from all other sites along Thai-Malay transect (Table 3.17).

**Table 3.16** Summary statistics for canonical variate analysis of populations of *Zeugodacus cucurbitae* collected from 17 sites along the Thai-Malay transect. Eigenvalues, percent variation and cumulative percent explained by each canonical variate.

| CV | Eigenvalues | % Variance | Cumulative % |
|---|---|---|---|
| 1 | 1.217 | 34.36 | 34.36 |
| 2 | 0.472 | 13.32 | 47.67 |
| 3 | 0.370 | 10.45 | 58.13 |
| 4 | 0.298 | 8.42 | 66.55 |
| 5 | 0.250 | 7.05 | 73.60 |
| 6 | 0.200 | 5.64 | 79.24 |
| 7 | 0.171 | 4.84 | 84.07 |
| 8 | 0.160 | 4.52 | 88.60 |
| 9 | 0.087 | 2.45 | 91.04 |
| 10 | 0.086 | 2.43 | 93.47 |
| 11 | 0.078 | 2.20 | 95.67 |
| 12 | 0.058 | 1.64 | 97.31 |
| 13 | 0.040 | 1.12 | 98.43 |
| 14 | 0.029 | 0.83 | 99.26 |
| 15 | 0.016 | 0.45 | 99.71 |
| 16 | 0.010 | 0.29 | 100.00 |

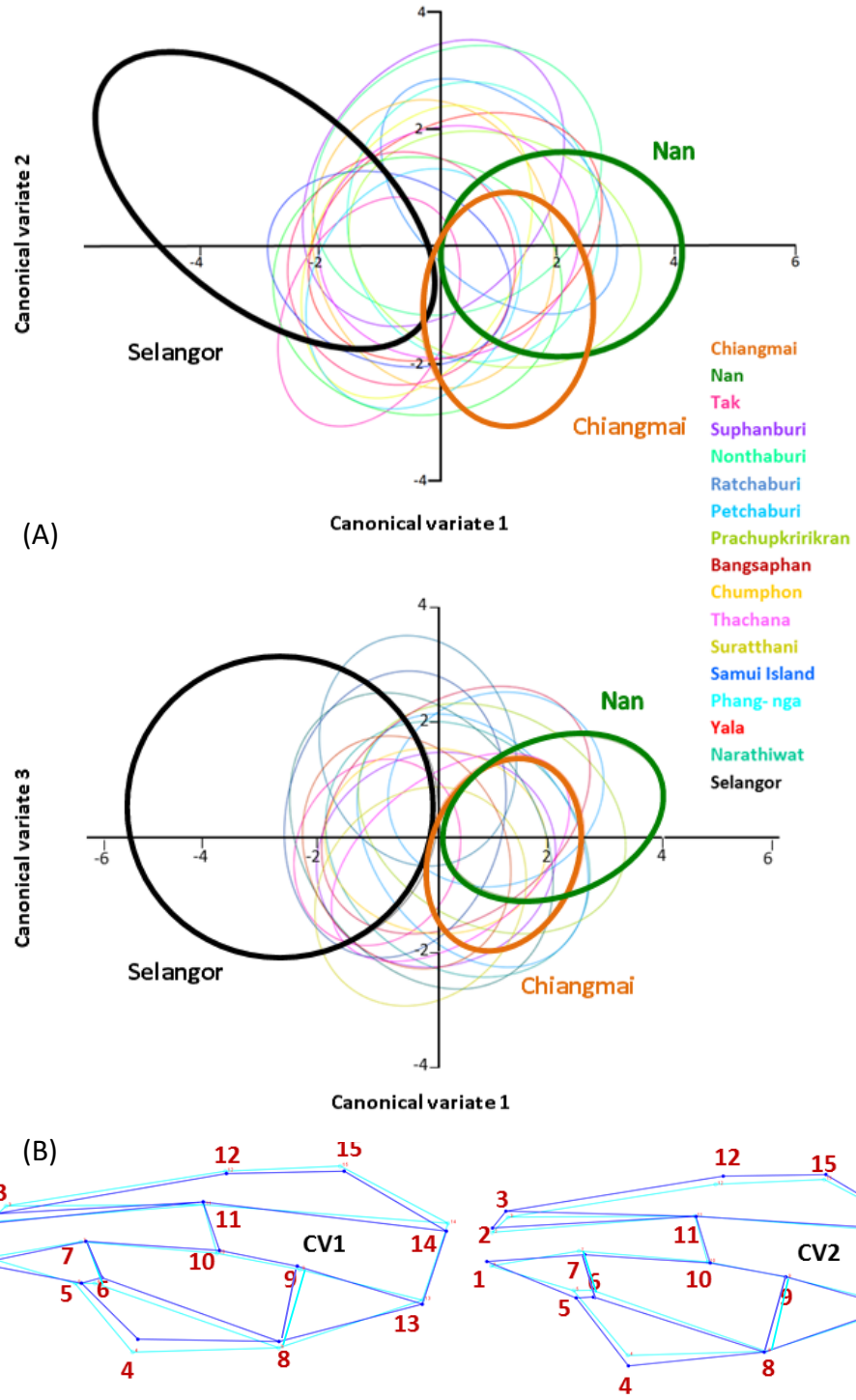



**Figure 3.23** (A) Plot of first three variates following canonical variate analysis of geometric morphometric wing shape data for *Zeugodacus cucurbitae* sampled from 17 sites along the Thai-Malay transect. Twenty wings were analysed per location; individual data have been removed for clarity. (B) Wireframe showing the wing shape variation (= visualization of landmark movement using wireframe for the canonical variates).

**Table 3.17** Pairwise Mahalanobis distances, based on *Zeugodacus cucurbitae* wing-shape analysis, between 17 sites along the Thai-Malay transect. Values above the diagonal are geographic distances (km). Values below the diagonal represent Mahalanobis distances as calculated from canonical variates analysis on all 17 sites. Values in bold were significant after Bonferroni correction for multiple tests, with the initial α=0.05 and the corrected $P < 0.0005$.

| | | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Chiangmai | - | 185 | 200 | 508 | 560 | 638 | 727 | 824 | 868 | 969 | 1065 | 1094 | 1075 | 1124 | 1424 | 1464 | 1791 |
| 2 | Nan | 1.920 | - | 243 | 559 | 530 | 633 | 718 | 815 | 864 | 967 | 1061 | 1085 | 1062 | 1133 | 1386 | 1417 | 1754 |
| 3 | Tak | **3.034** | **3.929** | - | 310 | 362 | 438 | 527 | 624 | 669 | 770 | 866 | 894 | 876 | 928 | 1224 | 1266 | 1591 |
| 4 | Suphanburi | **2.736** | **2.973** | **3.147** | - | 52 | 155 | 235 | 333 | 382 | 485 | 579 | 603 | 580 | 652 | 917 | 956 | 1285 |
| 5 | Nonthaburi | **2.871** | **2.819** | **3.037** | 2.020 | - | 121 | 192 | 287 | 337 | 440 | 533 | 556 | 532 | 609 | 866 | 904 | 1233 |
| 6 | Ratchaburi | **2.886** | **2.545** | **3.549** | **2.559** | 2.097 | - | 90 | 187 | 233 | 336 | 431 | 457 | 438 | 500 | 792 | 839 | 1157 |
| 7 | Petchaburi | **2.422** | **2.220** | 3.122 | 2.111 | 2.358 | 2.408 | - | 98 | 146 | 250 | 344 | 369 | 349 | 418 | 703 | 751 | 1067 |
| 8 | Prachupkririkran | **2.227** | **1.865** | **3.130** | **2.504** | 2.130 | 2.401 | 2.003 | - | 51 | 153 | 246 | 271 | 251 | 323 | 611 | 664 | 973 |
| 9 | Bangsaphan | **2.369** | **2.408** | **2.879** | **2.369** | 1.786 | 2.201 | 2.193 | 1.398 | - | 104 | 198 | 225 | 208 | 273 | 576 | 633 | 935 |
| 10 | Chumphon | **2.448** | **2.715** | **2.258** | 1.787 | 2.086 | 2.386 | **1.732** | 1.745 | 1.720 | - | 96 | 126 | 118 | 171 | 494 | 557 | 844 |
| 11 | Thachana | **2.307** | **2.636** | 2.971 | 2.278 | 2.175 | **2.678** | 1.964 | 2.145 | 2.011 | 2.152 | - | 35 | 53 | 99 | 410 | 478 | 752 |
| 12 | Suratthani | **2.654** | **3.259** | **2.929** | 2.281 | **2.415** | **2.590** | **3.206** | **2.491** | **2.308** | **2.030** | 2.106 | - | 36 | 106 | 374 | 443 | 719 |
| 13 | Samui Island | **1.864** | **3.343** | 2.243 | **2.492** | **2.557** | **2.641** | **2.597** | 2.503 | 4.668 | 1.837 | 2.192 | 2.091 | - | 142 | 375 | 439 | 728 |
| 14 | Phang- nga | **2.813** | **3.133** | **2.977** | **2.978** | **3.111** | **2.949** | 2.818 | **2.503** | 2.299 | **2.428** | **2.646** | 2.988 | 2.050 | - | 420 | 497 | 733 |
| 15 | Yala | **2.572** | **3.149** | 2.202 | **2.467** | **2.352** | **2.855** | 2.262 | **2.435** | 1.951 | 1.775 | 1.820 | 1.880 | 1.837 | 2.389 | - | 83 | 368 |
| 16 | Narathiwat | **2.544** | **3.190** | 2.588 | **2.592** | **2.752** | **2.818** | **2.457** | 2.319 | 2.161 | 1.797 | 2.275 | 2.120 | 1.788 | 2.437 | **1.925** | - | 344 |
| 17 | Selangor | **3.766** | **5.128** | **3.366** | **3.346** | **3.776** | **4.470** | **3.859** | **4.208** | **3.598** | **3.414** | **3.659** | **3.551** | **2.941** | **3.376** | **3.102** | **3.556** | - |

*Isolation by distance*

For wing shape, a significant, positive correlation was found between geographic distance and Mahalanobis distance for the 17 sites along the Thai-Malay transect ($R^2$ = 0.203, *P* < 0.05) (Figure 3.24).

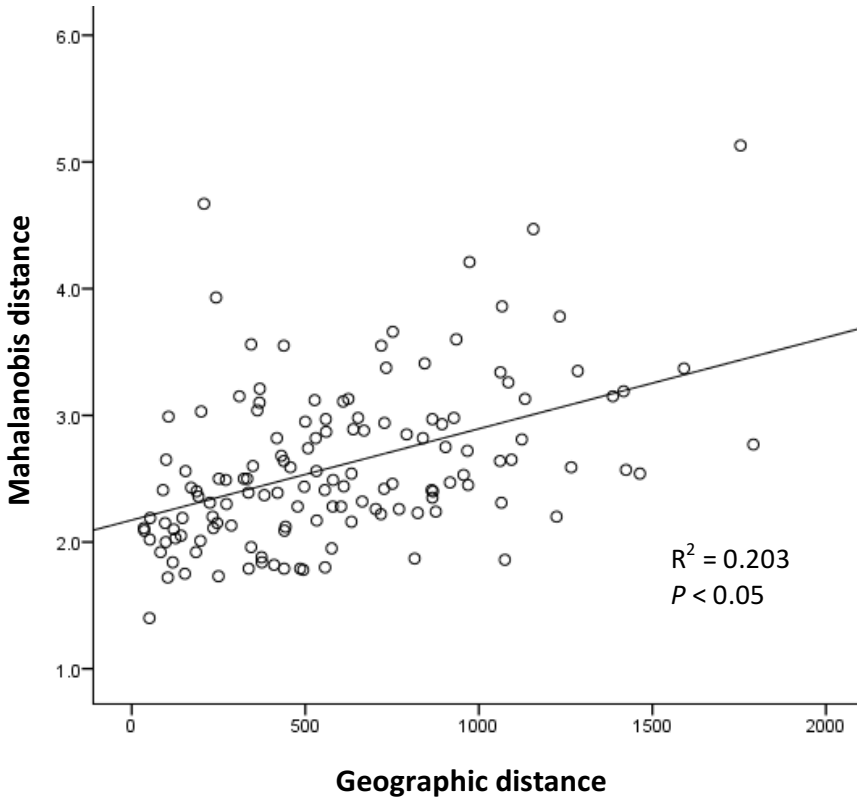



**Figure 3.24** Regression of Mahalanobis distances, calculated from wing shape analysis of *Zeugodacus cucurbitae* populations collected from 17 sites along the Thai-Malay transect, against geographic distance (km).

### 3.4.1.2 Aedeagus morphometric analysis

Aedeagus analysis was conducted on male genitalia from 340 specimens from 17 samples sites (20males/sites) along the Thai-Malay transect. There was a significant difference among populations for aedeagus length ($F_{16, 323}$ = 2.493; *P* < 0.05) (Figure 3.25), but no lattitudional correlation with aedeagus length ($R^2$ = 1.357E-4 *P* > 0.05) (Figure 3.26). When the samples were pooled among sites, there was no-significant difference in aedeagus length between the pooled populations

south of Isthmus of Kra and pooled populations north of Isthmus of Kra ($t_{1,338}$ = 1.041; $P > 0.05$).

There were significant differences in mean aedeagus length for populations north of the Isthmus of Kra ($F_{8, 171}$ = 4.014; $P < 0.05$), but no-significant differences between populations for flies south of the Isthmus of Kra ($F_{7, 152}$ = 1.413; $P > 0.05$). (Figure 3.25). For the northern populations, the mean aedeagal length of flies from Phetchaburi was significantly shorter than populations from Nonthaburi, Nan and Chiangmai, while no other populations differed from each other (Figure 3.25).

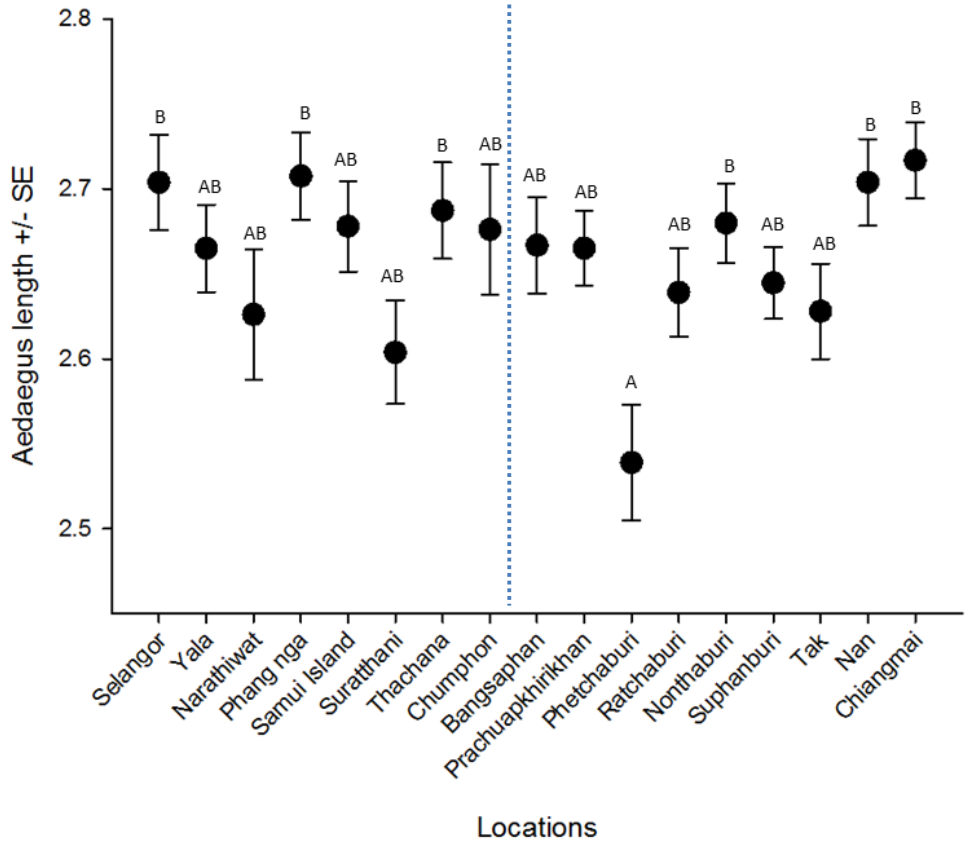



**Figure 3.25** Mean (± SE) aedeagus lengths of *Zeugodacus cucurbitae* populations from 17 sites along the Thai-Malay transect. Samples sharing the same letter are not statistically different from each other based on one-way ANOVA with Tukey *post hoc* test ($F_{16, 323}$ = 2.493; $P > 0.05$). The dotted blue line represents the Isthmus of Kra barrier that divides southern (left of line) and northern (right of line) Isthmus of Kra locations.

*Isolation by distance*

There was no significant relationship between aedeagus length and latitude ($R^2$= 1.357E-4, $P > 0.05$).

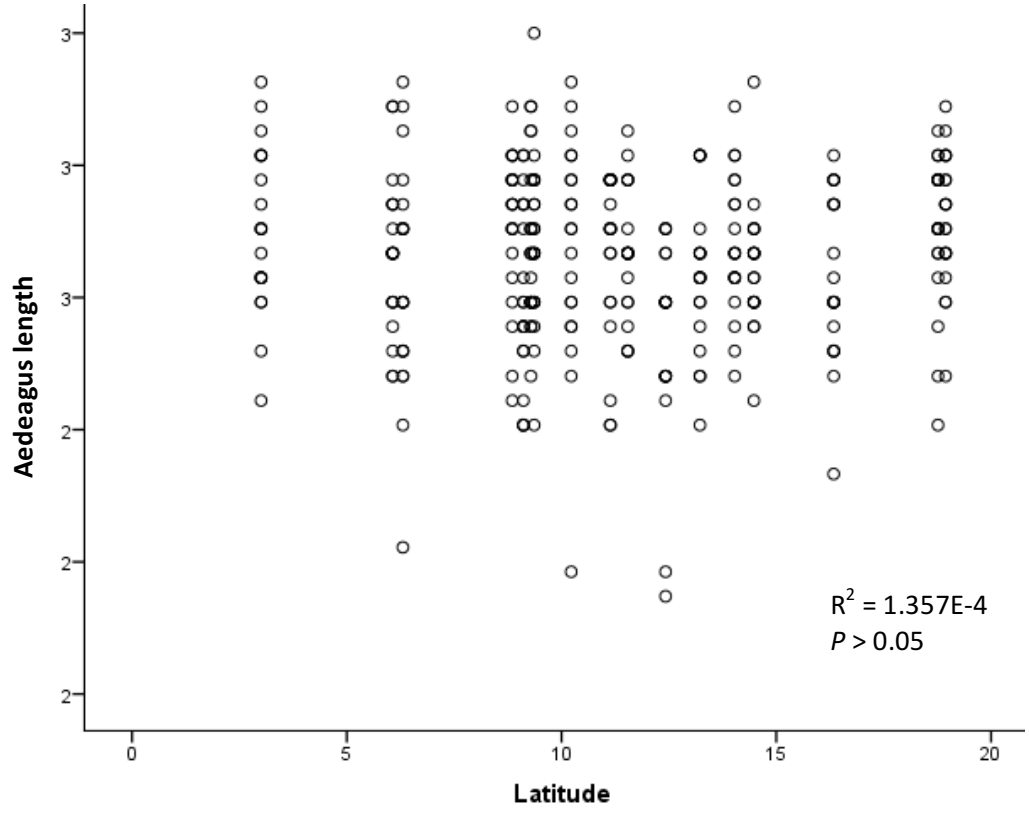



**Figure 3.26** Regression of aedeagus length (mm) of *Zeugodacus cucurbitae* from 17 sites along the Thai-Malay transect against latitude.

### 3.4.2 Molecular results

#### 3.4.2.1 Mitochondrial DNA (*Cox1*) analysis

*Gene diversity*

A total of 334 specimens of *Z. cucurbitae* was sequenced for *cox1* from 17 sites along the Thai-Malay transect. Thirty-seven unique haplotypes were identified in the span of 652 base pairs of the aligned sequences. Tachana (n=20) showed the greatest diversity with nine haplotypes whereas Selangor (n=18) showed the least diversity with two haplotypes.

Although *cox1* sequences varied throughout peninsular Thailand, there were no substantial, consistent differences between sites north and south of the Isthmus of Kra. The average number of unique haplotypes south of the Isthmus of Kra (4.87 ± 2.36 haplotypes) was almost identical for those sites North of the Isthmus (4.67 ± 1.87 haplotypes) (Table 3.18). Only 10 of 37 haplotypes were found in more than one site, with two of them (Haplotypes 1 and 2) found across most sites; 27 haplotypes were unique. Haplotype 1 (H1) was shared by all populations, while Haplotype 2 (H2) was shared by all populations except Selangor (Table 3.18).

The *cox1* median-joining (MJ) haplotype network revealed a central starburst-like pattern from the two common haplotypes (i.e., H1 and H2), with 35 singletons radiating from these haplotypes (Figure 3.27). There was no explicit spatial pattern between the haplotypes and their geographical distribution with regard to the Isthmus of Kra (Figure 3.28), and haplotypes from a given site were commonly distributed across the network.

**Table 3.18** Distribution of *Zeugodacus cucurbitae* mitochondrial *cox1* haplotypes from 17 sites along the Thai-Malay transect for sequence data new to this study. *Locations south of the Isthmus of Kra

| **Location** | Selangor* | Narathiwat* | Yala* | Phang-nga* | Samui Island* | Suratthani* | Tachana* | Chumphon* | Bangsaphan | Prachupkriri kran | Petchaburi | Ratchaburi | Nonthaaburi | Suphanburi | Tak | Nan | Chiangmai |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **No. samples** | 18 | 20 | 19 | 20 | 20 | 20 | 20 | 19 | 19 | 20 | 20 | 19 | 20 | 20 | 20 | 20 | 20 |
| Hap_1 | 17 | 16 | 14 | 13 | 16 | 12 | 10 | 15 | 13 | 11 | 13 | 9 | 9 | 13 | 13 | 16 | 7 |
| Hap_2 | - | 3 | 2 | 2 | 3 | 4 | 2 | 1 | 3 | 9 | 3 | 3 | 5 | 5 | 5 | 3 | 10 |
| Hap_3 | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - |
| Hap_4 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | - | - | - |
| Hap_5 | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - |
| Hap_6 | - | - | - | - | - | 1 | - | - | - | - | - | - | 1 | - | - | - | - |
| Hap_7 | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - |
| Hap_8 | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - |
| Hap_9 | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - |
| Hap_10 | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - |
| Hap_11 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - | 1 |
| Hap_12 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - | - |
| Hap_13 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - | - |
| Hap_14 | - | - | - | - | - | 1 | 2 | - | - | - | - | 3 | - | - | - | - | - |
| Hap_15 | - | - | - | 1 | - | - | 1 | - | - | - | 3 | - | - | - | - | - | - |
| Hap_16 | - | - | - | - | - | - | 2 | - | - | - | 1 | - | - | - | - | - | - |
| Hap_17 | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - |
| Hap_18 | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | 1 | - |
| Hap_19 | - | - | - | 1 | - | - | 1 | - | - | - | - | - | - | - | 1 | - | - |
| Hap_20 | - | 1 | - | - | 1 | 1 | - | - | - | - | - | - | - | - | - | - | - |
| Hap_21 | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| Hap_22 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_23 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_24 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_25 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| Hap_26 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hap_27 | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_28 | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_29 | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_30 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - |
| Hap_31 | - | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - |
| Hap_32 | - | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - |
| Hap_33 | - | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - |
| Hap_34 | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - |
| Hap_35 | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - |
| Hap_36 | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - |
| Hap_37 | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Total** | **2** | **3** | **5** | **7** | **3** | **6** | **9** | **5** | **5** | **2** | **4** | **7** | **8** | **4** | **4** | **3** | **5** |

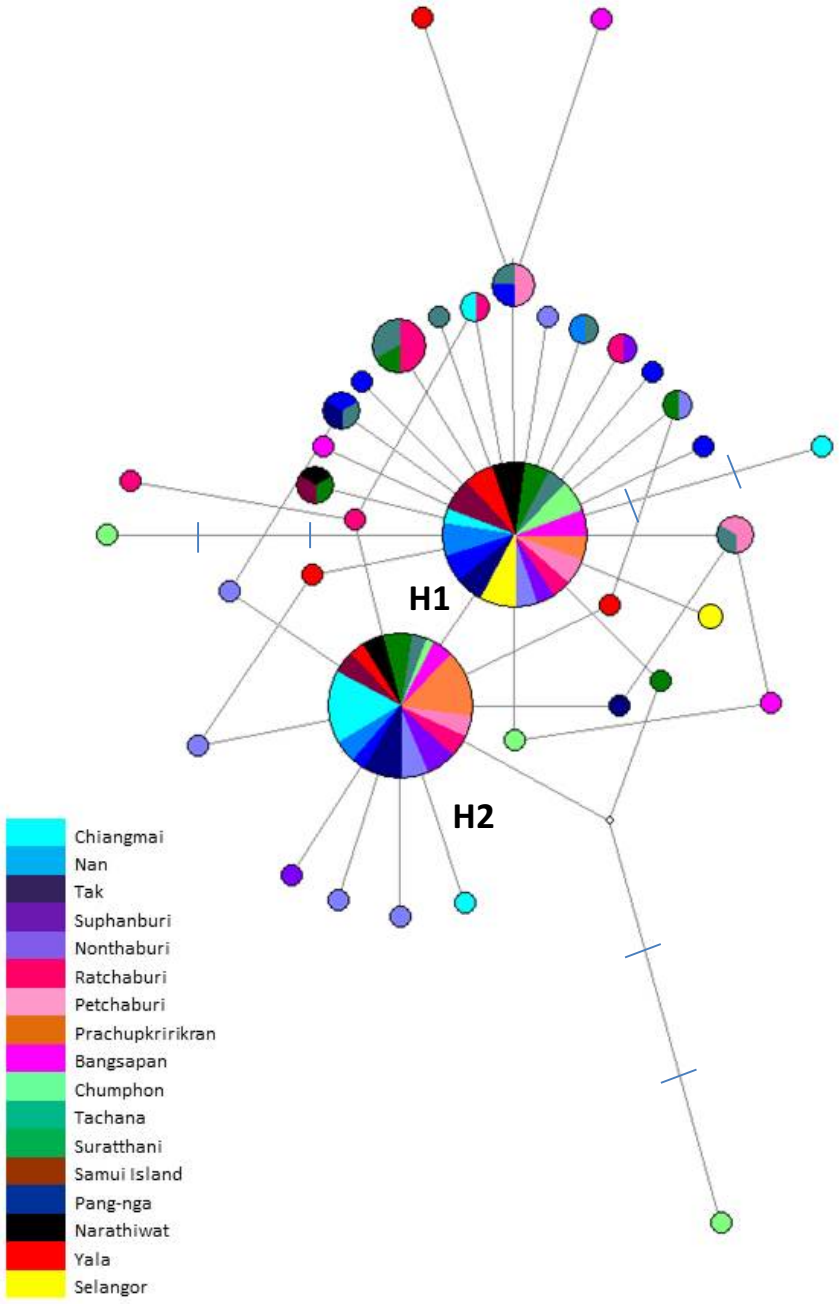



**Figure 3.27** Median-joining haplotype network generated from *Zeugodacus cucurbitae cox1* data collected from 17 sites along the Thai-Malay transect; 37 haplotypes are represented. Small white circle represents a median vector. Different colours represent different collecting locations. Sizes of nodes and pie segments are proportional to haplotype frequency. Length of branches is proportional to number of mutational changes between haplotypes.

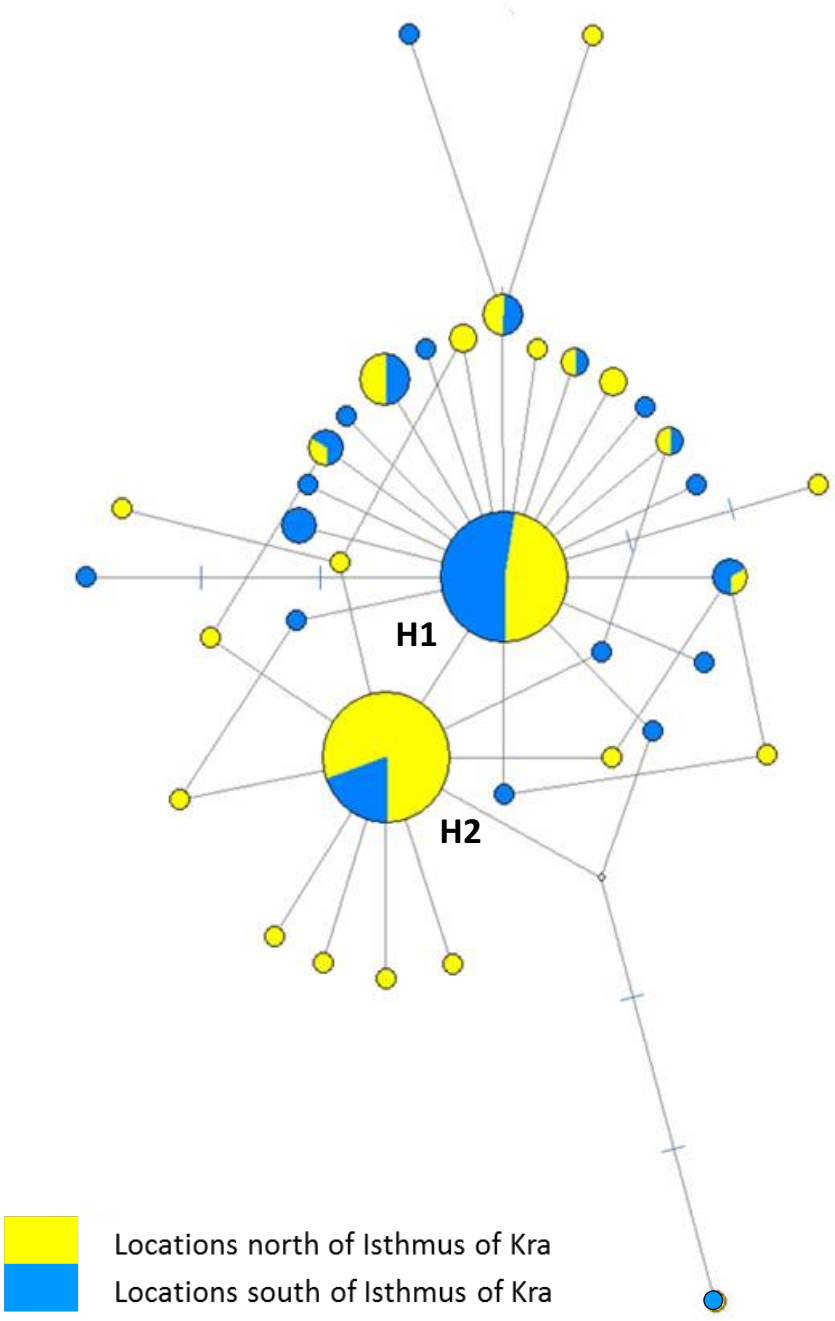



**Figure 3.28** Median-joining haplotype network generated from *Zeugodacus cucurbitae cox1* data collected from 17 sites along the Thai-Malay transect; 37 haplotypes are represented. Small white circle represents a median vector. Yellow represent individuals collected from north of the Isthmus of Kra, blue collected from south of the Isthmus. Sizes of nodes and pie segments are proportional to haplotype frequency. Length of branches is proportional to number of mutational changes between haplotypes.

Average genetic variability at sites north of the Isthmus of Kra was higher than at sites south of the Isthmus of Kra (0.58 ± 1.27 and 0.45 ± 0.20, respectively) (Table 3.19). Population genetic diversity ranged from 0.11 ± 0.094 (Selangor) to 0.75 ± 0.09 (Ratchaburi) and the genetic diversity parameter θπ ranged from 0.11 (Selangor) to 1.18 (Ratchaburi). The mean gene diversity of populations north of Isthmus of Kra (0.58 ± 1.27) was greater than populations south of the Isthmus (0.45 ± 0.20). Tajima's *D* tests of neutrality applied to the entire genetic data set were negative and statistically significant (D = -2.32, $P < 0.0001$) (Table 3.19).

*Genetic differentiation (cox1)*

Population pairwise $F_{ST}$ analysis revealed that 94% of pairwise population comparisons were not significantly different genetically (Table 3.20). Hierarchical analysis of molecular variance was conducted to test groupings of populations according to their site in relation to the Isthmus of Kra (based on *cox1* data). There was significant variation (4.25%, $F_{CT} = 0.042$, $P < 0.05$) between populations north and south of the Isthmus of Kra (Table 3.21). Only 2.14% of the variation was observed among populations within groups, whilst the remaining (93.60%) was found within populations.

*Isolation by distance*

The correlation between the genetic parameter $\Phi_{ST}$ and geographic distance (km) was significant ($R^2 = 0.140$, $P < 0.05$), indicating the presence of an isolation by distance effect (Figure 3.29).

**Table 3.19** Population genetics: summary statistics from *cox1* analysis of *Zeugodacus cucurbitae* from 17 sites along Thai-Malay transect. *Denotes site north of Isthmus of Kra, Thailand.

| Sample site | No of individuals | Gene Diversity | Θ¶ | Tajima's *D* | Tajima's *D P*-value | Fu'*Fs* | Fu'*Fs P*-value |
|---|---|---|---|---|---|---|---|
| Chiangmai* | 20 | 0.653 ± 0.076 | 0.921 | -1.165 | 0.156 | -1.326 | 0.106 |
| Nan* | 20 | 0.353 ± 0.123 | 0.368 | -1.545 | 0.227 | -0.775 | 0.189 |
| Tak* | 20 | 0.537 ± 0.104 | 0.642 | -1.532 | 0.297 | -1.006 | 0.127 |
| Suphanburi* | 20 | 0.537 ± 0.104 | 0.642 | -0.778 | 0.286 | -1.006 | 0.132 |
| Nonthaaburi* | 20 | 0.758 ± 0.081 | 1.121 | -0.763 | 0.085 | -4.504 | 0.003 |
| Ratchaburi* | 19 | 0.754 ± 0.088 | 1.181 | -1.165 | 0.353 | -3.050 | 0.010 |
| Petchaburi* | 20 | 0.558 ± 0.113 | 0.637 | -1.380 | 0.272 | -1.024 | 0.120 |
| Prachupkririkran* | 20 | 0.521 ± 0.042 | 0.521 | -1.723 | 0.967 | 1.467 | 0.721 |
| Bangsaphan* | 19 | 0.526 ± 0.126 | 0.807 | 0.000 | 0.030 | -1.739 | 0.055 |
| Chumphon | 19 | 0.386 ± 0.139 | 0.830 | -1.513 | 0.015 | -1.664 | 0.064 |
| Tachana | 20 | 0.747 ± 0.097 | 0.968 | -1.164 | 0.030 | -5.175 | 0.000 |
| Suratthani | 20 | 0.621 ± 0.109 | 0.737 | -1.704 | 0.060 | -3.242 | 0.002 |
| Samui Island | 20 | 0.353 ± 0.123 | 0.368 | 0.000 | 0.220 | -0.775 | 0.189 |
| Phang-nga | 20 | 0.584 ± 0.127 | 0.689 | -0.755 | 0.009 | -5.048 | 0.000 |
| Yala | 19 | 0.462 ± 0.136 | 0.702 | -1.704 | 0.041 | -2.111 | 0.014 |
| Narathiwat | 20 | 0.323 ± 0.123 | 0.368 | -1.704 | 0.232 | -0.775 | 0.205 |
| Selangor | 18 | 0.111 ± 0.094 | 0.111 | 0.000 | 0.146 | -0.794 | 0.098 |

**Table 3.20** Pairwise $F_{ST}$ distances between populations of *Zeugodacus cucurbitae* collected from 17 sites along the Thai-Malay transect. Values in bold are significant after Bonferroni correction for multiple tests, with the initial $P = 0.05$ and the corrected $P < 0.001$.

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Chiangmai | - | | | | | | | | | | | | | | | |
| 2 | Nan | 0.179 | - | | | | | | | | | | | | | | |
| 3 | Tak | 0.047 | 0.009 | - | | | | | | | | | | | | | |
| 4 | Suphanburi | 0.047 | 0.009 | -0.036 | - | | | | | | | | | | | | |
| 5 | Nonthaburi | -0.016 | 0.086 | -0.008 | -0.002 | - | | | | | | | | | | | |
| 6 | Ratchaburi | 0.067 | 0.039 | 0.016 | 0.011 | 0.039 | - | | | | | | | | | | |
| 7 | Petchaburi | 0.167 | 0.005 | 0.024 | 0.031 | 0.089 | 0.049 | - | | | | | | | | | |
| 8 | Prachupkririkran | -0.023 | 0.136 | -0.003 | -0.003 | -0.033 | 0.052 | **0.130** | - | | | | | | | | |
| 9 | Bangsaphan | 0.134 | -0.024 | -0.004 | 0.003 | 0.063 | 0.027 | -0.027 | 0.090 | - | | | | | | | |
| 10 | Chumphon | **0.169** | -0.016 | 0.029 | 0.029 | 0.093 | 0.042 | 0.008 | 0.130 | -0.019 | - | | | | | | |
| 11 | Thachana | **0.172** | -0.005 | 0.024 | 0.042 | 0.096 | 0.023 | -0.010 | **0.134** | -0.016 | 0.001 | - | | | | | |
| 12 | Suratthani | 0.109 | -0.024 | -0.014 | -0.014 | 0.038 | 0.005 | 0.005 | 0.062 | -0.018 | -0.013 | -0.003 | - | | | | |
| 13 | Samui Island | 0.179 | -0.038 | 0.010 | 0.009 | 0.086 | 0.039 | 0.005 | 0.137 | -0.024 | -0.016 | 0.002 | -0.033 | - | | | |
| 14 | Phang- nga | 0.187 | -0.017 | 0.028 | 0.035 | 0.100 | 0.048 | -0.012 | 0.148 | -0.019 | -0.013 | -0.011 | -0.005 | -0.017 | - | | |
| 15 | Yala | 0.141 | -0.026 | 0.003 | 0.003 | 0.057 | 0.029 | -0.021 | 0.097 | -0.027 | -0.012 | -0.004 | -0.027 | -0.026 | -0.020 | - | |
| 16 | Narathiwat | 0.179 | -0.038 | 0.010 | 0.009 | 0.086 | 0.039 | 0.005 | 0.137 | -0.024 | -0.016 | 0.002 | -0.033 | -0.053 | -0.017 | -0.026 | - |
| 17 | Selangor | **0.348** | 0.058 | **0.166** | 0.164 | **0.224** | 0.120 | 0.072 | **0.365** | 0.034 | 0.010 | 0.023 | 0.063 | 0.058 | 0.008 | 0.037 | 0.058 |

**Table 3.21** Results of the Analysis of Molecular Variance for the *cox1* dataset generated for *Zeugodacus cucurbitae* population sampled along the Thai-Malay transect. Samples were devided according to geographical location in relation to biogeographical barriers at the Isthmus of Kra. For geographical locations of 17 sites along Thai-Malay transect and potential Thai biogeographical refer to Figures 3.3 and Table 3.1.

| Source of variation | d.f. | Sum of square | Variance of components | % total variance | Fixation indices |
|---|---|---|---|---|---|
| Among groups | 1 | 3.102 | 0.01565 a | 4.25 | $F_{CT}$ = 0.0425* |
| Among populations within groups | 15 | 7.489 | 0.00788 b | 2.14 | $F_{ST}$ = 0.0639* |
| Within populations | 317 | 109.191 | 0.34445 c | 93.60 | $F_{SC}$ = 0.0224* |
| Total | 333 | 103.337 | 0.40548 | | |

* $P$ < 0.05

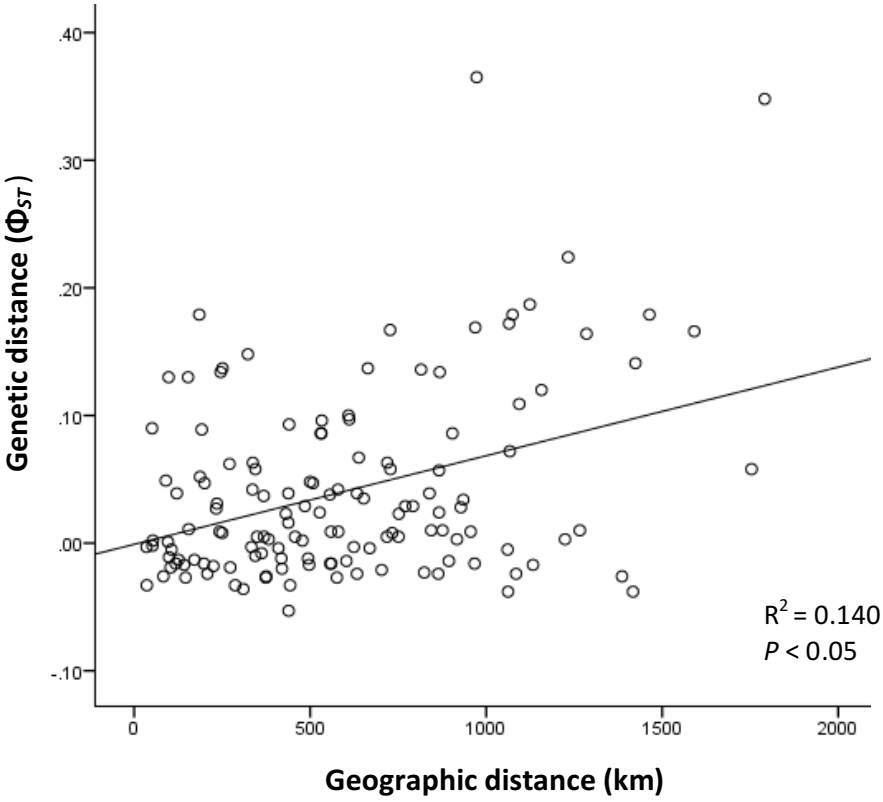



**Figure 3.29** Regression of *Zeugodacus cucurbitae* pairwise genetic distances $\Phi_{ST}$ against geographic distance (km) along 17 sites along Thai-Malay transect.

### 3.4.2.2 Microsatellite analysis

*Gene diversity*

Microsatellite diversity, Hardy-Weinberg and genotypic linkage equilibrium microsatellite markers (11 loci) were used to assess contemporary population structure in *Z. cucurbitae*, with a total of 336 individuals screened from 17 sites. Approximately 1% of the data were missing from the final data set, as a result of four PCR reactions that did not successfully amplify. The number of alleles per locus varied from 2.82 (Selangor) to 4.09 (Ratchaburi and Prachupkririkran), while the allelic richness ranged from 2.08 (Selangor) to 2.74 (Ratchaburi). The average number of alleles (3.79 ± 0.26) and allelic richness (2.63± 0.094) from populations north of the Isthmus of Kra is marginally higher than those to the south (3.59 ± 0.36 and 2.47 ± 0.18, respectively) (Table 3.22). Moderate to high levels of intra-population diversity were reflected in the observed heterozygosity, which varied from 0.28 (Selangor) to 0.46 (Prachupkririkran). Average gene diversity over all loci per site ranged from 0.41 ± 0.25 (Narathiwat) to 0.65 ± 0.39 (Tak) (Table 3.22).

**Table 3.22** Locations; sample sizes, microsatellite diversity estimates test values for *Zeugodacus cucurbitae* collected from 17 sites along a Thai-Malay transect.

| | Locations | No of individuals | Expected heterozygosity | Observed heterozygosity | No Alleles | Allelic Richness | Fis | H-W | Gene Diversity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Chiangmai | 20 | 0.4344 | 0.3971 | 3.545 | 2.506 | 0.088 | 0.0291 | 0.473 ± 0.262 |
| 2 | Nan | 20 | 0.4627 | 0.4096 | 3.636 | 2.571 | 0.118 | 0.0182 | 0.559 ± 0.308 |
| 3 | Tak | 19 | 0.4857 | 0.4296 | 3.818 | 2.679 | 0.119 | 0.0021 | 0.645 ± 0.393 |
| 4 | Suphanburi | 20 | 0.4708 | 0.4192 | 3.818 | 2.602 | 0.113 | 0.0651 | 0.536 ± 0.297 |
| 5 | Nonthaaburi | 20 | 0.4992 | 0.4093 | 3.909 | 2.710 | 0.184 | < 0.0001 | 0.486 ± 0.268 |
| 6 | Ratchaburi | 20 | 0.5139 | 0.3831 | 4.091 | 2.732 | 0.261 | < 0.0001 | 0.567 ± 0.312 |
| 7 | Petchaburi | 20 | 0.4578 | 0.4112 | 3.273 | 2.494 | 0.106 | 0.0292 | 0.476 ± 0.264 |
| 8 | Prachupkririkran | 20 | 0.5086 | 0.4567 | 4.091 | 2.744 | 0.105 | 0.0674 | 0.537 ± 0.293 |
| 9 | Bangsaphan | 20 | 0.4563 | 0.4094 | 3.909 | 2.601 | 0.107 | < 0.0001 | 0.483 ± 0.271 |
| 10 | Chumphon | 18 | 0.5018 | 0.4308 | 3.727 | 2.669 | 0.146 | < 0.0001 | 0.570 ± 0.319 |
| 11 | Tachana | 20 | 0.4787 | 0.4185 | 3.909 | 2.591 | 0.130 | < 0.0001 | 0.462 ± 0.257 |
| 12 | Suratthani | 20 | 0.4503 | 0.3684 | 3.909 | 2.550 | 0.187 | 0.0219 | 0.456 ± 0.275 |
| 13 | Samui Island | 20 | 0.4532 | 0.3260 | 3.636 | 2.518 | 0.287 | < 0.0002 | 0.437 ± 0.248 |
| 14 | Phang-nga | 20 | 0.4613 | 0.3830 | 3.818 | 2.512 | 0.174 | 0.0087 | 0.472 ± 0.262 |
| 15 | Yala | 20 | 0.4191 | 0.3732 | 3.364 | 2.335 | 0.112 | 0.5462 | 0.417 ± 0.232 |
| 16 | Narathiwat | 20 | 0.4505 | 0.3347 | 3.545 | 2.524 | 0.266 | 0.0265 | 0.411 ± 0.245 |
| 17 | Selangor | 19 | 0.3551 | 0.2834 | 2.818 | 2.081 | 0.207 | < 0.0001 | 0.413 ± 0.241 |

*Genetic differentiation and relationships among populations*

Population pairwise $R_{ST}$ analysis revealed that 78% of pairwise populations were not significantly different genetically (Table 3.23). However, Selangor and Yala were significantly different from all other sampled sites. AMOVA was conducted with populations separated into two groups (north and south of the Isthmus of Kra). The results indicated that 1.57% of the genetic variation was partitioned among the two groups, 2.64% among sampling sites within groups, and 95.79% within sampling sites. This was significant at all hierarchical levels and supports the hypothesis that there are differences between populations north and south of the Isthmus of Kra (Table 3.24).

*Isolation by distance*

Test of isolation by distance across the 17 sites identified significant correlation between geographic and genetic distances ($R_{ST}$) ($R^2$= 0.140, $P < 0.05$) (Figure 3.30).

**Table 3.23** Pairwise $R_{ST}$ distances between 17 sample sites of *Zeugodacus cucurbitae* collected from 17 sites along the Thai-Malay transect. Values in bold are significant after Bonferroni correction for multiple tests, with the initial $P = 0.05$ and the corrected $P < 0.001$.

| | | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Chiangmai | - | | | | | | | | | | | | | | | |
| 2 | Nan | 0.004 | - | | | | | | | | | | | | | | |
| 3 | Tak | -0.006 | 0.073 | - | | | | | | | | | | | | | |
| 4 | Suphanburi | -0.021 | -0.003 | 0.015 | - | | | | | | | | | | | | |
| 5 | Nonthaburi | 0.013 | 0.038 | 0.062 | 0.001 | - | | | | | | | | | | | |
| 6 | Ratchaburi | 0.005 | 0.02 | 0.057 | -0.008 | -0.020 | - | | | | | | | | | | |
| 7 | Petchaburi | 0.013 | 0.015 | 0.068 | -0.004 | -0.019 | -0.022 | - | | | | | | | | | |
| 8 | rachupkririkran | -0.007 | 0.026 | 0.009 | -0.01 | -0.012 | -0.013 | -0.006 | - | | | | | | | | |
| 9 | Bangsaphan | -0.009 | 0.047 | -0.013 | 0.001 | 0.037 | 0.033 | 0.038 | -0.005 | - | | | | | | | |
| 10 | Chumphon | 0.009 | 0.056 | 0.028 | 0.001 | -0.018 | -0.012 | -0.005 | -0.018 | 0.01 | - | | | | | | |
| 11 | Thachana | -0.013 | 0.042 | -0.011 | -0.01 | 0.015 | 0.014 | 0.023 | -0.007 | -0.014 | -0.006 | - | | | | | |
| 12 | Suratthani | -0.008 | -0.004 | 0.047 | -0.013 | 0.012 | 0.002 | -0.008 | -0.009 | 0.004 | 0.009 | 0.011 | - | | | | |
| 13 | Samui Island | -0.015 | 0.024 | 0.018 | -0.021 | -0.012 | -0.016 | -0.007 | -0.025 | 0.01 | -0.021 | -0.016 | 0.009 | - | | | |
| 14 | Phang- nga | -0.004 | 0.039 | -0.009 | 0.006 | 0.065 | 0.054 | 0.061 | 0.019 | -0.007 | 0.042 | -0.002 | 0.024 | 0.021 | - | | |
| 15 | Yala | **0.068** | **0.173** | 0.056 | **0.107** | **0.163** | **0.16** | **0.181** | **0.094** | **0.109** | **0.135** | **0.074** | **0.196** | **0.097** | **0.080** | - | |
| 16 | Narathiwat | 0.008 | 0.072 | 0.01 | 0.007 | 0.005 | 0.01 | 0.02 | -0.01 | 0.004 | -0.019 | -0.014 | 0.028 | -0.015 | 0.026 | 0.105 | - |
| 17 | Selangor | **0.061** | **0.127** | **0.088** | **0.091** | **0.145** | **0.134** | **0.154** | **0.091** | **0.132** | **0.134** | **0.091** | **0.182** | **0.085** | **0.087** | **0.02** | **0.11** |

**Table 3.24** Results of the Analysis of Molecular Variance (AMOVA) for the microsatellite dataset generated for *Zeugodacus cucurbitae* populations sampled along the Thai-Malay transect. Samples were constrained according to geographical location in relation to the biogeographical barrier of the Isthmus of Kra. For geographical location of collection sites refer to Figures 3.3 and Table 3.1.

| Source of variation | Sum of square | Variance of components | % total variance | Fixation indices |
|---|---|---|---|---|
| Among groups | 3744.509 | 10.17428 | 1.57003 | $F_{CT}$ = 0.01570* |
| Among populations within groups | 18403.694 | 17.08361 | 2.63623 | $F_{ST}$ = 0.04206* |
| Within populations | 372077.908 | 620.77384 | 95.79374 | $F_{SC}$ = 0.02678* |
| Total | 394226.111 | 648.03173 | | |

* $P < 0.05$

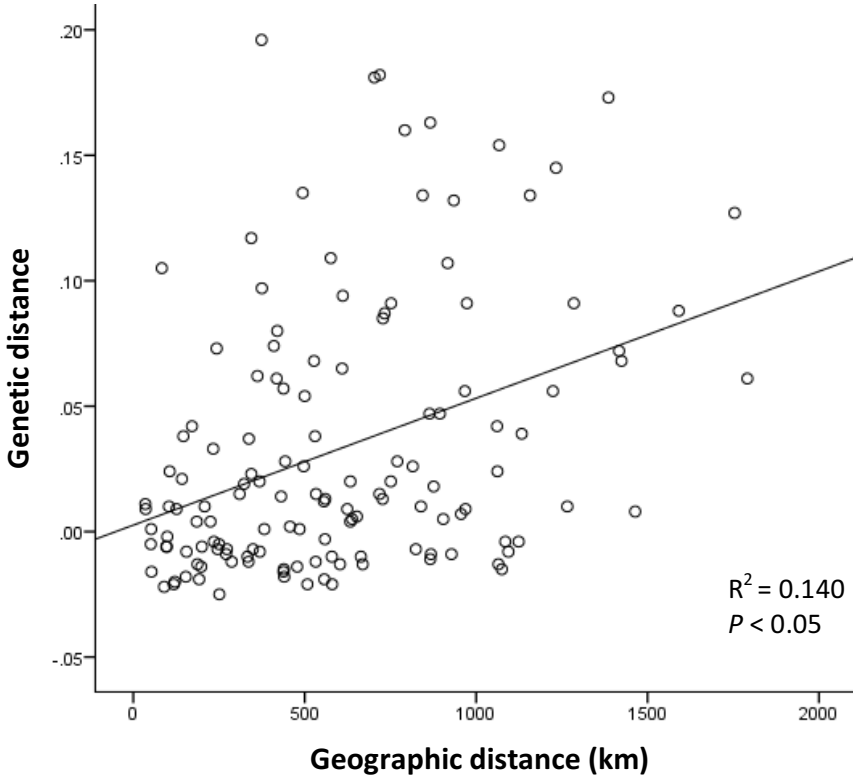
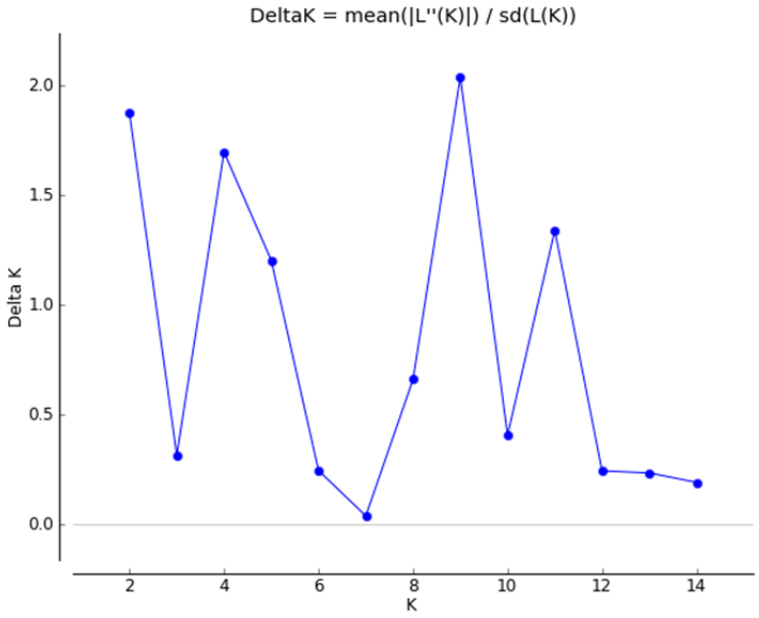



**Figure 3.30** Regression of pairwise genetic distances ($R_{ST}$) against geographic distance (km) calculated for *Zeugodacus cucurbitae* collected 17 sites along Thai-Malay transect.

*Population structure using Bayesian clustering*

The investigation of population differentiation using Bayesian clustering of the microsatellite data suggested that there were *K* = 9 groups within the sampled range according to the method of Evanno et al. (2005) (Figure 3.31). Evanno's method for estimating the optimal number of clusters (*K*) cannot calculate a Δ*K* value at *K* = 1, as it uses the second order rate of change. Examination of the log of the posterior probability of the data [ln P(D)] for each *K* value revealed the highest ln P(D) value at *K* = 1, an indication of the lack of population differentiation. Moreover, when the assignment of individuals based on *K*= 2 and *K* = 3 is summarised graphically (Figure 3.32), it can be seen clearly that the results from the Evanno method are misleading. In reality, only a single cluster dominated the ancestry of all individuals from the transect, which supports a lack of significant population differentiation. There is, however, some evidence for some population (Yala and Selangor) have slightly different patterns of admixture to the remaining sites (Figure 3.32).

(A)




(B)

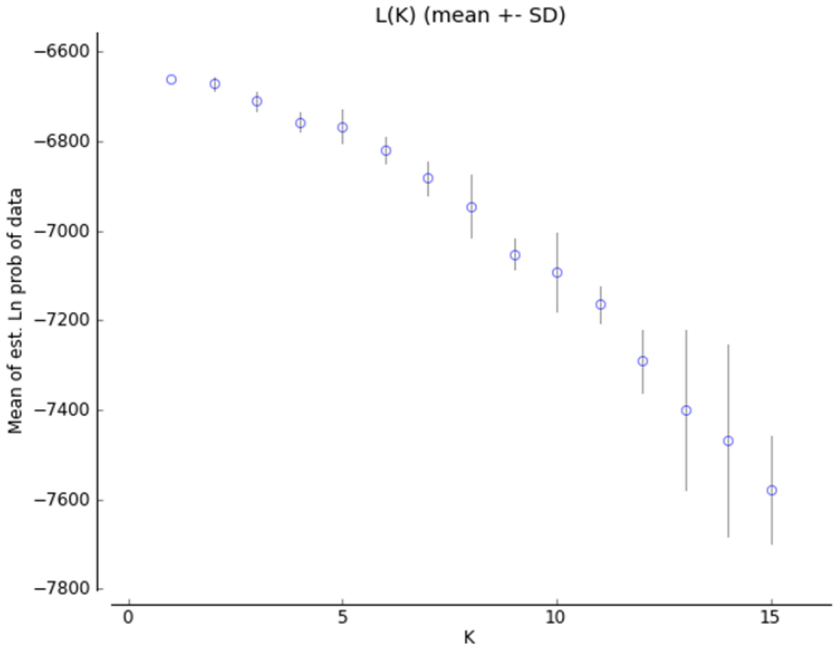



**Figure 3.31** Bayesian clustering results of *Zeugodacus curcurbitate* microsatellite data for 17 populations collected along the Thai-Malay transect; (A) plot of Δ*K* with the median value being that which is most highly supported as the optimum value of *K* for the analysed sample; (B) Mean of log probability of data (LnP (D)).

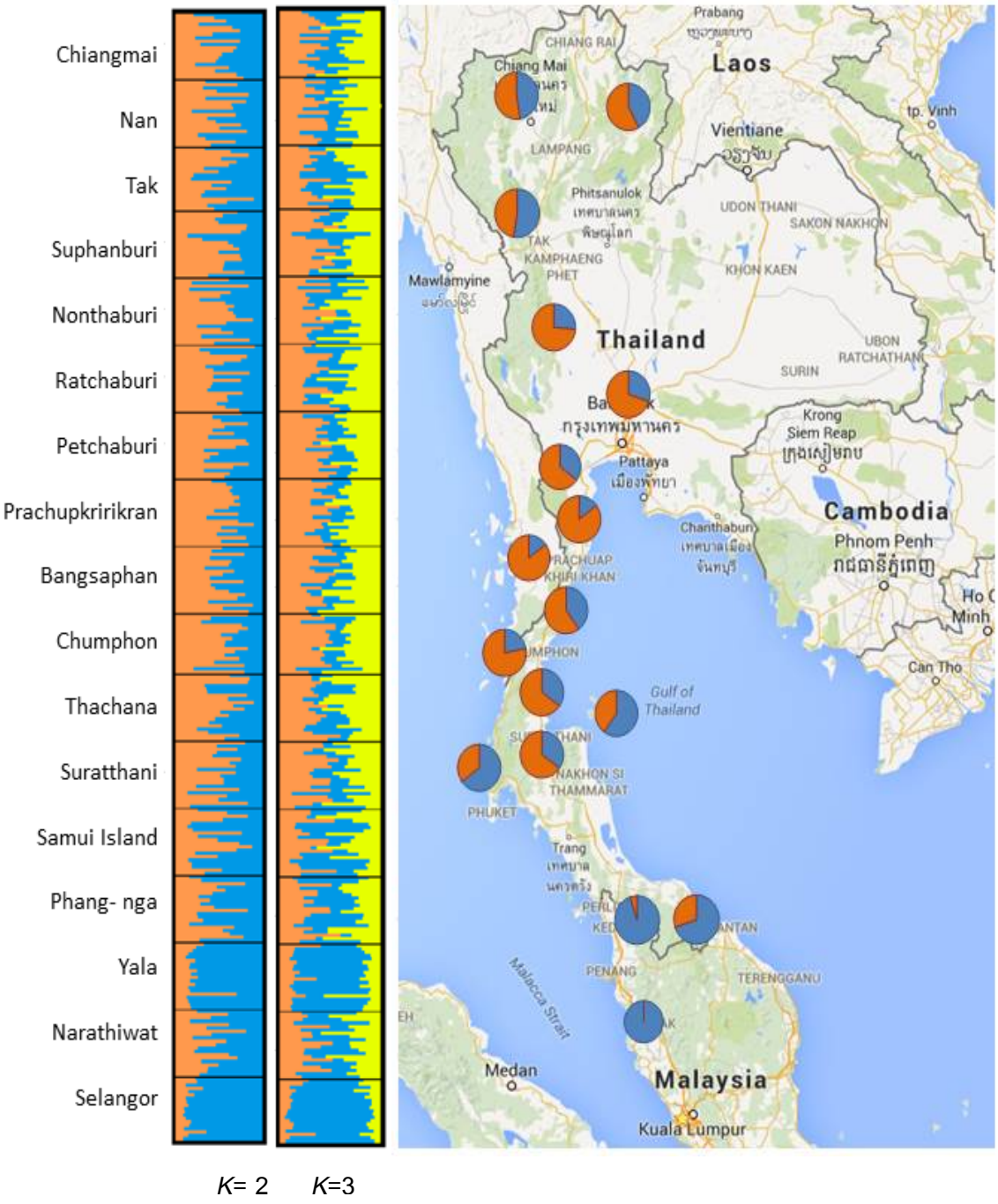



**Figure 3.32** Structure bar plot of Bayesian cluster analysis based on 11 microsatellite loci obtained from *Zeugodacus cucurbitae* collected from 17 sites along the Thai-Malay transect. The first two plots show population assignment results for values of *K* = 2 and *K* = 3. Each horizontal line represents a single individual and its probability of assignment to a particular cluster is given as a unique colour. Solid black lines separate individuals from each of the 17 sites. Pie charts represent assignment probability of individauls belonging to each of *K* = 2 clusters identified by structure based on microsatellite allele frequencies, with probability values normalized using CLUMPP.

The neighbour-joining tree of populations similarly showed no population structuring, with the exception of Selangor and Yala clustering separately from all other sites. No structure was evident between populations north or south of the Isthmus of Kra (Figure 3.33).

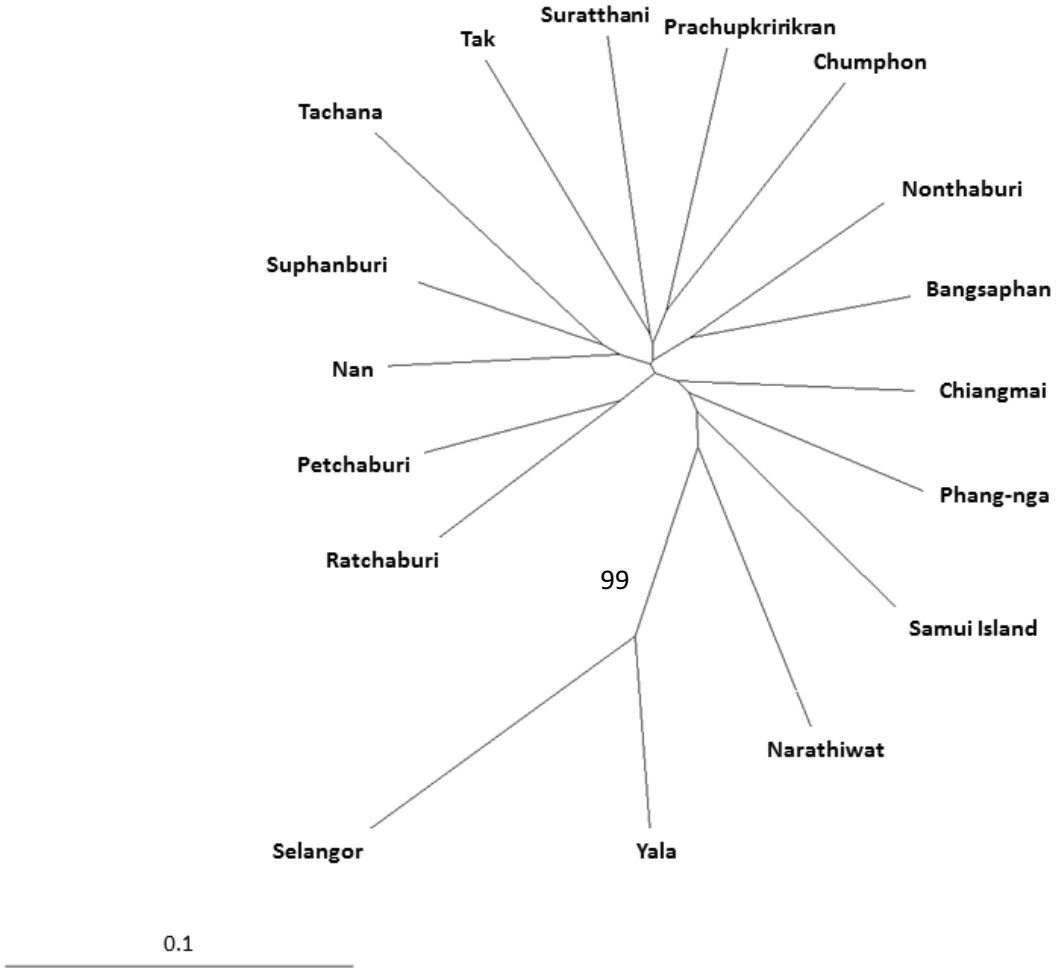



**Figure 3.33** Neighbour-joining unrooted tree of population relationships from the microsatellite dataset of *Zeugodacus cucurbitae* from 17 sites along the Thai-Malay transect based on Carvalli-Sforza & Edwards (1967) distances matrix. Bootstrap values were calculated using 1000 replications and are given as percentage, with only values greater than 50% shown.

Principal components analysis (PCA) of 11 loci reflected patterns observed in the Bayesian clustering and the neighbour-joining tree. The first two axes in the microsatellite PCA explained 52.42% of the variation in the data. The first principal component (PC) axis explained over 40.52% of the variation in the data and separated Yala and Selangor from all other populations. A secondary and more subtle pattern of structure among the remaining populations was explained by the second PC axis (11.90%), which separated several sites (Figure 3.34).

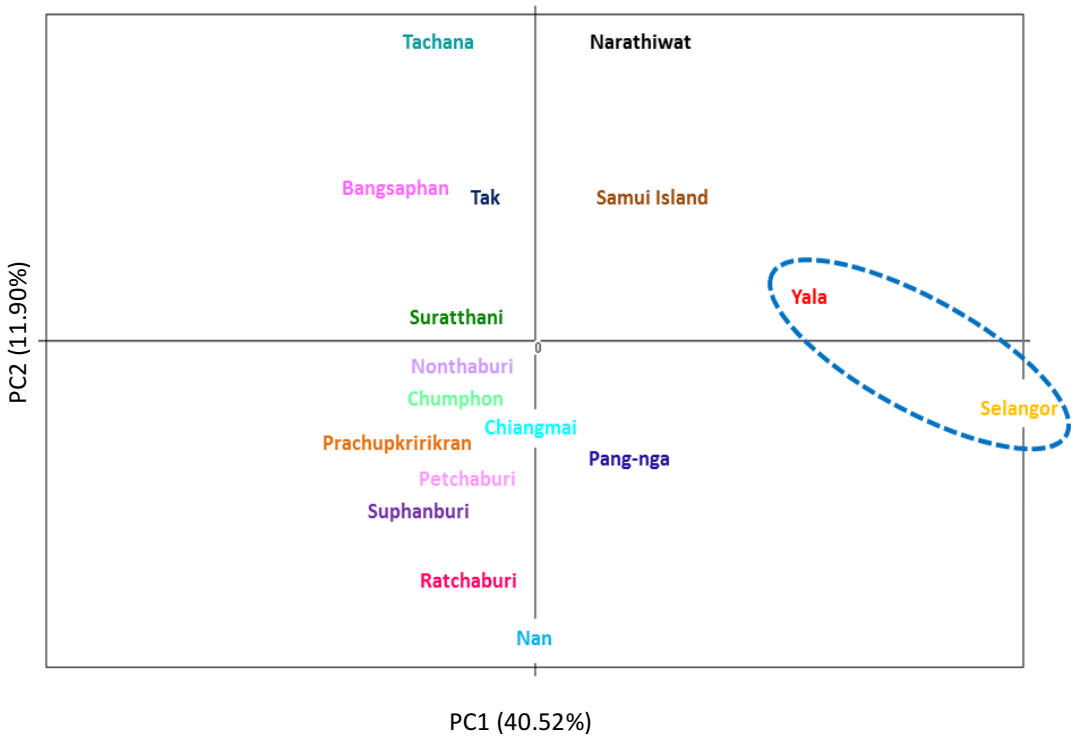



**Figure 3.34** Principal components analysis (PCA) plot based on 11 microsatellites loci of *Zeugodacus cucurbitae* collected from 17 sites along the Thai-Malay transect. The populations from Yala and Selangor which segregate from the rest of the populations are indicated by a dashed, blue line; these two populations also clustered independently using a Neighbour-joining unrooted tree.

## 3.5 DISCUSSION

Analysis of morphological and genetic variation in *Z. cucurbitae* in Thailand reveals that, with only some exceptions, there are minimal differences among populations from the majority of Thai biogeographical regions. The largest wing and aedeagal sizes were recorded from flies from the North and, overall, wing size and aedeagal length were significantly greater in northern populations than in populations from other sites. Microsatellite sequences suggest the distinctiveness of northeastern populations of *Z. cucurbitae*, while *cox1* and microsatellite data indicate differences between flies on the large islands of Samui and Chang and the adjacent mainland; these differences were not evident from morphometrics. Finally, a combination of morphometric and molecular data provides no support for the hypothesis that the Isthmus of Kra represents a biogeographic barrier for *Z. cucurbitae* populations. However, these data do suggest a biogeographic disjunction further to the south.

### 3.5.1 Biogeography of *Z. cucurbitae* across Thailand

Significant population structure in otherwise highly dispersive taxa often indicates the existence of concealed reproductive isolation (Knowlton, 2000) or may result from largely dynamic variation in the reproductive success of some individuals at particular sites (Schlosser & Angermeier, 1995). Similarly, evaluating associations between geographical distributions and genetic variation patterns can provide insights about how past and present-day patterns of gene flow have influenced genetic structure at various geographical scales and how this may have contributed to the persistence of extant populations (Avise et al., 1987). Contrary to patterns of obvious population structuring, population pairwise $F_{ST}$ and $R_{ST}$ values indicate an overall low level of genetic structuring among populations of *Z. cucurbitae* in Thailand. The results are consistent with many other population genetic studies on fruit flies in other parts of the world, which generally show low levels of population structuring in Southeast-Asia (Wu et al., 2009; Virgilio et al., 2010; Prabhakar et al., 2012), including a study which detected low levels of genetic structuring of melon fly in China (Hu et al., 2008).

The factor that may contribute substantially to the low level of genetic structuring in *Z. cucurbitae* in Thailand is its recent population history. Fu's *F*s and Tajima's *D* tests indicate a recent demographic expansion of *Z. cucurbitae* in Thailand, rather than their having been a genetic bottleneck in this species. Climatic conditions in tropical Asia, including Thailand, during the Pleistocene glaciations were thought to have become warm and humid about 18000 years ago, and this allowed the tropical forests to expand (Penny, 2001). The expansion in *Z. cucurbitae* is most likely associated with an expansion of host-plant distribution. The postulated historical population expansion in *Z. cucurbitae* after the return to warm and humid climatic conditions is consistent with the present-day seasonal abundance of this species, in which *Z. cucurbitae* becomes much more abundant in the monsoonal wet season of Southeast-Asia (Dhillon et al., 2015). The exception to the overall genetic homogeneity of *Z. cucurbitae* in Thailand is the significant differentiation in the microsatellites of the Northeastern population, and the less marked genetic differences between mainland flies and flies on the large islands of Samui and Chang.

### 3.5.2 Do island populations have less variation than mainland population?

Morphological and genetic variation between melon fly from the mainland regions of Thailand and the two large islands of Samui and Chang was not significant. The wing shape and aedeagal length of melon fly from Samui Island were similar to the wing shape and aedeagus length of flies from the nearby Lower and Upper South regions. Flies from the other large island, Chang Island, were similar in aedaegus length to flies from the northeastern region, which is the nearest mainland region. However, the island populations have lower levels of genetic variation than adjacent mainland populations (Samui Island compared with Upper South population, Chang Island and the Northeast population). Molecular results from *cox1* and microsatellite revealed that gene diversity of flies from Samui Island is low (e.g., only three *cox1* haplotypes compared to 16 haplotypes in the Upper South, see Table 3.7). Similarly, genetic variation of flies on Chang Island is lower than in populations in the nearest region of the mainland (i.e., the Northeast).

Lower genetic variation on Chang Island than in the Northeast region could indicate that the Chang (and possibly also Samui) populations have been derived from relatively few introductions and have remained separated for some time, rather than simply being a result of ongoing recent introductions from mainland locations. Minimal genetic variation in *cox1* and microsatellites compared to the mainland supports the hypothesis that loss of genetic variation and inbreeding are involved in the island populations. Genetic variation is correlated with island size, and because island populations are typically smaller than mainland populations they are predicted to have less genetic variation (Jaenike, 1973; Frankham, 1996). Nonetheless, the results demonstrated here revealed genetic variation in the island populations to be greater than some Thai mainland regions, (e.g., Lower South; Table 3.8). This may have occurred by chance due to sampling artefact, as a result of high migration rates, or separate migrations and dispersals of animals from already differentiated mainland populations (Frankham, 1997). For example, island populations of a bat (*Macrotus* sp.) and drosophilid fruit fly (*Drosophila* sp.) have high dispersal abilities and, paradoxically, have greater variation than mainland populations (Greenbaum & Baker, 1976). These mobile animals have particularly high levels of genetic variation and are considered to have experienced population bottlenecks each time they migrated to a new island (Carson et al., 1970; Giddings et al., 1989).

In contrast, some studies report that island populations of vertebrates have demonstrably lower levels of genetic variation than corresponding mainland populations, but invertebrates and birds commonly do not show significant differences (Nevo, 1978; Boag, 1988). All of these studies were based on a small data sets and there may not be sufficient data for definitive conclusions. Furthermore, the distance between islands and the closest mainland affects variation, as documented by studies on lizards (Soulé & Yang, 1973; Gorman & Kim, 1975) and mammal species (Kilpatrick, 1981; Schmitt et al., 1995).

Samui and Chang Island are each some distance from the mainland, but evidentally they are not far enough away for the water barrier to maintain clear biotic separation for *Z. cucurbitae* from the nearby mainland (Samui is 40 km from

the mainland; Chang Island only 10 km). In fact, it would be reasonable to expect more gene flow from the mainland populations to those islands resulting in less distinct populations. The differences that do exist may be due to the islands' long geographical isolation after sea levels rose and ended their connection to the mainland: this is most likely to have occurred at the end of the Pleistocene, 8000-10000 years ago (Ruttner, 1988).

The results from this study differ somewhat from previous studies which found the honey bee population from Samui Island to be strongly isolated from peninsular Thailand, northeast, north and central region based on genetic and morphometric data (Sylvester et al., 1998; Hepburn et al., 2001; Sittipraneed et al., 2001a, 2001b; Warrit et al., 2006). The more striking honey bee results might have arisen because the honey bee is a social insect and human-mediated dispersal is unlikely as they are difficult to move or bring to an island by accident. On the other hand, it is very easy to transport melon fly to these islands as larvae inside fruit.

### 3.5.3 The Northeast population

In general, there was no persuasive morphometric evidence for significant differences between Northeast populations of melon fly and other Thai populations. Certainly melon fly from the Northeast had large wings, but their aedeagi were relatively short in comparison to other sites (Figure 3.11). In contrast, molecular data revealed significant microsatellite differentiation of the Northeastern population (Figure 3.14). Several possible factors could be responsible for this population structure. Geographic barriers between regions may affect differentiation in this species, especially barriers dividing the Northeast (the Khorat Plateau) from other regions.

The northeast of Thailand is an important Mesozoic (252-66 MYA) geographical feature in Indochina, with a significant mountain range separating the Northeast from other regions of Thailand, thus defining the Khorat Plateau (Bunopas & Vella, 1978; Buffetaut & Ingavat, 1986). The Khorat Plateau resulted from erosion after the Quaternary tectonic event (uplifting and tilting) that changed the plateau geomorphically and delimited the southern-western margin by lowland (Rainboth 1996; Attwood & Johnston 2001; Carter & Bristow 2003; Glaubrecht &

Köhler, 2004). Other prehistoric events during the middle Pleistocene (700 KYA-5KYA) included extensive lava flows at the southeastern margin of the Khorat Plateau, resulting in its formation (Lukoschek et al., 2011). As a consequence of these geological events, the Northeastern part of Thailand is separated from Central Thailand by the Phetchabun mountain range and the Dong Phaya Yen Mountains in the west, the Sankamphaeng Range in the southwest and by the Dongrek Mountains in the south, all of which historically made access to the northeast difficult (Figure 3.35). These may inhibit or restrict dispersal by *Z. cucurbitae*. The geographic distances between the northeastern sites of this study and sites in other regions were far greater than the dispersal ability of an individual *Z. cucurbitae* (estimated at 2 km in 2 weeks; Peck et al., 2005) and the mountain ranges could also be an effective barrier for gene flow as has been reported in other fruit flies (Shi et al., 2005). An ecological study of *Z. cucurbitae* indicated that mountain ranges are effective geographic barriers to dispersal because this species occupies low altitude areas (Liu et al., 2013). Mountain ranges are also important geographic barriers to gene flow in other fruit flies (Shi et al., 2005; Meeyen et al., 2014; Kunprom et al., 2015).


**Figure 3.35** Map of the Korat Plateau region (Anonymous, 2015c).

*Local adaptation due to environmental variation*

Local adaptation due to environmental variation is assumed to influence population structure in most taxa (Wiszniewski et al., 2010; Perrier et al., 2011). The Northeast is distinguished from other regions by its poor soils, which are less favourable for agriculture than the soils of many other parts of Thailand.

Nevertheless, rice, sugar-cane and manioc are cultivated on a vast scale as, to a lesser extent, is rubber. The region consists mainly of the dry Khorat Plateau which in some parts is extremely flat, with a few low but rugged and rocky hills, principally the Phu Phan Mountains. The short monsoon season brings heavy flooding in the river valleys. Unlike the more fertile areas of Thailand, the Northeast has a long dry season, and much of the land is covered by sparse grasses. As noted above, mountains ring the plateau on the west and the south, and the Mekong delineates much of the northern and eastern rim. There are ample geographical and agricultural features which distinguish the Northeast from other parts of Thailand, and these may be the cause of the genetic distinctness of the flies from this region.

### 3.5.4 Patterns of population structure in *Z. cucurbitae* along the Thai-Malay Peninsula

The data from this study suggests that it is more accurate to characterize variation in *Z. cucurbitae* along the Thai-Malay transect as clinal, rather than dominated by a disjunction at the Isthmus of Kra. Certainly, the geometric-morphometric results for *Z. cucurbitae* wing shape from the northernmost and southernmost sites along the transect were considerably different from each other (Figure 3.23 and Table 3.17). Analyses of data collected from sites along the Thai-Malay transect revealed statistically significant, population-level variation in wing shape among some sampled populations south of the Isthmus. However, these southern populations also differed largely from each other, suggesting that the differentiation represents very localised population effects, rather than a simple north-south vicariance across the Kra Isthmus. Moreover, the cluster of distinct and related populations from Yala, Narathiwat and Selangor fits more with the hypothesis of a biogeographic barrier at the Kanger-Pattani line, rather than further north on the Kra Isthmus.

Isolation by distance (IBD) analysis of both morphological and molecular data revealed that the variation along the transect is correlated with geographic distance. IBD analysis of *Z. cucurbitae* aedeagal measurements revealed a pattern exactly opposite to that seen in *B. dorsalis* (Krosch et al., 2013). In this study, the aedeagus of *Z. cucurbitae* is smaller in flies from low latitudes (i.e., from the more

southerly sites), but in *B. dorsalis* the aedeagus is larger at low latitudes. The reason for this reversal of effect in the two fly species is unknown and further comparative studies with other species of tephritid are recommended.

AMOVA revealed some genetic differentiation among *Z. cucurbitae* populations among the populations from north and south of the Isthmus of Kra. However, the neighbor-joining mitochondrial haplotype network did not show any pattern of genetic differences between populations separated by this putative barrier. The genetic isolation between the northern and southern melon fly was relatively small, suggesting only weak, partial genetic isolation. However, there was a disjunction between populations from the southernmost sites (Yala, Narathiwat and Selangor) and all other sites. Taken together, the results support the currently accepted notion that *Z. cucurbitae* is a single species, but PCA, Bayesian analysis and population tree data for three of the southernmost sample sites indicate that in peninsular Thailand there is some distinction between populations. The genetic results demonstrated significant clinal characteristics along the Thai-Malay transect and this is clearly the most common pattern of population differentiation in this study. Genetic similarities between the southernmost populations (Yala and Narathiwat) suggest that these two populations were probably connected historically, and this facilitated gene flow between Yala and Narathiwat, and also Selangor. The pattern of the genetic cline along Thai-Malay transect might also reflect historic differences, which are currently eroding by natural or human-assisted dispersal.

The apparent affinities among the Yala, Narathiwat and Selangor populations are relevant to another major biogeographic transition: the zone between the Indochinese and Sundaic (Indomalayan) biotas. This transition approximates the Thai-Malay peninsula; indeed, there may actually be two major biogeographic transitions about 500 km apart in this region (Van Steenis, 1950; Good, 1964; Keng, 1970; Whitmore, 1984, 1998; Ashton, 1992; Baker et al., 1998; Wikramanayake et al., 2002; Lohman et al., 2011). Many zoologists are of the opinion that this transition occurs on the Isthmus of Kra between Chumpon (Thailand) on the east coast and Tavoy (Burma) on the west coast (Benzie, 1999;

Hepburn et al., 2001; Sittipraneed et al., 2001a, 2001b; Hughes et al., 2003; Round et al., 2003; de Bruyn et al., 2005; Crandall et al., 2007). This corresponds to the transition between seasonal evergreen and mixed moist deciduous forests. Others place more importance on transition at the Kangar-Pattani line near the Thai-Malay border (Woodruff et al., 2003). The Kangar-Pattani line runs from west to east between Kangar (Malaysia) and Pattani (Thailand) at 7 °N latitude along the Thai-Malay border (Whitemore, 1984). If this is taken as the dividing line between the Indochinese and Sundaic regions, Sundaland covers only a small portion of southern Thailand, including Pattani, Yala and Narathiwat (Choldumrongkul et al., 2007) (Figure 3.36).

### 3.5.5 Biogeographic barrier (Isthmus of Kra, Kanger-Pattani line)

The observed pattern of population structure resolved along the Thai-Malay Peninsula for both genetic and morphometric datasets was largely incongruent with the pattern expected for two independent populations bisected by a historical biogeographic barrier. In other words, there were no strong signals from the present study to support the existence of divergent melon fly populations on either side of the Isthmus of Kra barrier. This is consistent with studies of the Oriental fruit fly (*B. dorsalis*), for which populations are unstructured along the length of the Isthmus of Kra (Krosch et al., 2013; Akrawong et al., 2014).

Understanding of the biogeographic history of the Kra Isthmus is becoming increasingly complex. Studies of animal taxa across this region show a range of patterns and possible transition zones. These alternative biogeographic transition zones are possibly driven by historically unstable climate effects (Pauwels et al., 2003; Hughes et al., 2011) or multiple past marine transgressions into the Thai-Malay Peninsula (Woodruff, 2003; de Bruyn et al., 2005). Taken together, the data presented here add one more piece to the larger biogeographic puzzle by reporting a population-level study based on markers that also provide data relevant to biogeographic regions beyond the Thai-Malay peninsula.

It has been suggested that there was a trans-peninsular seaway that acted as a barrier and permitted divergence of the Indochinese and Sundaic biotas at the Isthmus of Kra (Woodruff, 2010). The transitions in some groups of animals lie north

of Kra in the northern peninsula, but these are not particularly well documented. A biogeographic barrier at the Isthmus of Kra is indicated by the zoogeography of several groups of animals, especially amphibians, fish and crustaceans, which cannot move far away from their habitat (Inger, 1966; Benzie, 1999; Inger, 1999; de Bruyn et al., 2005; Crandall et al., 2007). Studies of total regional faunas, e.g., birds (Hughes et al., 2003; Round et al., 2003) and honey bees (Rueppell et al., 2011), also support the concept of a single biogeographic barrier at the postulated Kra Seaway. However, other studies show no biogeographic barrier effect of the Kra Seaway (e.g., Pramual et al., 2005), nor do they identify alternate zones of faunal and/or population transition further north or further south (Wikramanayake et al., 2002; de Bruyn et al., 2005; Woodruff & Turner, 2009; Patou et al., 2010; Hughes et al., 2011). These latter faunistic studies, which downplay the significance of the Isthmus of Kra, correspond to geological data which suggests that the peninsula has never been dissected by a complete seaway (De Bruyn et al., 2005).

If not Kra, then are there other barriers? A cluster of genetically divergent and reciprocally related populations from Yala, Narathiwat and Selangor fits with the idea of a biogeographic barrier at the Kanger-Pattani line. Most historical explanations for the current distributions of Southeast-Asian biota invoke the existence of ancient seaways during periods of high sea levels, with these seaways forming barriers to dispersal by terrestrial organisms (Woodruff, 2003). This is not what is thought to have happened at the Kanger-Pattani line, with the marked turnover of species at the Kanger-Pattani line postulated to result from a corresponding change in environmental conditions (Whitmore 1984; Ashton, 1997; Lohman et al., 2011).

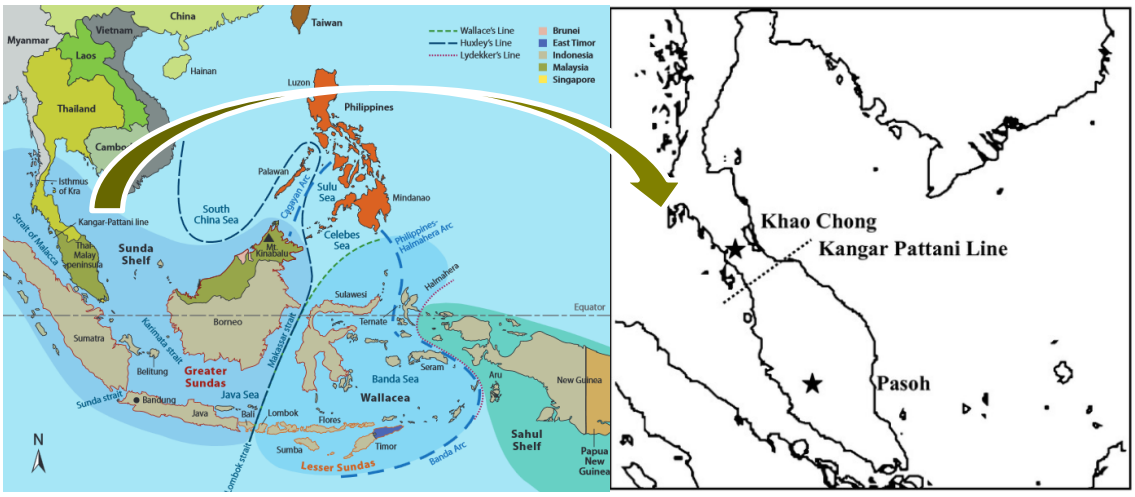



**Figure 3.36** Maps showing location of the Kanger-Pattani biogeographic barrier (modified from Lohman et al., 2011 and Baltzer et al., 2008).

The Kanger-Pattani transition is demonstrated by a major floristic and climatic transition from seasonal dry forest to aseasonal evergreen forest (Baltzer et al., 2008; Woodruff & Turner, 2009; Lohman et al., 2011). Approximately 575 plant genera reach their distributional limits at this line (Van Steenis, 1950; Lohman et al., 2011). Moreover, the distributions of many mammals, including bats, support a transition zone at the Kanger-Pattani line (Hughes et al., 2011). The close correspondence between species distributional limits and a rainfall seasonality transition has been proposed as a primary mechanism maintaining species distributional limits at the Kanger-Pattani line (Whitmore, 1984; Ashton, 1995; Richards, 1996; Lohman et al., 2011). Thus, both historical and environmental mechanisms could be invoked to explain the distributional pattern involving the Yala, Narathiwat and Selangor populations of *Z. cucurbitae*.

*Local adaptation due to environmental variation*

The morphology of flies from Nan and Chiangmai (northernmost populations) was different from others. The northern region is composed of several mountain ranges of high altitude, and this topography may separate populations of melon flies. As previously noted, there are some populations of guava fruitfly, *Bactrocera correcta*, in this region which differ from populations elsewhere in

Thailand (Kunprom et al., 2013). Kunprom et al. (2013) related the distinctive population of *B. correcta* from Phetchaboon province to environmental variables of the Phetchaboon range. Notwithstanding the subtle morphological differences of the northern melon flies and the genetic distinctness of melon fly from the northeast (noted above), *Z. cucurbitae* from the North and Northeast do not differ markedly from those from elsewhere in Thailand. The determining factor for the melon fly may be the fact that the cucurbit host-plants for *Z. cucurbitae* are very common in Thailand and effectively present a continuous habitat for the fly, promoting gene flow among populations.

Environmental factors could account for at least some of the affinities between the flies at Yala, Narathiwat and Selangor populations, which were significantly different from all other local populations. It is also worth noting that all of these three site were geographically adjacent to large tracts of natural evergreen forest. Thus, it could be that the characteristics which make flies from these sites distinctive from other populations, and similar to each other, were derived from flies developing in wild host plants. Many host-plant species reported for *Z. cucurbitae* (Allwood et al., 1999) are native plant species commonly found in the natural forest in Thailand.

**Conclusion and link to next chapter**

Two factors are most likely to account for the genetic homogeneity among populations of *Z. cucurbitae* in Thailand. First, *Z. cucurbitae* utilizes a wide range of host plants in Southeast-Asia, including 42 plant species belonging to 20 families (Allwood et al., 1999). Many of these host plants (e.g., cucurbits and beans) are commonly grown in Thailand, and so melon fly populations are likely to be geographically continuous. Second, human-mediated dispersal, such as local transportation of fruit and trade, may also facilitate movement of flies, as has been reported in other fruit fly species (Malacrida et al., 2007; Shi et al., 2012). This movement of flies with fruit would promote genetic exchange (i.e., gene flow) between populations. This gene flow would counter the effect of genetic drift or selection by lowering the level of genetic differentiation. The overall genetic structuring in Thailand was low except for the Northeastern population, which is

physically isolated and ecoclimatically distinct from other Thai regions. The haplotype shared between the mainland and islands most likely arose from long distance migration, which could be a result of either the historical population expansion or human-mediate gene flow.

These data do not suggest the presence of a cryptic species complex within *Z. cucurbitae* in Thailand. However, to conclusively test this, the next chapter examines host plant effects on morphological and genetic variance in *Z. cucurbitae*. Focusing on a single region in Thailand, it examines the variance of flies reared from known hosts, comparing particularly flies reared from cucurbits versus those reared from non-cucurbit hosts. It thus examines for the likelihood of host races occurring in this species.

# CHAPTER 4

## Cryptic host races in *Zeugodacus cucurbitae*: little evidence of host-related structure in Thailand

## 4.1 INTRODUCTION

### 4.1.1 Environment effects on insect variation

Organismal variability derives from the combined effects of the genotype and environmental influences and individual experiences during development (Beebee & Rowe, 2008). The environment an organism experiences can affect which genes are expressed, the extent to which they are expressed, or how genes interact to result in a particular phenotype (Nijhout, 1999; Beebee & Rowe, 2008). The most visible manifestation of this, especially for the insects, is represented by variation in morphology (Roff & Fairbairn, 1991; Zera & Denno, 1997; French et al., 1998). Environmental variables, such as temperature, nutrition, photophase and humidity, may also influence growth and development and so, ultimately, affect population fluctuations as well as phenotype (Moczek, 1998; Yin et al., 2007; Chown & Gaston, 2009). Temperature may play a particularly strong role in influencing insect body size (Partridge & French, 1996) with, for example, increased development time at lower temperatures typically resulting in larger adults (Stern & Emlen, 1999). In addition to this, nutrition during development is widely considered the key factor influencing adult size and allometry (Moczek, 1998; Awmack, & Leather, 2002; Shingleton et al., 2007). This effect may be intergenerational, as illustrated by the gypsy moth, *Lymantria dispar* (Linnaeus), in which the nutritional environment can significantly influence both the growth and reproductive potential of the subsequent generation (Lindroth et al., 1997).

Frugivorous tephritids oviposit into fruit, in which eggs hatch and larvae feed. Indeed, it is a specific characteristic of fruit fly larvae that they are internal feeders of fruit and that they develop in the host fruit selected for oviposition by the female parent (Averill & Prokopy, 1987; Novotny et al., 2005); as a result, other environmental effects on the larvae are limited and may play a relatively reduced role in development. Accordingly, the most significant environmental effect on tephritids comes from the host plant (Bush, 1974, 1992; Feder et al., 1988; Awmack, & Leather, 2002). Since the host plant is such a determining factor in the development of tephritids, these flies are prime candidates to diversify into genetic

lineages (be they host races and/or cryptic species) which are associated with different host fruit.

### 4.1.2 Host plant association, host race and cryptic species in fruit flies

Host-plant associations and shifts in host use can play a major role in the diversification of phytophagous insects (Bush, 1969; Guttman et al., 1981; Feder et al., 1988, Waring et al., 1990). Where a pattern of differential host affiliation is detected, the possibility of host races or unrecognised sibling species cannot be discounted (Abrahamson et al., 1989). A host race can be said to exist in a plant-feeding insect when different lineages exhibit strong preferences for different species or cultivars of a host plant, or for a range of closely related host plants (Diehl & Bush, 1984), and a lineage may be totally restricted to one or a few plant species or varieties. Importantly, host races may or may not be recognisable using traditional morphological characters (Feder et al., 1995).

Various mechanisms can promote the evolution of host races, such as geographical isolation or clonal reproductive strategies, as seen in aphids (Margaritopolous et al., 2009). Host races have been considered one of the principle mechanisms of sympatric speciation (Bush, 1975), and the adoption of a new host plant may stimulate rapid evolution (Carroll & Boyd, 1992; Dingle & Winchell, 1997). For this to be true, it is necessary to demonstrate that host races actually exist and to then determine if adaptations to a particular host lead to reproductive isolation. The existence of a continuous array of sympatric biotypes-from polymorphisms, through ecological or host races with increasing reproductive isolation, to good species-provides strong evidence for a continuous route to sympatric speciation and the emergence of cryptic species via natural selection. Lineages diverging as a result of behavioural changes (e.g., host shifts, courtship displays, or both) are often morphologically cryptic (Bush, 1969; Wood, 1980; Wood & Keese, 1990; Via, 1991; Wells & Henry, 1998; Emelianov et al., 2002; Bickford, 2006). The family Tephritidae includes classic examples of host races, e.g., in *Rhagoletis* (Bush, 1969; Berlocher, 2000; Filchak et al., 2000) and cryptic species e.g., the *Z. tau* complex (Baimai et al., 2000; Jamnongluk et al., 2003; Saelee et al., 2006; Kitthawee & Dujardin, 2010; Sumrandee et al., 2011; Kitthawee & Rungsri, 2011). I will explain these examples

further, before turning to the possibility of host races or cryptic species in *Z. cucurbitae.*

The best documented example of host races in tephritids is that of the apple maggot fly, *Rhagoletis pomonella* (Walsh) (Feder et al., 1997; Linn et al., 2004). When this temperate species, originally associated with native hawthorn fruits, came into association with novel hosts such as apple and cherry which ripened at different times of the year, selection of allele frequencies to adapt to these hosts was accompanied by habitat-specific mating and oviposition behaviours. This provoked rapid, sympatric, evolutionary divergence of the species into distinct races (Whitman & Agrawal, 2009) (Figure 4.1). The evolutionary outcome was that the adult flies of the apple or hawthorn races emerged at different times of the year and had limited temporal opportunity to interbreed, thus a mechanism for possible sympatric speciation was in place.

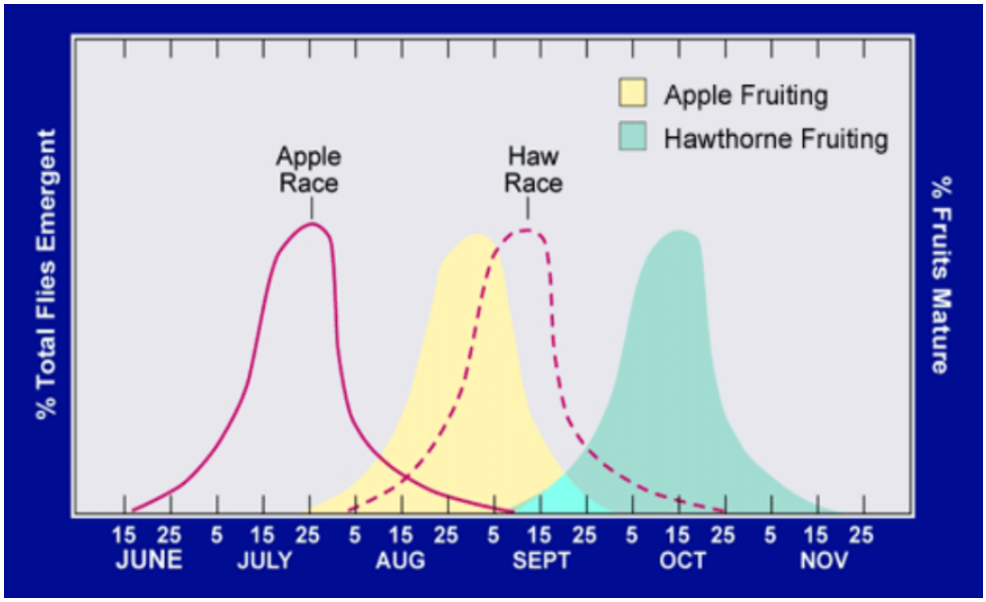



**Figure 4.1** Population graph illustrating the differential emergence times of apple and hawthorn races of *Rhagoletis pomonella*. Adults emerge from pupation prior to peak fruiting, so sexual maturity and mating coincides with fruiting (Bush, 1969).

The cluster of species referred to as the *Zeugodacus tau* group (Drew & Romig, 2013) is an example of multiple, closely related, cryptic species associated with different host plants. Perhaps some of these originated as host races, but the

precise mechanism for the emergence of reproductive isolation in this cluster of species is not as clear as in the case of *R. pomonella.* The correlation among the eight known *Z. tau* lineages and host plant preferences have been well characterised (Baimai et al., 2000; Jamnongluk et al., 2003). The evidence to substantiate that *Z. tau* is a complex of closely related species is substantial, comprising morphometric differences (especially in the shape of the wing), cytotaxonomy, allozyme electrophoresis, karyotype differences and variation in *cox1* sequences (Baimai et al., 2000; Jamnongluk et al., 2003; Sumrandee, et al., 2011). Host plant preferences and associations also indicate the existence of eight lineages. The *tau* group seems to comprise seven species, which have been labelled for some time in the literature as species A, B, C, D, E, F, G and I, with species A being *Z. tau* sensu stricto (Baimai et al., 2000; Jamnongluk et al., 2003; Saelee et al., 2006; Kitthawee & Dujardin, 2010; Sumrandee, et al., 2011; Kitthawee & Rungsri, 2011). Drew & Romig (2013) have described new species in the *Z. tau* complex, but have not linked their morphological species with the existing *Z. tau* code letters. With respect to *Z. tau* complex species, B has been found only in *Siponodon celastrineus* fruit, C and D occur allopatrically but attack the same host plant species, *Momordica cochinchinensis,* while F and G principly attack the same host, *Hydnocarpus anthelminthiscus*.

The long-running work on *R. pomonella* and the *Z. tau* complex has provided significant lessons for the investigation of host race and cryptic species in other tephrtids. Foremost among these lessons are the need for multiple lines of evidence, the need for evidence from multiple sites, and the advantage of analysing this evidence at different spatial scales.

### 4.1.3 Melon fly and cryptic species

The recognition that *Z. tau s.l.* is a complex of species has been one of the motivators of the present examination of *Z. cucurbitae.* It is a congener of *Z. tau* and, like *tau,* is an important pest. *Zeugodacus cucurbitae* and *Z. tau s.l.* share many host plants, especially cucurbits (Allwood et al., 1999). The melon fly is a polyphagous tephritid that has been reported to damage more than 125 species of host plants, especially in the families Cucurbitaceae and Solanaceae (Christenson &

Foote, 1960; Weems, 1964). *Zeugodacus cucurbitae* is typically oligophagous in Thailand, with a host range centered on cucurbits (Clarke et al., 2001). However, it appears to be more polyphagous in Southeast-Asia generally; over this geographical range it infests beans, rose-apples and 42 other plant species belonging to 20 families (Allwood et al., 1999). Thus, because *Z. cucurbitae* is known from such a range of plant families, with some seeming anomalies, there is *a priori* reason to suspect it may represent a complex of host-races: this is something yet to be adequately tested in its native range of Southeast-Asia, and notably in Thailand which lies in the geographical centre of the region.

### 4.1.4 Research objective

In this chapter, the characteristics of *Z. cucurbitae* associated with particular host plants are analysed in order to search for evidence of previously undetected host races or cryptic species. The study was restricted to the central part of Thailand to limit potentially confounding effects that may result from sampling across a broader geographic range. The apparent absence of geographical population structuring (see Chapter 3) makes central Thailand and ideal area for a search for population structure potentially correlated with host plants. Wing geometric morphometric, aedeagus length, *cox1* and microsatellite data are used to determine if host related variation occurs. The null hypothesis for this study is that there is no evidence of congruent morphological and molecular differentiation associated with host-reared material from different host plants.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Fruit fly collecting and sampling strategies

Melon fly host fruits were collected from the field from December 2012 to December 2013 throughout various regions of Thailand, including the Central, North, West, East, Northeast and South regions. This was undertaken principally by colleagues from the Insect Taxonomy group, Thai Department of Agriculture under my instruction. Species of Cucurbitaceae and Fabaceae are the most common host plants for the melon fly (see Chapter 1, Table 1.1), hence the majority of target fruit included hosts from these two families, e.g., angled gourd, ivy gourd, bitter melon, watermelon, silk squash and cucumber (Cucurbitaceae), and bean and yard long bean (Fabaceae). The precise types of host collected at different sites in the field depended on availability.

Previous studies have also reported melon fly from mango, star fruit, rose apple, avocado, orange, custard apple and okra. Therefore, in total, 22 fruit species in nine families (Cucurbitaceae, Fabaceae, Anacardiaceae, Averhoaceae, Myrtaceae, Lauraceae, Rutaceae, Annonaceae and Malvaceae) were collected in the field (Appendix 1 and Appendix 2) and transferred to the Insect Taxonomy laboratory, Plant Protection Research and Development Office, Department of Agriculture, Bangkok, Thailand.

In Thailand, a number of major and minor cucurbits are cultivated, and these share about 15% of the total vegetable production (DOAE, 2014). The crop is commercially cultivated as well as grown by home gardeners in every region in Thailand (Figure 4.2). However, most of the commercial cultivation areas in Thailand are subject to heavy applications of chemicals to control insect pests and diseases. Thus, commercial plantations were not suitable for collecting fruit flies. All specimens obtained as host reared material were collected from organic plantations or from plants grown in home gardens which used no or minimal pesticides. Moreover, the cucurbits and bean-growing areas have a very patchy geographical distribution, hence the number and variety of host plants utilized varied.


**Figure 4.2** Examples of host fruits from which *Zeugodacus cucurbitae* were collected from organic plantations and plants grown in home gardens. (A) angled gourd; *Luffa acutangula* (B) *Momordiaca charantia* (C) ivy gourd; *Coccinia grandis* (D) bitter melon; *Citrullus lanatus* (E) winter melon; *Beniscasa hispada* (F) melon; *Cucumis melo* (G) cucumber; *Cucumis sativus* (H-I) sponge gourd; *Luffa cylindrica* (J-K) bean: *Phaseolus vulgaris* (L) yard long bean; *Vigna unguiculata.*

The total weight of fruit collected from each site varied between 0.02 to 10.00 kg, depending on the number of fruits that could be found (Appendix 3). Larvae were permitted to complete development and adult flies were reared from these fruits. Emergent adults were kept alive for one week to allow cuticle sclerotization to preserve morphological characters (see detail in Chapter 2). Flies were preserved in 95% alcohol for genetic analysis. In total, in this study, melon fly were reared from ten host plants from ten sites from central Thailand, from six host plants from ten sites from the North, from only one host plant from five sites from the Northeast and from one host plant from one site from the South, West and East (Appendix 1-3). While I collected material from several regions of Thailand, subsequent analysis was conducted only on material collected from within one region: Central Thailand. This was because the Central Region represented the greatest diversity of host plants from which *Z. cucurbitae* were reared and, by focusing on one region, I minimized potentially confounding geographic effects.

Ultimately, melon flies emerged from ten species of host plants, those plants belonging to eight species in the family Cucurbitaceae (angled gourd, ivy gourd, bitter melon, watermelon, silk squash, cucumber, melon and sponge gourd) and two species of Fabaceae (bean, yard long bean) (Table 4.1, Figures 4.3 and 4.4). While previous studies have reported melon fly from mango, star fruit, rose apple, avocado, orange, custard apple and okra, in this study those plants were infested only with *B. dorsalis* and *Z. tau* (Appendix 3).

**Table 4.1** Collection data of host fruits sampled for the host-study of variation in *Zeugodacus cucurbitae*.

| | Family | Scientific name | Host Plants | Date of collection | Collectors | Locations | Latitude | Longitude |
|---|---|---|---|---|---|---|---|---|
| 1 | *Cucurbitae* | *Luffa acutangula* | Angled gourd | 9 -May-2013 | Sunadda Chaowarit | Suphanburi | 14.419 | 100.062 |
| 2 | *Cucurbitae* | *Coccinia grandis* | Ivy gourd | 14-May-2013 | Suppara Akkasarakul | Nakhonpathum | 13.952 | 99.992 |
| 3 | *Cucurbitae* | *Momordiaca charantia* | Bitter melon | 4-June- 2013 | Chamaiporn Buamas | Nakhonsawan | 15.698 | 101.114 |
| 4 | *Cucurbitae* | *Citrullus lanatus* | Water melon | 9-Aug-2013 | Sunadda Chaowarit | Lopburi | 14.958 | 100.694 |
| 5 | *Cucurbitae* | *Cucumis melo* | Melon | 30-Sep-2013 | Sunadda Chaowarit | Ayutthaya | 14.329 | 100.639 |
| 6 | *Cucurbitae* | *Beniscasa hispada* | Winter melon | 7-July-2013 | Sunadda Chaowarit | Saraburi | 14.563 | 100.952 |
| 7 | *Cucurbitae* | *Cucumis sativus* | Cucumber | 6-June-2013 | Sunadda Chaowarit | Chainat | 15.147 | 100.118 |
| 8 | *Cucurbitae* | *Luffa cylindrica* | Sponge gourd | 17-Dec-2013 | Chamaiporn Buamas | Phichit | 16.302 | 100.212 |
| 9 | *Fabaceae* | *Phaseolus vulgaris* | Bean | 28-Dec-2012 | Sunadda Chaowarit | Bangkok | 13.819 | 100.570 |
| 10 | *Fabaceae* | *Vigna unguiculata* | Yard long bean | 22-April-2013 | Samram Sukkul | Bangkok | 3.005 | 101.421 |

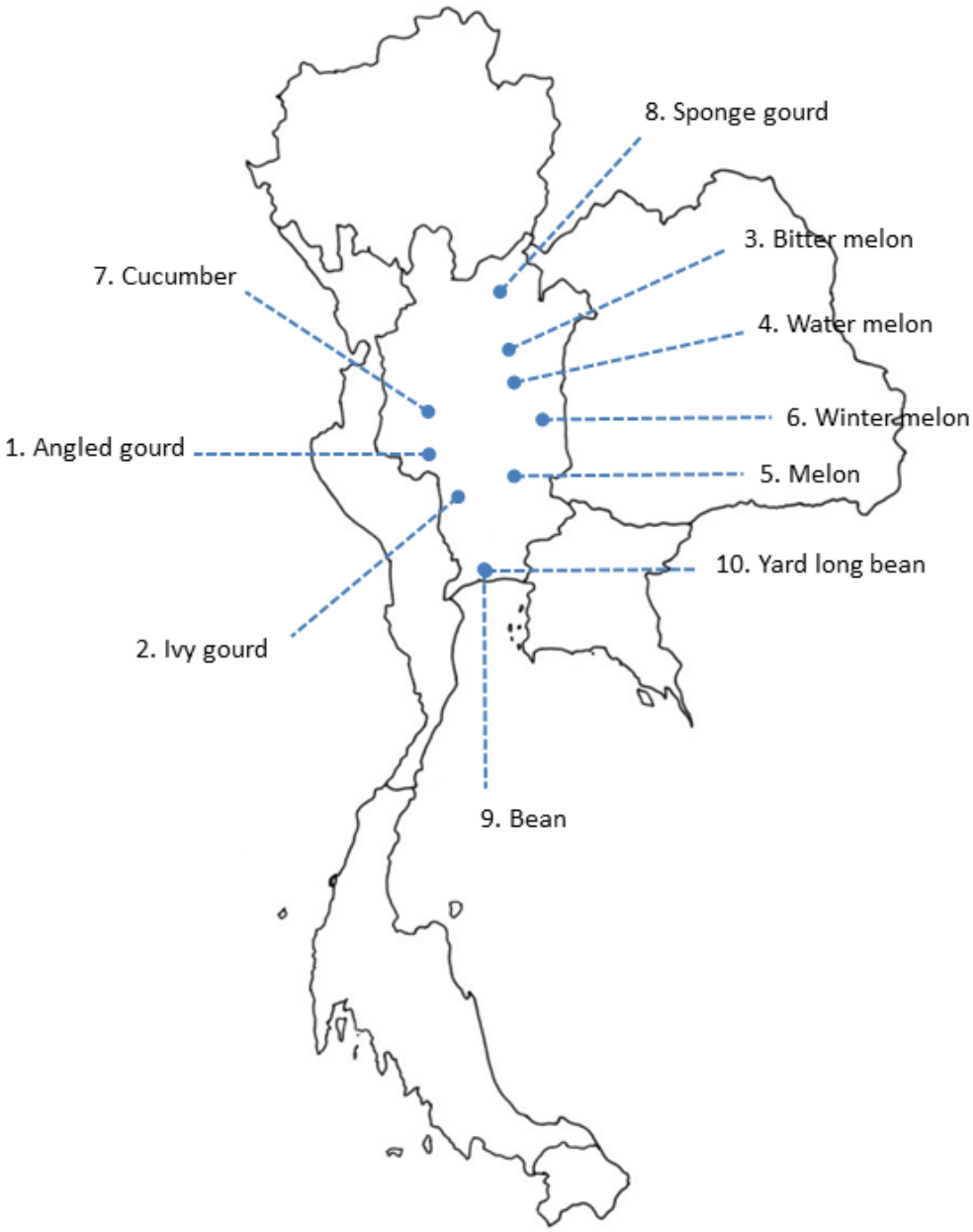



**Figure 4.3** Locations of sample sites in the Central Thailand regions at which *Zeugodacus cucurbitae* were reared from host plants. Specific collection data is presented in Table 4.1

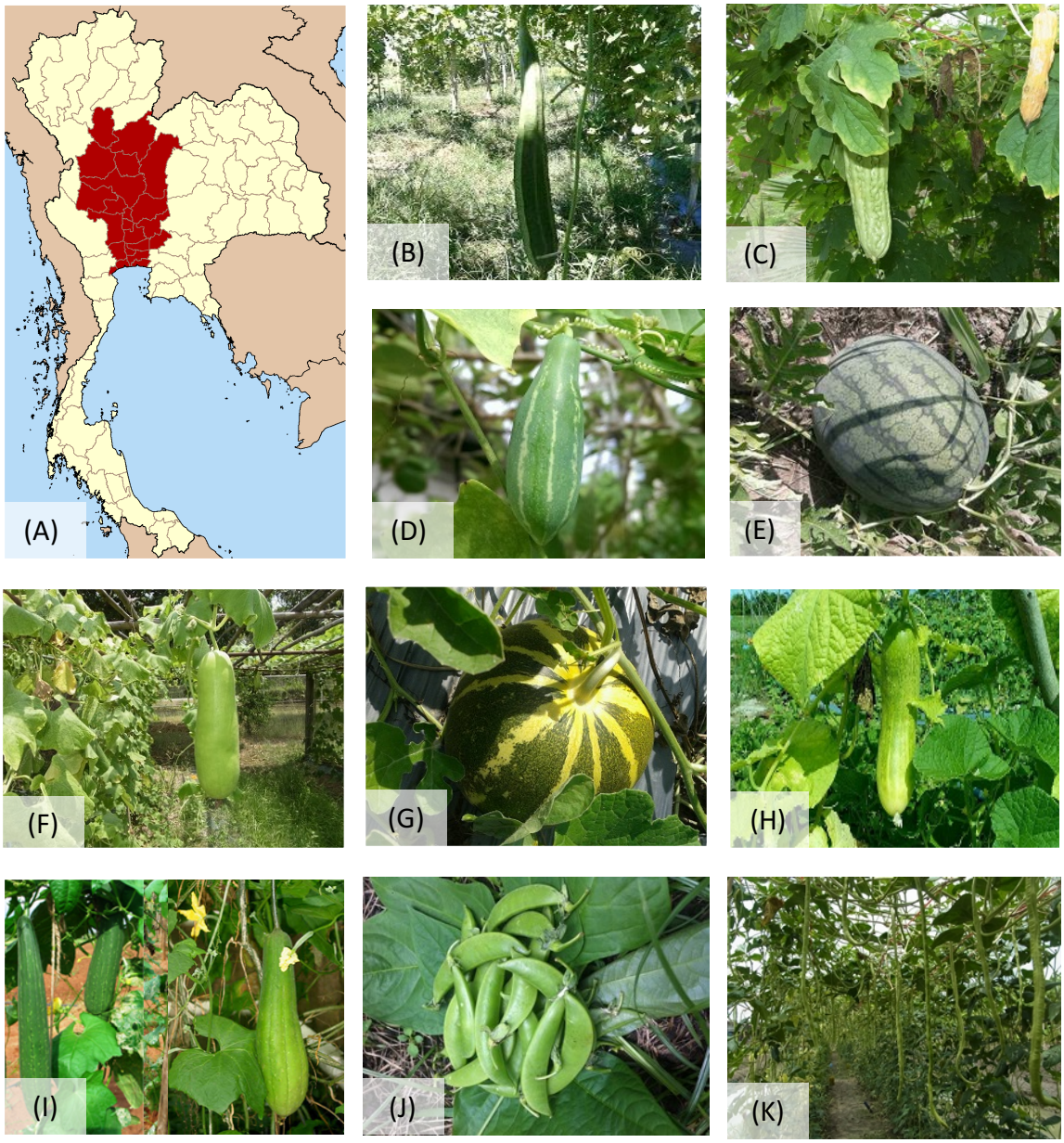



**Figure 4.4** Examples of fruit from which *Zeugodacus cucurbitae* were collected in Thailand. (A) Central Thailand, the region in which host plant collections were performed (B) angled gourd: *Luffa acutangula* (C) bitter melon: *Momordiaca charantia* (D) ivy gourd: *Coccinia grandis* (E) watermelon: *Citrullus lanatus* (F) winter melon: *Beniscasa hispada* (G) melon: *Cucumis melo* (H) cucumber: *Cucumis sativus* (I) *sponge* gourd: *Luffa cylindrica* (J) bean: *Phaseolus vulgaris* (K) yard long bean: *Vigna unguiculata*

### 4.2.2 Morphometric analyses

#### 4.2.2.1 Geometric morphometric analyses

Wings from 20 individuals from each of ten host plants (i.e., angled gourd, ivy gourd, bitter melon, watermelon, silk squash, bean, yard long bean, cucumber, melon and spring gourd) from the Central region of Thailand were used in the study. All details regarding specimen preparation, landmarking and Procrustes superimposition, and calculation of wing size are presented in Chapter 2.

*Wing size*: Individuals were assigned to groups based on host plant species. All data were first tested to confirm that they were consistent with assumptions of normality and homogeneity of variance before further analysis was undertaken. The data met assumptions, and one-way ANOVAs were performed to test for differences in centroid size between the *a priori* defined groups. ANOVA was performed to enable comparison of wing shape among flies reared from the ten different host plant species. A t-test was performed to compare between the groups of flies that were reared from different host plants families Cucurbitaceae and Fabaceae.

*Wing shape analyses*:

- Principal Component Analysis (PCA) was conducted to assess differences in wing shape between populations under a non-hypothesis scenario.

- Canonical variate analysis (CVA) was performed on Procrustes transformed data. The individuals were assigned to groups based on the hypotheses being tested. Canonical variates analysis (CVA) individuals were retained in their *a priori* defined groups for CVA carried out for ten different host plant species. Significant differences were determined via permutation tests (10000 permutations) for Mahalanobis distance among the *a priori* defined groups. It was also necessary to correct for multiple comparisons by performing Bonferroni corrections (Rice, 1989) throughout the analysis.

Canonical variate shape change transformation wireframes were produced for the first two canonical variates to depict relative changes in shape among the datasets.

#### 4.2.2.2 Aedeagal morphometrics

Males used for wing size and shape analysis were also examined for variation in aedeagus length. All details regarding the preparation of aedeagi are presented in Chapter 2.

*Statistical analysis of aedeagus length*: For the purposes of statistical analysis individuals were assigned to groups based on host plant species and data were analysed by ANOVA. The data met assumptions of normality and homogeneity, and one-way ANOVAs were performed to test for differences in aedeagus length among *a priori* defined groups for host plant species. A t-test was also performed to compare among the groups of melon flies reared from host plants from the families Cucurbitaceae and Fabaceae.

*Correlation between wing size and aedeagus length*: To assess the correlation between centroid size and aedeagus length, the centroid size (wing size) was regressed against the aedeagus length from the same individuals, using the combined dataset in SPSS.

### 4.2.3 Molecular procedure and analyses

#### 4.2.3.1 Mitochondrial DNA amplification, sequencing and analyses

Specimens used for morphological analyses were also utilised for molecular analysis. Two-hundred specimens from ten different host plants were examined. All details regarding *cox1* amplification, PCR protocol, reactions and sequencing, and analyses are presented in Chapter 2.

- **Mitochondrial DNA analyses**

Statistical analysis was undertaken to measure mitochondrial DNA gene diversity of *Z. cucurbitae* and also to investigate genetic differentiation. All details of basic statistical analyses employed are presented in Chapter 2.

*Statistical analysis of Molecular Variance*: An Analysis of Molecular Variance (AMOVA) was conducted in ARLEQUIN to assess partitioning of variation within and among host plants. Samples were constrained according to host plants evaluate the variation between host plant species.

#### 4.2.3.2 Microsatellite genotyping

**- Polymerase chain reaction (PCR) amplification sequencing and Sequence cleaning**

Eleven microsatellite loci that had been screened for variation in *Z. cucurbitae* were used for genotyping. Details regarding microsatellite loci, PCR protocol, reactions and analysis are also presented in Chapter 2.

**- Microsatellite analyses**

Statistical analysis was undertaken to measure microsatellite diversity of *Z. cucurbitae* and also to investigate genetic differentiation and population structure. All details of basic statistical analyses employed are presented in Chapter 2.

*Statistical analysis of Molecular Variance*: An analysis of molecular variance was conducted in ARLEQUIN to confirm population clusters and to differentiate the variation component among the melon fly from different host plants. The analysis was based on among-site $R_{ST}$ estimates.

## 4.3 RESULTS

### 4.3.1 Morphometric results

#### 4.3.1.1 Geometric morphometric wing shape analysis

*Centroid size calculation*

There were highly significant differences in centroid size among flies reared from the ten host plants ($F_{9, 190}$ = 3.482; $P$ < 0.001), yet also considerable overlap in wing size among flies reared from the different host plants. Flies reared from sponge gourd and yard long bean had the largest wings, although the wings of yard long wing wre not statistically larger than flies reared from many other host species. Flies from melon had wings smaller than flies reared from all other hosts. On average, flies reared from Fabaceae hosts were significantly larger (6.249 ± 0.355 mm) than those reared from cucurbits (5.944 ± 0.538 mm) ($t_{1, 198}$ = 3.411; $P$ < 0.001) (Figure 4.5).

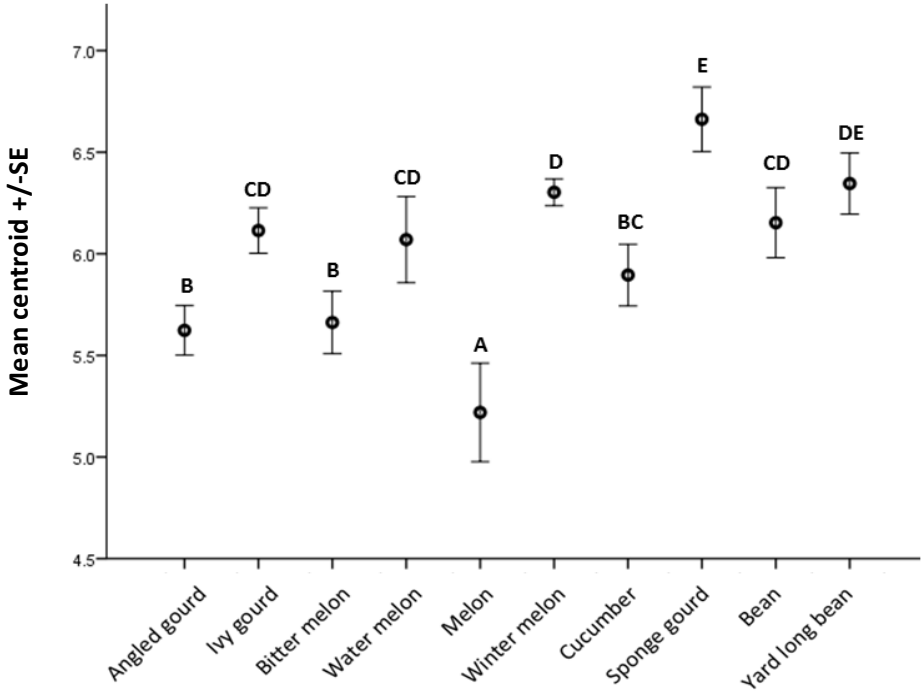



**Figure 4.5** Mean (± SE) wing centroid size of *Zeugodacus cucurbitae* reared from ten host plants in Central Thailand. Samples sharing the same letter are not statistically different from each other based on one-way ANOVA with a Tukey *post hoc* test ($F_{9, 190}$ = 3.482; $P$ < 0.001).

*Procrustes Superimposition*

The Procrustes sum of squares value was 0.2772 and the Tangent sums of squares value as 0.2754 (Table 4.2). The superimposition of coordinates showed that there were shape differences in wing venation among flies from different host plants. Relative shifts in landmarks four and 12 were greater compared to other landmarks (Figure 4.6).

**Table 4.2** Average positions on two axes of fifteen landmarks generated from Procrustes superimposition in two dimensions of *Zeugodacus cucurbitae* wings from individuals reared from ten host plants species across ten sites from central Thailand. The position of the landmarks in relation to a real wing is shown in Figure 2.4.

| Landmark | Axis 1 (X) | Axis 2 (Y) |
|---|---|---|
| 1 | -0.330891 | -0.012250 |
| 2 | -0.330562 | 0.047550 |
| 3 | -0.299293 | 0.074050 |
| 4 | -0.091098 | -0.161698 |
| 5 | -0.186799 | -0.049506 |
| 6 | -0.154226 | -0.048753 |
| 7 | -0.178750 | 0.018141 |
| 8 | 0.156158 | -0.154926 |
| 9 | 0.190643 | -0.030477 |
| 10 | 0.049605 | -0.000316 |
| 11 | 0.027251 | 0.077359 |
| 12 | 0.065739 | 0.133777 |
| 13 | 0.392027 | -0.080338 |
| 14 | 0.434891 | 0.046461 |
| 15 | 0.255305 | 0.140925 |

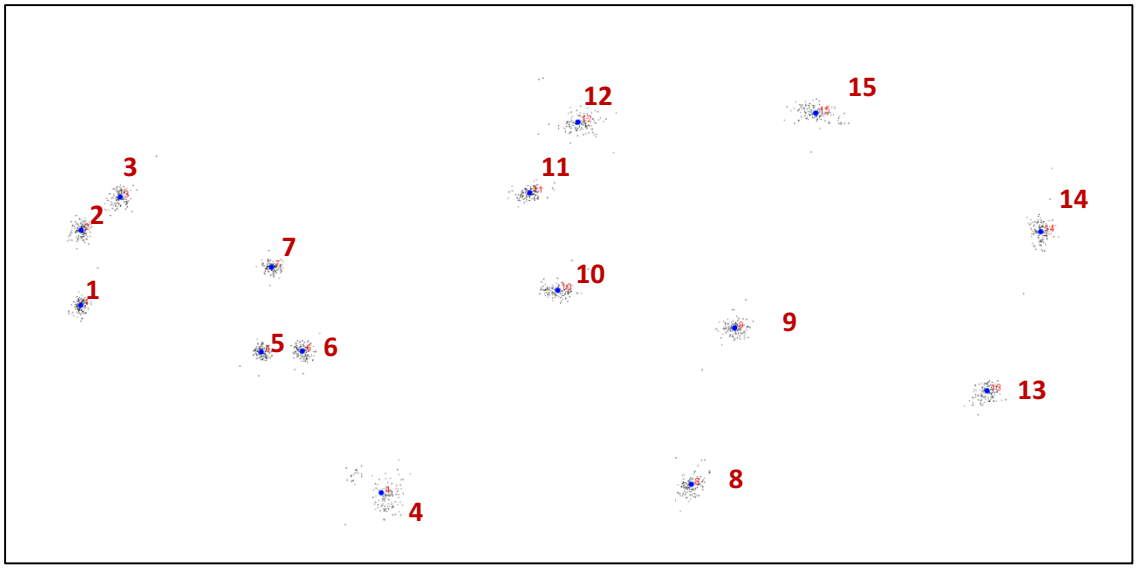



**Figure 4.6** Procrustes superimposition showing the variation of 15 landmarks of *Zeugodacus cucurbitae* wings from 200 individuals reared from ten host plant species. The figure shows the configurations of landmarks for which differences in position, scale and orientation have been removed. Numbered dots represent the average position for each landmark. Each 'cloud' of points around average landmark positions represent individual landmarks for each fly included in the analysis. The position of the landmarks in relation to a real wing is shown in Figure 2.4.

*Determination of allometric effect*

The regression of wing shape on centroid size revealed a statistically significant allometric effect ($P < 0.0001$), accounting for 8.21 % of shape variation (Figure 4.7). This allometric effect indicated there is a weak, significant relationship between size and shape. Subsequent multivariate analyses were conducted on data corrected to take the allometric effect into account.

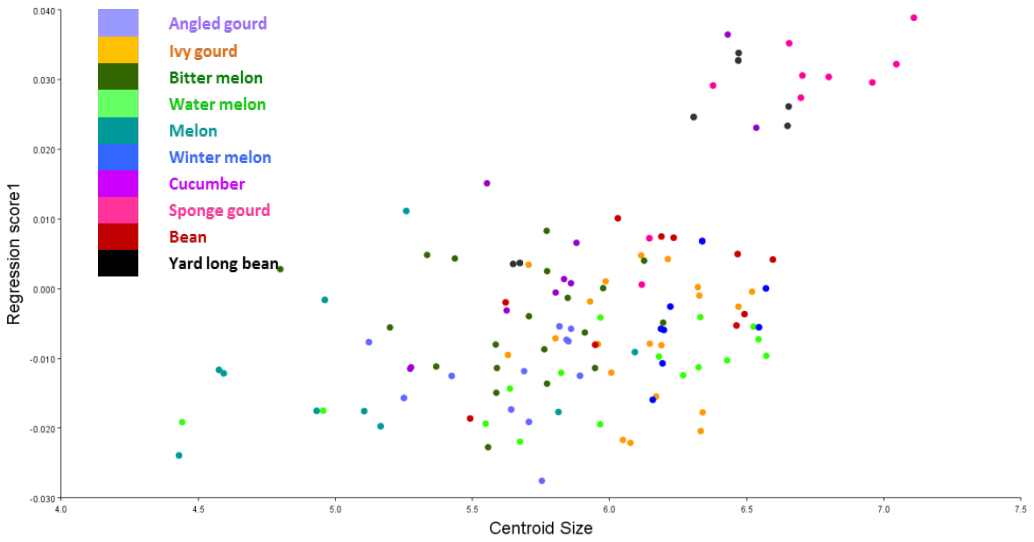


**Figure 4.7** Multiple regression of wing shape (regression score one) on centroid size for *Zeugodacus cucurbitae* reared from ten host plant species. Each coloured dot represents the wing of a fly from one of the listed hosts.

*Wing shape analyses*

- Principal component analysis

Principal component analysis was conducted on the 20 wing landmarks following Procrustes superimposition and correction for allometric effects. The first two Principal components accounted for 27% and 15% of the variance, respectively, for a total of 42% (Figure 4.8 and Table 4.3). While there was considerable overlap among groups of flies reared from different host plants, there are distinct differences in wing shape between flies reared from some fruit types. Flies reared from sponge gourd, for example, were somehow separated along the first principal component from those reared from winter melon, angled gourd, watermelon, ivy gourd, and melon; while overlapping at least in part with cucumber, bitter melon, yard long bean, and bean (Figure 4.9). Flies from melon in this case strongly overlap with flies from many other host plant species and do not, emerge as different, which is contrary to what is seen in centroid size data.

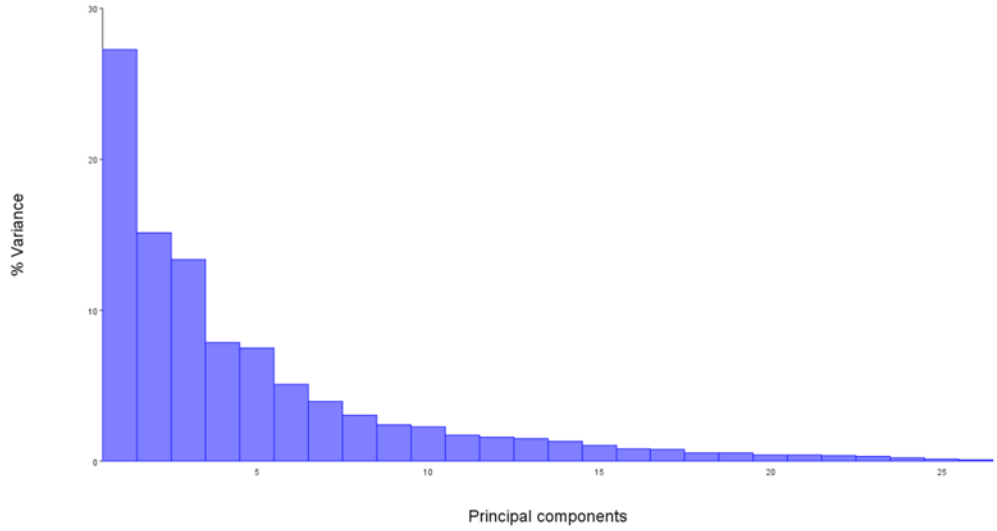



**Figure 4.8** Histogram illustrating the variances of all the principle components calculated from wing shape data of *Zeugodacus cucurbitae* reared from ten host plant species.

**Table 4.3** Summary statistics for principal component analysis of groups of *Zeugodacus cucurbitae* reared from ten host plants species. The table shows eigenvalues, percent variation and cumulative percentage of variance explained by each principal component axis (PC) for the entire dataset.

| PC | Eigenvalues | % Variance | Cumulative % |
|---|---|---|---|
| 1 | 0.00033230 | 27.237 | 27.237 |
| 2 | 0.00018493 | 15.157 | 42.394 |
| 3 | 0.00016324 | 13.380 | 55.774 |
| 4 | 0.00009596 | 7.865 | 63.639 |
| 5 | 0.00009159 | 7.507 | 71.146 |
| 6 | 0.00006196 | 5.079 | 76.225 |
| 7 | 0.00004815 | 3.947 | 80.172 |
| 8 | 0.00003705 | 3.037 | 83.209 |
| 9 | 0.00002960 | 2.426 | 85.635 |
| 10 | 0.00002763 | 2.265 | 87.900 |
| 11 | 0.00002106 | 1.726 | 89.626 |
| 12 | 0.00001969 | 1.614 | 91.240 |
| 13 | 0.00001858 | 1.523 | 92.763 |
| 14 | 0.00001605 | 1.316 | 94.079 |
| 15 | 0.00001308 | 1.072 | 95.151 |
| 16 | 0.00001033 | 0.847 | 95.998 |
| 17 | 0.00000941 | 0.772 | 96.769 |
| 18 | 0.00000708 | 0.580 | 97.349 |
| 19 | 0.00000684 | 0.560 | 97.910 |
| 20 | 0.00000540 | 0.442 | 98.352 |
| 21 | 0.00000527 | 0.432 | 98.784 |
| 22 | 0.00000460 | 0.377 | 99.160 |
| 23 | 0.00000402 | 0.329 | 99.490 |
| 24 | 0.00000294 | 0.241 | 99.731 |
| 25 | 0.00000188 | 0.154 | 99.884 |
| 26 | 0.00000141 | 0.116 | 100 |

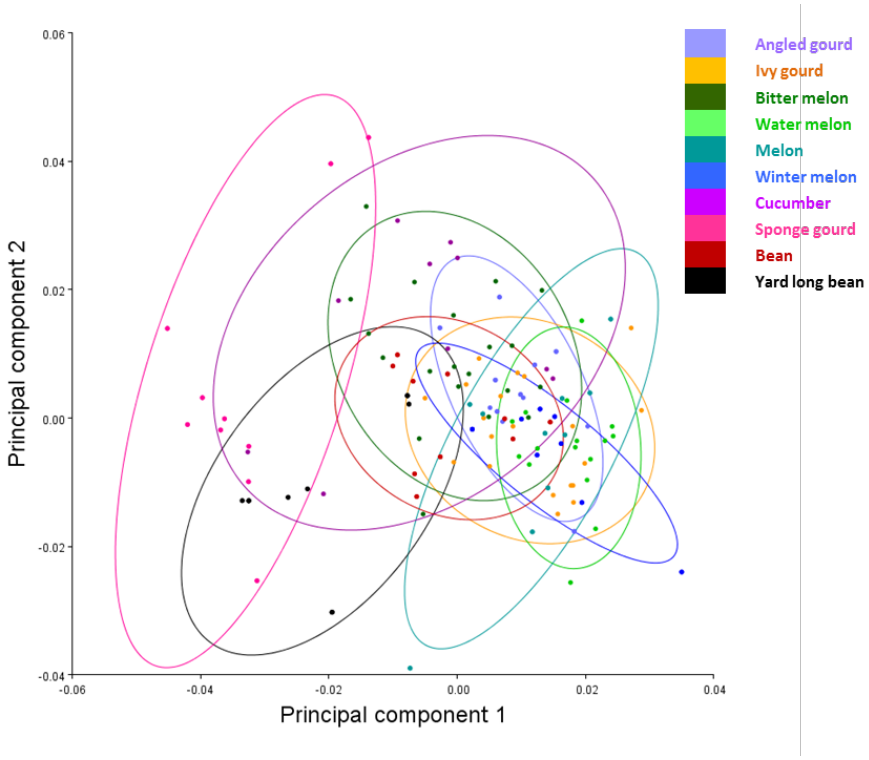



**Figure 4.9** First two principal components resulting from PCA of wing shape data of *Zeugodacus cucurbitae* reared from ten host plants species; 95% confidence ellipses are shown for each group. Each coloured dot represents the wing of a fly from one of the listed hosts.

- Canonical variates analysis

Nine canonical variates were obtained for wing shape data; the first three explained 76.41 % of the variation (Table 4.4 and Figure 4.10) and revealed varying degrees of separation of populations from the different host plants. Some groups are resolved from each other, and the same host plant groups are strongly separated from each other as in principal components analysis. There was a distinct separation of groups of flies from sponge gourd, winter melon and yard long bean (Figure 4.10A). Flies from yard long bean graphically stand apart on CV1 and CV2 and this is also supported by Mahalanobis distances. Group Mahalanobis distances were significantly different for all comparisons (Table 4.5). On the basis of Mahalanobis distances, the shape of the wings of flies from yard long bean were the most different to the shape of wings of flies from other host plant species, particularly flies from winter melon (Table 4.5). Wireframe deformations illustrate

relative shifts of the landmarks from the starting to the target shape and indicated the greatest movement of landmark four along the edge area of the wings in CV1 and CV2 (Figure 4.10B).

**Table 4.4** Summary statistics for canonical variates analysis of groups of *Zeugodacus cucurbitae* reared from ten host plant species. The table shows eigenvalues, percent variation and cumulative percent variation explained by each canonical variate axis for the entire dataset.

| CV | Eigenvalues | % Variance | Cumulative % |
|---|---|---|---|
| 1 | 4.816 | 39.26 | 39.26 |
| 2 | 2.806 | 22.88 | 62.14 |
| 3 | 1.750 | 14.27 | 76.41 |
| 4 | 1.051 | 8.57 | 84.98 |
| 5 | 0.817 | 6.66 | 91.64 |
| 6 | 0.523 | 4.26 | 95.91 |
| 7 | 0.285 | 2.33 | 98.23 |
| 8 | 0.130 | 1.06 | 99.30 |
| 9 | 0.086 | 0.71 | 100.00 |

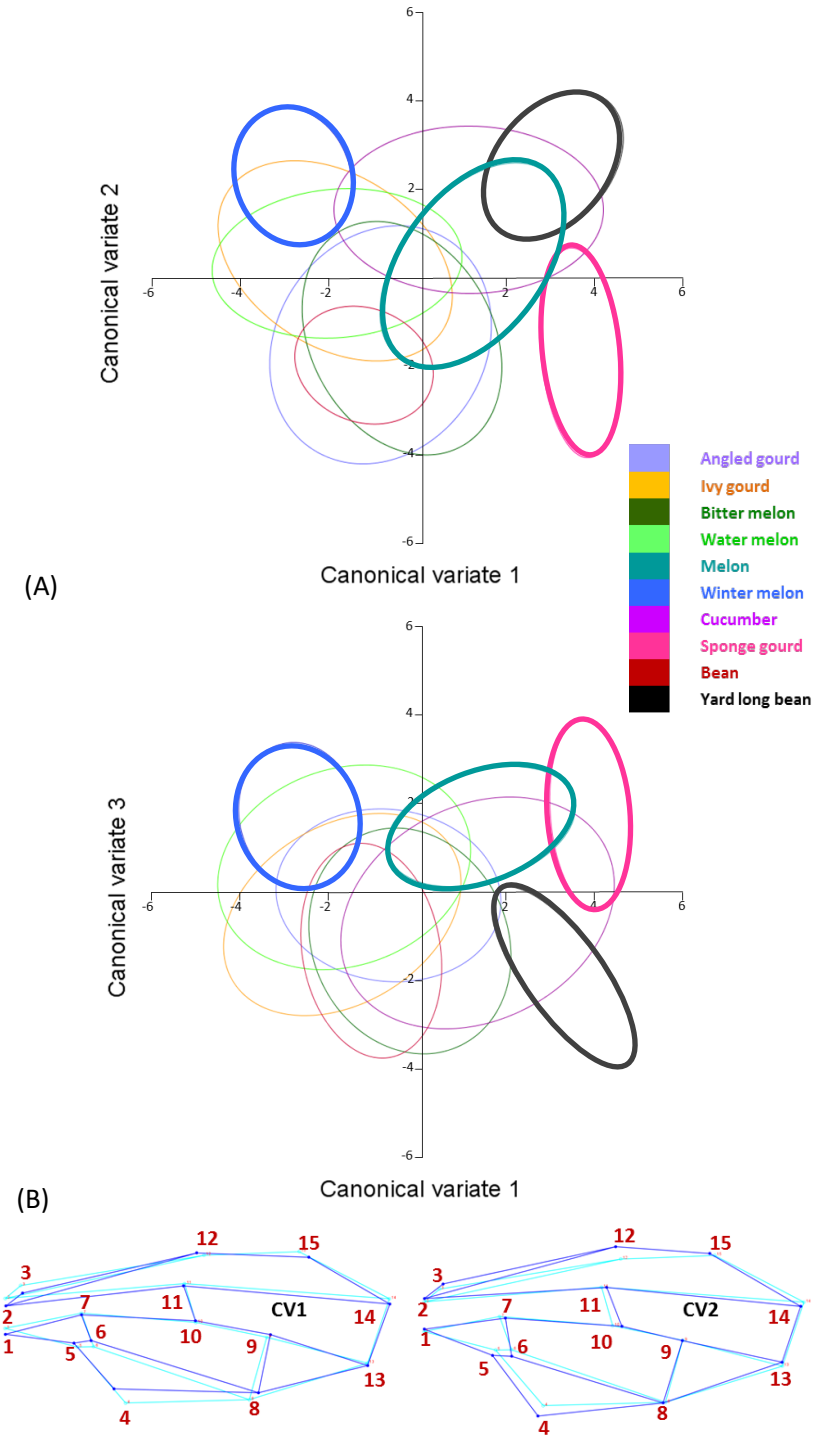



**Figure 4.10** A) Plots of the CV1 vs CV2 and CV2 vs CV3 based on *Zeugodacus cucurbitae* wing shape data subjected to Procrustes superimposition. 95% confidence ellipses are shown for each group, with individual data points removed for clarity. Individuals from yard long bean are shown in bold (B) Wireframe illustrations showing wing shape deformation along the first and second canonical variates.

**Table 4.5** Mahalanobis distances among ten groups of *Zeugodacus cucurbitae* reared from ten host plant species, as calculated from CVA. Values in bold represent significantly different groups following Bonferroni correction for multiple comparisons (initial α = 0.05 corrected to α = 0.001).

| | Host Plants | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Angled gourd | - | | | | | | | | |
| 2 | Ivy gourd | **2.820** | - | | | | | | | |
| 3 | Bitter melon | **2.637** | **3.486** | - | | | | | | |
| 4 | Watermelon | **3.175** | **2.188** | **3.684** | - | | | | | |
| 5 | Melon | **4.196** | **4.535** | **4.437** | **3.744** | - | | | | |
| 6 | Winter melon | **5.273** | **4.239** | **5.579** | **3.745** | **5.226** | - | | | |
| 7 | cucumber | **5.364** | **4.979** | **4.784** | **4.961** | **4.817** | **5.843** | - | | |
| 8 | Sponge gourd | **5.190** | **6.619** | **5.223** | **6.335** | **3.954** | **7.859** | **5.605** | - | |
| 9 | Bean | **3.679** | **4.370** | **3.355** | **4.169** | **4.769** | **5.912** | **5.870** | **6.362** | - |
| 10 | Yard long bean | **6.209** | **5.969** | **5.696** | **6.236** | **4.754** | **7.168** | **4.797** | **5.830** | **6.714** |

#### 4.3.1.2 Aedeagus morphometric analysis

Aedeagi significantly varied in length among the flies populations reared from the 10 plant species ($F_{9, 190}$ = 0.333; *P* < 0.001) (Figure 4.11). Aedeagi of flies from melon were significantly shorter than those from all other host plants except for winter melon, while the longest aedeagi were found in flies reared from sponge gourd, cucumber, watermelon, ivy gourd and the two bean hosts (Figure 4.11). There was a significant difference (*P* < 0.01) in aedeagus length for flies reared from the two host plant families Cucurbitaceae (2.487 ± 0.188 mm) and Fabaceae (2.629 ± 0.099 mm) ($t_{1, 198}$ = 4.608; *P* < 0.001) (Figure 4.11).

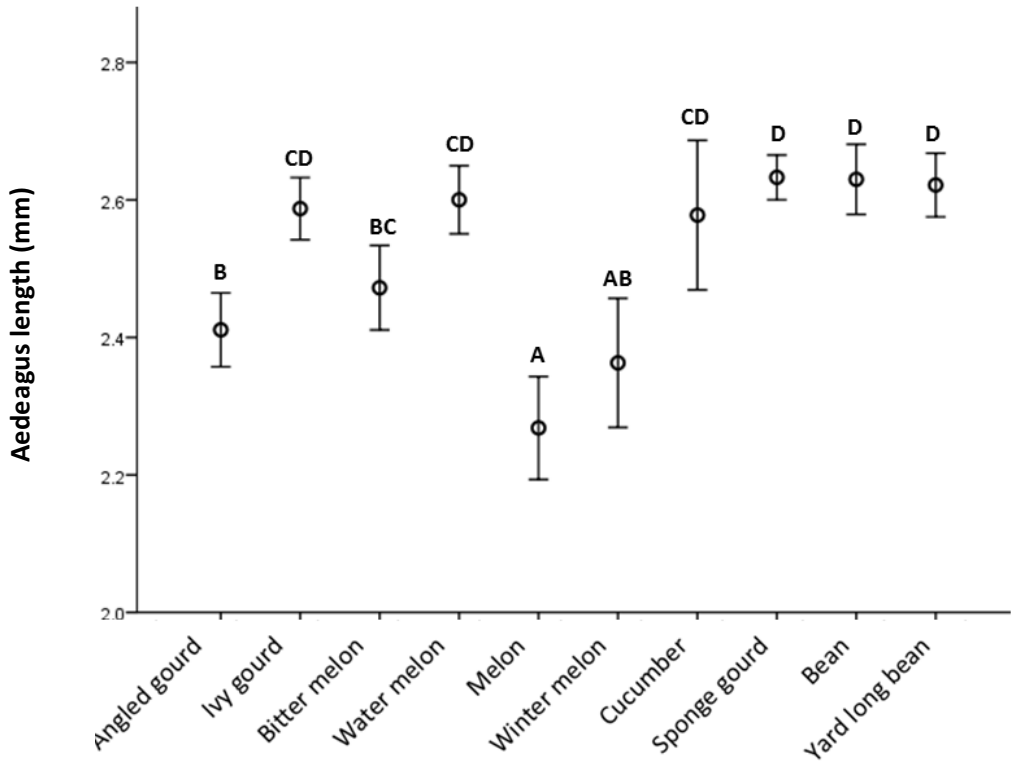



**Figure 4.11** Aedeagus length (mean ± SE) of *Zeugodacus cucurbitae* reared from ten host plant species from Thailand. Samples sharing the same letter are not statistically different from each other based on one-way ANOVA with Tukey *post hoc* test ($F_{9, 190}$ = 0.333; *P* < 0.001).

When aedeagal length was plotted against the wing size (centroid size), a significant, positive correlation was obtained ($R^2 = 0.226$; $P < 0.001$) (Figure 4.12).

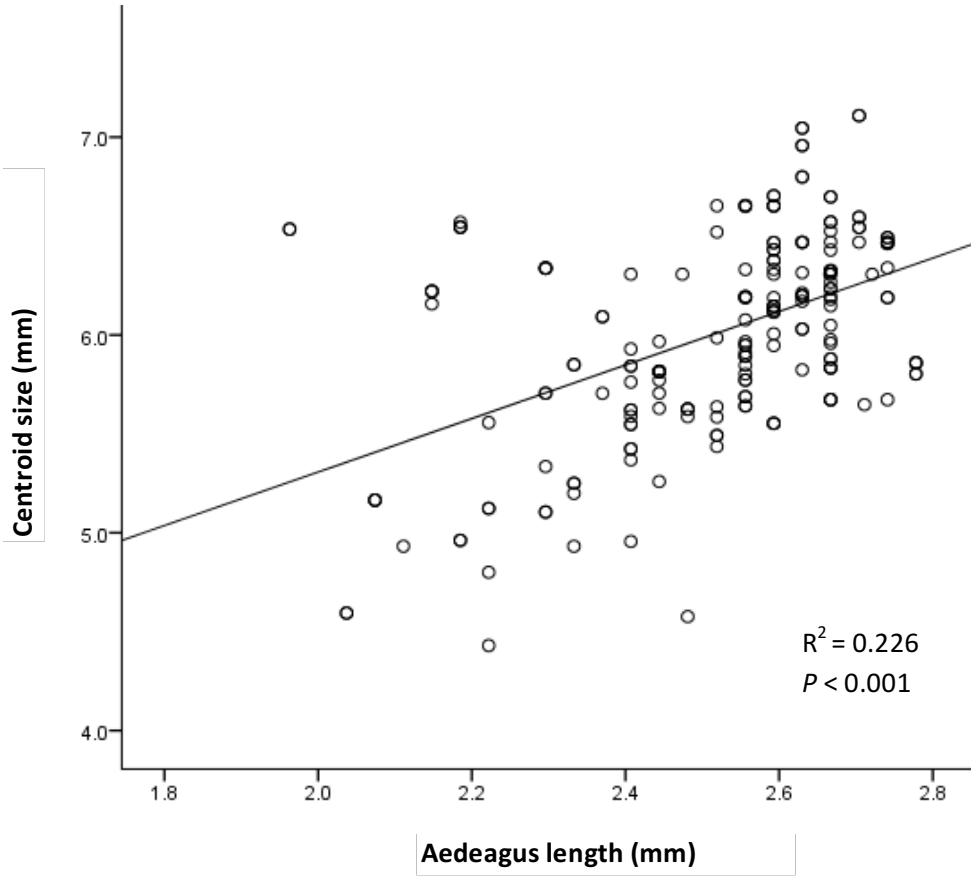



**Figure 4.12** Wing centroid size plotted against aedeagus length for *Zeugodacus cucurbitae* reared from ten host plant species.

### 4.3.2 Molecular results

#### 4.3.2.1 Mitochondrial DNA (*cox1*) analysis

*Gene diversity*

The same representatives of *Z. cucurbitae* from ten host plant species in the family Cucurbitaceae and Fabaceae that were used for morphometric analysis were sequenced at the *cox1* gene fragment (Table 4.6). The aligned sequences of *cox1* consisted of 652 characters and consisted of 13 unique haplotypes. The samples from bitter melon, cucumber and bean showed the greatest diversity with four haplotypes, with all other host plant species possessing only two or three haplotypes (Table 4.6). Only two of 13 haplotypes were found in more than one host plant species (Haplotypes 1 and 3), with Haplotype 3 found across all host plant species. Haplotype 1 (H1) was shared by all host plants except bean and yard long bean (Table 4.6).

Genetic diversity of *Z. cucurbitae* from melon was higher than in other host plants (gene diversity, 0.699 ± 0.043; population parameter θπ, 1.320; Table 4.7). *Zeugodacus cucurbitae* from bean had the lowest population genetic diversity (0.189 ± 0.108) and the lowest population parameter θπ (0.189). Tajima's *D* tests of neutrality were applied to the entire genetic data set, with the results negative and statistically significant (D = -1.015, $P < 0.0001$). Tests based on Fu's *Fs* were also negative and statistically significant across the data set ( F =-2.11, $P < 0.0001$) (Table 4.7).

**Table 4.6** Distribution of *cox1* haplotypes of *Zeugodacus cucurbitae* from ten host plant species collected from central Thailand, highlighted in grey haplotypes observed in more than one host plant.

| **Haplotype** | Angled gourd | Ivy gourd | Bitter melon | Watermelon | Melon | Winter melon | Cucumber | Sponge gourd | Bean | Yard long bean |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample size | 20 | 20 | 20 | 16 | 18 | 20 | 20 | 19 | 20 | 20 |
| Hap_1 | 12 | 12 | 4 | 3 | 6 | 10 | 11 | 10 | - | - |
| Hap_2 | 2 | - | - | - | - | - | - | - | - | - |
| Hap_3 | 6 | 8 | 12 | 11 | 7 | 10 | 4 | 5 | 18 | 8 |
| Hap_4 | - | - | 2 | - | - | - | - | - | - | - |
| Hap_5 | - | - | 2 | - | - | - | - | - | - | - |
| Hap_6 | - | - | - | 2 | - | - | - | - | - | - |
| Hap_7 | - | - | - | - | 5 | - | - | - | - | - |
| Hap_8 | - | - | - | - | - | - | - | - | 2 | - |
| Hap_9 | - | - | - | - | - | - | - | - | - | 8 |
| Hap_10 | - | - | - | - | - | - | - | - | - | 4 |
| Hap_11 | - | - | - | - | - | - | 3 | - | - | - |
| Hap_12 | - | - | - | - | - | - | 2 | - | - | - |
| Hap_13 | - | - | - | - | - | - | - | 4 | - | - |

**Table 4.7** Population genetics summary statistics from *cox1* analysis of *Zeugodacus cucurbitae* reared from ten host plant species collected in central Thailand.

| Host plant | number of individuals | Gene Diversity | Θ¶ | Tajima' s*D* | Tajima's D *P*-value | Fu' *Fs* | Fu'*Fs* *P*-value |
|---|---|---|---|---|---|---|---|
| Angled gourd | 20 | 0.568 ± 0.863 | 0.695 | 0.545 | 0.736 | 0.429 | 0.546 |
| Ivy gourd | 20 | 0.505 ± 0.056 | 0.505 | 1.430 | 0.945 | 1.409 | 0.698 |
| Bitter melon | 20 | 0.610 ± 0.103 | 0.716 | -0.410 | 0.370 | -0.764 | 0.253 |
| Water melon | 16 | 0.508 ± 0126 | 0.558 | -0.189 | 0.396 | -0.176 | 0.336 |
| Melon | 18 | 0.699 ± 0.043 | 1.320 | 1.424 | 0.923 | 1.795 | 0.839 |
| Winter melon | 20 | 0.526 ± 0.036 | 0.526 | 1.565 | 0.975 | 1.486 | 0.740 |
| Cucumber | 20 | 0.658 ± 0.092 | 0.979 | 0.421 | 0.716 | -0.058 | 0.479 |
| Sponge gourd | 19 | 0.643 ± 0.074 | 0.760 | 0.785 | 0.794 | 0.571 | 0.571 |
| Bean | 20 | 0.189 ± 0.108 | 0.189 | -0.592 | 0.234 | -0.097 | 0.220 |
| Yard long bean | 20 | 0.673 ± 0.049 | 0.842 | 1.158 | 0.879 | 0.834 | 0.666 |

A median-joining network of 193 sequences revealed no structuring of haplotypes according to host plant (Figure 4.13). Most haplotypes were connected with each other by branches of short lengths, except for a single haplotype recovered from flies reared from melon that was separated by two mutations. Overall, the network had a starburst-like pattern centered on two common haplotypes (H1 and H3), with 11 singletons radiating from these haplotypes. The network did not show any distinct pattern between the haplotypes and their host plant species or family (Table 4.8, Figure 4.13 and 4.14). However, haplotype 1 was not recovered from any flies from host plants in family Fabaceae. As for the morphometric data, the difference between the number of unique haplotypes of flies from the two host plant families might be driven by differences in sample sizes (Cucurbitaceae, n = 153; Fabaceae, n = 40).

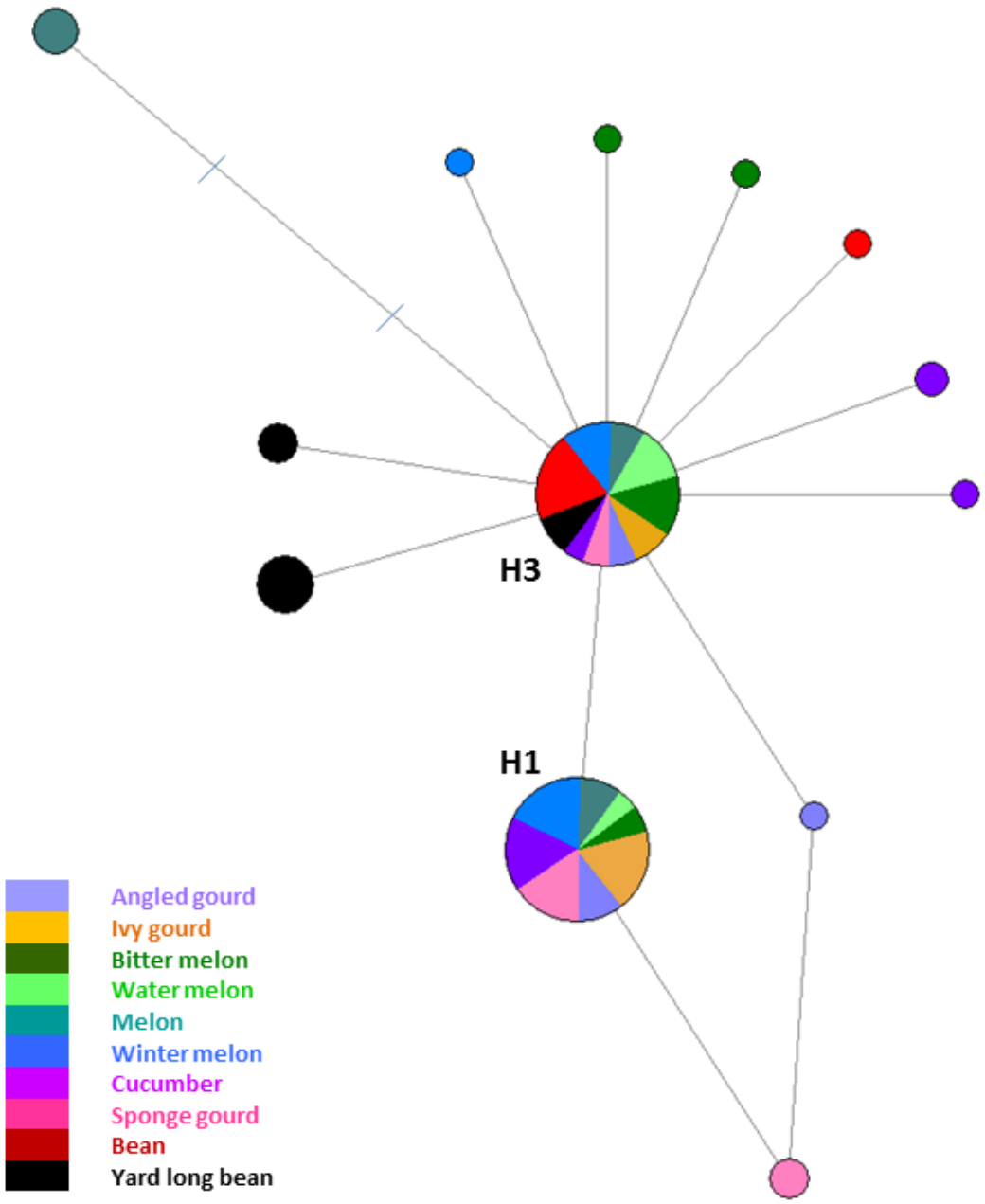



**Figure 4.13** Median-joining haplotype network generated from *Zeugodacus cucurbitae* collected from ten host plant species. The 13 *cox1* haplotypes are colour coded by plant species. Sizes of nodes and pie segments are proportional to haplotype frequency. Length of branches is proportional to a number of mutational changes between haplotypes.

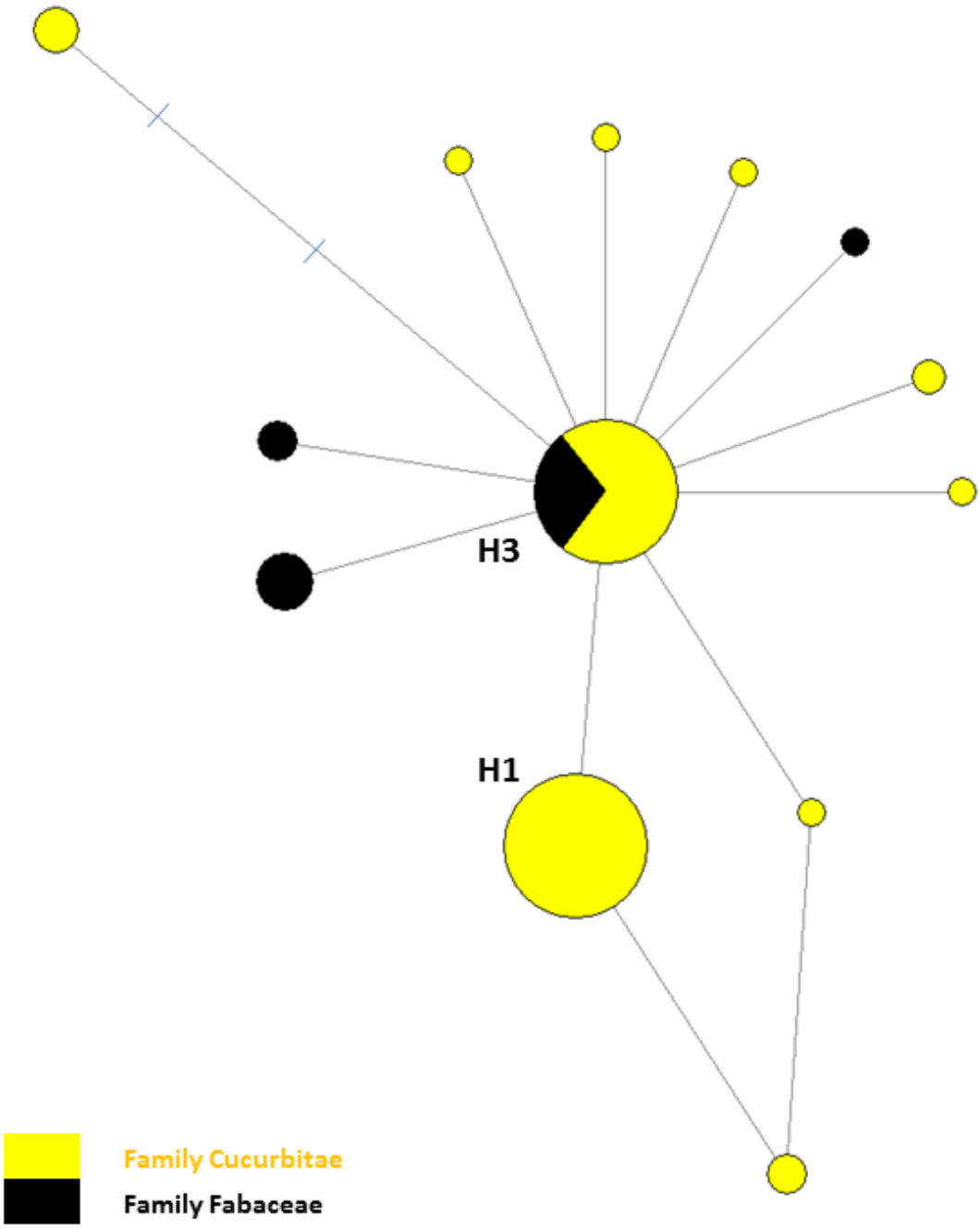



**Figure 4.14** Median-joining haplotype network generated from *Zeugodacus cucurbitae* collected form ten host plant species. The 13 *cox1* haplotypes are colour coded by plant family. Sizes of nodes and pie segments are proportional to haplotype frequency. Length of branches is proportional to a number of mutational changes between haplotypes.

*Genetic differentiation (cox1)*

Overall, pairwise $F_{ST}$ values among different host plants were generally low and the majority of pairwise comparisons were non-significant (Table 4.8). The proportion of variance among groups of ten different host plants was 7.29% (Table 4.9); however, this was not statistically significant. In contrast, both variations among populations within groups (18.48%), and within groups (74.24%) were significantly different.

**Table 4.8** Pairwise $F_{ST}$ distances among *Zeugodacus cucurbitae* reared from ten host plant species. Values in bold represent significantly different groups following Bonferroni correction for multiple comparisons (initial α = 0.05 corrected to α = 0.001).

| | Host plants | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Angled gourd | - | | | | | | | | |
| 2 | Ivy gourd | **-0.03449** | - | | | | | | | |
| 3 | Bitter melon | 0.18024 | 0.19694 | - | | | | | | |
| 4 | Watermelon | 0.20179 | 0.22825 | -0.00078 | - | | | | | |
| 5 | Melon | 0.15651 | **0.16728** | 0.12212 | **0.12234** | - | | | | |
| 6 | Winter melon | **-0.01755** | **-0.03158** | **0.11303** | **0.13331** | **0.13004** | - | | | |
| 7 | cucumber | 0.44759 | 0.50399 | 0.09458 | 0.10392 | 0.24639 | 0.40373 | - | | |
| 8 | Sponge gourd | 0.40808 | 0.43723 | 0.2201 | 0.22514 | 0.2768 | 0.37658 | 0.26051 | - | |
| 9 | bean | **0.00387** | **-0.00283** | 0.13539 | **0.14534** | 0.13247 | **-0.00359** | 0.35061 | 0.34902 | - |
| 10 | Yard long bean | **-0.00881** | **0.04657** | 0.29942 | 0.32624 | 0.22898 | **0.09547** | 0.55123 | 0.48128 | **0.07153** |

**Table 4.9** Results of the Analysis of Molecular Variance (AMOVA) for the *cox1* dataset generated for Thai *Zeugodacus cucurbitae* populations. Samples were constrained according to host plant species.

| Source of variation | d.f. | Sum of square | Variance of components | % total variance | Fixation indices |
|---|---|---|---|---|---|
| Among groups | 1 | 4.242 | 0.03481 a | 7.29 | $F_{CT}$ = 0.073 |
| Among populations within groups | 8 | 16.437 | 0.08824 b | 18.48 | $F_{ST}$ = 0.258* |
| Within populations | 183 | 64.881 | 0.35454 c | 74.24 | $F_{SC}$ = 0.199* |
| Total | 192 | 85.558 | 0.47759 | | |

*$P < 0.05$

### 4.3.2.2 Microsatellite analysis

*Gene diversity*

Microsatellite markers (11 loci) were used to assess contemporary population structure in *Z. cucurbitae*, among a total of 200 individuals screened from ten host plants. The number of alleles per locus varied from 2.55 (yard long bean) to 3.45 (ivy gourd) while the allelic richness ranged from 2.35 (yard long bean) to 3.14 (ivy gourd). Moderate to high levels of intra-population diversity were reflected in the observed heterozygosity, which varied from 0.40 (angled gourd) to 0.55 (ivy gourd). Moreover, average gene diversity over all loci per sample site ranged from 0.410 ± 0.235 (yard long bean) to 0.538 ± 0.297 (ivy gourd) (Table 4.10).

**Table 4.10** Host plant, sample sizes, microsatellite diversity estimates and Hardy-Weinberg (H-W) equilibrium test values for *Zeugodacus cucurbitae* populations reared from ten different host plant species. No values were significant after Bonferroni correction for multiple tests (initial $\alpha = 0.05$ corrected to $\alpha = 0.001$).

| Locations | Sample size | Expected heterozygosity | Observed heterozygosity | No Alleles | Allelic Richness | Fis | H-W | Gene Diversity |
|---|---|---|---|---|---|---|---|---|
| Angled gourd | 20 | 0.4848 | 0.4000 | 3.18 | 2.89 | 0.18 | < 0.0001 | 0.493 ± 0.272 |
| Ivy gourd | 20 | 0.5317 | 0.5545 | 3.45 | 3.14 | -0.04 | < 0.0001 | 0.538 ± 0.294 |
| Bitter melon | 20 | 0.4455 | 0.4606 | 3.27 | 2.87 | -0.03 | < 0.0001 | 0.436 ± 0.244 |
| Water melon | 20 | 0.4521 | 0.4364 | 3.18 | 2.89 | 0.04 | < 0.0001 | 0.463 ± 0.257 |
| Melon | 20 | 0.4659 | 0.5182 | 3.27 | 2.92 | -0.12 | < 0.0001 | 0.476 ± 0.264 |
| Winter melon | 20 | 0.3975 | 0.4295 | 2.91 | 2.63 | -0.08 | < 0.0001 | 0.425 ± 0.239 |
| Cucumber | 20 | 0.4323 | 0.4267 | 3.36 | 2.95 | 0.01 | < 0.0001 | 0.487 ± 0.273 |
| Sponge gourd | 20 | 0.4600 | 0.4591 | 3.00 | 2.68 | 0.00 | < 0.0001 | 0.500 ± 0.279 |
| Bean | 20 | 0.4017 | 0.4682 | 2.64 | 2.44 | -0.17 | < 0.0001 | 0.442 ± 0.247 |
| Yard long bean | 20 | 0.3859 | 0.4020 | 2.55 | 2.35 | -0.04 | 0.0019 | 0.410 ± 0.235 |

*Genetic differentiation and relationships among populations*

Population pairwise $R_{ST}$ analysis revealed that 55.55% of populations were not significantly different genetically (64.44%) (Table 4.11). Variation among groups of flies reared from ten different host plants was not significant ($F_{CT}$ = 0.004, $P$ > 0.05). The results indicated that 0.39% of the genetic variation was partitioned among the 10 melon fly populations from ten host plant species, 9.97% among ten different host plants within groups, and 90.42% within host plant (Table 4.12).

**Table 4.11** Pairwise $R_{ST}$ distances between populations of *Zeugodacus cucurbitae* reared from ten different host plant species. Values in bold represent significantly different groups following Bonferroni correction for multiple comparisons (initial α = 0.05 corrected to α = 0.001).

| | Host plants | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Angled gourd | - | | | | | | | | |
| 2 | Ivy gourd | **0.0197** | - | | | | | | | |
| 3 | Bitter melon | **0.0536** | **0.0335** | - | | | | | | |
| 4 | Water melon | **0.0164** | 0.0727 | **0.0250** | - | | | | | |
| 5 | Melon | 0.2252 | 0.2045 | 0.0934 | 0.1235 | - | | | | |
| 6 | Winter melon | 0.1810 | 0.1550 | 0.0568 | 0.1112 | **0.0274** | - | | | |
| 7 | Cucumber | 0.0797 | **0.0146** | **-0.0043** | **0.0729** | 0.1530 | 0.1022 | - | | |
| 8 | Sponge gourd | 0.0567 | **0.0526** | **-0.0029** | **0.0320** | 0.0945 | **0.0364** | **0.0293** | - | |
| 9 | Bean | **0.0107** | 0.0758 | **0.0776** | **0.0063** | 0.1769 | 0.1695 | 0.1068 | **0.0922** | - |
| 10 | Yard long bean | 0.1057 | 0.0692 | 0.0568 | **0.0932** | 0.0863 | **0.0833** | 0.0620 | 0.0540 | 0.1053 |

**Table 4.12** Analysis of Molecular Variance (AMOVA) among *Zeugodacus cucurbitae* collected from ten host plant species.

| Source of variation | Sum of square | Variance of components | % total variance | Fixation indices |
|---|---|---|---|---|
| Among groups | 2073.490 | -1.94064 | -0.39799 | $F_{CT}$ = -0.00398 |
| Among populations within groups | 16745.139 | 48.63927 | 9.97498 | $F_{ST}$ = 0.09577* |
| Within populations | 159439.272 | 440.91388 | 90.42300 | $F_{SC}$ = 0.09935* |
| Total | 178257.900 | 487.61251 | | |

*$P < 0.05$

*Population structure using Bayesian clustering*

Principal components analysis of 11 loci (Figure 4.15) and an unrooted neighbour-joining dendrogram (Figure 4.16) produced similar results. No distinct groups could be discerned that corresponded to any of the ten host plants. Principal components analysis, which explained 48.85% of the variation in the data, did not reveal any specific structuring of populations across two axes. The first principal component explained over 25.06% of the variation and the secondary, and more subtle pattern of structure among some of the remaining populations, was explained by the second principal component (23.79%). The data suggested some differentiation between flies from winter melon on principal components 1 and 2, and from sponge gourd and yard long bean on the first principal component, while flies from bean separated on the first and the second principal components.

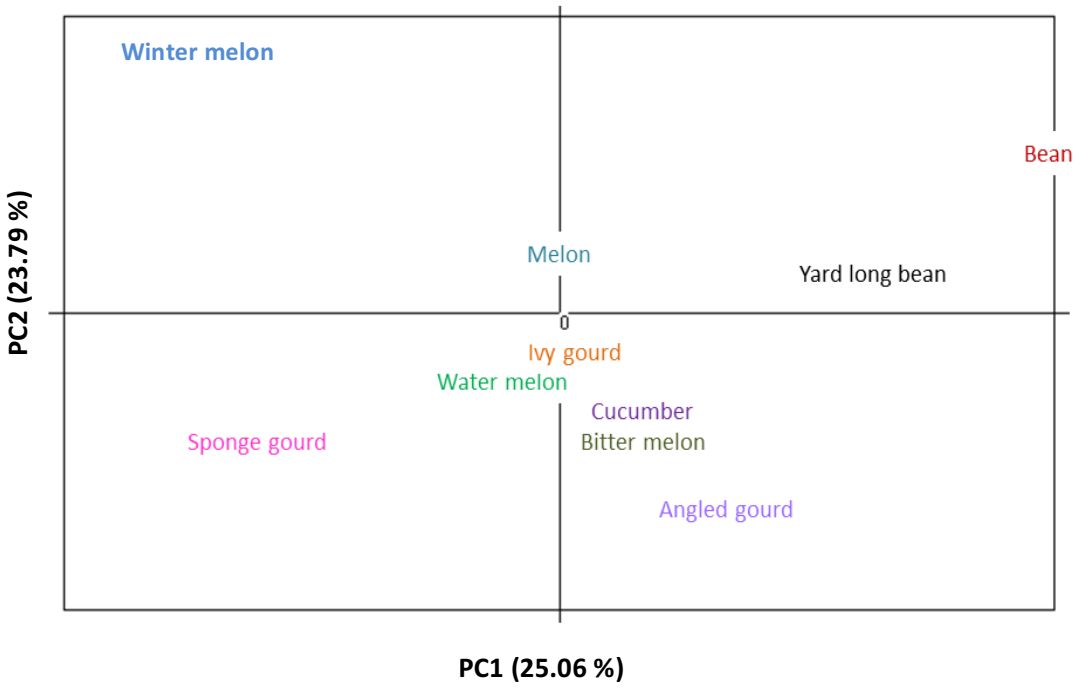



**Figure 4.15** Principal components analysis (PCA) plots based on 11 microsatellites loci. PCA of *Zeugodacus cucurbitae* based on allelic frequencies (11 loci) from ten host plant species. The population groups are indicated with different coloured lines corresponding to the host plant species identified using STRUCTURE.

The neighbor-joining tree was calculated based on genetic distance (Cavalli-Sforza & Edwards) from 11 loci among ten sampled melon fly groups. The relationships are generally consistent with the results of the analysis of the same microsatellite data using PCA. The unrooted neighbour-joining dendrogram did not reveal any clear pattern of structure among host plants; however, populations of melon fly from family Fabaceae (Yard long bean and bean) were strongly supported as sister groups (Figure 4.16).

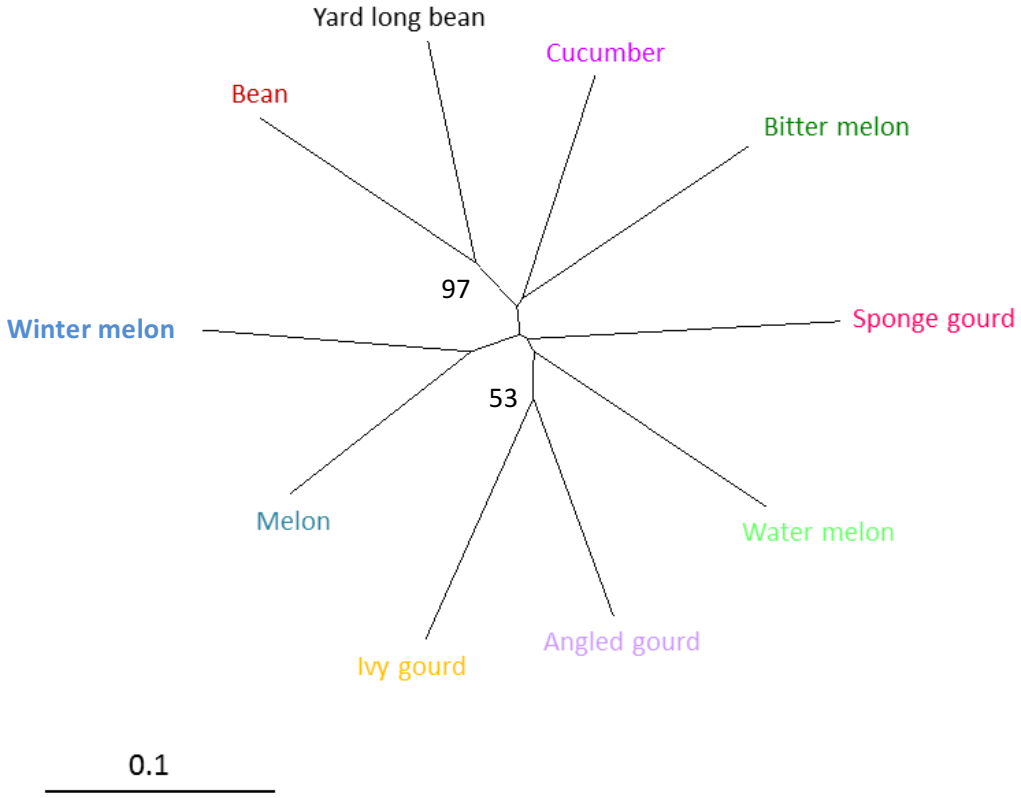
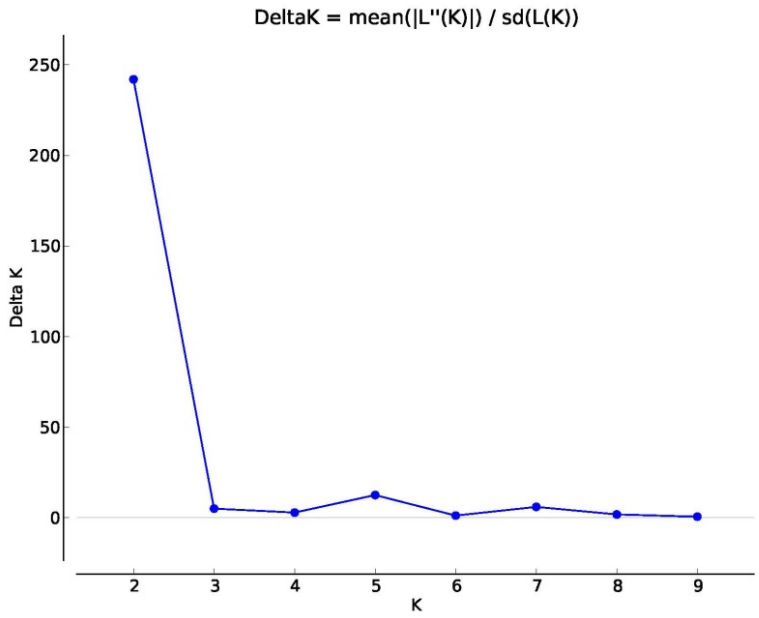



**Figure 4.16** Neighbour-joining unrooted tree of *Zeugodacus cucurbitae* reared from ten host plant species based on Carvalli-Sforza & Edwards (1967) distances matrix. Bootstrap values were calculated using 1000 replications and are given as percentage, with only values greater than 50% shown.

Population structure was analysed using the program STRUCTURE. As noted in the material and method section of Chapter 2, STRUCTURE requires users to decide which value of *K* suits their data best, guided by the relationships of L(*K*) and Δ*K* to each *K* value. Typically, either the *K* value at which L(*K*) begins to plateau, or that which corresponds to the median value of Δ*K* represents the optimum *K* value. However, it is also recommended that where hierarchical structure exists the highest value of *K* that contains biologically informative structure be used (Pritchard et al., 2000). The Evanno et al. (2005) method indicated the highest delta *K* as the optimal value of *K* given the data. This method clearly supported a *K*=2 scenario (Figure 4.17); however, in reality only a single large cluster dominated the ancestry of all sampled individuals (Figure 4.18). There was only weak evidence, even for the

winter melon population, as possessing ancestry from a second genetic cluster. In total, this analysis implied no genetic structure among populations from the 10 host plant species and suggested instead that all individuals sampled were of mixed ancestry.

(A)




(B)

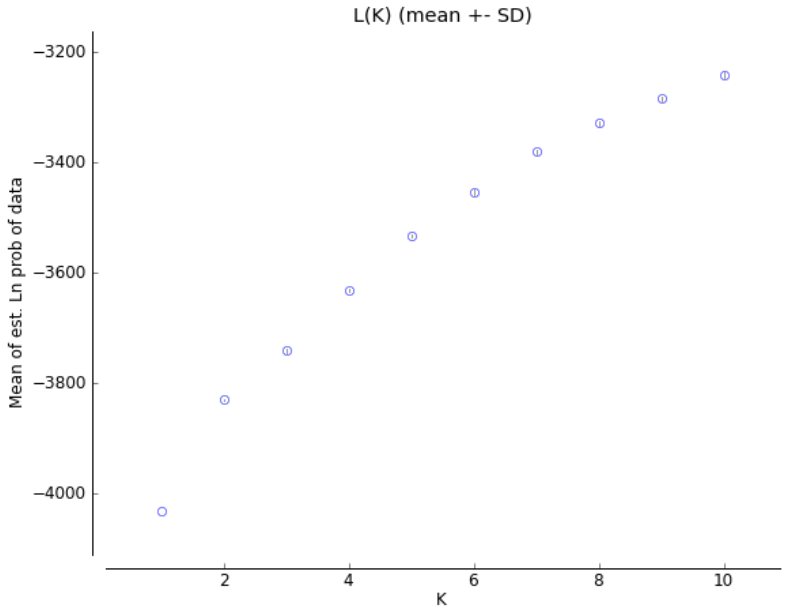



**Figure 4.17** Bayesian clustering results for populations of *Zeugodacus cucurbitae* collected from 10 host plants in central Thailand; (A) plot of Δ*K* with the median value which is most highly supported as the optimum value of *K* for the analysed sample (B) Mean of log probability of data (LnP (D)).

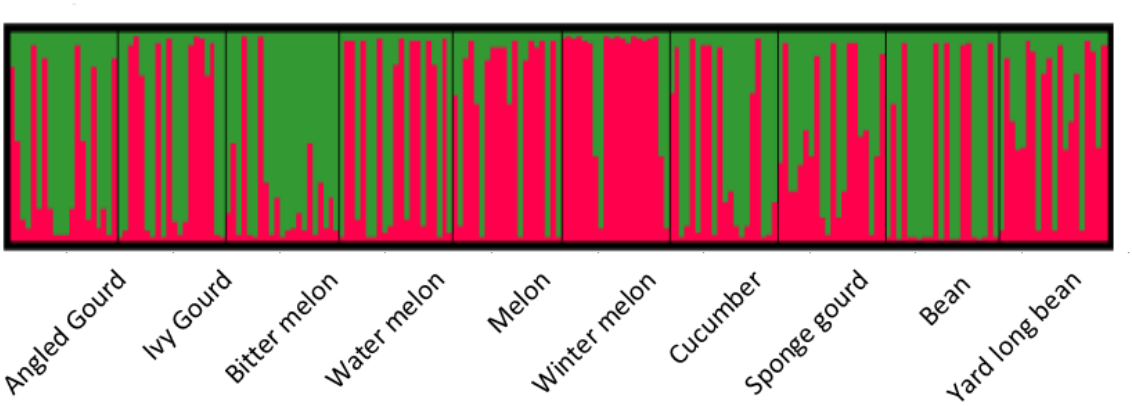



**Figure 4.18** Structure bar plot of Bayesian cluster analysis based on 11 microsatellite loci obtained from *Zeugodacus cucurbitae* collected from ten host plant species from central Thailand. The plots population assignment results for different values of *K* = 2. Each horizontal line represents a single individual and its cluster assignment to a particular cluster is given as a particular colour. Solid black lines separate individuals from each of ten host plant species.

## 4.4 DISCUSSION

The results revealed subtle morphological variability in melon flies from the various host plant species sampled, including variation in wing size, wing shape and aedeagus length. Wing centroid size of flies reared from sponge gourd was greater than all other host plants except yard long bean; while melon fly reared from melon is smallest and different from all other host plants. The wing shape of flies reared from sponge gourd, winter melon and yard long bean were different from the wing shapes of other flies. Aedeagus length of flies from melon was shorter than others except for winter melon. For genetic data, there was no obvious pattern in the *cox1* data. However, microsatellite sequences showed some structure in principal components analysis, with flies from winter melon, sponge gourd and bean possibly separated from flies other host plants. This corroborates the result from STRUCTURE which revealed winter melon flies to be slightly different to flies from other hosts.

### 4.4.1 Environment, phenotype and genotype

*Enviornmentally induced phenotypic variation*

Morphological variation within species is typically continuous, rather than discrete (Davidowitz & Nijhout, 2004). When affected by the environment this variation is known as “phenotypic plasticity” and, while this plasticity is influenced by intrinsic factors (gene expression) and a variety of extrinsic environmental factors (Raser & O'Shea, 2005), nutrition and temperature are considered the main drivers inducing such variation in insects (Chown & Gaston, 2009). In general, insects respond to temperature and nutritional resources in broadly similar ways. Thus, in addition to temperature, nutrition during development is a key factor influencing adult size in insects-commonly, warmer conditions and an abundance of food result in larger individuals.

It is not surprising to find intraspecific variation in tephritids, including variation in body size, because these insects are considered to be heavily influenced by host types phenology, and abiotic environmental conditions (Hooper, 1978; Krainacker et al., 1987). Developmental conditions experienced by tephritid larvae

may affect morphological variation and even behaviour in the adults (Bower, 1977; Shanmugam et al., 2002; Navarro-Campos et al., 2011). For example, the Mediterranean fruit fly, *C. capitata*, size is influenced by temperature and nutrition, with low temperatures and high nutrient food (such as increased sugar) during development, having a marked effect resulting in larger adults (Kaspi et al., 2000).

While variation in morphology and, particularly variation in wing shape, has provided fresh insight into biological differentiation within taxonomically challenging species complexes within the tephritids; such as the *B. dorsalis* (Krosch et al., 2013; Schutze et al., 2015a, 2015b) and *Z. tau* complexes (Dujardin & Kitthawee, 2013), it is perhaps inappropriate to apply this approach to *Z. cucurbitae*. The results from my study of *Z. cucurbitae* from central Thailand, showing host derived wing shape variation, is similar to those from a study of variation in wing size and shape in *B. dorsalis* (as *B. invadens*) reared from different host plants in Uganda (Isabir et al., 2013). Also while I detected significant differences in mean male aedeagus length between populations of flies from different hosts, and such differences have been used in *Bactrocera* as evidence for species delimitation (Drew & Hancock 1994; Drew et al., 2008), I also found a simple linear correlation between male genitalia and wing size (and thus body size) independent of rearing host. So this again suggests a direct environmental effect on trait variation, rather than evidence for underlying genetic variation. While my study also detected differences in wing size and aedeagus length of flies from the Cucurbitaceae and Fabaceae, this could be an artifact of differences in sample size (Cucurbitaceae, n = 160; Fabaceae, n = 40), and does not appear to be reflected in major differences at the host species level. As my sampling protocol controlled for geographic effect, and detected minimal host associated genetic differences (discussion following), I interpret the morphological variation found to be evidence of direct host (i.e., environment) effect, rather than evidence of host-associated races or cryptic taxa.

*Genetic variation*

Since morphological variation may not a reliable indicator of the existence of host races, evidence for host races through variation in *cox1* and microsatellite sequences was sought. Genetic results are consistent with the morphological data:

of all hosts, flies reared from winter melon displayed a low level of differentiation from flies reared from other hosts with respect to both wing shape and microsatellite variation. However, the median-joining network based on *cox1* haplotypes for flies from the ten different host plants revealed that most individuals were genetically closely related. The levels of genetic variation observed across host-plant species were low. An exception to this low level of genetic variation came from flies reared on beans (Fabaceae). While several haplotypes were shared by flies from different host plants, flies from beans did not share the common haplotype 1. As is the case for the morphometric data, the observed differences in the number of unique haplotypes of flies from the two host plant families might be driven by differences in sample sizes.

AMOVA based on *cox1* and microsatellite data revealed no genetic differentiation among *Z. cucurbitae* from different host plant species. These results indicate that *Z. cucurbitae* distribute themselves indiscriminately across these host plants in central Thailand. Population pairwise $F_{ST}$ values based on *cox1* and $R_{ST}$ from microsatellite sequences indicated an overall, low level of genetic structure in *Z. cucurbitae* in central Thailand; this suggests considerable gene flow among populations.

Tajima's *D* tests of neutrality were applied to the entire genetic data set, with the results negative and statistically significant. This could indicate that the sequences are under selection, or it could be that populations have expanded relatively recently in historical times. Tests based on Fu's *Fs* were also negative and statistically significant across the data set; this also suggests relatively recent, population expansion rather than the existence of a genetic bottleneck. Perhaps it is not surprising to find no intraspecific variation as all ten host-plants belong either to the Cucurbitaceae or the Fabaceae. Those host-plants are very common in central Thailand and they effectively present a continuous habitat for the fly and thus promote gene flow among populations. These results would be strengthened by the inclusion of sequence data of flies from other host plants, such as the families Capparidaceae, Solanaceae and Combretaceae that have been reported for *Z. cucurbitae* (Allwood et al., 1999). However, we did not include these host plant

families in this study because we could not collect fresh specimens, as they very rare hosts for this species (Clarke et al., 2001).

*Is there evidence for host races in Z. cucurbitae in Thailand?*

Host race formation via host shifts has been well documented in only a limited number of examples (see below). Using generally accepted concepts, if host race formation has occurred, then gene flow among host-associated populations should be low and genetic differentiation between sympatric, host-associated populations should be detectable (Feder et al., 1988; McPheron et al., 1988; Waring et al., 1990). However, in the present study, the analysis of *cox1* and microsatellite data did not provide unequivocal evidence of genetic differentiation of the flies reared from different hosts, which suggests that these are not representatives of host races.

*Are there any clues indicating host associated cryptic species of Z. cucurbitae in Thailand?*

The answer to this question is essentially the same as the response to the preceding question regarding host races, and draws upon the same evidence. Melon fly, *Z. cucurbitae* and the pumpkin fly, *Z. tau*, are both economically important pests that attack mainly cucurbit fruits (Singh et al., 2010). These two fruit fly species have similar geographical distributions; similar host ranges and occupy more or less the same ecological niche (Ohno et al., 2008). It seems clear, based on morphological and genetic data, that for the ancestor of the *Z. tau* complex, genetic adaptation to specific host plant species and geographic isolation have played important roles in species differentiation, including the emergence of closely related cryptic species (Jamnongluk et al., 2003; Saelee et al., 2006; Kitthawee & Dujardin, 2010; Sumrandee et al., 2011; Kitthawee & Rungsri, 2011; Drew & Romig, 2013). In stark contrast, the results of my study do not show any strong evidence of morphological and genetic differences that would indicate the existence of host associated cryptic species in melon fly.

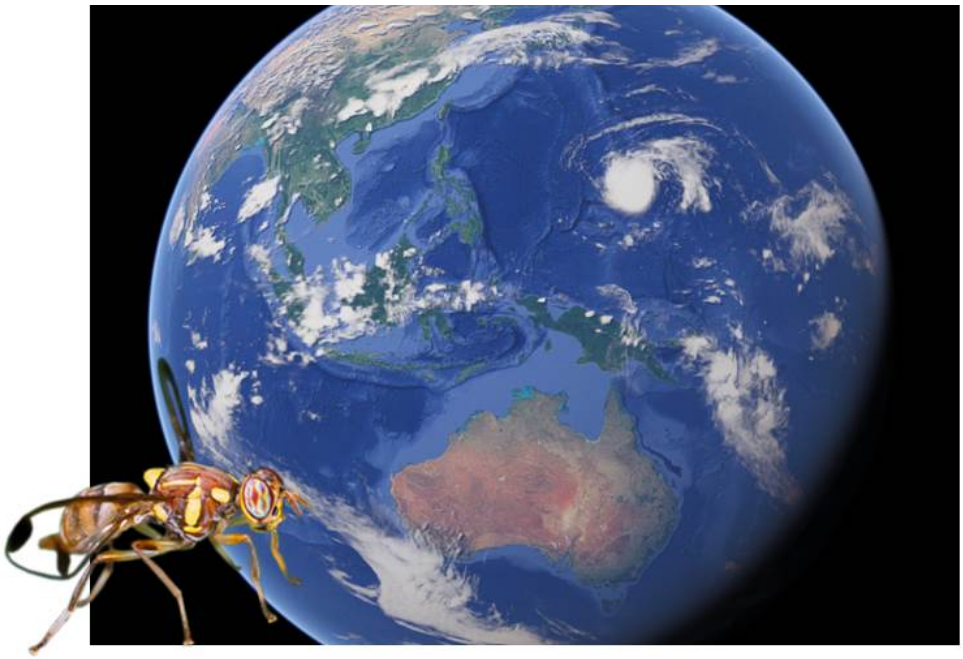
### 4.4.2 Summary and link to next chapter

Analysis of morphological, morphometric and molecular data for *Z. cucurbitae* revealed variation in shape and size of wings and in the length of the aedeagus but integration of these results did not show any strong evidence from either morphological or genetic differences that would constitute evidence for host-associated lineages or the existence of cryptic species in central Thailand. The present study confirmed that *Z. cucurbitae* populations from the ten different host plants comprised a monophyletic entity. Notably, the molecular data from this study showed inconsistent results and a low level of genetic differentiation between the flies reared from different host plants. Although the current dataset is not sufficient to address whether *Z. cucurbitae* has host races anywhere else across its geographical range, the data do suggest that in agricultural areas (such as central Thailand), where diverse hosts co-exist, host races do not arise or do not persist. When the results from this study are combined with the wider work that investigated melon flies in each of the Thai biogeographic regions and on the two large islands in Thailand (Chapter3), together, they shed important light on the taxonomy of *Z. cucurbitae.* The results discourage recognition of subdivisions of the species based on host associations, morphological, morphometric or molecular characteristics.

Having quantified the phenotypic and genotypic variation in *Z. cucurbitae* in Thailand, the next step of my work (Chapter 5) is to investigate variation in the fly at a broader geographic scale, covering the wider, presumed endemic region of the fly in Southeast-Asia, and invasive populations in Hawaii and the western Pacific.

# CHAPTER 5

**Signatures of invasion: an integrated approach to revealing the spread of melon Fly, *Zeugodacus cucurbitae* across Southeast-Asia and the West-Pacific**



## 5.1 INTRODUCTION

Invasion by an organism into a new area can be a significant threat to agriculture, human health or the natural environment, and increasing global commerce and travel make such invasions an all-too-common occurrence (Schrag & Wiener, 1995; Roderick & Howarth, 1997; Levine, 2008; Pimentel, 2011). Many species of tephritid fruit flies are invasive, and these have become an enormous threat because of their economic impact. Invasions by members of the family Tephritidae have been documented worldwide, presumed to be driven largely through fruit trade and non-commercial human carriage, despite strict quarantine procedures in many countries. Notable examples of these invasions are: *B. dorsalis*, an invasive species of Asian (likely Indian) origin and now well established in many parts of Africa (where its expansion has been recorded under the name *B. invadens*) (Drew et al., 2005; Drew & Romig, 2013) and other countries (where it has been recorded either as *B. papayae* or *B. philippinensis*) (Drew & Hancock, 1994; Drew & Romig, 2013); and *Ceratitis capitata,* which has expanded from its native range in sub-Saharan Africa to become an almost cosmopolitan species (Malacrida et al., 2007; De Meyer et al., 2008; Barr, 2009). Studies of intraspecific variation , and the processes of dispersal to new areas, may help to understand why different populations of invasive species seem to pose different pest management challenges. It can also help biosecurity scientists to infer the origin of invasive populations, reconstruct invasion pathways, and assess which potential exotic invasive species, or populations of already-established exotic species, represent quarantine risk.

Combined with quarantine interception data, molecular data at a population level can confirm historical or contemporary pathways for the introduction of invasive species (Corin et al., 2007). DNA-based studies of variation, including techniques based on RAPDs, AFLP, mtDNA (e.g., *cox1*) and microsatellites may all be useful for the study of variation; as can protein-based studies such as older allozyme approaches (Tajima, 1989; Clark et al., 1998; Rozas et al., 2003; Xu, 2012). Indeed, DNA markers are a powerful tool to characterize the genetic variability of populations of alien species and assign them to potential sources in their native

range (Cognato et al., 2005; Grapputo et al., 2005; Eastwood et al., 2006; Gwiazdowski et al., 2006; Havill et al., 2006; Corin et al., 2007; Puillandre et al., 2008).

An understanding of population structure (i.e., genetic variation) through population genetic studies can be used to untangle past dispersal events and identify potential invasion pathways (Estoup & Guillemaud, 2010; Virgilio et al., 2010; Zhang et al., 2012; Schutze et al., 2015b; Aketarawong et al., 2014b). Analysis of DNA sequence data may indicate which populations are similar and are likely to be related. If it can be inferred that one population is derived from another (e.g., from a phylogeny based on molecular sequences or Bayesian cluster assignments) it is reasonable to suppose that the species has dispersed from the location occupied by the ancestral population to that occupied by the derived population (Tsutsui et al., 2001; Scheffer & Grissell, 2003).

Population structure based on diversity indices and allele frequencies has also provided valuable information on the origin, colonization history, and dispersal trends of invasive species (Virgilio et al., 2010; Zhang et al., 2012). For example, if populations along a suspected dispersal pathway are most similar genetically to adjacent populations than more distant populations, it is reasonable to infer that a gradual, natural dispersal pattern along the pathway has taken place (Palumbi et al., 1997). However, if there is no such relationship, with more distant populations genetically similar, saltatorial dispersal may be more likely, perhaps due to natural events such as long-distance dispersal on the wind or via human-assisted transportation (Sved et al., 2003). Epicentres of genetic diversity may pinpoint places where there have been multiple invasions (i.e., invasion hotspots), and the amount of variation or presence of certain haplotypes may reveal the number of introductions (Cameron et al., 2008; Puillandre et al., 2008). Genetic diversity of invasive populations may give an idea of whether those populations have been present for a long time or have been established relatively recently, while also providing insight into the actual mechanism of dispersal (Corrie & Hoffmann, 2004) or host shifts (Scheffer & Grissell, 2003).

The preferred tools for recent studies of insect genetic variation have been *cox1* and microsatellites (Nabholz et al., 2009). For example, *cox1* was used to determine that *Dendrobaena octaedra* Savigny earthworms were introduced to the boreal forest of northern Alberta in Canada via multiple pathways (Cameron et al., 2008). Nevertheless, it is a challenge to establish the original range of invasive organisms which are now widely established. However, the variation in genetic diversity can provide strong clues: *cox1* sequences of the carpet sea squirt, *Didemnum vexillum* Kott are more diverse in Japan than in Northwestern Europe, western North America, and New Zealand. This agrees with the sparse, historical data which suggests that Japan lies within the native range of *D. vexillum* (Stefaniak et al., 2012). Among insects, *cox1* was used to investigate the genetic variation of red palm weevil, *Rhynchophorus ferrugineus* (Olivier) from 14 different countries in the Middle-East and the Mediterranean basin areas where this species had invaded. It was revealed that the Middle-East and the Mediterranean populations originated from different geographic source populations (EI-Mergawy et al., 2011). *Cox1* data also has suggested that the New Zealand population of the Argentine ant, *Linepithema humile* Mayr, most likely originated in Australia (Corin et al., 2007).

Present-day assemblages of more rapidly-evolving sequence information sometimes preserves historical information regarding population demographics. For example, microsatellite data from North American populations of the Eurasian spiny water flea, *Bythotrephes longimanus* Leydig, suggested an invasion bottleneck for at least one of the North American populations of this tiny aquatic predator from Europe. Microsatellites also revealed that overall *B. longimanus* genetic diversity in North America had been supplemented by gene flow from a number of different populations in Europe (presumably from multiple invasions), and that there was significant gene flow among populations in the North American range (Colautti et al., 2005). In another example, *cox1* and microsatellite data were used together to assess the genetic variability of populations and to trace the geographical origin of the horse chestnut leafminer, *Cameraria ohridella* Deschka & Dimic, from 88 localities from 22 different Central and Western European countries. These analyses revealed that the genetic diversity of *C. ohridella* populations across Europe is lower

compared with populations from the southern Balkans. Congruent, high haplotype diversity and low measures of nucleotide diversities indicate that *C. ohridella* has experienced rapid population expansion during its dispersal across Europe from its probable origin in the southern Balkans, near Greece (Valade, 2009). These parts of the genome have also proved informative for invasive tephritids: *cox1* and microsatellites have been examined in tandem in an invasive population of the olive fly, *B. oleae*, to resolve the history and colonization of this species in South and Central Africa, Pakistan, Mediterranean Europe, the Middle East, California and Mexico. Genetic similarity and assignment tests clustered the populations into two groups: i) Africa; and ii) a group which includes flies from the Mediterranean basin and the American region. The significantly greater diversity at microsatellite loci in Africa relative to the Mediterranean area indicates that Africa, and not the Mediterranean, is the origin of flies infesting cultivated olives. It also seems that the olive fly has invaded the Mediterranean region relatively recently and from there dispersed to the Americas (Nardi et al., 2005). These and other studies demonstrate the utility of genetic data, and *cox1* and microsatellites sequences in particular, towards unravelling invasion pathways and processes.

In addition to the molecular approach, geometric-morphometric analysis has been useful in resolving natural variation and inferring invasion pathways (Schutze et al., 2012b, 2015b). Geometric-morphometric analysis, with its demonstrated capacity to resolve fine-scale variation, can work additively with molecular data as an iterative approach for detection of population variation (Aytekin et al., 2007; Kitthawee & Rungsri, 2011; Schutze et al., 2012b; Krosch et al., 2013). It has also been used to determine affinities among populations and direction of dispersal. For example, Sri Lankan populations of *B. dorsalis* (at the time referred to as *B. invadens*) were related to African populations and also similar in wing shape to *B. dorsalis s.s* from Hawaii, despite the different ecological and biogeographical conditions in these locations (Khamis et al., 2012). Subsequent studies examining specimens over a broader geographical range have since revealed the wing shape of African populations to be more similiar to those from those sampled from Pakistan and Nepal rather than Sri Lanka; thereby, suggesting the African flies may have

instead originated from the northern Indian subcontinent (Schutze et al., 2015b). Among Southeast-Asian *B. dorsalis*, geometric morphometric data demonstrated a highly significant isolation-by-distance signal, thereby showing that populations which are geographically close tend to have similar-shaped wings relative to those at collected from greater distances away. This supports the notion that wing shape may be highly correlated with geographic origin and thereby a potentially powerful measure that can be used to infer the geographic origin of adventive populations.

Chapters 3 and 4 in this thesis revealed that Thai populations of *Z. cucurbitae* represent a single species and not a mixture of host races or cryptic species; and that, despite some population structure such as that observed for the Northeast, they are relatively homogeneous across the country. Northeastern flies differ with respect to microsatellite data from *Z. cucurbitae* from other parts of Thailand. Additionally, when the genetic structure of populations along the Isthmus of Kra was determined, populations from Yala and Narathiwat (which are located near the Thai-Malaysia border) were found to be very similar to flies from Malaysia. These results suggest two hypotheses which could be tested. First, it would be logical to explore whether flies from the northeast are more similar to populations from neighbouring countries (Vietnam, Laos and Cambodia) than to flies from elsewhere in Thailand. Second, suggested by the observation that southern populations along the peninsula are more variable in the size of wing and different genetically to populations from further north, it can be tested if they are then more similar to populations to the south (in Malaysia, Singapore and Indonesia). Indeed, these two ideas suggest a broader hypothesis, that Thailand may, in fact, be a transition zone between distinct clusters of populations within *Z. cucurbitae*. Notwithstanding the differences just noted, analysis of *Z. cucurbitae* within Thailand provides a robust case for the melon fly being a single biological species. However, the fine scale analyses undertaken in Chapters 3 and 4 also provide an understanding of *Z. cucurbitae* and a “sense” of what variation is possible within the species. That having been established, furthers studies on the population structure of *Z. cucurbitae* in Southeast-Asia (the native range of the species) are required,

especially for the clues that this study might reveal for dispersal to the West-Pacific (the invasive range of the species).

Despite its economic impact across Asia, Africa and the Pacific, *Z. cucurbitae* has not been as well studied as other species, for example *B. dorsalis*, in key regions where it naturally occurs (Weems et al., 1999; Dhillon et al., 2005). Having originated in the Indo-Oriental region (Drew & Hancock, 2000; Virgilio et al., 2010), *Z. cucurbitae* subsequently dispersed by either natural means and/or with human assistance into Indo-china, to the south through the Southeast-Asian archipelago, through Papua New Guinea and to the Pacific Islands, reaching as far as Hawaii (Wu et al., 2009; Virgilio et al., 2010). It is now present and widespread in many countries in the West-Pacific (i.e., its invasive range). Some of this dispersal has been documented in the historical record. This species was first detected in Hawaii in 1895. Subsequently, it was detected in Guam in 1936, in PNG in 1940 (although some authors seem to have overlooked the earlier record and give the year of detection as 1980) and the Solomon Islands in 1984 (Back & Pemberton, 1917; Eta, 1985; Waterhouse, 1993; Dhillon et al., 2005; Putulan, 2014; Vargas et al., 2015). The melon fly has also spread westwards into North and East Africa (Virgilio et al., 2010), again presumably moved in fruit with human assistance. Because of the substantial invasion history, and the difficulty of managing it in its introduced range, attention has been centred on contemporary biological invasions and on the role of humans in accelerating the introduction rate into new environments. However, a deeper understanding of the ecology and population structure of *Z. cucurbitae*, both in the native and the introduced ranges, is important to obtain a more complete and fundamental understanding of its invasion history (Malacrida et al., 2012).

Given this background, in this chapter I use an integrated approach (traditional morphometrics, geometric morphometrics, *cox1* and microsatellite) to understand the invasive pathways of *Z. cucurbitae* between Southeast-Asia and the West-Pacific. The current understanding of *Z. cucurbitae* population structure and invasion pathways is based on flies sampled predominantly from China, the Indian subcontinent, Africa and Hawaii (Virgilio et al., 2010; Wu et al., 2011; Prabhakar,

2012; Jacquard, 2013), with very little research undertaken on Southeast-Asian flies, or on flies from the invasive range of the West-Pacific. The lack of this data for *Z. cucurbitae* in this region is a key knowledge gap. This study has examined patterns of variation and the current population structure of *Z. cucurbitae*, evidence for historical barriers, and dispersal and invasion routes. Specifically, I test the hypotheses: (i) that there have been multiple and recent introductions of *Z. cucurbitae* from Southeast-Asia into the West Pacific, in which I predict that greater variation occurs in the native range of *Z. cucurbitae* in Southeast-Asia contrasted with reduced variation towards its invasive range in the West-Pacific; (ii) populations that have histories involving human movement or trade with Asian countries would be more genetically diverse than other populations because there had been more opportunities for multiple introductions; (iii) traditional morphology and geometric morphometrics provide additional insights in an invasion biology context when explicitly tested against genetic data (*cox1* and microsatellites) from the same flies.

Before beginning the research component of this chapter, I need to address the concept of isolation by distance (IBD; Wright, 1943), and an analytical decision I have taken in this chapter. IBD processes are very well understood theoretically and there is a large body of literature describing how patterns of IBD emerge and how IBD affects the distribution of variation over populations (Rousset, 1997). IBD is a measure of differences (genotypic/phenotypic) among populations as correlated with geographic distance (Wright, 1943). When organisms disperse and expand their distribution naturally, the process of change and build-up of differences among populations can take a long time. However, if there is sufficient time, regional differences accumulate and the result is a significant IBD signal (Vekemans & Hardy, 2004). However, if organisms are transported artificially to a remote, new location, their genetic/phenotypic signature will be closest to that of populations from where they originated (Meirmans, 2012). Therefore, including invasive organisms in IBD analyses will likely skew the result rendering it non-significant (i.e., instead of greater geographic distance being correlated with a large genetic/phenotypic difference, one would see the opposite). Among the sites

included in this study, Guam and Hawaii are geographically remote and the literature also contains many direct evidence indicating that melon fly arrived there by long-distance, human assistance and not by natural dispersal (Meixner et al., 2002; Vargas et al., 2015). Therefore, I have excluded Guam and Hawaii from IBD regression analyses of Southeast-Asia and the West-Pacific.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Sampling strategies

Adult male *Z. cucurbitae* were collected using cue lure traps from 14 sites across Southeast-Asia and the West-Pacific. Fruit fly traps were set up in ten sites in Southeast-Asia (Burma, Thailand, Laos, Cambodia, Vietnam, Malaysia, Singapore, Java, Sarawak and Philippines) and four sites from the Pacific (New Guinea, Solomon Islands, Guam and Hawaii) (Figure 5.1 and Table 5.1). All basic details regarding sample collection (adult trapping) are presented in Chapter 2.

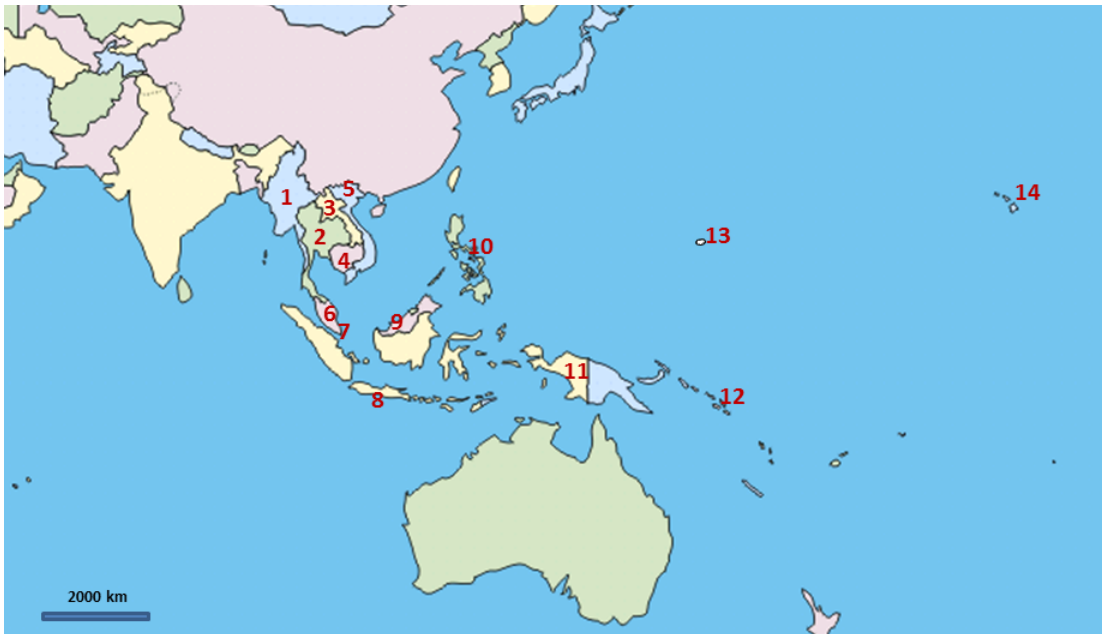



**Figure 5.1** Geographical locations across Southeast-Asia and the West-Pacific from which samples of *Zeugodacus cucurbitae* were collected. Specific collection data are presented in Table 5.1.

**Table 5.1** Locations and numbers of individuals used for wing shape, aedeagus length, *cox1* and microsatellite analyses for samples of male *Zeugodacus cucurbitae* collected from 14 sites across Southeast-Asia and the West-Pacific.

| | Location | Date | Latitude | Longitude | wing shape | aedeagus | *cox1* | microsatellite |
|---|---|---|---|---|---|---|---|---|
| 1 | Burma | 2-10 Sep 13 | 17.090 | 96.175 | 20 | 20 | 19 | 20 |
| 2 | Thailand | 11-15 May 12 | 14.478 | 100.050 | 20 | 20 | 20 | 20 |
| 3 | Laos | 2-9 May 12 | 18.011 | 102.643 | 20 | 20 | 20 | 19 |
| 4 | Cambodia | 20-26 Dec 12 | 11.277 | 105.583 | 20 | 20 | 19 | 20 |
| 5 | Vietnam | 1-15 May 14 | 21.016 | 105.846 | 20 | 20 | 20 | 20 |
| 6 | Peninsular Malaysia | 19-23 July 13 | 3.005 | 101.298 | 20 | 20 | 18 | 19 |
| 7 | Singapore | 3-8 May 12 | 1.356 | 103.781 | 20 | 20 | 19 | 20 |
| 8 | Java | 1-7 Oct 12 | -7.591 | 110.588 | 20 | 20 | 20 | 20 |
| 9 | Sarawak | 25-31 Jan 13 | 3.870 | 113.732 | 15 | 15 | 15 | 15 |
| 10 | Philippines | 8-15 March 13 | 10.542 | 122.546 | 20 | 20 | 20 | 19 |
| 11 | New Guinea* | 1-13 Jan 13 | -3.297 | 140.770 | 20 | 20 | 20 | 20 |
| 12 | Solomon Islands* | 2-15 Nov 13 | -9.434 | 159.943 | 20 | 20 | 20 | 19 |
| 13 | Guam* | 11-25 April 13 | 13.387 | 144.771 | 20 | 20 | 20 | 20 |
| 14 | Hawaii* | 11-15 Dec 12 | 23.145 | -157.684 | 20 | 20 | 19 | 12 |

***The West-Pacific locations

### 5.2.2 Morphometric analyses

#### 5.2.2.1 Morphometric analyses

Wings from 15-20 individuals from each of ten sites from Southeast-Asia and four sites from the West-Pacific were slide mounted prior to analysis. All details regarding specimen preparation, landmarking, Procrustes superimposition, calculation of wing centroid size, and general statistical analyses are presented in Chapter 2.

*Wing size*: Individuals were assigned to groups based on collection locations and the hypotheses being tested. All data were first tested to confirm that they were consistent with assumptions of normality and homogeneity of variance before further analysis was undertaken. The data met assumptions and one-way ANOVAs were performed to test for differences in centroid size among *a priori* defined groups for the 14 sites across Southeast-Asia and the West-Pacific. An additional independent-samples t-test was performed to compare between the group of Southeast-Asian flies (native range) and the group from the West-Pacific (invasive areas).

*Linear regression analysis*: Linear regression analyses were performed to assess whether wing size was significantly correlated with geographic distance (km) across Southeast-Asia and the West-Pacific. Regression was first conducted among the 10 sites from Southeast-Asia, and then by combining Southeast-Asian and the Pacific locations of Solomon Islands and New Guinea.

*Wing shape analyses*:

- Principal Component Analysis (PCA) was conducted to assess differences in wing shape between populations under a non-hypothesis scenario. In the present assessment of wing shapes, PCA can be used to reveal which shape changes are associated with the greatest variation or with the least variation, and to identify which shape features are particularly variable or particularly constant. In addition, to explore whether there were any differences between the native and invasive ranges, separate principal component analyses were performed, i.e., on flies from

Southeast-Asia (the native range) and flies from the West-Pacific (the invasive range).

- Canonical variate analysis (CVA) was performed on Procrustes transformed data. The individuals were assigned to groups based on the hypotheses being tested. Canonical variates analysis (CVA) Individuals were retained in their *a priori* defined groups for CVA and separate analyses were carried out for Southeast-Asia and the West-Pacific. Significant differences were determined via permutation tests (10000 permutations) for Mahalanobis distance among the *a priori* defined groups. It was also necessary to to correct for multiple comparisons by performing Bonferroni corrections (Rice, 1989) throughout the analysis.

To create the graphs appear as more realistic representations of the structures and to provide the additional information and depict relative changes in shape among the datasets. Wireframe graphs connecting the landmarks with straight lines were used to indicate the morphological context of the landmarks; a pair of superimposed wireframe graphs produced by the software MorphoJ was used to depict the starting and target shapes (Klingenberg, 2011).

*Linear regression analysis*: As for wing size, regression analysis was conducted for Mahalanobis distances against geographic distance (km) to determine if there was an isolation by distance effect regarding wing shape across Southeast-Asia and the West-Pacific.

**5.2.2.2 Aedeagus morphometrics**

Males used for wing shape analysis were also examined for variation in aedeagus length. All details regarding specimen preparation and statistical analysis of aedeagi are presented in Chapter 2.

*Statistical analysis of aedeagus length*: Data were tested to confirm that they were consistent with assumptions of normality and homogeneity of variance before further analysis was undertaken. The data met assumptions, and ANOVA was used to compare and test for differences in aedeagus length among *a priori* defined groups for all sites (10 sites from Southeast-Asia and four sites from the West-

Pacific) and for testing variation among 14 sites across Southeast-Asia and the West-Pacific. T-test was used to compare aedeagus length between those sites from the Southeast-Asia and the West-Pacific as native and invasive areas respectively.

*Linear regression analysis*: As for shape analysis above, aedeagus length was regressed against geographic distance (km) for specimens collected from the ten sites across Southeast-Asia and for Southeast-Asia combined with two sites from New Guinea and the Solomon Islands.

### 5.2.3 Molecular procedure and analyses

#### 5.2.3.1 Mitochondrial DNA amplification, sequencing and analyses

Specimens used for morphological analyses were also utilised for molecular analysis. One-hundred ninety-five specimens from Southeast-Asia and 80 specimens from the West-Pacific were examined. All details regarding *cox1* amplification, PCR protocol, reactions and sequencing, and analyses are presented in Chapter 2.

**- Mitochondrial DNA analyses**

Statistical analysis was undertaken to measure mitochondrial DNA gene diversity of *Z. cucurbitae* and also to investigate genetic differentiation. All details of basic statistical analyses employed are presented in Chapter 2.

*Statistical analysis of Molecular Variance*: An Analysis of Molecular Variance (AMOVA) was conducted in ARLEQUIN to assess partitioning of variation within and among sites. Samples were constrained according to sites within which the samples were collected to evaluate the variation in native and invasive areas.

*Isolation-by-distance*: Tests of isolation by distance (IBD) were conducted to assess whether geographical distance is correlated with genetic distance. IBD was assessed by linear regression analysis between geographic distance (km) and genetic distance among groups ($\Phi_{ST}$). For the same reasons as described for morphometric data, I performed regressions with sites from Southeast-Asia only and then Southeast-Asia plus New Guinea and the Solomon Islands.

Moreover, to obtain more sites from other continents where melon fly occur, 71 sequences from NCBI GenBank (from African countries and India (Virgilio, et al., 2010) and from China (Jiang et al., 2013, 2014)) (Appendix 4) were merged

with 262 sequences obtained in the present study to study the gene diversity with ARLEQUIN and through a median-joining (MJ) network.

#### 5.2.3.2 Microsatellite amplification, sequencing, and analyses

**- Polymerase chain reaction (PCR) amplification sequencing and Sequence cleaning**

Eleven microsatellite loci that had been screened for variation in *Z. cucurbitae* were used for genotyping. All details regarding microsatellite loci, PCR protocol, reactions are presented in Chapter 2.

**- Microsatellite analyses**

Statistical analysis was undertaken to measure microsatellite diversity of *Z. cucurbitae* and also to investigate genetic differentiation and population structure. All details of basic statistical analyses employed are presented in Chapter 2.

*Statistical analysis of Molecular Variance*: An analysis of molecular variance was conducted in ARLEQUIN to confirm population clusters and to differentiate the variation component among the population from 14 sites across Southeast-Asia and the West-Pacific. The analysis was based on among-site $R_{ST}$ estimates.

*Isolation-by-distance*: The hypothesis of IBD was assessed between geographic distance and genetic distance ($R_{ST}$) among populations by linear regression analysis. Thus, the pairwise values of genetic distance ($R_{ST}$) among 10 sites from Southeast-Asia were regressed against geographic distance for the Southeast-Asia locations. I also regressed genetic distance ($R_{ST}$) among populations from Southeast-Asia, including New Guinea and the Solomon Islands against geographic distance (km).

## 5.3 RESULTS

### 5.3.1 Morphomertic results

#### 5.3.1.1 Geometric morphometric wing shape analysis

*Centroid size calculation*

*Zeugodacus cucurbitae* wing size varied significantly among all sites across Southeast-Asia and the West-Pacific ($F_{13,\ 261} = 2.222$; $P < 0.05$) (Figure 5.2). Flies from Sarawak had the smallest wings overall (5.824 ± 0.590 mm), yet were not significantly different from other sites, except for Vietnam (6.421 ± 0.435 mm) and Singapore (6.437 ± 0.348 mm). Wings of flies from the latter two sites had the largest wings, but similarly, these were not significantly different from all other sites, except Sarawak. In comparing between native Southeast-Asia and the West-Pacific, West-Pacific wings (6.315 ± 0.049 mm) were less variable and significantly larger than Southeast-Asian wings (6.179 ± 0.192 mm) ($t_{1,\ 273} = 1.887$; $P < 0.05$) (Figure 5.2).

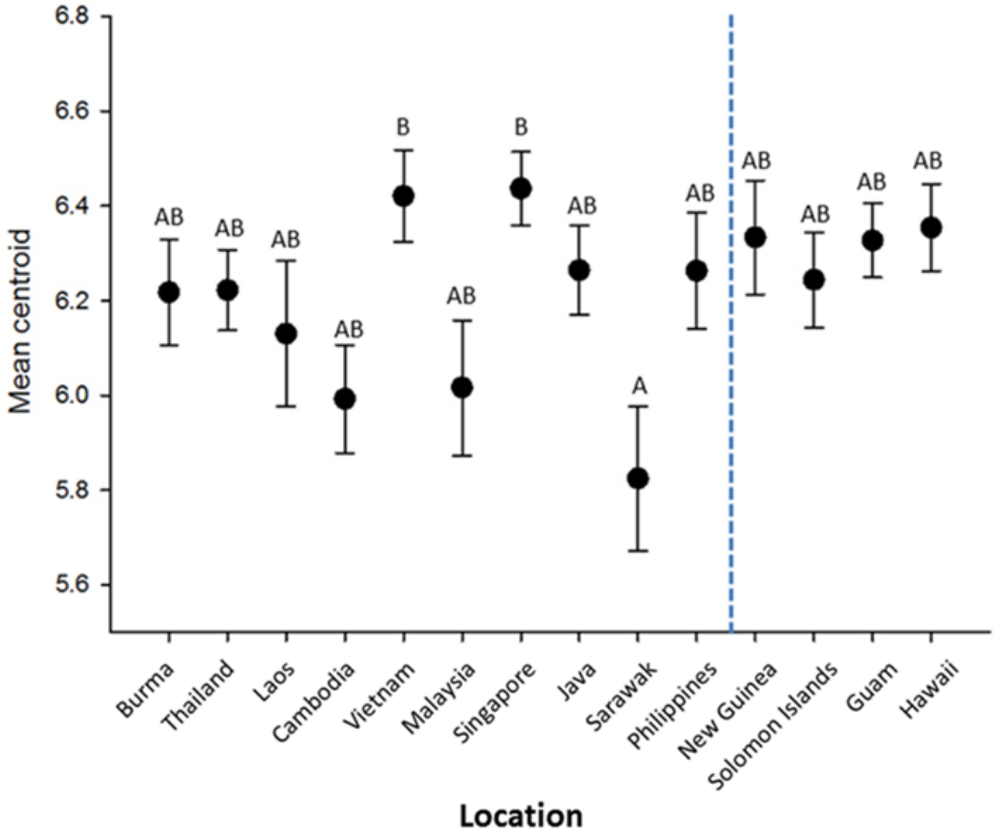
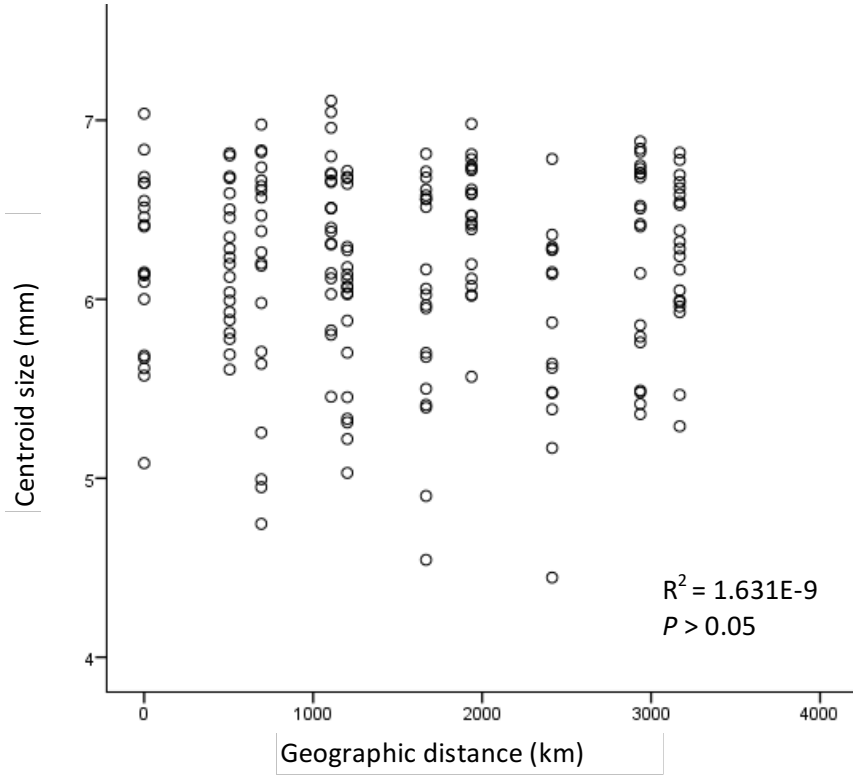


**Figure 5.2** Mean (± SE) wing centroid size of *Zeugodacus cucurbitae* from 14 sites across Southeast-Asia and the West-Pacific. Samples sharing the same letter are not statistically different from each other based on one-way ANOVA with a Tukey *post hoc* test ($F_{13, 261} = 2.222$; $P < 0.05$). The dotted blue line represents Wallace's line that divides Southeast-Asia and West-Pacific sites.

*Isolation by distance*

While there were differences in wing size among sites, there was no longitudinal trend from Southeast-Asia to the West-Pacific; i.e., no trend from the area presumed to have been occupied by natural dispersal to the area presumed to have been invaded with human assistance. The regression of the shape variable of centroid size against geographic distance across 10 sites from Southeast-Asia showed no significant relationship ($R^2 = 1.631 \times 10^{-9}$, $P > 0.05$) (Figure 5.3A). Likewise, there was no significant correlation between wing size and site across 12 sites from Southeast-Asia and the West-Pacific (New Guinea and Solomon Islands) ($R^2 = 0.010$, $P > 0.05$) (Figure 5.3B).

(A)




(B)

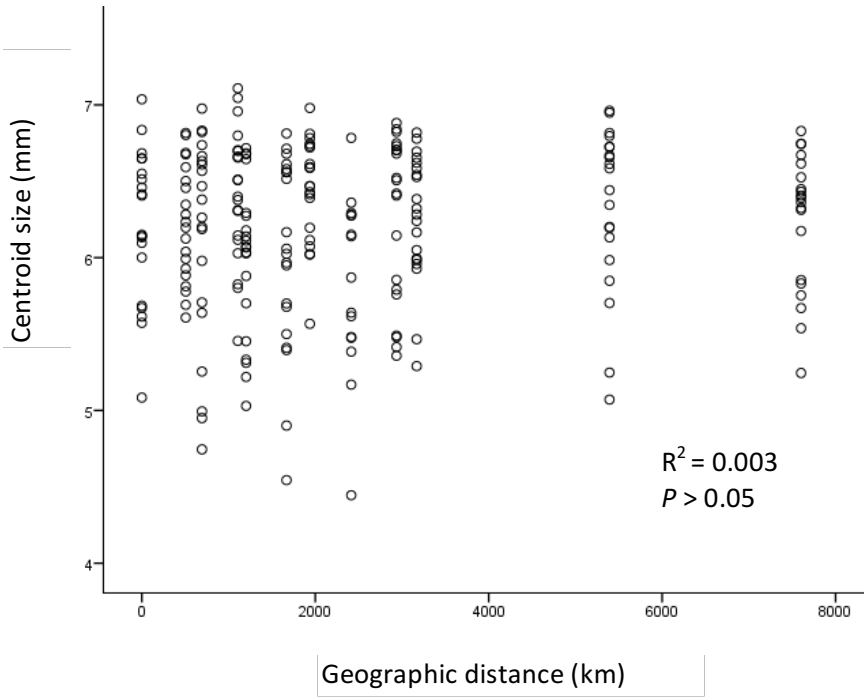



**Figure 5.3** Regression of wing centroid size (mm) of *Zeugodacus cucurbitae* from sites against geographic distance (km). (A) Across Southeast-Asia (B) Across Southeast-Asia and the West-Pacific (New Guinea and Solomon Islands).

*Procrustes Superimposition*

Two-hundred and seventy-five males from 14 sites (15-20 samples/sites) across Southeast-Asia (ten sites) and the West Pacific (four sites) were examined for wing size and shape variation using geometric morphometric analysis. Generalized Procrustes superimposition produced a new set of co-ordinate data for each of the individuals used in the study (Figure 5.4). Fifteen landmarks in two dimensions were explored and the average shape rendered in two axes. The Procrustes sum of squares was 0.2291 and the Tangent sums of squares was 0.2289 (Table 5.2).

**Table 5.2** Average position on two axes of fifteen landmarks generated from Procrustes superimposition in two dimensions of *Zeugodacus cucurbitae* wings from individuals collected from 14 sites across Southeast-Asia and the West-Pacific. The position of the landmarks in relation to a real wing is shown in Figure 2.4

| Landmark | Axis 1 (X) | Axis 2 (Y) |
|---|---|---|
| 1 | -0.3300713 | -0.0119772 |
| 2 | -0.3297722 | 0.0490179 |
| 3 | -0.3004105 | 0.0755929 |
| 4 | -0.0899007 | -0.1640178 |
| 5 | -0.1853510 | -0.0505075 |
| 6 | -0.1523665 | -0.0501087 |
| 7 | -0.1767059 | 0.0178712 |
| 8 | 0.1517639 | -0.1583646 |
| 9 | 0.1871692 | -0.0284486 |
| 10 | 0.0500580 | -0.0010596 |
| 11 | 0.0278540 | 0.0793278 |
| 12 | 0.0630610 | 0.1343168 |
| 13 | 0.3927302 | -0.0816007 |
| 14 | 0.4359293 | 0.0471541 |
| 15 | 0.2560126 | 0.1428042 |

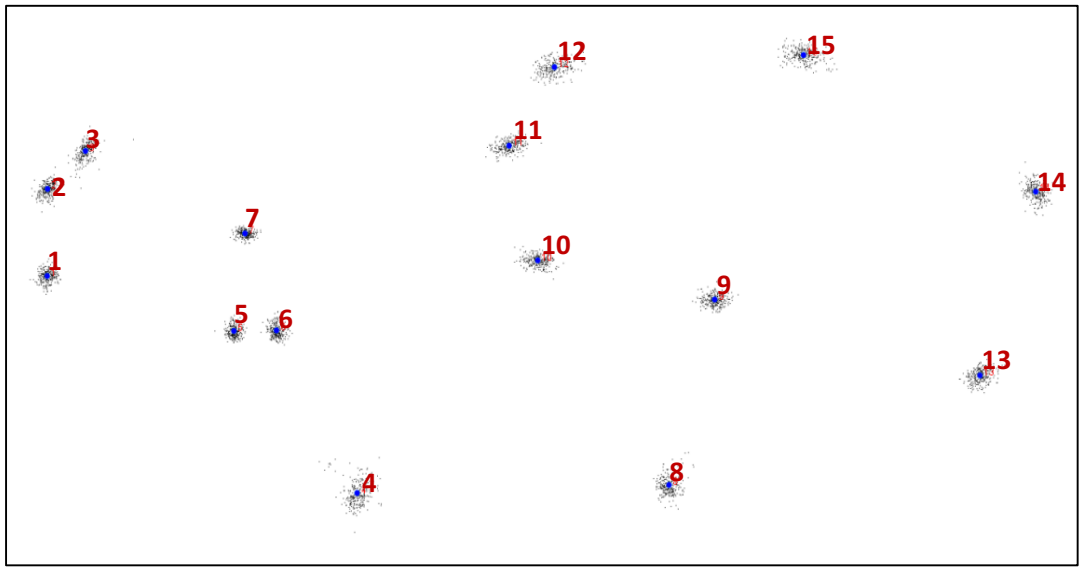



**Figure 5.4** Procrustes superposition showing the variation of 15 landmarks of *Zeugodacus cucurbitae* wings from 275 individuals collected from 14 sites across Southeast-Asia and the West-Pacific. The figure shows the configurations of landmarks for which differences in position, scale and orientation have been removed. Numbered dots represent the average position for each landmark. Each 'cloud' of points around average landmark positions represent individual landmarks for each fly included in the analysis. The position of the landmarks in relation to a real wing is shown in Figure 2.4

*Determination of allometric effect*

Multiple regression of wing shape on centroid size showed a significant allometric effect ($P < 0.0001$) and accounted for 3.86 % of shape variation (Figure 5.5). Hence, the subsequent CVA was conducted on data corrected to take allometric effect into account

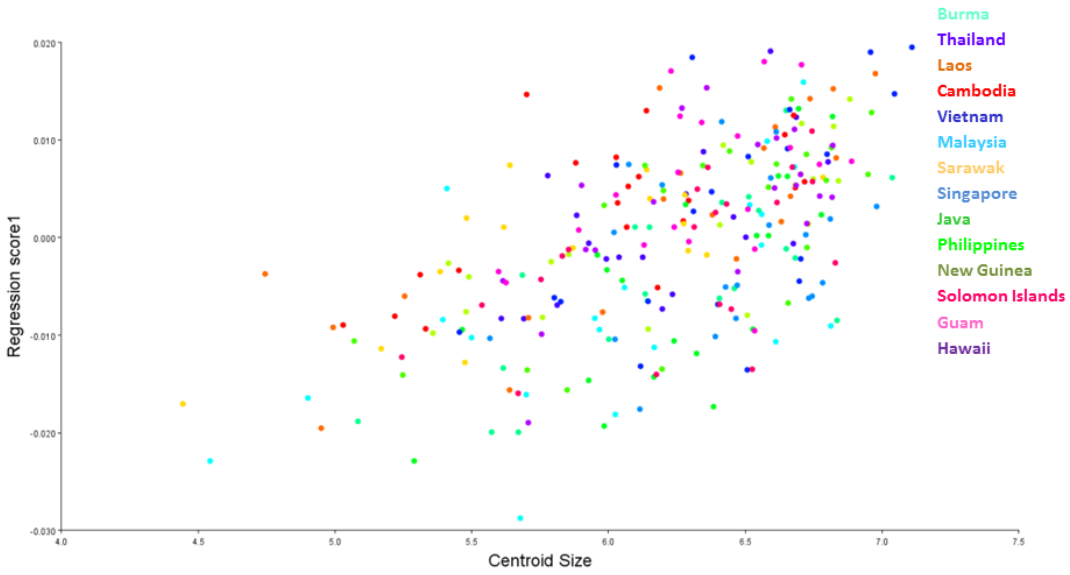



**Figure 5.5** Multiple regression of wing shape (regression score one) on centroid size (a measure of wing size) from 14 sites between Southeast-Asia and the West-Pacific. Each coloured dot represents the wing of a fly from one of the listed locations.

*Wing shape analyses*

Analysis of wing shape of melon fly from Southeast-Asia (native range)

- Principal component analysis

The first two principal components account for 41.94% of the total shape variation, and therefore, they did not provide a reasonable contribution of the total variation (Table 5.3 and Figure 5.6). The first two principal axes showed partial or indistinct separation of the populations (Figure 5.7). The principal component analysis showed variation in the wing shape of melon flies, but the scatter plots did not reveal discrete groups.

**Table 5.3** Summary statistics for principal component analysis of groups of *Zeugodacus cucurbitae* collected from ten sites across Southeast-Asia. The table shows eigenvalues, percent variation and cumulative percentage of variance explained by each principal component axis (PC) for the entire dataset.

| PC | Eigenvalues | % Variance | Cumulative % |
|---|---|---|---|
| 1 | 0.00024997 | 28.988 | 28.988 |
| 2 | 0.00011171 | 12.954 | 41.942 |
| 3 | 0.00008685 | 10.072 | 52.014 |
| 4 | 0.00008564 | 9.932 | 61.945 |
| 5 | 0.00005899 | 6.841 | 68.786 |
| 6 | 0.00004988 | 5.784 | 74.571 |
| 7 | 0.00003576 | 4.146 | 78.717 |
| 8 | 0.00003054 | 3.541 | 82.258 |
| 9 | 0.00002416 | 2.802 | 85.06 |
| 10 | 0.00001644 | 1.906 | 86.967 |
| 11 | 0.00001601 | 1.857 | 88.823 |
| 12 | 0.00001392 | 1.614 | 90.438 |
| 13 | 0.00001236 | 1.434 | 91.871 |
| 14 | 0.00001156 | 1.341 | 93.212 |
| 15 | 0.00000872 | 1.011 | 94.224 |
| 16 | 0.00000857 | 0.994 | 95.217 |
| 17 | 0.00000783 | 0.908 | 96.125 |
| 18 | 0.00000634 | 0.735 | 96.86 |
| 19 | 0.00000587 | 0.681 | 97.541 |
| 20 | 0.00000450 | 0.522 | 98.063 |
| 21 | 0.00000425 | 0.493 | 98.556 |
| 22 | 0.00000346 | 0.402 | 98.958 |
| 23 | 0.00000321 | 0.373 | 99.33 |
| 24 | 0.00000249 | 0.289 | 99.619 |
| 25 | 0.00000172 | 0.200 | 99.819 |
| 26 | 0.00000156 | 0.181 | 100 |

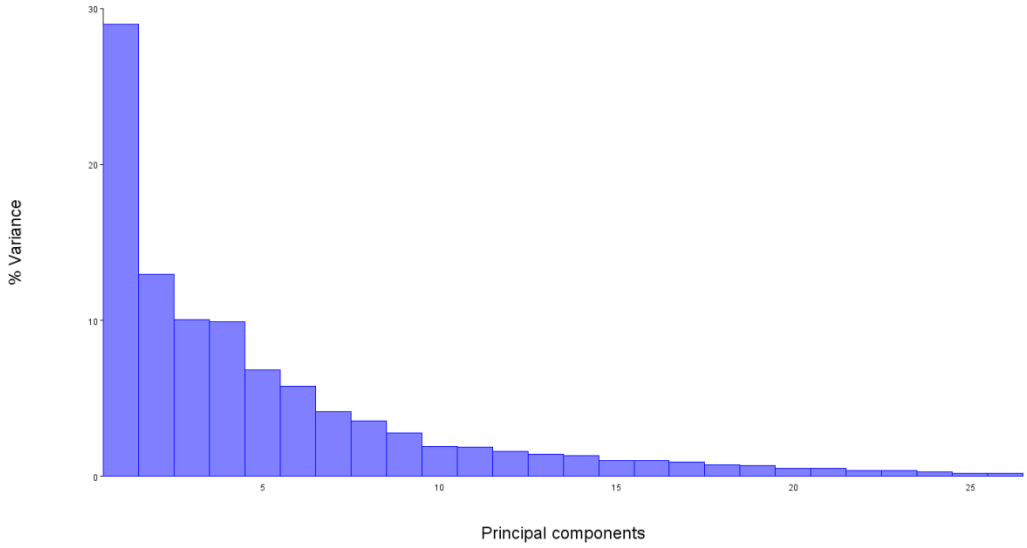



**Figure 5.6** Histogram illustrating the variances of all the principle components calculated from wing shape data of *Zeugodacus cucurbitae* collected from ten sites across Southeast-Asia.

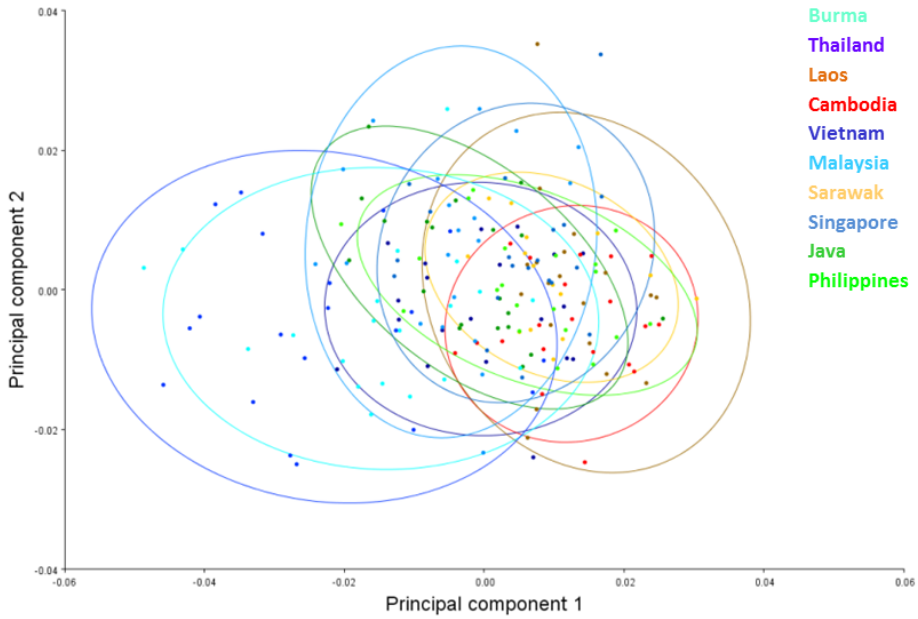



**Figure 5.7** First two principal components resulting from PCA of wing shape data *Zeugodacus cucurbitae* from ten sites across Southeast-Asia; 95% confidence ellipses are shown for each group. Each coloured dot represents the wing of a fly from one of the listed locations.

- Canonical variate analysis

Canonical variate analysis for samples only from Southeast-Asia sites revealed nine canonical variates, of which the first two accounted for 57.34% of variation (Table 5.4). Based on the third canonical variates, all Southeast-Asian groups were closest to their neighbors except Vietnam, which pulled away from others groups along the first canonical variate (Figure 5.8). Mahalanobis distances were generally the greatest between Vietnam and other sites in Southeast-Asia, as compared to among other sites in the region (Table 5.5).

**Table 5.4** Summary statistics for canonical variates analysis of groups of *Zeugodacus cucurbitae* collected from ten sites across Southeast-Asia. The table shows eigenvalues, percent variation and cumulative percent variation explained by each canonical variate axis for the entire dataset.

| CV | Eigenvalues | % Variance | Cumulative % |
|---|---|---|---|
| 1 | 2.38 | 36.51 | 36.51 |
| 2 | 1.36 | 20.83 | 57.34 |
| 3 | 0.86 | 13.19 | 70.53 |
| 4 | 0.56 | 8.53 | 79.06 |
| 5 | 0.44 | 6.75 | 85.81 |
| 6 | 0.33 | 5.10 | 90.91 |
| 7 | 0.24 | 3.70 | 94.60 |
| 8 | 0.21 | 2.34 | 97.84 |
| 9 | 0.14 | 2.16 | 100.00 |

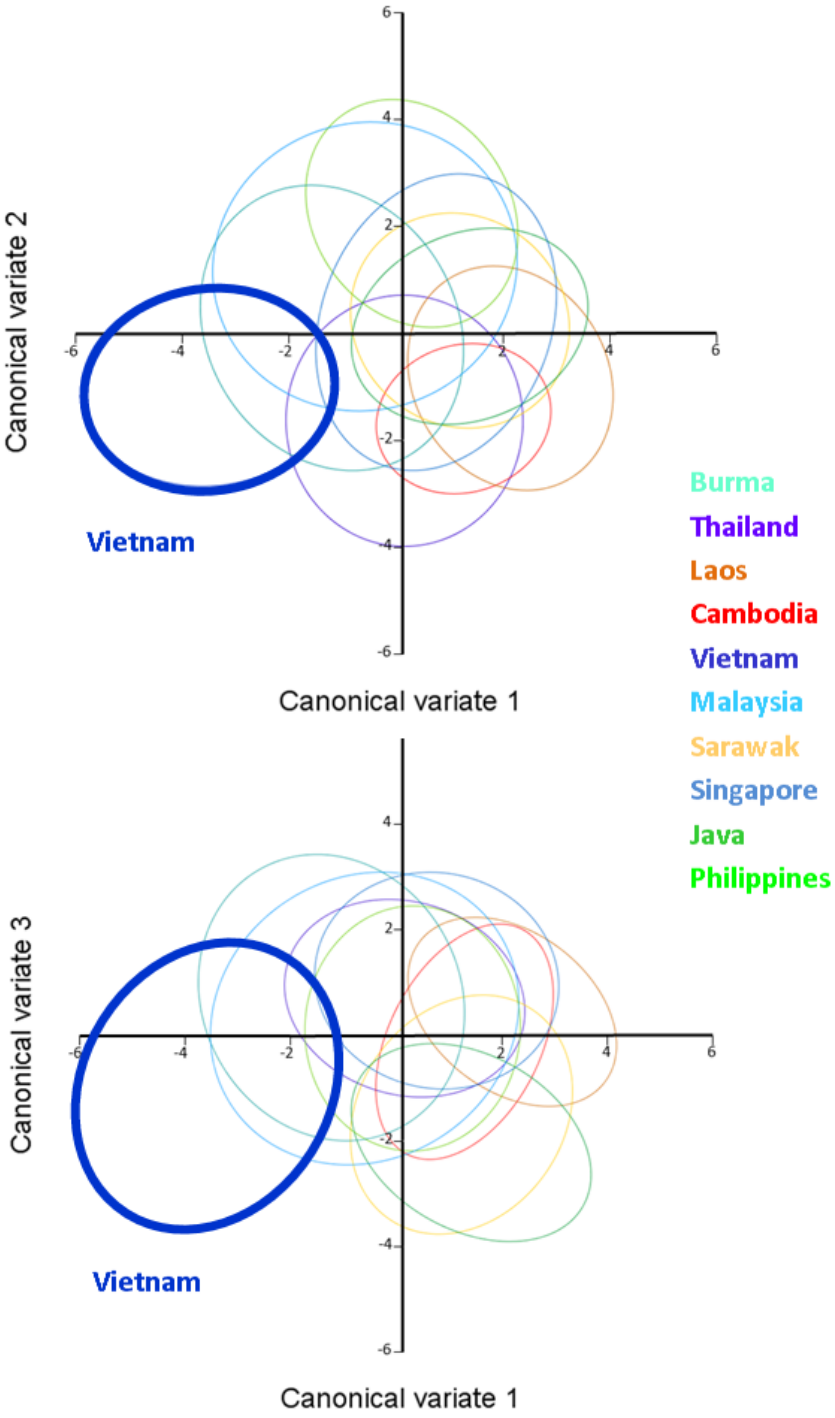



**Figure 5.8** Plot of the first three variates following canonical variate analysis of geometric morphometric wing shape data for *Zeugodacus cucurbitae* sampled from ten sites across Southeast-Asia. Fifteen to twenty wings were analysed per location; 95% confidence ellipses are represented, individual data points have been removed for clarity.

**Table 5.5** Mahalanobis distances among ten groups of *Zeugodacus cucurbitae* collected across Southeast-Asia, as calculated from CVA. Values below the diagonal represent Mahalanobis distances as calculated from canonical variates analysis on wing shape data from all ten sites. Values above the diagonal are geographic distances (km) between sample sites. Note that all Mahalanobis pairwise comparisons were significantly different between groups following Bonferroni correction for multiple comparisons (initial $\alpha = 0.05$ corrected to $\alpha = 0.001$).

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Burma | - | 506 | 693 | 1202 | 1106 | 1668 | 1937 | 3169 | 2414 | 2936 |
| 2 | Thailand | 3.058 | - | 481 | 697 | 951 | 1285 | 1516 | 2715 | 1908 | 2480 |
| 3 | Laos | 4.076 | 3.230 | - | 813 | 474 | 1674 | 1856 | 2977 | 1982 | 2298 |
| 4 | Cambodia | 3.497 | 2.906 | 2.746 | - | 1083 | 1028 | 1121 | 2170 | 1218 | 1854 |
| 5 | Vietnam | 3.661 | 4.477 | 2.847 | 5.038 | - | 2059 | 2198 | 3223 | 2088 | 2130 |
| 6 | Malaysia | 2.812 | 3.536 | 3.894 | 3.850 | 4.143 | - | 320 | 1556 | 1370 | 2477 |
| 7 | Singapore | 3.333 | 3.277 | 2.847 | 3.012 | 4.848 | 2.798 | - | 1249 | 1140 | 2311 |
| 8 | Java | 3.058 | 4.122 | 3.887 | 4.167 | 5.111 | 2.575 | 3.098 | - | 1321 | 2412 |
| 9 | Sarawak | 3.704 | 3.697 | 3.136 | 2.884 | 5.017 | 3.373 | 3.171 | 3.172 | - | 1223 |
| 10 | Philippines | 3.983 | 3.590 | 3.245 | 3.194 | 5.202 | 3.529 | 3.533 | 3.421 | 2.405 | - |

*Isolation by distance*

Regression of pairwise Mahalanobis distances against geographical distance (km) across ten samples sites from Southeast-Asia (i.e., the native range) revealed a strongly significant IBD effect ($R^2 = 0.146$, $P < 0.001$) (Figure 5.9).

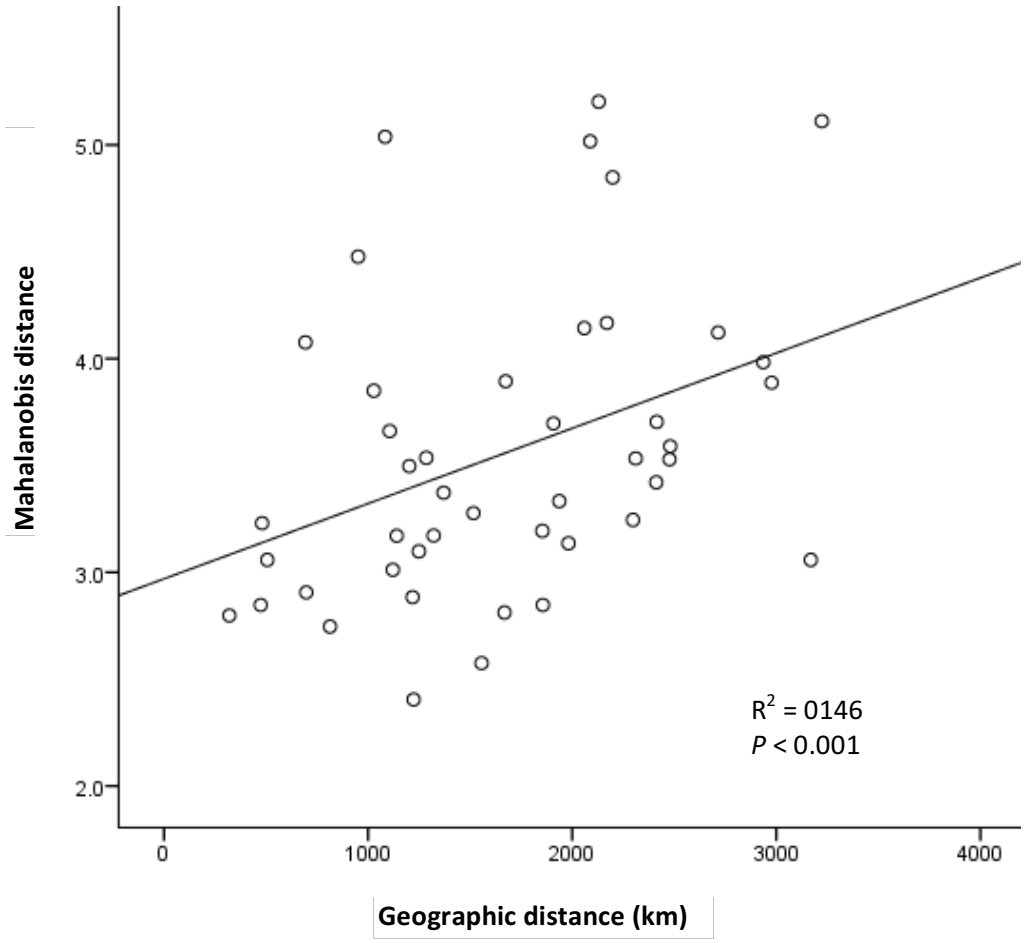



**Figure 5.9** Regression of Mahalanobis distances between groups based on sample location following CVA on shape data generated from *Zeugodacus cucurbitae* wings against geographic distance (km) across Southeast-Asia.

Analysis of wing shape of melon fly from West-Pacific

Wing shape variation was examined using geometric morphometric analysis in a data set for 80 males from four sites from the West-Pacific; New Guinea, Solomon Islands, Guam and Hawaii.

- Principal component analysis

The first three principal components accounted for 43.40% of the total shape variation (Table 5.6 and Figure 5.10). Plots on the first two principal axes showed limited separation of the populations. The principal component analysis showed some pattern in the shape variation in the wings of melon flies from the West-Pacific (New Guinea, Solomon Islands, Guam and Hawaii). It revealed that the wing shape of flies from New Guinea is different to wing shape of flies from Hawaii, being separated along the first and second PCs (Figure 5.11).

**Table 5.6** Summary statistics for principal component analysis of groups of *Zeugodacus cucurbitae* collected from four sites across the West-Pacific. The table shows eigenvalues, percent variation and cumulative percentage of variance explained by each principal component axis (PC) for the entire dataset.

| PC | Eigenvalues | % Variance | Cumulative % |
|---|---|---|---|
| 1 | 0.00020154 | 27.307 | 27.307 |
| 2 | 0.00011884 | 16.102 | 43.409 |
| 3 | 0.00008488 | 11.501 | 54.910 |
| 4 | 0.00007628 | 10.335 | 65.245 |
| 5 | 0.00004720 | 6.395 | 71.641 |
| 6 | 0.00004095 | 5.549 | 77.190 |
| 7 | 0.00003206 | 4.344 | 81.534 |
| 8 | 0.00002258 | 3.059 | 84.593 |
| 9 | 0.00001763 | 2.388 | 86.981 |
| 10 | 0.00001694 | 2.296 | 89.277 |
| 11 | 0.00001285 | 1.741 | 91.018 |
| 12 | 0.00001090 | 1.476 | 92.494 |
| 13 | 0.00000885 | 1.199 | 93.693 |
| 14 | 0.00000810 | 1.098 | 94.791 |
| 15 | 0.00000691 | 0.936 | 95.727 |
| 16 | 0.00000536 | 0.727 | 96.454 |
| 17 | 0.00000504 | 0.683 | 97.137 |
| 18 | 0.00000475 | 0.644 | 97.780 |
| 19 | 0.00000406 | 0.550 | 98.331 |
| 20 | 0.00000324 | 0.439 | 98.770 |
| 21 | 0.00000223 | 0.302 | 99.072 |

| | | | |
|---|---|---|---|
| 22 | 0.00000192 | 0.260 | 99.332 |
| 23 | 0.00000156 | 0.211 | 99.543 |
| 24 | 0.00000131 | 0.177 | 99.72 |
| 25 | 0.00000111 | 0.150 | 99.87 |
| 26 | 0.00000096 | 0.130 | 100 |

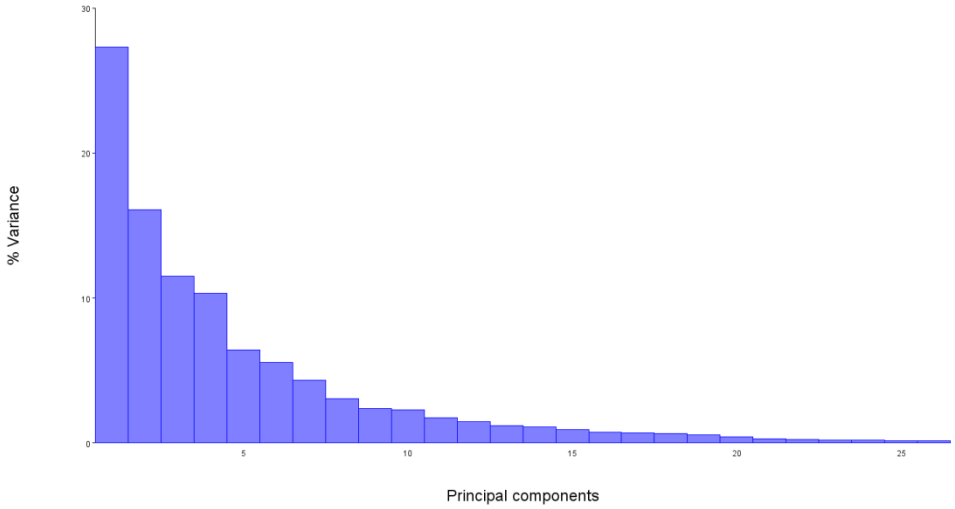



**Figure 5.10** Histogram illustrating the variances of all the principle components calculated from wing shape data of *Zeugodacus cucurbitae* collected from four sites across the West-Pacific.

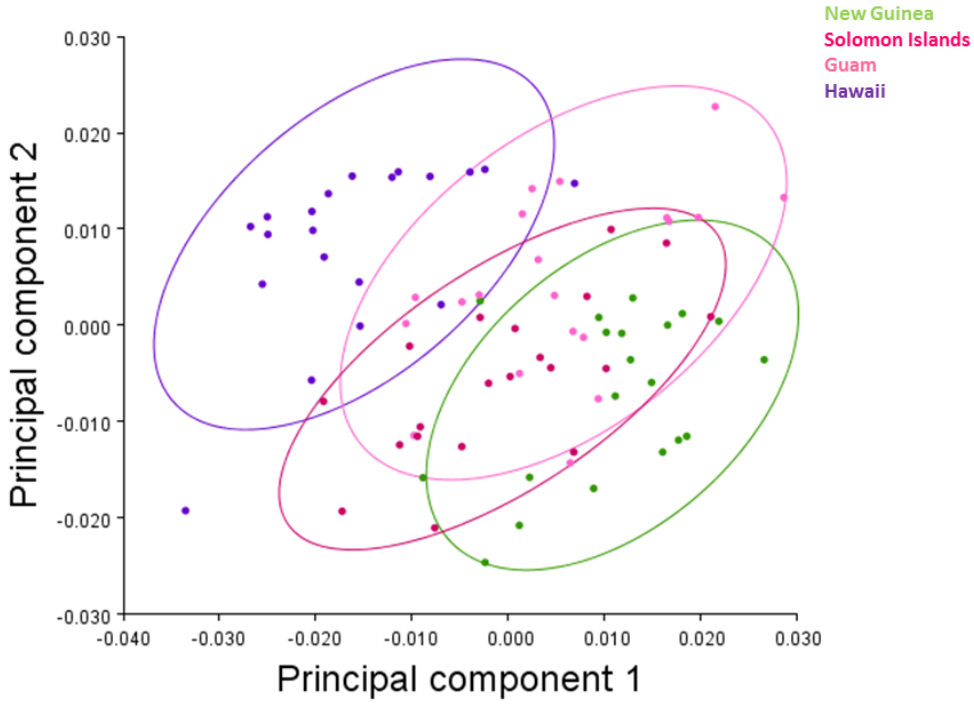



**Figure 5.11** First two principal components resulting from PCA of wing shape data *Zeugodacus cucurbitae* collected from four sites across the West-Pacific; 95% confidence ellipses are shown for each group. Each coloured dot represents the wing of a fly from one of the listed locations.

- Canonical variate analysis

Canonical variate analysis of the dataset based on the four sites from the West-Pacific groups resulted in three canonical variates, with the first two accounting for 88.57% of the variation (Table 5.7). Differentiation among groups was particularly evident along the first canonical axis which explained 59.25% of the variation, resulting a clear pattern in which Hawaii separated from the remaining sites; Solomon Islands was strongly separated from Guam and New Guinea along the second canonical variate (Figure 5.12). This pattern is reflected in the pairwise Mahalanobis distances between flies from four sites (Table 5.8).

**Table 5.7** Summary statistics for canonical variates analysis of groups of *Zeugodacus cucurbitae* collected from ten sites across Southeast-Asia. The table shows eigenvalues, percent variation and cumulative percent variation explained by each canonical variate axis for the entire dataset.

| CV | Eigenvalues | % Variance | Cumulative % |
|---|---|---|---|
| 1 | 8.27 | 59.25 | 59.25 |
| 2 | 4.09 | 29.32 | 88.57 |
| 3 | 1.56 | 11.43 | 100.00 |

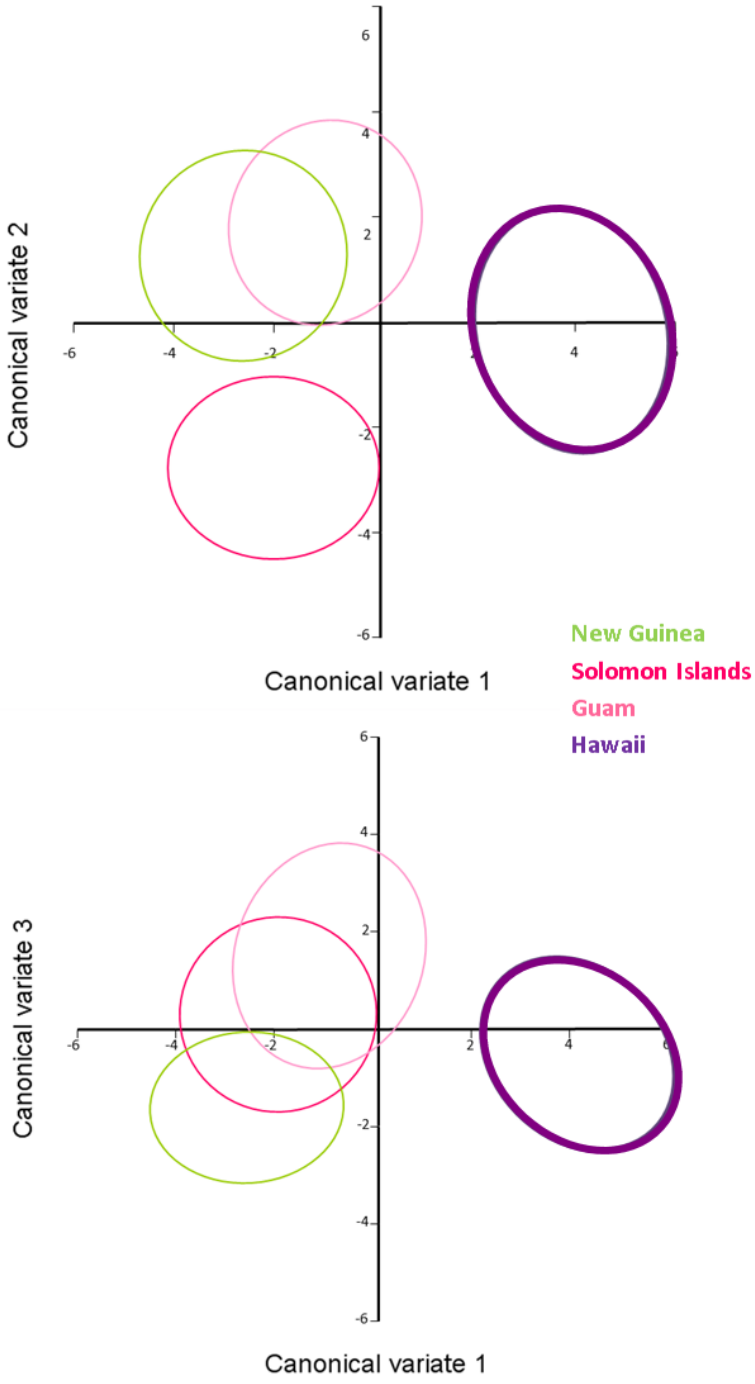



**Figure 5.12** First three principal components resulting from PCA of wing shape data *Zeugodacus cucurbitae* collected from four sites across the West-Pacific. 95% confidence ellipses are shown for each group. Individual data points have been removed for clarity.

**Table 5.8** Mahalanobis distances (below diagonal) as calculated from CVA based on wing shape data of *Zeugodacus cucurbitae* from four sites across the West-Pacific. All pairwise comparisons were significantly different following Bonferroni correction for multiple comparisons (initial α = 0.05 corrected to α = 0.001).Values above the diagonal are geographic distances (km) between sample sites.

| | | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| 1 | New Guinea | - | 1907 | 2225 | 7283 |
| 2 | Guam | **3.86** | - | 3040 | 6135 |
| 3 | Solomon Islands | **4.95** | **7.14** | - | 5862 |
| 4 | Hawaii | **7.37** | **6.19** | **5.40** | - |

*Isolation by distance*

Regression of pairwise Mahalanobis distances against geographical distance (km) across four sites from the West-Pacific (i.e., invasive range) revealed a significant IBD effect ($R^2 = 0.378$, $P < 0.05$) (Figure 5.13).

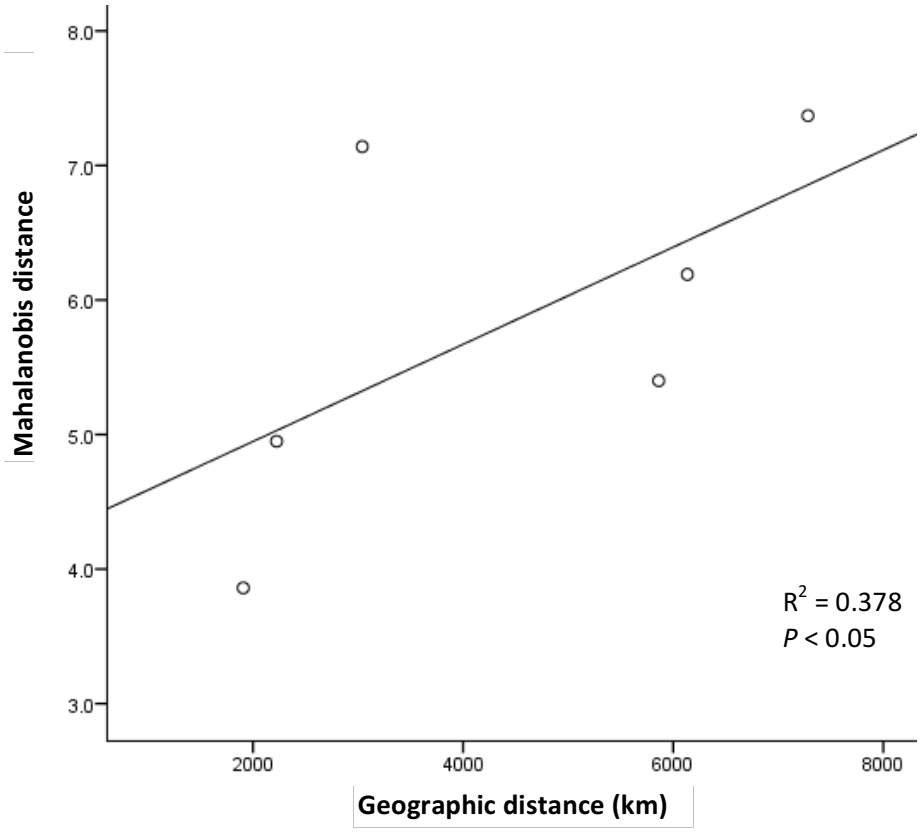



**Figure 5.13** Regression of Mahalanobis distances between groups based on sites following CVA on shape data generated from *Zeugodacus cucurbitae* wings against geographic distance (km) between the West-Pacific sample sites.

Analysis of wing shape of melon fly from Southeast-Asia and the West-Pacific

- Principal component analysis

The first two principal components accounted for 40.63% of the total shape variation (Table 5.9 and Figure 5.14). The first two principal axes did not show any appreciable separation of the melon fly populations (Figure 5.15).

**Table 5.9** Summary statistics for principal component analysis of groups of *Zeugodacus cucurbitae* collected from 14 sites across Southeast-Asia and the West-Pacific. The table shows eigenvalues, percent variation and cumulative percentage of variance explained by each principal component axis (PC) for the entire dataset.

| CV | Eigenvalues | % Variance | Cumulative % |
|---|---|---|---|
| 1 | 0.0002292 | 27.437 | 27.437 |
| 2 | 0.0001103 | 13.197 | 40.634 |
| 3 | 0.0000828 | 9.914 | 50.548 |
| 4 | 0.0000761 | 9.106 | 59.653 |
| 5 | 0.0000663 | 7.937 | 67.591 |
| 6 | 0.0000521 | 6.231 | 73.822 |
| 7 | 0.0000385 | 4.611 | 78.433 |
| 8 | 0.0000287 | 3.439 | 81.872 |
| 9 | 0.0000230 | 2.753 | 84.625 |
| 10 | 0.0000186 | 2.23 | 86.855 |
| 11 | 0.0000147 | 1.764 | 88.619 |
| 12 | 0.0000131 | 1.573 | 90.192 |
| 13 | 0.0000123 | 1.468 | 91.66 |
| 14 | 0.0000116 | 1.39 | 93.05 |
| 15 | 0.0000089 | 1.064 | 94.114 |
| 16 | 0.0000082 | 0.983 | 95.097 |
| 17 | 0.0000074 | 0.883 | 95.98 |
| 18 | 0.0000065 | 0.774 | 96.753 |
| 19 | 0.0000057 | 0.688 | 97.441 |
| 20 | 0.0000043 | 0.514 | 97.955 |
| 21 | 0.0000041 | 0.491 | 98.446 |
| 22 | 0.0000038 | 0.459 | 98.904 |
| 23 | 0.0000033 | 0.399 | 99.304 |
| 24 | 0.0000024 | 0.292 | 99.596 |
| 25 | 0.0000018 | 0.218 | 99.814 |
| 26 | 0.0000016 | 0.186 | 100 |

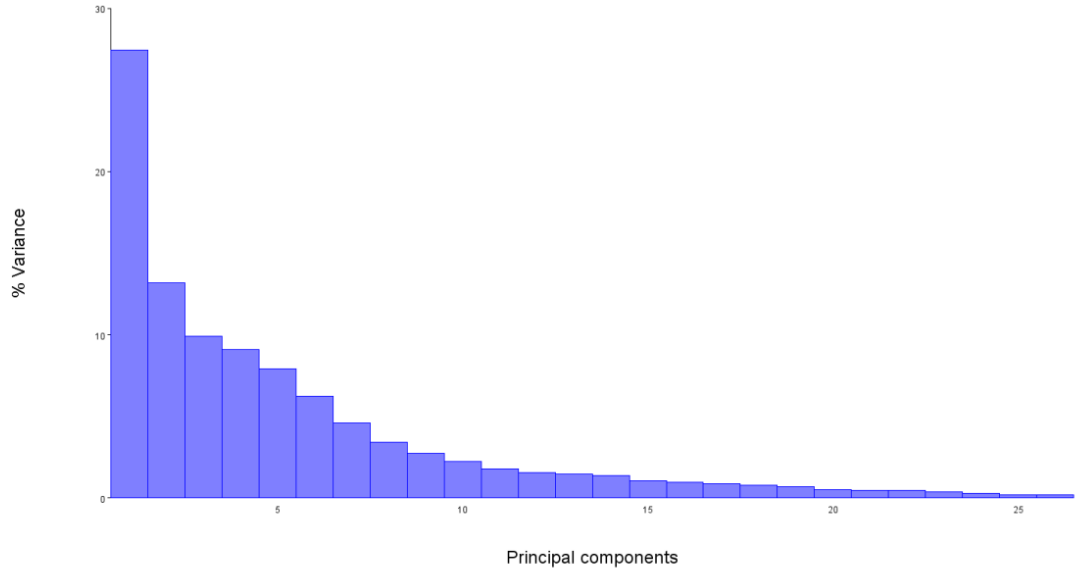



**Figure 5.14** Histogram illustrating the variances of all the principle components calculated from wing shape data of *Zeugodacus cucurbitae* collected from 14 sites across Southeast-Asia and the West-Pacific.

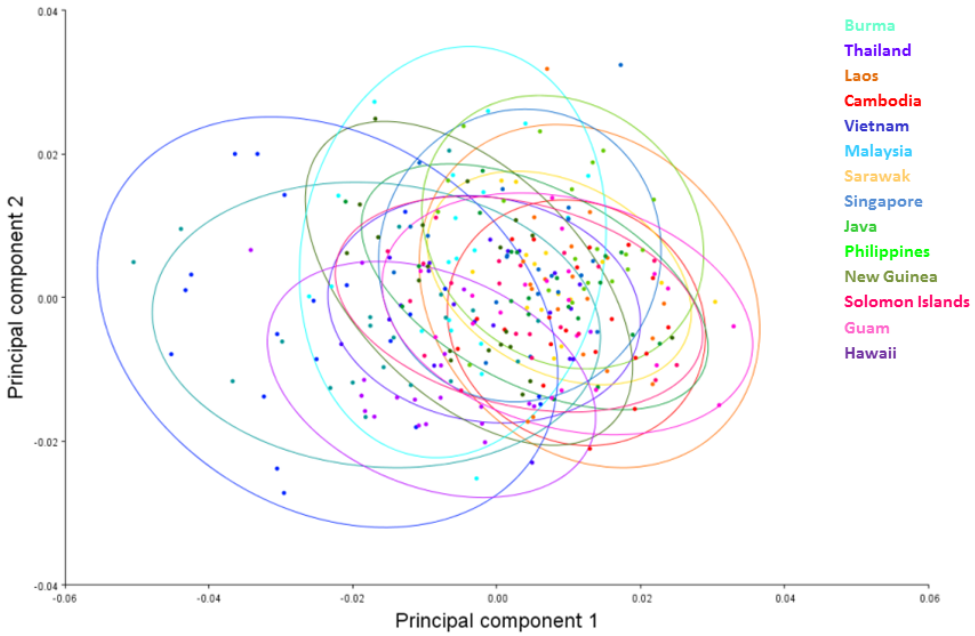



**Figure 5.15** First two principal components resulting from PCA of wing shape data *Zeugodacus cucurbitae* collected from 14 sites across Southeast-Asia and the West-Pacific; 95% confidence ellipses are shown for each group. Each coloured dot represents the wing of a fly from one of the listed locations

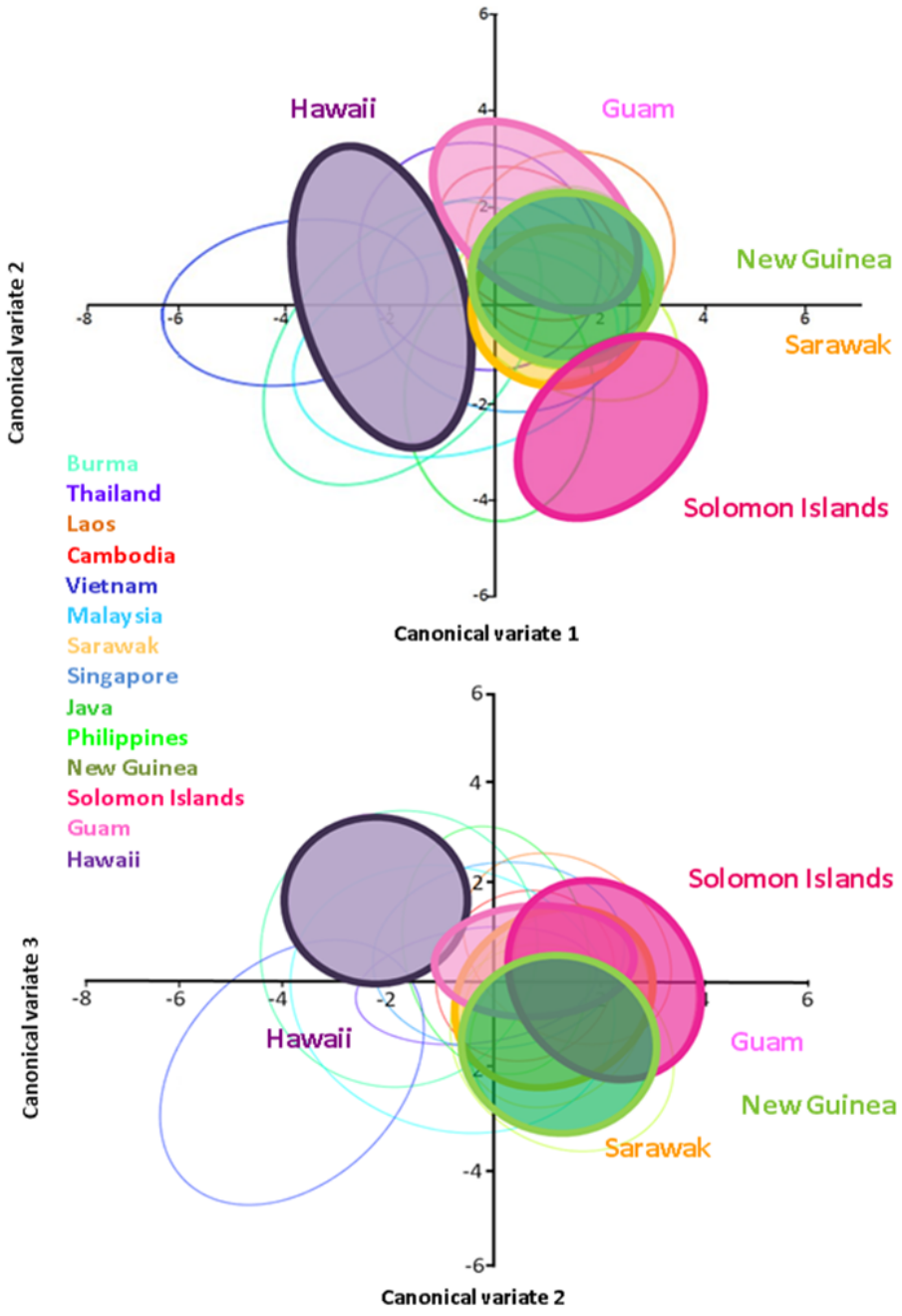
- Canonical variate analysis

Canonical variate analysis following correction for allometric effects (see above) yielded 13 canonical variates, for which the first two accounted for 53.19% of the variation (Table 5.10). The West-Pacific group heavily overlapped with Southeast-Asian sites based on the first two canonical variates. However, West-Pacific populations were particularly resolved from each other as per the individual analysis of Wes-Pacific populations above (i.e., Hawaii separated along CV1; Solomon Islands along CV2) (Figure 5.16A). The majority of Mahalanobis distance comparisons between groups were significantly different based on Bonferroni corrections for all comparisons ($P < 0.0005$), except Hawaii which did not significantly differ from Burma (Table 5.11). The greatest differences in wing shape, based on Mahalanobis distances, were between Vietnamese wings and the remaining sites (Table 5.11); average Mahalanobis distances between Vietnam and all other sites was 5.23 ± 0.83. Moreover, West Pacific wings (New Guinea, Guam and Solomon Islands) were highly similar in shape to wings from Sarawak, with an average Mahalanobis distance of 2.86 ± 0.58 (Table 5.11). Variation in wing shape was depicted as a landmark movement from a consensus configuration using wireframe; greatest deformation along the first canonical variate occurred at landmark four; while the second canonical variate revealed deformation about landmarks four and eight (Figure 5.16B).

**Table 5.10** Summary statistics for canonical variates analysis of groups of *Zeugodacus cucurbitae* collected from 14 sites across Southeast-Asia and the West-Pacific. The table shows eigenvalues, percent variation and cumulative percent variation explained by each canonical variate axis for the entire dataset.

| CV | Eigenvalues | % Variance | Cumulative % |
|---|---|---|---|
| 1 | 2.71 | 34.77 | 34.77 |
| 2 | 1.44 | 18.42 | 53.19 |
| 3 | 0.95 | 12.13 | 65.32 |
| 4 | 0.61 | 7.88 | 73.20 |
| 5 | 0.53 | 6.85 | 80.05 |
| 6 | 0.38 | 4.86 | 84.92 |
| 7 | 0.32 | 4.16 | 89.07 |
| 8 | 0.26 | 3.30 | 92.38 |
| 9 | 0.25 | 3.16 | 95.53 |
| 10 | 0.13 | 1.69 | 97.23 |
| 11 | 0.11 | 1.39 | 98.61 |
| 12 | 0.08 | 0.96 | 99.57 |
| 13 | 0.03 | 0.43 | 100.00 |

(A)


Hawaii
Guam
New Guinea
Sarawak
Solomon Islands
Canonical variate 2
Canonical variate 1
Burma
Thailand
Laos
Cambodia
Vietnam
Malaysia
Sarawak
Singapore
Java
Philippines
New Guinea
Solomon Islands
Guam
Hawaii
Canonical variate 3
Canonical variate 2

(B)

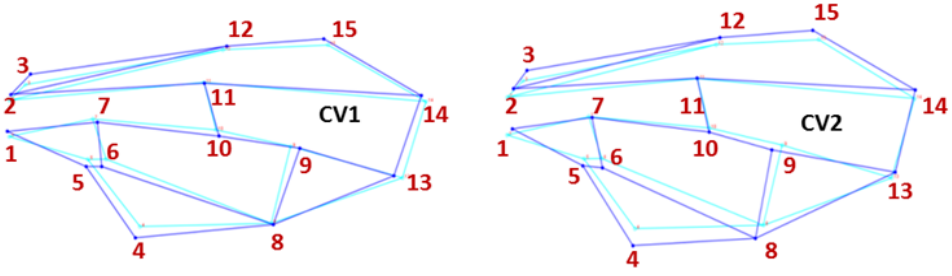

CV1
CV2

**Figure 5.16** Plot of the first three variates following canonical variate analysis of geometric morphometric wing shape data for *Zeugodacus cucurbitae* sampled from ten sites across Southeast-Asia and the West-Pacific. Fiteen to twenty wings were analysed per site; individual data points have been removed for clarity. (A) The four West-Pacific sites most geographically distant from Southeast-Asia (New-Guinea, Guam, Solomon Islands and Hawaii) are shown in bold. The yellow circle represents the wing shape of flies from Sarawak, for which the Mahalanobis distance is the closest with wing shape of flies from the West-Pacific sites; New Guinea, Solomon Islands and Guam. (B) Wireframe illustratrations are depicted for wing shape deformation along the first and second canonical variates.

**Table 5.11** Mahalanobis distances (below diagonal) among 14 groups of *Zeugodacus cucurbitae* collected across Southeast-Asia and the West-Pacific, as calculated from CVA. Values above the diagonal are geographic distances (km) between sites. Values in bold represent significantly different groups following Bonferroni correction for multiple comparisons (initial $\alpha = 0.05$ corrected to $\alpha = 0.001$).

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Burma | - | 506 | 693 | 1202 | 1106 | 1668 | 1937 | 3169 | 2414 | 2936 | 5393 | 5217 | 7602 | 10831 |
| 2 | Thailand | **3.111** | - | 481 | 697 | 951 | 1285 | 1516 | 2715 | 1908 | 2480 | 4903 | 4820 | 7118 | 10587 |
| 3 | Laos | **4.342** | **3.219** | - | 813 | 474 | 1674 | 1856 | 2977 | 1982 | 2298 | 4803 | 4530 | 6990 | 10169 |
| 4 | Cambodia | **3.693** | **2.963** | **2.819** | - | 1083 | 1028 | 1121 | 2170 | 1218 | 1854 | 4216 | 4259 | 6436 | 10191 |
| 5 | Vietnam | **3.804** | **4.740** | **6.136** | **5.400** | - | 2059 | 2198 | 3223 | 2088 | 2130 | 4667 | 4210 | 6809 | 9726 |
| 6 | Malaysia | **2.925** | **3.510** | **3.989** | **3.849** | **4.329** | - | 320 | 1556 | 1370 | 2477 | 4429 | 4900 | 6629 | 10986 |
| 7 | Singapore | **3.427** | **3.272** | **2.928** | **3.069** | **5.076** | **2.768** | - | 1249 | 1140 | 2311 | 4144 | 4704 | 6333 | 10820 |
| 8 | Java | **3.181** | **3.958** | **3.849** | **4.152** | **5.323** | **2.655** | **3.007** | - | 1321 | 2412 | 3374 | 4437 | 5427 | 10514 |
| 9 | Sarawak | **3.972** | **3.644** | **3.061** | **2.883** | **5.436** | **3.438** | **3.205** | **3.271** | - | 1223 | 3108 | 3568 | 5330 | 9694 |
| 10 | Philippines | **4.182** | **3.532** | **3.213** | **3.116** | **5.476** | **3.597** | **3.525** | **3.475** | **2.465** | - | 2537 | 2437 | 4695 | 8513 |
| 11 | New-Guinea | **4.373** | **3.541** | **3.181** | **3.313** | **5.594** | **3.529** | **3.509** | **4.104** | **2.286** | **3.021** | - | 1907 | 2225 | 7283 |
| 12 | Guam | **4.300** | **3.320** | **3.026** | **3.023** | **5.728** | **4.127** | **3.441** | **4.309** | **2.853** | **3.660** | **3.190** | - | 3040 | 6135 |
| 13 | Solomon | **4.598** | **4.837** | **4.485** | **4.551** | **6.889** | **4.485** | **4.349** | **3.630** | **3.454** | **3.680** | **4.109** | **5.000** | - | 5862 |
| 14 | Hawaii | 2.248 | **3.953** | **4.429** | **4.072** | **4.192** | **3.772** | **3.836** | **3.918** | **4.278** | **5.085** | **4.931** | **4.064** | **5.605** | - |

*Isolation by distance*

Tests of IBD were conducted to assess whether geographical distance is correlated with Mahalanobis distance (wing shape). IBD tests of combined invasive and native populations were conducted to determine whether observed wing shape is associated with geographical distance. Analyses were conducted on both Southeast-Asian and West-Pacific populations (New Guinea and Solomon Islands). Regression of pairwise Mahalanobis distances against geographical distance (km) across 12 sites revealed a significant but weak IBD effect ($R^2$ = 0.057, $P$ < 0.05) (Figure 5.17).

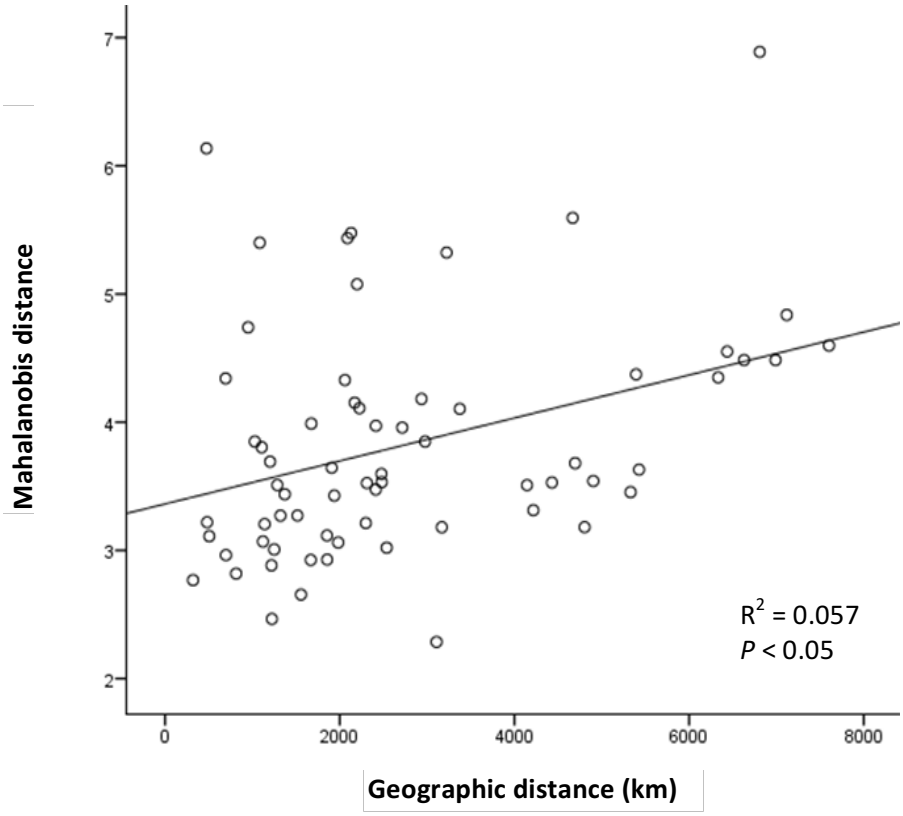



**Figure 5.17** Regression of Mahalanobis distances between groups based on site following CVA on shape data generated from *Zeugodacus cucurbitae* wings against geographic distance (km), across Southeast-Asia and the West-Pacific (New Guinea and Solomon Islands).

### 5.3.1.2 Aedeagus morphometric analysis

Two-hundred and seventy-five males from 14 sites (15-20 males/site) were dissected for measurement of their aedeagi. There was a strongly significant difference in aedeagus length among all sites ($F_{13, 261} = 5.398$; $P < 0.001$) (Figure 5.18). Southeast-Asian and the West Pacific flies were significantly different in terms of male genitalia length ($t_{1, 273} = 4.608$; $P < 0.05$), with the West-Pacific flies possessing significantly longer aedeagi but with slightly less variation than aedeagi from Southeast-Asian flies (2.747 ± 0.118 mm, 2.663 ± 0.126 mm, respectively); this result is similar to wing size, even though this difference was not significant in that case. Wing size of flies from Sarawak was significantly different to flies from Vietnam and Singapore, but the aedeagus length was not significantly different from these sites ($P > 0.05$). Wing shape of Vietnamese flies was relatively different from other sites from both Southeast-Asia and the West-Pacific (see above), yet Vietnamese aedeagus length was not significantly different from any other site (Figure 5.18).

Significant aedeagus length variation was observed within Southeast-Asian sites ($F_{9, 185} = 3.147$; $P < 0.001$). Aedeagi ranged from 2.185 to 3.043 mm; while Sarawak files have the smallest wings (and therefore smallest overall body size), their aedeagi were not especially long or short relative to other sites (2.655 ± 0.125 mm; Figure 5.18). Moreover, males from Laos (which had average wing size) had significantly shorter aedeagi than males from Burma, Malaysia, Singapore, Java and the Philippines ($P < 0.05$; Figure 5.18).

There were significant differences in aedeagi length among West-Pacific sites ($F_{3, 76} = 3.290$; $P < 0.05$), with aedeagi ranging from 2.689 ± 0.087 mm from Guam to 2.78 ± 0.106 mm (Solomon Islands). However, this was driven by significantly smaller aedeagi in flies from Guam relative to other West Pacific sites (i.e., New Guinea and Solomon Islands; and Hawaii = 2.767 ± 0.147 mm). Moreover, the aedeagi of Hawaiian flies were similar in length to aedeagi of flies from Guam, New Guinea and the Solomon Islands ($P > 0.05$), although aedeagi from Guam flies were significantly shorter than those from New Guinea and the Solomon Islands ($P < 0.05$).

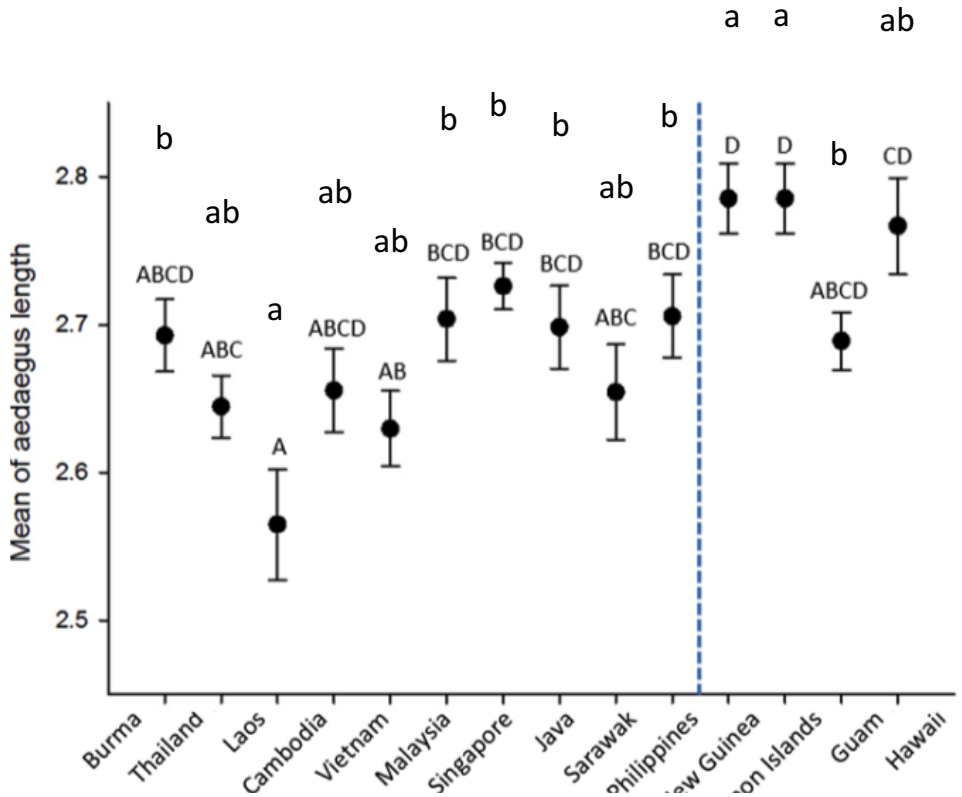



**Figure 5.18** Aedeagus length (mean ± SE) of *Zeugodacus cucurbitae* from 14 sites across Southeast-Asia and the West-Pacific. Uppercase letters indicate differences when compared among all sites; the lower case letters indicate differences within sitesof Southeast Asia and the West-pacific. Samples sharing the same letters are not statistically different from each other based on one-way ANOVA with Tukey *post hoc* test ($P > 0.05$). The dotted blue line divides Southeast-Asia and West-Pacific sites.

*Isolation by distance*

While the regression of wing shape against geographic distance (km) (within Southeast-Asia) was not significant (see above), the regression of aedeagus length revealed a weak but significant relationship between aedeagus length and geographic distance ($R^2 = 0.030$, $P < 0.05$; Figure 5.19A). However, when two sites from the invasive area (New Guinea and Solomon Islands), were added to the dataset the result showed a relationship between aedeagus length and geographic distance ($R^2 = 0.132$, $P < 0.05$) (Figure 5.19B).

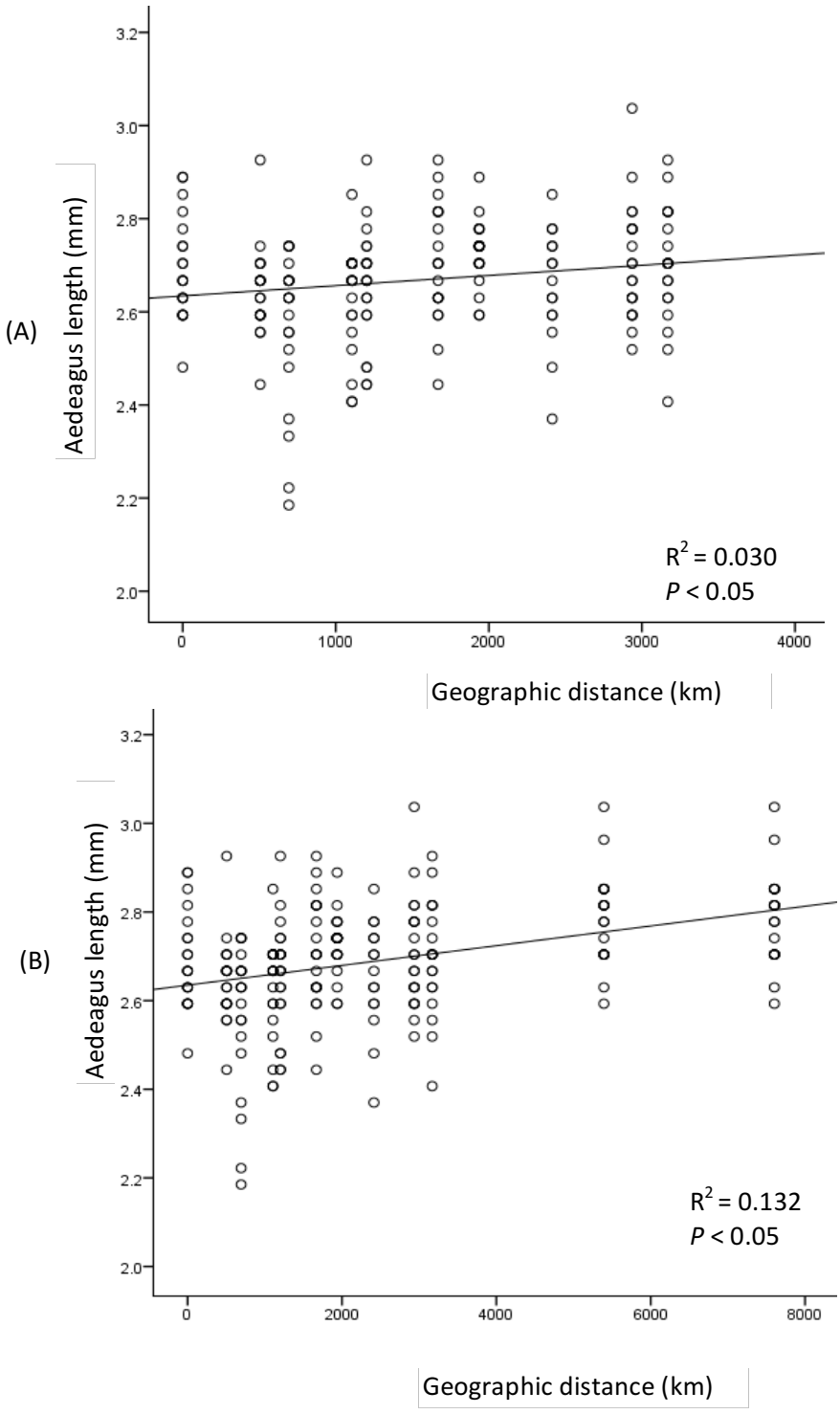



**Figure 5.19** Regression of aedeagus length pairwise geographic localities against geographic distance (in km). (A) Across Southeast-Asia (B) Across Southeast-Asia and the West-Pacific (New Guinea and Solomon Islands).

### 5.3.2 Molecular results

#### 5.3.2.1 Mitochondrial DNA (*cox1*) analysis

*Gene diversity*

A total of 262 specimens of *Z. cucurbitae* were sequenced for the *cox1* gene from 14 sites across Southeast-Asia and the West-Pacific (Table 5.1). After sequence alignment, 28 unique haplotypes were observed in a span of 652 base pairs of the aligned sequences. The samples from Laos showed the most diversity with 12 haplotypes (n=20), followed by Singapore with five haplotypes (n=20) and only one haplotype was found in each of Sarawak and Guam (n=15 and 20, respectively). The average number of unique haplotypes sampled from Southeast-Asian sites was greater than the West-Pacific (4.1 ± 3.034 and 2.5 ± 1.290 haplotypes; Table 5.12). Only five of 28 haplotypes were found in more than one site (Haplotypes 1, 5, 8, 12 and 14), with two common haplotypes (H1 and H2) found across most sites; 23 haplotypes were restricted to a single sites. Haplotype 1 was shared by all populations except the Solomon Islands and Guam, while H2 was shared among all populations except Malaysia and Sarawak (Table 5.12).

Genetic variability in Southeast-Asia was higher than the West-Pacific (Table 5.13). Population genetic diversity ranged from 0.1000 ± 0.0880 (Burma) to 0.8895 ± 0.0548 (Laos) and the population parameter θπ ranged from 0.100 (New Guinea) to 1.584 (Laos). The mean gene diversity of Southeast-Asian populations (0.4188 ± 0.3173) was greater than West-Pacific populations (0.2335 ± 0.2438). Although gene diversity in West-Pacific populations was generally very low, diversity in the Hawaiian population was considerably higher than other sites in this region (genetic diversity 0.555 ± 0.130 and θπ = 0.62).

Tajima's *D* tests of neutrality the entire genetic data set were negative and statistically significant (D = -2.245, $P < 0.0001$). Tests based on Fu's *Fs* were also negative and statistically significant across the data set (F = -2.31, $P < 0.0001$) (Table 5.13).

The *cox1* haplotypes median-joining (MJ) network revealed a central starburst-like pattern from the two common haplotypes (i.e., H1 and H2), with

numerous (26) singletons radiating from these haplotypes (Figures 5.20 and 5.21). There was no distinct pattern between the haplotypes and their geographical distribution and haplotypes from a given site were commonly distributed across the network. Haplotype 1 was shared by all sites from Southeast-Asia except Java and two sites (PNG and Hawaii) from the West-Pacific. Haplotype 2 was shared with all sites from Southeast-Asia except Malaysia and Sarawak, and was found in all West Pacific sites (Table 5.13, Figure 5.20). Southeast-Asian populations possessed greater variation and endemicity (19 endemic haplotypes) than the West-Pacific (4 endemic haplotypes). Of the four endemic West Pacific haplotypes, two of these were found only in Hawaii and two were sampled only from the Solomon Islands. Both common haplotypes were represented in West-Pacific populations: Hawaii and West Papua included both common haplotypes (H1 and H2), whereas Guam and the Solomon Islands shared only H1 (Figure 5.21).

Furthermore, 71 sequences from NCBI GenBank (e.g., African countries, India and China) were merged with 262 sequences for median-joining (MJ) network. India and China shared the common haplotype H1 with most other sites. China possessed four endemic haplotypes and shared haplotypes 2 and 28 (H2 and H28) with the Southeast Asian populations. On the other hand, African populations possessed two unique haplotypes not shared with Southeast-Asian populations. One of the African haplotypes is much more common than the other (H29), is shared among all sites, and is connected to two Southeast Asian haplotypes plus H1 by only a single base change (Table 5.14, Figure 5.22).

**Table 5.12** Distribution of *cox1* haplotypes of *Zeugodacus cucurbitae* from 14 sites across Southeast-Asia and the West-Pacific as generated from this study. Highlighted in grey haplotypes observed in more than one site.

| Haplotype: | Burma | Thailand | Laos | Cambodia | Vietnam | Malaysia | Singapore | Java | Sarawak | Philippines | New Guinea | Solomon | Guam | Hawaii |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 19 | 20 | 20 | 19 | 20 | 18 | 19 | 20 | 15 | 20 | 20 | 20 | 20 | 19 |
| Hap_1 | 18 | 5 | 6 | 7 | 10 | - | 1 | 17 | - | 1 | 1 | 18 | 20 | 12 |
| Hap_2 | 1 | 13 | 4 | 10 | 6 | 17 | 13 | - | 15 | 18 | 19 | - | - | 5 |
| Hap_3 | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - |
| Hap_4 | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - |
| Hap_5 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| Hap_6 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| Hap_7 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| Hap_8 | - | - | 1 | - | - | - | - | 1 | - | - | - | - | - | - |
| Hap_9 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| Hap_10 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| Hap_11 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| Hap_12 | - | 1 | 1 | - | - | - | - | - | - | - | - | - | - | - |
| Hap_13 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| Hap_14 | - | - | 1 | - | - | - | - | - | - | 1 | - | - | - | - |
| Hap_15 | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - |
| Hap_16 | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - |
| Hap_17 | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - |
| Hap_18 | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - |
| Hap_19 | - | - | - | - | - | - | 2 | - | - | - | - | - | - | - |
| Hap_20 | - | - | - | - | - | - | 2 | - | - | - | - | - | - | - |
| Hap_21 | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| Hap_22 | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| Hap_23 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - |
| Hap_24 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - |
| Hap_25 | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_26 | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - |
| Hap_27 | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - |
| Hap_28 | - | - | - | - | 2 | - | - | - | - | - | - | - | - | - |

**Table 5.13** Distribution of *cox1* haplotypes of *Zeugodacus cucurbitae* from 14 sites across Southeast-Asia, West-Pacific and India, China and Africa countries (obtained from Genbank; see Appendix 4 for accession numbers).

| Location | Burma | Thailand | Laos | Cambodia | Vietnam | Malaysia | Singapore | Java | Sarawak | Philippines | New Guinea | Solomon | Guam | Hawaii | India | China | Kenya | Tanzania | Sudan | Reunion |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | 19 | 20 | 20 | 19 | 20 | 18 | 19 | 20 | 15 | 20 | 20 | 20 | 20 | 19 | 4 | 33 | 19 | 7 | 1 | 7 |
| Hap_1 | 18 | 5 | 6 | 7 | 10 | - | 1 | 17 | - | 1 | 1 | 18 | 20 | 12 | 3 | 16 | - | - | - | - |
| Hap_2 | 1 | 13 | 4 | 10 | 6 | 17 | 13 | - | 15 | 18 | 19 | - | - | 5 | - | 7 | - | - | - | - |
| Hap_3 | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_4 | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_5 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_6 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - |
| Hap_7 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_8 | - | - | 1 | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_9 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_10 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_11 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_12 | - | 1 | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_13 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_14 | - | - | 1 | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - |
| Hap_15 | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_16 | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_17 | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_18 | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_19 | - | - | - | - | - | - | 2 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_20 | - | - | - | - | - | - | 2 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_21 | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - |
| Hap_22 | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - |
| Hap_23 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - |
| Hap_24 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - |
| Hap_25 | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_26 | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_27 | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap_28 | - | - | - | - | 2 | - | - | - | - | - | - | - | - | - | - | 5 | - | - | - | - |
| Hap_29 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 19 | 6 | 1 | 7 |
| Hap_30 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - |
| Hap_31 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - | - |

**Table 5.13** continued

| Location | Burma | Thailand | Laos | Cambodia | Vietnam | Malaysia | Singapore | Java | Sarawak | Philippines | New Guinea | Solomon | Guam | Hawaii | India | China | Kenya | Tanzania | Sudan | Reunion |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | 19 | 20 | 20 | 19 | 20 | 18 | 19 | 20 | 15 | 20 | 20 | 20 | 20 | 19 | 4 | 33 | 19 | 7 | 1 | 7 |
| Hap_32 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - |
| Hap_33 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - |
| Hap_34 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - |
| Hap_35 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - |

**Denoted Locations** China, India, Kenya, Tanzania, Sudan, Reunion from NCBI GenBank

**Table 5.14** Population genetics summary statistics from *cox1* analysis of *Zeugodacus cucurbitae* from 14 sites across Southeast-Asia and the West-Pacific.

| Sample site | number of individuals | Gene Diversity | Θ¶ | Tajima's*D* | Tajiam's *D* *P*-value | Fu'*Fs* | Fu'*Fs* *P*-value |
|---|---|---|---|---|---|---|---|
| Burma | 19 | 0.105 ± 0.092 | 0.105 | -1.165 | 0.152 | -0.838 | 0.075 |
| Thailand | 20 | 0.779 ± 0.085 | 1.221 | -1.545 | 0.054 | -5.641 | 0.000 |
| Laos | 20 | 0.889 ± 0.055 | 1.584 | -1.532 | 0.040 | -9.449 | 0.000 |
| Cambodia | 19 | 0.614 ± 0.075 | 0.842 | -0.778 | 0.228 | -0.453 | 0.318 |
| Vietnam | 20 | 0.679 ± 0.080 | 1.058 | -0.763 | 0.234 | -0.971 | 0.201 |
| Malaysia | 18 | 0.111 ± 0.096 | 0.111 | -1.165 | 0.142 | -0.794 | 0.074 |
| Singapore | 19 | 0.532 ± 0.130 | 0.608 | -1.380 | 0.087 | -2.504 | 0.007 |
| Java | 20 | 0.284 ± 0.128 | 0.300 | -1.723 | 0.018 | -2.749 | 0.003 |
| Sarawak | 15 | 0.000 ± 0.000 | 0.000 | 0.000 | 1.000 | 0.000 | N.A. |
| Philippines | 20 | 0.195 ± 0.114 | 0.200 | -1.513 | 0.038 | -1.863 | 0.010 |
| New Guinea | 20 | 0.100 ± 0.088 | 0.100 | -1.164 | 0.134 | -0.879 | 0.079 |
| Solomon | 20 | 0.278 ± 0.075 | 0.293 | -1.704 | 0.007 | -5.064 | 0.000 |
| Guam | 20 | 0.000 ± 0.000 | 0.000 | 0.000 | 1.000 | 0.000 | N.A. |
| Hawaii | 19 | 0.556 ± 0.103 | 0.620 | -0.755 | 0.253 | -1.135 | 0.092 |

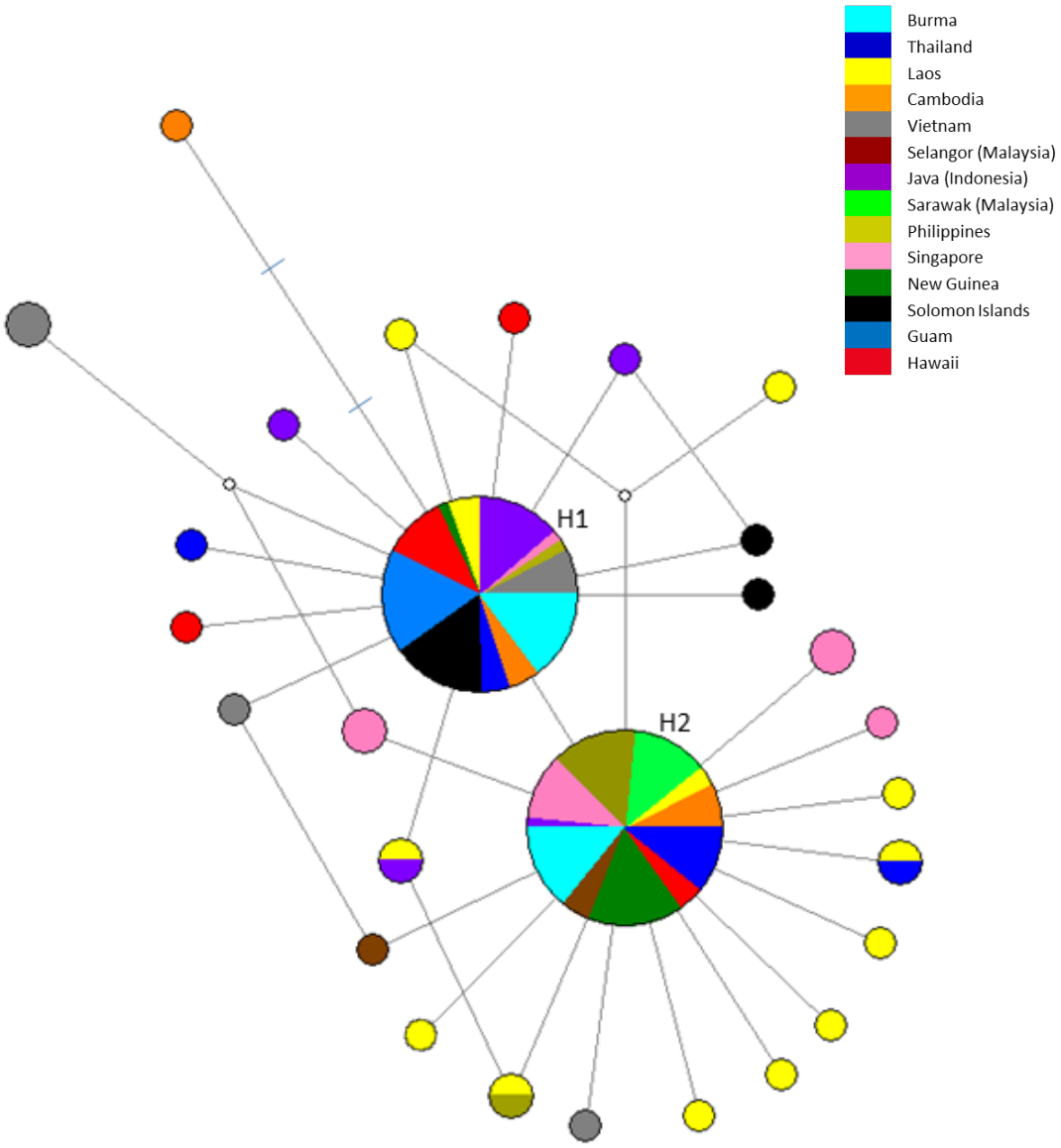



**Figure 5.20** Median-joining haplotype network generated from *cox1* data from *Zeugodacus cucurbitae* collected from 14 sites in Southeast-Asia and the West-Pacific; 28 haplotypes are represented. Small white circles are median vectors. Different colours represent different collecting sites. Sizes of nodes and pie segments are proportional to haplotype frequency. Length of branches is proportional to number of mutational changes between haplotypes.

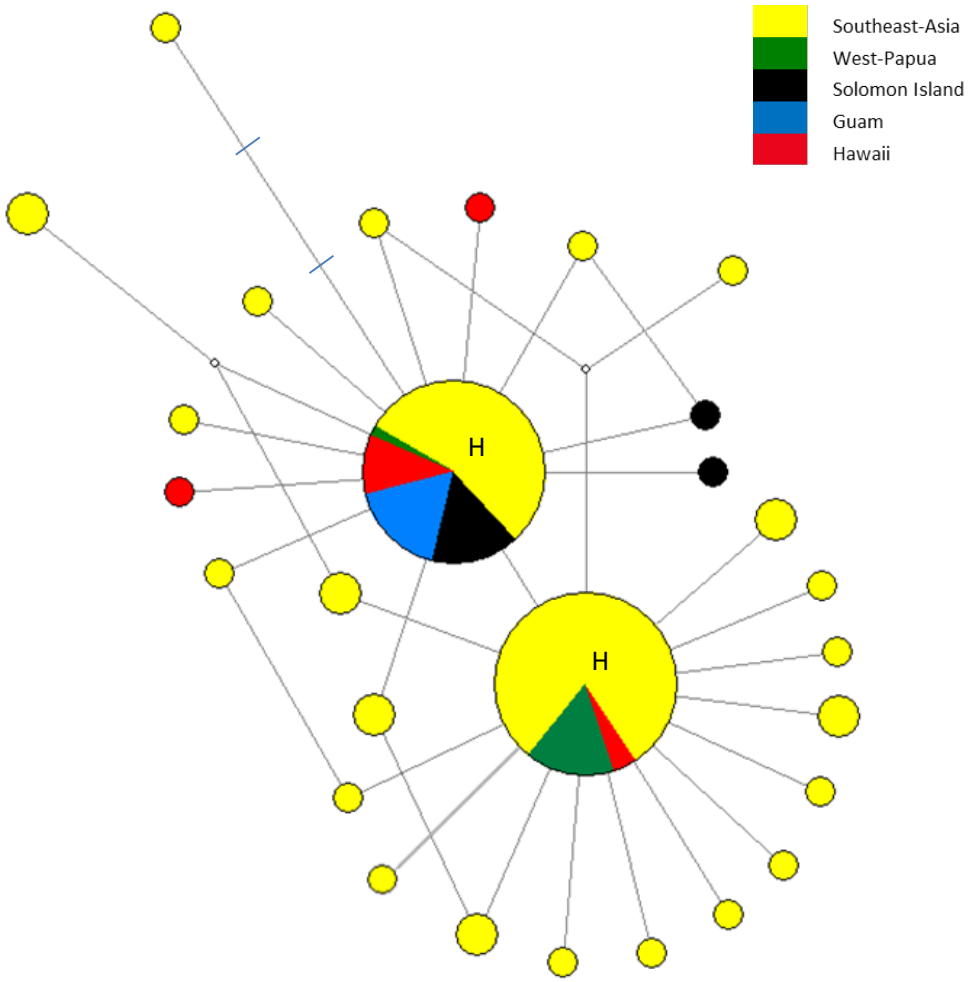



**Figure 5.21** Median-joining haplotype network generated from *cox1* data from *Zeugodacus cucurbitae* collected from 14 sites in Southeast-Asia and the West-Pacific; 28 haplotypes are represented. Small white circles are median vectors. Different colours represent different collecting sites, with all Southeast-Asian sites coloured yellow. Sizes of nodes and pie segments are proportional to haplotype frequency. Length of branches is proportional to number of mutational changes between haplotypes.

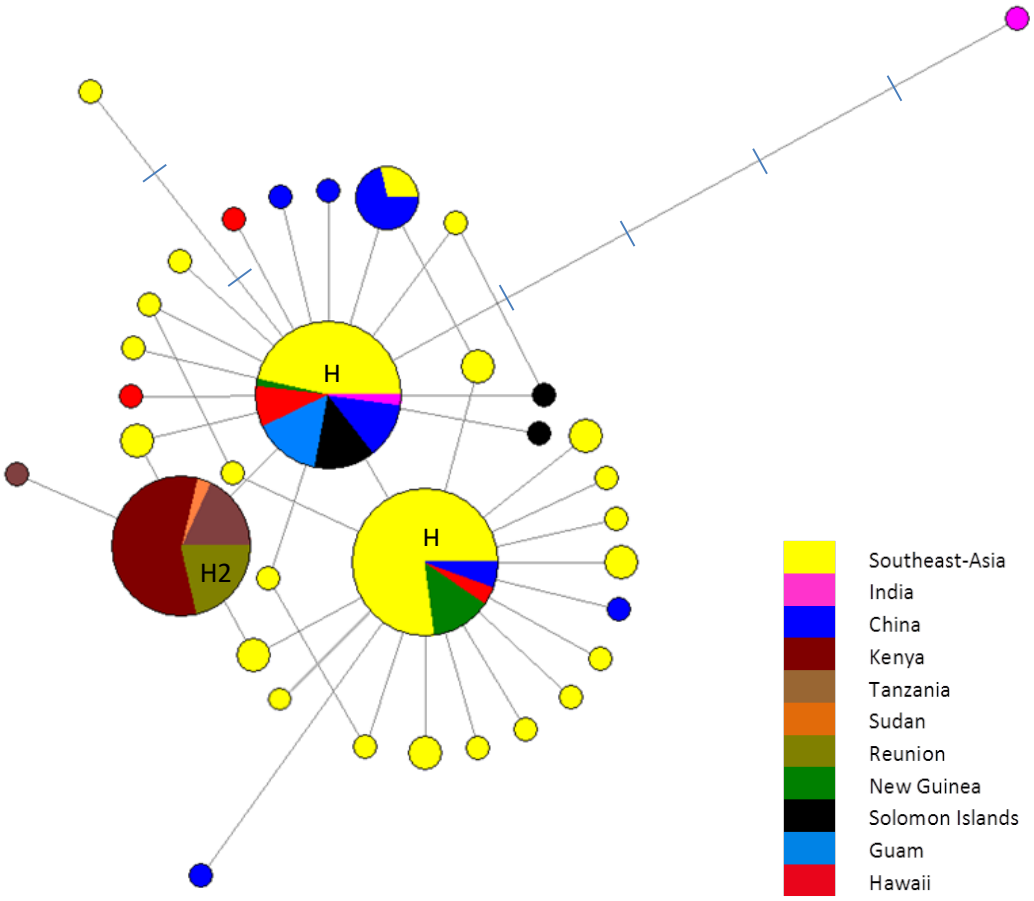



**Figure 5.22** Median-joining haplotype network generated from *cox1* data from *Zeugodacus cucurbitae* collected from 14 sites in Southeast-Asia and the West-Pacific in addition to 71 sequences from NCBI Genbank for African, Chinese, and Indian sites; 35 haplotypes are represented. Small white circles are median vectors. Different colours represent different collecting sites. Sizes of nodes and pie segments are proportional to haplotype frequency. Length of branches is proportional to number of mutational changes between haplotypes.

*Genetic differentiation (cox1)*

Population pairwise $F_{ST}$ analysis revealed that 28.57% of populations were not significantly different genetically (Table 5.15). Analysis of molecular variance (AMOVA) was conducted to assess partitioning of variation within and among hierarchical levels (within populations, among population within groups and among groups). Populations were grouped according to whether they formed part of the native or invasive range (Southeast-Asia and the West-Pacific); 3.90% of the variation was between groups, but this was not statistically significant. The variation within populations was 57.60%, among populations within groups was 38.50% and both results were statistically significant (Table 5.16).

**Table 5.15** Pairwise $F_{ST}$ distances between population of *Zeugodacus cucurbitae* collected from 14 sites across Southeast-Asia and the West-Pacific. Values in bold represent significantly different groups following Bonferroni correction for multiple comparisons (initial α = 0.05 corrected to α = 0.001).

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Burma | - | | | | | | | | | | | | |
| 2 | Thailand | **0.515** | - | | | | | | | | | | | |
| 3 | Laos | **0.253** | -0.008 | - | | | | | | | | | | |
| 4 | Cambodia | **0.304** | 0.008 | -0.010 | - | | | | | | | | | |
| 5 | Vietnam | **0.122** | **0.115** | 0.043 | 0.016 | - | | | | | | | | |
| 6 | Malaysia | **0.892** | **0.165** | **0.147** | **0.301** | **0.403** | - | | | | | | | |
| 7 | Singapore | **0.694** | **0.086** | **0.102** | **0.187** | **0.290** | 0.029 | - | | | | | | |
| 8 | Java | -0.001 | **0.504** | **0.270** | **0.319** | **0.151** | **0.826** | **0.669** | - | | | | | |
| 9 | Sarawak | **0.938** | **0.165** | **0.136** | **0.302** | **0.403** | -0.011 | 0.021 | **0.853** | - | | | | |
| 10 | Philippines | **0.839** | 0.104 | **0.107** | **0.243** | **0.361** | -0.001 | 0.017 | **0.782** | -0.015 | - | | | |
| 11 | West Papua | **0.886** | 0.116 | **0.122** | **0.263** | **0.381** | 0.000 | 0.020 | **0.818** | -0.015 | -0.035 | - | | |
| 12 | Guam | 0.003 | **0.598** | **0.311** | **0.392** | **0.186** | **0.950** | **0.754** | 0.000 | **1.000** | **0.900** | **0.947** | - | |
| 13 | Solomon | -0.001 | **0.532** | **0.287** | **0.340** | **0.161** | **0.864** | **0.695** | -0.010 | **0.897** | **0.818** | **0.857** | 0.000 | - |
| 14 | Hawaii | 0.078 | **0.211** | **0.082** | 0.057 | -0.006 | **0.588** | **0.432** | **0.115** | **0.602** | **0.532** | **0.566** | **0.164** | **0.128** |

**Table 5.16** Results of the Analysis of Molecular Variance (AMOVA) for the *cox1* dataset generated for Thai *Zeugodacus cucurbitae* popluations. Samples were constrained according to geographical region in relation Southeast-Asia and the West-Pacific. For locations of sites and biogeographic zones see Figures 5.1 and Table 5.1.

| Source of variation | d.f. | Sum of square | Variance of components | % total variance | Fixation indices |
|---|---|---|---|---|---|
| Among groups | 1 | 5.055 | 0.01583 a | 3.90 | $F_{CT}$ = 0.039 |
| Among populations within groups | 12 | 38.727 | 0.15611 b | 38.50 | $F_{ST}$ = 0.424* |
| Within populations | 255 | 59.555 | 0.23355 c | 57.60 | $F_{SC}$ = 0.400* |
| Total | 268 | 103.337 | 0.40548 | | |

* $P < 0.05$

*Isolation by distance*

Analyses were conducted on two datasets: one comprising only Southeast-Asian populations, and the second comprising both Southeast-Asian and West-Pacific populations. IBD tests of combined invasive and native populations were conducted to determine whether observed genetic structure is associated with geographical distance. The results from both analyses showed that regression relationship was non-significant (Southeast-Asia only $R^2 = 0.056$, $P > 0.05$; combined $R^2 = 0.035$, $P > 0.05$) (Figure 5.23).

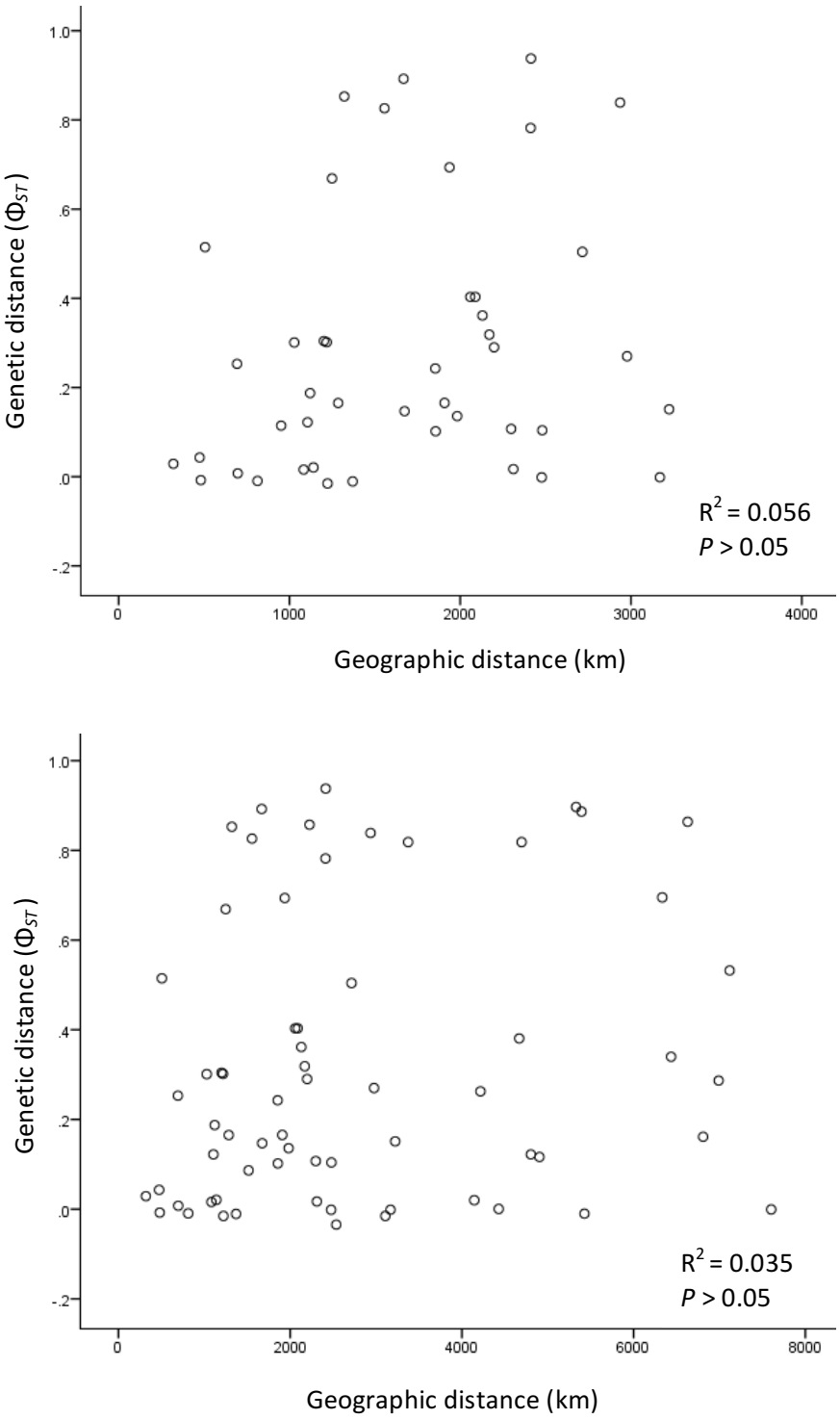



**Figure 5.23** Regression of pairwise genetic distances $\Phi_{ST}$ against geographic distance (km) across Southeast-Asia.

### 5.3.2.2 Microsatellite analysis

*Gene diversity*

Eleven microsatellite loci were screened for 280 individuals from 14 geographic locations. Approximately 6.4% of the data were missing in the final data set, a result of 18 PCR reactions that did not successfully amplify for certain individuals.

The number of alleles per locus varied from 1.91 (Hawaii) to 3.82 (Burma, Thailand and Cambodia) while the allelic richness ranged from 0.129 (Hawaii) to 1.492 (Burma). The average of number of alleles and allelic richness from Southeast-Asia (3.292 ± 0.515 and 1.421 ± 0.053, respectively) is comparatively higher than the West-Pacific (2.567 ± 0.532 and 1.358 ± 0.049, respectively) (Table 5.17). Moderate to high levels of intra-population diversity were reflected in the observed heterozygosity, which varied from 0.2197 (Hawaii) to 0.4192 (Thailand). Moreover, average gene diversity over all loci per sample site ranged from 0.297 ± 0.189 (Philippines) to 0.536 ± 0.297 (Thailand) (Table 5.17).

**Table 5.17** Locations, sample sizes, microsatellite diversity estimates and Hardy-Weinberg (H-W) equilibrium test values for *Zeugodacus cucurbitae* populations from 14 sites from Southeast-Asia and the West-Pacific. No values were significant after Bonferroni correction for multiple tests (initial $\alpha = 0.05$ corrected to $\alpha = 0.001$).

| Locations | Sample size | Expected heterozygosity | Observed heterozygosity | No Alleles | Allelic Richness | Fis | H-W | Gene Diversity |
|---|---|---|---|---|---|---|---|---|
| Burma | 20 | 0.4915 | 0.3753 | 3.82 | 1.492 | 0.243 | 0.000 | 0.527 ± 0.319 |
| Thailand | 20 | 0.4708 | 0.4192 | 3.82 | 1.471 | 0.113 | 0.067 | 0.536 ± 0.297 |
| Laos | 19 | 0.4361 | 0.4185 | 3.55 | 1.436 | 0.042 | 0.867 | 0.490 ± 0.279 |
| Cambodia | 20 | 0.4618 | 0.3995 | 3.82 | 1.462 | 0.139 | 0.006 | 0.478 ± 0.265 |
| Vietnam | 20 | 0.4301 | 0.3701 | 3.55 | 1.430 | 0.143 | 0.022 | 0.465 ± 0.262 |
| Malaysia | 19 | 0.3551 | 0.2834 | 2.82 | 1.355 | 0.207 | 0.106 | 0.414 ± 0.241 |
| Java | 20 | 0.4222 | 0.3596 | 3.18 | 1.443 | 0.210 | > 0.001 | 0.422 ± 0.241 |
| Sarawak | 15 | 0.3796 | 0.2690 | 2.73 | 1.422 | 0.152 | 0.019 | 0.368 ± 0.233 |
| Philippines | 19 | 0.3572 | 0.3022 | 2.36 | 1.380 | 0303 | 0.466 | 0.297 ± 0.189 |
| Singapore | 19 | 0.4430 | 0.3524 | 3.27 | 1.327 | 0.285 | 0.152 | 0.418 ± 0.249 |
| New Guinea | 20 | 0.3273 | 0.2360 | 2.45 | 1.357 | 0.159 | 0.002 | 0.299 ± 0.190 |
| Solomon Is | 19 | 0.4001 | 0.3223 | 3.18 | 1.400 | 0.199 | 0.001 | 0.467 ± 0.273 |
| Guam | 20 | 0.3850 | 0.4207 | 2.73 | 1.385 | -0.095 | > 0.001 | 0.348 ± 0.204 |
| Hawaii | 12 | 0.2899 | 0.2197 | 1.91 | 1.290 | 0.258 | 0.108 | 0.000 ± 0.000 |

*Genetic differentiation and relationships among populations*

Population pairwise $R_{ST}$ analysis revealed that 37.36% populations were genetically not significantly different (Table 5.18). Analysis of molecular variance (AMOVA) was performed based on two groups (Southeast-Asian; native range and West-Pacific populations; invasive range). Only 13.40% of the variation was observed among populations within groups, and the remaining (88.35%) was found within populations. There was not significant variation ($F_{CT}$ = - 0.0157, $P$ > 0.05) between native Test of Isolation-By-Distance (IBD) among all Southeast-Asian samples sites were significant between genetic distances ($R_{ST}$) and geographic distance (km) ($R^2$ = 0.253, $P$ < 0.05), while IBD among site in Southeast-Asia and the West-Pacific also was significant ($R^2$ = 0.248, $P$ < 0.05) (Figure 5.24).

**Table 5.18** Pairwise $R_{ST}$ distances between populations of *Zeugodacus cucurbitae* collected from 14 sites across Southeast-Asia and the West-Pacific. Values in bold represent significantly different groups following Bonferroni correction for multiple comparisons (initial α = 0.05 corrected to α = 0.001).

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Burma | - | | | | | | | | | | | | |
| 2 | Thailand | -0.003 | - | | | | | | | | | | | |
| 3 | Laos | 0.002 | -0.018 | - | | | | | | | | | | |
| 4 | Cambodia | 0.007 | -0.008 | -0.021 | - | | | | | | | | | |
| 5 | Vietnam | -0.027 | -0.005 | 0.002 | -0.002 | - | | | | | | | | |
| 6 | Malaysia | 0.066 | 0.065 | 0.085 | **0.125** | **0.072** | - | | | | | | | |
| 7 | Singapore | **0.049** | **0.084** | 0.088 | **0.120** | **0.055** | 0.013 | - | | | | | | |
| 8 | Java | **0.102** | **0.142** | **0.148** | **0.184** | **0.123** | 0.025 | -0.019 | - | | | | | |
| 9 | Sarawak | **0.249** | **0.222** | **0.194** | **0.254** | **0.235** | 0.139 | **0.119** | **0.063** | - | | | | |
| 10 | Philippines | **0.112** | **0.175** | **0.172** | **0.201** | **0.131** | **0.082** | 0.007 | 0.000 | **0.133** | - | | | |
| 11 | New Guinea | **0.062** | **0.327** | **0.295** | **0.336** | **0.294** | **0.082** | **0.169** | **0.108** | **0.191** | **0.062** | - | | |
| 12 | Guam | **0.076** | 0.026 | **0.061** | **0.073** | 0.034 | **0.138** | **0.225** | **0.268** | **0.493** | **0.315** | **0.615** | - | |
| 13 | Solomon | **0.233** | **0.233** | **0.204** | **0.257** | **0.222** | **0.159** | 0.077 | 0.017 | 0.026 | **0.048** | **0.088** | **0.530** | - |
| 14 | Hawaii | -0.183 | -0.249 | -0282 | -0.260 | -0.288 | -0.142 | -0.063 | -0.027 | **0.253** | -0.002 | **0.464** | 0.062 | **0.348** |

**Table 5.19** Results of Analysis of Molecular Variance for microsatellite data for population from Southeast-Asia and the West-Pacific. Samples were constrained according to geographical location in relation to the native (Southeast-Asia) and invasive (The West-Pacific) populations. For geographical locations of 14 sites across Southeast-Asia and the West-Pacific refer to Figures 5.1 and Table 5.1.

| Source of variation | Sum of square | Variance of components | % total variance | Fixation indices |
|---|---|---|---|---|
| Among groups | 2248.114 | -12.70766 | -1.75188 | $F_{CT}$ = -0.01752 |
| Among populations within groups | 41211.127 | 97.24245 | 13.40582 | $F_{ST}$ = 0.11654* |
| Within populations | 256759.914 | 640.83995 | 88.34605 | $F_{SC}$ = 0.13175* |
| Total | 300219.155 | 725.37474 | | |

* $P < 0.05$

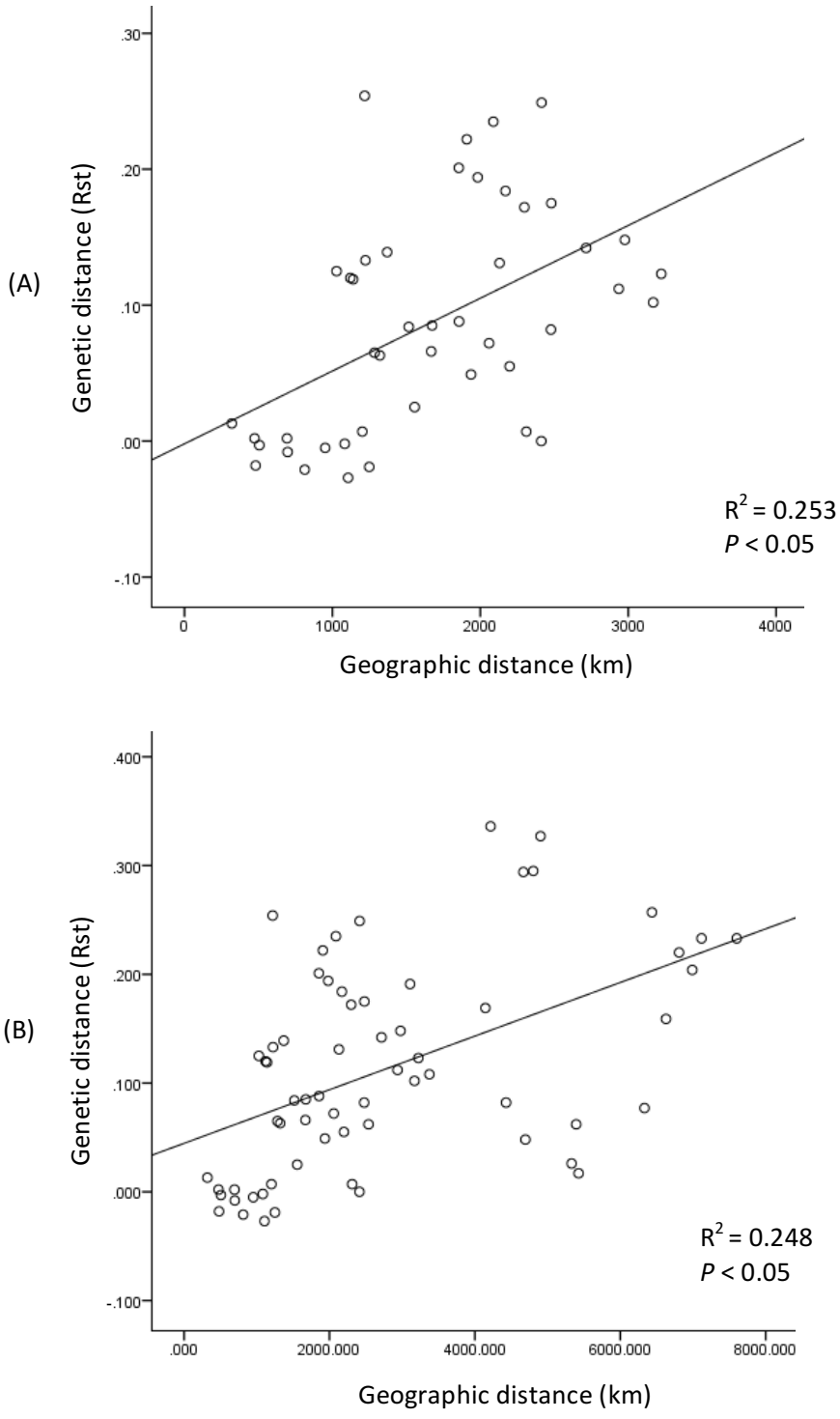
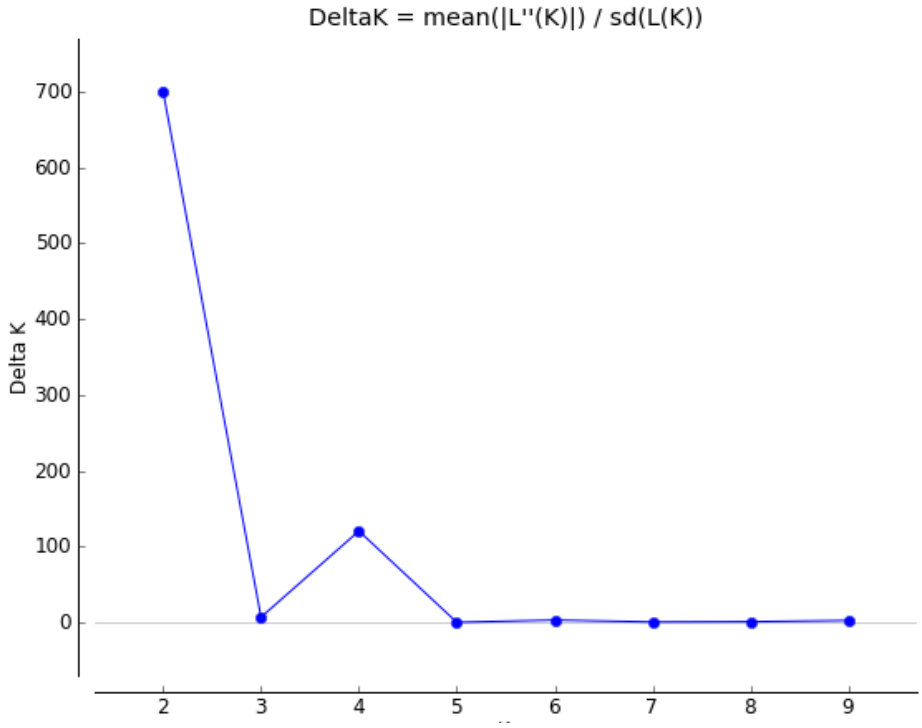



**Figure 5.24** Regression of pairwise genetic distances ($R_{ST}$) against geographic distance (km). (A) Across Southeast-Asia (B) Across Southeast-Asia and the West-Pacific.

*Population structure using Bayesian clustering*

Structure requires users to decide which value of *K* suits their data best, guided by the relationships of L(*K*) and Δ*K* to each *K* value (Figure 5.25). However, it is also recommended that where hierarchical structure exists the highest value of *K* that contains biologically informative structure be used (Evanno et al., 2005). Although L(*K*) appears to plateau at *K* = 4, the median Δ*K* value corresponds with *K* = 2, with a spike at *K* = 4 (Figure 5.25A). Bayesian clustering at *K* = 2 suggested that Guam and Hawaii shared ancestry with mainland Southeast-Asian populations, while New Guinea and Solomon Islands shared ancestry with archipelagic Southeast-Asian sites (Figure 5.26A). However, further increasing *K* in Structure showed additional, distinct and biologically meaningful subdivisions. Analysis under *K* = 3 separated mainland Southeast Asian populations (Burma, Thailand, Laos, Cambodia and Vietnam [red]), from archipelagic Southeast Asian populations (Malaysia, Singapore, Java, Sarawak, Philippines [purple]), West Papua and Solomon Islands (purple), and Guam and Hawaii (red/green) (Figure 5.26B). Analysis under *K* = 4 further differentiated Peninsular Malaysia, Singapore and Solomon Islands as separate from Java, Sarawak, Philippines and New Guinea, with Guam and Hawaii also supported as distinct (Figure 5.26C).

(A)




(B)

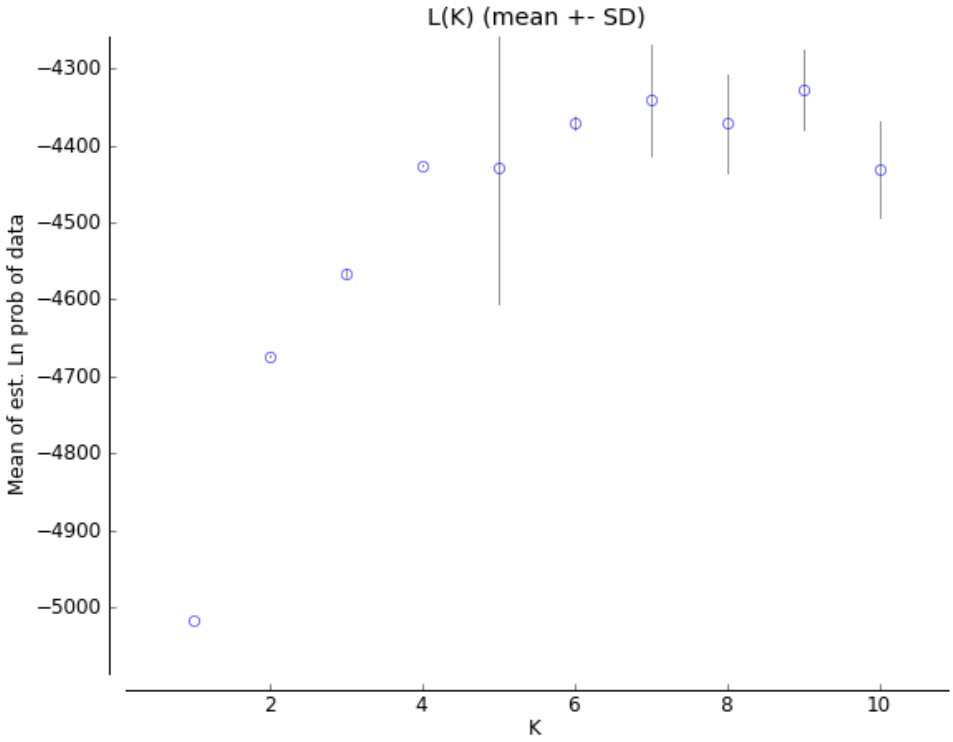



**Figure 5.25** Bayesian clustering results for microsatellite data of *Zeugodacus cucurbitae* collected from 14 sites across Southeast-Asia and the West-Pacific; (A) plot of Δ*K* with the median value is that which is most highly supported as the optimum value of *K* for the analysed sample (B) Mean of log probability of data (LnP (*D*)).

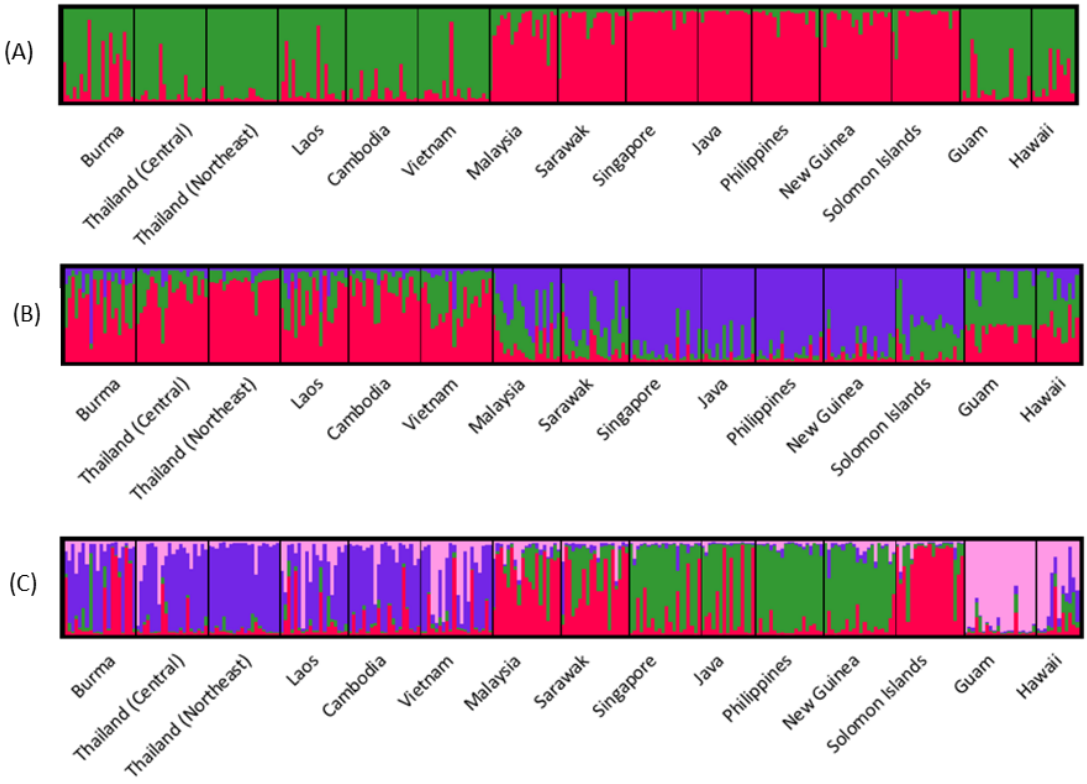



**Figure 5.26** Structure bar plot of Bayesian cluster analysis based on 11 microsatellite loci obtained from *Zeugodacus cucurbitae* collected from 14 sites across Southeast-Asia and the West-Pacific. The first four plots show population assignment results for different values of *K*: (A) *K* = 2; (B) *K* = 3; (C) *K* = 4. Each vertical line represents a single individual and its cluster assignment to a particular cluster is given as a particular colour. Solid black lines separate individuals from each of the 14 sites.

The relationships between sampled melon fly populations are consistent with the results of analysis of the same microsatellite data using STRUCTURE (*K*=4). The branching of the tree was congruent with the population co-ancestry relationships. The pie charts depict the partitioning into four groups according to STRUCTURE results. The result revealed three clusters, with the first group comprising Southeast-Asian mainland (Burma, Thailand, Laos, Cambodia, and Vietnam); the second group comprising Southeast-Asian Islands (Java, Sarawak, Philippines) and West Papua; and the third group made up of the central and west Pacific Islands of Hawaii and Guam. Each of these is a rather homogeneous group, except that the populations of Peninsular Malaysia, Singapore and the Solomon

Islands are differentiated from the populations from archipelagic Southeast-Asia (Java, Sumatra, the Philippines and New Guinea. The third group comprised Guam and Hawaiian populations (Figure 5.27).

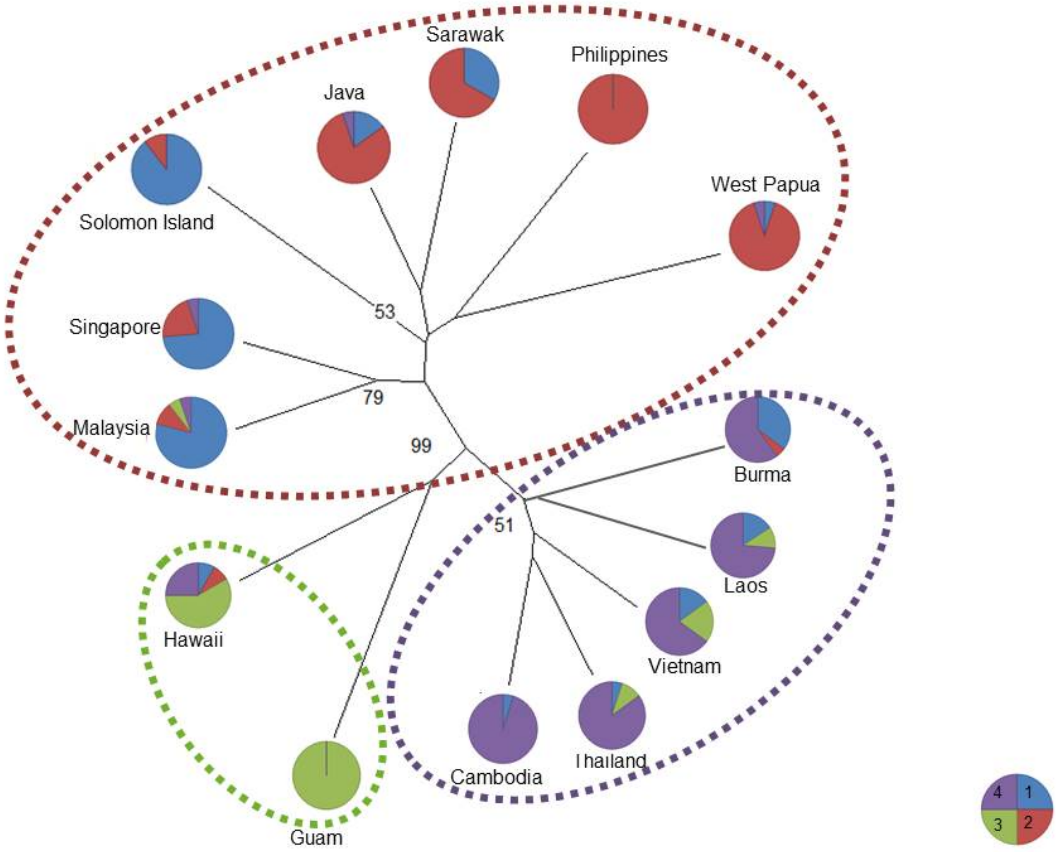



**Figure 5.27** Neighbour-joining unrooted tree of *Zeugodacus cucurbitae* from Southeast-Asia and the West-Pacific based on Carvalli-Sforza & Edwards (1967) distances matrix. Bootstrap values were calculated using 1,000 replications and are given as percentage, with only values greater than 50% showed. The four coloured segments in the pies represent the coancestry distribution of the 262 flies in four hypothetical clusters (*K* = 4). The dashed lines correspond to the three clusters identified using Bayesian cluster analysis results from Structure (*K* = 3).

Principal components analysis of 11 loci reflected patterns also observed in STRUCTURE. The first two axes in the microsatellite PCA explained 63.36% of the variation in the data (PC1 = 40.95%; PC2 = 22.41%; Figure 5.28). Consistent with the results from STRUCTURE, the PCA revealed two well-separated clusters, the first comprising Southeast-Asian mainland (Burma, Thailand, Laos, Cambodia, and Vietnam) and Hawaiian and Guam populations, and the second comprising the Islands of Southeast-Asia (Java, Sarawak, Philippines, Singapore), Peninsular Malaysia and two populations from West-Pacific New Guinea and Solomon Islands).

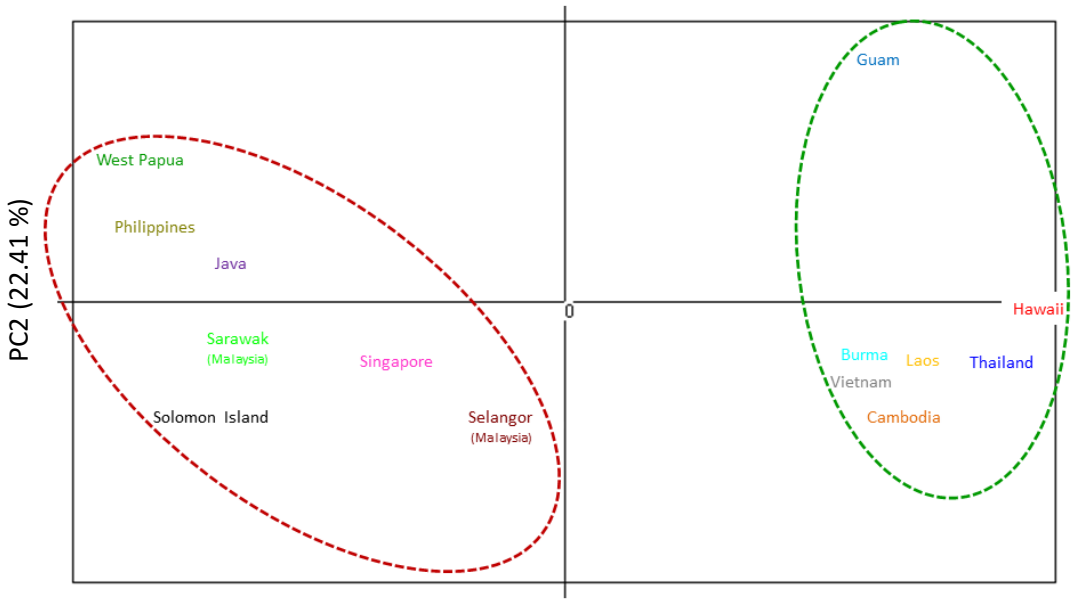



**Figure 5.28** Principal components analysis (PCA), plots based on 11 microsatellites loci, for *Zeugodacus cucurbitae* collected from 14 sites across Southeast-Asia and theWest-Pacific. The green and the red dashed lines correspond to the two clusters identified using Bayesian cluster analysis results from Structure (*K* = 2).

## 5.4 DISCUSSION

### 5.4.1 Comparison of variation in Southeast-Asia (native range) and the West-Pacific (invasive range)

Previous research suggests *Z. cucurbitae* may have originated in India (Bezzi, 1913) or Central Asia (Virgilio et al., 2010) and then dispersed westwards to the African continent, eastwards to Southeast-Asia and thence further eastwards to the West-Pacific (Vargas et al., 2015). The present study has demonstrated that, for the populations sampled, the greatest variation in *Z. cucurbitae* exists in Southeast-Asia, which suggests that this region is at least part of the native range of the melon fly. Evidence also suggests that, over many thousands of years, genetic diversity in mainland Southeast-Asia gradually increased through local diversification and occasional, ongoing accession of flies from South or Central Asia. Dispersal westwards from South or Central Asia into Africa and eastwards across the more easterly parts of the Southeast-Asian region and the West-Pacific came much later (Bess et al., 1961; Virgilio et al., 2010; Vargas et al., 2015).

Morphometric and molecular data from my study reveal distinct differences in variation between Southeast-Asian and West-Pacific populations; wing shape, aedeagal length and gene diversity all are highly variable in Southeast-Asia, while samples from the West-Pacific exhibit a relatively low level of variability. High levels of gene diversity in the native Southeast-Asian range contrast to low diversity and few haplotypes in the invasive range that is presumably inhabited by more recently established populations (Hu et al., 2008; Prabhakar et al., 2012). Likewise, the total number of haplotypes and the number of unique haplotypes were greater in Southeast-Asia than in the West-Pacific, and the low genetic diversity in West-Pacific samples supports the notion that *Z. cucurbitae* is a recent introduction into this area. Genetic diversity was particularly pronounced in mainland Southeast-Asia (Thailand, Laos, Cambodia, Vietnam and Singapore). This is consistent with Wu et al. (2011) who found greater genetic variation in Southeast-Asian populations (Burma and Thailand) than in China, which also seems to be part of the invasive range of the species.

The average mitochondrial gene diversity of melon fly populations from Southeast-Asian populations is generally high; however, the diversity of populations from Burma, Malaysia, Philippines, Java and Sarawak is relatively low (Table 5.14). Apart from Burma, these latter regions are geographically more distant from the presumed origin of India, and hence the more recent dispersal towards the Indo-Malay Archipelago may explain such reduced generic variability compared to mainland Southeast-Asian sites closer to the subcontinent. In general, high genetic uniformity and low overall genetic variability are usually associated with population processes such as far-range expansion and invasion (Meixner et al., 2002; Nardi et al., 2005; Malacrida et al., 2007). When mitochondrial data obtained in this thesis from all populations were analysed using Tajima's *D* tests of neutrality, it was negative and statistically significant. This may indicate that they are under selection, or it could be that populations have expanded in relatively recent historical times. Tests based on Fu's *Fs* were also negative and statistically significant; this also suggests relatively recent population expansion rather than the existence of a genetic bottleneck created by some other factor. Therefore, an invasion of the West-Pacific by the melon fly has probably been a gradual, invasion process.

Even though Burma is very close to the postulated area of origin of the melon fly (Central or South Asia), it has low gene diversity. This may be due to few and small effective populations of melon fly in Burma. Burma does not have as extensive agricultural areas as Thailand, and much of its arable land is given over to field crops such as rice and pulses, rather than crops that are hosts of melon fly (Allendorf et al., 2006; Adas, 2011). The relative paucity of host plants may render the country less suitable for melon fly than neighboring countries such as Thailand. Indeed, hot and humid regions of India and Thailand are more suitable areas for melon fly than are horticultural areas which are cooler (Hu et al., 2010), which is the case in the northern ranges and the tablelands of Burma (King et al., 2001). It is also possible that a relatively small number of individuals were initially introduced to Burma, and this may be the reason for the low gene diversity. In addition, natural dispersal from the Bengal region in the west, and from Thailand and Laos in the east, has always been impeded by forested mountain barriers (Aung-Thwin & Aung-

Thwin, 2012); consequently, human-assisted dispersal may have been limited. For most of the period since the end of the Second World War, trade of commodities into Burma has been further restricted by political and economic barriers; this may also have reduced the likelihood of multiple introductions (Aung-Thwin & Aung-Thwin, 2012).

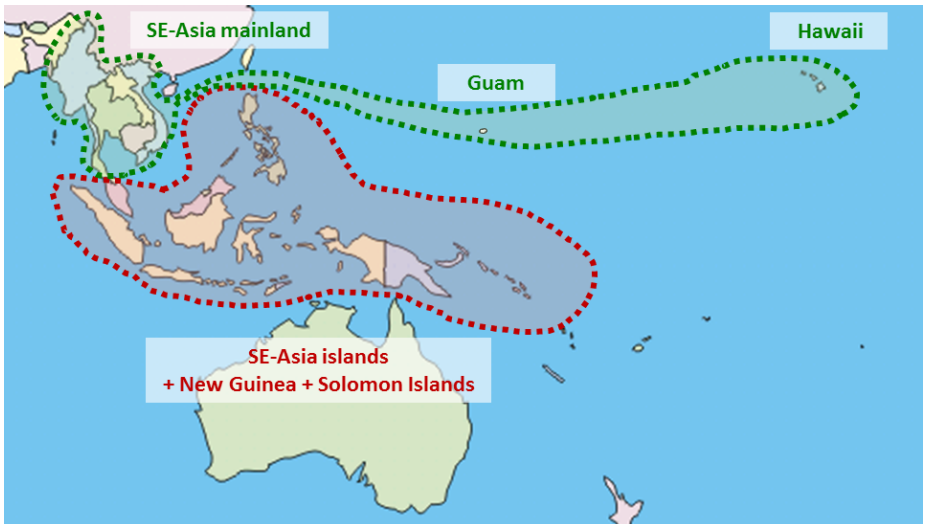
### 5.4.2 Invasive pathway of melon fly in Southeast-Asia and the West-Pacific (Isolation by distance and natural dispersal)

Isolation by distance analysis of both wing shape and aedeagus data for *Z. cucurbitae* were statistically significant and indicated that the difference in wing shape between sites increased according to the geographical distance between those sites. These results conform with a scenario of natural and gradual dispersal, as flies become increasingly biologically differentiated the further apart populations become. This is especially demonstrated in the Southeast-Asian analysis, yet is further shown when combined with geographically close Pacific sites (Figure 5.16). As a consequence, we see that flies from Sarawak (Malaysia) possess a highly similar wing shape to flies from nearby West-Pacific sites (New Guinea, Solomon Islands and Guam). These data suggest the possibility that melon fly from Sarawak are closely related to those in nearby West-Pacific sites, and that Sarawak (or a nearby unsampled site) may have been the site from which this species dispersed into the Pacific.

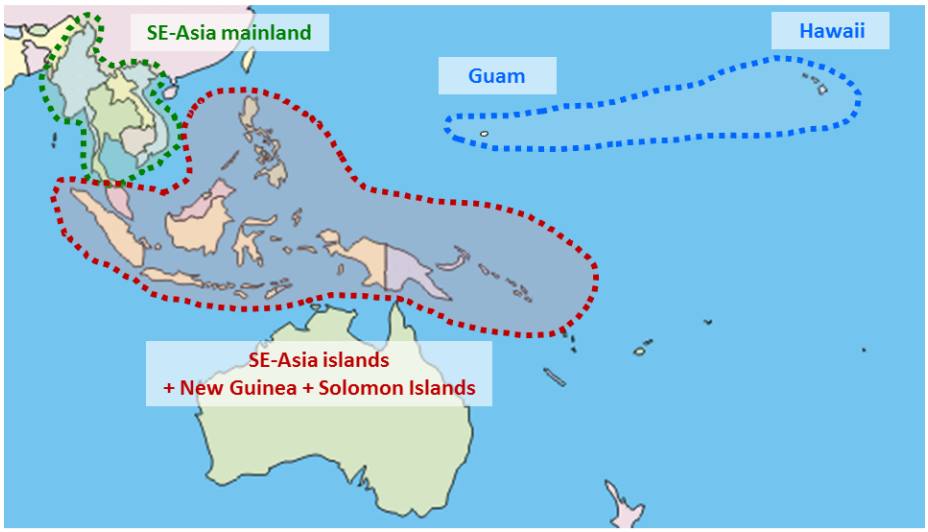
Affinity between Southeast-Asian mainland populations and flies from Guam and Hawaii was supported by genetic data (Figures 5.26A and 5.29A). It has been suggested that melon fly was accidentally introduced by anthropogenic activities from Southeast-Asia to Hawaii (Severin et al., 1914; Bess et al., 1961; Virgilio et al., 2010; Vargas et al., 2015). Wherever the flies came from, it seems certain that the adventive population was relatively large and polymorphic. It is likely that this was the case for the invasion of Guam, where there is likewise no evidence of a genetic bottleneck in the present-day population. Guam is geographically remote but has been a transportation hub, especially for US military planes and vessels, since the end of World War II. Thus, it is possible that melon fly arrived in fruit with human assistance, very likely with the US armed services. Allwood et al. (1999) were of the

opinion that it was probable that melon fly were introduced by imported contaminated hosts from Asia.

(A)




(B)




(C)

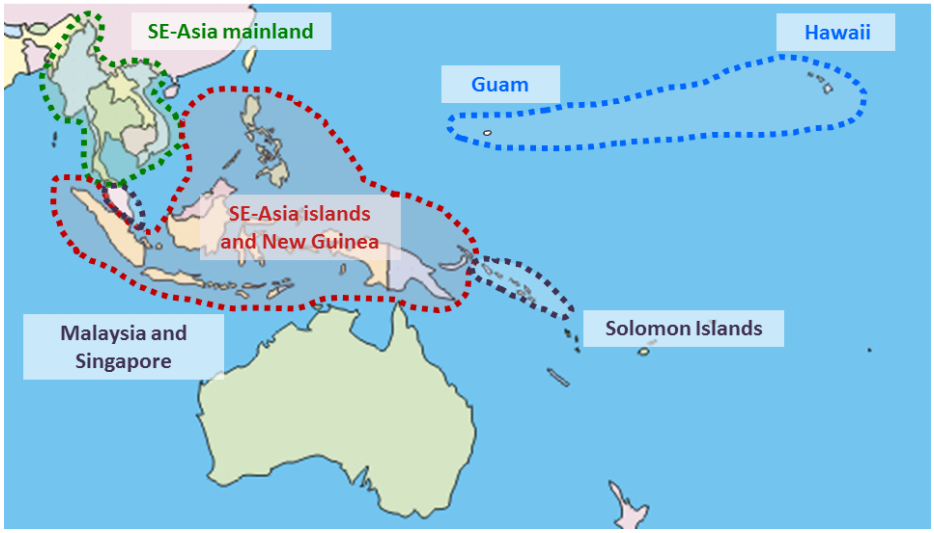



**Figure 5.29** Population structure of *Zeugodacus cucurbitae*, the dashed lines correspond to

population cluster identified using Bayesian clustering from Structure, assignment results for(A) *K* = 2, (B) *K* = 3 and (C) *K* = 3 scenarios.

Additionally, the present study suggests a recent expansion of melon fly into Papua New Guinea and the Solomon Islands. My results showed that the pattern of population structure in the Solomon Islands is different from that in New Guinea. Solomon Island flies are, in fact, more similar to flies from Malaysia and Singapore populations, whereas New Guinean flies are more closely affiliated with populations from Indonesia, Sarawak and the Philippines (Figure 5.29B-C). This suggests that melon fly in the Solomon Islands came from Malaysia or Singapore. This result contrasts with a previous study that assumed (without evidence) melon fly had been moved from Papua New Guinea (recorded since 1940) to the western province of the Solomon Islands in 1980 (Allwood & Drew, 1996).

Human mediated dispersal is the most plausible explanation for the large-scale dispersion of *Z. cucurbitae*, particularly for the colonization of the islands of Guam and Hawaii. Human mediated transport could also explain the occurrence of introduced individuals in the West Pacific. While it is relatively easy to implicate human-mediated transport as a promoter of the inter-regional dispersion of *Z. cucurbitae*, it is more difficult to define a timeline for the different introduction events.

### 5.4.3 Multiple introductions in Hawaii

While mitochondrial gene diversity is very low in the West-Pacific, populations from Hawaii showed relatively higher diversity and a larger number of haplotypes. Together with the haplotype network and analysis of population genetic structure using Bayesian cluster assignments, this supports a hypothesis of multiple introductions from Southeast-Asia into Hawaii. One previous study proposed independent introductions of melon flies into Hawaii from different source populations based on shared microsatellite alleles (Meixner et al., 2002). Moreover, aedeagus length of melon fly from Hawaii is also more variable than in West-Pacific sites (Figure 5.18). Hawaiian flies possibly came from many potential sites in Southeast-Asia (i.e., Thailand, Laos, Cambodia, Vietnam and even the Philippines), possibly by commerce in the nineteenth and early twentieth centuries.

The present findings are also supported by the phylogenetic results of Prabhakar et al. (2012), who found Hawaiian populations belonged to the same group as Southeast-Asian populations. Variation in microsatellite markers in the Hawaiian population was also observed by Virgilio et al. (2010), who suggested that the adventive population must have been relatively large and polymorphic as the invasion of Hawaii did not appear to be associated with a genetic bottleneck.

Multiple introductions have increased the potential for adaptation in the melon fly population and possibly greater invasion success in Hawaii (Kolbe et al., 2004). These multiple introductions may be attributable to the fruit trade, or they may have occurred in some other way, with flies as hitch-hikers associated with movement of humans or goods. Moreover, this scenario can be related to historical records of its invasion across the region. Melon fly was first detected in Hawaii in 1895 (Bess et al., 1961; Vargas et al., 1989), and the fly was well-established there by 1897 (Severin et al., 1914). It is probable that populations established primarily in lowland areas (Vargas et al., 1990) that possessed cultivated as well as wild cucurbits (Jackson et al., 2003; Vargas et al., 2004). While Hawaii seems to have undergone multiple invasions, the islands of New Guinea and the Solomon Islands seem to have experienced just a single introduction of *Z. cucurbitae* from Southeast-Asia.

### 5.4.4 Global distribution of *Z. cucurbitae*

In contrast to the situation in Southeast-Asia, mitochondrial gene diversity is very low in the West-Pacific (the postulated invasive range of the melon fly) except for populations from Hawaii. Since it has been suggested previously that the melon fly originated in India (Bezzi, 1913) or Central Asia (Virgilio et al., 2010) and dispersed to Africa, Southeast-Asia and the West-Pacific, publicly available sequences from India, Africa and China were included in this study. The pattern of relationships among these regions fits with coalescence theory, which predicts that in the case of a recent invasion by a limited number of founders, we should expect to detect a single common haplotype and a few haplotypes linked with few mutations to the common haplotype (Slatkin & Hudson, 1991; Avise, 2000). So, the hypothesis of the melon fly being a recent invader in the West-Pacific is supported

by the low level of overall genetic variability and the specific shape of the haplotype network.

The hypothesis of an Indian origin of *Z. cucurbitae* proposes that there has been a recent expansion of melon fly to other parts of Asia, the West-Pacific and the African continent (with the first record from Tanzania in 1936 and more recent records in many other African countries (Virgilio et al., 2012). The haplotype network also showed that Africa does not share common haplotypes with Southeast-Asia. However, India and China shared some common haplotypes with Southeast-Asia, and India possessed a haplotype which is derived by many steps (mutations) from the rest of the sampled haplotypes. However, due to the small number of sequences from India included in this study, it is difficult to confirm here that India is the origin of melon fly. The most that I can say is that the pattern does not contradict an Indian origin. Moreover, the Chinese population had a higher number of haplotypes than many of the West pacific sites, and China shares three haplotypes with Southeast-Asian populations. This suggests that melon fly might have invaded China from Southeast-Asia several times, although there is no evidence as to whether this is a recent occurrence or not. This result corroborates the findings of Hu et al. (2008), who suggest that the introduction of melon fly to China is a relatively recent event from Southeast-Asia.

### 5.4.5 Conclusion

The integrative morphological and molecular study of *Z. cucurbitae* from across Southeast-Asia and the West-Pacific supports the hypotheses that *Z. cucurbitae* are more variable phenotypically and genotypically in the native population in Southeast-Asia than the invasive populations in the West-Pacific. Moreover, the current study demonstrates the usefulness of geometric morphometric analyses in an invasion biology context, especially when combined with genetic data on the same flies.

My data are largely in accordance with evidence from previous studies. Melon fly populations from mainland Southeast-Asian countries (Thailand, Laos, Burma, Cambodia and Vietnam) are similar, most likely because they are part of the old, native distribution range of the specie. There is likely ongoing mixture of flies

among these countries, at least in border regions. Other Southeast-Asian countries (Malaysia, Singapore, Java, Sarawak and Philippines) might have acquired melon fly more recently compared to mainland Southeast-Asia. Dispersal into the Melanesian arc (New Guinea, Solomon Islands) seems to have been more recent, perhaps by natural means with assistance of some long-distance, human-assisted dispersal. Dispersal into remote Pacific Islands (Guam, Hawaii) has been long-distance and human assisted. Introductions to these islands are likely to have been relatively large and to have included appreciable genetic diversity.

# CHAPTER 6

## GENERAL DISCUSSION

## INTRODUCTION

This final discussion draws links between the key findings from each chapter of my thesis. The combined results confirm the value of integrative taxonomy for understanding variation in melon fly, and the approach's applicability to research questions other than taxonomy. In this chapter I first summarise key findings of the thesis, discuss the relevance of new insights to Thai Agriculture, provide advice for future research into further resolving melon fly population-level variation, and conclude by discussing how the results may help us to better understand not only melon fly, but other insect pest systems in the region.

## 6.1 SUMMARY OF RESULTS

I analysed genetic and morphological variation in *Z. cucurbitae*, a wide-ranging, serious fruit fly pest in its native range and parts of its invasive range which had not been sampled previously. I also resolved population structure of *Z. cucurbitae* across the geographical regions of Thailand, and in relation to host associations in horticulturally-intensive, central Thailand. Overall, genetic data were highly informative, especially microsatellite information; wing shape also provided insight, but genitalia demonstrated reduced utility due to being inconsistently variable and closely correlated with body size. The thesis illustrates that individual approaches, taken by themselves, can be revealing, but that integration of these datasets is a more powerful analytical tool for understanding population structure and exploring for possible cryptic species.

Genetic diversity of *Z. cucurbitae* is relatively low when all sampled populations of this species are taken into account, but genetic diversity is still higher in the native range and remarkably low in the invasive range. I have determined that some population structuring correlates to biogeographic transition zones. Analysis of morphological and genetic variation of *Z. cucurbitae* in Thailand revealed that the Northeast population differs from other Thai populations, presumably because significant mountain ranges (Dong Paya Yen, Phetchabun Range and Samkambeng Range) form natural barriers separating the northeast from other regions. I did not find any evidence for a biogeographic barrier at the Isthmus of

Kra, but there is evidence of a biological transition zone in the area around the Thai-Malaysian border at the Kanger-Pattani line.

I found no population structure within the horticulturally intensive central region of Thailand, which may reflect that the flies move freely across the landscape, utilising hosts wherever they find them: an important insight from a management perspective. Nevertheless, there was some very slight sub-structuring among hosts that may reflect the potential for melon fly to develop into host races in the future. The results also revealed morphological variability among groups of flies reared from different host plants which may be due to environmental factors (e.g., host quality or abiotic factors). However, I can confirm that there is no evidence for any discrete lineage or cryptic species associated with any geographical area or host fruit in Thailand.

Analysis of population structure of *Z. cucurbitae* from Southeast-Asia and the West-Pacific confirmed that Southeast-Asian populations were more variable in morphology and genetic make-up than West-Pacific populations, as expected considering Southeast-Asia is part of the native range of *Z. cucurbitae*. In the invasive range, Hawaii stands out from West-Pacific sites. The Hawaiian population is more variable than populations from West-Pacific sites, suggesting that there have been multiple, separate introductions over time into that island chain. Genetic signature and morphological variation indicate that single (or very few) introductions have occurred to islands of the West-Pacific.

## 6.2 INTEGRATIVE TAXONOMY AND THAI AGRICULTURE

Analysis of variation of melon fly throughout the Kingdom of Thailand and adjacent countries provides lessons that are applicable to the management of fruit fly, and other arthropod pests, in Thailand. These lessons are relevant to domestic agriculture, quarantine within Thailand and international market access.

### 6.2.1 Domestic quarantine

The present study, based on a synthesis of morphological, molecular and host use data, in the absence of species limits preconceptions, and drawing on sampling from throughout the region, confirms that *Z. cucurbitae* is a single species likely

utilizing numerous alternate hosts and moving freely from one host to another. In central Thailand, individuals disperse sufficiently through agricultural areas for there to be considerable sharing of genes among nearby populations. Effective management of the impact of melon fly on a crop will, therefore, depend on effective management of populations in nearby tracts of land and on alternate hosts. Control measures directed solely at the target crop are unlikely to be successful as topographical and climatic characteristics of Thailand, and cultivation practices, allow this mobility in this polyphagous pest. We may expect similar mobility in other vagile, polyphagous, horticultural pests, such as whiteflies and lepidopteran pests (Vreysen et al., 2007). Furthermore, a mutation which increased the fitness of melon flies, or any other polyphagous pest for that matter (e.g., a gene for pesticide-resistance), might spread rapidly through central Thailand. By the same token, and all things being equal, a deleterious gene or a *Wolbachia* introduced for pest management also could be expected to move freely among pest populations in the region.

Thailand has no domestic quarantine arrangements. Despite this, however, evidence from the present study suggests that there is a natural barrier to gene flow between melon fly populations in the central Chao Praya valley and the Northeast region. There may be some value in ensuring that this barrier between the Northeast and the remainder of Thailand is not compromised in the future by trade in commodities, especially as Thai government initiatives seek to promote increased production and purchasing power in the Northeast. However, the present study indicates that there are no easily observable, morphological markers distinguishing north eastern flies from the rest of Thailand. Accordingly, it may not be economically feasible, or biologically justifiable, to invest heavily in intercepting flies on fruit travelling between the two regions. It may be more practical to periodically monitor for changes in populations on either side of the barrier than to run a traditional trapping program based on identification of individual flies.

### 6.2.2 International quarantine and market access

Thailand is one of the world's major exporters of tropical fruit (Somsri, 2011). Maintaining this international market access in the future will depend in no

small measure on the continuing credibility of Thailand's pest lists and on its capacity to interpret detections of "novelties" that affect pest status critically and effectively.

- **6.2.2.1 Thai exports of fresh fruit, existing and new markets**

Assessment of the risk associated with international trade in agricultural commodities relies heavily on the credibility of pest lists of exporting and importing countries (e.g., ISPMs 02, 11 and 21) (International Plant Protection Convention, 2015). It also relies on the accuracy and precision with which biological attributes, such as host plant preferences, correlate with species (e.g., are they truly polyphagous, or a collection of specialized monophages?). Importing countries compare the list of pests that could be associated with the commodity proposed for importation and with the list of pests present in the importing country. Pests present on the exporting country's list but not established in the importing country or under official control in the importing country could represent a phytosanitary risk to the importing country. Clearly, it is essential that the pest lists of both countries are based on reliable taxonomy and that there is consensus on that taxonomy. The outcome of modern taxonomic work poses significant implications for trade. Changes in nomenclature as a result of taxonomic work can be problematic for regulatory agencies, but changes in taxonomic concepts require reassessment of phytosanitary risk and have the potential to disrupt trade arrangements (Hendrichs et al. in press).

The present study confirms the taxonomic status of the melon fly in Thailand, in that it is a single, slightly variable, biological species. Thai melon fly are conspecific with melon fly in all contiguous countries (Burma, Laos, Cambodia and Malaysia) and in near-neighbouring China and Vietnam. Further, there is no evidence of cryptic species or host races in melon fly. On the face of it, confirming the *status quo* might seem to be an inconsequential result. However, this is far from the case. Trade in commodities linked to melon fly within and among the listed countries is burgeoning because of increased demand, increased capacity to pay for imported products (especially in China, Thailand and Malaysia), and improving transport infrastructure (e.g., new, all-weather, fast highways connecting southern

Vietnam, southern Laos and eastern Thailand). The phytosanitary risk associated with this unprecedented flow of commodities deserves renewed scrutiny. The present study demonstrates that this trade does not create increased phytosanitary risk, at least not in relation to melon fly.

By way of comparison, the implications of taxonomic research in recent years on *Z. tau* in Thailand are not so straightforward. Research has revealed *Z. tau* to be a complex of at least eight species (Saelee et al., 2006; Kitthawee & Dujardin, 2010; Kitthawee & Rungsri, 2011; Sumrandee, et al., 2011; Drew & Romig, 2013). Because of this, the distribution, host associations, and economic impact of these eight species should now attract the closer attention of countries which trade in commodities that are "*tau*" hosts. In fact, the eight species previously listed have largely different host associations (S. Srikachar, personal communication, October 9, 2015), demonstrating that the phytosanitary risk to an importing country will vary depending on the host plant imported. The important point to note in the context of this thesis is that taxonomic changes prompt the need for re-assessment of phytosanitary risk.

Another scenario is playing out following the recent determination that several of the "species" within the *B. dorsalis* complex are, in fact, the same biological species as *B. dorsalis* sensu stricto (Schutze et al., 2015a, 2015b). For example, *B. dorsalis* is well established in China, and it has previously been considered that the closely related *B. philippinensis* and *B. papayae* were not (occurring in the Philippines and southern Southeast-Asia, respectively). Accordingly, countries wishing to export fruit to China were required to demonstrate that they were free of either of these two species. Recent integrative taxonomic work on the *B. dorsalis* complex has determined that flies previously regarded as *B. philippinensis* and *B. papayae* are conspecific with *dorsalis*. Thus, there is now no scientific justification for China to require trading partners to demonstrate freedom from either of these taxa (Dohino et al., unpublished).

It remains to be seen whether trading partners will insist in the future that pest lists be based on species concepts developed through integrative taxonomic approaches and thorough exploration for geographical segregates and host races, lineages or sibling species. Nevertheless, the accumulation of integrative datasets

provides a more robust foundation for trade negotiations than the application of any single line of enquiry (e.g., morphology alone).

**- 6.2.2.2 Assessing unusual host plant records**

How should we respond if flies that superficially look like *Z. cucurbitae* are reared from a fruit not previously recorded as a host of the melon fly? This scenario may occur on a farm remote from the international border, or on fruit intercepted at an international checkpoint. *Zeugodacus cucurbitae* is neither a regulated pest in Thailand nor a quarantine pest for the Kingdom. There remains, however, a risk that the detection of *Z. cucurbitae* in a novel host could be dismissed as a mere extension of the known range of the species and a record of no particular concern to Thailand. If the flies were reared from a host for which market access protocols had already been negotiated with trading partners (e.g., mangoes which are not a known host of melon fly), the maintenance of the trade would likely depend upon a thorough analysis of the record. An integrative taxonomic approach may provide the means to analyse the record and support an argument for the continuation of the export of mangoes.

How should an insect taxonomist who has the responsibility for diagnostics of pests deal with this situation?

As a result of this thesis, we now have a substantial data set that characterises melon fly from different parts of its geographical range and from several host plants across more than one plant family. We also possess a suite of sophisticated, analytical tools for making sense of these data. Drawing on this, a systematic evaluation of the “novel host record” scenario might proceed as follows:

i) First, because the host has not been recorded previously, we may suspect that we are dealing with a new, cryptic species closely related to the well-known pest. The question becomes: are they melon fly or something different? We would initially assess this using external morphology and colour pattern to determine if the fly from the novel host conforms to the currently accepted morphological definition of melon fly. We would avoid examining other traditional morphometric characters

(e.g., aedeagus length) because, as demonstrated in this thesis, this character is of little informative diagnostic value.

ii) We would use the same individuals to obtain geometric morphometric and molecular data. The geometric morphometric data, *cox1* and microsatellite data used in this thesis may be used to determine whether the flies from the novel host represent a previously undetected segregate or an outlier. For example, the detection of a unique haplotype known previously only from a remote location would suggest that the flies from the novel host may have originated from a human-assisted, long-distance dispersal. If the haplotypes were shared with flies from other hosts in nearby locations, then we might suspect that the novelty was "home grown". Microsatellite data may also indicate likely place of origin for the novelty based on similarities to other known populations of the pest. Similarly, wing shape data may point to the potential origin of the fly; however, we would need to be aware of the potentially confounding effects of environmental factors influencing the fly's phenotype.

iii) If the investigation described in (ii) indicated that the novel host record had resulted from an incursion, then the pathway by which the incursion had taken place becomes a matter of interest. If the novel host record comes from a location at, or near a port of entry or border, the next step should be to investigate whether there are pathways by which the fly may already have dispersed or been transported to additional locations (i.e., we would need to perform a "trace forward" analysis to determine additional locations to which the fly might have gone). If the location is far from a port or border area, then we should determine whether there are pathways by which the fly could have arrived at the location (i.e., we perform "trace back" analysis to determine where the fly could have come from) (McMaugh, 2005). Survey of the novel and familiar hosts of the melon fly based on the trace forward or trace back analyses would be warranted and flies analysed using the same procedures as used in (ii).

iv) The outcomes of (ii) and (iii) will provide critical information for deciding what management response is warranted, feasible and cost effective, if indeed a response is to be made at all.

In the context of this thesis, the important points to note are that:

A) The diagnostics and evaluation described above is possible because we now have workable, morphometric and molecular profiles of the melon fly from many Asia-Pacific locations; and

B) The reliability of the diagnostic process and the evaluation would be enhanced by having a more comprehensive and representative set of profiles (e.g., including data from all known hosts and additional locations).

## 6.3 POTENTIAL AND FUTURE RESEARCH, AND INITIATIVES: THAILAND

The literature review and the results of this thesis and the discussion in the preceding section, taken together, identify areas for future study in our understanding of melon fly as relevant to Thai biosecurity and Thailand's overall biosecurity and market access strategy.

### 6.3.1 Research priorities for melon fly in Thailand

As mentioned elsewhere, this thesis has examined taxonomically informative aspects of *Z. cucurbitae* (traditional morphology, geometric morphometric and genetic studies). However, there are other characteristics that could further elucidate the population structure of this fly, especially mating compatibility and chemical ecology. The results from this thesis have defined *Z. cucurbitae* as one species, yet genetic results revealed populations from the northeast and from the southernmost extremity of Thailand as distinct from other Thai populations. Mating compatibility and chemical ecology (pheromone or CHCs) of these populations, in particular, may shed further light on population structure of *Z. cucurbitae* across this region. Further study of mating compatibility, chemical ecology and pheromones is warranted because there may yet be undetected variability in this key aspect of the melon fly.

This thesis has applied an integrative taxonomic approach using specimens from many sites from Southeast-Asia and the West-Pacifc, including specimens reared from many host plant species. These results demonstrate that melon fly is a single species. This critical information sets a framework for management of melon fly in Thailand and specifically defines the background for Sterile Insect Technique (SIT) (e.g., there are neither distinct genetic lineages nor host races) (Vreysen et al.,

2007; Hendrichs et al., in press). Consequently, the Thai government may confidently explore options for area-wide management of melon fly in Thailand under this single-species scenario, and these options may include the release of sterile insects to suppress local populations. In fact, if area-wide management strategies are to be considered in Thailand for any pest fruit flies, microsatellite data will be required to understand how the pest spreads through the landscape and to assist with the release of sterile flies which are most compatible with target populations.

### 6.3.2 Application of integrative taxonomy to other systems

Owing to the facilities generally available in Thailand, traditional taxonomy based on external morphology will remain the initial means by which most scientists and technicians will perform species-level identifications. Accordingly, there is an urgent requirement for an up-to-date key to tephritid species of interest to Thailand. This is especially pressing because of recent taxonomic changes resulting from work on the *B. dorsalis* complex by Schutze et al., (2015a, 2015b) and the monographic revisionary work of Drew & Romig (2013) which, for example, increased the number of *Z. tau* complex species to twenty-one. Such a key to genera and species should include species present in Thailand, species present in neighbouring countries, and species of biosecurity concern. A traditional, dichotomous key may be acceptable initially, but a random-access, digital key in Lucid (or other) format should be the goal, similar to that recently produced for the African fruit flies (Virgilio et al., 2014). There are opportunities to collaborate with regional fruit fly specialists on such a project, including opportunities to work with a new Australian initiative investigating systematic relationships within the *Bactrocera* and closely related groups. This would also facilitate the updating of tephritid entries in two widely used information resources, namely the *PaDIL* image library (Padil, 2015) and the CABI Crop Compendium (CABI, 2015).

The *Z. tau* complex is an obvious candidate among the tephritids for the application of integrative taxonomy, but there are many other groups of arthropods that include important pest species in Thailand. For example, the application of geometric morphometrics to the complex, three dimensional structure of the pronotum in membracid leafhoppers, the wings of mosquitoes (Jaramilo et al.,

2015), whiteflies and leaf-mining agromyzid flies (Shiao, 2004) which appear to have extended their geographical range in Southeast-Asia in recent times, or to stingless bees (social species which can be difficult to differentiate) (Combey et al., 2013), could all provide interesting insights into species and population structure. The assembly of *cox1* libraries for major pests present in Thailand is another readily achievable and worthy objective. DNA barcoding (Hajibabaei et al., 2007) would be a viable companion to any of the projects suggested for geometric morphometrics. For example, cryptic species and biotypes have been demonstrated in whiteflies in other countries but not in Thailand (Xu et al., 2010; Liu et al., 2012; Wang et al., 2012). Whitefly research in Thailand urgently requires the application of new technologies (e.g., *cox1* and microsatellite) to support taxonomic studies and pest management. DNA sequencing is becoming more and more economical and streamlined and there may be opportunities in the future to collaborate with global projects on deep sequencing, which enable exploration for new, taxonomically informative parts of the insect genome. However, irrespective of the wealth of DNA data that will become available and the discovery of new molecular markers, sequence data should be used in concert with other data (e.g., morphological data) and not in isolation.

Integrative taxonomy is crucial to integrated pest management (IPM) and other research (Dayrat, 2005; Schlick-Steiner et al., 2010; Yeates et al., 2011). In an academic context, the integrative approach is valuable in terms of coordinating collaboration among research groups. It can establish a framework for allocating work on molecular characteristics, morphology, host use, and mating behavior to laboratories and teams expert in respective fields of enquiry. Thus, it is not only the integration of approaches that is critical, but also the integration of laboratories that focus on different aspects of a central research problem. In Thailand, for example, an integrated approach to research by tephritologists would draw together laboratories of the Department of Agriculture, the Department of Agriculture Extension, the Office of Atoms for Peace and several universities, in order to reach common goals. This would be in contrast to the current situation, where all these groups operate as isolated teams. As has been done in *B. dorsalis*

complex (Schutze et al., 2015a, 2015b), so it should be done in *Z. cucurbitae* (in an intraspecific context) and *Z. tau* (in an interspecific context).

Regrettably, there has been little in the way of overall coordination of taxonomic work in Thailand and no clear identification of national priorities. A national or regional strategy for fruit fly research may catalyse collaboration in fruit fly research as an exemplar strategy for other pest groups. A strategy for pest fruit flies is required to identify the key management, quarantine, and market access challenges, and devise a range of cost-effective responses to these challenges. I suggest that a National Fruit Fly Strategy for Thailand could be structured around management of established pest fruit flies (tools and extension), quarantine procedures and treatments, surveillance, diagnostics, information management and responding to outbreaks. This strategy should include a broad-based plan for targeted research and development (R&D), including integrative taxonomic research (CRC, 2014). The present study has underlined the close relationships among populations of melon flies in the Asia-Pacific region and especially the interrelatedness of melon fly in countries such as Thailand, Laos, Cambodia and Vietnam. This suggests that it may ultimately be more effective to develop not just national but also regional strategies for fruit fly R&D and management, and ultimately for other major pests in the Southeast-Asian region.

# BIBLIOGRAPHY

Abrahamson, W. G., Brown, J. M., Roth, S. K., Sumerford, D. V., Horner, J. D., Hess, M. D., ... Itami, J. (1993). Gallmaker speciation: an assessment of the roles of host-plant characters, phenology, gallmaker competition, and natural enemies. *The Ecology and Evolution of Gall Forming Insects.* North Central Forest Experimental Station, Forest Service, USDA, St. Paul, MN, 208-222.

Adas, M. (2011). *The Burma delta: economic development and social change on an Asian rice frontier, 1852-1941*. University of Wisconsin Press.

Aketarawong, N., Bonizzoni, M., Malacrida, A. R., Gasperi, G., & Thanaphum, S. (2006). Seventeen novel microsatellite markers from an enriched library of the pest species

Aketarawong, N., Bonizzoni, M., Thanaphum, S., Gomulski, L.M., Gasperi, G., Malacrida, A. R., & Gugliemino, C. R. (2007). Inferences on the population structure and colonization process of the invasive oriental fruit fly, *Bactrocera dorsalis* (Hendel). *Molecular Ecology, 16*(17), 3522-3532.

Aketarawong, N., Chinvinijkul, S., Orankanok, W., Guglielmino, C.R., Franz, G., Malacrida, A. R., & Thanaphum, S. (2011). The utility of microsatellite DNA markers for the evaluation of area-wide integrated pest management using SIT for the fruit fly, *Bactrocera dorsalis* (Hendel), control programs in Thailand. *Genetica, 139*(1), 129-140.

Aketarawong, N., Isasawin, S., & Thanaphum, S. (2014a). Evidence of weak genetic structure and recent gene flow between *Bactrocera dorsalis* ss and *B. papayae*, across Southern Thailand and West Malaysia, supporting a single target pest for SIT applications. *BMC Genetics, 15*(1), 70.

Aketarawong, N., Guglielmino, C. R., Karam, N., Falchetto, M., Manni, M., Scolari, F., ... & Malacrida, A. R. (2014b). The oriental fruitfly *Bactrocera dorsalis* ss in East Asia: disentangling the different forces promoting the invasion and shaping the genetic make-up of populations. *Genetica*, *142*(3), 201-213.

AliNiazee, M. T. (1974). The Western cherry fruit fly, *Rhagoletis indifferens* (Diptera: Tephritidae): Aggressive behavior. *The Canadian Entomologist, 106*(11), 1201-1204.

Allendorf, T., Swe, K. K., Oo, T., Htut, Y., Aung, M., Aung, M., ... & Wemmer, C. (2006). Community attitudes toward three protected areas in Upper Myanmar (Burma). *Environmental Conservation*, *33*(4), 344-352.

Allwood, A. J., Chinajariyawong, A., Drew, R. A. I., Hamacek, E. L., Hancock, D. L., Hengsawad, C., Jinapin, J. C., Jirasurat, M., KongKrong, C., Kritsaneepaiboon,

S., Leong, C. S. T., Vijaysegaran, S., (1999). Host plant records of fruit flies (Diptera: Tephritidae) in Southeast Asia. *The Raffles Bulletin of Zoology. Suppl*, *7*, 92 pp.

Allwood, A. J. & Drew, R. A. I. (1996). Management of fruit flies in the Pacific. *ACIAR Proceedings No. 76*. Canberra, Australia. ACIAR, Canberra.

Allwood, A. J., Chinajariyawong, A., Drew, R. A. I., Hamacek, E. L., Hancock, D. L., Hengsawad, C., Jinapin, J. C., Jirasurat, M., Kong Krong, C., Kritsaneepaiboon, S., Leong, C. T. S. & Vijaysegaran, S. (1999). Host plant records for fruit flies (Diptera: Tephritidae) in Southeast Asia. *Raffles Bulletin of Zoology, 7*, 1-92.

Aluja, M., & Norrbom, A. (1999). *Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior*. Crc Press.

Anonymous (2015a). *Bactrocera*. Retrieved from http://www.discoverlife.org/mp/20q

Anonymous (2015b). Thailand. Retrieved from http://www.asean.fta.govt.nz/thailand-overview

Anonymous (2015c). Thailand and climate change Retrieved from https://climatechangethailand.wordpress.com/geography

Anonymous (2015d). Template: Location map Thailand. Retrieved from https://en.wikipedia.org/wiki/Template:Location_map_Thailand

Armstrong, J. W., Silva, S. T., & Shishido, V. M. (1995). Quarantine cold treatment for Hawaiian carambola fruit infested with Mediterranean fruit fly, melon fly, or oriental fruit fly (Diptera: Tephritidae) eggs and larvae. *Journal of Economic Entomology, 88*(3), 683-687.

Armstrong, K. F., & Ball, S. L. (2005). DNA barcodes for biosecurity: invasive species identification. *Philosophical Transactions of the Royal Society of London B: Biological Sciences, 360*(1462), 1813-1823.

Ashton, P. S. (1992). Plant conservation in the Malaysian region. In harmony with nature: Proceedings of the International Conference of Tropical Biodiversity (eds Y.S. Kheong and L.S.Win), pp. 86-93. Malayan Nature Society, Kuala Lumpur.

Ashton, P. S. (1995). Towards a regional forest classification for the humid tropics of Asia. Vegetation Science in Forestry (eds E.O.Box,R.K.Peet, T.Masuzawa, T.Yamada, K.Fujiwara & P.F.Maycock), pp. 453-464. Kluwer Academic Publishers, Amsterdam.

Ashton, P. S. (1997). South Asian evergreen forests: some thoughts toward biogeographic reevaluation. *Tropical Ecology*, *38*, 171-180.

Asokan, R., Rebijith, K. B., Singh, S. K., Sidhu, A. S., Siddharthan, S., Karanth, P. K., & Ramamurthy, V. V. (2011). Molecular Identification and Phylogeny of

*Bactrocera* Species (Diptera: Tephritidae). *Florida Entomologist, 94*, 1026-1035.

Attwood, S. W., & Johnston, D. A. (2001). Nucleotide sequence differences reveal genetic variation in *Neotricula aperta* (Gastropoda: Pomatiopsidae), the snail host of schistosomiasis in the lower Mekong Basin. *Biological Journal of the Linnean Society*, *73*(1), 23-41.

Aung-Thwin, M., & Aung-Thwin, M. (2013). *A history of Myanmar since ancient times: traditions and transformations*. London, Reaktion Books.

Averill, A. L., & Prokopy, R. J. (1987). Intraspecific competition in the tephritid fruit fly *Rhagoletis pomonella. Ecology*, 878-886.

Avise, J. C. (2000). *Phylogeography: The History and Formation of Species*. Harvard university press.

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., ... Saunders, N. C. (1987). Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics*, 489-522.

Awmack, C. S., & Leather, S. R. (2002). Host plant quality and fecundity in herbivorous insects. *Annual Review of Entomology*, *47*(1), 817-844.

Ayasse, M., Paxton, R. J., & Tengö, J. (2001). Mating behavior and chemical communication in the order Hymenoptera. *Annual Review of Entomology*, *46*(1), 31-78.

Aytekin, M. A., Terzo, M., Rasmont, P., & Çağatay, N. (2007). Landmark based geometric morphometric analysis of wing shape in *Sibiricobombus* Vogt (Hymenoptera: Apidae: *Bombus* Latreille). In *Annales de la Société Entomologique de France* (Vol. 43, No. 1, pp. 95-102). Taylor & Francis Group.

Back, E. A., & Pemberton, C. E. (1917). The melon fly in Hawaii. *USDA Bulletin, 491*, 1-64.

Baimai, V., Phinchongsakuldit, J., Sumrandee, C., & Tigvattananont, S. (2000). Cytological evidence for a complex of species within the taxon *Bactrocera tau* (Diptera: Tephritidae) in Thailand. *Biological Journal of the Linnean Society, 69*, 399-409.

Baimai, V., Trinachartvanit, W., Grote, P. J., Kijchalao, U., Tigvattananont, S., & Poramarcom, R. (1995). Metaphase karyotypes of fruit flies of Thailand. I. Five sibling species of the *Bactrocera dorsalis* complex. *Genome, 38*(5), 1015-1022.

Baker, W. J., Coode, M. J. E., Dransfield, J., Dransfield, S., Harley, M. M., Hoffmann, P., & Johns, R. J. (1998). Patterns of distribution of Malesian vascular plants. *Biogeography and Geological Evolution of SE Asia*, 243-258.

Bale, J. S. (2002). Insects and low temperatures: from molecular biology to distributions and abundance. *Philosophical Transactions of the Royal Society B: Biological Sciences, 357*(1423), 849-862.

Balloux, F., & Goudet, J. (2002). Statistical properties of population differentiation estimators under stepwise mutation in an infinite island model. *Molecular Ecology, 11*, 771-783.

Baltzer, J. L., Davies, S. J., Bunyavejchewin, S., & Noor, N. S. M. (2008). The role of desiccation tolerance in determining tree species distributions along the Malay-Thai Peninsula. *Functional Ecology*, *22*(2), 221-231.

Bandelt, H. J., Forster, P. & Röhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution, 16*(1), 37-48.

Barr, N. B. (2009). Pathway analysis of *Ceratitis capitata* (Diptera: Tephritidae) using mitochondrial DNA. *Journal of Economic Entomology*, *102*(1), 401-411.

Barr, N. B., Copeland, R. S., De Meyer, M., Masiga, D., Kibogo, H.G., Billah, M.K., ... McPheron, B. A. (2006). Molecular diagnostics of economically important *Ceratitis* fruit fly species (Diptera: Tephritidae) in Africa using PCR and RFLP analyses. *Bulletin of Entomological Research, 96*(5), 505-521.

Beebee, T. J. C., & Rowe, G. (2008). *An Introduction to Molecular Ecology*. New York, USA, Oxford University Press.

Benzie, J. A. H. (1999). Major genetic differences between crown-of-thorns starfish (*Acanthaster planci*) populations in the Indian and Pacific Oceans. *Evolution, 53*, 1782-1795.

Berlocher, S. H. (2000) Radiation and divergence in the *Rhagoletis pomonella* species group: inferences from allozymes. *Evolution*, *54*, 543-557.

Bernays, E. A. (1998). Evolution of feeding behavior in insect herbivores. *Bioscience*, *48(*1), 35-44.

Bess, H. A., Van Den Bosch, R., & Haramoto, F. H. (1961). Fruit fly parasites and their activities in Hawaii.

Bezault, E., Balaresque, P., Toguyeni, A., Fermon, Y., Araki, H., Baroiller, J. F., & Rognon, X. (2011). Spatial and temporal variation in population genetic structure of wild Nile tilapia (*Oreochromis niloticus*) across Africa. *BMC Genetics*, *12*(1), 102.

Bezzi, M. (1913). Indian Tephritids (fruit flies) in the collection of the Indian Museum, Calcutta. *Memoirs of the Indian Museum, 3*, 153-175.

Bickford, D., Lohman, D. J., Dohdi, N. S., Ng, P. K. L., Meier, R., Winker, K. et al. (2006) Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution*, *22,* 148-155.

Bigler, F., Bale, J. S., Cock, M. J. W., Dreyer, H., Greatrex, R., Kuhlmann, U., ... Van Lenteren, J. C. (2005). Guidelines on information requirements for import and release of invertebrate biological control agents in European countries. *Biocontrol News and Information, 26*(4), 115N-123N.

Blomquist, G. J., & Bagnères, A. G. (Eds.). (2010). *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology*. New York, Cambridge University Press.

Boag, P. T. (1988). The genetics of island birds. *Acta XIX Congressus Internationalis Ornithologici. National Museum of Natural Sciences, Ottawa*, 1550-1563.

Bookstein, F. L. (1991). *Morphometric Tools for Landmark Data: Geometry and Biology*. Cambridge, UK, Cambridge University Press.

Bossart, J. L., & Prowell, D. P. (1998). Genetic estimates of population structure and gene flow: limitations, lessons and new directions. *Trends in Ecology & Evolution*, *13*(5), 202-206.

Bower, C. C. (1977). Inhibition of larval growth of the Queensland fruit fly, *Dacus tryoni* (Diptera: Tephritidae) in apples. *Annals of the Entomological Society of America, 70*, 97-100.

Boykin, L. M., Armstrong, K. F., Kubatko L., & D. B. P. (2012). Species delimitation and global biosecurity. *Evolutionary Bioinformatics, 8,* 1-37.

Brízová, R., Mendonça, A. L., Vanícková, L., Mendonça, A. L., Eduardo Da Silva, C., Tomcala, A., ... Do Nascimento, R. R. (2013). Pheromone analyses of the *Anastrepha fraterculus* (Diptera: Tephritidae) cryptic species complex. *Florida Entomologist, 96*(3), 1107-1115.

Bruford, M. W., & Wayne, R. K. (1993). Microsatellites and their application to population genetic studies. *Current Opinion in Genetics & Development, 3*, 939-943.

Brunner, P. C., Chatzivassiliou, E. K., Katis, N. I., & Frey, J. E. (2004). Host-associated genetic differentiation in *Thrips tabaci* (Insecta; Thysanoptera), as determined from mtDNA sequence data. *Heredity*, *93*(4), 364-370.

Buffetaut, E. R. I. C., & Ingavat, R. U. C. H. A. (1986). The succession of vertebrate faunas in the continental Mesozoic of Thailand. *Geological Society of Malaysia Bulletin*, *19*, 167-172.

Bunopas, S. A. N. G. A. D., & Vella, P. (1978, November). Late Palaeozoic and Mesozoic structural evolution of northern Thailand, a plate tectonic model. In *Proceedings of the Third Regional Conference on Geology and Mineral Resources of Southeast Asia, Bangkok* (Vol. 12, pp. 133-140).

Busch-Petersen, E., Ripfel, J., Pyrek, A., & Kafu, A. (1988). Isolation and mass rearing of a pupal genetic sexing strain of the Mediterranean fruit fly, *Ceratitis capitata* (Wied.). *Modern Methods of Insect Control: Nuclear Techniques and Biotechnology*, 211-219.

Bush, G. L. (1969). Sympatric host race formation and speciation in frugivorous flies of the genus *Rhagoletis* (Diptera, Tephritidae). *Evolution*, 237-251.

Bush, G. L. (1974). The mechanism of sympatric host race formation in the true fruit flies (Tephritidae). In *Genetic Mechanisms of Speciation in Insects* (pp. 3-23). Springer Netherlands.

Bush, G. L. (1975). Modes of animal speciation. *Annual Review of Ecology and Systematics*, 339-364.

Cabi (2015). Cabi. Retrieved from. http://www.cabi.org/

Cáceres , C., Segura, D. F., Vera, M. T., Wornoayporn, V., Cladera, J. L., Teal, P., Sapountzis, P., Zacharopoulou, K., & Robinson, A.S. (2009). Incipient speciation revealed in *Anastrepha fraterculus* (Diptera; Tephritidae) by studies on mating compatibility, sex pheromones, hybridization, and cytology. *Biological Journal of the Linnean Society, 97*, 152-165.

Cameron, E. K., Bayne, E. M., & Coltman, D. W. (2008). Genetic structure of invasive earthworms *Dendrobaena octaedra* in the boreal forest of Alberta: insights into introduction mechanisms. *Molecular Ecology*, *17*(5), 1189-1197.

Cameron, E. C., Sved, J. A., & Gilchrist, A. S. (2010). Pest fruit fly (Diptera: Tephritidae) in northwestern Australia: one species or two? *Bulletin of Entomological Research, 100*, 197-206.

Capula, M. (1994). Evolutionary relationships of Podarcis lizards from Sicily and the Maltese Islands. *Journal of Zoological Systematics and Evolutionary Research*, *32*(3), 180-192.

Carlson, D. A., & Yocom, S. R. (1986). Cuticular hydrocarbons from six species of tephritid fruit flies. *Archives of Insect Biochemistry and Physiology, 3*(4), 397-412.

Carroll, S. P., & Boyd, C. (1992). Host race radiation in the soapberry bug: natural history with the history. *Evolution*, *46*(4), 1052-1069.

Carson, H. L., Hardy, D. E., Spieth, H. T., & Stone, W. S. (1970). *The Evolutionary Biology of the Hawaiian Drosophilidae* (pp. 437-543). Springer US.

Carter, A., & Bristow, C. S. (2003). Linking hinterland evolution and continental basin sedimentation by using detrital zircon thermochronology: a study of the Khorat Plateau Basin, eastern Thailand. *Basin Research*, *15*(2), 271-285.

Cassens, I., Mardulyn, P., & Milinkovitch, M. C. (2005). Evaluating intraspecific "network" construction methods using simulated sequence data: do existing algorithms outperform the global maximum parsimony approach?. *Systematic Biology, 54*(3), 363-372.

Cavalli-Sforza, L. L., & Edwards, A. W. (1967). Phylogenetic analysis. Models and estimation procedures. *American Journal of Human Genetics, 19*(3), 233.

Cheng, C. C., & Lee, W. Y. (1991). Fruit flies in Taiwan. In S. Vijaysegaran & A.G. Ibrahim (Eds.), *First International Symposium on Fruit Flies in the Tropics, Kuala Lumpur* (pp.152-160). Kuala Lumpur, Malaysian Agricultural Research and Development Institute.

Chinajariyawong, A., Kritsaneepaiboon, S., & Drew, R. A. I. (2003). Efficacy of protein bait sprays in controlling fruit flies (Diptera: Tephritidae) infesting angled luffa and bitter gourd in Thailand. *Raffles Bulletin of Zoology, 51*(1), 7-16.

Choldumrongkul, S., Tubtim, N., & Ratanachan, S. (2011). Geographical distribution and status of Actias Moths in Thailand. *Walailak Journal of Science and Technology (WJST)*, *4*(2), 203-213.

Chown, S. L., & Gaston, K. J. (2009). Body size variation in insects: A macroecological perspective. *Biological Reviews, 85*, 139-169.

Christenson, L. D., & Foote, R. H. (1960). Biology of fruit flies. *Annual Review of Entomology, 5*, 171-192.

Chua, T. H., Chong, Y. V., & Lim, S. H. (2010). Species determination of Malaysian *Bactrocera* pests using PCR-RFLP analyses (Diptera: Tephritidae). *Pest Management Science*, *66*(4), 379-384.

Clark, A. G., Weiss, K. M., Nickerson, D. A., Taylor, S. L., Buchanan, A., Stengård, J., & Sing, C. F. (1998). Haplotype structure and population genetic inferences from nucleotide-sequence variation in human lipoprotein lipase. *The American Journal of Human Genetics, 63*, 595-612.

Clarke, A. R., & Schutze, M. K. (2014). The complexities of knowing what it is you are trapping. In T. Shelly, N. Epsky, E. B. Jang, J. Reyes-Flores & R. Vargas (Eds.), *Trapping and the Detection, Control, and Regulation of Tephritid Fruit Flies* (pp. 611-632). Springer Netherlands.

Clarke, A. R., Armstrong, K. F., Carmichael, A. E., Milne, J. R., Raghu, S., Roderick, G. K., & Yeates, D. K. (2005). Invasive phytophagous pests arising through a

recent tropical evolutionary radiation: the *Bactrocera dorsalis* complex of fruit flies. *Annual Review of Entomology, 50*, 293-319.

Clarke, A. R., Allwood, A., Chinajariyawong, A., Drew, R. A. I., Hengsawad, C., Jirasurat, M., Krong, C. K., Kritsaneepaiboon, S., & Vijaysegaran, S. (2001). Seasonal abundance and host use patterns of seven *Bactrocera* Macquart species (Diptera: Tephritidae) in Thailand and Peninsular Malaysia. *Raffles Bulletin of Zoology, 49*, 207-220.

Cognato, A. I., Sun, J-H., Anducho-Reyes, M. A., & Owen, D. R. (2005). Genetic variation and origin of red turpentine beetle (*Dendroctonus valens* LeConte) introduced to the People's republic of China. *Agricultural and Forest Entomology*, *7,* 87-94.

Colautti, R. I., Manca, M., Viljanen, M., Ketelaars, H. A., Buergi, H., Macisaac, H. J., & Heath, D. D. (2005). Invasion genetics of the Eurasian spiny waterflea: evidence for bottlenecks and gene flow using microsatellites*. Molecular Ecology, 14*(7), 1869-1879.

Collins, N. M., Sayer, J. A., & Whitmore, T. C. (1991). The conservation atlas of tropical forests: Asia and the Pacific. International Union for Conservation of Nature and Natural Resources.

Condon, M. A., & Steck, G. J. (1997). Evolution of host use in fruit flies of the genus *Blepharoneura* (Diptera: Tephritidae): cryptic species on sexually dimorphic host plants. *Biological Journal of the Linnean Society*, *60*(4), 443-466.

Condon M. A., & Norrbom A. L. (1999). Behavior of flies in the genus *Blepharoneura* Blepharoneurinae). In M. Aluja & A. L. Norrbom (Eds.), *Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior* (pp.135-156). Boca Raton, FL: CRC Press.

Condon, M., Adams, D. C., Bann, D., Flaherty, K., Gammons, J., Johnson, J., ... Swensen, S. (2008). Uncovering tropical diversity: six sympatric cryptic species of *Blepharoneura* (Diptera: Tephritidae) in flowers of *Gurania spinulosa* (Cucurbitaceae) in eastern Ecuador. *Biological Journal of the Linnean Society, 93*(4), 779-797.

Corin, S. E., Lester, P. J., Abbott, K. L., & Ritchie, P. A. (2007). Inferring historical introduction pathways with mitochondrial DNA: the case of introduced Argentine ants (*Linepithema humile*) into New Zealand. *Diversity and Distributions*, *13*, 510-518.

Corrie, A. M., & Hoffmann, A. A. (2004). Fine-scale genetic structure of grape phylloxera from the roots and leaves of Vitis. *Heredity*, *92*(2), 118-127.

Craig, T. P., Horner, J. D., & Itami, J. K. (1997). Hybridization studies on the host races of *Eurosta solidaginis*: implications for sympatric speciation. *Evolution*, 1552-1560.

Craig, T. P., Itami, J. K., Abrahamson, W. G., & Horner, J. D. (1993). Behavioral evidence for host-race formation in *Eurosta solidaginis*. *Evolution*, 1696-1710.

CRC Plant Biosecurity. (2015). The National Fruit Fly Research, Development & Extension Plan. Retrieved from http://www.pbcrc.com.au/sites/default/files/managedfiles/National%20Fruit%20Fly%20RD&E%20Plan.pdf

Crandall, E. D. et al. (2007). Contrasting demographic history and phylogeographical patterns in two Indo-Pacific gastropods. *Molecular Ecology, 17*, 611-626.

Crisci, J. V. (2006). One-dimensional systematist: perils in a time of steady progress. *Systematic Botany*, *31*(1), 217-221.

Crow, J. F. (1957). Genetics of insect resistance to chemicals. *Annual Review of Entomology, 2*(1), 227-246.

Curtis, S., Sztepanacz, J. L., White, B. E., Dyer, K. A., Rundle, H. D., & Mayer, P. (2013). Epicuticular compounds of *Drosophila subquinaria* and *D. recens*: identification, quantification, and their role in female mate choice. *Journal of Chemical Ecology, 39*(5), 579-590.

d'Ettorre, P., & Lenoir, A. (2010). Nestmate recognition. *Ant Ecology*, 194-209.

Davidowitz, G., & Nijhout, H. F. (2004). The physiological basis of reaction norms: The interaction among growth rate, the duration of growth and body size. *Integrative and Comparative Biology, 44*, 443-449.

Dayrat, B. (2005). Towards integrative taxonomy. *Biological Journal of the Linnean Society, 85*(3), 407-415.

De Bruyn, M., Nugroho, E., Hossain, M. M., Wilson, J. C., & Mather, P. B. (2005). Phylogeographic evidence for the existence of an ancient biogeographic barrier: the Isthmus of Kra Seaway. *Heredity*, *94*(3), 370-378.

De Meyer, M., Robertson, M. P., Peterson, A. T., & Mansell, M. W. (2008). Ecological niches and potential geographical distributions of Mediterranean fruit fly (*Ceratitis capitata*) and Natal fruit fly (*Ceratitis rosa*). *Journal of Biogeography*, *35*(2), 270-281.

De Meyer, M., Robertson, M. P., Mansell, M. W., Ekesi, S., Tsuruta, K., Mwaiko, W., .... Peterson, A. T. (2010). Ecological niche and potential geographic distribution of the invasive fruit fly *Bactrocera invadens* (Diptera, Tephritidae). *Bulletin of Entomological Research, 100*(1), 35-48.

De Meyer, M., Delatte, H., Mwatawala, M., Quilici, S., Vayssieres, J. F., & Virgilio, M. (2015). A review of the current knowledge on *Zeugodacus cucurbitae* (Coquillett)(Diptera, Tephritidae) in Africa, with a list of species included in Zeugodacus. *ZooKeys*, *(540)*, 539.

De Queiroz, K. (1998). The general lineage concept of species, species criteria, and the process of speciation: a conceptual unification and terminology recommendations. In D. J. Howard & S. H. Berlocher (Eds.), *Endless Forms: Species and Speciation* (pp 57-75). Oxford University Press, New York.

De Queiroz, K. (1999). The general lineage concept of species and the defining properties of the species category. In R. A. Wilson (Eds.), *Species: New Interdisciplinary Essays* (pp 48-89). MIT Press, Cambridge, Massachusetts.

De Queiroz, K. (2007a). Species concepts and species delimitation. *Systematic Biology*, *56*(6), 879-886.

De Queiroz, K. (2007b). Toward an integrated system of clade names. *Systematic Biology*, *56*(6), 956-974.

Debat, V., Béagin, M., Legout, H., & David, J. R. (2003). Allometric and nonallometric components of *Drosophila* wing shape respond differently to developmental temperature. *Evolution, 57*, 2773-2784.

Defra (2002). Bluetongue disease control strategy for the United Kingdom. London, UK: DEFRA. Retrieved from http://www.defra.gov.uk/animalh/diseases/ notifiable/disease/bluetongue_control_strategy.pdf

Delatte, H., Virgilio, M., Simiand, C., Risterucci, A. M., De Meyer, M., & Quilici, S. (2010). Isolation and characterization of microsatellite markers from *Bactrocera cucurbitae* (Coquillett). *Molecular Ecology Resource*, *10*, 576-579.

Department of Agriculture. (2010). 2009 annual report of Plant protection and development office, Department of Agriculture, Thailand. Retrieved from http://www.doa.go.th/research/files/1282_2552.pdf

Department of Agriculture. (2012). 2009 annual report of Plant protection and development office, Department of Agriculture, Thailand. Retrieved from http://www.doa.go.th/research/files/2200_2554.pdf

Dhillon, M. K., Singh, R., Naresh, J. S., & Sharma, H. C. (2005). The melon fruit fly, *Bactrocera cucurbitae*: A review of its biology and management. *Journal of Insect Science*, *5*(1), 40.

Diamantidis, A. D., Carey, J. R., & Papadopoulus, N. T. (2008). *Life-history of An Invasive Tephritid*. Department of Agriculture, Crop Production and Rural Environmental, University of Thessaly, Magnisias, Greece.

Diehl, S. R., & Bush, G. L. (1984). An evolutionary and applied perspective of insect biotypes. *Annual Review of Entomology*, *29*(1), 471-504.

Dingle, H., & Winchell, R. (1997). Juvenile hormone as a mediator of plasticity in insect life histories. *Archives of Insect Biochemistry and Physiology*, *35*(4), 359-373.

Doharey, K. L. (1983). Bionomics of fruit flies (*Dacus* spp.) on some fruits. *Indian Journal of Entomology, 45*, 406-413.

Downie, D. A. (2002). Locating the sources of an invasive pest, grape phylloxera, using a mitochondrial DNA gene genealogy. *Molecular Ecology*, *11*, 2013-2026.

Drake, A. G., & Klingenberg, C. P. (2008). The pace of morphological change: historical transformation of skull shape in St. Bernard dogs. *Proceedings of the Royal Society of London, B Biological Sciences, 275*, 71-76.

Drès, M., & Mallet, J. (2002). Host races in plant-feeding insects and their importance in sympatric speciation. *Philosophical Transactions of the Royal Society of London B*: *Biological Sciences*, *357*(1420), 471-492.

Drew, R. A. I., Tsuruta, K., & White, I. M. (2005). A new species of pest fruit fly (Diptera: Tephritidae: Dacinae) from Sri Lanka and Africa. *African Entomology*, *13*(1), p-149.

Drew, R. A. I. & Hancock, D. L. (1994). The *Bactrocera dorsalis* complex of fruit flies (Diptera: Tephritidae: Dacinae) in Asia. *Bulletin of Entomological Research Supplement Series, 2,* 1-68.

Drew, R. A. I., & Hancock, D. L. (2000). Phylogeny of the tribe Dacini (Dacinae) based on morphological, distributional, and biological data. In Aluja, M. & Norrbom, A. L. (Eds.) *Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior* (pp. 491-504). Florida, USA, CRC Press.

Drew, R. A. I., & Lambert, D. M. (1986). On the Specific Status of *Dacus* (*Bactrocera*) *aquilonis* and *D.* (*Bactrocera*) *tryoni* (Diptera: Tephritidae). *Annals of the Entomological Society of America, 79*, 870-878.

Drew, R. A. I., & Romig, M. C. (2013). *Tropical Fruit Flies (Tephritidae Dacinae) of South-East Asia: Indomalaya to North-West Australasia*. CABI.

Drew, R. A. I. (1989). The tropical fruit flies (Diptera: Tephritidae: Dacinae) of the Australasian and Oceania Regions. *Memoirs of the Queensland Museum*, *26*, 1-521.

Drew, R. A. I. (2004). Biogeography and speciation in the Dacini (Diptera: Tephritidae: Dacinae). *Bishop Museum Bulletin in Entomology, 12*, 165-178.

Drew, R. A. I., Hooper, G. H. S., & Bateman, M. A. (1982). *Economic Fruit Flies of the South Pacific Region* (2nd ed.). Department of Primary Industries, Brisbane, Queensland.

Drew, R. A. I., Raghu, S., & Halcoop, P. (2008). Bridging the morphological and biological species concepts: studies on the *Bactrocera dorsalis* (Hendel) complex (Diptera: Tephritidae: Dacinae) in Southeast-Asia. *Biological Journal of the Linnaean Society, 93*, 217-226.

Dryden, I. L., & Mardia, K. V. (1998). *Statistical Shape Analysis*. Chichester, GB, Wiley.

Dujardin, J. P., & Kitthawee, S. (2013). Phenetic structure of two *Bactrocera tau* cryptic species (Diptera: Tephritidae) infesting Momordica cochinchinensis (Cucurbitaceae) in Thailand and Laos. *Zoology*, *116*(2), 129-138.

Dyck, V. A., Hendrichs, J., & Robinson, A. S. (2005). *Sterile Insect Technique Principles and Practice in Rea-Wide Integrated Pest Management Dordrecht* (5th ed.). Vienna, Austria.

E Linn, C., Dambroski, H., Nojima, S., Feder, J. L., Berlocher, S. H., & Roelofs, W. L. (2005). Variability in response specificity of apple, hawthorn, and flowering dogwood-infesting *Rhagoletis* flies to host fruit volatile blends: implications for sympatric host shifts. *Entomologia Experimentalis et Applicata*, *116*(1), 55-64.

Earl, D. A., & vonHoldt, B. M. (2011). STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources, 4*, 359-361.

Eastwood, R., Boyce, S. L., & Farrell, B. D. (2006). The provenance of Old world swallowtail butterflies, *Papilio demoleus* (Lepidoptera: Papilionidae), recently discovered in the new world. *Annals of the Entomological Society of America*, *99*, 165-167.

El-Mergawy, R. A. A. M., Faure, N., Nasr, M. I., Avand-Faghih, A., Rochat, D., & Silvain, J. F. (2011). Mitochondrial genetic variation and invasion history of red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), in the Middle-East and Mediterranean Basin. *International Journal of Agricultural and Biological Engineering, 13,* 631-637.

Emelianov, I., Simpson, F., Narang, P., & Mallet, J. (2002). Host races in plant-feeding insects and their importance in sympatric speciation. *Journal of Evolutionary Biology*, *16*, 208-218.

Enkerlin, W. (2003). Economics of area-wide SIT control programs. In: Recent Trends on Sterile Insect Technique and Area-Wide Integrated Pest Management – Economic Feasibility, Control Projects, Farmer Organization and *Bactrocera*

*dorsalis* Complex Control Study. Research Institute for Subtropics, Naha, Japan, 1-10.

Ensing, D., P. A. Prodohl, P. McGinnity, P. Boylan, N. O'Maoileidigh & W. W. Crozier. (2011). "Complex pattern of genetic structuring in the Atlantic salmon (*Salmo salar* L.) of the River Foyle system in northwest Ireland: disentangling the evolutionary signal from population stochasticity. *Ecology and Evolution, 1*(3), 359-72.

Erwin, T. L. (1982). Tropical forests: their richness in Coleoptera and other arthropod species. *Coleopterists Bulletin, 36*, 74-75.

Erwin, T. L. (1991). How many species are there?. *Conservation Biology, 5*, 1-4.

Estoup, A., & Guillemaud, T. (2010). Reconstructing routes of invasion using genetic data: why, how and so what?. *Molecular Ecology*, *19*(19), 4113-4130.

Eta, C. R. (1985). Eradication of the melon fly from Shortland Islands, Western Province, Solomon Islands (special report). *Solomon Islands Agricultural Quarantine Service, Annual Report.* Ministry of Agriculture and Lands, Honiara, 14-23.

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology, 14*, 2611-2620.

Excoffier, L., Laval, G., & Schneider, S. (2005). Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online, 1*, 47.

Excoffier, L., Smouse, P. E., & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics, 131*(2), 479-491.

Falush, D., Stephens, M., & Pritchard, J. K. (2003). Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics, 164*(4), 1567-1587.

Falush, D., Stephens, M., & Pritchard, J. K. (2007). Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes, 7*(4), 574-578.

Feder, J. L. (1988). Genetic differentiation between sympatric host races of the apple maggot fly. *Nature*, *336*, 3.

Feder, J. L. (1995). The effects of parasitoids on sympatric host races of *Rhagoletis pomonella* (Diptera: Tephritidae). *Ecology*, 801-813.

Feder, J. L. (1998). The apple maggot fly, *Rhagoletis pomonella*. *Endless Forms: Species and Speciation.* Oxford University. Press, New York, 130-144.

Feder, M. E., & Krebs, R. A. (1997). Ecological and evolutionary physiology of heat shock proteins and the stress response in *Drosophila*: complementary insights from genetic engineering and natural variation. In *Environmental Stress, Adaptation and Evolution* (pp. 155-173). Birkhäuser Basel.

Feder, J. L., Roethele, J. B., Filchak, K., Niedbalski, J., & Romero-Severson, J. (2003). Evidence for inversion polymorphism related to sympatric host race formation in the apple maggot fly, *Rhagoletis pomonella*. *Genetics, 163*(3), 939-953.

Fedina, T. Y., Kuo, T. H., Dreisewerd, K., Dierick, H. A., Yew, J. Y., & Pletcher, S. D. (2012). Dietary effects on cuticular hydrocarbons and sexual attractiveness in *Drosophila*. *PloS one*, *7*(12), 49799.

Filchak, K. E., Roethele, J. B., & Feder, J. L. (2000). Natural selection and sympatric divergence in the apple maggot *Rhagoletis pomonella*. *Nature*, *407,* 739-742.

Fitzpatrick, M. J., & Gray, D. A. (2001). Divergence between the courtship songs of the field crickets *Gryllus texensis* and *Gryllus rubens* (Orthoptera, Gryllidae). *Ethology*, *107*(12), 1075-1085.

Fitzpatrick, B. M., Fordyce, J. A., & Gavrilets, S. (2009). Pattern, process and geographic modes of speciation. *Journal of Evolutionary Biology*, *22*(11), 2342-2347.

Fletcher, B. S. (1987). The biology of Dacine fruit flies. *Annual Review of Entomology, 32*, 115-144.

Fletcher, M. T., & Kitching, W. (1995). Chemistry of fruit flies. *Chemical Reviews, 95*(4), 789-828.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular marine biology and biotechnology*, *3*(5), 294-299.

Francoy, T. M., Grassi, M. L., Imperatriz-Fonseca, V. L., de Jesús May-Itzá, W., & Quezada-Euán, J. J. G. (2011). Geometric morphometrics of the wing as a tool for assigning genetic lineages and geographic origin to *Melipona beecheii* (Hymenoptera: Meliponini). *Apidologie*, *42*(4), 499-507.

Frankham, R. (1996). Relationship of genetic variation to population size in wildlife. *Conservation Biology*, 1500-1508.

Frankham, R. (1997). Do island populations have less genetic variation than mainland populations?. *Heredity*, *78*(3), 311-327.

Freeland, J. R., Petersen, S.D., & Heather, K. (2011). *Molecular Ecology*. Chicester, UK, John Wiley & Sons Ltd.

French, V., Feast, M., & Partridge, L. (1998). Body size and cell size in *Drosophila*: the developmental response to temperature. *Journal of Insect Physiology, 44*(11), 1081-1089.

Fu, Y. X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics, 147*(2), 915-925.

Fu, Y. X., & Li, W. H. (1993). Statistical tests of neutrality of mutations. *Genetics, 133*(3), 693-709.

Futuyma, D. J. (1983). *Science on Trial: The Case for Evolution* (p. 82). Pantheon Books.

Futuyma, D. J., & Peterson, S. C. (1985). Genetic variation in the use of resources by insects. *Annual Review of Entomology*, *30*(1), 217-238.

Gasparich, G. E., Silva, J. S., Han, & H-Y et al. (1997). Population genetic structure of Mediterranean fruit fly (Diptera: Tephritidae) and implications for worldwide colonization patterns. *Annals of the Entomological Society of America, 90*, 790-797.

Gibbs, A. G. (2011). Thermodynamics of cuticular transpiration. *Journal of Insect Physiology, 57*(8), 1066-1069.

Giddings, L. V., Kaneshiro, K. Y., & Anderson, W. W. (Eds.). (1989). *Genetics, Speciation, and the Founder Principle*. New York, New York: Oxford University Press.

Gilchrist, A. S., Dominiak, B., Gillespie, P. S., & Sved, J. A. (2006). Variation in population structure across the ecological range of the Queensland fruit fly, *Bactrocera tryoni*. *Australian Journal of Zoology*, *54*(2), 87-95.

Gilchrist, A. S. & Crisafulli, D. C. A. (2006). Using variation in wing shape to distinguish between wild and mass-reared individuals of Queensland fruit fly, *Bactrocera tryoni*. *Entomologia Experimentalis et Applicata*, *119*, 175-178.

Glaubitz, J. C. (2004). Convert: A user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages. *Molecular Ecology Notes, 4*(2), 309-310.

Glaubrecht, M., & Kohler, F. (2004). Radiating in a river: systematics, molecular genetics and morphological differentiation of viviparous freshwater gastropods endemic to the Kaek River, central Thailand (Cerithioidea, Pachychilidae). *Biological Journal of the Linnean Society*, *82*(3), 275-311.

Goh, S. H., Ooi, K. E., Chuah, C. H., Yong, H. S., Khoo, S. G., & Ong, S. H. (1993). Cuticular hydrocarbons from two species of Malaysian *Bactrocera* fruit flies. *Biochemical Systematics and Ecology, 21*(2), 215-226.

Goldstein, D. B., & Schlötterer, C. (1999). *Microsatellites: Evolution and Applications*. New York, USA. Oxford University Press.

Good, R. (1964). *The Geography of the Flowering Plants*, 3rd edn. Longman, London.

Gordh, G., & McKirdy, S. (2013). *The Handbook of Plant Biosecurity: Principles and Practices for the Identification, Containment and Control of Organisms that Threaten Agriculture and the Environment Globally*. Springer Science & Business Media.

Gorman, G. C., & Kim, Y. J. (1975). Genetic variation and genetic distance among populations of Anolis lizards on two Lesser Antillean island banks. *Systematic Biology*, *24*(3), 369-373.

Goudet, J. (2001). FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3).

Goudet, J. (2005). PCAGEN 1.2. Available at: http://www2.unil.ch/popgen/softwares/pcagen.htm (accessed 1 January 2014)

Grapputo, A., Boman, S., Lindström, L., Lyytinen, A., Mappes, J. (2005). The voyage of an invasive species across continents: genetic diversity of North American and European colorado potato beetle populations. *Molecular Ecology*, *14*, 4207-4219.

Gray, D. A. (2005). Does courtship behavior contribute to species-level reproductive isolation in field crickets?. *Behavioral Ecology*, *16*(1), 201-206.

Greenbaum, I. F., & Baker, R. J. (1976). Evolutionary relationships in *Macrotus* (Mammalia: Chiroptera): biochemical variation and karyology. *Systematic Biology*, *25*(1), 15-26.

Guamán Sarango (2009). Monitoring and pest control of Fruit flies in Thailand. Retrieved from http://stud.epsilon.slu.se/699/

Guillem, R. M., Drijfhout, F. P., & Martin, S. J. (2012). Using chemo-taxonomy of host ants to help conserve the large blue butterfly. *Biological Conservation, 148*(1), 39-43.

Gullan, P. J., Kaydan, M. B., & Hardy, N. B. (2010). Molecular phylogeny and species recognition in the mealybug genus *Ferrisia* Fullaway (Hemiptera: Pseudococcidae). *Systematic Entomology, 35*, 329-339.

Gullette, G. (2013). The role of urbanity, status, and identity on migrant remittance management and rural development potentials in Thailand. *Human Organization, 72*(2), 132-143.

Guttman, C. M., DiMarzio, E. A., & Hoffman, J. D. (1981). Modelling the amorphous phase and the fold surface of a semicrystalline polymer-the Gambler's Ruin method. *Polymer*, *22*(11), 1466-1479.

Gwiazdowski, R. A., Van Driesche, R. G., Desnoyers, A. et al. (2006). Possible geographic origin of beech scale, *Cryptococcus fagisuga* (Hemiptera: Eriococcidae), an invasive pest in North America. *Biological Control*, *39*, 9-18.

Hajibabaei, M., Singer, G. A., Hebert, P. D., & Hickey, D. A. (2007). DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. TRENDS in Genetics, *23*(4), 167-172.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *In Nucleic Acids Symposium Series, 41*, 95-98.

Hancock, J. M. (1999). Microsatellites and other simple sequences: genomic context and mutational mechanisms. In D. B. Goldstein& C. Schlotterer (Eds.), *Microsatellites: Evolution and Applications* (pp.287-289). Oxford, UK, Oxford University Press.

Hardy, D. E. (1973). The fruit flies (Tephritidae-Diptera) of Thailand and bordering countries. *Pacific Insects Monograph, 31*, 1-353.

Havill, N. P., Montgomery, M. E., Yu, G., Shiyake, S., & Caccone, A. (2006). Mitochondrial DNA from hemlock woolly adelgid (Hemiptera: Adelgidae) suggests cryptic speciation and pinpoints the source of the introduction to eastern North America. *Annals of the Entomological Society of America, 99*, 195-203.

Heather, N. W., & Hallman, G. J. (2008). *Pest Management and Phytosanitary Trade Barriers*. CABI.

Hebert, P. D. N, Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B: Biological Sciences, 270*, 313-321. doi: 10.1098/rspb. 2002.2218.

Hendrichs, J. (2000). Use of the sterile insect technique against key insect pests. *Sustainable Development International, 2*, 75-79.

Hendrichs J., Vera M.T., De Meyer M., Clarke A.R.. Resolving cryptic species complexes of major tephritid pests. In: De Meyer M, Clarke AR, Vera MT, Hendrichs J (Eds) Resolution of Cryptic Species Complexes of Tephritid Pests to Enhance SIT Application and Facilitate International Trade. ZooKeys: in press.

Hepburn, H. R., Smith, D. R., Radloff, S. E., Otis, G. W. (2001). Infraspecific categories of *Apis cerana*: Morphometric, allozymal and mtDNA diversity. *Apidologie*, 32, 3-23.

Hepburn, H. R., & Radloff, S. E. (2011). Biogeography. In *Honeybees of Asia* (pp. 51-67). Springer Berlin Heidelberg.

Hernández-Ortiz, V., Bartolucci, A. F., Morales-Valles, P., Frías, D., & Selivon, D. (2012). Cryptic species of the *Anastrepha fraterculus* complex (Diptera: Tephritidae): a multivariate approach for the recognition of South American morphotypes. *Annals of the Entomological Society of America*, *105*(2), 305-318.

Hernández-Ortiz, V., Gómez-Anaya, J. A., Sánchez, A., McPheron, B. A., & Aluja, M. (2004). Morphometric analysis of Mexican and South American populations of the *Anastrepha fraterculus* complex (Diptera: Tephritidae) and recognition of a distinct Mexican morphotype. *Bulletin of Entomological Research, 94*(6), 487-499.

Hollingsworth, R. G., Vagalo, M., & Tsatsia, F. (1997). Biology of melon fly, with special reference to Solomon Islands. In A.J. Allwood and R.A.I. Drew (Eds.), Management of Fruit Flies in the Pacific: *ACIAR Proceedings* (pp. 140-144). Australian Centre for International Agricultural Research.

Hooper, W. C., & Haritt, W. H. (1978). The Influence of Cathodic Polarization Upon Fatigue of Notched Structural Steel in Sea Water. *Corrosion*, *34*(9), 320-324.

Hu, J., Zhang, J. L., Nardi, F., & Zhang, R. J. (2008). Population genetic structure of the melon fly, *Bactrocera cucurbitae* (Diptera: Tephritidae), from China and Southeast Asia. *Genetica*, *134*(3), 319-324.

Huemer, P., Mutanen, M., Sefc, K. M., & Hebert, P. D. (2014). Testing DNA barcode performance in 1000 species of European Lepidoptera: large geographic distances have small genetic impacts. *PloS one*, *9*(12), e115774.

Hufbauer, R. A., Bogdanowicz, S. M., & Harrison, G. (2004). The population genetics of a biological control introduction: mitochondrial DNA and microsatellite variation in native and introduced populations of *Aphidus ervi,* a parasitoid wasp. *Molecular Ecology*, *13*, 337-348.

Hughes, A. C., Satasook, C., Bates, P. J., Bumrungsri, S., & Jones, G. (2011). Explaining the causes of the zoogeographic transition around the Isthmus of Kra: using bats as a case study. *Journal of Biogeography*, *38*(12), 2362-2372.

Hughes, J. B., Round, P. D., & Woodruff, D. S. (2003). The Indochinese-Sundaic faunal transition at the Isthmus of Kra: an analysis of resident forest bird species distributions. *Journal of Biogeography, 30*(4), 569-580.

Hughes, T. P., Baird, A. H., Bellwood, D. R., Card, M., Connolly, S. R., Folke, C., ... Roughgarden, J. (2003). Climate change, human impacts, and the resilience of coral reefs. *Science*, *301*(5635), 929-933.

Hurst, G. D., & Jiggins, F. M. (2005). Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. *Proceedings of the Royal Society of London B: Biological Sciences*, *272*(1572), 1525-1534.

Ibrahim, R. I. (1990). *Handbook on Identification of Fruit Flies in the Tropics*. Penerbit University Pertanian Malaysia.

Inger, R. F. (1966). Systematics and zoogeography of the Amphibia of Borneo. *Field. Zoo, 52*, 1-402.

Inger, R. F. (1999). Distribution of amphibians in southern Asia and adjacent islands. *Patterns of Distribution of Amphibians: A Global Perspective*, 445-482.

International Plant Protection Convention. (2015). ISPMs. Retrieved from https://www.ippc.int/en/core-activities/standards-setting/ispms/

Isabirye B. E., Masembe, C., Nankinga, C. K., Muyinza, H., & Akol, A. M. (2013). Geometric Morphometrics of Geographic and Host-Associated Population Variations of *Bactrocera invadens* in Uganda. Agriculture and Biology Journal of North America. ISSN Print: 2151-7517, ISSN Online: 2151-752.

Iwahashi, O., & Routhier, W. (2001). Aedeagal length and its variation of the peach fruit fly, *Bactrocera zonata* (Saunders) (Diptera: Tephritidae), which recently invaded Egypt. *Applied Entomology and Zoology, 36*(1), 13-17.

Iwaizumi, R., Kaneda, M., & Iwahashi, O. (1997). Correlation of length of terminalia of males and females among nine species of *Bactrocera* (Diptera, Tephritidae) and differences among sympatric species of *B. dorsalis* complex. *Annals of the Entomological Society of America, 90,* 664-666.

Jacquard, C., Virgilio, M., David, P., Quilici, S., De Meyer, M., & Delatte, H. (2013). Population structure of the melon fly, *Bactrocera cucurbitae*, in Reunion Island. *Biological Invasions*, *15*(4), 759-773.

Jaenike, J. R. (1973). A steady state model of genetic polymorphism on islands. *American Naturalist*, 793-795.

Jakobsson, M., & Rosenburg, N. A. (2007). CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics*, *23*, 1801-1806.

Jamnongluk, W., Baimai, V., & Kittayapong, P. (2003a). Molecular phylogeny of tephritid fruit flies in the *Bactrocera tau* complex using the mitochondrial COI sequences. *Genome, 46*, 112-118.

Jamnongluk, W., Baimai, V., & Kittayapong, P. (2003b). Molecular evolution of tephritid fruit flies in the genus *Bactrocera* based on the cytochrome oxidase I gene. *Genetica, 119*(1), 19-25.

Jamnongluk, W., Kittayapong, P., Baimai, V., & O'neill, S. L. (2002). *Wollbachia* infections of tephritid fruit flies: molecular evidence for five distinct strains in a single host species. *Current Microbiology, 45*(4), 255-260.

Jarmillo-O, N., Dujardin, J.P., Calle-Londono, D., & Fonseca-Gonzales, I. (2015). Geometric morphometrics for the taxonomy of 11 species of *Anopheles* (*Nyssorhynchus*) mosquitoes. *Medical and veterinary entomology*, *29*(1), 26-36.

Jennings, J. H., Etges, W. J., Schmitt, T., & Hoikkala, A. (2014). Cuticular hydrocarbons of *Drosophila montana*: Geographic variation, sexual dimorphism and potential roles as pheromones. *Journal of Insect Physiology, 61*, 16-24.

Jiang, F., Li, Z. H., Deng, Y. L., Wu, J. J., Liu, R. S., & Buahom, N. (2013). Rapid diagnosis of the economically important fruit fly, *Bactrocera correcta* (Diptera: Tephritidae) based on a species-specific barcoding cytochrome oxidase I marker. *Bulletin of entomological research*, *103*(03), 363-371.

Jiang, F., Jin, Q., Liang, L., Zhang, A. B., & Li, Z. H. (2014). Existence of species complex largely reduced barcoding success for invasive species of Tephritidae: a case study in *Bactrocera* spp. *Molecular ecology resources*, *14*(6), 1114-1128.0

Johnson, M. S. (1988). Founder effects and geographic variation in the land snail *Theba pisana*. *Heredity*, *61*(1), 133-142.

Jörger, K. M., & Schrödl, M. (2013). How to describe a cryptic species? Practical challenges of molecular taxonomy. *Frontiers in Zoology*, *10*(10), 1742-9994.

Kapoor, V. C. (2006). Taxonomy and biology of economically important fruit flies of India. *Israel Journal of Entomology, 35*, 459-475.

Karsten, M., van Vuuren, B. J., Barnaud, A., & Terblanche, J. S. (2013). Population Genetics of *Ceratitis capitata* in South Africa: Implications for Dispersal and Pest Management. *PLoS One, 8*, e54281.

Karsten, M., Jansen van Vuuren, B., Addison, P., & Terblanche, J. S. (2015). Deconstructing intercontinental invasion pathway hypotheses of the Mediterranean fruit fly (*Ceratitis capitata*) using a Bayesian inference approach: are port interceptions and quarantine protocols successfully preventing new invasions?. *Diversity and Distributions*, *21*(7), 813-825.

Kaspi, R., Taylor, P. W., & Yuval, B. (2000). Diet and size influence sexual advertisement and copulatory success of males in Mediterranean fruit fly leks. *Ecological Entomology, 25*, 279-284.

Kather, R., & Martin, S. J. (2012). Cuticular hydrocarbon profiles as a taxonomic tool: advantages, limitations and technical aspects. *Physiological Entomology, 37*(1), 25-32.

Keng, H. (1970). Size and affinities of the flora of the Malay Peninsula. *Journal of Tropical Geography,* 31, 43-56.

Kerr, K. C. R., Lijtmaer, D. A., Barreira, A. S., Hebert, P. D. N., & Tubaro, P. L. (2009). Probing Evolutionary Patterns in Neotropical Birds through DNA Barcodes *PLoS One, 4*, e4379. doi:10.1371/journal.pone.0004379.

Khamis, F. M., Masiga, D. K., Mohamed, S. A., Salifu, D., de Meyer, M., & Ekesi, S. (2012). Taxonomic identity of the invasive fruit fly pest, *Bactrocera invadens*: concordance in morphometry and DNA barcoding.

Khamis, F. M., Karam, N., Ekesi, S., De Meyer, M., Bonomi, A., Gomulski, L. M., Scolari, F., Gabrieli, P., Siciliano, P., Masiga, D., Kenya, E. U., Gasperi, G., Malacrida, A. R., & Guglielmino, C. R. (2009). Uncovering the tracks of a recent and rapid invasion: the case of the fruit fly pest *Bactrocera invadens* (Diptera: Tephritidae) in Africa. *Molecular Ecology, 18*, 4798-4810.

Kilpatrick, C. W. (1981). Genetic structure of insular populations. In: Smith, M. H. and Joule, J. *(eds) Mammalian Population Genetics,* pp. 28-59. University of Georgia Press, Athens, GA.

King, B., Buck, H., Ferguson, R., Fisher, T., Goblet, C., Nickel, H., & Suter, W. (2001). Birds recorded during two expeditions to North Myanmar (Burma). *Forktail*, 29-40.

Kittayapong, P., Milne, J. R., Tigvattananont, S., & Baimai, V. (2000). Distribution of the reproduction-modifying bacteria, *Wolbachia*, in natural populations of tephritid fruit flies in Thailand. *Science Asia, 26*, 93-103.

Kitthawee, S., & Dujardin, J. P. (2010). The geometric approach to explore the *Bactrocera tau* complex (Diptera: Tephritidae) in Thailand. *Zoology, 113*, 243-249.

Kitthawee, S., & Rungsri, N. (2011). Differentiation in wing shape in the *Bactrocera tau* (Walker) complex on a single fruit species in Thailand. *Science Asia, 37*, 308-313.

Klingenberg, C. P. (2009). Morphometric integration and modularity in configurations of landmarks: tools for evaluating a-priori hypotheses. *Evolution & Development, 11*, 405-421.

Klingenberg, C. P. (2011). MorphoJ: an integrated software package for geometric morphometrics. *Molecular Ecology Resources*, *11*, 353-357.

Klingenberg, C. P., & McIntyre, G. S. (1998). Geometric morphometrics of developmental instability: analyzing patterns of fluctuating asymmetry with Procrustes methods. *Evolution*, 1363-1375.

Knowlton, N. (2000). Molecular genetic analyses of species boundaries in the sea. *Hydrobiologia*, *420*(1), 73-90.

Kodandaramaiah, U. (2009). Vagility: the neglected component in historical biogeography. *Evolutionary Biology*, *36*(3), 327-335.

Kong, G., Rodoni, B., Zheng, L., Geering, A., Hailstones, D., Flack, M., ... Beasley, D. (2012). Project Plant biosecurity: technological research and training for improved pest diagnostics in Thailand and Australia.

Koyama, J., Kakinohana, H., & Miyatake, T. (2004). Eradication of the melon fly, *Bactrocera cucurbitae*, in Japan: importance of behavior, ecology, genetics, and evolution. *Annual Reviews in Entomology, 49*, 331-349.

Krainacker, D. A., Carey, J. R., & Vargas, R. I. (1987). Effect of larval host on life history traits of the Mediterranean fruit fly, *Ceratitis capitata*. *Oecologia*, *73*(4), 583-590.

Krongkaew, M. (1985). Agricultural development, rural poverty, and income distribution in Thailand. *The Developing Economies, 23*(4), 325-346.

Krosch, M. N., Schutze, M. K., Armstrong, K. F., Graham, G. C., Yeates, D. K., & Clarke, A. R. (2012). A molecular phylogeny for the Tribe Dacini (Diptera: Tephritidae): systematic and biogeographic implications. *Molecular Phylogenetics and Evolution, 64*(3), 513-523.

Krosch, M. N., Schutze, M. K., Armstrong, K. F., Boontop, Y., Boykin, L. M., Chapman, T. A., Englezou, A., Cameron, S. L., & Clarke, A. R. (2013). Piecing together an integrative taxonomic puzzle: microsatellite, wing shape and aedeagus length analyses of *Bactrocera dorsalis* s.l. (Diptera: Tephritidae) find no evidence of multiple lineages in a proposed contact zone along the Thai/Malay Peninsula. *Systematic Entomology, 38*, 2-13.

Kume, T., Amano, E., Nakanishi, T. M., & Chino, M. (2002). Economic scale of utilization of radiation (II): Agriculture comparison between Japan and the USA. *Journal of Nuclear Science and Technology, 39*, 1106-1113.

Kunprom, C., Sopaladawan, P. N., & Pramual, P. (2015). Population genetics and demographic history of guava fruit fly *Bactrocera correcta* (Diptera: Tephritidae) in northeastern Thailand. *European Journal of Entomology*, *112*(2), 227.

Langella, O. (1999). POPULATIONS 1.2.28: Population genetic software: individuals or populations distances based on allelic frequencies, phylogenetic trees, file conversions.

Lavine, B. K., Carlson, D. A., & Calkins, C. O. (1992). Classification of tephritid fruit fly larvae by gas chromatography/pattern recognition techniques. *Microchemical Journal, 45*(1), 50-57.

Lawson, A. E., McGuire, D. J., Yeates, D. K, Drew R. A. I., & Clarke, A. R. (2003). *Dorsalis*: An interactive identification tool to fruit flies of the *Bactrocera dorsalis* complex. CD-ROM publication, Griffith University, Brisbane, Australia.

Lefébure, T., Douady, C. J., Gouy, M., & Gibert, J. (2006). Relationship between morphological taxonomy and molecular divergence within Crustacea: proposal of a molecular threshold to help species delimitation. *Molecular Phylogenetics and Evolution*, *40*(2), 435-447.

Lekagul, B., Round, P. D., Wongkalasin, M., & Komolphalin, K. (1991). *A Guide to the Birds of Thailand.* Saha Karn Bhaet.

Levine, J. M. (2008). Biological invasions. *Current Biology*, *18*(2), R57-R60.

Liu, S. S., Colvin, J., & De Barro, P. J. (2012). Species concepts as applied to the whitefly *Bemisia tabaci* systematics: how many species are there?. *Journal of Integrative Agriculture*, *11*(2), 176-186.

Librado, P., & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics, 25*(11), 1451-1452.

Lim, L. S., Mohd-Adnan, A., Zubaid, A., Struebig, M. J., & Rossiter, S. J. (2014). Diversity of Malaysian insectivorous bat assemblages revisited. *Journal of Tropical Ecology*, *30*(02), 111-121.

Lim, P. E., Tan, J., Suana, I. W., Eamsobhana, P., & Yong, H. S. (2012). Distinct genetic lineages of *Bactrocera caudata* (Insecta: Tephritidae) revealed by COI and 16S DNA sequences. *PloS one, 7*(5), e37276.

Lincoln, R. J., Boxshall, G. A., & Clark, P. F. (1982). *A Dictionary of Ecology, Evolution and Systematics*. Cambridge, UK. Cambridge University Press.

Lindroth, R. L., Klein, K. A., Hemming, J. D., & Feuker, A. M. (1997). Variation in temperature and dietary nitrogen affect performance of the gypsy moth (*Lymantria dispar* L.). *Physiological Entomology*, *22*(1), 55-64.

Linn, C., Feder, J. L., Nojima, S., Dambroski, H. R., Berlocher, S. H., & Roelofs, W. (2003). Fruit odor discrimination and sympatric host race formation in *Rhagoletis. Proceedings of the National Academy of Sciences*, *100*(20), 11490-11493.

Linn, C., Nojima, S., & Roelofs, W. (2005). Antagonist effects of non-host fruit volatiles on discrimination of host fruit by *Rhagoletis* flies infesting apple (*Malus pumila*), hawthorn (*Crataegus* spp.), and flowering dogwood (*Cornus florida*). *Entomologia Experimentalis et Applicata*, *114*(2), 97-105.

Liu, L., Liu, J., Wang, Q., Ndayiragije, P., Ntahimpera, A., Nkubaye, E., ... Li, Z. (2013). Identification of *Bactrocera invadens* (Diptera: Tephritidae) from Burundi, based on morphological characteristics and DNA barcode. *African Journal of Biotechnology, 10*(62), 13623-13630.

Lohman, D. J., de Bruyn, M., Page, T., von Rintelen, K., Hall, R., Ng, P. K., ... von Rintelen, T. (2011). Biogeography of the Indo-Australian archipelago. *Annual Review of Ecology, Evolution, and Systematics*, *42*, 205-226.

Long, B. (1973). Research on Tropical Fruits: Proposal for an International Tropical Fruits Centre in Asia (Thailand).

Lukoschek, V., Osterhage, J. L., Karns, D. R., Murphy, J. C., & Voris, H. K. (2011). Phylogeography of the Mekong mud snake (*Enhydris subtaeniata*): the biogeographic importance of dynamic river drainages and fluctuating sea levels for semiaquatic taxa in Indochina. *Ecology and Evolution*, *1*(3), 330-342.

Lux, S. A., Copeland, R. S., White, I. M., Manrakhan, A., & Billah, M. K. (2003). A new invasive fruit fly species from the *Bactrocera dorsalis* (Hendel) group detected in East Africa. *International Journal of Tropical Insect Science*, *23*(04), 355-361.

Mahmood, K. (2004). Identification of pest species in Oriental Fruit Fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) species complex. *Pakistan Journal of Zoology, 36*, 219-230.

Malacrida, A. R., Gomulski, L. M., Bonizzoni, M., Bertin, S., Gasperi, G., & Guglielmino, C. R. (2007). Globalization and fruitfly invasion and expansion: the medfly paradigm. *Genetica*, *131*(1), 1-9.

Margaritopoulos, J. T., Kasprowicz, L., Malloch, G. L., & Fenton, B. (2009). Tracking the global dispersal of a cosmopolitan insect pest, the peach potato aphid. *BMC Ecology*, *9*(1), 13.

Marod, D., & Kutintara, U. (2012). Biodiversity observation and monitoring in Thailand. In The Biodiversity Observation Network in the Asia-Pacific Region (pp. 53-63). Springer Japan.

Martin, S. J., Vitikainen, E., Helanterä, H., & Drijfhout, F. P. (2008). Chemical basis of nest-mate discrimination in the ant *Formica exsecta*. *Proceedings of the Royal Society of London B: Biological Sciences, 275*(1640), 1271-1278.

Martin, S., & Drijfhout, F. (2009). A review of ant cuticular hydrocarbons. *Journal of Chemical Ecology, 35*(10), 1151-1161.

Maynard, G. V., Hamilton, J. G., & Grimshaw, J. F. (2004). Quarantine-phytosanitary, sanitary andincursion management: an Australian entomological perspective. *Australian Journal of Entomology, 43*, 318-328.

McAlpine, D. K., & Schneider, M. A. (1978). A systematic study of *Phytalmia* (Diptera, Tephritidae) with description of a new genus. *Systematic Entomology*, *3*(2), 159-175.

McMaugh, T. (2005). *Guidelines for surveillance for plant pests in Asia and the Pacific*. Canberra, Australia: Australian Centre for International Agricultural Research.

McPheron, B. A., Smith, D. C., & Berlocher, S. H. (1988). Genetic differences between host races of *Rhagoletis pomonella*. *Nature*, *336*(6194), 64-66.

Meats, A., & Smallridge, C. J. (2007). Short-and long-range dispersal of medfly, *Ceratitis capitata* (Diptera, Tephritidae), and its invasive potential. *Journal of Applied Entomology*, *131*(8), 518-523.

Medina, F. I. S., Carillo, P. A. V., Gregorio, J. S., & Aguilar, C. P. (1998). The mating compatibility between *Bactrocera philippinensis* and *Bactrocera dorsalis*. In *Abstracts, 5th International Symposium on Fruit Flies of Economic Importance: 1-5 June*.

Meeyen, K., Nanork Sopaladawan, P., & Pramual, P. (2014). Population structure, population history and DNA barcoding of fruit fly *Bactrocera latifrons* (Hendel) (Diptera: Tephritidae). *Entomological Science, 17*(2), 219-230.

Meirmans, P. G. (2012). The trouble with isolation by distance. *Molecular Ecology*, *21*(12), 2839-2846.

Meirmans, P. G. (2015). Seven common mistakes in population genetics and how to avoid them. *Molecular Ecology, 24,* 3223-3231.

Meixner, M. D., McPheron, B. A., Silva, J. G., Gasparich, G. E., & Sheppard, W. S. (2002). The Mediterranean fruit fly in California: evidence for multiple introductions and persistent populations based on microsatellite and mitochondrial DNA variability. *Molecular Ecology*, *11*(5), 891-899.

Meksongsee, M., Liewvanich, A., & Jirasuratana, M. (1991). Fruit flies in Thailand. In S. Vijaysegaran & A.G. Ibrahim (Eds.), *First International Symposium on Fruit Flies in the Tropics, Kuala Lumpur* (pp.83-98). Kuala Lumpur, Malaysian Agricultural Research and Development Institute.

Miner, B. G., Sultan, S. E., Morgan, S. G., Padilla, D. K., & Relyea, R. A. (2005). Ecological consequences of phenotypic plasticity. *Trends in Ecology & Evolution*, *20*(12), 685-692.

Mitter, C., & Futuyma, D. J. (1983). An evolutionary-genetic view of host-plant utilization by insects. *Variable Plants and Herbivores in Natural and Managed Systems*, 427-459.

Mitteroecker, P., Gunz, P., Windhager, S., & Schaefer, K. (2013). A brief review of shape, form, and allometry in geometric morphometrics, with applications to human facial morphology. *Hystrix, The Italian Journal of Mammalogy*, *24*(1), 59-66.

Miyatake, T. (1997). Correlated responses to selection for developmental period in *Bactrocera cucurbitae* (Diptera: Tephritidae): time of mating and daily activity rhythms. *Behavior Genetics, 27*(5), 489-498.

Moczek, A. P. (1998). Horn polyphenism in the beetle *Onthophagus taurus*: larval diet quality and plasticity in parental investment determine adult body size and male horn morphology. *Behavioral Ecology*, *9*(6), 636-641.

Moritz, C., Dowling, T. E., & Brown, W. M. (1987). Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annual Review of Ecology and Systematics, 18*, 269-292.

Morrow, J., Scott, L., Congdon, B., Yeates, D., Frommer, M., & Sved, J. (2000). Close genetic similarity between two sympatric species of tephritid fruit fly reproductively isolated by mating time. *Evolution*, *54*, 899-910.

Mousseau, T. A., & Dingle, H. (1991). Maternal effects in insect life histories. *Annual Review of Entomology, 36*, 511-534.

Muminhanov, H. (2008). Country report on the state of plant genetic resources for food and agriculture. *Republic of Tajikistan. FAO*.

Muraji, M., & Nakahara, S. (2002). Discrimination among pest species of Bactrocera (Diptera: Tephritidae) based on PCR-RFLP of the mitochondrial DNA. *Applied Entomology and Zoology*, *37*(3), 437-446.

Murray, J. M., Watson, G. J., Giangrande, A., Licciano, M., & Bentley, M.G. (2012). Managing the marine aquarium trade: revealing the data gaps using ornamental polychaetes. *PloS one, 7*(1), e29543.

Nabholz, B., Glémin, S., & Galtier, N. (2009). The erratic mitochondrial clock: variations of mutation rate, not population size, affect mtDNA diversity across birds and mammals. *BMC Evolutionary Biology.* doi:10.1186/1471-2148-9-54.

Nadler, S. A., & DE LEÓN, G. P. P. (2011). Integrating molecular and morphological approaches for characterizing parasite cryptic species: implications for parasitology. *Parasitology, 138*(13), 1688-1709.

Narayanan E. S. (1953). Seasonal pests of crops. *Indian Farming, 3*(4): 8-11 & 29-31.

Narayanan, E. S., & Batra, H. N. (1960). *Fruit Flies and Their Control*. Indian Council of Agricultural Research. India.

Nardi, F., Carapelli, A., Dallai, R., Roderick, G. K., & Frati, F. (2005). Population structure and colonization history of the olive fly, *Bactrocera oleae* (Diptera, Tephritidae). *Molecular Ecology, 14*(9), 2729-2738.

Nason, J. D., Heard, S. B., & Williams, F. R. (2002). Host-associated genetic differentiation in the goldenrod elliptical-gall moth, *Gnorimoschema gallaesolidaginis* (Lepidoptera: Gelechiidae). *Evolution*, *56*(7), 1475-1488.

Nation, J. L. (1990). Biology of pheromone release by male Caribbean fruit flies, *Anastrepha suspensa* (Diptera: Tephritidae). *Journal of Chemical Ecology*, *16*(2), 553-572

Navarro-Campos, C., Martinez-Ferrer, M. T., Campos, J. M., Fibla, J. M., Alcaide, J., Bargues, L., & Garcia-Mari, F. (2011). The influence of host fruit and temperature on the body size of adult *Ceratitis capitata* (Diptera: Tephritidae) under laboratory and field conditions. *Environmental Entomology, 40*, 931-938.

Nevo, E. (1978). Genetic variation in natural populations: patterns and theory. *Theoretical Population Biology*, *13*(1), 121-177.

Nijhout, H. F. (2003). The control of body size in insects. *Developmental Biology, 261*, 1-9.

Nojima, S., Sakuma, M., Nishida, R., & Kuwahara, Y. (1999). A glandular gift in the German cockroach, *Blattella germanica* (L.)(Dictyoptera: Blattellidae): the courtship feeding of a female on secretions from male tergal glands. *Journal of Insect Behavior*, *12*(5), 627-640.

Novotny, V., Clarke, A. R., Drew, R. A., Balagawi, S., & Clifford, B. (2005). Host specialization and species richness of fruit flies (Diptera: Tephritidae) in a New Guinea rain forest. *Journal of Tropical Ecology*, *21*, 67-77.

OAE (2008). *Agricultural Statistics of Thailand: Crop Year 2006/2007.* Office of Agricultural Economics, Agricultural Statistics, Ministry of Agriculture and Cooperatives. Bangkok, Thailand.

Ohno, S., Tamura, Y., Haraguchi, D., & Kohama, T. (2008a). First detection of the pest fruit fly, *Bactrocera tau* (Diptera: Tephritidae), in the field in Japan:

Evidence of multiple invasions of Ishigaki Island and failure of colonization. *Applied Entomology and Zoology*, *43*(4), 541-545.

Orankanok, W., Chinvinijkul, S., Thanaphum, S., Sitilob, P., & Enkerlin, W. R. (2007). Area-wide integrated control of oriental fruit fly *Bactrocera dorsalis* and guava fruit fly *Bactrocera correcta* in Thailand. In *Area-Wide Control of Insect Pests* (pp. 517-526). Springer Netherlands.

Padial, J. M., Miralles, A., Riva, I. De la & Vences, M. (2010). Review integrative future of taxonomic. *Frontiers in Zoology*, *7*, 16-19.

Padil (2015). Padil. Retrieved from http://www.padil.gov.au/

Panuwet, P., Siriwong, W., Prapamontol, T., Ryan, P. B., Fiedler, N., Robson, M. G., & Barr, D. B. (2012). Agricultural pesticide management in Thailand: status and population health risk. *Environmental Science & Policy*, *17*, 72-81.

Papadopoulos, N. T. (2014). Fruit Fly Invasion: Historical, Biological, Economic Aspects and Management. In *Trapping and the Detection, Control, and Regulation of Tephritid Fruit Flies* (pp. 219-252). Springer Netherlands.

Pareek, B. L., & Kavadia, V. S. (1994). Relative preference of fruit fly, *Dacus cucurbitae* Coquillett on different cucurbits. *Indian Journal of Entomology*, *56*, 72-75.

Parenti, L. R., & Humphries, C. J. (2004). Historical biogeography, the natural science. *Taxon*, *53*(4), 899-904.

Park, S. D. E. (2001). *Trypanotolerance in West African Cattle and the Population Genetic Effects of Selection*. Ph. D. thesis, University of Dublin.

Partridge, L., & French, V. (1996). Thermal evolution of ectotherm body size: why get big in the cold. *Animals and Temperature: Phenotypic and Evolutionary Adaptation*, *59*, 265.

Patou, M. L., Wilting, A., Gaubert, P., Esselstyn, J. A., Cruaud, C., Jennings, A. P., ... Veron, G. (2010). Evolutionary history of the *Paradoxurus* palm civets-a new model for Asian biogeography. *Journal of Biogeography*, *37*(11), 2077-2097.

Pauls, S. U., Blahnik, R. J., Zhou, X., Wardwell, C. T., & Holzenthal, R. W. (2010). DNA barcode data confirm new species and reveal cryptic diversity in Chilean Smicridea (Smicridea) (Trichoptera: Hydropsychidae). *Journal of the North American Benthological Society*, *29*(3), 1058-1074.

Palumbi, S. R., Grabowsky, G., Duda, T., Geyer, L., & Tachino, N. (1997). Speciation and population genetic structure in tropical Pacific sea urchins. *Evolution*, 1506-1517

Pauwels, O. S., David, P., Chimsunchart, C., & Thirakhupt, K. (2003). Reptiles of Phetchaburi Province, Western Thailand: a list of species, with natural

history notes, and a discussion on the biogeography at the Isthmus of Kra. *The Natural History Journal of Chulalongkorn University*, *3*(1), 23-53.

Peck, S. L., McQuate, G. T., Vargas, R. I., Seager, D. C., Revis, H. C., Jang, E. B., & McInnis, D. O. (2005). Movement of sterile male *Bactrocera cucurbitae* (Diptera: Tephritidae) in a Hawaiian agroecosystem. *Journal of Economic Entomology*, *98*(5), 1539-1550.

Penny, D. (2001). A 40,000 year palynological record from north-east Thailand; implications for biogeography and palaeo-environmental reconstruction. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *171*(3), 97-128.

Perkins, M.V., Fletcher, M.T., Kitching, W., Drew, R.A.I., & Moore, C. J. (1990). Chemical studies of rectal gland secretions of some species of *Bactrocera dorsalis* complex of fruit flies (diptera: Tephritidae). *Journal of Chemical Ecology, 16*(8), 2475-2487.

Perrier, C., Guyomard, R., BAGLINIERE, J. L., & Evanno, G. (2011). Determinants of hierarchical genetic structure in Atlantic salmon populations: environmental factors vs. anthropogenic influences. *Molecular Ecology*, *20*(20), 4231-4245.

Pimentel, D. (Ed.). (2011). *Biological Invasions: Economic and Environmental Costs of Alien Plant, Animal, and Microbe Species*. CRC Press.

Piñero, J. C., Ja´come, I., Vargas, R., & Prokopy, R. J. (2006). Response of female melon fly, *Bactrocera cucurbitae*, to host-associated visual and olfactory stimuli. *Entomologia Experimentalis et Applicata*, *121*, 261-269.

Plummer, M. G., & Yue, C. S. (2009). Realizing the ASEAN Economic Community: a comprehensive assessment. Institute of Southeast Asian Studies.

Polaszek, A., Manzari, S., & Quicke, D. L. (2004). Morphological and molecular taxonomic analysis of the *Encarsia meritoria* species-complex (Hymenoptera, Aphelinidae), parasitoids of whiteflies (Hemiptera, Aleyrodidae) of economic importance. *Zoologica Scripta, 33*(5), 403-421.

Posadas, P., Crisci, J. V., & Katinas, L. (2006). Historical biogeography: a review of its basic concepts and critical issues. *Journal of Arid Environments*, *66*(3), 389-403.

Post, E., Stenseth, N. C., Langvatn, R., & Fromentin, J. M. (1997). Global climate change and phenotypic variation among red deer cohorts. *Proceedings of the Royal Society of London B: Biological Sciences*, *264*(1386), 1317-1324.

Poulin, R., & Thomas, F. (1999). Phenotypic Variability Induced by Parasites: Extent and Evolutionary Implications. *Parasitology Today*, *15*(1), 28-32.

Prabhakar, C.S., Mehta, P.K., Sood, P., Singh, S.K., Sharma, P., & Sharma, P.N. (2012). Population genetic structure of the melon fly, *Bactrocera cucurbitae*

(Coquillett) (Diptera: Tephritidae) based on mitochondrial cytochrome oxidase (COI) gene sequences. *Genetica*, *140*, 83-91.

Pramual, P., Kuvangkadilok, C., Baimai, V., & Walton, C. (2005). Phylogeography of the black fly *Simulium tani* (Diptera: Simuliidae) from Thailand as inferred from mtDNA sequences. *Molecular Ecology*, *14*(13), 3989-4001.

Pritchard, J. K., Stephens, M. & Donnely, P. (2000). Inference of population structure using multilocus genotype data. *Genetics, 155*, 945-959.

Puillandre, N., Dupas, S., Dangles, O., Zeddam, J. L., Capdevielle-Dulac, C., Barbin, K., ... & Silvain, J. F. (2008). Genetic bottleneck in invasive species: the potato tuber moth adds to the list. *Biological Invasions*, *10*(3), 319-333.

Putulan, D., Sar, S., Drew, R. A., Raghu, S., & Clarke, A. R. (2004). Fruit and vegetable movement on domestic flights in Papua New Guinea and the risk of spreading pest fruit-flies (Diptera: Tephritidae). *International Journal of Pest Management*, *50*(1), 17-22.

Rainboth, W. J. (1996). *The Taxonomy, Systematics, and Zoogeography of Hypsibarbus, A New Genus of Large Barbs (Pisces, Cyprinidae) from the Rivers of Southeastern Asia* (Vol. 129). University of California Press.

Ramadan, M. M., & Messing, R. H. (2003). A survey for potential biocontrol agents of *Bactrocera cucurbitae* (Diptera: Tephritidae) in Thailand.

Ranganath, H. R., Suryanarayana, M. A., & Veenakumari, K. (1997). Management of melon fly (*Bactrocera* (*Zeugodacus*) *cucurbitae* Coquillett) in cucurbits in South Andaman. *Insect Environment*, *3*(2), 32-33.

Raser, J. M., & O'Shea, E. K. (2005). Noise in gene expression: origins, consequences, and control. *Science, 309*, 2010-2013.

Rattanapun, W., Amornsak, W., & Clarke, A. R. (2010). Is a mango just a mango? Testing within-fruit oviposition site choice and larval performance of a highly polyphagous fruit fly. *Arthropod-Plant Interactions*, *4*(1), 35-44.

Rice, W. R. (1989). Analyzing tables of statistical tests. *Evolution,* 223-225.

Richards, P. W. (1996). *The Tropical Rain Forest: An Ecological Study*, 2nd edn. Cambridge University Press, Cambridge.

Roderick, G. K., & Howarth, F. G. (1997). Invasion genetics: natural colonizations, exotic pests, and classical biological control. In: *Biological Invasions of Ecosystems by Pests and Beneficial Organisms (eds Miyazaki M, Andow D),* pp. 349-357. National Institute of Agro-Environmental Sciences, Tsukuba, Japan.

Roff, D. A., & Fairbairn, D. J. (1991). Wing dimorphisms and the evolution of migratory polymorphisms among the Insecta. *American Zoologist*, *31*(1), 243-251.

Rohlf, F., & Marcus, L. F. (1993). A revolution morphometrics. *Trends in Ecology & Evolution, 8*, 129-132.

Rohlf, F. J. (1999). Shape statistics: Procrustes superimpositions and tangent spaces. *Journal of Classification*, *16*, 197-223.

Rohlf, F. J. (2013). tpsDig, digitize landmarks and outlines. Department of Ecology and Evolution, State University of New York at Stony Brook, NY, USA. Available online at http://life.bio.sunysb.edu/morph/(accessed March 2013).

Rosenburg, N. A. (2004). Distruct: a program for the graphical display of population structure. *Molecular Ecology Notes, 4*, 137-138.

Rottler, A. M., Schulz, S., & Ayasse, M. (2013). Wax lipids signal nest identity in bumblebee colonies. *Journal of Chemical Ecology*, *39*(1), 67-75

Round, P. D., Hughes, J. B., & Woodruff, D. S. (2003). Latitudinal range limits of resident forest birds in Thailand and the Indochinese-Sundaic zoogeographic transition. *Natural History Bulletin of the Siam Society*, *51*, 69-96.

Rousset, F. (1997). Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, *145*(4), 1219-1228.

Rozas, J., Sánchez-DelBarrio, J. C., Messeguer, X., & Rozas, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, *19*(18), 2496-2497.

Rubinoff, D., Cameron, S., & Will, K. (2006). A genomic perspective on the shortcomings of mitochondrial DNA for “barcoding” identification. *Journal of Heredity*, *97*(6), 581-594.

Rueppell, O., Hayes, A. M., Warrit, N., & Smith, D. R. (2011). Population structure of *Apis cerana* in Thailand reflects biogeography and current gene flow rather than Varroa mite association. *Insectes Sociaux*, *58*(4), 445-452.

Ruiz-Montoya, L., Nunez-Farfan, J., & Vargas, J. (2003). Host-associated genetic structure of Mexican populations of the cabbage aphid *Brevicoryne brassicae* L. (Homoptera: Aphididae). *Heredity*, *91*(4), 415-421.

Ruttner, F. (1988). Biogeography and Taxonomy of Honeybees. Retrieved from http://ae.oxfordjournals.org/content/ae/36/1/58.full.pdf

Saelee, A., Tigvattananont, S., & Baimai, V. (2006). Allozyme electrophoretic evidence for a complex of species within the *Bactrocera tau* group (Diptera: Tephritidae) in Thailand. *Songklanakarin Journal of Science and Technology*, *28*, 249-259.

Scheffer, S. J., & Grissell, E. E. (2003). Tracing the geographical origin of *Megastigmus transvaalensis* (Hymenoptera: Torymidae): an African wasp feeding on a South American plant in North America. *Molecular Ecology*, *12*, 415-421.

Schlick-Steiner, B. C., Steiner, F. M., Seifert, B., Stauffer, C., Christian, E., & Crozier, R. H. (2010). Integrative taxonomy: a multisource approach to exploring biodiversity. *Annual Review of Entomology*, *55*, 421-438.

Schlosser, I. J., & Angermeier, P. L. (1995). Spatial variation in demographic processes of lotic fishes: conceptual models, empirical evidence, and implications for conservation. In *American Fisheries Society Symposium* (Vol. 17, pp. 392-401).

Schmitt, L. H., Kitchener, D. J., & How, R. A. (1995). A genetic perspective of mammalian variation and evolution in the Indonesian archipelago: biogeographic correlates in the fruit bat genus *Cynopterus*. *Evolution*, *49*, 399-412.

Schrag, S., & Wiener, P. (1995). Emerging infectious disease: what are the relative roles of ecology and evolution?. *Trends in Ecology and Evolution*, *10,* 319-324.

Schutze, M. K., Yeates, D. K., Graham, G. C., & Dodson, G. (2007). Phylogenetic relationships of antlered flies, *Phytalmia* Gerstaecker (Diptera: Tephritidae): the evolution of antler shape and mating behaviour. *Australian Journal of Entomology*, *46*(4), 281-293.

Schutze, M. K., Jessup, A., & Clarke, A. R. (2012a). Wing shape as a potential discriminator of morphologically similar pest taxa within the *Bactrocera dorsalis* species complex (Diptera: Tephritidae). *Bulletin of entomological research*,*102*(01), 103-111.

Schutze, M. K., Krosch, M. N., Armstrong, K. F., Chapman, T. A., Englezou, A., Chomič, A., .... Clarke, A. R. (2012b). Population structure of *Bactrocera dorsalis ss, B. papayae* and *B. philippinensis* (Diptera: Tephritidae) in Southeast Asia: evidence for a single species hypothesis using mitochondrial DNA and wing-shape data. *BMC Evolutionary Biology, 12*(1), 130.

Schutze, M. K., Jessup, A., Ul-Haq, I., Vreysen, M. J. B., Wornoayporn, V., Vera, M. T., & Clarke, A. R. (2013). Mating compatibility among four pest members of the *Bactrocera dorsalis* fruit fly species complex (Diptera: Tephritidae). *Journal of Economic Entomology, 106*(2), 695-707.

Schutze, M. K., Aketarawong, N., Amornsak, W., Armstrong, K. F., Augustinos, A. A., Barr, N., ... Clarke, A. R. (2015a). Synonymization of key pest species within the *Bactrocera dorsalis* species complex (Diptera: Tephritidae): taxonomic

changes based on a review of 20 years of integrative morphological, molecular, cytogenetic, behavioural and chemoecological data. *Systematic Entomology, 40*(2), 456-471.

Schutze, M. K., Mahmood, K., Pavasovic, A., Bo, W., Newman, J., Clarke, A. R., ... Cameron, S. L. (2015b). One and the same: integrative taxonomic evidence that *Bactrocera invadens* (Diptera: Tephritidae) is the same species as the Oriental fruit fly *Bactrocera dorsalis*. *Systematic Entomology, 40*(2), 472-486.

Shiao, S. F. (2004). Morphological diagnosis of six *Liriomyza* species (Diptera: Agromyzidae) of quarantine importance in Taiwan. *Applied Entomology and Zoology*, *39*(1), 27-39.

Segura, M. D., Callejas, C., Fernandez, M. P., & Ochando, M. D. (2006). New contributions towards the understanding of the phylogenetic relationships among economically important fruit flies (Diptera: Tephritidae). *Bulletin of Entomological Research*, *96*(03), 279-288.

Selivon, D., Perondini, A. L. P., & Morgante, J. S. (2005). A genetic-morphological characterization of two cryptic species of the *Anastrepha fraterculus* complex (Diptera: Tephritidae). *Annals of the Entomological Society of America, 98*(3), 367-381.

Severin, H. H. P., Severin, M. A., & Hartung, W. J. (1914). The ravages, life history, weights of stages, natural enemies, and methods of control of the melon fly (*Dacus cucurbitae*). *Annals of the Entomological Society of America, 7*, 177-207.

Shah, M. I., Batra, H. N., & Ranjhen, P. L. (1948). Notes on the biology of *Dacus* (*strumeta*) *ferrugineus* fab. and other fruit flies in the North-West Frontier Province. *Indian Journal Entomology*, *10*, 249-266.

Shanmugam, V., Walter, G., & Drew, R. A. I. (2002). Influence of adult diet on the development of the reproductive system and mating ability of Queensland fruit fly *Bactrocera tryoni* (Froggatt)(Diptera: Tephritidae). *Journal of Tropical Agriculture and Food Science, 30*, 119-136.

Shelly, T. E., & Dewire, A. L. M. (1994). Chemically mediated mating success in male oriental fruit flies (Diptera: Tephritidae). *Annals of the Entomological Society of America*, *87*(3), 375-382.

Shelly, T. E., Whittier, T. S., & Villalobos, E. M. (1996). Trimedlure affects mating success and mate attraction in male Mediterranean fruit flies. *Entomologia Experimentalis et Applicata*, *78*(2), 181-185.

Shelly, T. E., & Villalobos, E. M. (1995). Cue lure and the mating behaviour of male melon flies (Diptera: Tephritidae). *Florida Entomologist*, *78*, 473-473.

Shelly, T. E., Pahio, E., & Edu, J. (2004). Synergistic and inhibitory interactions between methyl eugenol and cue lure influence trap catch of male fruit flies, *Bactrocera dorsalis* (Hendel) and *B. cucurbitae* (Diptera: Tephritidae). *Florida Entomologist*, 87, 481-486.

Shen, K., Hu, J., Wu, B., An, K., Zhang, J., Liu, J., & Zhang, R. (2014). Competitive Interactions between Immature Stages of *Bactrocera cucurbitae* (Coquillett) and *Bactrocera tau* (Walker) (Diptera: Tephritidae) under Laboratory Conditions. *Neotropical Entomology*, *43*(4), 335-343.

Shi, W., Kerdelhue, C., & Ye, H. (2005). Population genetics of the oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae), in Yunnan (China) based on mitochondrial DNA sequences. *Environmental Entomology*, *34*(4), 977-983.

Shi, W., Kerdelhué, C., & Ye, H. (2010). Population genetic structure of the Oriental Fruit Fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) from Yunnan province (China) and nearby sites across the border. *Genetica*, *138*, 377-385.

Shi, W., Kerdelhué, C., & Ye, H. (2012). Genetic structure and inferences on potential source areas for *Bactrocera dorsalis* (Hendel) based on mitochondrial and microsatellite markers.

Shingleton, A. W., Frankino, W. A., Flatt, T., Nijhout, H. F., & Emlen, D. (2007). Size and shape: the developmental regulation of static allometry in insects. *BioEssays*, *29*(6), 536-548.

Sidlauskas, B. L., Mol, J. H., & Vari, R. P. (2011). Dealing with allometry in linear and geometric morphometrics: a taxonomic case study in the *Leporinus cylindriformis* group (Characiformes: Anostomidae) with description of a new species from Suriname. *Zoological Journal of the Linnean Society, 162*(1), 103-130.

Siebert, J. (1999). Update on the economic impact of Mediterranean fruit fly on California agriculture. *Subtropical Fruit News*, *7*(1), 16-8.

Simon, C. (1991). Molecular systematic at the species boundary: exploiting conserved and variable regions of the mitochondrial genome of animals via direct sequencing from amplified DNA. In: G.M. Hewitt, AWB Johnston & JPW Young (Eds.), *Molecular Techniques in Taxonomy* (pp 33-71). Springer-Verlag.

Singh, R., & Tewari, R. R. (2012). Genetic Variation Among fruit flies of the Genus *Bactrocera* (Diptera: Tephritidae: Dacinae). *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *82*(3), 361-364.

Sites, J. W., & Marshall, J. C. (2003). Delimiting species: a Renaissance issue in systematic biology. *Trends in Ecology & Evolution*, *18*(9), 462-470.

Sittipraneed, S., Laoaroon, S., Klinbunga, S., & Wongsiri, S. (2001a). Genetic differentiation of the honey bee (*Apis cerana*) in Thailand: Evidence from microsatellite polymorphism. *Journal Apiculture Research*, *40*, 9-16.

Sittipraneed, S., Sihanuntavong, D., & Klinbunga, S. (2001b). Genetic differentiation of the honey bee (*Apis cerana*) in Thailand revealed by polymorphism of a large subunit of mitochondrial ribosomal DNA. *Insectes Sociaux*, *48*(3), 266-272.

Sivinski, J., & Pereira, R. (2005). Do wing markings in fruit flies (Diptera: Tephritidae) have sexual significance?. *Florida entomologist*, *88*(3), 321-324.

Sivinski, J., Burk, T., & Webb, J. C. (1984). Acoustic courtship signals in the Caribbean fruit fly, *Anastrepha suspensa* (Loew). *Animal Behaviour, 32*(4), 1011-1016.

Slatkin, M., & Hudson, R. R. (1991). Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics*, *129*(2), 555-562.

Smith-Caldas, M. R. B., McPheron, B. A., Silva, J. G., & Zucchi, R. A. (2001). Phylogenetic relationships among species of the *fraterculus* group (*Anastrepha*: Diptera: Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase I. *Neotropical Entomology, 30*, 565-573.

Snustad, D. P., Simmons, M. J., Jenkins, J. B., & Crow, J. F. (2000). *Principles of Genetics*. John Wiley.

Somsri, S. (2011, December). Tropical and Subtropical Fruit Production in Thailand: GAP. In *International Symposium on Tropical and Subtropical Fruits 1024* (pp. 25-37).

Soule, M., & Yang, S. Y. (1973). Genetic variation in side-blotched lizards on islands in the Gulf of California. *Evolution*, 593-600.

STDF: Standards and Trade Development Facility (2010). A coordinated multi-stakeholder approach to control fruit fly in West Africa. Retrieved from http://www.standardsfacility.org/Files/Briefings/STDF_Briefing_No4_EN_web.pdf

Steck, G. J. (1999). Taxonomic status of *Anastrepha fraterculus*. In International Atomic Energy Agency (Eds.), *The South American Fruit Fly, Anastrepha fraterculus (Wied.); Advances in Artificial Rearing, Taxonomic Status and Biological Studies* (pp. 13-20). IAEA, Vienna, Austria.

Stefaniak, L., Zhang, H., Gittenberger, A., Smith, K., Holsinger, K., Lin, S., & Whitlatch, R. B. (2012). Determining the native region of the putatively invasive ascidian *Didemnum vexillum* Kott, 2002. *Journal of Experimental Marine Biology and Ecology*, *422*, 64-71.

Stern, D. L., & Emlen, D. J. (1999). The developmental basis for allometry in insects. *Development, 126*, 1091-1101.

Steyskal, G.C. (1972). The meaning of the term 'sibling species. *Systematic Zoology*, *21*, 446.

Stone, A. (1942). The fruit flies of the genus *Anastrepha*. United States Department of Agriculture Miscellaneous Publications*, 439*, 1-112.

Stonehouse, J. M., Mumford, J., & Mustafa, G. (1998). Economic losses to tephritid fruit flies (Diptera: Tephritidae) in Pakistan. *Crop Protection*, *17*, 159-164.

Stork, N. E. (1993). How many species are there?. *Biodiversity and Conservation*, *2*, 215-232.

Sumrandee, C., Milne, J. R., & Baimai, V. (2011). Ovipositor morphology and host relations of the *Bactrocera tau* complex (Diptera: Tephritidae) in Thailand. *Sonklanakarin Journal of Science and Technology, 33*(3), 247.

Sunnucks, P. (2000). Efficient genetic markers for population biology. *Trends in Ecology and Evolution*, *15*, 199-203.

Sutantawong, M., Orankanok, W., Enkerlin, W. R., Wornoayporn, V., Caceres, C., & Barnes, B. N. (2004). The sterile insect technique for control of the oriental fruit fly, *Bactrocera dorsalis* (Hendel), in mango orchards in Ratchaburi Province, Thailand. In B. N. Barnes (Eds.), *Proceedings, Symposium: 6th International Symposium on Fruit Flies of Economic Importance* (pp. 223-232). Stellenbosch, South Africa.

Sutton, B. D., & Steck, G. J. (1994). Discrimination of Caribbean and Mediterranean fruit fly larvae (Diptera: tephritidae) by cuticular hydrocarbon analysis. *Florida Entomologist, 77*(2), 231-237.

Sved, J. A., Yu, H., Dominiak, B., & Gilchrist, A. S. (2003). Inferring modes of colonization for pest species using heterozygosity comparisons and a shared-allele test. *Genetics*, *163*(2), 823-831.

Sylvester, H. A., Limbipichai, K., Wongsiri, S., Rinderer, T. E., & M. Mardan. (1998). Morphometric studies of *Apis cerana* in Thailand and the Malaysian Peninsula. *Journal of Apicultural Research, 37*, 137-145.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, *123*, 585-595.

Tajima, F. (1989). The effect of change in population size on DNA polymorphism. *Genetics*, *123*(3), 597-601.

Tajima, F. (1993). Measurement of DNA polymorphism. In: Mechanisms of Molecular Evolution. Introduction to Molecular Paleopopulation Biology,

edited by Takahata, N. and Clark, A. G., Tokyo, Sunderland, MA: Japan Scientific Societies Press, Sinauer Associates, Inc., p. 37-59.

Takezaki, N., & Nei, M. (1996). Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics, 144*(1), 389-399.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, *24*, 1596-1599.

Tan, K. H. (2003). Interbreeding and DNA analysis of sibling species within the *Bactrocera dorsalis* complex. In *Recent Trends on Sterile Insect Technique and Area-Wide Integrated Pest Management-Economic Feasibility, Control Projects, Farmer Organization and Bactrocera dorsalis Complex Control Study*. 2003:113-122. 12.

Tan, K. H., & Nishida, R. (1996). Sex pheromone and mating competition after methyl eugenol consumption in the *Bactrocera dorsalis* complex. *Fruit Fly Pests: A World Assessment of their Biology and Management.* St. Lucie Press, Delray Beach, Florida, 147-153

Tan, K. H., & Nishida, R. (2012). Methyl eugenol: its occurrence, distribution, and role in nature, especially in relation to insect behavior and pollination. *Journal of Insect Science*, *12*(1), 56.

Tan, K. H., Tokushima, I., Ono, H., & Nishida, R. (2011). Comparison of phenylpropanoid volatiles in male rectal pheromone gland after methyl eugenol consumption, and molecular phylogenetic relationship of four global pest fruit fly species: *Bactrocera invadens*, *B. dorsalis*, *B. correcta* and *B. zonata*.*Chemoecology*, *21*(1), 25-33.

Tan, K. H., Wee, S. L., Ono, H., & Nishida, R. (2013). Comparison of methyl eugenol metabolites, mitochondrial COI, and rDNA sequences of *Bactrocera philippinensis* (Diptera: Tephritidae) with those of three other major pest species within the *dorsalis* complex. *Applied entomology and zoology*, *48*(3), 275-282.

Tauber, C. A., & Tauber, M. J. (1989). Sympatric speciation in insects: perception and perspective. *Speciation and its Consequences*, 307-344.

Tautz, D., & Schlötterer, C. (1994). Simple sequences. *Current Opinion in Genetics & Development, 4*, 832-837.

Templeton, A. R., Hemmer, H., Mace, G., Seal, U. S., Shields, W. M., & Woodruff, D. (1986). Local adaptation, coadaptation, and population boundaries. *Zoo Biology, 5*(2), 115-125.

Tigvattananont, S. (1986). The importance of *Dacus tau* (Walker) in Thailand. *Kaen Kaset, 14*, 114-118.

Tixier, M-S., Tsolakis, H., Ragusa, S., Poinso, A., Ferraro, M., Okassa., M., & Kreiter, S. (2011). Integrative taxonomy demonstrates the unexpected synonymy between predatory mite species: *Cydnodromus idaeus* and *C. picanus* (Acari: Phytoseeidae). *Invertebrate Systematics*, *25*, 273-281.

Travis, J. (1980). Phenotypic variation and the outcome of interspecific competition in hylid tadpoles. *Evolution*, 40-50.

Tsutsui, N. D., Suarez, A. V., Holway, D. A., & Case, T. J. (2001). Relationships among native and introduced populations of the Argentine ant (*Linepithema humile*) and the source of introduced populations. *Molecular Ecology*, *10*(9), 2151-2161.

Uchida, G. K., Vargas, R. I., Beardsley, J. W., & Liquido, N. J. (1990). Host suitability of wild cucurbits for melon fly, *Dacus cucurbitae* Coquillett, in Hawaii, with notes on their distribution and taxonomic status.

Valade, R., Kenis, M., Hernandez-lopez, A., Augustin, S., Mari Mena, N., Magnoux, E., ... Lopez-vaamonde, C. (2009). Mitochondrial and microsatellite DNA markers reveal a Balkan origin for the highly invasive horse-chestnut leaf miner *Cameraria ohridella* (Lepidoptera, Gracillariidae). *Molecular Ecology*, *18*(16), 3458-3470.

Van Herrewege, J., & DAVID, J. R. (1997). Starvation and desiccation tolerances in *Drosophila*: comparison of species from different climatic origins. *Ecoscience,* 151-157.

Van Sauers-Muller, A. (2005). Host plants of carambola fruit fly, *Bactrocera carambolae* Drew & Hancock (Diptera: Tephritidae), in Suriname, South America. *Neotropical Entomology*, *34*(2), 203-214.

Van Steenis, C. G. G. J. (1950). The delimitation of Malaysia and its main plant geographical divisions. *Flora Malesiana*, *1*(1).

Vaníčková, L., Virgilio, M., Tomčala, A., Břízová, R., Ekesi, S., Hoskovec, M., ... De Meyer, M. (2014). Resolution of three cryptic agricultural pests (*Ceratitis fasciventris, C. anonae, C. rosa,* Diptera: Tephritidae) using cuticular hydrocarbon profiling. *Bulletin of Entomological Research, 104*(5), 631-638.

Vargas, R. I., Long, J., Miller, N. W., Delate, K., Jackson, C. G., Uchida, G. K., ... Harris, E. J. (2004). Releases of *Psyttalia fletcheri* (Hymenoptera: Braconidae) and sterile flies to suppress melon fly (Diptera: Tephritidae) in Hawaii. *Journal of Economic Entomology*, *97*(5), 1531-1539.

Vargas, R. I., Stark, J. D., & Nishida, T. (1989). Abundance, distribution, and dispersion indices of the oriental fruit fly and melon fly (Diptera: Tephritidae) on Kauai, Hawaiian Islands. *Journal of Economic Entomology*, *82*(6), 1609-1615.

Vargas, R. I., Stark, J. D., & Nishida, T. (1990). Population dynamics, habitat preference, and seasonal distribution patterns of oriental fruit fly and melon fly (Diptera: Tephritidae) in an agricultural area. *Environmental Entomology*, *19*(6), 1820-1828.

Vargas, R. I., Piñero, J. C., & Leblanc, L. (2015). An Overview of Pest Species of *Bactrocera* Fruit Flies (Diptera: Tephritidae) and the Integration of Biopesticides with Other Biological Approaches for Their Management with a Focus on the Pacific Region. *Insects*, *6*(2), 297-318.

Vayssières, J. F., Rey, J. Y., & Traoré, L. (2007). Distribution and host plants of *Bactrocera cucurbitae* in West and Central Africa. *Fruits, 62*(6), 391-396.

Vekemans, X., & Hardy, O. J. (2004). New insights from fine-scale spatial genetic structure analyses in plant populations. *Molecular Ecology*, *13*(4), 921-935.

Vera, M. T., Cáceres, C., Wornoayporn, V., Islam, A., Robinson, A. S., De la Vega, M. H., Hendrichs, J., & Cayol, J. P. (2006). Mating incompatibility among populations of the South American fruit fly *Anastrepha fraterculus* (Diptera: Tephritidae). *Annals of the Entomological Society of America, 99*, 387-397.

Via, S. (1984). The quantitative genetics of polyphagy in an insect herbivore. I. Genotype-environment interaction in larval performance on different host plant species. *Evolution*, 881-895.

Via, S. (1991). The genetic structure of host plant adaptation in a spatial patchwork: demographic variability among reciprocally transplanted pea aphid clones. *Evolution*, *45*, 827-852.

Virgilio, M., Delatte, H., Backeljau, T., & DeMeyer, M. (2010). Macrogeographic population structuring in the cosmopolitan agricultural pest *Bactrocera cucurbitae (*Diptera: Tephritidae). *Molecular Ecology*, *19*, 2713-2724.

Virgilio, M., Jordaens, K., Breman, F. C., Backeljau, T., & De Meyer, M. (2012). Identifying insects with incomplete DNA barcode libraries, African Fruit flies (Diptera: Tephritidae) as a test case. *PLoS One, 7*(2), e31581.

Virgilio, M., White, I., & De Meyer, M. (2014). A set of multi-entry identification keys to African frugivorous flies (Diptera, Tephritidae). ZooKeys, (*428*), 97.

Virgilio, M., Jordaens, K., Verwimp, C., White, I. M., & De Meyer, M. (2015). Higher phylogeny of frugivorous flies (Diptera, Tephritidae, Dacini): Localised

partition conflicts and a novel generic classification. *Molecular Phylogenetics and Evolution, 85*, 171-179.

Vreysen, M. J., Robinson, A. S., & Hendrichs, J. (2007). Area-wide control of insect pests: from research to field implementation. Springer Science & Business Media. Dordrecht, Netherlands.

Waage, J. K., & Mumford, J. D. (2008). Agricultural biosecurity. *Philosophical Transactions of the Royal Society B: Biological Sciences, 363*(1492), 863-876.

Walter, G. H. (2003). Insect Pest Management and Ecological Research. Cambridge University Press, Cambridge.

Wang, Y., Yu, H., Raphael, K., & Gilchrist, A. S. (2003). Genetic delineation of sibling species of the pest fruit fly *Bactrocera* (Diptera: Tephritidae) using microsatellites. *Bulletin of Entomological Research, 93*, 351-360.

Wang, P., Sun, D. B., Qiu, B. L., & Liu, S. S. (2011). The presence of six cryptic species of the whitefly *Bemisia tabaci* complex in China as revealed by crossing experiments. *Insect Science*, *18*(1), 67-77.

Waring, G. L., Abrahamson, W. G., & Howard, D. J. (1990). Genetic differentiation among host-associated populations of the gallmaker *Eurosta solidaginis* (Diptera: Tephritidae). *Evolution*, 1648-1655.

Warrit, N., Smith, D. R., & Lekprayoon, C. (2006). Genetic subpopulations of Varroa mites and their *Apis cerana* hosts in Thailand. *Apidologie*, *37*(1), 19.

Wasserthal, L. T. (1997). The pollinators of the Malagasy star orchids *Angraecum sesquipedale, A. sororium* and *A. compactum* and the evolution of extremely long spurs by pollinator shift. *Botanica Acta*, *110*(5), 343-359.

Waterhouse, D. F. (1993). *The Major Arthropod Pests and Weeds of Agriculture in Southeast Asia*. ACIAR.

Wee, S. L., & Tan, K. H. (2005). Evidence of natural hybridization between two sympatric sibling species of *Bactrocera dorsalis* complex based on pheromone analysis. *Journal of Chemical Ecology, 31*(4), 845-858.

Weems, H. V. (1964). Melon fly (*Dacus cucurbitae* Coquillett)(Diptera: Tephritidae). *Entomology Circular, Division of Plant Industry, Florida Department of Agriculture and Consumer Services*, *21*, 1-2.

Weems, H. V. Jr., & Heppner, J. B. (1999). Oriental fruit fly, *Bactrocera* (= *Dacus*) *dorsalis* (Hendel) (Insecta: Diptera: Tephritidae). Florida Department of Agriculture and Consumer Services, Division of Plant Industry, and T.R. Fasulo, University of Florida. University of Florida Publication EENY-083. *Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior* (M. Aluja & A. L. Norrbom eds). New York, USA, CRC Press.

Weems Jr, H. V., Heppner, J. B., & Fasulo, T. R. (2001). Melon fly, *Bactrocera cucurbitae* Coquillet (Insecta: Diptera: Tephritidae). Institute of Food and Agricultural Services Publication EENY-119. University of Florida, Gainsveille, FL, USA. Retrieved from http://edis. ifas. ufl. edu/IN356.

Weir, B. S., & Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution,* 1358-1370.

Wells, M. M., & Henry, C. S. (1998). Songs, reproductive isolation, and speciation in cryptic species of insects. *Endless Forms* (ed. by D. J. Howard and S. H. Berlocher), pp. 217-233. Oxford University Press, New York.

Wen, H. C. (1985). Field studies on melon fly (*Dacus cucurbitae*) and attractant experiment in Southern Taiwan. *Journal of Agricultural Research China, 34*, 228-235.

White, I. M., & Elson-Harris, M. M. (1992). *Fruit Flies of Economic Significance: Their Identification and Bionomics*. CAB International.

White, I. M., & Hancock, D. L. (1997). CABIKEY to the Dacini (Diptera, Tephritidae) of the Asia Pacific Australasian regions. Wallingford, UK: CAB International.

Whitman, D. W., & Agrawal, A. A. (2009). What is phenotypic plasticity and why is it important. *Phenotypic Plasticity of Insects*, *10,* 1-63.

Whitmore, T. C. (1984). *Tropical Rainforests of the Far East*, 2nd edn. Oxford University Press, Oxford.

Whitmore, T. C. (1998) *An Introduction to Tropical Rain Forests*. Oxford University Press, New York.

Whitworth, T. L., Dawson, R. D., Magalon, H., & Baudry, E. (2007). DNA barcoding cannot reliably identify species of the blowfly genus *Protocalliphora* (Diptera: Calliphoridae). *Biological Sciences*, *274*(1619), 1731-1739.

Wicker-Thomas, C. (2007). Pheromonal communication involved in courtship behavior in Diptera. *Journal of Insect Physiology*, *53*(11), 1089-1100.

Wiemers, M., & Fiedler, K. (2007). Does the DNA barcoding gap exist? - a case study in blue butterflies (Lepidoptera: Lycaenidae). *Frontiers in Zoology*, *4*(8), 1-16.

Wiens, J. J., & Donoghue, M. J. (2004). Historical biogeography, ecology and species richness. *Trends in Ecology & Evolution*, *19*(12), 639-644.

Wikramanayake, E. D. (2002). Terrestrial ecoregions of the indo-pacific: A conservation assessment. Washington D.C: Island Press. (Covelo and London).

Wilcox, A. J. (2006). Looking Back, and Looking Forward. Epidemiology, *17*(1), 1.

Will, K. W., Mishler, B. D., & Wheeler, Q. D. (2005). The perils of DNA barcoding and the need for integrative taxonomy. *Systematic Biology*, *54*(5), 844-851.

Wilson, J. J. (2012). DNA barcodes for insects. In *DNA Barcodes* (pp. 17-46). Humana Press.

Wilson, S. W., Mitter, C., Denno, R. F., & Wilson, M. R. (1994). Evolutionary patterns of host plant use by delphacid planthoppers and their relatives. In F.D. Robert & T. J. Perfect (Eds.), *Planthoppers* (pp. 7-113). US: Springer.

Wiszniewski, J., Beheregaray, L. B., Allen, S. J., & Möller, L. M. (2010). Environmental and social influences on the genetic structure of bottlenose dolphins (*Tursiops aduncus*) in Southeastern Australia. *Conservation Genetics*, *11*(4), 1405-1419.

Wong, T. Y., Cunningham, R. T., McInnis, D. O., & Gilmore, J. E. (1989). Spatial distribution and abundance of *Dacus cucurbitae* (Diptera: Tephritidae) in Rota, Commonwealth of the Northern Mariana Islands. *Environmental Entomology*, *18*, 1079-1082.

Wood, T. K. (1980). Divergence in the *Enchenopa binotata* Say complex (Homoptera: Membracidae) effected by host plant adaptation. *Evolution*, *34*, 147-160.

Wood, T. K., & Keese, M. C. (1990). Host-plant-induced assortative mating in *Enchenopa* treehoppers. *Evolution*, *44*, 619-628.

Woodruff, D. S. (2003). Neogene marine transgressions, palaeogeography and biogeographic transitions on the Thai-Malay Peninsula. *Journal of Biogeography*, *30*(4), 551-567.

Woodruff, D. S. (2003). The location of the Indochinese-Sundaic biogeographic transition in plants and birds. *Natural History Bulletin of the Siam Society*, *51*, 97-108.

Woodruff, D. S. (2010). Biogeography and conservation in Southeast Asia: how 2.7 million years of repeated environmental fluctuations affect today's patterns and the future of the remaining refugial-phase biodiversity. *Biodiversity and Conservation*, *19*(4), 919-941.

Woodruff, D. S., & Turner, L. M. (2009). The Indochinese-Sundaic zoogeographic transition: a description and analysis of terrestrial mammal species distributions. *Journal of Biogeography*, *36*(5), 803-821.

Woolley, S. M., Posada, D., & Crandall, K. A. (2008). A comparison of phylogenetic network methods using computer simulation. *PLoS One, 3*(4), e1913.

Wright, S. (1943). Isolation by distance. *Genetics*, *28*(2), 114.

Wu, Y., Li, Z. H., & Wu, J. J. (2009). Polymorphic microsatellite markers in the Melon fruit fly, *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae). *Molecular Ecology Resources, 9*, 1404-1406.

Wu, Y., Li, Y., Ruiz-Arce, R., McPheron, B., Wu, J., & Li, Z. (2011). Microsatellite markers reveal population structure and low gene flow among collections of *Bactrocera cucurbitae* (Diptera: Tephritidae) in Asia. *Journal of Economic Entomology*, *104*, 1065-1074.

Wyatt, T. D. (2003). *Pheromones and Animal Behaviour: Communication by Smell and Taste*. Cambridge University Press.

Xu, J., De Barro, P. J., & Liu, S. S. (2010). Reproductive incompatibility among genetic groups of *Bemisia tabaci* supports the proposition that the whitefly is a cryptic species complex. *Bulletin of entomological research*, *100*(03), 359-366.

Xu, D., Li, S., Lou, B., Zhang, Y., Zhan, W., & Shi, H. (2012). Genetic diversity in two Japanese flounder populations from China seas inferred using microsatellite markers and COI sequences. *Chinese Journal of Oceanology and Limnology, 30*, 604-610.

Yeates, D. K., Seago, A., Nelson, L., Cameron, S. L., Joseph, L. E. O., & Trueman, J. W. (2011). Integrative taxonomy, or iterative taxonomy?. *Systematic Entomology*, *36*(2), 209-217.

Yin, Y., Wang, B., & Yuan, M. (2007). Effect of Different Environmental Factors on the Growths and Developments of *Bactrocera dorsalis* (Hendel)[J]. *Jiangxi Plant Protection*, *4*, 009.

Zelditch, M. L., Swiderski, D. L., & Sheets, H. D. (2012). *Geometric Morphometrics for Biologists: A Primer*: Academic Press.

Zera, A. J., & Denno, R. F. (1997). Physiology and ecology of dispersal polymorphism in insects. *Annual Review of Entomology*, *42*(1), 207-230.

Zhang, B., Edwards, O. R., Kang, L., & Fuller, S. J. (2012). Russian wheat aphids (*Diuraphis noxia*) in China: native range expansion or recent introduction?. *Molecular Ecology*, *21*(9), 2130-2144.

Zhang, D. X., & Hewitt, G. M. (2003). Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Molecular Ecology, 12*, 563-584.

Zheng, X., Zeng, D., Sun, A., Luo, Y., Wang, Q., & Wang, F. (2008). Network-based analysis of Beijing SARS data. In *Biosurveillance and Biosecurity* (pp. 64-73). Springer Berlin Heidelberg.

Zhou, A., & Thomson, E. (2009). The development of biofuels in Asia. *Applied Energy, 86*, S11-S20.

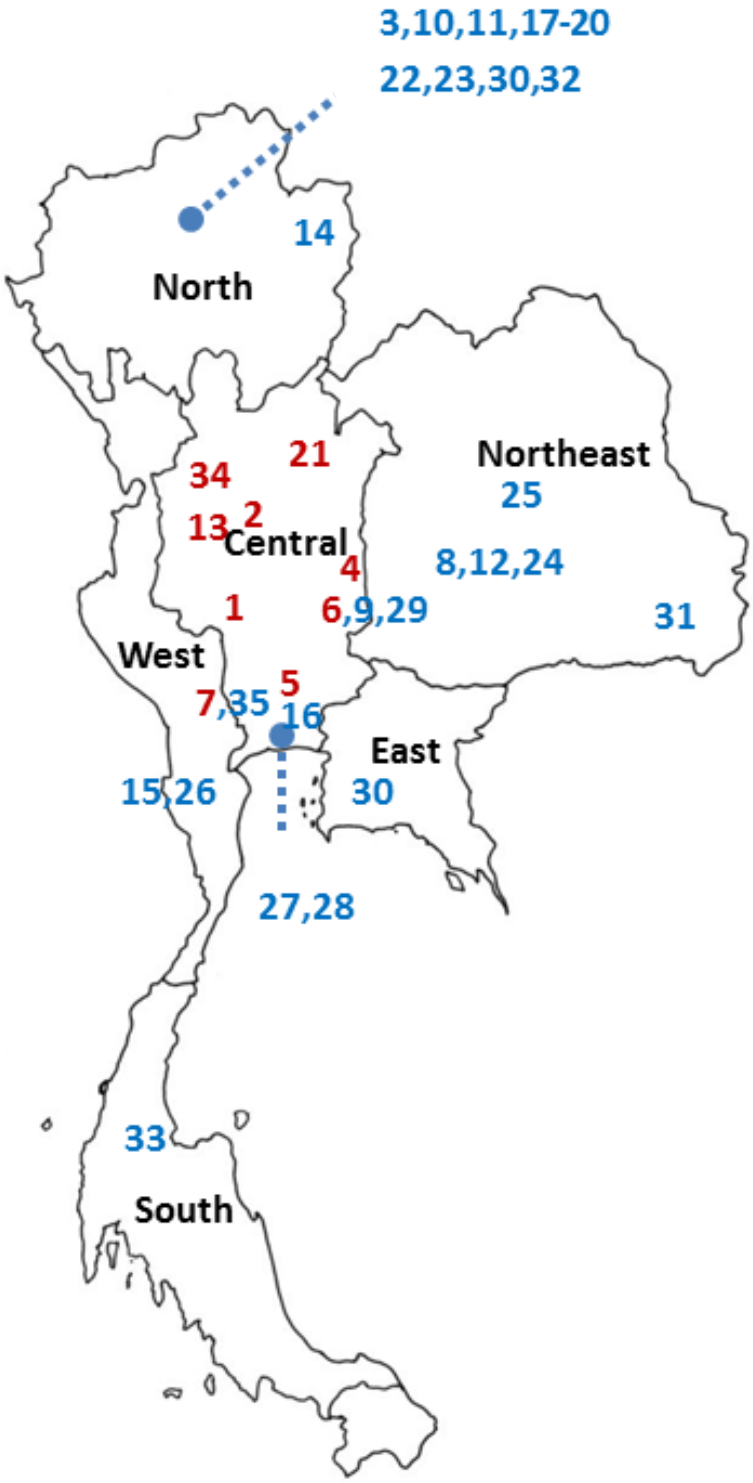
**Appendix 1** Fruit collection data showing hosts sampled, date of collection, collector, locations and latitude and longitude.

| No | Plant Family | Scientific name | Host plants | Date of collection | Collector | Locations | Regions | Latitude | Longitude |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Cucurbitaceae | *Luffa acutangula** | Angled gourd | 9 -May-2013 | Sunadda Chaowarit | Suphanburi* | Central | 14.419 | 100.062 |
| 2 | Cucurbitaceae | *Momordiaca charantia** | Bitter melon | 4-June- 2013 | Chamaiporn Buamas | Nakhonsawan* | Central | 15.698 | 101.114 |
| 3 | Cucurbitaceae | *Momordiaca charantia* | Bitter melon | 12-June-2013 | Sunadda Chaowarit | Chiangmai | North | 18.515 | 98.455 |
| 4 | Cucurbitaceae | *Citrullus lanatus** | Water melon | 9-Aug-2013 | Sunadda Chaowarit | Lopburi* | Central | 14.958 | 100.694 |
| 5 | Cucurbitaceae | *Cucumis melo** | Melon | 30-Sep-2013 | Sunadda Chaowarit | Ayutthaya* | Central | 14.329 | 100.639 |
| 6 | Cucurbitaceae | *Luffa* sp. * | Silk squash | 7-July-2013 | Sunadda Chaowarit | Saraburi* | Central | 14.563 | 100.952 |
| 7 | Cucurbitaceae | *Coccinia grandis** | Ivy gourd | 14-May-2013 | Suppara Akkasarakul | Nakhonpathum* | Central | 13.952 | 99.992 |
| 8 | Cucurbitaceae | *Coccinia grandis* | Ivy gourd | 4 –June-2013 | Sunadda Chaowarit | Nakhonaschasima | Northeast | 13.342 | 99.403 |
| 9 | Cucurbitaceae | *Coccinia grandis* | Ivy gourd | 17-Dec-2012 | Chamaiporn Buamas | Saraburi | Central | 14.408 | 101.032 |
| 10 | Cucurbitaceae | *Coccinia grandis* | Ivy gourd | 12-June-2013 | Samram Sukkul | Chiangmai | North | 18.491 | 98.552 |
| 11 | Cucurbitaceae | *Coccinia grandis* | Ivy gourd | 12-June-2013 | Samram Sukkul | Chiangmai | North | 18.542 | 98.433 |
| 12 | Cucurbitaceae | *Cucumis sativus* | cucumber | 24-June-2013 | Chamaiporn Buamas | Nakhonratchasima | Northeast | 14.508 | 101.173 |
| 13 | Cucurbitaceae | *Cucumis sativus** | Cucumber | 6-June-2013 | Sunadda Chaowarit | Chainat* | Central | 15.191 | 99.983 |
| 14 | Cucurbitaceae | *Cucumis sativus* | Cucumber | 4-June-2013 | Sunadda Chaowarit | Nan | North | 19.053 | 100.551 |
| 15 | Cucurbitaceae | *Cucumis sativus* | Cucumber | 27-June-2013 | Sunadda Chaowarit | Ratchaburi | Central | 14.527 | 101.393 |
| 16 | Cucurbitaceae | *Cucumis sativus* | Cucumber | 11-June-2013 | Sunadda Chaowarit | Pathumthani | Central | 19.234 | 98.807 |
| 17 | Cucurbitaceae | *Cucurbita L. var. cylindica* | Zuchini | 11-June-2013 | Sunadda Chaowarit | Chiangmai | North | 18.318 | 98.324 |
| 18 | Cucurbitaceae | *Cucurbita L. var. cylindica* | Zuchini | 12-June-2013 | Jantiwa Thadeewong | Chiangmai | North | 18.313 | 98.348 |
| 19 | Cucurbitaceae | *Cucurbita L. var. cylindica* | Zuchini | 12-June-2013 | Sunadda Chaowarit | Chiangmai | North | 18.491 | 98.552 |
| 20 | Cucurbitaceae | *Cucurbita L. var. cylindica* | Zuchini | 12-June-2013 | Sunadda Chaowarit | Chiangmai | North | 18.499 | 98.552 |
| 21 | Cucurbitaceae | *Luffa cylindrica** | Sponge gourd | 17-Dec-2012 | Chamaiporn Buamas | Phichit* | Central | 16.302 | 100.212 |
| 22 | Cucurbitaceae | *Momordiaca cochinchinesis* | Spring bitter | 13-June-2013 | Sunadda Chaowarit | Chiangmai | North | 18.343 | 98.524 |
| 23 | Cucurbitaceae | *Cucurbita moschata* | Pumpkin | 12-June-2013 | Sunadda Chaowarit | Chiangmai | North | 18.318 | 98.348 |

**Appendix 1** Continued

| No. | Plant Family | Scientific name | Host plants | Date of collection | Collector | Locations | Regions | Latitude | Longitude |
|---|---|---|---|---|---|---|---|---|---|
| 24 | Cucurbitaceae | *Cucurbita moschata* | Pumpkin | 4-June-2013 | Sunadda Chaowarit | Nakhonraschasima | Northeast | 14.643 | 101.493 |
| 25 | Cucurbitaceae | *Cucurbita moschata* | Pumpkin | 5-June-2013 | Sunadda Chaowarit | Khon Kaen | Northeast | 16.396 | 102.537 |
| 26 | Cucurbitaceae | *Sechium edule* | Chayote | 28-Dec-2012 | Sunadda Chaowarit | Ratchaburi | West | 13.289 | 99.160 |
| 27 | Fabaceae | *Phaseolus vulgaris* * | Bean | 28-Dec-2012 | Sunadda Chaowarit | Bangkok* | Central | 13.819 | 100.570 |
| 28 | Fabaceae | *Vigna unguiculata** | Yard long bean | 22-April-2013 | Samram Sukkul | Bangkok* | Central | 3.005 | 101.421 |
| 29 | Anacardiaceae | *Magifera indica* | Mango | 26-March-2013 | Sunadda Choawarit | Saraburi | Central | 14.323 | 101.044 |
| 30 | Averhoaceae | Averrhoa carambola | Star fruit | 14-June-2013 | Sunadda Choawarit | Chantaburi | East | 12.906 | 102.054 |
| 31 | Myrtaceae | *Syzygium jambos* | Rose apple | 20-July-2012 | Sunadda Choawarit | Ubonratchatani | Northeast | 15.140 | 105.012 |
| 32 | Lauraceae | *Persea americana* | Avocado | 13-June-2013 | Sunadda Choawarit | Chiangmai | North | 18.491 | 98.552 |
| 33 | Rutaceae | *Citrus sinensis* | Orange | 1-June-2013 | Sunadda Choawarit | Chumphon | South | 10.156 | 99.007 |
| 34 | Annonaceae | *Anona reticulate* | Custard apple | 29-July-2012 | Jantiwa Thadeewong | Kamphaenphet | Central | 16.460 | 99.411 |
| 35 | Malvaceae | *Abelmoschus esculentus* | Okra | 19-Sep-2013 | Sunadda Choawarit | Nakhonpathom | Central | 13.721 | 100.249 |

*specimens for Chapter3




**Appendix 2** Locations of sample sites in the Central Thailand regions at which *Zeugodacus cucurbitae* were collected. Specific collection data is presented in Table 3.1. Red numbers denote locations used for analyses.

**Appendix 3** Fruit collection data showing hosts sampled, date of collection, collector, locations and latitude and longitude.

| No. | Plant Family | Scientific name | Host plants | Weight of fruits (kg) | Number of fruits | No of Pupae | Emergence (%) | *Z. cucurbitae* | *Z. tau* | *B. dorsalis* |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Cucurbitaceae | *Luffa acutangula* variety 1 | Angled gourd | 2.000 | 25 | 217 | 87.56 | 190* | - | - |
| 2 | Cucurbitaceae | *Momordiaca charantia* | Bitter melon | 1.890 | 200 | 749 | 94.93 | 711* | - | - |
| 3 | Cucurbitaceae | *Momordiaca charantia* | Bitter melon | 2.960 | 300 | 170 | 58.82 | - | - | 100 |
| 4 | Cucurbitaceae | *Citrullus lanatus* | Chinese water melon | 7.662 | 15 | 176 | 85.79 | 151* | - | - |
| 5 | Cucurbitaceae | *Cucumis melo* | Melon | 3.500 | 18 | 192 | 76.56 | 147* | - | - |
| 6 | Cucurbitaceae | *Luffa* sp. | Silk squash (Sponge) | 2.000 | 21 | 58 | 89.55 | 52* | - | - |
| 7 | Cucurbitaceae | *Coccinia grandis* | Ivy gourd | 1.104 | 80 | 30 | 90.00 | 270* | - | - |
| 8 | Cucurbitaceae | *Coccinia grandis* | Ivy gourd | 0.252 | 20 | 9 | 88.89 | 8 | - | - |
| 9 | Cucurbitaceae | *Coccinia grandis* | Ivy gourd | 0.150 | 36 | 21 | 85.71 | 18 | - | - |
| 10 | Cucurbitaceae | *Coccinia grandis* | Ivy gourd | 0.024 | 2 | 5 | 100.00 | - | - | 5 |
| 11 | Cucurbitaceae | *Coccinia grandis* | Ivy gourd | 0.220 | 10 | 13 | 100.00 | 13 | - | - |
| 12 | Cucurbitaceae | *Cucumis sativus* | Cucumber | 1.100 | 30 | 17 | 88.23 | 15 | - | - |
| 13 | Cucurbitaceae | *Cucumis sativus* | Cucumber | 0.870 | 26 | 89 | 96.63 | 73* | 13 | - |
| 14 | Cucurbitaceae | *Cucumis sativus* | Cucumber | 0.695 | 17 | 10 | 60.00 | - | 6 | - |
| 15 | Cucurbitaceae | *Cucumis sativus* | Cucumber | 3.360 | 35 | 71 | 85.91 | 45 | 16 | - |
| 16 | Cucurbitaceae | *Cucumis sativus* | Cucumber | 1.490 | 20 | 145 | 96.55 | 140 | - | - |
| 17 | Cucurbitaceae | *Cucurbita L. var. cylindica* | Zucchini | 0.987 | 30 | 139 | 93.52 | - | - | 130 |
| 18 | Cucurbitaceae | *Cucurbita L. var. cylindica* | Zucchini | 0.340 | 20 | 152 | 86.18 | - | - | 131 |
| 19 | Cucurbitaceae | *Cucurbita L. var. cylindica* | Zucchini | 0.809 | 3 | 55 | 70.90 | | - | 39 |
| 20 | Cucurbitaceae | *Cucurbita L. var. cylindica* | Zucchini | 1.184 | 12 | 46 | 82.61 | - | - | 38 |
| 21 | Cucurbitaceae | *Luffa cylindrica* | Sponge gourd | 0.712 | 11 | 39 | 79.49 | 32* | - | - |
| 22 | Cucurbitaceae | *Momordiaca cochinchinesis* | Spring bitter cucumber | 0.379 | 3 | 2 | 50.00 | 0 | - | - |
| 23 | Cucurbitaceae | *Cucurbita moschata* | Pumpkin | 0.618 | 1 | 30 | 90.90 | - | 30 | - |

**Appendix 3** Continued

| No. | Plant Family | Scientific name | Host plants | Weight of fruits (kg) | Number of fruits | No of Pupae | Emergence (%) | *Z. cucurbitae* | *Z. tau* | *B. dorsalis* |
|---|---|---|---|---|---|---|---|---|---|---|
| 24 | Cucurbitaceae | *Cucurbita moschata* | Pumpkin | 8.638 | 20 | 20 | 100.00 | - | 20 | - |
| 25 | Cucurbitaceae | *Cucurbita moschata* | Pumpkin | 1.789 | 4 | 60 | 91.67 | - | 55 | - |
| 26 | Cucurbitaceae | *Sechium edule* | Chayote | 1.287 | 10 | 42 | 66.67 | - | - | 28 |
| 27 | Fabaceae | *Phaseolus vulgaris* * | Bean | 2.000 | - | 44 | 47.73 | 21* | - | - |
| 28 | Fabaceae | *Vigna unguiculata* | Yard long bean | 5.000 | 200 | 120 | 37.5 | 45* | - | - |
| 29 | Anacardiaceae | *Magifera indica* | Mango | 10.000 | 50 | 732 | 93.58 | - | - | 685 |
| 30 | Averhoaceae | Averrhoa carambola | Starfruit | 5.200 | 67 | 46 | 67.39 | - | - | 31 |
| 31 | Myrtaceae | *Syzygium jambos* | Rose apple | 10.000 | 89 | 420 | 89.52 | - | - | 376 |
| 32 | Lauraceae | *Persea americana* | Avocado | 4.988 | 36 | 31 | 41.62 | - | - | 13 |
| 33 | Rutaceae | *Citrus sinensis* | Orange | 5.200 | 43 | 173 | 87.28 | - | - | 151 |
| 34 | Annonaceae | *Anona reticulate* | Custard apple | 10.000 | 97 | 163 | 61.96 | - | - | 101 |
| 35 | Malvaceae | *Abelmoschus esculentus* | Okra | 2.950 | - | 32 | 53.125 | - | - | 17 |

*specimens for Chapter3

**Appendix 4** GenBank accession numbers of *Zeugodacus cucurbitae* sequences used in the analyses

| Number | Accession numbers |
|---|---|
| 1 | GQ154128 |
| 2 | GQ154129 |
| 3 | GQ154130 |
| 4 | GQ154131 |
| 5 | GQ154132 |
| 6 | GQ154133 |
| 7 | GQ154134 |
| 8 | GQ154135 |
| 9 | GQ1541106 |
| 10 | GQ1541107 |
| 11 | GQ1541108 |
| 12 | GQ1541109 |
| 13 | GQ1541110 |
| 14 | JQ692821 |
| 15 | JQ692803 |
| 16 | JQ692783 |
| 17 | JQ692751 |
| 18 | JQ692734 |
| 19 | JQ692696 |
| 20 | JQ692685 |
| 21 | JQ692642 |
| 22 | JQ692772 |
| 23 | jQ692685 |
| 24 | JQ692740 |
| 25 | JQ692751 |
| 26 | JQ692740 |
| 27 | JQ692696 |
| 28 | GQ154120 |
| 29 | GQ154119 |
| 30 | GQ154118 |
| 31 | GQ154114 |
| 32 | GQ154115 |
| 33 | GQ154116 |
| 34 | GQ154117 |
| 35 | GQ154100 |
| 36 | GQ154106 |
| 37 | GQ154107 |
| 38 | GQ154107 |
| 39 | GQ154108 |
| 40 | GQ154109 |

**Appendix 4** Continued

| Number | Accession numbers |
|---|---|
| 40 | JX266418 |
| 41 | JX266419 |
| 42 | JX266420 |
| 43 | JX266421 |
| 44 | KF660031 |
| 45 | KF660032 |
| 46 | KF660033 |
| 47 | KF660034 |
| 48 | KF660035 |
| 49 | KF660036 |
| 50 | KF660037 |
| 51 | KF660038 |
| 52 | KF660039 |
| 53 | KF660040 |
| 54 | KF660041 |
| 55 | KF660042 |
| 56 | KF660043 |
| 57 | KF660044 |
| 58 | KF660045 |
| 59 | KF660046 |
| 60 | KF660047 |
| 61 | KF660048 |
| 62 | KF660049 |
| 63 | KF660050 |
| 64 | KF660051 |
| 65 | KF660052 |
| 66 | KF660053 |
| 67 | KF660054 |
| 68 | KF660055 |
| 69 | KF660056 |
| 70 | KF660057 |
| 71 | KF660058 |

# Supplementary Attachment

Supplementary Attachment 1. Abstracts of poster and oral presentation relevant to this research project that were presented in scientific conferences.

The Ninth international International symposium Symposium on fruit Fruit flies Flies of economic Economic importance Importance (9th ISFFEI)

12-16 May 2014

Bangkok, Thailand

**Poster: Natural Variation and Biogeography of *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae) in South-East Asia and the West-Pacific**

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*Background*: The melon fly, *Bactrocera cucurbitae*, infests the fruit of over 125, predominantly cucurbit, plant species. Its geographic distribution includes a native range of the Indian Subcontinent and South-East Asia, and an invasive range which extends into Africa, the West Pacific and parts of Oceania. This paper examines regional variation of *B.cucurbitae* from South-East Asia to the West-Pacific, to better understand the biogeography of this species particularly with respect to populations from native versus invasive ranges.

*Methods*: Morphological variation of *B. cucurbitae* was examined. Aedeagus length and wing shape and size (using geometric morphometric analysis of 15 landmarks) were investigated at two geographic scales: i) across Thailand (as a native area) and ii) throughout South-East Asia and the West-Pacific (invasive range).

*Results*: Wing centroid size significantly differed among Thai locations ($P < 0.001$), but with only a weak latitudinal trend ($r2=0.063$). Wing shape was similar across most locations, but there were wing shape differences for flies from Nan (far north) and Prachapkririkhan (peninsular Thailand). Males from northern locations had longer aedeagi than those from the south; however, while significant, this

relationship was very weak (r2 = 0.013, P < 0.001). The broad-scale geographic study revealed the wings of flies from Yala (Thailand) to be significantly smaller than wings from all other locations (P < 0.001). Aedeagus lengths of flies from South-East Asia are shorter than those from the West-Pacific (P < 0.001). CVA revealed significant differences in wing shape among locations: flies from among South-East Asian mainland locations tended to have the most similar wing shape; with the greatest differences found between these locations and geographically more distant locations such as the Solomon Islands.

*Conclusions*: Patterns of morphometric variation of *B. cucurbitae* revealed in this study have demonstrated fine scale variation within Thailand and broader scale differences across South-East Asia and the West-Pacific; patterns which are particularly distinct between the native range and invasive range. These data will be combined with a population genetic study, applied within an integrative taxonomic framework, to address specific biogeographic hypotheses and better understand invasion patterns in this pest species.

Keywords: Melon fly, morphometric structure, shape analysis, native and invasive

**Supplementary material 1.** Continued.

# Natural Variation and Biogeography of *Bactrocera cucurbitae* (Coquillett) in South-East Asia and the West-Pacific

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## Introduction

The melon fly, *Bactrocera cucurbitae*, infests the fruit of over 125, predominantly cucurbit, plant species.[1] Its geographic distribution includes a native range of the Indian Subcontinent and South-East Asia and a recent invasive expansion into Africa, Oceania, and the West Pacific, where it was first recorded from Hawaii in 1895, Guam in 1936, and the Solomon Islands in 1984.[2]

This study examines regional morphometric variation of *B. cucurbitae* from South-East Asia to the West-Pacific at two geographic scales. The broad-scale study compares morphometric variation between its native and invasive ranges under the expectation of reduced variability in the West Pacific; and a fine scale study across Thai-Malay peninsula.

These results will be integrated with genetic analyses (*mt*DNA and microsatellite data) to demonstrate the effectiveness of morphometric analyses, particularly wings shape, to further address alternate biogeographic hypotheses and better understand invasion patterns in this pest species.

## Materials & Method

Morphometric variation of *B. cucurbitae* was examined via aedeagus length and geometric morphometric analysis of wing shape & centroid size (Figs 1 & 2). Aedeagi and wings were dissected from wild material trapped in cue-lure traps.

Analyses were conducted at two geographic scales:

i) Broad scale: throughout South-East Asia and the West-Pacific; 12 sample sites, 20 individuals per site (Fig. 3)
ii) Fine scale: Thailand; 17 sample sites, 20 individuals per site (Fig. 4).

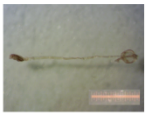

**Figure 1** Dissected aedeagus of *B. curcubitae*

**Figure 2** Right wing of *B. curcubitae* individual showing 15 landmarks.

**Figure 3** Geographic location of sample sites between Southeast Asia and West Pacific were collected.

**Figure 4** Geographic location of sample sites along Thai/Malay peninsular were collected.

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| | |
|---|---|
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## Results and Discussion

### Southeast Asia – West Pacific

While aedeagus length was significantly different among locations ($F_{11,222}$ = 1.890; P < 0.05), there was considerable overlap in size between South-East Asian and West-Pacific locations (Fig. 5). However, West Pacific aedeagi were on average longer than those from South-East Asian males (2.76 ± 0.12 and 2.66 ± 0.132).

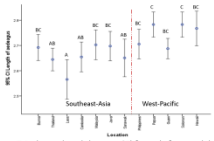


**Figure 5** Aedeagus length (mean ± SE) for male from each locations.

Wing size similarly varied significantly among locations ($F_{11,222}$ = 5.253; P < 0.05); however, as for aedeagus length there was significant difference between South-East Asian and West Pacific populations. Notably, there was reduced variability in wing size in the West Pacific flies relative to those from South-East Asia (6.31 ± 0.44; 6.12 ± 0.44, respectively; Fig. 6).

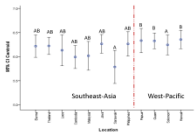


**Figure 6** Centroid size(mean ± SE) for male from each locations.

Wings from all South-East Asian populations were significantly different from each other ($F_{7, 146}$ = 1.632; P < 0.05). While significant difference were found between almost all locations, the greatest difference were between Southeast-Asia and West-Pacific location. No evidence for isolation by distance was found from South-East Asia to the West Pacific ($r^2$ = 0.01, P > 0.05), with populations from Hawaii, Guam, Papua, and the Solomon Islands emerging equally from South-East Asian populations (Fig. 7).

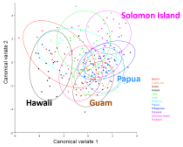


**Figure 7** Confidence ellipses (95%) of groups for canonical variates 1 and 2 calculated from wing shape data for *B. cucurbitae* from 13 sites across Southeast-Asia and West-Pacific.

### Thai- Malay Peninsula

Aedeagus length differenced among locations ($F_{16, 323}$ = 2.493; P < 0.05; Fig. 8); however there was no significant latitudinal trend or clear difference between populations north or south of the Isthmus of Kra (Fig. 9).

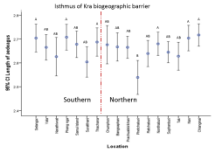


**Figure 8** Centroid size (mean ± SE) for male from each locations.

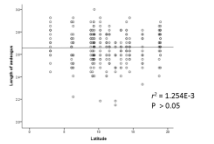


**Figure 9** Regression of aedeagus lengths (mm) from each locations against a latitude gradient from northern Thailand to Peninsular Malaysia.

While there was no significant isolation by distance effect in wing shape across Thailand/Malaysia ($r^2$ = 0.001, P> 0.05). Average Mahalanobis distance among Sotheast-Asia was 3.63 ± 0.48 and average Mahalanobis distance among Sotheast-Asia was 3.90 ± 0.73); the most extreme locations (northern Nan and southern Selangor) were strongly separated along the first canonical variates (Mahalanobis distance; P < 0.001 (Fig. 12).

As for aedeagi, populations sampled for the fine scale study were significantly different for wing size ($F_{16, 323}$= 3.437; P < 0.05). In this case, however, flies from north of the Isthmus of Kra had, on average, larger wings which were also less variable than those from the south (6.26 ± 0.42 and 6.05 ± 0.67, respectively, Fig. 10); this resulted in a significant latitudinal trend (Fig. 11).

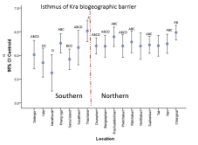


**Figure 10** Centroid size (mean ± SE) for male from each locations.

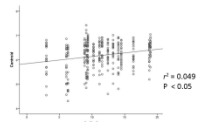


**Figure 11** Regression of aedeagus lengths (mm) from each locations against a latitude gradient from northern Thailand to Peninsular Malaysia.

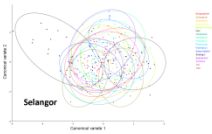


**Figure 12** Confidence ellipses (95%) of groups for canonical variates 1 and 2 calculated from wing shape data for *B. cucurbitae* from 13 sites across Southeast-Asia and West-Pacific.

Broad scale morphometric analysis of *B. cucurbitae* reveals signatures of multiple and recent introductions into the West Pacific: i) wing size is less variable in the invasive range; and ii) there is no IBD signal in wing shape but rather a pattern reflective of independent invasion events. Fine scale morphometric analyses show evidence of differences north and south of the Isthmus of Kra biogeographic barrier, particularly in wing size and shape for which there is also a weak but significant IBD trend. This may be the consequence of historical vicariance events, *e.g.*, elevated sea levels flooding the Isthmus of Kra.

**References**

Anonymous. (2002) Melon fly, *Bactrocera cucurbitae*. Retrieved March 17, 2014. http://www.spc.int/Pacifly/Species_profiles/B_cucurbitae.htm. Pacific fruit fly.

Piñero, J.C., **Le corre,** L., **Vargas, R. & Prokopy, R.J.** (2006) Response of female melon fly, *Bactrocera cucurbitae*, to host-associated visual and olfactory stimuli. *Entomologia Experimentalis et Applicata* **121**, 261-269.

**Supplementary material 1.** Continued.

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**Oral Presentation: Signatures of invasion: an integrated approach to revealing the spread of Melon fly, Bactrocera cucurbitae, (Diptera: Tephritidae) across SE Asia and the West-Pacific**

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Multidisciplinary approaches greatly enhance our understanding of invasion pathways, particularly via tools that resolve population structure over geographic and temporal scales. This study uses such tools to investigate population structure in the Melon fly, *Bactrocera cucurbitae*, a widely distributed horticultural pest with a native range including the Indian Subcontinent and Asia. Melon fly has subsequently invaded Africa, the West Pacific and parts of Oceania. While its population structure is partially documented, this is poorly understood in SE Asia and the West Pacific.

We have used molecular (mtDNA-*cox1*) and morphological data (geometric morphometric wing shape and size; aedeagus morphometrics) to resolve invasive signatures of Melon fly from 13 SE Asian and West Pacific locations. This information is interpreted in relation to historical records of its invasion across the region. We report distinct differences in genetics diversity in SE Asia relative to the West-Pacific. Furthermore, wing size, shape and aedeagal length differ between mainland SE Asia and to the West-Pacific.

We discuss these data under a hypothesis of multiple and recent introductions from SE Asia into the West Pacific. Greatest diversity in Laos suggests this as the origin of Melon fly with dispersal into and across the region. Increased diversity in Hawaii supports the notion of multiple introductions to some Islands. These results reflect the value of integrating multiple data sets to develop understanding of dispersal pathways in a serious, invasive pest.