ELECTROPHORETIC STUDY OF THE BACTROCERA TAU COMPLEX (DIPTERA : TEPHRITIDAE) IN CERTAIN POPULATIONS IN THAILAND

ANCHALEE SAELEE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(ENVIRONMENTAL BIOLOGY)
FACULTY OF GRADUATE STUDIES
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The *Bactrocera* (*Zeugodacus*) *tau*-like flies are some of the most important agricultural pests of south-east Asian cucurbit crops and form a complex of species. There has always been taxonomic confusion regarding the separation of species within this species complex. This is because it is difficult to determine if differences between *B. tau* flies are due to genetic variation within species or genetic differentiation between species. Morphological observations coupled with cytological evidence has revealed at least seven genetic species within this taxon, temporarily designated as species A (=*B. tau*), C, D, E, F, G and I. In this thesis electrophoretic evidence is used to verify the existence of these seven genetic species within the *B. tau* complex. Genetic markers for species separation and phylogenetic relationships of these species are also described.

The genetic variability of and differences among 43 collected samples of seven species of the *B. tau* complex were evaluated electrophoretically. Nine enzyme systems, which were composed of twelve loci, were used. Twenty-eight populations of *B. tau* were characterized by low genetic variability as indicated by low values of average heterozygosity ($\overline{H}_o$) and mean genetic distance ($\overline{D}$). No geographical pattern relationships were displayed because the level of genetic differentiation was similar between populations in the same geographical region and between populations originating from different geographical regions. In the same manner, populations from the same host plant species had similar levels of genetic differentiation as those from different host species. Single diagnostic alleles with specific relative mobilities, which can be used as genetic markers for species classification within the *B. tau* complex, were discovered only in species D. Although, no single allele was diagnostic for the other six species, the use of more than two alleles permitted the correct classification of nearly all individuals of even the two most closely related species. A phylogenetic tree was estimated by using a UPGMA clustering of Nei's unbiased genetic distance. The tree indicates that three main lineages exist in the *B. tau* complex. The first group consists of a complex of extremely similar species (*B. tau*, species E, F and G). The second group consists of species C and I and the last group consists of species D.

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อัญชลี แซ่หลี : การศึกษากลุ่มประชากรแมลงวันผลไม้ *Bactrocera tau* complex (Diptera: Tephritidae) ในประเทศไทย โดยใช้เทคนิคอิเล็กโทรฟอริซีส (ELECTROPHORETIC STUDY OF THE *BACTROCERA TAU* COMPLEX (DIPTERA: TEPHRITIDAE) IN CERTAIN POPULATIONS IN THAILAND). คณะกรรมการควบคุมวิทยานิพนธ์ : วิสุทธิ์ ใบไม้, Ph.D., John Milne, Ph.D., สังวรณ์ กิจทวี, Ph. D., ปรีชา ประเทพา, Ph.D. 124 หน้า. ISBN 974-663-006-7

แมลงวันผลไม้กลุ่ม *Bactrocera* (*Zeugodacus*) *tau* เป็นแมลงศัตรูพืชที่สำคัญของพืชตระกูลแตง (cucurbit) ในประเทศไทย นอกจากนี้ยังก่อให้เกิดความสับสนแก่นักอนุกรมวิธาน เนื่องจากแมลงวันผลไม้กลุ่มนี้มีความแตกต่างแปรผันทางรูปร่างลักษณะภายนอก ซึ่งยากต่อการตัดสินใจว่าเป็นความแปรผันทางสันฐานภายในสปีชีส์เดียวกัน หรือเป็นความแตกต่างระหว่างสปีชีส์ การศึกษาทางสันฐานวิทยาและเซลล์พันธุศาสตร์ได้ศึกษาและจำแนกแมลงวันผลไม้กลุ่มนี้ ออกเป็น 7 สปีชีส์ คือ สปีชีส์ A (=*B. tau*), C, D, E, F, G และ I

งานวิจัยนี้ได้ศึกษาความแปรผันทางพันธุกรรมของแมลงวันผลไม้กลุ่ม *B. tau* complex โดยใช้เทคนิคโพลิอะคริลาไมด์เจล อิเล็กโทรฟอริซีสในแนวราบ ของเอนไซม์ 9 ระบบ ซึ่งประกอบด้วย 12 โลไซ เพื่อค้นหาเครื่องหมายทางพันธุกรรม (genetic markers) ที่สามารถใช้ในการจำแนกสปีชีส์ของแมลงวันผลไม้กลุ่มนี้ นอกจากนี้ยังศึกษาความสัมพันธ์ทางพันธุกรรมเชิงวิวัฒนาการระหว่างสปีชีส์อีกด้วย จากผลการวิเคราะห์ความแปรผันทางพันธุกรรมระหว่างประชากรของแมลงวันผลไม้ *B. tau* ทั้ง 28 ประชากร ในประเทศไทย พบว่า ค่าเฉลี่ยเฮเทโรไซโกซิตี ($\overline{H}_o$) และค่าเฉลี่ยความแตกต่างทางพันธุกรรม ($\overline{D}$) มีค่าต่ำมาก และไม่มีความสัมพันธ์กับชนิดของพืชอาศัยและภูมิศาสตร์ นอกจากนี้พบว่ามีเอนไซม์บางโลไซ ซึ่งมีระยะทางการเคลื่อนที่บนเจล เปรียบเทียบกับมาตรฐาน เป็นแบบเฉพาะ สามารถใช้ในการจำแนกสปีชีส์ *B. tau* complex ได้ สำหรับบางสปีชีส์ซึ่งมีการแสดงอัลลีลร่วมกันอยู่ในบางโลไซ สามารถใช้โลไซมากกว่า 2 โลไซขึ้นไปร่วมกัน เพื่อจำแนกสปีชีส์ได้ในระดับความเชื่อมั่นสูง จากการวิเคราะห์ค่าความสัมพันธ์เชิงวิวัฒนาการของ *B. tau* complex โดยใช้เทคนิค UPGMA cluster analysis พบว่าสามารถแบ่งกลุ่มแมลงวันผลไม้ตามความสัมพันธ์เชิงวิวัฒนาการ ออกเป็น 3 กลุ่มหลัก คือ กลุ่มที่หนึ่ง ประกอบด้วย กลุ่มแมลงวันผลไม้ที่มีความสัมพันธ์ใกล้เคียงกันมาก (*B. tau*, สปีชีส์ E, F และ G) กลุ่มที่สอง ประกอบด้วย สปีชีส์ C และ I กลุ่มสุดท้ายคือสปีชีส์ D

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LIST OF ABBREVIATIONS

Bis acrylamide	= N,N' methylene bis acrylamide
EDTA	= ethylene diamiamine tetra acetic acid
NAD^+	= nicotinamide adenine dinucleotide
$NADP^+$	= nicotinamide adenine dinucleotide phosphate
NBT	= nitro blue tetrazolium dye
PMS	= phenazine methosulfate
Tris	= tris (hydroxy methyl) aminomethane
TEMED	= N,N,N',N'-tetramethylethylene diamine
U	= international enzyme units (1 unit refers to the ability of the enzyme to convert one micromole of substrate to product at 25°C and optimum pH)

CHAPTER I

INTRODUCTION

The true fruit flies (Tephritidae: Diptera) are one of the most economically important agricultural pests throughout the world (1). The female of many species oviposits her eggs via a long extensible ovipositor into fleshy tissues of fruits and/or flower heads (1). The larvae hatch from the eggs and feed on the fruits or flowers, thereby resulting in the production of low quality fruits (2). Taxonomically, there is considerable confusion due to lumping of species with overlapping external morphological characteristics into the one species taxon in early taxonomic studies. Later studies of these taxa often revealed sibling species complexes. Some tephritid fruit fly species in Thailand are suspected to be complexes of sibling species (3). Detailed investigation of the ecology, biology and control of these pest species is hampered by the inability to distinguish species.

Sibling species are increasingly being detected among populations by genetic variation studies. The methods commonly used to measure genetic variation, and hence differentiation between populations, include polytene chromosomes, laboratory crossing experiments, enzyme electrophoresis, mitrochondrial and ribosomal DNA restriction fragment length polymorphisms (mtDNA RFLP and rDNA RFLP), and random amplified DNA polymorphisms generated by random amplified polymorphic DNA (RAPD-PCR) in taxonomic studies (4).

Among the various approaches to the study of the genetic structure of living organisms, enzyme electrophoresis is the most useful procedure yet devised for revealing genetic variation. This technique came into widespread use in the late 1960s (5). Extensive enzyme variation has been found in virtually all natural populations studied by electrophoresis, including bacteria, plants, insects, and vertebrates (6). One advantage of allozymes over that of DNA methods (e.g. PCR) is that they are codominant characters, so those heterozygous individuals can be distinguished from homozygotes. Other advantages are that they are relatively inexpensive and easy to assay (5).

Enzymes are of particular value in basic genetic studies, because they are the product of genes, and because most of them are under the control of nuclear genes, which undergo a straight forward Mendelian pattern of inheritance. Hence, enzymes can provide basic information on genetic variation and differentiation among populations.

Enzyme electrophoresis has been utilized for extensive genetic investigations of host-associated variation, and the evolutionary and geographic origins of sympatric host races of the apple maggot fly, *Rhagoletis pomonella*, in the United States and Canada (7, 8, 9, 10). Studies of genetic differentiation and phylogenetic relationships among *Rhagoletis* sibling species in North America have also been investigated using enzyme electrophoresis (11, 12, 13). This technique was also used to distinguish *Bactrocera opiliae* from other sibling species in the *B. dorsalis* complex from northern Australia (14). In Malaysia, Yong (15, 16, 17) utilized electrophoresis of different enzyme systems to study variation among *B. dorsalis* populations. Ooi (18),

another Malaysian scientist, tried to differentiate species in two sympatric *B. dorsalis* taxa, based on different mobilities of several enzyme systems.

Although several tephritid fruit fly investigations have been conducted in many regions thoughout the world, the genetic structure of populations of the major fruit fly pest species of cucurbit crops, *B. tau*, has not been reported before. In 1997, Drew and Romig (19) suggested that the *B. tau* taxon represented a large group of sibling species, which were major pests of cucurbit crops in southeast Asia. Recently, Baimai *et al* (2) provided cytogenetical evidence for the existence of nine species within the *B. tau* taxon in Thailand. However, no evidence of genetic variation among species of this complex in the area has been reported. Therefore, the research reported here seeks to determine if electrophoretic evidence supports the division, by Baimai *et al* (2) and S. Tigvattananont (unpublished), of the *B. tau* taxon into these species.

CHAPTER II

OBJECTIVES

The objectives of this research were:

(1) to determine levels of genetic variation in natural populations of *Bactrocera tau*,

(2) to determine if there are different species among *B. tau* natural populations,

(3) to establish genetic markers from enzyme electrophoresis of natural populations of the *B. tau* complex to be used as biochemical characters for species identification, and

(4) to determine phylogenetic relationships among different populations of the *B. tau* complex.

CHAPTER III

LITERATURE REVIEW

3.1 Fruit Fly Distribution

The family Tephritidae includes about 4,000 species arranged in 500 genera (20). It is the largest family of Diptera and one of the most economically important (1). Tephritid fruit flies occupy habitats in extremes of climates from cold temperate latitudes to tropical equatorial regions (1). Further, they attack many parts of plants e.g. stems, growing tips, leaves, flowers and bamboo shoots (20). About 35% of all species attack soft fruits including many commercial fruits. They often cause serious damage to all kinds of fleshy tropical and subtropical fruit including some vegetables. Besides attacking soft fruit, the larvae of about 40% of species are associated with the flowers of Asteraceae. The remaining 25% of species develop in the flowers of families other than Asteraceae, or other kinds of plant tissue e.g. leaf, stem or root tissue (1).

The taxonomy of tephritid fruit flies in south-east Asia has been revised extensively in recent years (3, 21). Consequently, many species names have been changed. Throughout this thesis, I will use the species names given by Drew (21) and Drew and Hancock (3).

The distribution of the genus *Bactrocera* is described by White and Elson-Harris (1) and is summarized in the following. This genus consists of 440 species,

which attack a wide range of fruits in the tropical and warm temperate regions of the old world. About 23 subgenera are found in tropical Asia, the South Pacific and Australia, with only five subgenera occurring in Africa and only *B. oleae* in Southern Europe. Forty-five plant families contain the recorded hosts of the genus, *Bactrocera.* Most of the pest species attack fruits belonging to several unrelated families of plants, their larvae developing in the fruit flesh. Exceptions are a few pest species of the subgenus *Zeugodacus* that are primarily associated with the fruits and flowers of a single family, Cucurbitaceae. Temperate species with a narrow host range, such as *Rhagoletis* spp., are usually univoltine, i.e., they only have one generation per year. However, tropical pest species of *Bactrocera* are typically multivotine, i.e. they have several generations per year.

The subgenus *Zeugodacus* includes about 70 species that have been found in tropical Asia, Australia, Indonesia and Papua New Guinea. In contrast to most other members of the genus *Bactrocera*, most species of the *B. (Zeugodacus)* subgenera have a strong preference for attacking Cucurbitaceae, often attacking the flowers rather than the fruit (1).

3.2 Fruit Fly Biology and Ecology

The flies of the family Tephritidae are moderately large. Individual flies vary in body length from 1 to over 20 mm. The wings of most species are patterned with yellow, brown or black stripes or spots or a combination of both (20). One of the characteristic features of this family is the long extensible ovipositor of the females. The female fruit fly uses this structure to deposit eggs within the host fruit, or host

flower (1). If the flower is being used, then the female usually oviposits into the ovaries (20).

The typical developmental cycle commences when adult females insert their eggs beneath the skin of suitable hosts, especially in ripening or ripe fruits and vegetables. Normally, there are three larval instars, although some flower associated species complete the first instar before emerging from the egg (22). Most fruit feeders drop to the ground after reaching the third instar and move into the soil where they form a puparium, but most flower feeding tephritids pupariate within the host tissue. The larvae of many fruit feeders can jump along the ground to find suitable pupariation sites (20). Under normal conditions, pupariation sites for most species are rarely deeper than 1 to 2 inches. Emerging adults tend to crawl upward through the soil. Newly emerged adults require a few days to at least a week to reach sexual maturity. After mating, the new developmental cycle is initiated again (20).

A variety of food sources are utilized by adult fruit flies including glandular secretions of plants, nectar, and plant sap exuding from trunk, stem, leaf or fruit injuries caused by insect feeding, disease or mechanical damage. Rotting fruits, bird dung, decaying insect bodies and honeydew secreted by homopterans are also food sources. Water is required for survival of all species and, in nature, fruit flies obtain water from liquid foods, dew or rain drops (20).

The adults feed within the host tree on certain bacteria which supply nutrients to permit egg maturation. The fruit flies alight on the host tree when fruit are at the right stage for oviposition and are primarily immature females and sexually mature males (23). The flies introduce bacteria onto fruit surfaces, e.g., *Pseudomonas*

savastanoi, a bacterial pathogen of olive trees that has a symbiotic relationship with *Bactrocera oleae* (23). Within 7-14 days most fruits have a heavy growth of "fruit fly type" bacteria. The nutrient supply for such bacterial colonization is provided by "leachates", which are chemicals that occur within a plant and which exude onto the plant surface. Leaching is prominent on soft fleshy fruits when they begin to ripen and heavy leaching occurs under conditions of wetting during light rain, dew and cloud mist (1).

3.3 The Economic Fruit Fly Problem and Its Management

Within southeast Asia and the Pacific region, fruit flies are regarded as the major insect pests of fruit and vegetable crops. Their economic importance can be summarized as: (*i*) they attack commercially produced fruit, (*ii*) some species are spreading and may become pest problems in these new areas, (*iii*) quarantine regulations of importing countries have to be imposed to limit further spread of fruit fly pests and can either deny a producing country a potential export market, or force the producer to carry out expensive disinfestation treatment (1).

Fruit flies cause four types of economic losses. First, ovipositional punctures may cause various types of surface defects of the fruits including discoloration or formation of abnormal growth around the puncture. Second, ovipositional punctures may provide entrance points for decay organisms, resulting in fermentation and decomposition of the fruit. Third, the entire fruit can be lost to maggots feeding on and tunneling in its fleshy tissue. Fourth, the indirect loss comes from an obstruction of expansion and development of various fruit crops for export because of restrictions placed on fruit imports by importing countries (1).

In the developing countries of southeast Asia and the Pacific region, loss of fruit and vegetable crops is a major concern, as it causes serious reductions in the availability of essential fruit-based nutrients. However, the major economic losses due to fruit flies are those related to loss of export trade.

In the past, post-harvest disinfestation of fruit for export relied largely on fumigation. However, the only effective chemical fumigant, ethylene dibromide, has been banned because of its carcinogenic effects. Other techniques such as heat treatment, either with hot vapor or hot water, cold treatments, insecticidal dipping and irradiation are either unacceptable to consumers or ineffective. Without effective and acceptable post-harvest disinfestation, prevention of initial fruit infestations becomes extremely important and seems to be the best strategy (1). Preventing crops from being attacked in fruit fly infested areas where labor costs are low may be achieved by wrapping large fruit individually in paper or cloth before they reach a suitable stage for fruit fly attack (1). This is impractical in areas where labor costs are high, so, eradication and suppression programs have been developed.

Eradication and suppression programs involve the integration, at various levels, of techniques that differ in efficiency depending on the fly population density and the physical features of the area. Eradication is the complete destruction of populations with an area. Eradication can not be used in large areas, e.g., Thailand, because it would be too expensive. In large areas, suppression is practiced. Bateman (24) listed three main procedures, namely (*i*) bait spraying, (*ii*) male annihilation and (*iii*) sterile insect release. Bait sprays work on the principle that both male and female tephritids are strongly attracted to a protein source. They can be applied as a spot

treatment so that the flies are attracted to the insecticide and there is minimal impact on natural enemies. Bait sprays may be used for either eradication or suppression, the distinction being that eradication refers to procedures designed to protect a single farm or island while suppression refers to procedures covering a large area.

Male annihilation utilizes the attraction of males of many species to chemical lures (methyl eugenol and cue lure for *Bactrocera* spp.) and was used to eradicate *B. dorsalis* from the Northern Ryukyu Island, Japan. This technique has also been used against *B. cucurbitae* and *Ceratitis capitata* in Hawaii, where it did have some impact on population size (1). However, Bateman (24) noted that male lures could only be used for eradication when traps (or fiber board impregnated with bait plus insecticide) were set at very high density over the entire range of the target population.

Sterile insect release has been used to eradicate some populations of fruit flies. The sterile insect technique (SIT) requires the release of millions of sterile flies into the wild population so that there is a strong likelihood of wild females mating with sterile males. SIT was used to eradicate *B. dorsalis* from the Ogasawara Islands and *B. cucurbitae* from Kume Island in Japan. SIT has been used against *C. capitata* in California, Costa Rica, Hawaii, Italy, Mexico, Nicaragua, Peru, Spain and Tunisia. This technique has also been tried against *Anastrepha ludens*, but no major control program has been carried out. SIT depends on the ability to mass rear millions of sterile flies. Combinations of the above three techniques may also be used. A combination of bait spraying and male annihilation was used to eradicate two successive outbreaks of

B. tryoni in Easter Island. Similarly, it is current policy to use a combination of bait spraying and SIT against *C. capitata* outbreaks in the USA (1).

3.4 The Sibling Species Concept

Sibling, isomorphic or cryptic species, as they are called, are species that are so similar in their morphology that species separation is unreliable or can not be done at all by their external morphology (25). Sibling species, although morphologically indistinguishable, are complete species. They are reproductively isolated from one another, and are thus true biological species (4).

The recognition of cryptic species in a species complex is important. For example, Muller (26) has found prezygotic and postzygotic isolation mechanisms among plant host races of aphid species, suggesting that some biotypes (genetic and niche-specific different types) may in actuality be sibling species (27).

The prudent approach to identifying unknown cryptic taxa is to designate a particular population to serve as a reference taxon. This population should be characterized in terms of its genetic makeup (chromosome types, allozyme frequencies and DNA variability patterns) and ecological, biological and possibly physiological attributes. Comparison of populations with the reference taxon using this type of information then provides the basis to identify and characterize variant population structures and new taxonomic entities (4).

3.5 The Study of Species Complexes in Diptera

Modern approaches to species problems include cytogenetic, ecological, behavioral and biochemical methods, but only biochemical methods permit large scale screening of natural populations for genetic attributes which have simple Mendelian

properties. These genetic characteristics provide markers for closely related species, even those which are morphologically indistinguishable.

The electrophoresis diagnostic method has been successfully employed to distinguish among species of the *Drosophila pseudoobscura* group (28, 29, 30). Electrophoresis of 28 enzyme loci was used to study the phylogeny of *D. subobscura* in Yugoslavia (31). Ayala and Powell (25) used allozyme allelic frequencies as diagnostic characters for distinguishing six sibling species of *Drosophila* from Northern, Central and South America. Genetic variation in natural populations of five species in the *D. willistoni* group was studied using allozyme electrophoresis by Ayala *et al* (32).

In North America, the apple maggot *Rhagoletis pomonella* was studied extensively because it caused serious damage to numerous cultivated crops such as apple, cherry, plum and pear, and was suspected of being a species complex (33). It was difficult to distinguish each of the sympatric sibling species in the *pomonella* group, i.e., *R. pomonella*, *R. mandax* and *R. cornivora* when only morphological characters applied (34).

Berlocher (11) presented the electromorph patterns of enzymes superoxide dismutase, malate dehydrogenase, aldolase, alcohol dehydrogenase-1 and fumarase as an electrophoretic key which permits individual larvae, pupae and adults of the *R. pomonella* complex to be identified to species. Berlocher and Bush (12) determined phylogenetic relationships of 22 *Rhagoletis* species based on 15 enzyme loci. Berlocher *et al* (13) revised the phylogeny of the seven taxa of the *R. pomonella* species complex from North America based on data for genetic differentiation at 29

enzyme loci, an example of the application of gel electrophoresis to populations and possible species complexes.

With the application of modern techniques such as gel electrophoresis, pheromone analysis, the study of acoustic signals and cytogenetics, it has been possible to recognize species limits among the many varied populations of tephritid fruit flies that have been observed in tropical Asia and Australia (21). For the *Bactrocera dorsalis* complex, Drew and Hardy (14) recognized *B. opiliae* as a new sibling species from Northern Australia. This species was described based on electrophoretic characters.

One of the studies on sibling species in the southeast Asia and the Pacific region is from Drew and Lambert (35) in which two closely related allopatric species in the *tryoni* complex were distinguished on the basis of morphology, cytology, enzyme electrophoresis, mating and sterility test and chemical analysis of male pheromones.

Yong (36) quantified genetic variability of *B. umbrosa* from four localities in Peninsular Malaysia using the proportion of polymorphic loci and heterozygosity based on 13 gene-enzyme systems comprising 18 loci. Furthermore, *B. dorsalis* also was analyzed via horizontal starch-gel electrophoresis for isocitrate dehydrogenase, phosphogluconate dehydrogenase, dimeric esterase and malate dehydrogenase (15, 16, 17).

In Thailand, there have been few studies made of tephritid fruit flies. A lack of information on the accurate identification is prominent and this will certainly affect other detailed investigations such as geographical distribution, genetic variation

and sexual behavior, etc. and especially control measures. Little work has been done on the genetics of natural populations of fruit flies in Thailand despite the strong indication of species complexes being present in the taxa of economical importance.

Drew and Hancock (3) have found fourteen species of the *B. dorsalis* complex in Thailand based mainly on morphological characters, including scanning electron micrography of the aculeus. In addition, Satayalai (37) reported electrophoretic evidence of some new genetic species of the *B. dorsalis* complex and their genetic variations in natural populations in Thailand.

3.6 Electrophoresis

Electrophoresis is the process of moving charged molecules in solution by applying an electrical field across the solution. Because molecules in an electrical field move with a speed dependent on their charge, shape, and size, electrophoresis has been extensively developed for molecular separations. As an analytical tool, electrophoresis is simple and relatively rapid. It is used chiefly for analysis of very large molecules such as proteins and nucleic acids, but can be applied to simple charged molecules, including charged sugars, amino acids, peptides, nucleotides and simple ions. Highly sensitive detection methods have been developed to monitor and analyze electrophoretic separations.

Electrophoresis of macromolecules is normally carried out by applying a thin layer of a sample to a porous matrix. Under the influence of an applied voltage, different molecules in the sample move through the matrix at different velocities. At the end of the separation, the different species are detected as bands at different positions in the matrix. A matrix is required because the electric current passing

through the electrophoresis solution generates heat, which cause diffusion and connective mixing of the bands in the absence of a stabilizing medium. The matrix can be composed of a number of different materials, including paper, cellulose acetate, or gels made of polyacrylamide, agarose or starch. In acrylamide gels, the matrix also acts as a size-selective sieve in the separation. At the end of the run the separated molecules can be detected in position in the gel by staining or autoradiography, quantitated by scanning with a densitometer, and the gel dried for permanent storage.

Proteins are amphoteric (or zwitterionic) compounds and are therefore either positively or negatively charged because they contain both acidic and basic residues. Most of the charge of a protein comes from the pH-dependent ionization of side-chain carboxyl and amino groups of amino acids. For each protein species, there is a pH at which the molecule has no net charge. At this pH, called the isoelectric point or pI, the weak acids and bases are titrated to the point at which there is an equal number of positive and negative charges on the molecule. Each protein has a unique pI. In a solution with a pH above the pI, a protein has a net negative charge and migrates toward the positive electrode (anode) in an electric field. When in a solution below a protein's pI, the protein is positive and migrates toward the negative electrode (cathode) (38).

For electrophoretic protein separations based on the mobility of different species, the pH of the solution must be kept constant to maintain the charge and, hence, the mobility of proteins. Therefore, because electrolysis of water generates H^+ at the anode and OH^- at the cathode, the solutions used in electrophoresis must be buffered.

If an enzyme in an individual has an amino acid substitution that leads to a difference in the enzyme's overall ionic charge, then this enzyme will have an altered electrophoretic mobility, that means it will move through the gel at a different rate.

3.7 The *Bactrocera tau* Complex

Bactrocera tau was previously known under several names such as *Chaetodacus tau* (Walker), *Dacus caudacus* var. *nubilus* Hendel, *Dacus hageni* De Meijere, *D. nubilus* Hendel, and *D. tau* (Walker), *Dasyneura tau* Walker, *Zeugodacus bezzianus* Hering and *Z. nubilus* (Hendel) (1). *Bactrocera nubilus* from Taiwan was separated from *B. tau* by Hardy (39). Morphologically, *B. nubilus* is similar to *B. tau*, but can be distinguished by the basal segment of the ovipositor and the apex of the aculeus (39).

Bactrocera tau is the most common species of *Zeugodacus* found in southeast Asia. It is widespread throughout the Oriental region and infests a wide range of hosts (39). It has been recorded from 34 hosts in southeast Asia (19), including several genera of cucurbits and an assortment of fleshy fruits such as jack fruit, star fruit, guava, mango, chico (Sapodilla) and wax apple (39).

Drew and Romig (19) suggested that there was a large complex of sibling species of major pests of cucurbit crops in southeast Asia called the *tau*-complex. Recent cytogenetic evidence suggests that there are at least nine species in this complex in Thailand (2). These species have been designated as species A to I by Baimai *et al* (2), with species A being *B. tau*. This species complex requires intensive systematic and ecological studies in order to define the pest species in commercial or

edible fruit crops and to understand their phylogenetic relationships and evolutionary processes.

CHAPTER IV

MATERIALS AND METHODS

4.1 Sample Collection

Larval samples belonging to the *Bactrocera tau* complex (species A, C, D, E, F, G and I) were obtained from a wide variety of infested fruits. Species B and H were omitted in this report because sample sizes were too small for reliable electrophoretic study. The infested fruits were brought back to Mahidol University and assigned collection codes according to locality, date of collection and host-plant species. Larvae from each collection were either used in chromosome studies or reared to adult for electrophoresis studies and for voucher specimens (Fig. 1). For mitotic chromosome study, some third instar larvae were randomly sampled from each collection. Fruits containing larvae were placed in plastic boxes with some sawdust to allow pupation of the remaining larvae. Newly emerged adults were fed with water and sugar mixed with yeast hydrolyzate for at least two weeks to ensure the production of good electromorphs. Live adults from each collection were temporarily immobilized by freezing (2 to 5 min) and then sorted, by morphology, into two groups, members of the *B. tau* complex and other flies using the keys of White and Elson-Harris (1) and Hardy (39). Flies of the *B. tau* complex were further sorted into species A to I based on morphological characters (S. Tigvattananont, unpublished descriptions). The adults were either utilized immediately for electrophoresis or kept in liquid nitrogen (-196°C)

until required for electrophoresis (Fig. 1). Some voucher specimens were kept for formal taxonomic studies.

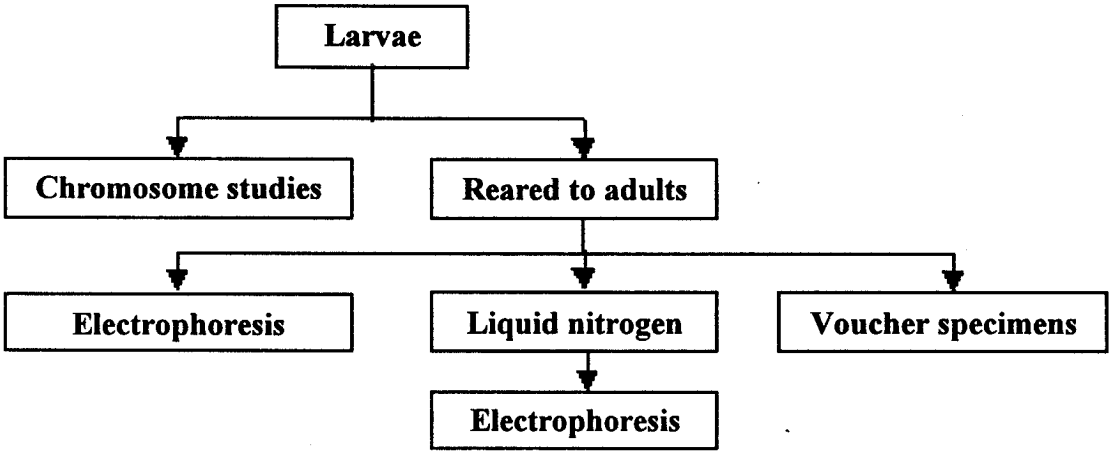

Figure 1 Fate of larvae in this study of the *Bactrocera tau* complex derived from infested fruit.

4.2 Electrophoresis

4.2.1 Apparatus

The horizontal polyacrylamide gel electrophoresis apparatus described by Steiner and Joslyn (40) was used with some modifications. It consisted of two electrode chambers containing a buffered electrolyte solution (section 4.2.3). Each electrode chamber contained a platinum electrode, which was attached to a plug set in one end of the chamber. The pair of chambers held one gel. About 250 ml of electrolyte solution was placed in each chamber for each electrophoresis run.

The gel was connected to each electrode buffer by wicks made of paper towels. The system was cooled (to about 4 ^{o}C) by a refrigerated circulating water bath (LKB 2209 Multitemp, Frigomix 1495 B. Braun and LKB Multitemp thermostatic circulator).

Electrophoresis runs were conducted using the power supply (Bio-Rad model 3000 Xi, Pharmacia EPS 600 and E-C Apparatus Corporation EC 575).

4.2.2 Gel Mold

The mold was prepared using glass plates, 20 cm long and 19 cm wide. A glass strip (19 cm long, 1 cm wide and 1.5 mm thick) was glued on each 19 cm side of a glass plate to provide a gel thickness of about 1.5 mm (Fig. 2a). The cassette for gel polymerization was prepared by arranging four of the molds into a stack and covering them with a glass plate of the same size (Fig. 2a). The cassette was then inserted into a plastic bag (Fig. 2b). The cassette in the plastic bag was sandwiched between two glass plates and held together with masking tape. The top side of the plastic bag was left open for pouring the gel solution (Fig. 2c).

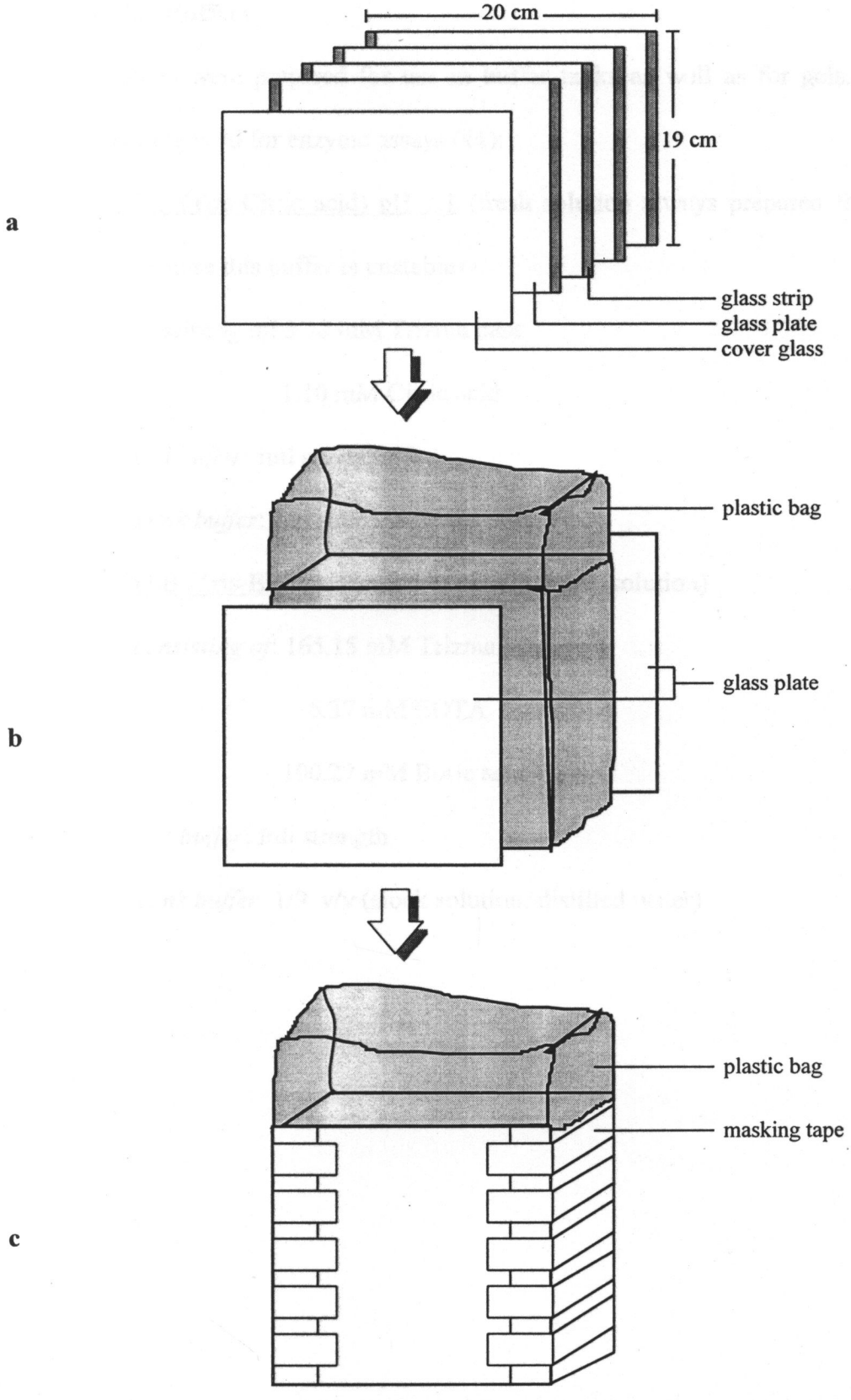

Figure 2 Diagram of mold used for preparing four 1.5 mm thick polyacrylamide gels.

4.2.3 Buffers

Buffers were prepared for use in buffer tanks as well as for gels. Two buffer systems were used for enzyme assays (41):

I. TC (Tris-Citric acid) pH 7.1 (fresh solution always prepared for use because this buffer is unstable)

consisting of: 3.38 mM Trizma base

1.10 mM Citric acid

Gel buffer: full strength

Tank buffer: full strength

II. TEB (Tris-EDTA-Boric acid) pH 8.5 (stock solution)

consisting of: 165.15 mM Trizma base

5.37 mM EDTA

100.27 mM Boric acid

Gel buffer: full strength

Tank buffer: 1/9 v/v (stock solution: distilled water)

4.2.4 Gel Preparation

Two types of acrylamide gels were prepared, one gel for use with each tank buffer system: (*i*) the TC gel was prepared with a 5.5% w/v gel matrix and the TC buffer system and (*ii*) the TEB gel with a 6.0% w/v gel matrix and the TEB buffer system. Both kinds of gel have 3% w/w of bis acrylamide to acrylamide. The components of each gel type are as follows:

I. TC gel

TC buffer	280.00 ml
Acrylamide	14.94 g
Bis acrylamide	0.46 g
Sucrose	7.00 g
Kodak photo flo 600	1.40 ml
TEMED	0.21 ml
Ammonium persulfate	0.21 g

II. TEB gel

TEB buffer (stock solution)	7.00 ml
Distilled water	273.00 ml
Acrylamide	16.30 g
Bis acrylamide	0.50 g
Sucrose	7.00 g
Kodak photo flo 600	1.40 ml
TEMED	0.21 ml
Ammonium persulfate	0.21 g

Each gel was prepared by dissolving the required amounts of acrylamide, bis acrylamide and sucrose in buffer. Photo flo, TEMED and ammonium persulfate were then rapidly added and mixed thoroughly. The gel solution was poured into the prepared cassette and overlaid with distilled water. The gel was completely polymerized at room temperature in 30 minutes. It was then refrigerated overnight.

4.2.5 Sample Preparation

A drop (approximately 60 µl) of cool deionized water was placed on the ground surface of a cool glass slide. A living or preserved fly was placed in the drop and ground up by using a glass rod. Homogenate from each fly was absorbed onto small rectangular pieces (1.5 × 3 mm) of filter paper (Whatman no. 1).

4.2.6 Electrophoresis Run

When required for electrophoresis, a polymerized gel was removed from the glass cassette and two sets of slots were made, one at 3 cm and the other at 11 cm from one end, using a plastic comb. One gel plate could, therefore, be used for staining two enzyme systems. The gels were covered with plastic sheet, to prevent water evaporation, and placed on cold brass plates (at about 4 ^{o}C).

Each piece of filter paper with its homogenate was inserted into a cut slot of a gel. Forty two samples, including the reference *B. dorsalis* standard flies (from laboratory colonies with known monomorphic electromorphs), plus a front marker, were applied to each gel plate.

The front marker was a mixture of bromophenol blue (for TC gels) and hemoglobin (for TEB gels). The marker consisted of 20 µl of 1% bromophenol blue solution mixed with 20 µl of whole human blood. The mixture was absorbed onto

filter paper and inserted into the first slot of the gel plate in the same way as the fly homogenates.

After sample application, each gel was removed from its glass mold and sandwiched between two plastic sheets. The buffer tanks were then filled and the gel was connected to the electrode buffer by paper towel bridges at both ends of the gel. The gel held between the sheets was then sandwiched between two cool brass plates. The electric cords of the power supply were connected to the electrode tanks. The electrophoresis run was carried out at constant voltage (450 V DC) until bromophenol blue migrated 5.0 cm in a TC gel or hemoglobin migrated 4.5 cm in a TEB gel. The power supply was then switched off and the gels were prepared for histochemical staining.

4.2.7 Preparation for Enzyme Assay

Nine enzyme systems were used for electrophoretic study, namely AAT, ADH, G3PDH, GPI, IDH, MDH, ME, PGD and SOD. Each enzyme was visualized using histochemical stains specific to each enzyme system. Enzyme names, enzyme commission numbers, their abbreviations and the specific buffer systems are shown in Table 1.

Staining methods and recipes were modified from Harris and Hopkinson (42), Berlocher (11), Satayalai (37) and Steiner and Joslyn (40) (Table 1). All chemicals used for enzyme assay were supplied by Sigma Chemical Company. The staining procedure for each enzyme is summarized in Appendix 1. The staining solutions for each enzyme were prepared before hand and kept in a refrigerator at 5 ^{o}C until use.

Table 1 Enzyme names, commission (E.C.) numbers and abbreviations, and the buffer systems used for their analysis. Details of staining systems are given in Appendix 1.

Enzyme name	E.C. number	Abbreviation	Buffer system & pH	Staining reference
1. Aspartate aminotransferase	2.6.1.2	AAT	TEB pH 8.5	Steiner and Joslyn, 1979
2. Alcohol dehydrogenase	1.1.1.1	ADH	TEB pH 8.5	Satayalai, 1995
3. Glyceral-3-phosphate dehydrogenase	1.1.1.8	G3PDH	TC pH 7.1	Satayalai, 1995
4. Glucose phosphate isomerase	1.3.1.9	GPI	TC pH 7.1	Satayalai, 1995
5. Isocitrate dehydrogenase	1.1.1.42	IDH	TC pH 7.1	Satayalai, 1995
6. Malate dehydrogenase	1.1.1.37	MDH	TC pH 7.1	Satayalai, 1995
7. Malic enzyme	1.1.1.4	ME	TC pH 7.1	Satayalai, 1995
8. Phosphogluconate dehydrogenase	1.1.1.44	PGD	TC pH 7.1	Harris and Hopkinson, 1976
9. Superoxide dismutase	1.15.1.1	SOD	TEB pH 8.5	Berlocher, 1980

After electrophoresis was completed, each gel plate was cut along the line where samples were first applied and placed into a 13.5×19.0×2.5 cm plastic box lined with plastic wrap and containing the appropriate staining solution. The gels were incubated at 37 °C for 30 minutes. Then PMS was added and the gels were reincubated. After the stained bands were strong enough to be seen clearly, the stain was poured off and replaced by fixer (25:16:250, acetic acid: ethyl alcohol: water) for at least 15 minutes. Then the fixer was poured off, replaced by tap water and left over night. For a permanent record, the gel was sandwiched between cellophane plates and air dried.

4.3 Nomenclature

Electromorphs were identified using the *B. dorsalis* bands as reference standards on each gel. The most frequent allele in *B. dorsalis* was taken as the "100" reference allele, and the mobility of each allele was calculated in relation to this standard allele (41).

The loci were termed "polymorphic" using the criterion that the frequency of the most abundant allele was less than 0.99 (43). Alleles with frequencies less than 0.005 in each species were regarded as rare and their frequencies were pooled (6). The term "population" in this study means all individuals of each fruit fly species that emerge from the same host species in the particular area.

4.4 Analysis of Electrophoretic Data

After scoring, the frequency of different genotypes found for each locus in populations of each species of the *B. tau* complex was used as input data for the computer program POPGENE Version 1.1 (44). Allele frequencies were computed

from the initial data input. Allele frequencies of 12 enzyme loci in each population of seven species in the *B. tau* complex are presented in Appendix 2.

Genetic variation statistics, namely percentage of polymorphic loci (P), mean number of alleles per locus (A), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F) and the Hardy-Weinberg equilibrium test were calculated for each population. Genetic differentiation between populations was analyzed using Wright's F-statistic; the total genetic variability (F_{IT}) was partitioned into within (F_{IS}) and between (F_{ST}) population variation (45, 46). Gene flow (N_m) was estimated from F_{ST} (47, 48). Genetic distances were used to estimate phylogenetic relationships (49), using *B. cucurbitae* as an outgroup species.

Percent of polymorphic loci (P) for a population is defined as the mean of all loci investigated. The average number of alleles per locus (A) is calculated with an arithmetic mean. The expected heterozygosity (H_e) is obtained by applying Hardy-Weinberg' Law. This number is compared to the observed heterozygosity (H_o) to identify deviations from a population in equilibrium. The difference between the observed and expected heterozygosity can be tested for statistical significance by applying a χ^2 test for goodness of fit. The more the observed values deviate from the expected ones, the more the values of χ^2 increases. If χ^2 equal zero, it means the observed values equal expected values (43). The values of fixation index ranges from +1.000, total absence of heterozygotes in mixtures of two or more heterozygotes, to -1.000, total absence at homozygotes (6).

The F-statistics are defined as "inbreeding coefficients" but they differ according to the reference populations. F_{IS} is concerned with inbreeding in individuals

(I), relative to the subpopulation (S) to which they belong; F_{ST} is concerned with inbreeding in subpopulations (S), relative to the total population (T) of which they are a part; and F_{IT} is concerned with inbreeding individuals (I), relative to the total population (T). F_{IT} is the most inclusive measure of inbreeding in that it takes into account both the effects of nonrandom mating within subpopulations (F_{IS}) and the effects of population subdivision (F_{ST}). F_{IS} and F_{ST} are the estimated inbreeding coefficients due to nonrandom mating within subpopulations and population subdivisions, respectively. Thus, F_{IT} is the amount of inbreeding due to the combined effects of nonrandom mating within subpopulations and to random genetic drift among subpopulations (6).

Values of F_{IS} ranges from +1.000 to –1.000. Values of F_{ST} is always greater than (or equal to) zero. If all subpopulations are in Hardy-Weinberg equilibrium, $F_{ST} = 0$ (6). Qualitatively, a range of 0.05 to 0.15 for the F_{ST} indicates moderate differentiation, while a range of 0.15 to 0.25 indicates great differentiation. An F_{ST} of more than 0.25 is indicative of very great differentiation (4).

The parameter of gene flow is N_m, where N is the effective population size and m is the proportion of migrants exchanged between populations per generation. Gene flow, which is reported as N_m, is the number of migrants per generation. Low mean private allele frequencies in a species indicate a high rate of gene flow, while high mean frequencies of rare alleles indicate low gene flow. The N_m values greater than one is considered high (43).

Nei's (49) unbiased genetic identity (I) and genetic distance (D) were used to quantify the amount of genetic differentiation among populations of each species.

I may range from zero (no alleles in common at any locus) to one (the same alleles in identical frequencies at all loci). *D* may range from zero to infinity (31). A further step to demonstrate the genetic differentiation among populations was performance of cluster analysis. The unweighted pair-group method with arithmetic averaging (UPGMA) was selected to calculation based on Nei's (49) unbiased genetic distance.

CHAPTER V

RESULTS

Seven species of the *Bactrocera tau* complex including 28 populations of *B. tau* and 15 populations of closely related species (species C, D, E, F, G and I) were obtained from 16 provinces throughout Thailand from February, 1996 to January, 1998 (Table 2, Fig. 3).

5.1 Distribution and Host Fruit Preferences

Ten host plant species from four families were found to be infested with larvae of the *B. tau* complex (Table 2). *Bactrocera tau* was a common species infesting fruit of a wide range of host plants, mostly the family Cucurbitaceae, but also one species in the family Dioscoreaceae. Four species of the *B. tau* complex appeared to be host specific, as follows (Table 2):

(*i*) Species C specifically occurred in *Momordica cochinchinensis* (Fak Khao) at Kanchanaburi, Phetchaburi and Ratchaburi provinces. Additionally, in Ratchaburi province [RB(B)4], *B. tau* was found with species C in the same host fruit.

(*ii*) Species E was found only in *Strychnos thorelii* (Salangjai Thoa) in Ranong province.

(*iii*) Species F and (*iv*) G were found in the same species of host plant, *Hydnocarpus anthelminthicus* (Krabao Yai), but in different localities. Species F occurred in Ranong province [RN(M/2)223] while species G was found only in

Table 2 Collection details for samples of seven species of the *Bactrocera tau* complex collected for electrophoretic study in Thailand.

Species	Pop. nos.	Collection code	Host Plant Species	Sample size	Date of collection
A	1	CM(X)2	*Trichosanthes tricuspidata*[1]	20	Dec. 96
(=*B. tau*)	2	CM(X)9	*Trichosanthes tricuspidata*[1]	29	Dec. 96
	3	CM(X)19	*Cucurbita moschata*[1]	60	Dec. 96
	4	CM(W)2	*Trichosanthes cucumerina*[1]	50	Dec. 96
	5	CM(Y)1	*Trichosanthes tricuspidata*[1]	60	Dec. 96
	6	CR(T)1	*Trichosanthes tricuspidata*[1]	40	Dec. 96
	7	NA(D)7	*Trichosanthes tricuspidata*[1]	111	Dec. 96
	8	NA(D)8	*Luffa cylindrica*[1]	20	Dec. 96
	9	NA(D)10	*Trichosanthes cucumerina*[1]	60	Dec. 96
	10	NA(F)3	*Trichosanthes cucumerina*[1]	60	Dec. 96
	11	PY(F)1	*Lagenaria siceraria*[1]	60	Dec. 96
	12	UD(D)1	*Luffa cylindrica*[1]	60	Dec. 96
	13	UD(E)1	*Luffa cylindrica*[1]	69	Dec. 96
	14	NK(B)2	*Luffa cylindrica*[1]	40	Oct. 97
	15	NM(C)1	*Luffa cylindrica*[1]	38	Oct. 97
	16	MH(D)2	*Luffa cylindrica*[1]	40	Oct. 97
	17	MH(E)1	*Trichosanthes tricuspidata*[1]	40	Oct. 97
	18	PH(B)3	*Trichosanthes tricuspidata*[1]	63	Feb. 98
	19	RB(B)4	*Momordica cochinchinensis*[1]	33	Jan. 98
	20	RN(M/2)139	*Trichosanthes cordata*[1]	21	Feb. 96
	21	RN(M/2)503	*Dioscorea membranacea*[2]	21	Feb. 97
	22	RN(A)20	*Trichosanthes tricuspidata*[1]	23	Feb. 97
	23	YL(C)5	*Trichosanthes tricuspidata*[1]	84	Nov. 97
	24	PL(C)1	*Trichosanthes tricuspidata*[1]	38	Nov. 97
	25	PN(B)1	*Trichosanthes cordata*[1]	29	Nov. 97
	26	PN(B)2	*Trichosanthes tricuspidata*[1]	85	Nov. 97
	27	SO(D)2	*Trichosanthes cordata*[1]	50	Nov. 97
	28	SO(D)4	*Trichosanthes cordata*[1]	90	Nov. 97

Table 2 (ctd.) Collection details for samples of seven species of the *Bactrocera tau* complex collected for electrophoretic study in Thailand.

Species	Pop. nos.	Collection code	Host Plant Species	Sample size	Date of collection
C	29	KB(S)50	*Momordica cochinchinensis*[1]	48	Nov. 97
	30	PH(B)1	*Momordica cochinchinensis*[1]	71	Jan. 98
	31	RB(B)4	*Momordica cochinchinensis*[1]	70	Jan. 98
D	32	RN(H)22	*Trichosanthes tricuspidata*[1]	18	Jan. 97
	33	PL(C)1	*Trichosanthes tricuspidata*[1]	34	Nov. 97
	34	YL(C)5	*Trichosanthes tricuspidata*[1]	58	Nov. 97
	35	SO(D)2	*Trichosanthes cordata*[1]	12	Nov. 97
	36	SO(D)4	*Trichosanthes cordata*[1]	15	Nov. 97
E	37	RN(M/2)492	*Strychnos thorelii*[3]	32	Jan. 97
	38	RN(M/2)499	*Strychnos thorelii*[3]	60	Feb. 97
F	39	RN(M/2)223	*Hydnocarpus anthelminthicus*[4]	51	Apr. 96
G	40	KB(S)3	*Hydnocarpus anthelminthicus*[4]	33	Feb. 97
I	41	YL(C)5	*Trichosanthes tricuspidata*[1]	58	Nov. 97
	42	PN(B)2	*Trichosanthes tricuspidata*[1]	60	Nov. 97
	43	SO(D)2	*Trichosanthes cordata*[1]	47	Nov. 97

[1] Family Cucurbitaceae, [2]Family Dioscoreaceae, [3] Family Strychnaceae,

[4] Family Flacourtiaceae

North: Chiang Mai (CM), Chiang Rai (CR), Nan (NA), Phayao (PY), Uttaradit (UD)

Northeast: Nong Khai (NK), Nakhon Phanom (NM), Mukda Han (MH)

Central: Phetchaburi (PH), Ratchaburi (RB)

West: Kanchanaburi (KB)

South: Ranong (RN), Yala (YL), Phatthalung (PL), Pattani (PN), Songkhla (SO)

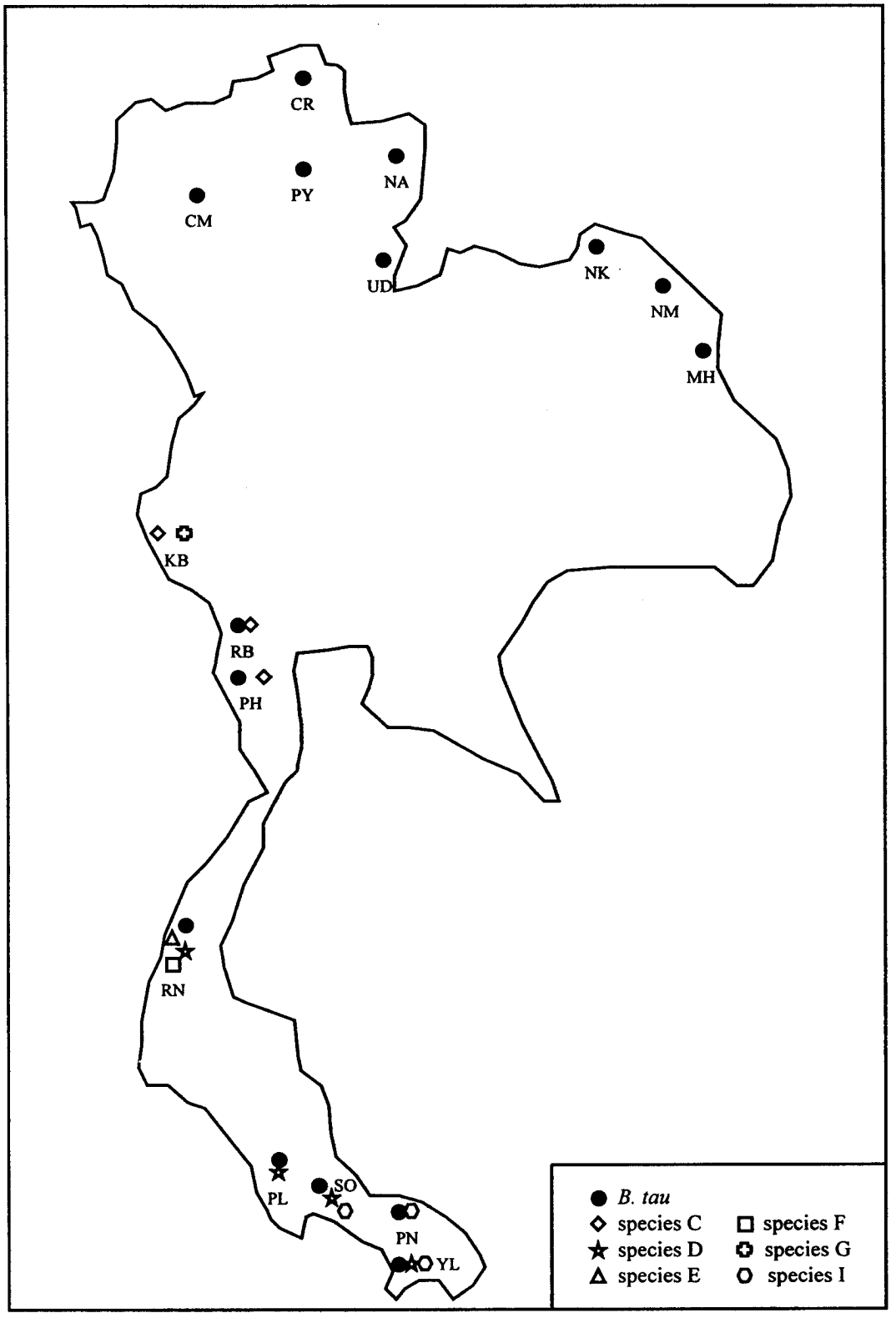

Figure 3 Map of Thailand showing collection localities of seven species of the *Bactrocera tau* complex. See Table 2 for provinces abbreviated on map.

Kanchanaburi province [KB(S)3]. These two species may therefore occur as allopatric populations.

Both species D and I were found in two hosts, *Trichosanthes cordata* (Kheeka Khao) and *T. tricuspidata* (Kheeka Dang), and appear to be not as specific as other species. The shape and color of these hosts' fruit was very similar, both being round and red, and this may account for them being attacked by both fruit fly species. *Bactrocera tau* also infested fruit of both these hosts and was found with species D in the same fruit of *T. tricuspidata* at Phatthalung [PL(C)1] and *T. cordata* at Songkhla [SO(D)4] province (Table 2). Species I only occurred in southern Thailand, i.e., Yala, Pattani and Songkhla provinces. *Bactrocera tau* also occurred sympatrically with species I [PN(B)2]. All three species occurred together in *T. tricuspidata* fruit at Yala [YL(C)5] and *T. cordata* fruit at Songkhla [SO(D)2].

5.2 Description of Banding Patterns

I. Aspartate aminotransferase (AAT)

Aspartate aminotransferase was a special histochemical staining gel, which was represented by dark brown bands on the light red brown background. The *Aat* electromorphs migrated anodally in TEB gel (Fig. 4a). It was found that *Aat* codes for a dimeric enzyme and consists of six alleles (Table 3).

II. Alcohol dehydrogenase (ADH)

Three loci of ADH gave dimeric enzymes consist of *Adh-1* and *Adh-2*, which migrated anodally, and *Adh-3*, which migrated cathodally in most cases, (except for some individuals of species C and D in which it migrated anodally). *Adh-1* migrated more slowly than *Adh-2*. Both *Adh-1* and *Adh-2* comprised four alleles

whereas eight alleles were expressed at the *Adh-3* locus (Table 3). Identification of *Adh-1* and *Adh-2* was unambiguous in all species excepting species C, E and I in which no bands were seen for these two loci (Fig. 4b, 4c).

III. Glyceral-3-phosphate dehydrogenase (G3PDH)

Though this enzyme was not shown to be so variable (Fig. 4d), there were three alleles discovered from a dimeric locus (Table 3).

IV. Glucose phosphate isomerase (GPI)

There were seven alleles in a dimeric locus (Table 3). The *Gpi* alleles proved difficult to distinguish because of close relative mobilities in gels (Fig. 5a).

V. Isocitrate dehydrogenase (IDH)

The IDH system always developed very consistently with clear and scorable bands (Fig. 5b). There were seven alleles observed for *Idh*, which is a dimeric enzyme (Table 3).

VI. Malate dehydrogenase (MDH)

MDH migrated anodally in TC gel and consisted of nine alleles (Table 3). The dimeric locus of *Mdh* also developed very consistently (Fig. 5c).

VII. Malic enzyme (ME)

A dimeric locus of *Me*, in which there were seven alleles was discovered (Table 3). *Me* was the most difficult staining system. Whenever it developed, it was nice and easy to score electromorph, but this locus did not consistently develop in species C (Fig. 6a).

VIII. Phosphogluconate dehydrogenase (PGD)

There were two dimeric loci expressed in phosphogluconate dehydrogenase gel. *Pgd-2* migrated faster than *Pgd-1*. Twelve alleles occurred in *Pgd-1* and five alleles in *Pgd-2* (Table 3). Both loci showed complicated banding patterns, i.e., some heterozygous alleles of *Pgd-1* had in the same mobilities as *Pgd-2* alleles (Fig. 6b).

IX. Superoxide dismutase (SOD)

Superoxide dismutase had a special histochemical staining gel, which showed white banding patterns on dark blue background. A dimeric enzyme, *Sod*, migrated anodally (Fig. 6c). There were eight alleles represented in this locus (Table 3).

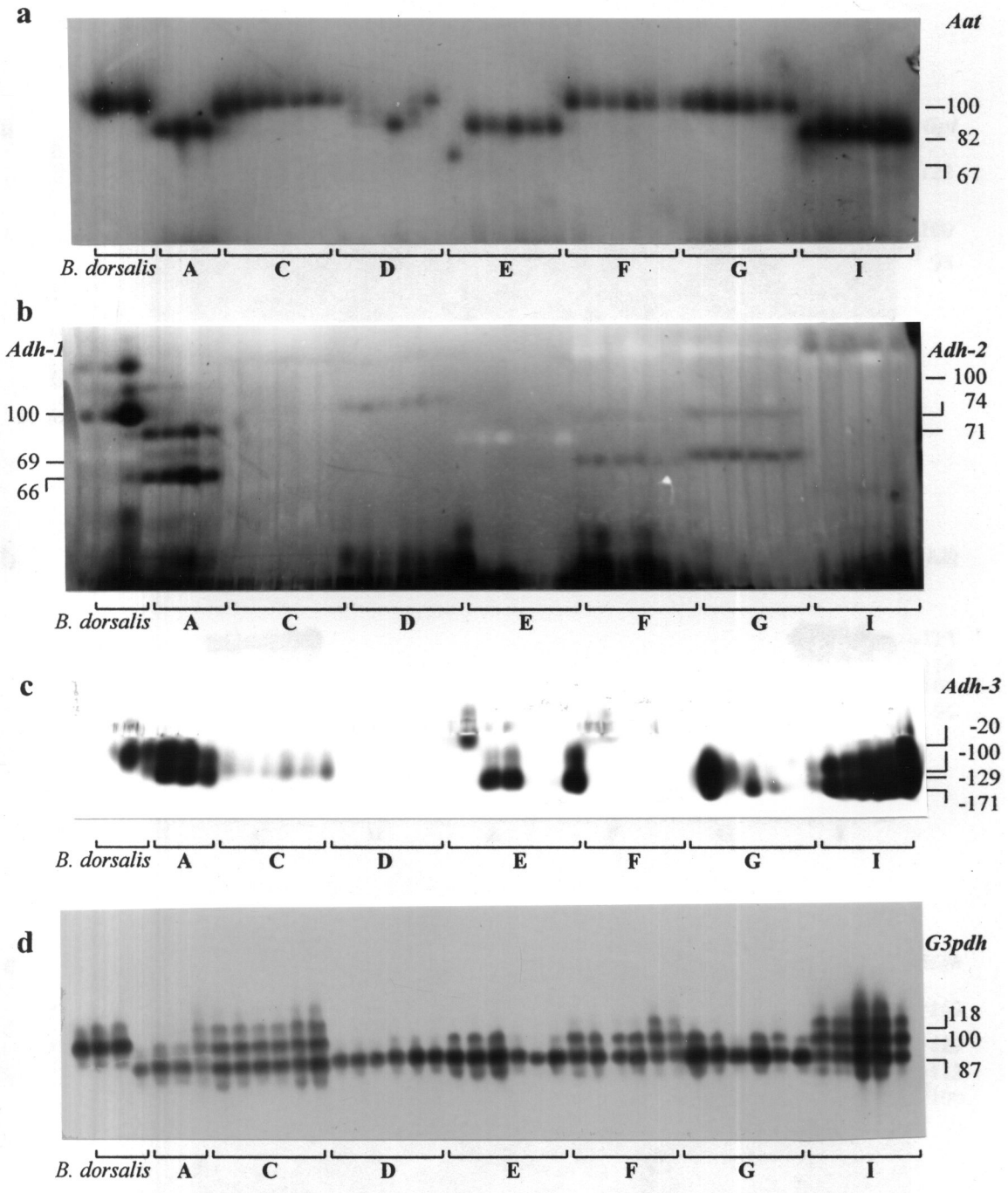

Figure 4 Examples of enzyme electromorphs in seven species of the *Bactrocera tau* complex (a) aspartate aminotransferase (AAT), (b) alcohol dehydrogenase (ADH) illustrating two loci, *Adh-1* and *Adh-2*, (c) alcohol dehydrogenase (ADH) illustrating one locus, *Adh-3* and (d) glyceral-3-phosphate dehydrogenase (G3PDH).

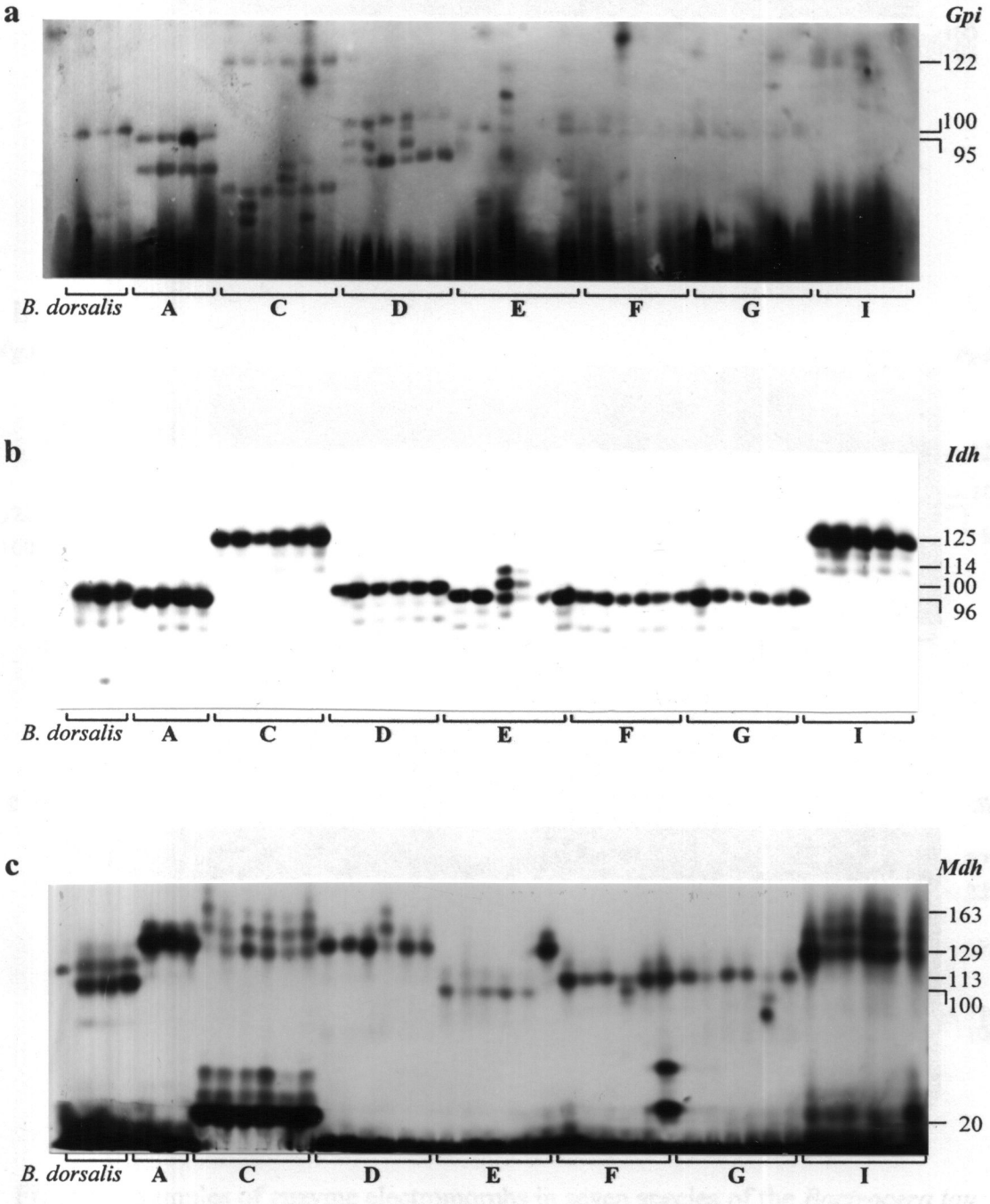

Figure 5 Examples of enzyme electromorphs in seven species of the *Bactrocera tau* complex (a) glucose phosphate isomerase (GPI), (b) isocitrate dehydrogenase (IDH) and (c) malate dehydrogenase (MDH).

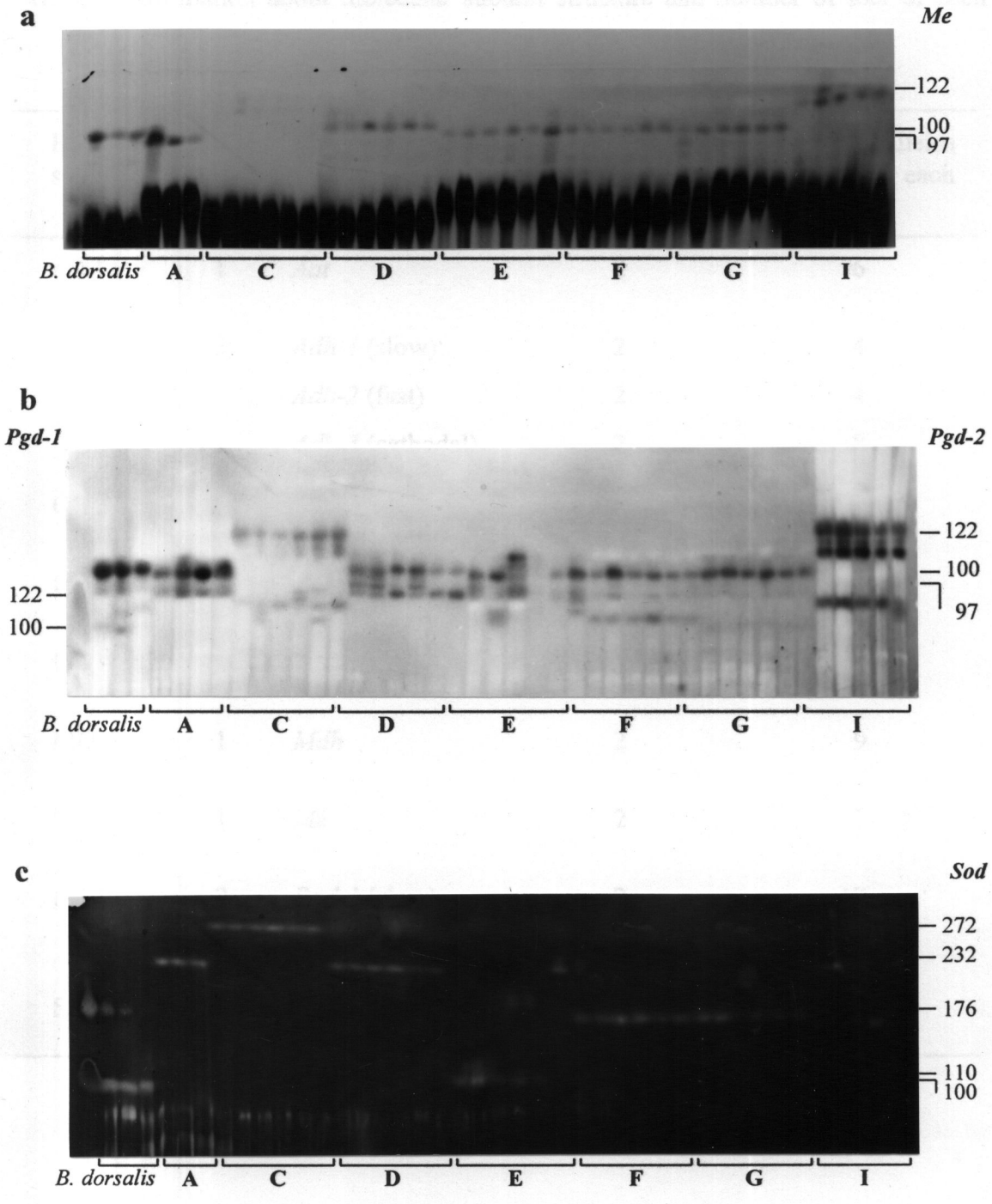

Figure 6 Examples of enzyme electromorphs in seven species of the *Bactrocera tau* complex (a) malic enzyme (ME), (b) phosphogluconate dehydrogenase (PGD) illustrating two loci, *Pgd-1* and *Pgh-2*, and (c) superoxide dismutase (SOD).

Table 3 Information about molecular subunit structure and number of loci of each enzyme system investigated.

Enzyme system	Number of loci	Locus symbol	Number of molecular subunits	Number of alleles observed for each loci
AAT	1	*Aat*	2	6
ADH	3	*Adh-1* (slow)	2	4
		Adh-2 (fast)	2	4
		Adh-3 (cathodal)	2	8
G3PDH	1	*G3pdh*	2	3
GPI	1	*Gpi*	2	7
IDH	1	*Idh*	2	7
MDH	1	*Mdh*	2	9
ME	1	*Me*	2	7
PGD	2	*Pgd-1* (slow)	2	12
		Pgd-2 (fast)	2	5
SOD	1	*Sod*	2	8

5.3 Genetic Differentiation between Species

5.3.1 Genetic Markers

Of the seven investigated species in the *B. tau* complex, only one species, species D, showed unique fixed alleles (absolutely monomorphic) (Table 4). These alleles ($Adh\text{-}1^{100}$, $Adh\text{-}2^{100}$, Gpi^{100}, Me^{100} and $Pgd\text{-}2^{113}$) can be used as genetic markers for distinguishing this species from the other six.

Although no single diagnostic allele was detected for distinguishing unambiguously between species A, C, E, F, G and I, allele frequency differences over multiple loci were sufficient to distinguish individuals of each species in various complex patterns, with a high degree of probability (Table 4).

Two enzyme loci, *Mdh* and *Sod*, are useful in diagnosing species E. Mdh^{100} appears to be the only electromorph specific to species E, with a typical frequency of 0.958. If Mdh^{129} or Mdh^{88}, which are shared with species F and G, occur, then the presence of Sod^{110} would unambiguously identify the specimen as species E. If Sod^{100} only were present, then there is still a high probability that the species is species E (Table 4).

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

Species A, F and G formed a group in which each species shared two or more of the following alleles: $Adh\text{-}1^{66, 69}$ and $Adh\text{-}2^{71, 74}$. Species A was separated from species F and G by alleles at the *Aat* and *Mdh* loci. If a specimen had Aat^{82} or Mdh^{129} and did not have Aat^{100} or Mdh^{113}, then there is a high probability of it being species A.

A specimen of species F and G may be distinguished from species A by the reverse. The most difficult separation in the *B. tau* complex is species F and G, since they share the same alleles at all loci, although at different frequencies. However, *Adh-3* may be of use in distinguishing these species. From Table 4, $Adh\text{-}3^{-20}$ is possessed by species F (91.1%) but not by species G. The remaining shared alleles ($Adh\text{-}3^{-71}$ and $Adh\text{-}3^{-129}$) could cause misidentification in a mixed samples of species F and G. $Adh\text{-}3^{-129}$ would occur at a probability of 75% in species G but only 3.3% in species F (Table 4). The most confusing electromorph is $Adh\text{-}3^{-71}$, with expected 5.6% in species F and 9.4% in species G.

The seven species can be divided into four main groups (Fig. 7) based on allele frequencies: (*i*) species D distinguished by a single diagnostic allele for each of five loci; (*ii*) species E distinguished from other species by allele Mdh^{100}; (*iii*) species C and I were distinguishable by the possession of two unique alleles (Idh^{125} and $Pgd\text{-}2^{122}$) and (*iv*) species A (=*B. tau*), F and G distinguished from other species by possessing unique alleles at two loci ($Adh\text{-}1^{66, 69}$ and $Adh\text{-}2^{71, 74}$). An electrophoretic key, which permits these seven species of the *B. tau* complex to be distinguished, is given in Appendix 3.

Table 4 Allele frequencies, observed and expected heterozygosity (H_o and H_e) at 12 loci of the seven species (A, C, D, E, F, G and I) of the *Bactrocera tau* complex. The common alleles are shown in bold type (N = sample size; * indicates that no electromorphs were detected).

Locus	A	C	D	E	F	G	I
Aat							
N	1,393	183	101	92	51	33	165
122	.000	.036	.015	.000	.000	.000	.000
111	.000	.000	.005	.000	.000	.000	.000
100	.010	**.751**	.297	.000	**.961**	**.985**	.000
90	.000	.213	.000	.000	.000	.000	.000
82	**.980**	.000	**.683**	**.973**	.039	.015	**.982**
67	.008	.000	.000	.027	.000	.000	.012
Rare alleles	.002	.000	.000	.000	.000	.000	.006
H_o	.040	.210	.356	.033	.078	.030	.036
H_e	.039	.390	.445	.053	.075	.030	.036
Adh-1							
N	1,394	189	137	92	51	33	165
100	.000	*	**1.000**	*	.000	.000	*
69	.000	*	.000	*	**1.000**	**.515**	*
66	**.998**	*	.000	*	.000	**.485**	*
Rare alleles	.002	*	.000	*	.000	.000	*
H_o	.005	*	.000	*	.000	.000	*
H_e	.004	*	.000	*	.000	.500	*
Adh-2							
N	1,394	189	137	92	51	33	165
100	.000	*	**1.000**	*	.000	.000	*
74	.000	*	.000	*	**1.000**	**.515**	*
71	**.998**	*	.000	*	.000	**.485**	*
Rare alleles	.002	*	.000	*	.000	.000	*
H_o	.005	*	.000	*	.000	.000	*
H_e	.004	*	.000	*	.000	.500	*

Table 4 (ctd.) Allele frequencies, observed and expected heterozygosity (H_o and H_e) at 12 loci of the seven species (A, C, D, E, F, G and I) of the *Bactrocera tau* complex. The common alleles are shown in bold type (N = sample size; * indicates that no electromorphs were detected).

Locus	A	C	D	E	F	G	I
Adh-3							
N	1,386	189	76	92	45	32	165
150	.000	.111	.000	.000	.000	.000	.000
50	.000	.000	**.421**	.000	.000	.000	.000
-20	.009	.000	.138	.119	**.911**	.000	.000
-71	.051	.000	.000	.000	.056	.094	.000
-100	.010	.000	**.441**	.000	.000	.062	.000
-129	**.923**	**.889**	.000	.087	.033	**.750**	.036
-171	.005	.000	.000	**.761**	.000	.094	**.964**
-190	.000	.000	.000	.033	.000	.000	.000
Rare alleles	.002	.000	.000	.000	.000	.000	.000
H_o	.013	.000	.197	.174	.044	.000	.000
H_e	.145	.197	.609	.398	.166	.416	.069
G3pdh							
N	1,394	189	137	92	51	33	165
118	.000	.000	.000	.022	.000	.000	.000
87	**.995**	**.997**	**1.000**	**.978**	**1.000**	**1.000**	**.973**
63	.000	.000	.000	.000	.000	.000	.021
Rare alleles	.005	.003	.000	.000	.000	.000	.006
H_o	.010	.005	.000	.044	.000	.000	.018
H_e	.009	.006	.000	.043	.000	.000	.053
Gpi							
N	1,394	154	137	92	51	33	165
130	.000	.000	.000	.000	.000	.000	.012
122	.000	**1.000**	.000	.000	.000	.000	**.988**
108	.006	.000	.000	.049	**1.000**	**.515**	.000
100	.000	.000	**1.000**	.011	.000	.000	.000
95	**.991**	.000	.000	**.940**	.000	**.485**	.000
Rare alleles	.003	.000	.000	.000	.000	.000	.000
H_o	.011	.000	.000	.098	.000	.000	.024
H_e	.018	.000	.000	.114	.000	.500	.024

Table 4 (ctd.) Allele frequencies, observed and expected heterozygosity (H_o and H_e) at 12 loci of the seven species (A, C, D, E, F, G and I) of the *Bactrocera tau* complex. The common alleles are shown in bold type (N = sample size; * indicates that no electromorphs were detected).

Locus	A	C	D	E	F	G	I
Idh							
N	1,394	189	137	91	51	33	165
125	.000	**1.000**	.000	.000	.000	.015	**1.000**
114	.005	.000	.000	.011	.000	.000	.000
109	.005	.000	.022	.055	.000	.000	.000
100	.000	.000	**.978**	.000	.000	.000	.000
96	**.985**	.000	.000	**.907**	**1.000**	**.970**	.000
84	.000	.000	.000	.016	.000	.015	.000
74	.000	.000	.000	.011	.000	.000	.000
Rare alleles	.005	.000	.000	.000	.000	.000	.000
H_o	.029	.000	.044	.180	.000	.061	.000
H_e	.030	.000	.043	.174	.000	.059	.000
Mdh							
N	1,394	189	137	72	51	33	128
163	.000	.000	.011	.000	.000	.000	.008
135	.012	.000	.014	.000	.000	.000	.000
129	**.980**	.000	**.971**	.035	.019	.000	**.992**
113	.000	.000	.000	.000	**.971**	**.849**	.000
100	.000	.000	.000	**.958**	.000	.000	.000
88	.000	.000	.000	.007	.000	.151	.000
20	.000	**1.000**	.000	.000	.010	.000	.000
Rare alleles	.008	.000	.004	.000	.000	.000	.000
H_o	.041	.000	.051	.020	.020	.242	.016
H_e	.039	.000	.057	.081	.057	.256	.016
Me							
N	1,373	189	137	59	15	33	165
130	.000	.000	.000	.000	.000	.000	.009
122	.000	**1.000**	.000	.000	.000	.000	**.991**
108	.000	.000	.000	.042	.000	.000	.000
100	.000	.000	**1.000**	.000	.000	.000	.000
97	**.993**	.000	.000	**.949**	**1.000**	**1.000**	.000
86	.000	.000	.000	.009	.000	.000	.000
Rare alleles	.007	.000	.000	.000	.000	.000	.000
H_o	.013	.000	.000	.102	.000	.000	.018
H_e	.014	.000	.000	.098	.000	.000	.018

Table 4 (ctd.) Allele frequencies, observed and expected heterozygosity (H_o and H_e) at 12 loci of the seven species (A, C, D, E, F, G and I) of the *Bactrocera tau* complex. The common alleles are shown in bold type (N = sample size; ∗ indicates that no electromorphs were detected).

Locus	A	C	D	E	F	G	I
Pgd-1							
N	1,394	107	137	92	51	33	165
136	.009	.060	.000	.006	.000	.000	.021
128	.000	.156	.000	.000	.000	.000	.000
122	**.972**	**.559**	**.920**	**.978**	**1.000**	**1.000**	**.973**
115	.006	.041	.000	.000	.000	.000	.000
107	.009	.166	.069	.016	.000	.000	.000
100	.000	.000	.007	.000	.000	.000	.000
90	.000	.009	.000	.000	.000	.000	.000
68	.000	.009	.000	.000	.000	.000	.000
Rare alleles	.004	.000	.004	.000	.000	.000	.006
H_o	.044	.459	.153	.022	.000	.000	.055
H_e	.055	.630	.149	.043	.000	.000	.053
Pgd-2							
N	1,392	189	137	69	51	33	165
122	.000	**1.000**	.000	.000	.000	.000	**1.000**
113	.010	.000	**1.000**	.058	.000	.000	.000
97	**.985**	.000	.000	**.942**	**1.000**	**1.000**	.000
Rare alleles	.005	.000	.000	.000	.000	.000	.000
H_o	.029	.000	.000	.116	.000	.000	.000
H_e	.030	.000	.000	.109	.000	.000	.000
Sod							
N	1,356	189	137	92	51	33	165
272	.000	**1.000**	.000	.000	.000	.000	.000
244	.000	.000	.022	.000	.000	.000	.000
232	**.872**	.000	**.967**	.000	.000	.000	**.685**
176	.119	.000	.007	.000	**.980**	**.970**	.267
165	.006	.000	.000	.000	.000	.000	.048
110	.000	.000	.000	**.804**	.000	.000	.000
100	.000	.000	.000	.196	.020	.030	.000
Rare alleles	.003	.000	.004	.000	.000	.000	.000
H_o	.201	.000	.022	.000	.039	.061	.388
H_e	.225	.000	.064	.315	.039	.058	.457

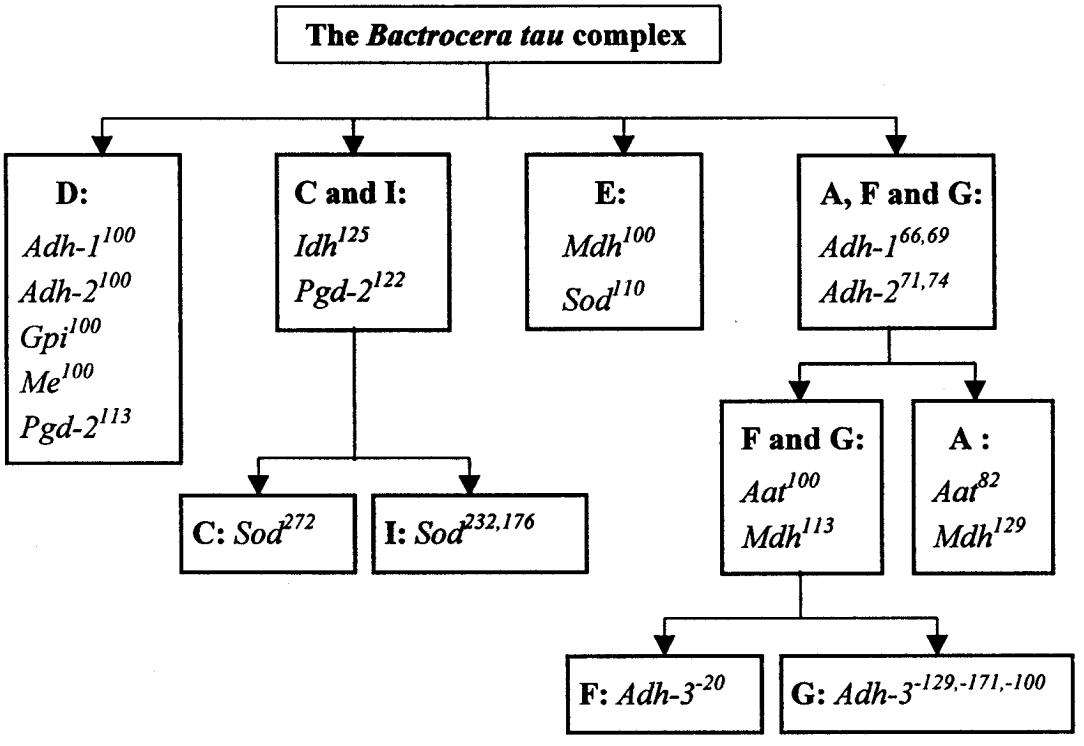

Figure 7 Electromorphs used as genetic markers for identification of seven species of the *Bactrocera tau* complex.

5.3.2 *F*- statistics Analysis

Table 5 shows the results of the *F*- statistics analysis as well as an indirect estimate of gene flow (N_m) in seven *B. tau* complex species. The mean value of F_{IT} over all loci of the 43 populations of the *B. tau* complex was very high (F_{IT} = 0.811). Genetic differentiation among populations (F_{ST} = 0.754) was also very high. The F_{IT} values of each species of the *B. tau* complex show moderate to great genetic variability (0.090-0.332). Partitioning of each species resulted in substantially smaller values of F_{ST} for each species. The F_{ST} values of species F and G can not be calculated because there is only one population in each species. The value of F_{ST} of species A (0.043) is close to zero indicating that the populations within species A were very similar. The same was found for species I (F_{ST}= 0.018). Moderate genetic differentiation occurred among populations within each of species C, D and E with F_{ST} values of 0.067, 0.148 and 0.070, respectively. In addition, the pair of most closely related allopatric species (F and G), which infest the same host-plant species show a moderate value of variation between them (F_{ST} = 0.131).

There was a little genetic differentiation within populations of species A and D, with F_{IS} = 0.060 and 0.073, respectively. The F_{IS} values of species C, E, F, G and I are equal to 0.172, 0.292, 0.111, 0.332 and 0.162, respectively, and suggest strong genetic differentiation within populations.

Average F_{IS} was higher than F_{ST} in species C, E and I, suggesting the genetic differentiation across these populations may be a consequence of nonrandom mating in a local population being greater than the subdivision of the population. *Bactrocera tau* had values of F_{IS} and F_{ST} nearly equal to zero, suggesting that random mating occurs within and among populations in *B. tau*. For species D, F_{IS} was lower

than F_{ST}, the simplest interpretation being that random mating occurs within populations but that most genetic differentiation may be a consequence of geographic variation.

According to Crow and Aoki (50), gene flow values (N_m) of less than 1.00 should be interpreted as little or no gene flow. Thus the average value, N_m = 0.80, observed between the *B. tau* complex populations would indicate extremely rare migratory events between these species. High gene flow value between populations within *B. tau* (N_m=8.44) related to F_{ST} value (0.043). When *B. tau* populations showed a high rate of gene flow, resulting in the low level of genetic difference between populations (F_{ST}). In other word, a high rate of gene flow indicates random mating between populations within *B. tau* species.

Table 5 *F*-statistics (F_{IS}, F_{ST} *and* F_{IT}) and an indirect estimate of gene flow (N_m) based on 12 loci of the seven species of the *Bactrocera tau* complex (standard error in parenthesis). I, all populations; II, each species; III, the two most closely related allopatric species, i.e., species F and G.

Criterion	No. of pops.	F_{IS}	F_{ST}	F_{IT}	N_m
I. All populations	43	**0.272**	**0.754**	**0.811**	**0.80**
II. Species A	28	0.060	0.043	0.090	8.44
Species C	3	0.172	0.067	0.215	N/A
Species D	5	0.073	0.148	0.175	N/A
Species E	2	0.292	0.070	0.316	N/A
Species F	1	0.111	-	0.111	N/A
Species G	1	0.332	-	0.332	N/A
Species I	3	0.162	0.018	0.172	N/A
Mean*		**0.152** (0.093)	**0.069** (0.049)	**0.194** (0.082)	**N/A**
III. Sp. F and G	2	**0.334**	**0.131**	**0.343**	**N/A**

* Calculated from five species (species A, C, D, E and I); N/A: estimate of gene flow is not available due to a very small, a negative or no estimate of F_{ST}.

5.3.3 Genetic Distance and Cluster Analysis

Table 6 presents the mean genetic distances among the *B. tau* complex species. The mean genetic distance between populations within species ranged from 0.002 (species I) to 0.050 (species E). The highest mean genetic distance between species was between species C and D (1.857), while the lowest mean genetic distance between species was between species F and G (0.136).

Calculating from Table 6, the average values of mean genetic distance for comparisons between each of seven *B. tau* complex species and the other six are as follows: species A = 0.818, species C = 1.413, species D = 1.387, species E = 0.957, species F = 1.057, species G = 0.865, species I = 1.019. The average of the mean genetic distance was considerably less for species A than for any of the other six species. Species C showed the greatest genetic distance from other species in the group.

Using UPGMA cluster analysis, a possible phylogenetic tree of the *B. tau* complex was constructed and is shown in Figure 8. The population, NA(D)7, was used to represent all *B. tau* (=species A). The populations of seven species within the complex can be divided into three major clusters. The first cluster contains *B. tau, B. cucurbitae* (outgroup species), and species E, F and G. The second cluster consists of species C and I. Species D populations form the last cluster and have the same ancestor as species C and I. For all members of the species complex, populations belonging to the same species cluster together.

Table 6 Mean Nei's unbiased genetic distances within and between species of the *Bactrocera tau* complex based on 12 loci (* indicates mean genetic distance within a species; standard error in parentheses).

Species	No. of populations	A	C	D	E	F	G	I
A	28	0.004* (0.006)						
C	3	1.528 (0.161)	0.025* (0.012)					
D	5	0.979 (0.071)	1.857 (0.219)	0.039* (0.022)				
E	2	0.389 (0.053)	1.811 (0.234)	1.397 (0.139)	0.050* (-)			
F	1	0.804 (0.026)	1.566 (0.139)	1.612 (0.147)	0.700 (0.072)	-		
G	1	0.383 (0.017)	1.193 (0.120)	1.546 (0.146)	0.531 (0.063)	0.136 (-)	-	
I	3	0.822 (0.015)	0.520 (0.044)	0.932 (0.076)	0.916 (0.020)	1.522 (0.037)	1.399 (0.049)	0.002* (0.001)

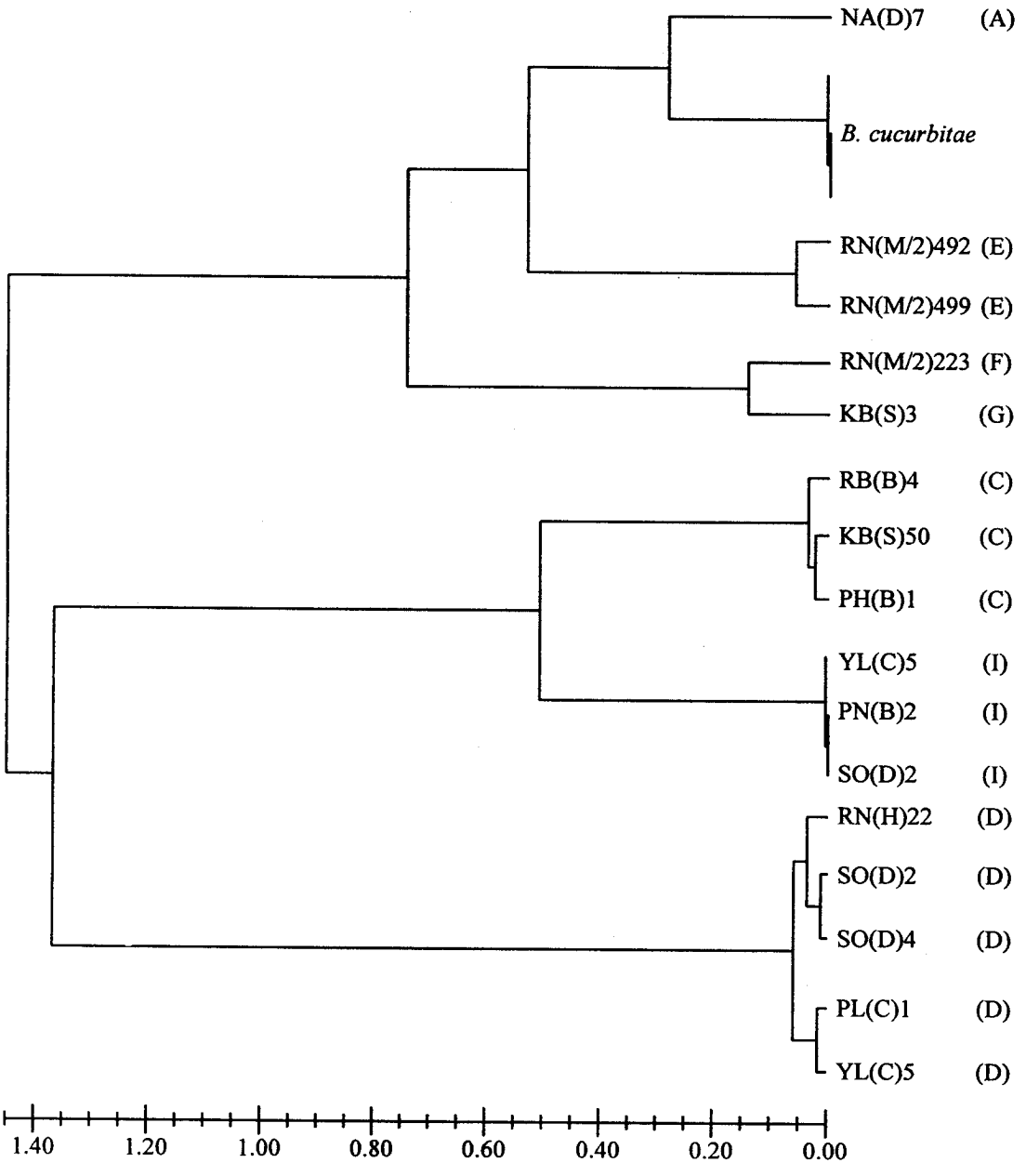

Figure 8 A phenogram of the *Bactrocera tau* complex. Three populations of *B. cucurbitae* were used as outgroups.

5.4 Genetic Variation between Populations within *Bactrocera tau*

The genetic variability in 28 populations of *B. tau* was evaluated using allele frequencies for twelve loci of nine gene-enzyme systems. In this study, we only focus on intra-specific variation within one species, i.e., *B. tau* (=species A), because the numbers of populations of other species within the *B. tau* complex were less than or equal to five and, thus, not high enough to assess intra-specific genetic variation.

5.4.1 Genetic Variability

The measures used for determining intra-specific genetic variation, were expressed by the parameters, P (percentage of polymorphic loci), A (mean number of alleles per locus), $\overline{H}_o$ and $\overline{H}_e$ (mean observed and expected heterozygosity per locus) and F (mean fixation index per locus).

The percentage of polymorphic loci in each population ranged from 8.33 to 83.33. Populations 7 and 10 had the highest value, while population 7 also showed the highest mean number of alleles per locus (A = 3.08). Population 20 had the lowest P and A values (A = 1.17) (Table 7).

The values of $\overline{H}_o$ and $\overline{H}_e$ calculated in each population, ranged from 0.007 (populations 18) to 0.082 (population 19) and 0.011 (populations 13) to 0.092 (population 12), respectively (Table 7). The average observed and expected heterozygosity of all individuals of *B. tau* are 0.037 and 0.051, respectively (Table 8).

Major departure from Hardy-Weinberg expectation in almost all the populations examined were due to heterozygote deficiency as indicated by positive value of fixation index.

Table 7 Values of genetic variability measures for each of 28 populations of *Bactrocera tau* (=species A) based on 12 loci.

Population	Percentage of polymorphic loci (*P*)	Mean number of alleles per locus (*A*)	Mean observed heterozygosity per locus (H_o)	Mean expected heterozygosity per locus (H_e)	Mean fixation index per locus (*F*)
1	50.00	1.50	0.038	0.072	0.129
2	41.67	1.42	0.029	0.038	0.074
3	75.00	1.83	0.042	0.062	0.095
4	25.00	1.25	0.030	0.028	-0.014
5	41.67	1.75	0.035	0.036	0.017
6	50.00	1.58	0.046	0.049	0.006
7	83.33	3.08	0.053	0.070	0.128
8	50.00	1.67	0.050	0.049	-0.011
9	58.33	1.92	0.045	0.046	0.005
10	83.33	2.17	0.028	0.035	0.077
11	50.00	1.83	0.043	0.047	0.011
12	66.67	2.00	0.057	0.092	0.058
13	25.00	1.33	0.009	0.011	0.081
14	16.67	1.17	0.021	0.019	-0.010
15	16.67	1.33	0.029	0.027	-0.011
16	33.33	1.33	0.015	0.026	0.102
17	25.00	1.25	0.029	0.028	-0.012
18	25.00	1.33	0.007	0.012	0.041
19	33.33	1.42	0.082	0.068	-0.043
20	8.33	1.17	0.016	0.050	0.057
21	75.00	1.83	0.060	0.083	0.059
22	58.33	1.67	0.044	0.064	0.104
23	75.00	2.33	0.045	0.071	0.098
24	16.67	1.33	0.022	0.038	0.070
25	16.67	1.25	0.029	0.031	0.071
26	75.00	2.25	0.053	0.072	0.077
27	33.33	1.75	0.030	0.067	0.138
28	66.67	2.00	0.055	0.052	0.059

Table 8 Values of genetic variability measures for each locus for all individuals from 28 populations of *Bactrocera tau* (=species A).

Locus	Observed Heterozygosity (H_o)	Expected heterozygosity (H_e)	Fixation index (F)
Aat	0.040	0.039	-0.014
Adh-1	0.005	0.004	-0.002
Adh-2	0.005	0.004	-0.002
Adh-3	0.013	0.145	0.911
G3pdh	0.010	0.009	-0.004
Gpi	0.011	0.018	0.071
Idh	0.029	0.030	0.039
Mdh	0.041	0.039	-0.015
Me	0.013	0.014	-0.004
Pgd-1	0.044	0.055	0.102
Pgd-2	0.029	0.030	0.037
Sod	0.201	0.225	0.083
Mean	0.037	0.051	0.100

A negative value of fixation index signified heterozygote excess for that population or enzyme locus. The coefficient for heterozygote deficiency or excess (fixation index) is reported in both Tables 7 and 8.

Many populations showed a deficiency of heterozygote, with positive fixation index greater from 0.050 (population 1-3, 7, 10, 12-13, 16 and 20-28). The remaining populations showed both positive and negative fixation indexes that were close to zero, indicating that these populations were close to Hardy-Weinberg equilibrium (Table 7).

5.4.2 Genetic Homogeneity and Heterogeneity

Seven of the twelve loci (*Aat*, *Adh*-3, *Idh*, *Mdh*, *Pgd-1*, *Pgd-2* and *Sod*) were polymorphic using the criterion that the frequency of the most abundant allele is less than 0.99 for pooled populations of species A (Table 4).

These seven loci were, therefore, selected for testing homogeneity of genotype frequencies using Hardy-Weinberg test, within populations. Some loci were monomorphic within a population, and, so, could not be tested, but genotypic frequencies of such loci are, by definition, homogeneous. For polymorphic loci within a population, homogeneity of observed genotype frequency has been computed for the deviation from Hardy-Weinberg equilibrium using Chi-square goodness-of-fit tests at the 0.01 significant level. The Chi-square value and fixation indexes at 28 populations of *B. tau* are presented in Table 9.

Aat, *Idh* and *Mdh* were homogeneous under Hardy-Weinberg equilibrium across all populations. Ten populations (population 4, 5, 6, 8, 9, 11, 14, 15, 17 and 19) were in equilibrium at all seven loci. Significant Hardy-Weinberg disequilibrium, i.e.,

heterozygote deficiency, was observed at *Adh-3* in many populations and *Sod* in some populations, but there was no apparent pattern; (*i*) neither *Adh-3* nor *Sod* was in disequilibrium across all populations and (*ii*) eighteen populations were in disequilibrium at least at one of seven loci.

Fixation index (*F*) also showed large heterozygote deficiency in many cases (Table 9). Most of the Hardy-Weinberg disequilibrium groups displayed the highest value for fixation index ($F = 1.000$). The results of fixation index and Hardy-Weinberg tests are mostly parallel; when fixation index proved to be large positive at a given locus, the populations were generally not at Hardy-Weinberg equilibrium.

Table 9 Chi-square tests (χ^2) for deviation from Hardy-Weinberg equilibrium and Wright's fixation index (F) at seven polymorphic loci of 28 populations of the *Bactrocera tau* (N: sample size, H-W: departures from Hardy-Weinberg equilibrium, E: Hardy-Weinberg equilibrium, D: Hardy-Weinberg disequilibrium; significant at 0.01 level, - locus monomorphic, so, no test done).

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Aat														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
χ^2	-	0.000	0.026	0.688	0.137	0.013	0.100	-	0.009	0.009	-	0.090	-	0.000
H-W	-	E	E	E	E	E	E	-	E	E	-	E	-	E
F	-	0.000	-0.011	-0.114	-0.032	-0.013	-0.021	-	-0.009	-0.004	-	-0.028	-	0.000
Adh-3														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
χ^2	22.415	38.038	64.965	-	-	-	479.925	-	-	71.427	-	140.652	137.007	-
H-W	D	D	D	-	-	-	D	-	-	D	-	D	D	-
F	1.000	1.000	1.000	-	-	-	1.000	-	-	1.000	-	1.000	1.000	-
Idh														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
X^2	0.027	-	0.009	-	-	0.000	0.047	0.027	6.668	0.009	0.090	0.009	-	-
H-W	E	-	E	-	-	E	E	E	E	E	E	E	-	-
F	-0.027	-	-0.009	-	-	0.000	-0.012	-0.013	0.215	-0.004	-0.021	-0.009	-	-
Mdh														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
χ^2	-	-	0.000	-	0.196	0.040	0.135	-	0.090	0.000	0.090	-	-	-
H-W	-	-	E	-	E	E	E	-	E	E	E	-	-	-
F	-	-	0.000	-	-0.038	-0.026	-0.023	-	-0.028	0.000	-0.035	-	-	-
Pgd-1														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
X^2	0.027	0.117	0.009	-	0.137	0.000	0.014	0.086	-	0.026	-	0.026	0.023	-
H-W	E	E	E	-	E	E	E	E	-	E	-	E	E	-
F	-0.027	-0.057	-0.009	-	-0.028	0.000	-0.006	-0.036	-	-0.011	-	-0.017	-0.010	-

Table 9 (ctd.) Chi-square tests (χ^2) for deviation from Hardy-Weinberg equilibrium and Wright's fixation index (F) at seven polymorphic loci of 28 populations of the *Bactrocera tau* (N: sample size, H-W: departures from Hardy-Weinberg equilibrium, E: Hardy-Weinberg equilibrium, D: Hardy-Weinberg disequilibrium; significant at 0.01 level, - locus monomorphic, so, no test done).

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Pgd-2														
N	20	29	60	50	60	38	111	20	60	60	60	60	69	40
χ^2	0.027	-	0.009	-	-	0.149	0.135	0.027	0.026	0.000	0.090	0.137	-	-
H-W	E	-	E	-	-	E	E	E	E	E	E	E	-	-
F	-0.027	-	-0.009	-	-	-0.039	-0.020	-0.027	-0.017	0.000	-0.028	-0.032	-	-
Sod														
N	20	29	59	50	60	40	111	20	59	60	60	60	69	40
χ^2	12.324	0.117	2.373	0.168	6.605	0.897	80.019	0.027	0.452	0.196	3.116	2.867	0.046	0.565
H-W	D	E	E	E	E	E	D	E	E	E	E	E	E	E
F	0.655	-0.057	0.198	-0.054	0.306	0.147	0.155	-0.027	-0.084	-0.054	0.224	-0.217	-0.023	-0.114

Table 9 (ctd.) Chi-square tests (χ^2) for deviation from Hardy-Weinberg equilibrium and Wright's fixation index (F) at seven polymorphic loci of 28 populations of the *Bactrocera tau* (N: sample size, H-W: departures from Hardy-Weinberg equilibrium, E: Hardy-Weinberg equilibrium, D: Hardy-Weinberg disequilibrium; significant at 0.01 level, - locus monomorphic, so, no test done).

Population	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Aat														
N	38	40	40	63	33	21	21	23	84	38	29	85	49	90
χ^2	-	0.000	0.082	0.000	-	-	0.000	0.023	0.000	-	-	0.018	0.032	-
H-W	-	E	E	E	-	-	E	E	E	-	-	E	E	-
F	-	0.000	-0.040	0.000	-	-	0.000	-0.012	0.000	-	-	-0.008	-0.011	-
Adh-3														
N	38	40	40	63	25	21	21	23	84	38	29	85	50	90
χ^2	-	52.693	-	-	2.811	22.716	23.550	30.049	355.704	-	57.018	258.022	113.511	-
H-W	-	D	-	-	E	D	D	D	D	-	D	D	D	-
F	-	1.000	-	-	-0.300	0.685	0.868	1.000	1.000	-	1.000	1.000	1.000	-
Idh														
N	38	40	40	63	33	21	21	23	84	38	29	85	50	90
X^2	-	-	-	-	-	-	0.000	0.000	0.006	-	-	0.037	-	0.058
H-W	-	-	-	-	-	-	E	E	E	-	-	E	-	E
F	-	-	-	-	-	-	0.000	0.000	-0.006	-	-	-0.018	-	-0.016
Mdh														
N	38	40	40	63	33	21	21	23	84	38	29	85	50	90
χ^2	-	-	0.141	-	0.175	-	-	0.000	0.095	-	-	0.006	-	0.169
H-W	-	-	E	-	E	-	-	E	E	-	-	E	-	E
F	-	-	-0.054	-	-0.067	-	-	0.000	-0.023	-	-	-0.003	-	-0.032
Pgd-1														
N	38	40	40	63	33	21	21	23	84	38	29	85	50	90
X^2	0.331	0.000	-	0.000	0.000	-	-	-	15.730	75.077	-	0.062	32.348	0.058
H-W	E	E	-	E	E	-	-	-	E	D	-	E	D	E
F	-0.075	0.000	-	0.000	0.000	-	-	-	0.130	0.456	-	-0.017	0.385	-0.018

Table 9 (ctd.) Chi-square tests (χ^2) for deviation from Hardy-Weinberg equilibrium and Wright's fixation index (*F*) at seven polymorphic loci of 28 populations of the *Bactrocera tau* (N: sample size, H-W: departures from Hardy-Weinberg equilibrium, E: Hardy-Weinberg equilibrium, D: Hardy-Weinberg disequilibrium; significant at 0.01 level, - locus monomorphic, so, no test done).

Population	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Pgd-2														
N	38	40	40	63	33	21	21	23	84	38	29	85	50	90
χ^2	-	-	-	-	-	-	0.000	0.000	0.000	-	-	0.018	-	179.006
H-W	-	-	-	-	-	-	E	E	E	-	-	E	-	D
F	-	-	-	-	-	-	0.000	0.000	0.000	-	-	-0.012	-	1.000
Sod														
N	38	40	40	35	25	21	21	23	84	38	29	85	50	90
χ^2	0.230	2.377	0.141	69.031	0.558	-	0.300	1.571	2.247	8.671	1.117	56.421	5.919	3.464
H-W	E	E	E	D	E	-	E	E	E	E	E	D	E	E
F	-0.060	0.229	-0.054	0.489	-0.143	-	-0.111	0.254	0.076	0.389	-0.152	0.009	0.276	-0.180

5.4.3 *F*-statistics Analysis

Table 10 summarizes the results of the *F*-statistics and indirect estimate of gene flow (N_m) at 28 populations of *B. tau*. In this study, *F*-statistics values were calculated to quantify the degree of inter-host variation. The overall F_{IT} value showed a relatively moderate level of genetic variability (F_{IT} = 0.090). The overall value of F_{IS} and F_{ST} obtained for all *B. tau* populations are 0.060 and 0.043, respectively. The F_{IS} value is slightly greater than F_{ST} suggesting that genetic differentiation is according to nonrandom mating more than population subdivision. So it is not surprising that indirect gene flow among all *B. tau* populations was quite high (8.44). Partitioning each population by host plant species reduces F_{ST} to a mean of 0.028 and suggests that out-crossing between local individuals occurs across subpopulations, probably by immigration from the same host fruit preference population. Thus, it was found that *B. tau* exists within Thailand as a large population with a relative high migration rate among and within host plant species. Considering *B. tau* populations in the same host plant species, nonrandom mating is the main cause acting on genetic differentiation of the populations ($F_{IS}>F_{ST}$).

Table 10 Summary of *F*-statistics (F_{IS}, F_{ST} *and* F_{IT}) and indirect estimate of gene flow (N_m) for host plant subpopulations of *Bactrocera tau* (=species A) based on 12 loci (standard error in parenthesis).

Criterion	No. of pops.	F_{IS}	F_{ST}	F_{IT}	N_m
I. All subpopulations	28	**0.060**	**0.043**	**0.090**	**8.44**
II. Host-plant: *Cucurbita moschata*	1	0.089	-	0.089	N/A
Luffa cylindrica	6	0.057	0.035	0.073	N/A
Lagenaria siceraria	1	0.007	-	0.007	N/A
Dioscorea membranacea	1	0.042	-	0.042	N/A
Momordica cochinchinensis	1	- 0.048	-	- 0.048	N/A
Trichosanthes cordata	4	0.156	0.039	0.175	N/A
Trichosanthes cucumerina	3	0.073	0.014	0.084	N/A
Trichosanthes tricuspidata	11	0.096	0.025	0.113	N/A
Mean*		**0.096**	**0.028**	**0.111**	**N/A**
		(0.043)	(0.011)	(0.046)	N/A

* Calculated from four host plant species: *Luffa cylindrica, T. cordata, T. cucumerina* and *T. tricuspidata*; N/A: estimate of gene flow is not available due to a very small, a negative or no estimate of F_{ST}.

5.4.4 Genetic Distance and Cluster Analysis

I values for 28 populations of *B. tau* range from 0.973 to 1.000 (*D* = 0.000-0.027). The highest genetic identity with *I* = 1.000 was found between populations 3 and 22, 6 and 11, 10 and 16, 22 and 26. The lowest genetic identity found (*I* = 0.973) was between populations 6 and 20. *I* and *D* values for all pair of populations are presented in Appendix 5.

When populations were partitioned by host plant, the genetic distances between *B. tau* populations within and among host plant species remained small, with mean genetic distance values ranging from 0.0009 to 0.0129 (Table 11). The highest mean *D* values observed were in comparisons between *B. tau* populations in *T. cordata* and the populations in each of the other seven host plant species is 0.0064 - 0.0086 (Table 11). The highest mean *D* value both between and within host plant species was that within *T. cordata* (0.0129, Table 11). This indicates that the populations that infest *T. cordata* have the strongest genetic distance both within and among host plant species. The average genetic distances within any host plant species were similar or even higher than those averages between host plant species, indicating that differentiation of populations between host plant species was not stronger than between populations within host plant species (Table11). Figure 9 is a phenogram constructed from the calculated values of genetic distance between populations of *B. tau*. It showed a similar pattern, i.e., populations from the same host plant were not clustered into a single branch.

Table 11 Mean Nei's unbiased genetic distances between *Bactrocera tau* (=species A) populations of each host plant species based on 12 loci (* indicates mean genetic distance between populations that infest the same host plant species; standard error in parentheses).

	Host-plant	No. of populations	I	II	III	IV	V	VI	VII	VIII
I.	*Cucurbita moschata*	1	-							
II.	*Luffa cylindrica*	6	0.0027 (.0018)	0.0040* (.0052)						
III.	*Lagenaria siceraria*	1	0.0011 (-)	0.0027 (.0041)	-					
IV.	*Dioscorea membranacea*	1	0.0013 (-)	0.0033 (.0010)	0.0037 (-)	-				
V.	*Momordica cochinchinensis*	1	0.0046 (-)	0.0062 (.0012)	0.0058 (-)	0.0042 (-)	-			
VI.	*Trichosanthes cordata*	4	0.0065 (.0117)	0.0080 (.0097)	0.0076 (.0130)	0.0064 (.0083)	0.0079 (.0061)	0.0129* (.0128)		
VII.	*Trichosanthes. cucumerina*	3	0.0023 (.0007)	0.0025 (.0040)	0.0012 (.0008)	0.0033 (.0011)	0.0061 (.0009)	0.0077 (.0103)	0.0009* (.0004)	
VIII.	*Trichosanthes tricuspidata*	11	0.0016 (.0013)	0.0028 (.0033)	0.0014 (.0010)	0.0030 (.0013)	0.0053 (.0010)	0.0086 (.0109)	0.0017 (.0012)	0.0020* (.0013)

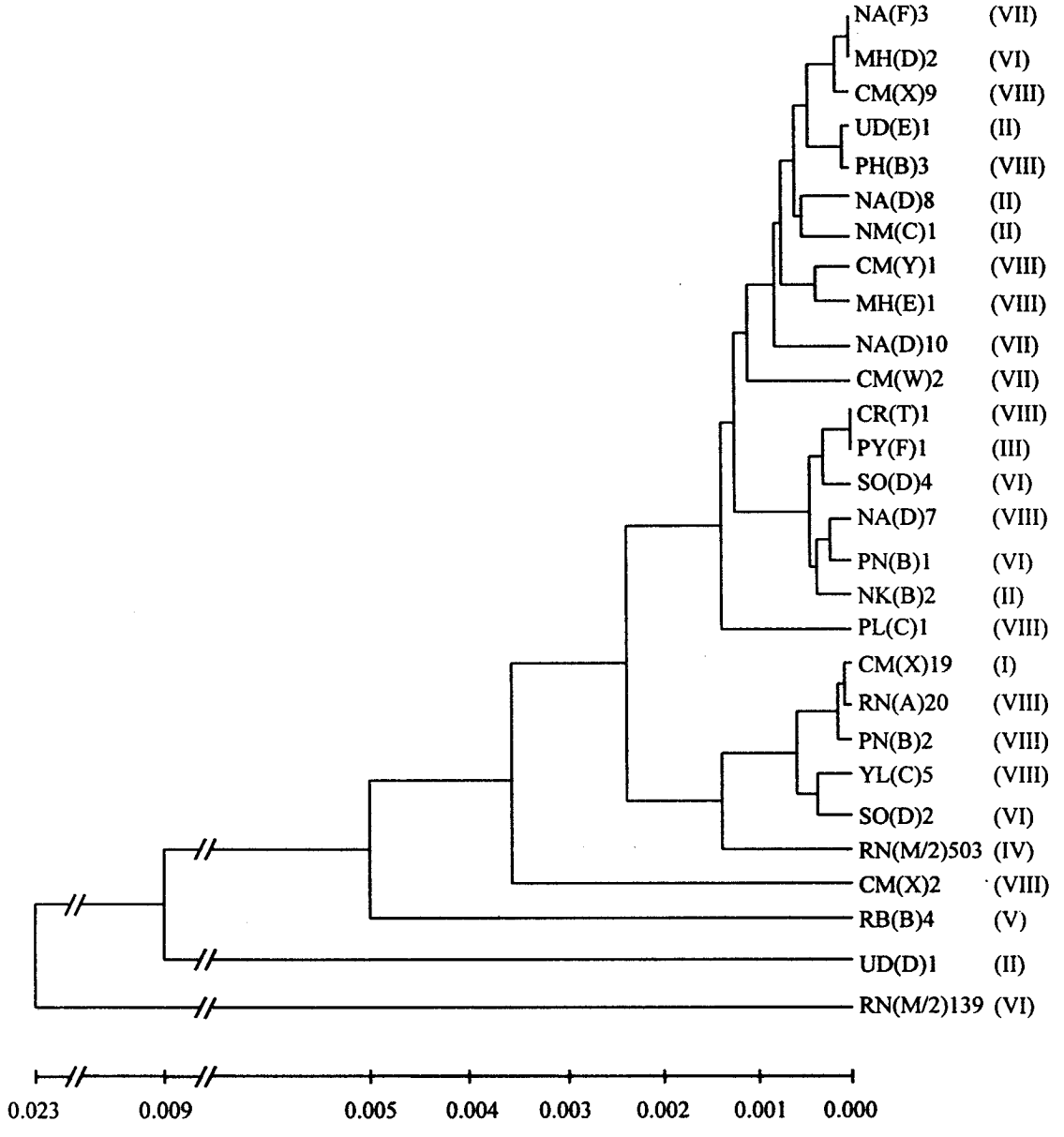

Figure 9 A phenogram of 28 *Bactrocera tau* (=species A) populations (Roman numbers in parentheses refer to host plant species as giving in Table 11).

CHAPTER VI

DISCUSSION

Although several tephritid fruit fly investigations have been conducted in many regions thoughout the world, the genetic make up information of the major fruit fly pest species of cucurbit crops in southeast Asia, *Bactrocera tau*, has not been reported. Drew and Romig (19) suggested that the *B. tau* complex represented a large group of sibling species. Recently, Baimai *et al* (2) found cytogenetical evidence for the existence of several species within the *B. tau* taxa from Thailand. However, no genetic variation evidence for this species complex in the area was reported. Therefore, this work is the first attempt to estimate genetic and phylogenetic relationships among species of the *B. tau* complex using enzyme electrophoresis.

Bactrocera tau is a common species and mainly infests the family Cucurbitaceae (51). In this study, members of the *B. tau* complex infested three families in addition to Cucurbitaceae, namely, Dioscoreaceae, Strychnaceae, Flacourtiaceae. Fruits of ten host plant species from these four families were infested by members of this complex. *Bactrocera tau* (species A) is a polyphagous species which mostly infested cucurbit crops. The other *B. tau* complex species tended to be monophagous species (host-specific species). Feder *et al* (8) suggested several factors which could be responsible for observed genetic differences between different host plant specific species. Among the possibilities are: (*i*) post mating reproductive isolation between host plant species, (*ii*) a genetic bottleneck associated with the

founding of the host race, (*iii*) differential larval survivorship associated with the host fruit environment, (*iv*) differential host recognition by adult flies and, (*v*) temporal differences in the timing of adult emergence. Thus, more ecological and biological means such as oviposition choice, host attractants analysis and life cycle are required to support these factors.

6.1 Banding Patterns

Nine enzyme systems, which comprised 12 loci, were used in this study. All nine enzyme systems utilized in this study have also been used in other dipteran studies. Therefore, comparison of electromorph patterns is possible, though electrophoretic conditions, such as pH of buffer, ionic strength, electrical power and supporting media differed among studies. In what follows, I compare the similarities and differences between electromorph patterns of this study with those of others.

The AAT system of Thai tephritid fruit flies found in this study consisted of one anodal locus. This is similar to the AAT system of the *Anopheles dirus* complex in Thailand (52). In contrast, the AAT system of *Rhagoletis* spp. in North America comprised *Aat-1* (cathodal) and *Aat-2* (anodal) (8, 9, 10, 13).

The most difficult enzyme to study in this research was alcohol dehydrogenase (ADH), because all species showed weak or no bands for *Adh-1* and *Adh-2*. Nevertheless, identification of the three *Adh* loci was unambiguous in the four species, A, D, F and G. The other three species, C, E and I have only one zone of very strong ADH activity, i.e., *Adh-3*. There are several possible explanations for the lack of *Adh-1* and *Adh-2* bands for these three species. The three isozymes may have the same mobilities and be superimposed on each other on the gel. They may be silenced, either

by a mutation at the active site or by regulation. Or the substrate affinity of the enzyme may have changed so drastically that it no longer is the oxidized form at all. *Adh-1* and *Adh-2* bands were also absent from *R. striatella*, one of the *Rhagoletis* spp. in the United States (12).

The ADH system of *Rhagoletis* spp. comprised two different loci. *Adh-1* (=*Adh-A*, cathodal) stained more intensively than *Adh-2* (=*Adh-B*, anodal) (11, 12, 13).The cathodally migrating ADH of *Rhagoletis* spp. (*Adh-1*, *Adh-A*) is probably *Adh-3* in this study, whereas the anodally migrating *Adh-2* (*Adh-B*) in *Rhagoletis* spp. may be *Adh-1* or *Adh-2* in *Bactrocera* spp.

Epi-enzymes, the regularly occurring electrophoretic patterns which are not a direct expression of structural genes were observed for *G3pdh* (*α-G3pdh* in *Rhagoletis* spp.) and *Idh*. Epi-enzymes or subbands of the electrophoretic pattern result from post-translation modification of proteins which could be caused by denaturation, deamination, phosphorelation, sulphation, oxidation, reduction, addition of other molecules, aggregation or cleavage of polypeptides (53). Residual ammonium persulfate in acrylamide gel was also found to produce artifacts by causing either band splitting which gave rise to subbands or disappearance of bands (54, 55). The reasons for these extra bands observed in *G3pdh* and *Idh* loci require further detailed investigation.

The number of enzyme subunits was reported from the electrophoresis of *Anopheles leucosphyrus* group from East Malaysia where GPI was reported as a monomeric enzyme (56) instead of being a dimeric enzyme like this study.

Only one locus was found for the IDH (=ICD) system in my study as well as from starch gel electrophoresis studies of both *Rhagoletis* spp. (13) and *Bactrocera* spp. (15, 17, 18). In contrast, two loci for the IDH system was recorded in polyacrylamide gel electrophoresis of *Aedes* (*Ochlerotatus*) *stimulans* (57); a slow locus (*Idh-1*) and a fast locus (*Idh-2*). Three loci of the IDH system were discovered from starch gel electrophoresis of the *Anopheles leucosphyrus* group (56), in which a locus (*Idh-3*) was identified as a product of locus *Idh-2* gene duplication.

Electromorph patterns of the MDH system of *Rhagoletis* spp. (11) and *Bactrocera* spp. i.e., *B. dorsalis* and *B. umbosa* (17, 36), respectively showed a cathodal locus, designated as *Mdh-1*. Locus *Mdh-1* is the mitochondrial form (11). An anodal locus (*Mdh-2*) was also found and is the cytosolic form of this enzyme (11). In addition, locus *Mdh-1* was found in *Anopheles minimus* (41). However, only one locus of MDH (*Mdh*, anodal) was revealed from my polyacrylamide gel electrophoresis study of the *B. tau* complex. The discrepancy in number of loci expressed between the *Bactrocera* spp. in my study and those of other studies might have resulted from the difference in electrophoretic conditions employed in each study, e.g., buffer pH or ionic strength.

One locus was discovered for ME in *Rhagoletis* spp. (8, 9, 10, 13), two taxa of *B. dorsalis* in Malaysia (18) and in some mosquito species related to *Aedes* (*Ochlerotatus*) *stimulans* as well as the *B. tau* complex in Thailand (this study).

Starch gel electrophoresis of *B. dorsalis* provided a locus of PGD (18, 36) corresponding to locus *Pgd* of polyacrylamide gel electrophoresis in this study.

Berlocher (11) reported that superoxide dismutase (SOD) is seen for all dehydrogenase stains as white zones on the blue background of the gel, and is apparently the same enzyme referred to in the older literature as indophenol oxidase or tetrazolium oxidase. It is best seen on malate dehydrogenase gels, especially if they are over stained. In this study, SOD electromorphs were observed by exposing the gel to strong light during the staining reaction. Berlocher (11) and Ooi (18) also reported a locus of the SOD enzyme system in *B. dorsalis* and *Rhagoletis* spp., respectively.

6.2 The Electrophoretic Separation of Species within the *Bactrocera tau* Complex

Analysis of enzyme banding patterns indicated that ten of the twelve loci (*Aat*, *Adh-1*, *Adh-2*, *Adh-3*, *Gpi*, *Idh*, *Mdh*, *Me*, *Pgd-2* and *Sod*) can be used to distinguish species of the *B. tau* complex (Fig. 7). The other two loci *G3pdh* and *Pgd-1* were not useful for discriminating among species. *G3pdh* was monomorphic and did not differ significantly in allele frequency among species. *Pgd-1* differed in allele frequency among species, but the alleles were not discriminating.

The seven species of the *B. tau* complex could be reliably distinguished by electrophoretic data, which provided *B. tau*, species C, D, E and I with 100% level of correct separation. The level of correct separation of species F and G is 91.1%. Furthermore, since those individuals contributing inaccuracy error are identifiable, they can be tested for ecological distribution, morphological and cytogenetic studies against samples which are unambiguously distinguished.

There are at least three situations in which morphological data alone is sometimes inadequate for defining species boundaries: (*i*) sympatric species, (*ii*) allopatric species and (*iii*) parapatric species (5).

In the first situation, two species may be sympatric (overlapping), but be so similar in morphology that their specific status goes undetected. Although, it is noted that several species of the *B. tau* complex, which have been found in the same host fruit, i.e., *B. tau* and species C in *Momordica cochinchinensis* [RB(B)4], *B. tau* and species D in *T. tricuspidata* [PL(C)1], *B. tau* and species I in *T. tricuspidata* [PN(B)2], *B. tau*, species D and I in *T. tricuspidata* [YL(C)5], can now be distinguished morphologically (S. Tigvattananont unpublished). There are a few characteristics that can be used to distinguish among some species such as the patterns of marking on the head used to separate species C and I apart from the *B. tau* complex. Different species usually have a fixed allelic difference at some of the loci screened in electrophoretic studies. Thus, for predominantly outcrossing species, the presence of sympatric cryptic species can be tested by looking for variable loci that lack heterozygotes.

The second situation, two allopatric (geographically separate) populations may be morphologically similar which could be solved by assessing the extent of genetic divergence between the populations being tested in relation to geographic variation within species. In this studies, species F and G occur in allopatric populations although they infest fruits of the same species, i.e., *Hydnocarpus anthelminthicus*. Species F occurs in Ranong province while species G has been found only in Kanchanaburi province, about 500 kilometers north of Ranong (Fig. 3). No single fixed allelic difference was observed between these two allopatric species but there were a number of gene frequency differences (Table 4). The average Nei's unbiased genetic distance between species F and G was estimated as 0.136 (Table 6) and this combined with genetic variation of 13.1% (Table 5), supports the separation of these two groups

as being different species. It seems likely that these two species arose from a common ancestor through the process of allopatric speciation (Fig. 8).

The third situation, two parapatric populations may be morphologically distinct, but show clinical variation or broad hybridization, and the fourth, two morphologically distinct forms, which may represent polymorphism, occurring within a single interbreeding population, do not appear in this study.

In terms of classification, the results of electrophoretic study correlated closely with both external morphology (S. Tigvattananont, unpublished) and cytogenetic study (2). The unexpected placement of the *B. tau* complex and *B. cucurbitae* (outgroup species) is probably due to the enormous disparity between their morphological and electromorphic divergence. The close electromorphic phylogenetic relationship between *B. tau* and *B. cucurbitae*, although these species are morphologically distinct, suggests that different evolutionary forces act on protein polymorphisms and morphological variation. Whereby, morphological variation is known to be influenced by both the genetics of individuals and the environment in which they develop. The finding that the electrophoretic classification of *B. tau* and *B. cucurbitae* differs from the conventional classification is not especially significant, since the disagreements can be resolved after more thorough analysis of the morphology and karyology. Several ovipositor features (Phinchonsakuldit, personal communication) and the unique karyotypes of members of the *B. tau* complex (2) can provide unambiguous separation.

The members of the *B. tau* complex, especially *B. tau*, species E, F and G were, until recently, morphologically indistinguishable and so were considered to be

cryptic species. *Bactrocera tau* and species E showed very slight difference in sex chromosomes (2), but electrophoretic data has provided an unambiguous means of distinguishing these two species. Species F and G are hard to separate morphologically (S. Tigvattananont, personal communication) and electrophoretically (this study) but they can be recognized by the appearance of autosome number 4 and the Y chromosome (2). In the group of species D, C and I, species C and D have been found to exhibit similar mitotic karyotypes based on pericentric heterochromatin in the autosomes and both sex chromosomes, but they can be distinguished by external morphology (S. Tigvattananont, unpublished) and electrophoretic patterns (this study).

6.3 A Possible Phylogenetic History of the *Bactrocera tau* Complex

Bactrocera tau is, on the average, the species most similar to all the others. One the other hand, the mean genetic distance is considerably less for *B. tau* than for any of other six species. Genetic evolution is likely to be for the most part divergent, rather than convergent (31). Thus, in the case of electrophoretically detectable allelic variation, evolutionary change is more likely to lead to genetic differentiation than to genetic similarity. If this view is accepted, it follows that *B. tau* is the most similar to the ancestral species of all the species in this study of the *B. tau* complex. Under the same view, species E is more similar than species F and G to the ancestral species.

A likely phylogenetic history of the seven species based on their phylogenetic (Fig. 8) and host plant relationships (Table 2) is as follows. From the ancestral *B. tau*-like species lineage, species like species I branched out. Both *B. tau* and species I infest *T. tricuspidata*. So it is possible that this plant was also the host of the *B. tau*-like and I-like species. The I-like species may have also infested *T. cordata*

and may have branched out and formed species D-like that infests *T. tricuspidata* and *T. cordata*. The other lineage split and from this host race, a C-like species, which infests *M. cochinchinensis*, eventually diverged.

The other *B. tau*-like lineage consists of a complex of extremely similar species. This lineage split and formed the G-like species, which infests *H. anthelminthicus*. The G-like species lineage finally separated by allopatric speciation to species F and G. The shift of the *B. tau*-like species from its native host (*T. tricuspidata*) to *Strychnos thorelii* may have resulted in another host race that finally developed to species E (Fig. 8). However, this phylogeny is only tentative and incomplete because it only looks at the *B. tau* complex in Thailand ignoring species in the *B. tau* complex that occur outside of Thailand.

6.4 Migration, Selection and Genetic Drift within *Bactrocera tau*

The genetic structure of the *B. tau* (species A) populations was evaluated by using genetic variability values (*P*, *A*, $\overline{H}_o$, $\overline{H}_e$, and *F*), Hardy-Weinberg test, *F* statistics analysis, genetic distance and cluster analysis. Populations of *B. tau* from different regions of Thailand showed a wide range in the percent of polymorphic loci (Table 7) ranging from 8.33 to 83.33%.

In an effort to understand more about genetic variation in natural populations, population geneticists have measured levels of polymorphism and heterozygosity and have tried to relate these measurements to the position of the population in the species range, to environmental variation, to predictability of trophic resources and to niche breadth (58). A preliminary attempt to correlate the polymorphic status of each *B. tau* population to their fruit hosts or geographical

regions was without success. Therefore, the explanation for this variation may be obtained if the detailed ecological situations of each population were investigated.

Mitton (58) reported that greater phenotypic diversity of side-blotch lizard and macaques is associated with a greater number of genotypes. In addition, a diversity observation suggests that higher levels of heterozygosity resulted in lower levels of phenotypic variance. Similarly, within the *B. tau* population, a high level of phenotypic variation existed with a low level of heterozygosity (mean = 0.037 per locus) (Table 8). This phenomenon is in good agreement with the high variation of thorax and abdomen patterns of *B. tau* in preliminary morphological observations.

Eighteen of the 28 populations of *B. tau* showed deviation from Hardy-Weinberg expectation for at least one locus, due to deficiencies in the number of heterozygotes, especially at loci *Adh-3* and *Sod* (Table 9). It may be possible that the presence of rare alleles in the homozygous state was the cause of this deviation. Alternatively, it could be due to the morphological misidentification of some flies.

The other analyses on genetic structure of the investigated *B. tau* populations (*F*-statistics, genetic distances, cluster analysis) generated similar results. Populations showed little genetic differentiation and these small differences were not according to geographical variation. In other words, populations in different regions do not differ to a greater extent than populations in the same region.

In theory, the lack of geographic pattern in genetic variation can be the consequence of three different forces: (*i*) intensive migration between regions, (*ii*) similar effects of selection in all regions and (*iii*) genetic drift (59). Both intensive migration between regions and the same selection forces should result in homogeneous

allele frequencies across populations. On the other hand, genetic drift would lead to heterogeneity in frequencies. The significant differences from expected in allele frequencies only at four (*Adh-3*, *Pgd-1*, *Pgd-2* and *Sod*) of twelve loci (Table 9) of all *B. tau* populations, suggest that the main evolutionary forces act on allozyme variation in these populations are both intensive migration between regions and similar effects of selection in all regions.

CHAPTER VII

CONCLUSIONS

Genetic variation and differentiation of 43 collected samples of seven species of the *Bactrocera tau* complex, obtained from geographically distinct localities were evaluated electrophoretically using nine systems in this study.

Species A, which has been designated as *Bactrocera tau*, infested various species of host plant, mostly in the cucurbit family. Four species were host plant specific in this study. Thus, species C and E infested *Momordica cochinchinensis* and *Strychnos thorelii*, respectively, and species F and G both infested *Hydnocarpus anthelminthicus*. Species F and G that infested *H. anthelminthicus* may have arisen as a result of allopatric speciation in different geographical populations of the ancestral species. Species C was specific to a host plant species and at the same time overlapped with other species in the same hosts, e.g., population 31 and *B. tau* (population 19) both infested *M. cochinchinensis*.

A single locus that could be used as a genetic marker for species separation was established only for species D. Although, no single locus was diagnostic for the other six species because of the overlapping allele frequencies in some alleles, the use of more than one allele permitted correct separation of all species, except for the two most closely related species (species F and G) in which 91.1% of individuals could be correctly separated. Separations of ambiguous species were clear-cut when subject to morphological and cytogenetic analysis. Thus, multiple approaches ensure correct

identification. The phylogenetic analysis suggests that three main lineages exist in the *B. tau* species group. One lineage consists of a complex of very closely related species (*B. tau*, species E, F and G). An other distinct group is species C and I, and the remainder is species D.

Genetic variability estimation among populations of *B. tau* (species A) indicated that populations of *B. tau* from different regions of Thailand and from the greatest variety of host fruits exhibited the least variation of all species in the *B. tau* complex as observed from the high average value of genetic similarity (I=0.996) and the low genetic differentiation among populations (F_{ST}=0.043). The high gene flow (N_m= 8.44) between *B. tau* populations ensures that populations do not genetically differentiate. This conclusion is reinforced by the low value of F_{ST} when populations were partitioned according to host plant species (F_{ST}=0.028, Table 10). The phenogram of *B. tau* populations is unrelated to its host plant by electrophoretic analysis. However, if host plant species are important, then a phenogram may be resolved by further systematic genetic analysis of populations on different host plant species.

Although, allozyme electrophoresis can distinguish species within a species complex, it is still not known what levels of polymorphism and heterozygosity are characteristic of entire genomes. So, genetic markers are not yet available that might detect relations between fruit flies and their host plants. However, the absolute amount of genetic variation is now amenable to study at the DNA level, either directly by means of DNA sequencing or indirectly by examining variation in restriction sites. Thus, many interesting aspects concerning species in the *B. tau* complex in Thailand require more detailed investigations and confirmations. With this information, further

correlation between the genetic data and other factors, e.g., fruit host preference could be firmly established.

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APPENDICES

Appendix 1. Enzyme staining procedure of the *Bactrocera tau* complex.

I. Aspartate aminotransferase (AAT)

1. Mixed the follows chemicals together

-Aspartic acid	200.0	mg
-Alpha ketoglutaric acid	100.0	mg
-Pyridoxal-5-phosphate	15.0	mg
2. Add distilled water to	40.0	ml
3. Adjust to pH 7-8 with 5.0 N NaOH		
4. Add 1.0 M Tris-HCl buffer pH 8.0	2.5	ml
5. After incubate, add Fast Blue BB	120.0	mg

6. Shaking for about 5 minutes and fix when the electromorphs fully developed.

II. Alcohol dehydrogenase (ADH)

1. Mixed the follows chemicals until ethanol well dissolved

-0.2 M Tris-HCl pH 8.5	40.0	ml
-Absolute ethanol	5.0	ml
2. Add NAD^+	10.0	mg
3. Add NBT	10.0	mg
4. After incubate, add PMS	5.0	mg

Appendix 1. (ctd.)

III. Glyceral-3-phosphate dehydrogenase (G3PDH)

1. Mixed the follows chemicals together

-0.2 M Tris-HCl pH 8.0	50.0	ml
-DL-alpha-Glycerophosphate	1.0	g
-0.1 M Magnesium chloride	1.0	ml

2. Adjust to pH 8.0 with 5.0 N NaOH

3. Add NAD^+	10.0	mg
4. Add NBT	5.0	mg
5. After incubate, add PMS	5.0	mg

IV. Glucose phosphate isomerase (GPI)

1. Mixed the follows chemicals together

-Sodium fructose-6-phosphate	10.0	mg
-0.1 M Magnesium chloride	2.5	ml
-1.0 M Tris-HCl pH 8.5	2.5	ml
-$NADP^+$	5.0	mg
-NBT	7.5	mg
2. Add distilled water to	20.0	ml
3. Add Glucose-6-phosphate dehydrogenase	10.0	U
4. After incubate, add PMS	5.0	mg

Appendix 1. (ctd.)

V. Isocitrate dehydrogenase (IDH)

1. Mixed the follows chemicals together

-0.06 M Sodium isocitrate	0.5	ml
-0.1 M Magnesium chloride	2.5	ml
-1.0 M Tris-HCl pH 8.5	2.5	ml
-$NADP^+$	7.5	mg
-NBT	7.5	mg

2. Add distilled water to 40.0 ml

3. After incubate, add PMS 5.0 mg

VI. Malate dehydrogenase (MDH)

1. Mixed the follows chemicals together

-1.0 M Sodium malate pH 7.0	0.5	ml.
-1.0 M Tris-HCl pH 8.5	5.0	ml.
-NAD^+	7.5	mg.
-NBT	12.5	mg.

2. Add distilled water to 50.0 ml.

3. After incubate, add PMS 5.0 mg

Appendix 1. (ctd.)

VII. Malic enzyme (ME)

1. Mixed the follows chemicals together

-1.0 M Sodium malate pH 7.0	0.5	ml
-0.1 M Magnesium chloride	1.25	ml
-1.0 M Tris-HCl pH 8.5	2.5	ml
-$NADP^+$	10.0	mg
-NBT	7.5	mg

2. Add distilled water to 50.0 ml
3. Add PMS 5.0 mg
4. After incubate, add PMS 5.0 mg

VIII. Phosphogluconate dehydrogenase (PGD)

1. Mixed the follows chemicals together

-0.2 M Tris-HCl pH 8.5	20.0	ml
-Sodium (or Barium) - 6 phosphogluconate	3.0	mg
-Magnesium chloride	28.0	mg
-$NADP^+$	7.0	mg
-NBT	7.0	mg

2. After incubate, add PMS 5.0 mg

Appendix 1. (ctd.)

IX. Superoxide dismutase (SOD)

1. Mixed the follows chemicals together

-0.2 M Tris-HCl pH 8.0	50.0	ml
-NBT	20.0	mg
-PMS	5.0	mg

2. During staining, exposure to strong light at room temperature 30 min

Appendix 2. Allele frequencies of 12 enzyme loci in 43 populations of the *Bactrocera tau* complex (referred to Table 2) and populations 44-46 are *B. cucurbitae* (=outgroup species). N= sample size.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Aat														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
111	.000	.000	.008	.000	.017	.000	.000	.000	.000	.008	.000	.000	.000	.000
100	.000	.017	.000	.110	.000	.000	.010	.000	.000	.008	.000	.033	.000	.000
82	1.000	.983	.975	.890	.950	.975	.968	1.000	.983	.984	1.000	.959	1.000	.988
67	.000	.000	.017	.000	.033	.025	.022	.000	.017	.000	.000	.008	.000	.012
Adh-1														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
66	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Adh-2														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
71	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Adh-3														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
-20	.000	.000	.000	.000	.000	.000	.009	.000	.000	.000	.000	.033	.000	.000
-100	.200	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
-71	.000	.069	.100	.000	.000	.000	.036	.000	.000	.050	.000	.333	.015	.000
-129	.800	.931	.900	1.000	1.000	1.000	.928	1.000	1.000	.950	1.000	.634	.986	1.000
-171	.000	.000	.000	.000	.000	.000	.027	.000	.000	.000	.000	.000	.000	.000

Appendix 2. (ctd.)

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14
G3pdh														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
118	.000	.000	.000	.010	.017	.000	.036	.000	.000	.008	.000	.000	.000	.000
87	1.000	1.000	1.000	.990	.983	1.000	.964	1.000	1.000	.984	1.000	1.000	1.000	1.000
63	.000	.000	.000	.000	.000	.000	.000	.000	.000	.008	.000	.000	.000	.000
Gpi														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
113	.000	.000	.000	.000	.000	.000	.004	.025	.008	.000	.000	.008	.000	.000
108	.050	.000	.025	.000	.000	.000	.014	.000	.017	.008	.017	.008	.000	.000
95	.950	.983	.975	1.000	1.000	1.000	.982	.975	.975	.992	.975	.984	1.000	1.000
86	.000	.017	.000	.000	.000	.000	.000	.000	.000	.000	.008	.000	.000	.000
Idh														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
114	.000	.000	.017	.000	.000	.012	.004	.025	.017	.008	.017	.017	.000	.000
109	.050	.000	.000	.000	.000	.000	.004	.025	.050	.008	.017	.000	.000	.000
96	.950	1.000	.983	1.000	1.000	.988	.978	.950	.933	.984	.958	.983	1.000	1.000
84	.000	.000	.000	.000	.000	.000	.014	.000	.000	.000	.008	.000	.000	.000
Mdh														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
135	.000	.000	.008	.000	.033	.037	.023	.000	.000	.008	.042	.000	.000	.000
129	1.000	1.000	.992	1.000	.942	.963	.964	1.000	.959	.992	.958	1.000	1.000	1.000
113	.000	.000	.000	.000	.000	.000	.009	.000	.033	.000	.000	.000	.000	.000
107	.000	.000	.000	.000	.025	.000	.004	.000	.008	.000	.000	.000	.000	.000

Appendix 2. (ctd.)

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Me														
N	20	10	60	50	60	40	109	20	60	60	60	60	69	40
113	.000	.000	.000	.000	.000	.000	.000	.050	.008	.000	.000	.000	.000	.000
108	.000	.000	.017	.000	.000	.000	.005	.000	.017	.008	.008	.008	.000	.000
97	1.000	1.000	.983	1.000	1.000	1.000	.990	.950	.975	.992	.992	.992	1.000	1.000
86	.000	.000	.000	.000	.000	.000	.005	.000	.000	.000	.000	.000	.000	.000
Pgd-1														
N	20	29	60	50	60	40	111	20	60	60	60	60	69	40
136	.000	.069	.000	.000	.008	.012	.004	.025	.000	.017	.000	.025	.007	.000
122	.950	.931	.983	1.000	.950	.988	.987	.925	1.000	.975	1.000	.975	.978	1.000
115	.000	.000	.000	.000	.017	.000	.009	.050	.000	.000	.000	.000	.000	.000
107	.050	.000	.017	.000	.025	.000	.000	.000	.000	.000	.000	.000	.015	.000
95	.000	.000	.000	.000	.000	.000	.000	.000	.000	.008	.000	.000	.000	.000
Pgd-2														
N	20	29	60	50	60	38	111	20	60	60	60	60	69	40
113	.050	.000	.017	.000	.000	.040	.009	.050	.025	.008	.033	.033	.000	.000
97	.950	1.000	.983	1.000	1.000	.934	.964	.950	.975	.992	.959	.950	1.000	1.000
92	.000	.000	.000	.000	.000	.000	.013	.000	.000	.000	.000	.000	.000	.000
84	.000	.000	.000	.000	.000	.026	.014	.000	.000	.000	.008	.017	.000	.000
Sod														
N	20	29	59	50	60	40	111	20	59	60	60	60	69	40
244	.000	.000	.000	.000	.000	.000	.005	.000	.000	.000	.000	.000	.000	.000
232	.925	.931	.805	.940	.950	.825	.824	.950	.915	.942	.850	.817	.971	.888
176	.075	.069	.195	.060	.050	.175	.153	.050	.085	.058	.150	.183	.029	.112
165	.000	.000	.000	.000	.000	.000	.014	.000	.000	.000	.000	.000	.000	.000
100	.000	.000	.000	.000	.000	.000	.004	.000	.000	.000	.000	.000	.000	.000

Appendix 2. (ctd.)

Population	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Aat														
N	38	40	40	63	33	21	21	23	84	38	29	85	49	90
111	.000	.012	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
100	.000	.000	.050	.000	.000	.000	.024	.022	.006	.000	.000	.006	.010	.000
90	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.010	.000
82	1.000	.988	.950	.992	1.000	1.000	.976	.956	.994	1.000	1.000	.982	.970	1.000
67	.000	.000	.000	.008	.000	.000	.000	.022	.000	.000	.000	.012	.010	.000
Adh-1														
N	38	40	40	63	33	21	21	23	84	38	29	85	50	90
66	1.000	1.000	1.000	1.000	1.000	1.000	.952	1.000	1.000	1.000	1.000	1.000	1.000	.972
36	.000	.000	.000	.000	.000	.000	.048	.000	.000	.000	.000	.000	.000	.028
Adh-2														
N	38	40	40	63	33	21	21	23	84	38	29	85	50	90
71	1.000	1.000	1.000	1.000	1.000	1.000	.952	1.000	1.000	1.000	1.000	1.000	1.000	.972
43	.000	.000	.000	.000	.000	.000	.048	.000	.000	.000	.000	.000	.000	.028
Adh-3														
N	38	40	40	63	25	21	21	23	84	38	29	85	50	90
150	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.060	.000
-20	.000	.000	.000	.000	.240	.095	.024	.000	.012	.000	.000	.000	.000	.000
-71	.000	.050	.000	.000	.000	.000	.190	.000	.107	.000	.034	.117	.120	.000
-100	.000	.000	.000	.000	.000	.476	.000	.000	.000	.000	.000	.000	.000	.000
-129	1.000	.950	1.000	1.000	.740	.429	.786	1.000	.845	1.000	.966	.871	.820	1.000
-171	.000	.000	.000	.000	.020	.000	.000	.000	.036	.000	.000	.012	.000	.000
G3pdh														
N	38	40	40	63	33	21	21	23	84	38	29	85	50	90
118	.000	.000	.000	.000	.000	.000	.000	.022	.000	.000	.000	.000	.000	.000
87	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.978	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 2. (ctd.)

Population	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Gpi														
N	38	40	40	63	33	21	21	23	84	38	29	85	50	90
108	.000	.000	.000	.000	.000	.000	.024	.000	.006	.000	.000	.000	.000	.000
95	1.000	1.000	1.000	1.000	1.000	1.000	.976	.000	.994	1.000	1.000	.988	1.000	.994
86	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.012	.000	.006
Idh														
N	38	40	40	63	33	21	21	23	84	38	29	85	50	90
125	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.017
114	.000	.000	.000	.000	.000	.000	.000	.000	.012	.000	.000	.000	.000	.000
109	.000	.000	.000	.000	.000	.000	.024	.022	.000	.000	.000	.000	.000	.000
96	1.000	1.000	1.000	1.000	1.000	1.000	.976	.978	.988	1.000	1.000	.977	1.000	.972
84	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.023	.000	.011
Mdh														
N	38	40	40	63	33	21	21	23	84	38	29	85	50	90
135	.000	.000	.062	.000	.000	.000	.000	.022	.024	.000	.000	.000	.000	.033
129	1.000	1.000	.938	1.000	.924	1.000	1.000	.978	.964	1.000	1.000	.988	1.000	.956
113	.000	.000	.000	.000	.076	.000	.000	.000	.000	.000	.000	.006	.000	.000
107	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.006	.000	.011
95	.000	.000	.000	.000	.000	.000	.000	.000	.012	.000	.000	.000	.000	.000
Me														
N	38	40	40	63	33	21	21	23	84	38	29	85	50	90
108	.000	.000	.000	.000	.000	.000	.024	.000	.994	1.000	1.000	.977	1.000	1.000
97	1.000	1.000	1.000	1.000	1.000	1.000	.976	1.000	.006	.000	.000	.000	.000	.000
86	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.023	.000	.000

Appendix 2. (ctd.)

Population	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Pgd-1														
N	38	40	40	63	33	21	21	23	84	38	29	85	50	90
136	.079	.012	.000	.000	.000	.000	.000	.000	.024	.000	.000	.000	.000	.000
128	.000	.000	.000	.008	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
122	.908	.988	1.000	.992	.985	1.000	1.000	1.000	.928	.868	1.000	.970	.950	.972
115	.013	.000	.000	.000	.000	.000	.000	.000	.018	.000	.000	.018	.020	.006
107	.000	.000	.000	.000	.015	.000	.000	.000	.030	.066	.000	.012	.030	.022
95	.000	.000	.000	.000	.000	.000	.000	.000	.000	.066	.000	.000	.000	.000
Pgd-2														
N	38	40	40	63	33	21	21	23	84	38	29	85	50	90
113	.000	.000	.000	.000	.000	.000	.024	.022	.006	.000	.000	.000	.000	.011
97	1.000	1.000	1.000	1.000	1.000	1.000	.976	.978	.994	1.000	1.000	.982	1.000	.989
84	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.018	.000	.000
Sod														
N	38	40	40	35	25	21	21	23	84	38	29	85	50	90
275	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.012	.000	.000
244	.000	.000	.000	.000	.000	.000	.000	.000	.006	.882	.000	.018	.000	.000
232	.921	.913	.938	.943	.860	1.000	.881	.783	.827	.000	.828	.770	.800	.833
176	.066	.087	.062	.028	.140	.000	.119	.217	.143	.000	.138	.200	.180	.156
165	.013	.000	.000	.029	.000	.000	.000	.000	.024	.013	.034	.000	.020	.011
100	.000	.000	.000	.000	.000	.000	.000	.000	.000	.105	.000	.000	.000	.000

Appendix 2. (ctd.)

Population	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
Aat															
N	48	71	64	18	34	49	0	0	32	60	51	33	58	60	47
122	.021	.007	.078	.000	.000	.031	.000	.000	.000	.000	.000	.000	.000	.000	.000
111	.000	.000	.000	.028	.000	.000	.000	.000	.000	.000	.000	.015	.000	.000	.000
100	.604	.845	.758	.639	.073	.326	.000	.000	.000	.000	.961	.985	.008	.000	.000
90	.375	.148	.164	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.008	.000
82	.000	.000	.000	.333	.927	.643	.000	.000	.969	.975	.039	.000	.983	.975	.989
67	.000	.000	.000	.000	.000	.000	.000	.000	.031	.025	.000	.000	.009	.017	.011
Adh-1															
N	-	-	-	18	34	58	12	15	-	-	51	33	-	-	-
100	-	-	-	1.000	1.000	1.000	1.000	1.000	-	-	.000	.000	-	-	-
69	-	-	-	.000	.000	.000	.000	.000	-	-	1.000	.515	-	-	-
66	-	-	-	.000	.000	.000	.000	.000	-	-	.000	.485	-	-	-
Adh-2															
N				18	34	58	12	15	-	-	51	33	-	-	-
100	-	-	-	1.000	1.000	1.000	1.000	1.000	-	-	.000	.000	-	-	-
74	-	-	-	.000	.000	.000	.000	.000	-	-	1.000	.515	-	-	-
71	-	-	-	.000	.000	.000	.000	.000	-	-	.000	.485	-	-	-

Appendix 2. (ctd.)

Population	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
Adh-3															
N	48	71	70	0	27	49	0	0	32	60	45	32	58	60	47
150	.000	.113	.186	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
50	.000	.000	.000	.000	.407	.429	.000	.000	.000	.000	.000	.000	.000	.000	.000
-20	.000	.000	.000	.000	.074	.173	.000	.000	.250	.050	.911	.000	.000	.000	.000
-71	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.056	.094	.000	.000	.000
-100	.000	.000	.000	.000	.519	.398	.000	.000	.000	.000	.000	.062	.000	.000	.000
-129	1.000	.887	.814	.000	.000	.000	.000	.000	.172	.042	.033	.750	.103	.000	.000
-171	.000	.000	.000	.000	.000	.000	.000	.000	.547	.875	.000	.094	.897	1.000	1.000
-190	.000	.000	.000	.000	.000	.000	.000	.000	.031	.033	.000	.000	.000	.000	.000
G3pdh															
N	48	71	70	18	34	58	12	15	32	60	51	33	58	60	47
118	.000	.000	.000	.000	.000	.000	.000	.000	.000	.033	.000	.000	.017	.000	.000
87	1.000	1.000	.993	1.000	1.000	1.000	1.000	1.000	1.000	.967	1.000	1.000	.974	.950	1.000
63	.000	.000	.007	.000	.000	.000	.000	.000	.000	.000	.000	.000	.009	.050	.000
Gpi															
N	48	36	70	18	34	58	12	15	32	60	51	33	58	60	47
130	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.009	.000	.032
122	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.991	1.000	.968
108	.000	.000	.000	.000	.000	.000	.000	.000	.000	.075	1.000	.515	.000	.000	.000
100	.000	.000	.000	1.000	1.000	1.000	1.000	1.000	.000	.017	.000	.000	.000	.000	.000
95	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.908	.000	.485	.000	.000	.000
86	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000

Appendix 2. (ctd.)

Population	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
Idh															
N	48	71	70	18	34	58	12	15	31	60	51	33	58	60	47
125	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.015	1.000	1.000	1.000
114	.000	.000	.000	.000	.000	.000	.000	.000	.032	.000	.000	.000	.000	.000	.000
109	.000	.000	.000	.000	.088	.000	.000	.000	.000	.083	.000	.000	.000	.000	.000
100	.000	.000	.000	1.000	.912	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000	.000
96	.000	.000	.000	.000	.000	.000	.000	.000	.920	.900	1.000	.970	.000	.000	.000
84	.000	.000	.000	.000	.000	.000	.000	.000	.016	.017	.000	.015	.000	.000	.000
74	.000	.000	.000	.000	.000	.000	.000	.000	.032	.000	.000	.000	.000	.000	.000
Mdh															
N	48	71	70	18	34	58	12	15	32	40	51	33	58	60	10
163	.000	.000	.000	.083	.000	.000	.000	.000	.000	.000	.000	.000	.017	.000	.000
135	.000	.000	.000	.000	.000	.000	.000	.133	.000	.000	.000	.000	.000	.000	.000
129	.000	.000	.000	.917	1.000	1.000	1.000	.834	.078	.000	.019	.000	.983	1.000	1.000
113	.000	.000	.000	.000	.000	.000	.000	.033	.000	.000	.971	.849	.000	.000	.000
100	.000	.000	.000	.000	.000	.000	.000	.000	.906	1.000	.000	.000	.000	.000	.000
88	.000	.000	.000	.000	.000	.000	.000	.000	.016	.000	.010	.151	.000	.000	.000
20	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
Me															
N	48	71	70	18	34	58	12	15	18	41	15	33	58	60	47
130	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.032
122	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	1.000	.968
113	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
108	.000	.000	.000	.000	.000	.000	.000	.000	.000	.061	.000	.000	.000	.000	.000
100	.000	.000	.000	1.000	1.000	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000	.000
97	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.927	1.000	1.000	.000	.000	.000
86	.000	.000	.000	.000	.000	.000	.000	.000	.000	.012	.000	.000	.000	.000	.000

Appendix 2. (ctd.)

Population	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
Pgd-1															
N	40	67	0	18	34	58	12	15	32	60	51	33	58	60	47
136	.000	.097	.000	.000	.000	.009	.000	.000	.000	.008	.000	.000	.000	.050	.011
128	.000	.254	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.008	.000
122	.750	.440	.000	1.000	.809	.965	.792	1.000	1.000	.967	1.000	1.000	1.000	.934	.989
115	.107	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
107	.095	.209	.000	.000	.191	.009	.208	.000	.000	.025	.000	.000	.000	.008	.000
100	.000	.000	.000	.000	.000	.017	.000	.000	.000	.000	.000	.000	.000	.000	.000
90	.024	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
68	.024	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
Pgd-2															
N	48	71	70	18	34	58	12	15	32	37	51	33	58	60	47
122	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	1.000	1.000
113	.000	.000	.000	1.000	1.000	1.000	1.000	1.000	.000	.108	.000	.000	.000	.000	.000
97	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.892	.000	.000	.000	.000	.000
84	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
Sod															
N	48	71	70	18	34	58	12	15	32	60	51	33	58	60	47
294	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
275	.000	.000	.000	.000	.009	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
272	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
244	.000	.000	.000	.000	.029	.035	.000	.000	.000	.000	.000	.000	.000	.000	.000
232	.000	.000	.000	1.000	.971	.940	1.000	1.000	.000	.000	.000	.000	.595	.742	.723
176	.000	.000	.000	.000	.000	.017	.000	.000	.000	.000	.980	.970	.284	.250	.266
165	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.121	.008	.011
110	.000	.000	.000	.000	.000	.000	.000	.000	.438	1.000	.000	.000	.000	.000	.000
100	.000	.000	.000	.000	.000	.000	.000	.000	.562	.000	.020	.030	.000	.000	.000

Appendix 2. (ctd.)

Population	44	45	46
Aat			
N	37	38	37
82	1.000	1.000	1.000
Adh-3			
N	37	38	37
50	1.000	1.000	1.000
G3pdh			
N	37	38	37
87	1.000	1.000	1.000
Idh			
N	37	38	37
114	.000	.026	.014
109	.000	.000	.013
96	.973	.921	.865
84	.027	.053	.108
Mdh			
N	37	37	37
129	.987	.946	.973
100	.013	.054	.027

Population	44	45	46
Me			
N	37	38	37
113	.013	.000	.000
108	.000	.000	.027
97	.973	.947	.865
86	.014	.053	.108
Gpi			
N	37	38	37
95	1.000	.947	1.000
86	.000	.053	.000
Pgd-1			
N	37	38	37
100	1.000	1.000	1.000
Pgd-2			
N	37	38	37
113	.013	.013	.041
97	.973	.947	.851
84	.014	.040	.108
Sod			
N	37	38	37
294	1.000	1.000	1.000

Appendix 3 Electrophoretic key of the *Bactrocera tau* complex.

In order to use the key, the unknown specimen is electrophoresed side by side with standard i.e., *B. dorsalis* (mobility=100).

1. ***Glyceral-3-phosphate dehydrogenase*** (Appendix 4V)

G3pdh $^{87/87}$	(*B. tau* complex) 2
G3pdh mobility other than that of *G3pdh* $^{87/87}$	not *B. tau* complex

2. ***Alcohol dehydrogenase-1*** (Appendix 4II)

Adh-1 mobility same as standard	Species D
Adh-1 mobility slower than standard	3

3. ***Isocitrate dehydrogenase*** (Appendix 4VII)

Idh $^{125/125}$	4
Idh mobility slower than *Idh* $^{125/125}$	5

4. ***Superoxide dismutase*** (Appendix 4XII)

Sod $^{272/272}$	Species C
Sod $^{232/232}$ and *Sod* $^{176/176}$	Species I

5. ***Malate dehydrogenase*** (Appendix 4VIII)

Mdh mobility same as standard	Species E
Mdh mobility faster or slower than standard	6

6. ***Superoxide dismutase*** (Appendix 4XII)

Sod $^{110/110}$	Species E
Sod mobility faster or slower than *Sod* $^{110/110}$	7

Appendix 3 (ctd.)

7. *Alcohol dehydrogenase-2* (Appendix 4III)

Adh-2 $^{71/71}$ or *Adh-2* $^{74/74}$ 8

Adh-2 mobility same as standard Species D

8. *Aspartate aminotransferase* (Appendix 4I)

Aat $^{82/82}$ (=*B. tau*) Species A

Aat mobility same as standard 9

9.*Alcohol dehydrogenase-3 (Appendix 4IV)

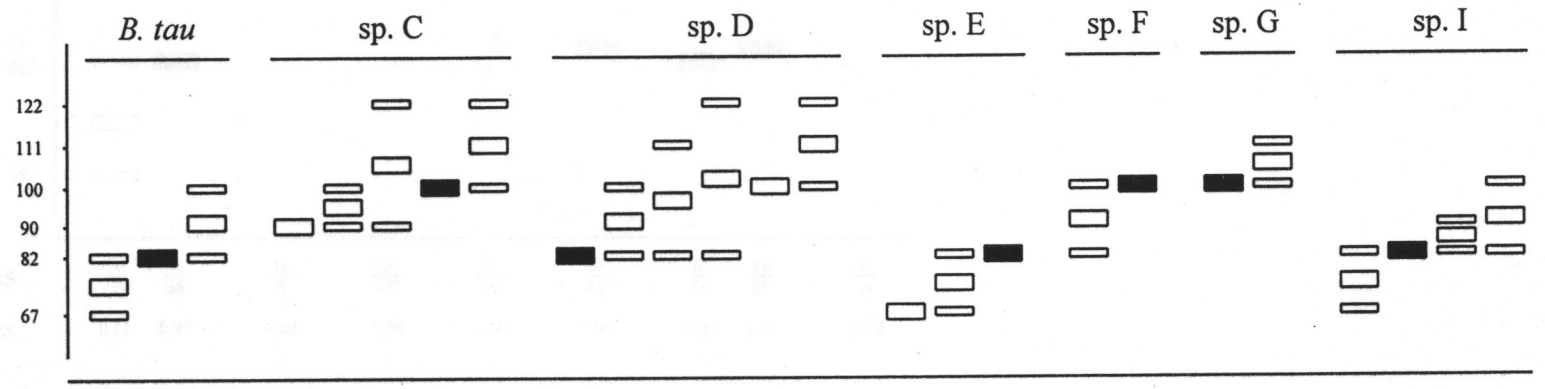
Adh-3 $^{-20/-20}$, *Adh-3* $^{-20/-71}$ or *Adh-3* $^{-20/-129}$ Species F

Adh-3 $^{-129/-129}$, *Adh-3* $^{-171/-171}$ or *Adh-3* $^{-100/-100}$ Species G

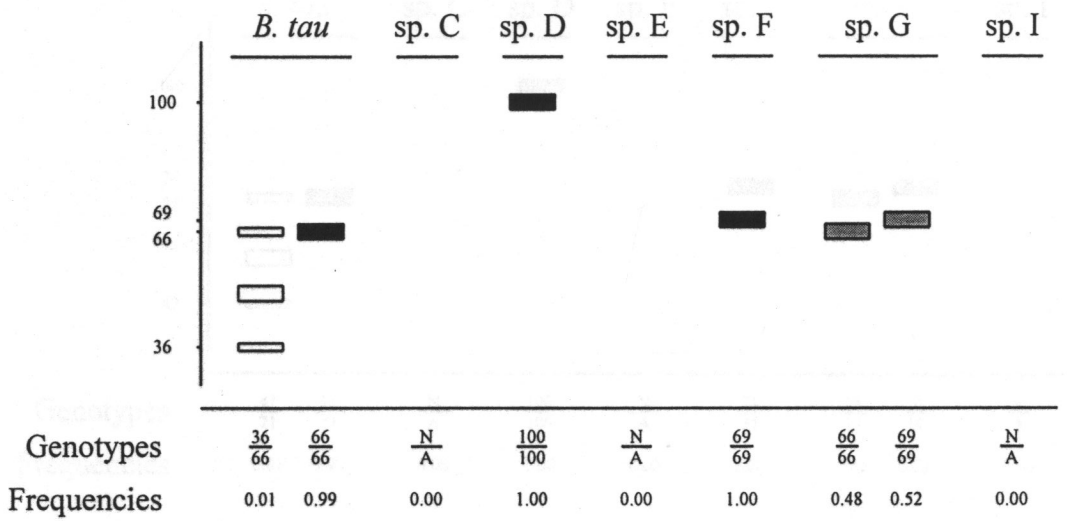
*Comments: There is 2% probability that a *Adh-3* $^{-129/-129}$ homozygote will occur in species G. When *Adh-3* $^{-71/-71}$ homozygote occur, it will be species F with 4% probability and species G with 9% probability.

Appendix 4. Diagrams of electromorphs showing genotypic patterns and frequencies of each locus investigated in the *Bactrocera tau* complex.

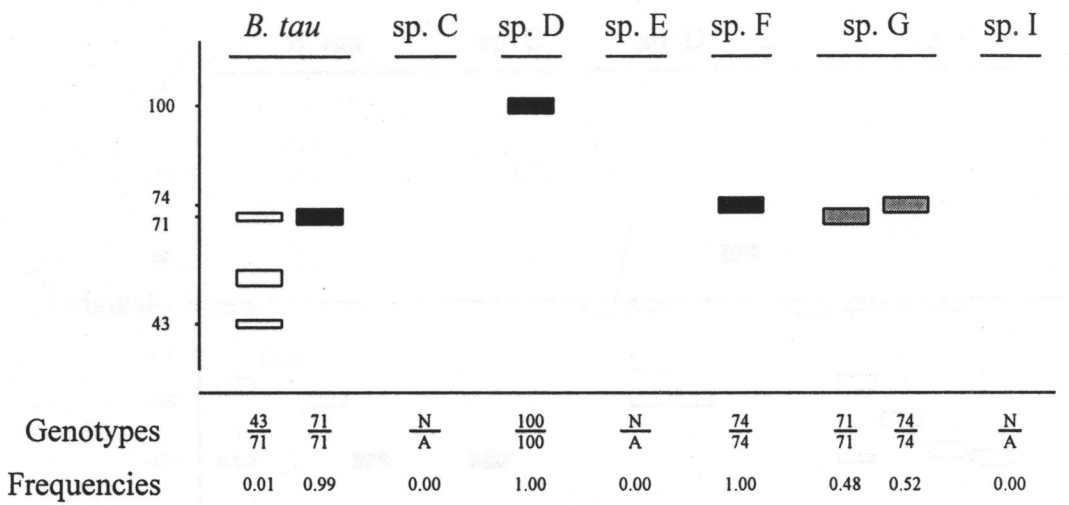
I. Aspartate aminotransferase (*Aat*)



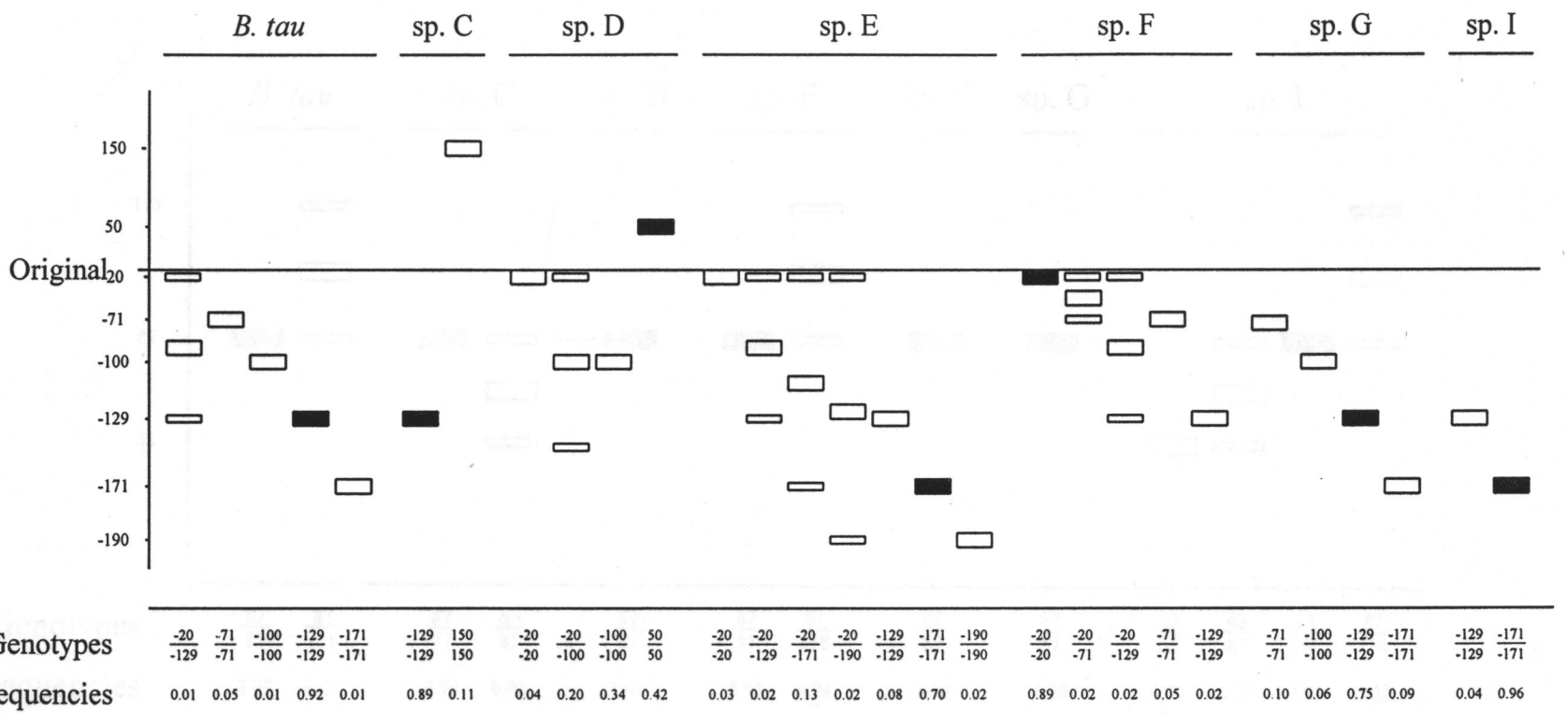
II. Alcohol dehydrogenase (*Adh-1*)



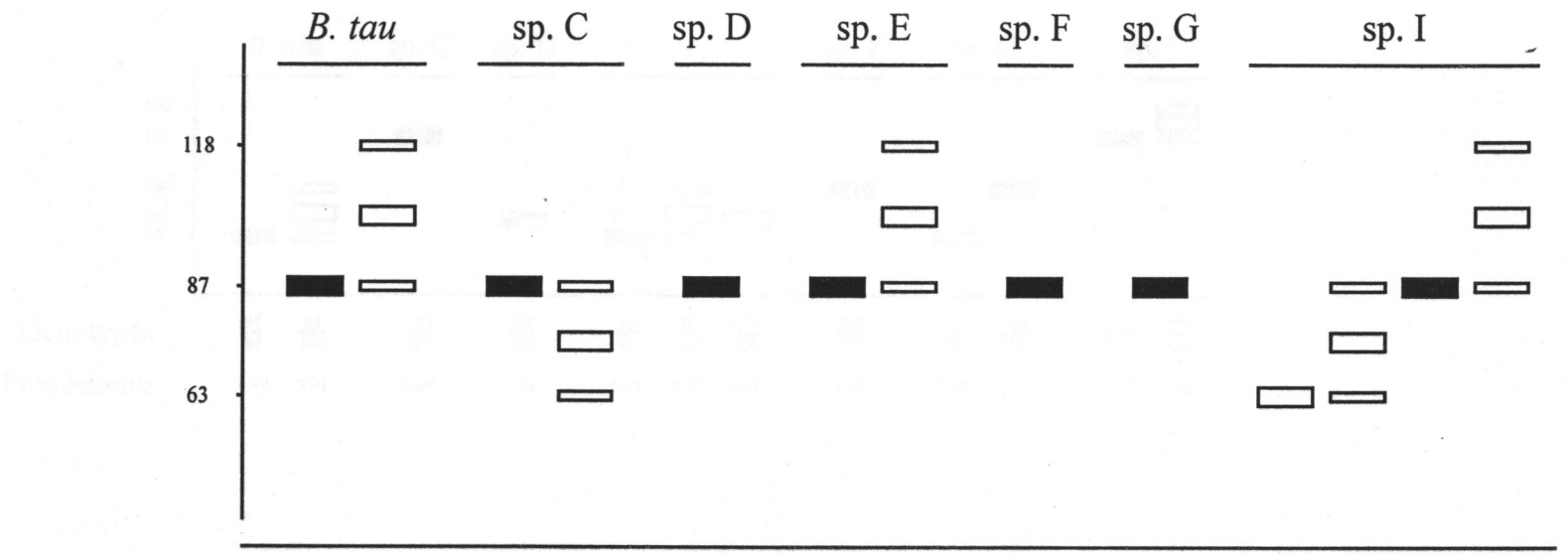
III. Alcohol dehydrogenase (*Adh-2*)



IV. Alcohol dehydrogenase (*Adh-3*)

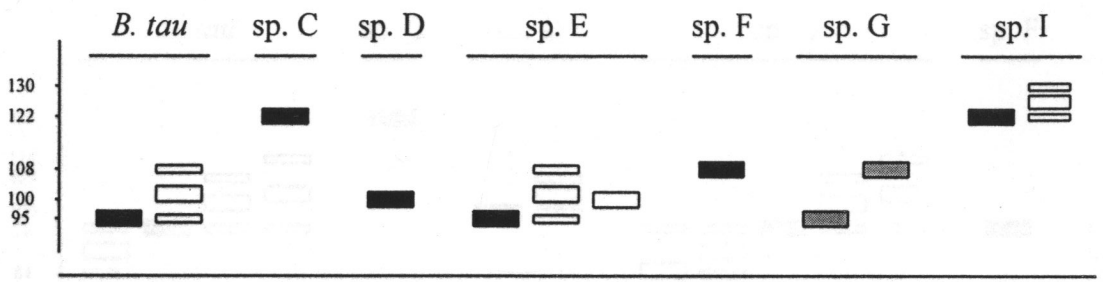


V. Glyceral-3-phosphate dehydrogenase (*G3pdh*)



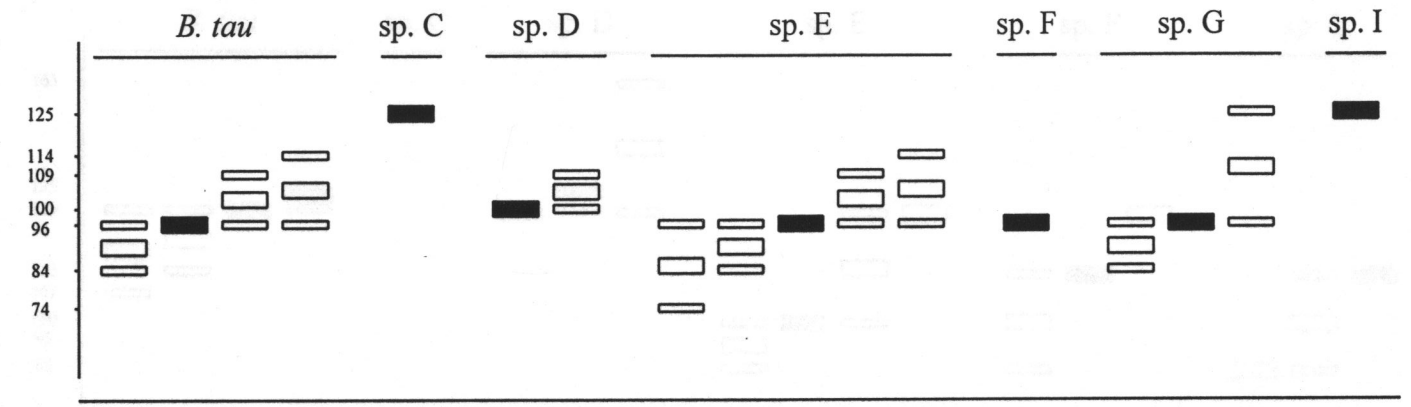
Genotypes	87/87	87/118	87/87	63/87	87/87	87/87	87/118	87/87	87/87	63/63	63/87	87/87	87/118
Frequencies	0.99	0.01	0.99	0.01	1.00	0.96	0.04	1.00	1.00	0.02	0.01	0.96	0.01

VI. Glucose phosphate isomerase (*Gpi*)



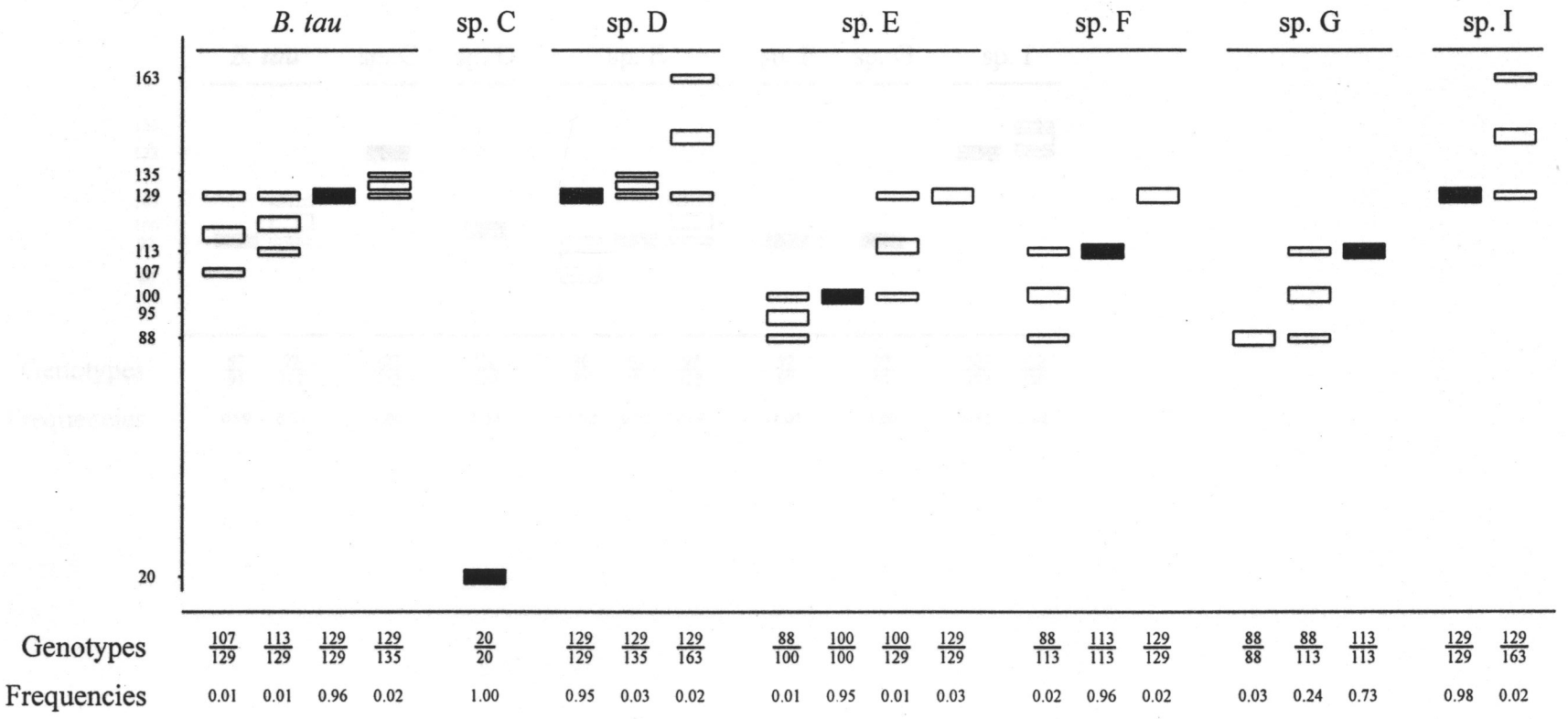
	B. tau		sp. C	sp. D	sp. E			sp. F	sp. G		sp. I	
Genotypes	95/95	95/108	122/122	100/100	95/95	95/108	100/100	108/108	95/95	108/108	122/122	122/130
Frequencies	0.99	0.01	1.00	1.00	0.89	0.10	0.01	1.00	0.48	0.52	0.98	0.02

VII. Isocitrate dehydrogenase (*Idh*)

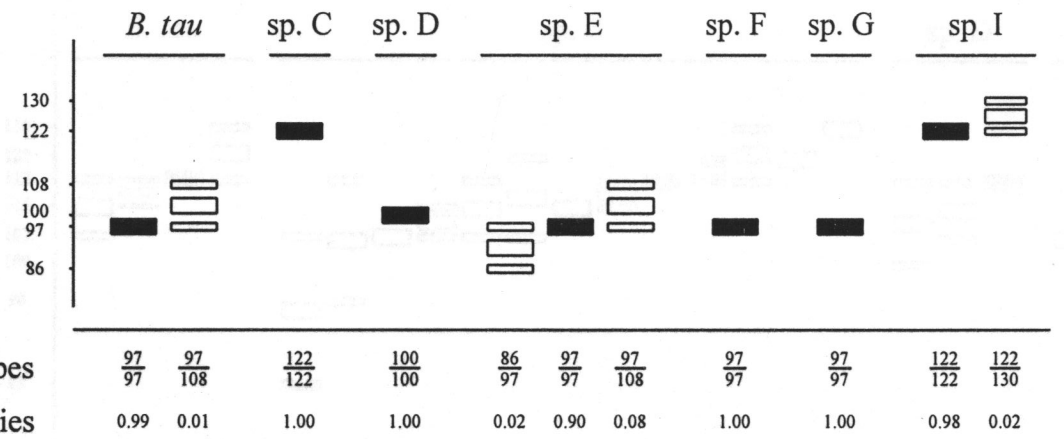


Genotypes	84/96	96/96	96/109	96/114	125/125	100/100	100/109	74/96	84/96	96/96	96/109	96/114	96/96	84/96	96/96	96/125	125/125
Frequencies	0.01	0.97	0.01	0.01	1.00	0.96	0.04	0.02	0.03	0.82	0.11	0.02	1.00	0.03	0.94	0.03	1.00

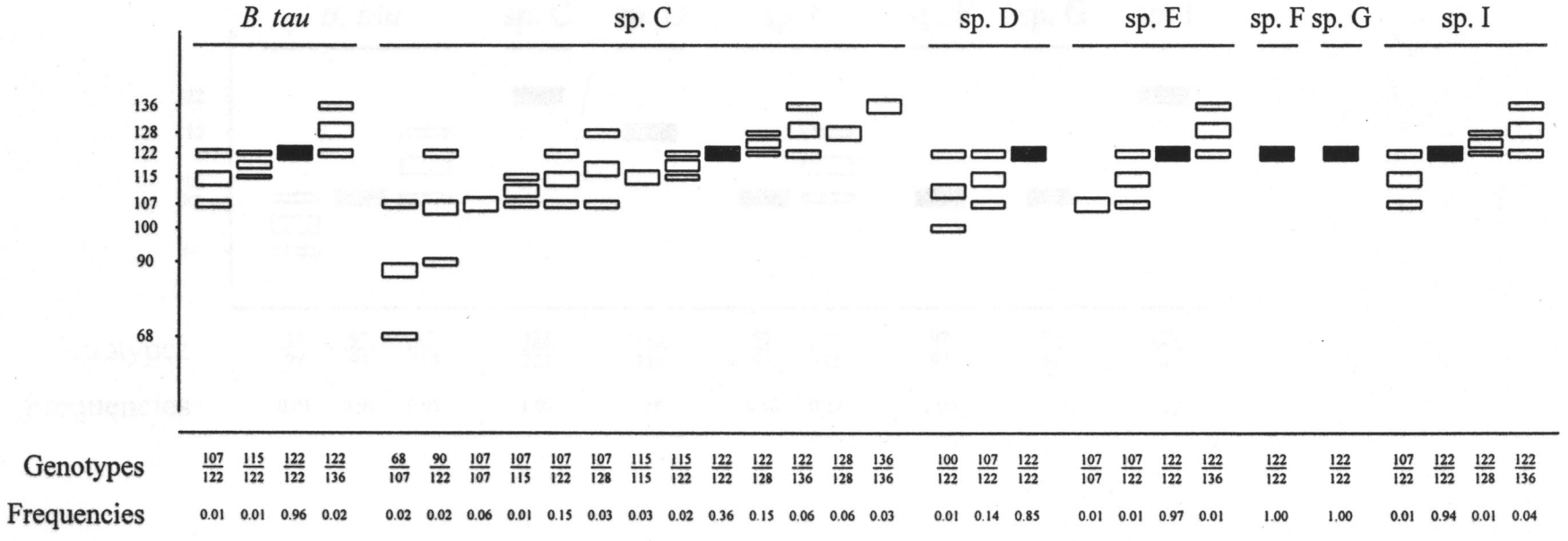
VIII. Malate dehydrogenase (*Mdh*)



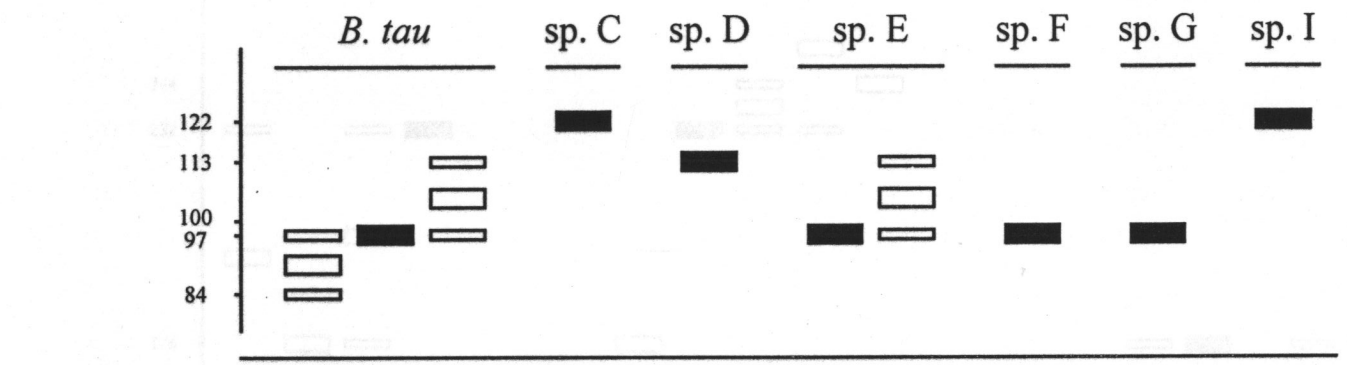
IX. Malic enzyme (*Me*)



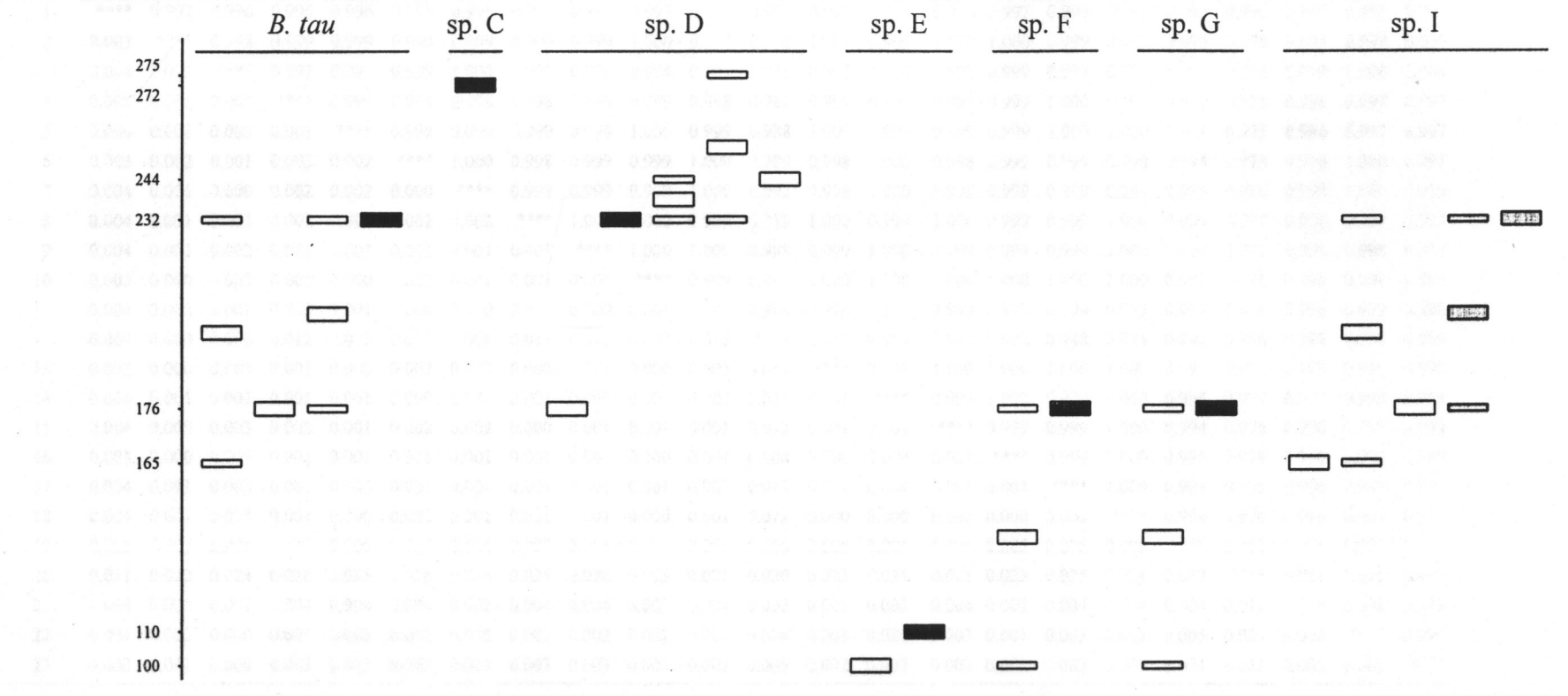
X. Phosphogluconate dehydrogenase (*Pgd-1*)



XI. Phosphogluconate dehydrogenase (*Pgd-2*)



XII. Superoxide dismutase (*Sod*)



Genotypes	165/232	176/176	176/232	232/232	272/272	176/176	232/232	232/244	232/275	244/244	100/100	110/110	100/176	176/176	100/176	176/176	165/165	165/232	176/176	176/232	232/232
Frequencies	0.01	0.02	0.19	0.78	1.00	0.01	0.96	0.01	0.01	0.01	0.20	0.80	0.04	0.96	0.06	0.94	0.02	0.05	0.10	0.34	0.49

Appendix 5. Nei's unbiased genetic identity (above diagonal) and unbiased genetic distance (below diagonal) from 12 enzyme loci of 43 population of the *Bactrocera tau* complex. Three population of *B. cucurbitae* (population nos. 44, 45 and 46) are used as outgroup species.

Pop. ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	****	0.997	0.996	0.995	0.996	0.996	0.996	0.997	0.997	0.997	0.996	0.991	0.997	0.996	0.996	0.997	0.996	0.997	0.995	0.990	0.997	0.996	0.997
2	0.003	****	0.998	0.999	0.999	0.998	0.999	0.999	0.999	1.000	0.999	0.992	1.000	0.999	1.000	1.000	0.999	0.999	0.995	0.978	0.998	0.998	0.999
3	0.004	0.002	****	0.997	0.997	0.999	1.000	0.997	0.998	0.998	0.999	0.995	0.997	0.999	0.998	0.999	0.997	0.997	0.995	0.976	0.999	1.000	1.000
4	0.005	0.001	0.003	****	0.999	0.998	0.998	0.998	0.999	0.999	0.998	0.988	0.999	0.999	0.999	0.999	1.000	0.999	0.993	0.975	0.996	0.997	0.997
5	0.004	0.001	0.003	0.001	****	0.998	0.999	0.999	0.999	1.000	0.999	0.988	1.000	0.999	0.999	0.999	1.000	1.000	0.994	0.975	0.996	0.997	0.997
6	0.005	0.002	0.001	0.002	0.002	****	1.000	0.998	0.999	0.999	1.000	0.989	0.998	1.000	0.998	0.999	0.999	0.998	0.994	0.973	0.996	1.000	0.998
7	0.004	0.001	0.000	0.002	0.002	0.000	****	0.998	0.999	0.999	1.000	0.992	0.998	1.000	0.998	0.999	0.999	0.999	0.996	0.976	0.998	1.000	0.999
8	0.004	0.001	0.003	0.002	0.001	0.002	0.002	****	1.000	1.000	0.999	0.988	1.000	0.999	1.000	0.999	0.999	1.000	0.993	0.975	0.996	0.997	0.997
9	0.004	0.001	0.002	0.001	0.001	0.001	0.001	0.001	****	1.000	1.000	0.988	0.999	1.000	0.999	0.999	0.999	1.000	0.994	0.975	0.996	0.998	0.997
10	0.003	0.000	0.002	0.001	0.000	0.002	0.001	0.001	0.001	****	0.999	0.991	1.000	1.000	1.000	1.000	1.000	1.000	0.995	0.978	0.998	0.998	0.999
11	0.004	0.002	0.001	0.002	0.001	0.000	0.000	0.001	0.000	0.001	****	0.989	0.999	1.000	0.999	0.999	0.999	0.999	0.994	0.973	0.996	0.999	0.998
12	0.009	0.008	0.006	0.012	0.013	0.011	0.008	0.013	0.012	0.009	0.011	****	0.989	0.989	0.988	0.992	0.988	0.988	0.992	0.980	0.998	0.994	0.995
13	0.003	0.000	0.003	0.001	0.000	0.002	0.002	0.000	0.001	0.000	0.001	0.011	****	0.999	1.000	1.000	1.000	1.000	0.994	0.977	0.997	0.997	0.998
14	0.004	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.000	0.000	0.000	0.011	0.001	****	0.999	1.000	0.999	1.000	0.995	0.975	0.997	0.999	0.998
15	0.004	0.000	0.003	0.002	0.001	0.002	0.002	0.000	0.001	0.001	0.001	0.012	0.001	0.001	****	0.999	0.999	1.000	0.994	0.975	0.996	0.997	0.998
16	0.003	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001	0.008	0.000	0.000	0.001	****	0.999	1.000	0.995	0.978	0.998	0.999	0.999
17	0.004	0.001	0.003	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.012	0.001	0.001	0.001	0.001	****	1.000	0.994	0.975	0.996	0.998	0.997
18	0.004	0.001	0.003	0.001	0.000	0.002	0.001	0.001	0.001	0.000	0.001	0.012	0.000	0.000	0.001	0.000	0.000	****	0.994	0.976	0.996	0.997	0.998
19	0.005	0.005	0.005	0.007	0.006	0.006	0.004	0.007	0.006	0.005	0.006	0.008	0.006	0.005	0.006	0.005	0.006	0.006	****	0.983	0.996	0.995	0.997
20	0.011	0.022	0.024	0.026	0.025	0.028	0.024	0.026	0.026	0.023	0.027	0.020	0.023	0.025	0.025	0.023	0.025	0.024	0.017	****	0.981	0.975	0.979
21	0.004	0.002	0.001	0.004	0.004	0.004	0.002	0.004	0.004	0.002	0.004	0.002	0.003	0.003	0.004	0.002	0.004	0.004	0.004	0.019	****	0.998	0.999
22	0.004	0.002	0.000	0.003	0.003	0.001	0.000	0.003	0.002	0.002	0.001	0.006	0.003	0.001	0.003	0.001	0.003	0.003	0.005	0.025	0.002	****	0.999
23	0.003	0.001	0.000	0.003	0.003	0.002	0.001	0.003	0.003	0.002	0.002	0.005	0.002	0.002	0.002	0.001	0.003	0.003	0.004	0.021	0.001	0.001	****

Appendix 5. (ctd.) (above diagonal)

Pop. ID	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
1	0.996	0.996	0.995	0.996	0.996	0.248	0.215	0.171	0.389	0.435	0.418	0.360	0.361	0.709	0.642	0.444	0.671	0.451	0.443	0.451	0.763	0.759	0.754
2	0.999	0.999	0.998	0.998	0.998	0.256	0.223	0.180	0.377	0.410	0.397	0.347	0.349	0.709	0.639	0.440	0.676	0.441	0.432	0.440	0.760	0.755	0.749
3	0.998	1.000	1.000	1.000	0.999	0.259	0.224	0.179	0.374	0.408	0.396	0.345	0.348	0.716	0.646	0.458	0.693	0.446	0.435	0.443	0.763	0.758	0.753
4	0.998	0.998	0.996	0.996	0.998	0.271	0.237	0.191	0.383	0.405	0.398	0.351	0.354	0.705	0.634	0.449	0.690	0.436	0.427	0.434	0.748	0.743	0.738
5	0.999	0.999	0.996	0.996	0.999	0.262	0.227	0.183	0.371	0.403	0.391	0.344	0.347	0.708	0.637	0.437	0.677	0.433	0.424	0.432	0.751	0.746	0.741
6	0.999	1.000	0.998	0.998	1.000	0.267	0.231	0.186	0.373	0.406	0.395	0.344	0.348	0.713	0.642	0.448	0.691	0.442	0.430	0.439	0.755	0.750	0.746
7	0.998	1.000	0.999	0.999	0.999	0.260	0.224	0.179	0.372	0.404	0.393	0.341	0.345	0.716	0.646	0.452	0.691	0.444	0.433	0.441	0.759	0.754	0.749
8	0.999	0.998	0.996	0.996	0.998	0.263	0.229	0.186	0.384	0.419	0.405	0.355	0.357	0.700	0.630	0.426	0.665	0.446	0.436	0.444	0.752	0.747	0.743
9	0.998	0.999	0.997	0.996	0.999	0.268	0.231	0.186	0.381	0.414	0.401	0.351	0.355	0.708	0.638	0.442	0.681	0.446	0.435	0.443	0.749	0.744	0.739
10	0.999	0.999	0.998	0.998	0.999	0.259	0.224	0.179	0.379	0.412	0.399	0.350	0.352	0.709	0.639	0.438	0.674	0.443	0.434	0.441	0.754	0.750	0.744
11	0.999	1.000	0.998	0.997	1.000	0.268	0.232	0.186	0.376	0.410	0.398	0.346	0.350	0.712	0.642	0.447	0.687	0.446	0.434	0.442	0.753	0.748	0.743
12	0.988	0.991	0.995	0.996	0.989	0.238	0.206	0.161	0.384	0.416	0.405	0.353	0.356	0.720	0.652	0.465	0.686	0.449	0.441	0.449	0.773	0.768	0.763
13	0.999	0.999	0.996	0.996	0.998	0.260	0.225	0.181	0.379	0.412	0.399	0.350	0.352	0.708	0.638	0.433	0.670	0.443	0.434	0.441	0.752	0.747	0.742
14	0.999	1.000	0.998	0.998	1.000	0.264	0.228	0.183	0.374	0.407	0.395	0.345	0.348	0.712	0.641	0.444	0.683	0.443	0.432	0.440	0.754	0.749	0.744
15	0.999	0.999	0.997	0.997	0.999	0.259	0.226	0.184	0.371	0.406	0.392	0.343	0.344	0.707	0.636	0.434	0.673	0.438	0.429	0.436	0.758	0.753	0.748
16	0.999	1.000	0.998	0.998	0.999	0.259	0.224	0.179	0.377	0.410	0.398	0.348	0.350	0.713	0.642	0.442	0.679	0.444	0.434	0.442	0.757	0.752	0.746
17	0.998	0.999	0.996	0.996	0.999	0.268	0.233	0.188	0.377	0.405	0.395	0.345	0.350	0.711	0.640	0.446	0.686	0.437	0.427	0.435	0.749	0.744	0.739
18	0.999	0.999	0.997	0.996	0.999	0.263	0.227	0.182	0.377	0.410	0.398	0.348	0.351	0.709	0.639	0.435	0.672	0.442	0.432	0.440	0.752	0.747	0.742
19	0.994	0.995	0.995	0.996	0.994	0.244	0.211	0.166	0.374	0.410	0.399	0.343	0.347	0.732	0.659	0.481	0.689	0.445	0.437	0.445	0.768	0.763	0.758
20	0.974	0.976	0.976	0.979	0.973	0.212	0.181	0.138	0.391	0.447	0.430	0.361	0.364	0.717	0.651	0.447	0.645	0.449	0.446	0.453	0.767	0.762	0.757
21	0.995	0.998	0.999	0.999	0.996	0.253	0.219	0.173	0.390	0.422	0.411	0.358	0.361	0.722	0.653	0.460	0.684	0.454	0.445	0.454	0.762	0.757	0.752
22	0.998	1.000	1.000	1.000	0.999	0.263	0.228	0.182	0.374	0.405	0.395	0.343	0.347	0.718	0.648	0.463	0.701	0.444	0.432	0.441	0.762	0.757	0.752
23	0.998	0.999	1.000	1.000	0.998	0.251	0.219	0.175	0.370	0.406	0.393	0.341	0.343	0.721	0.653	0.452	0.686	0.444	0.435	0.443	0.770	0.765	0.759

Appendix 5. (ctd.) (below diagonal)

Pop. ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
24	0.004	0.001	0.002	0.002	0.001	0.002	0.002	0.001	0.002	0.001	0.002	0.012	0.001	0.001	0.001	0.001	0.002	0.001	0.007	0.027	0.005	0.003	0.002
25	0.004	0.001	0.000	0.002	0.002	0.000	0.000	0.002	0.001	0.001	0.000	0.009	0.001	0.000	0.001	0.000	0.001	0.001	0.005	0.025	0.002	0.000	0.001
26	0.005	0.002	0.000	0.004	0.004	0.002	0.001	0.004	0.003	0.003	0.002	0.005	0.004	0.002	0.003	0.002	0.004	0.004	0.005	0.024	0.002	0.000	0.001
27	0.004	0.002	0.000	0.004	0.004	0.003	0.001	0.004	0.004	0.003	0.003	0.004	0.004	0.002	0.003	0.002	0.004	0.004	0.004	0.021	0.001	0.001	0.000
28	0.004	0.002	0.001	0.002	0.001	0.000	0.001	0.002	0.001	0.001	0.000	0.011	0.002	0.000	0.001	0.001	0.001	0.001	0.006	0.027	0.004	0.001	0.002
29	1.393	1.362	1.350	1.306	1.341	1.319	1.347	1.336	1.318	1.353	1.317	1.437	1.346	1.331	1.352	1.350	1.316	1.337	1.410	1.551	1.374	1.336	1.381
30	1.535	1.499	1.496	1.439	1.483	1.464	1.495	1.476	1.464	1.497	1.463	1.580	1.490	1.478	1.488	1.496	1.455	1.482	1.558	1.708	1.521	1.480	1.520
31	1.766	1.713	1.722	1.654	1.699	1.683	1.722	1.683	1.685	1.719	1.684	1.824	1.709	1.699	1.695	1.718	1.673	1.702	1.798	1.981	1.756	1.706	1.741
32	0.946	0.977	0.983	0.959	0.991	0.985	0.990	0.958	0.966	0.970	0.978	0.957	0.971	0.983	0.992	0.976	0.975	0.975	0.984	0.939	0.943	0.984	0.993
33	0.834	0.892	0.897	0.903	0.908	0.900	0.906	0.869	0.882	0.887	0.891	0.877	0.886	0.898	0.901	0.892	0.904	0.891	0.891	0.804	0.863	0.903	0.901
34	0.874	0.924	0.927	0.921	0.940	0.930	0.935	0.904	0.913	0.918	0.922	0.903	0.918	0.928	0.936	0.923	0.929	0.922	0.918	0.844	0.890	0.930	0.935
35	1.022	1.058	1.065	1.049	1.068	1.068	1.076	1.035	1.048	1.051	1.062	1.042	1.050	1.064	1.071	1.057	1.063	1.055	1.070	1.019	1.027	1.070	1.076
36	1.018	1.053	1.057	1.040	1.060	1.057	1.065	1.030	1.037	1.043	1.050	1.034	1.043	1.056	1.067	1.049	1.049	1.046	1.058	1.010	1.018	1.059	1.070
37	0.343	0.344	0.334	0.350	0.346	0.338	0.334	0.357	0.346	0.345	0.340	0.329	0.345	0.339	0.347	0.339	0.341	0.343	0.311	0.333	0.325	0.332	0.327
38	0.443	0.448	0.437	0.456	0.451	0.443	0.436	0.462	0.450	0.449	0.443	0.428	0.450	0.445	0.452	0.443	0.446	0.449	0.417	0.430	0.426	0.435	0.427
39	0.813	0.820	0.782	0.800	0.829	0.802	0.793	0.853	0.817	0.825	0.805	0.765	0.836	0.812	0.835	0.816	0.808	0.833	0.731	0.805	0.778	0.769	0.793
40	0.399	0.392	0.366	0.371	0.390	0.370	0.370	0.408	0.384	0.394	0.375	0.377	0.401	0.382	0.397	0.388	0.377	0.397	0.372	0.438	0.380	0.355	0.377
41	0.797	0.819	0.808	0.829	0.837	0.817	0.812	0.809	0.809	0.815	0.809	0.801	0.815	0.814	0.826	0.813	0.828	0.816	0.810	0.801	0.789	0.812	0.811
42	0.815	0.839	0.833	0.852	0.858	0.844	0.837	0.829	0.832	0.836	0.835	0.819	0.836	0.839	0.847	0.835	0.851	0.839	0.829	0.808	0.809	0.839	0.833
43	0.797	0.822	0.814	0.834	0.840	0.824	0.818	0.812	0.814	0.818	0.816	0.801	0.818	0.820	0.831	0.817	0.833	0.821	0.810	0.791	0.790	0.819	0.815
44	0.270	0.275	0.271	0.290	0.286	0.281	0.276	0.285	0.290	0.282	0.284	0.257	0.285	0.282	0.277	0.279	0.289	0.285	0.264	0.265	0.272	0.272	0.262
45	0.276	0.281	0.277	0.297	0.293	0.287	0.282	0.291	0.296	0.288	0.290	0.264	0.292	0.289	0.283	0.285	0.296	0.292	0.270	0.272	0.279	0.279	0.268
46	0.283	0.289	0.284	0.304	0.300	0.294	0.289	0.297	0.302	0.295	0.297	0.270	0.299	0.296	0.291	0.293	0.303	0.299	0.278	0.279	0.285	0.286	0.275

Appendix 5. (ctd.)

Pop. ID	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
24	****	0.999	0.997	0.997	0.999	0.257	0.225	0.185	0.366	0.402	0.388	0.338	0.338	0.707	0.636	0.436	0.676	0.435	0.425	0.433	0.762	0.758	0.752
25	0.001	****	0.999	0.999	1.000	0.262	0.226	0.181	0.371	0.406	0.393	0.341	0.344	0.717	0.646	0.449	0.687	0.444	0.432	0.441	0.760	0.755	0.749
26	0.003	0.001	****	1.000	0.998	0.257	0.222	0.178	0.370	0.405	0.393	0.340	0.343	0.719	0.649	0.457	0.694	0.446	0.435	0.443	0.767	0.763	0.758
27	0.003	0.001	0.000	****	0.998	0.251	0.218	0.175	0.371	0.405	0.393	0.342	0.344	0.720	0.650	0.457	0.691	0.443	0.432	0.440	0.771	0.766	0.761
28	0.001	0.000	0.002	0.003	****	0.269	0.233	0.188	0.371	0.406	0.393	0.341	0.344	0.718	0.647	0.449	0.689	0.445	0.433	0.441	0.756	0.751	0.746
29	1.357	1.338	1.359	1.384	1.315	****	0.983	0.962	0.218	0.167	0.193	0.170	0.184	0.216	0.191	0.228	0.330	0.638	0.613	0.612	0.097	0.098	0.099
30	1.493	1.485	1.503	1.524	1.457	0.017	****	0.981	0.207	0.149	0.175	0.151	0.155	0.184	0.162	0.225	0.320	0.613	0.591	0.589	0.098	0.099	0.101
31	1.689	1.708	1.728	1.746	1.673	0.039	0.019	****	0.159	0.111	0.132	0.111	0.111	0.135	0.116	0.178	0.264	0.581	0.562	0.558	0.100	0.102	0.103
32	1.006	0.990	0.993	0.992	0.992	1.523	1.576	1.836	****	0.944	0.973	0.970	0.974	0.255	0.248	0.241	0.263	0.391	0.393	0.401	0.204	0.203	0.210
33	0.911	0.903	0.904	0.904	0.902	1.790	1.901	2.198	0.058	****	0.989	0.938	0.932	0.293	0.285	0.179	0.188	0.429	0.432	0.439	0.296	0.297	0.305
34	0.947	0.933	0.935	0.935	0.935	1.645	1.742	2.025	0.027	0.011	****	0.954	0.955	0.284	0.273	0.224	0.229	0.417	0.418	0.426	0.275	0.275	0.283
35	1.085	1.075	1.078	1.073	1.076	1.770	1.893	2.199	0.030	0.065	0.047	****	0.994	0.206	0.201	0.171	0.185	0.356	0.361	0.367	0.187	0.186	0.194
36	1.084	1.066	1.071	1.068	1.067	1.692	1.862	2.201	0.027	0.071	0.046	0.006	****	0.227	0.221	0.193	0.209	0.360	0.363	0.371	0.171	0.171	0.178
37	0.347	0.333	0.329	0.328	0.331	1.531	1.695	2.003	1.366	1.228	1.259	1.579	1.482	****	0.951	0.523	0.615	0.399	0.388	0.399	0.589	0.586	0.572
38	0.453	0.437	0.432	0.431	0.436	1.657	1.822	2.157	1.394	1.255	1.300	1.606	1.508	0.050	****	0.472	0.562	0.406	0.400	0.411	0.537	0.536	0.523
39	0.829	0.801	0.783	0.782	0.800	1.479	1.493	1.727	1.424	1.722	1.498	1.768	1.647	0.649	0.750	****	0.873	0.225	0.210	0.220	0.339	0.335	0.318
40	0.391	0.375	0.366	0.370	0.372	1.108	1.141	1.331	1.336	1.673	1.474	1.685	1.563	0.486	0.576	0.136	****	0.259	0.235	0.247	0.500	0.496	0.482
41	0.832	0.812	0.807	0.815	0.811	0.449	0.490	0.544	0.938	0.846	0.875	1.033	1.022	0.920	0.902	1.491	1.350	****	0.997	0.998	0.282	0.283	0.288
42	0.855	0.839	0.833	0.839	0.838	0.490	0.526	0.576	0.934	0.840	0.872	1.018	1.013	0.948	0.917	1.563	1.447	0.003	****	1.000	0.277	0.278	0.283
43	0.838	0.820	0.813	0.820	0.819	0.491	0.530	0.584	0.915	0.824	0.854	1.001	0.992	0.919	0.890	1.513	1.399	0.003	0.001	****	0.282	0.283	0.288
44	0.271	0.275	0.265	0.260	0.280	2.336	2.324	2.299	1.592	1.219	1.292	1.676	1.764	0.530	0.621	1.082	0.693	1.266	1.283	1.266	****	1.000	0.998
45	0.278	0.282	0.271	0.266	0.286	2.320	2.309	2.283	1.593	1.215	1.290	1.681	1.767	0.534	0.624	1.093	0.701	1.264	1.281	1.264	0.000	****	0.999
46	0.285	0.289	0.277	0.273	0.294	2.309	2.298	2.273	1.559	1.188	1.261	1.642	1.729	0.559	0.648	1.144	0.731	1.244	1.261	1.244	0.002	0.001	****

BIOGRAPHY

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