VV BIOSYSTEMATICS OF THE *COTESIA FLAV/PES* COMPLEX

(HYM: BRACONIDAE), PARASITOIDS OF GRAMINEOUS STEMBORERS. "

By

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1994

DECLARATION

I Susan Wangari Kimani hereby declare that the work presented in this thesis is my own and has not been submitted for a degree in any other University.

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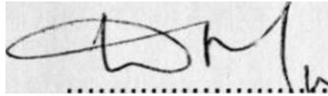

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DEDICATION

To my husband Njogu Gituiku

and our son Gituiku Njogu who

had to accept second place during

the course of this work.

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ABSTRACT

The species of the *Cotesia flavipes* complex are gregarious endoparasitoids of lepidopteran stemborers of gramineous plants. The complex consists of three morphologically similar species: C. *flavipes,* in the Indo-Australian Region, C. *sesamiae* in sub-saharan Africa, and *C. chilonis* in Japan and China.

The systematics of the *Cotesia flavipes* complex were studied using: morphology, morphometries, cuticular components pattern analysis, allozyme electrophoresis, Random Amplified Polymorphic DNA-Polymerase Chain Reaction, mating behaviour, reciprocal crossing and volatile pheromones.

The external morphological characters examined showed a high degree of intraspecific variation, particularly in colour, density of setae and surface sculpture. A survey of five morphological characters; scored from sixteen populations indicated that a combination of the following characters: the number of hairs on the scutellum, the scuto-scuteller sulcus and the rugosity on the propodeum could be used to separate the females and males in the complex. The shape of the male genitalia was valid for separating the populations of C. *flavipes* from the other two species. The male genitalia of the Mauritius *C. flavipes* population differed in various aspects from the other populations of C. *flavipes.*

Morphometric studies of eleven allopatric populations of the *Cotesia flavipes* species complex were conducted. Sixteen characters were measured. Canonical Variate analysis separated the complex into three distinct clusters with populations from Africa clustering together, populations from the Indo-Australian region and the Neotropics forming a second cluster, and material from China and Japan forming a third cluster. The Mahalanobis squared distances between the three clusters were nearly equal.

Cuticular component pattern analysis demonstrated that the pattern of cuticular components in the three species was not distinct and therefore could not be used to separate the species. No distinct separations were obtained using either principal components analysis or canonical variates. The Mahalanobis distance between the groups indicated that *C. flavipes* was closer to C. *chilonis* than to *C. sesamiae.* It is suggested that the surface chemistry of parasitoids may be influenced by their lepidopterous hosts since their fatty acids serve as precursors in the parasitoid hydrocarbon biosynthesis.

Allozyme analysis indicated that esterase, hexokinase, sorbitol dehydrogenase and 6-phosphogluconicacid dehydrogenase had fixed alleles for the three species and could be used to distinguish them. C. *chilonis* and *C. sesamiae,* which have similar male genitalia and partial mating compatibility are distinct species (Nei's genetic identity, I = 0.587). Average heterozygosity

was very low for all the populations (0.016). Cladistic analysis of the allozyme data indicated that *C. flavipes* may not be a monophyletic group. RAPD-PCR produced specific banding patterns of the three species and *C. g/omerata* L.

Mating behaviour, volatile pheromones and reciprocal breeding studies were conducted. Wing fanning and antennal vibration were the initial courtship signals from the males. Antennal stroking by the male was also an important contact signal and a prerequisite to successful mounting and copulation. Interspecific crosses revealed that males of *C. flavipes* exhibited courtship behaviour, mounted and copulated with females of *C. chilonis* and *C. sesamiae,* and transferred sperms, but progeny from these crosses did not include females. Males of *C. sesamiae* copulated with females of *C. chilonis* and the progeny included viable females. The progeny backcrosses of the hybrid females to male parents also included viable females. Sex pheromone experiments were conducted in a Y-tube olfactometer and in large field cages. Males and females of *C. flavipes* perceived and responded to odours emitted by the opposite sex. There was no significant response to odours from conspecific individuals of the same sex in any of the three species. Pheromone bioassays in field cages using sticky traps baited with live virgin *C. flavipes* females attracted conspecific males.

It is concluded that *Cotesia sesamiae* and *Cotesia chilonis* are two distinct species and can be separated using esterase, hexokinase and sorbitol dehydrogenase allozymes.

CHAPTER ONE

1 GENERAL INTRODUCTION

The species of the *Cotesia flavipes* complex (Hymenoptera: Braconidae) are primary gregarious endoparasitoids of Lepidoptera larvae. Current literature indicates that the *Cotesia flavipes* complex consists of three putative species. The three species have been imported and released in classical biological control programmes against various cereal stem borers in over 40 countries (Polaszek & Walker, 1991). The species included in this complex are: *Cotesia flavipes* Cameron, *Cotesia sesamiae* (Cameron) and *Cotesia chilonis* (Matsumura). These species were previously placed in the genus *Apanteles,* but Mason (1981) rearranged the classification and transferred the three species to the genus *Cotesia.*

Cotesia flavipes is an important parasitoid of gramineous stem-borers and is indigenous to the Indo-Australian region (Polaszek and Walker, 1991) where it predominantly attacks pyralid and noctuid stem borers (Mohyuddin 1971, 1990). C. *flavipes* has been successfully introduced for classical biological control in a number of countries. Among other successful introductions, it was reportedly imported from Japan to Punjab and Kashmir states in Pakistan to control *Chilo partellus* (Swinhoe) on maize in 1961 (Alam *et al.* 1972; Attique

et al. 1980). It was also introduced in several areas of the Neotropics against the sugarcane borer *Diatraea saccharalis* (F), (Gifford and Mann 1967; Fuchs *et at.* 1979; Macedo *et at.* 1984). *C. flavipes* was also introduced into Madagascar from Mauritius to control *Chilo sacchariphagus* (Mohyuddin 1971).

Cotesia sesamiae is an important parasitoid of stem borers in sub-saharan Africa (Mohyuddin 1971). It has been recorded from *C. partellus, Eldana saccharina* (Walker), *Maliarpha separatella* Ragonot (Pyralidae), *Sesamia ca/amistis* Hampson and *Busseola fusca* (Fuller) (Noctuidae) (Mohyuddin and Greathead 1970; Mohyuddin 1971). *C. sesamiae* has also been used in classical biological control programmes. It was successfully introduced into Mauritius from Kenya in 1951-52 to control *Sesamia ca/amistis* (Greathead 1971). Girling (1976) reported that *C. sesamiae* was the only parasitoid of *Eldana saccharina* in East Africa.

Cotesia chiionis occurs in Japan, China and Malaysia. It has been reported to attack *Chilo auricilius, Chilo polychrysus, Chilo suppressalis, Chilo terenellus* and *Sesamia* species. No successful introductions of C. *chiionis* have been reported. Currently there are no morphological characters known which separate *C. chiionis* from C. *sesamiae* (Polaszek and Walker 1991).

That *C. flavipes* is a valid species is supported by the fact that its male genitalia are distinct from the other two in the complex (Rao and Nagaraja 1967; Sigwalt and Pointel 1980; Polaszek and Walker 1991). Additionally, attempts to interbreed *C. sesamiae* and *C. flavipes* have resulted in the production of all male progeny, indicative of sexual incompatibility (Shami 1990). Mohyuddin (1971) stated that it is likely that because of its discontinuous distribution and conflicting host records, *Cotesia flavipes* may in fact comprise a number of strains, sub-species or sibling species. Reciprocal crosses of different strains from different localities (Shami 1990) did not show hybrid sterility. Mohyuddin *et al.* (1981) suggested that biotypes may also exist that are morphologically identical and sexually compatible, but prefer different habitats.

A review of the systematics of these important parasitoids and specific diagnosis of the species and / or strains in relation to locality and host preference is therefore necessary.

Chi/o partellus, an introduced pest from Pakistan, is among the most serious stem borer pests of cereal crops in Kenya, especially in the Coast province (Warui and Kuria 1983). It is for this reason that a classical biological control programme is underway to control this pest. *Cotesia flavipes,* imported from Pakistan, is being evaluated for its potential as a biological control agent of *C. partellus* in Kenya.

One of the fundamental prerequisites to a successful biological control programme is an accurate identification of both the target pest and its natural enemies. Success in many programmes has been significantly delayed by misidentifications of pests or natural enemies (Cock 1986). The major aim of this study, which is part of a classical biological control programme against C. *partellus,* is to clarify the taxonomy and biosystematics of the parasitoids. Currently *C. sesamiae* and *C. chilonis* can only be separated by geographical regions. *C. flavipes* which consists of various allopatric populations can only be separated from C. *sesamiae* and *C. chilonis* by the shape of the male genitalia. Host specific strains have been reported in *C. flavipes* but their biosystematics have not been studied. It is therefore necessary to clarify whether *C. sesamiae* and *C. chilonis* are distinct species and whether different populations of *C. flavipes* are indeed the same species. Also an increased understanding of the biosystematics of the species, subspecies, host strains or populations in the C. *flavipes* complex will not only lead to more host specific choices of the biocontrol agent but also provide information on the interactions of the species and possible consequences of introducing them into each others geographical areas.

1.1 OBJECTIVES

The purpose of the study was:

1. to identify non-overlapping diagnostic characters through the study of the morphology of adult and immature stages of selected populations of the C. *flavipes* complex.

2. to identify diagnostic alleles and / or loci within and between species of *Cotesia flavipes* complex through electrophoretic and isoeletric electric focusing analyses.

3. to assess the taxonomic value of cuticular components in *Cotesia* species.

4. to test the validity of the three putative species using the biological species concept.

5. to compare the above techniques and evaluate the feasibility of applying them to routine taxonomy.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Morphology

The species of the *Cotesia flavipes* complex are gregarious endoparasitoids of lepidopteran stem borers of gramineous plants. The complex is currently believed to include three species; *C. flavipes,* in the Indo-Australian Region, *C. sesamiae* in Africa, and C. *chilonis* in Japan. The three putative species have been imported and released in classical biological control programmes against various cereal stem borers in over 40 countries (Polaszek & Walker 1991) (figure 2.1). In some cases, a species has been introduced into an area of endemism of one of the other two species. For example, *C. flavipes* has been introduced several times into various countries in Africa where C. *sesamiae* is indigenous (Mohyuddin 1971; Breniere & Bordat 1982; Skoroszewski and Van Hamburg 1987; Overholt *et at.* 1994).

The three *Cotesia* species are extremely difficult, and sometimes impossible, to distinguish using external morphological characters.

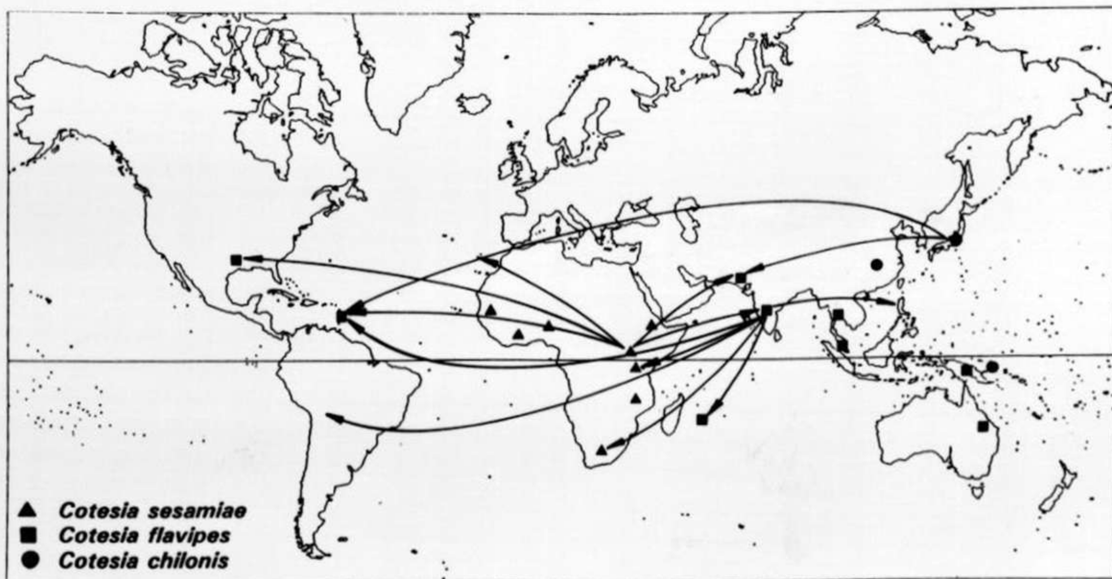


Fig. 2.1

Several workers have proposed various morphological characters to separate the *C. flavipes* complex species, primarily based on coloration, sculpturing, and male genitalia (Wilkinson 1932; Rao and Nagaraja 1967; Nagaraja 1971; Alam *et at.* 1972; Sigwalt and Pointel 1980). With the exception of male genitalia, these characters have not proven to be completely reliable (Polaszek and Walker 1991). Using male genitalia, Polaszek and Walker (1991) separated the species in the *C. flavipes* complex into two morphospecies: the *C. sesamiae/C. chilonis* subcomplex and *C. flavipes.* At present, except for very subjective morpholgical features, no valid characters have been found to distinguish accurately between *C. sesamiae* and *C. chilonis.*

The absence of clear diagnostic characters may have confounded past efforts to assess the impact of introductions of the *C. flavipes* complex. A biological control programme against *Chilo partellus* Swinhoe (Lep: Pyralidae) in East Africa made releases of *C. flavipes* from 1968-72. The identification of recovered *Cotesia* species was based on the coloration of the hind coxae and antennae, and the density of hairs on the antennae (Mohyuddin 1971). In Mauritius, assessment of parasitization by *C. flavipes* and *C. sesamiae* on *Chilo sacchariphagus* Bojer and *Sesamia calamistis* Hampson (Lep: Noctuidae) was based on the same morphological characters (Rajabalee and Govendasamy 1988). These characters are now known to be highly variable and unreliable for separating *C. flavipes* and *C. sesamiae* (Sigwalt and Pointel 1980; Polaszek

and Walker 1991). In South Africa, where *C. flavipes* was released against *C. partel/us,* recoveries were recorded from both C. *partellus* and *Busseola fusca* Fuller (Lep. Noctuidae) (Skoroszewski and Van Hamburg 1987). However, Mohyuddin (1971) reported that *B. fusca* was not a suitable host for *C. flavipes,* and this has been corroborated by recent work in our laboratory (Ngi-Song, *et at.* 1994), suggesting that there may have been misidentifications.

C. sesamiae and *C. chilonis* are ecologically and morphologically similar and if it were not for their non-overlapping distributions and differences in host records, would pass for a single species based on morphology (Polaszek and Walker 1991). C. *chilonis* is an important parasite of *Chilo suppressalis* (Walker) in Japan, while *C. sesamiae* is a widespread parasite of *B. fusca, Chilo* spp and *Sesamia* spp. in Africa. Morphological studies by Nagaraja (1971), Sigwalt and Pointel (1980) and Polaszek and Walker (1991) have shown only slight variation in the thoracic terga and the genitalia, which the authors agree, are subject to intraspecific variation. Considering the wide geographical separation between the African and Asian populations of the *chilonis/sesamiae* sub-complex, and the widespread presence of C. *flavipes* in between, further studies are essential before any decisions are made regarding the taxonomic status of C. *chilonis* and C. *sesamiae.*

Clearly the use of morphological characters alone is not sufficient to separate species in this complex and should be supplemented by other techniques. This view is also held by Polaszek and Walker (1991) who suggested that it was necessary to clarify the following ambiguities:

determine whether C. *chilonis,* with a distribution range from Australia to Japan, is a distinct species from C. *sesamiae* which is restricted to Africa.

determine whether C. *chilonis* is restricted to Japan,

determine whether specimens conforming to the *chilonis/sesamiae* morphology from outside Japan are either C. *sesamiae* or a different species.

2.2 Morphometries

Morphometries is the measurement and analysis of form (Daly 1985). It is a formal treatment of our ideas about dissimilarity of geometrical form among biological objects. Morphometric methods are powerful research tools when used in the context of sound biological knowledge. They allow us to summarize morphological data numerically and graphically, to express and test hypothetical relationships, and with multivariate techniques, to examine relationships in many dimensions in a manner not otherwise possible.

Techniques for the analysis of morphometric characters in systematics have been thoroughly treated in several reviews (Gould and Johnson 1972; Sokal 1965). Sneath and Sokal (1973) listed over one hundred taxonomic studies on insects where numerical methods had been applied. Daly (1985) reviewed the applications and interpretations of morphometries in insect biology and concluded that it is a useful tool especially where appropriate background knowledge of an insects' life history is available.

Multivariate analysis

Multivariate statistical analysis exploits pre-existing knowledge of dissimilarities among objects. As it is not possible to plot more than three characters simultaneously, the relationship of several measurements can be summarised by ordination (multivariate analysis). The techniques of ordination commonly used in multivariate morphometric studies are canonical variate analysis and principle component analysis. Applications of these techniques to systematics have been discussed by several authors, most recently by Marcus (1990) and Reyment (1990). In this study both principal component and canonical variate analyses were used.

Principal component analysis

Principal components represent a coordinate system, constructed as linear functions of the original data. The first principal component is that dimension through the original data that expresses the most possible variance. The second principal component expresses the next greatest amount of variance in the data, given the important constraint that it is orthogonal to the first. The third contains as much of the remaining variance as possible and is orthogonal to the first two and so on. There are as many principal components as the original variables in the data. Most of the variance in the data will be captured in the first few principal components. Therefore, plotting the observations on the first two or three principal components is a way of representing as much of the information as possible in a reduced set of maximally informative dimensions. Principal components are constructed without any reference to *a priori* assignment of the observations into groups.

Canonical variate analysis

Canonical variate analysis is a discriminant method, which presupposes that the data will fall into a given number of preassigned groups and then maximises inter-group covariance and minimises within-group variation. This analysis has particular value where taxonomic groups are very close or overlap

(Sneath and Sokal 1973). The analysis provides the latent roots, percentage discrimination and centroids for each canonical axis. The latent roots provide a measure of the discriminatory power associated with each canonical variate. The first canonical variate, which is the best discriminating axis attempts to maximise the distance between the centroids of all the groups. The second canonical variate provides maximal discrimination, subject to the constraint that it is orthogonal to the first and so on. The number of canonical variates that can be contructed are equivalent to the number of class variables minus one. The first two canonical variate axes account for a large proportion of the variation among groups. The distance between any two centroids in canonical variate space is termed Mahalanobis distance, usually presented in the squared form. Mahalanobis distance provides the best multivariate measure of the relative distance between groups, taking proper account of the variation within each of them. In practice, the two most different groups will generally be separated on the first canonical variate, the next most different group will be separated from the first two groups on the second canonical variate, and so forth. Albrecht (1980) provided an exellent analogy: "it is as if the coordinate system defined by the original variables is suspended in air such that the investigator can walk around it until the most favourable vantage point is located for viewing the differences among the populations." One can think of principal components analysis this way also, except that here the best vantage point is found for viewing maximum amount of variance in the data.

2.3 Cuticular Components Pattern Analysis

The use of surface hydrocarbons of insects as taxonomic characters was first suggested by Lockey (1976) and Jackson and Blomquist (1976). Insect cuticular components provide an essential barrier to moisture diffusion and can serve as recognition factors and pheromones (Howard and Blomquist 1982; Blomquist and Dillwith 1985; Lockey 1991). Since most of these components are synthesized by the insects, cuticular components have been utilised as a taxonomic character by several workers to distinguish between closely related species. Lockey (1991) reviewed the use of hydrocarbon chemotaxonomy in nine insect groups including sibling species, and showed that hydrocarbon composition not only separates species, but also reflects links between species and higher taxa. Espelie *et al.* (1990) analysed the pattern of cuticular hydrocarbons of geographically isolated populations of *Rhopalicuspu/chripennis* (Hymenoptera: Pteromalidae) and found differences in the hydrocarbon composition of individuals from the two regions. Cuticular hydrocarbons of arthropods are stable end products of genetically controlled synthesis, and hence may be used to identify dead preserved museum specimens especially when live or fresh material is not available (Carlson and Service 1980; Kaib *et al.* 1991).

2.4 Allozyme electrophoresis

The value of electrophoresis as a tool in systematics is being increasingly recognised by taxonomists (Avise 1974; Menken and Ulenberg 1987). However, there have been few published studies concerning insect parasitoids. Electrophoresis can be particularly useful for the recognition of sibling species, which often differ in their protein composition (Menken and Ulenberg 1987) or in allozyme frequencies (Berlocher 1984).

Propp (1986) used starch gel electrophoresis to examine the inter- and intraspecific isoenzyme variation in three species of *Spalangia (S. cameroni* Perk., S. *endius* Wlk., 5. *nigroaenea* Curt.) and two *Muscidifurax* species (S. *raptor* Gir., *M. zaraptor* Kog.), pteromalid parasitoids attacking the pupae of muscoid flies. The three *Spalangia* species were readily distinguishable at 8 of the 15 loci examined and the two *Muscidifurax* species at 3 of 10 loci. Calculation of Rogers' Similarity Index (Rogers 1972) from this data gave values below 0.3 for *Spalangia* species, but a value of 0.65 for the *Muscidifurax* species, indicative of a genetic similarity associated with sibling species (Propp 1986). The electrophoretic results were in agreement with existing morphological and biological characters used to identify these particular species.

The most extensive enzymatic study of parasitic Hymenoptera has been done on *Trichogramma,* a genus in which species identification using morphological characteristics is frequently difficult. Examination of enzyme banding patterns, especially of esterases, from numerous cultures has allowed clarification of inter- and intraspecific relationships, including the recognition of new species and the provision of evidence of species hybridisation (Jardak *et al.* 1979; Pintureau and Babault 1980, 1981, 1982; Pintureau and Voegele 1980; Hung 1982; Hung and Huo 1985). Referring to differences at nine esterase loci, Jardak *et al.* (1979) separated *Trichogramma o/eae* from *T. evanescens* Westw., whilst Pintureau and Babault (1981) provided enzymatic data to support the separation of *Trichogramma maidis* from *T. evanescens.* Esterases, with supplementary data from malate dehydrogenase, readily permitted recognition of other related species of *Trichogramma,* but tetrazolium oxidase distinguished only distant species (Pintureau and Babault 1982). A major problem encountered with electrophoretic data is the fact that the majority of amino acid substitutions are undetected under standard electrophoretic conditions (Menken 1989). The technique of isoelectric focusing with immobilised pH gradients enables the separation of proteins up to 0.003 of a pH unit (Cossu and Righetti 1987). Both techniques were used in this study.

2.5 Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR)

Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) was first described by Williams *et a/.* (1990) who demonstrated that the technique could reveal polymorphisms in the genomes of a wide variety of species. Black era/. (1992) showed that RAPD-PCR could be used to identify polymorphisms within and among four aphid species, *Schizaphis graminum, Diuraphis noxia, Arcythposiphon pisum* and *Uroleucon ambrosiae,* from single plants. They also demonstrated polymorphisms within two endoparasitic wasps *Lysiphlebus testaceipes* and *Diaretiella rapae* which had been reported to exhibit low allozyme variability (Nevo *et a/.* 1984). Perring *et at.* (1993) were able to classify two whitefly types, which due to morphological similarity had been identified as strains of a single species, into two distinct species using allelic frequencies and RAPD-PCR. Puterka *et at.* (1993) used allozyme and RAPD-PCR markers to examine genetic relationships among populations of the Russian wheat aphid *(Diuraphis noxia).* Three of the twenty allozyme loci were polymorphic. Cluster analysis of the polymorphic loci was only able to separate thirty-five populations into five groups. In contrast, cluster analysis with RAPD-PCR polymorphisms placed populations into the same five groups, but further separated populations within each group. Roehrdanz *et at.* (1993) used PCR and short random primers to obtain banding patterns that could be used as

genetic markers suitable for species and strain identification in parasitic Hymenoptera. They examined five aphid parasitoids; *Diaeretiella rapae, Aphidius matricariae, Aphidius picipes, Aphidius colemani* and *Lysiphlebus testaceipes.* Within *D. rapae* and *A. matricariae,* primers were found that produced banding patterns that distinguished laboratory-reared colonies derived from collections at different geographic locations. DNA markers that differentiate populations of these insects could have immediate practical use in monitoring the success of released parasitoid strains in classical biocontrol programmes, and monitoring the genetics of laboratory-reared strains. Alleles at arbitrarily primed loci segregate as dominant markers which prevents their use in studies of random mating and other aspects of insect genetics. Hunt and Page (1992) reported that 90% of alleles segregated as dominant markers in *Apis me/lifera.* However, this (dominance) does not apply to the Hymenoptera with haploid males. Black (1993) reviewed the use of RAPD-PCR in separating insect species and pointed out shortcomings, such as changes in the numbers, sizes and intensities of bands in response to changes in PCR buffers and amplification parameters, and suggested precautions that could be taken to ensure repeatability and consistency of the amplification products.

2.6 Mating behaviour, volatile pheromones and reciprocal crossing

Behavioural characteristics have not been used much in the systematics of parasitic Hymenoptera. The degree of sexual isolation between closely related populations is a measure of ethological barriers preventing successful mating between the individuals of such populations. Direct observation of the mating preferences of males and females may therefore provide a simple means of distinguishing between species. A detailed study by Gordh and Debach (1978) showed that courtship and mating behaviour are both qualitatively and quantitatively different in such closely related species as *Aphytis Ungnanensis* (Hym: Aphelinidae) and *Aphytis melinus* (Hym: Aphelinidae). Discrimination between species in some groups of parasitic Hymenoptera is facilitated by the elaborate and protracted behavioural interaction between males and females before copulation takes place and sometimes even afterwards (Van den Assem 1974). More recently, Walter (1993) studied the mating behaviour of two closely related species in the ochraceus-group of *Coccophagus* Westwood (C. *bartletti* and *C. lutescens* (Hym.: Aphelinidae). They found that the mating behaviour of *C. bartletti* was consistently and significantly different from that of *C. lutescens,* which was useful in distinguishing between the species.

The mating behaviour of the family Braconidae was summarised by Matthews (1974) as typically involving attraction, recognition, orientation, wing

fanning, mounting, antennation, copulation and post-copulatory grooming. Kamano *et at* (1989) studied the mating behaviour of the egg-larval parasitoid *Ascogaster reticulatus* Watanabe (Hym: Braconidae) and observed a similar sequence of events. In addition they observed that a hexane extract from the female body elicited male antennal searching.

Sex pheromones in parasitic Hymenoptera are summarized by Eller *et at.* (1984) in which the evidence of sex pheromones was shown in seven families; Aphelinidae, Chalcididae, Pteromalidae, Scelionidae, Braconidae and Ichneumonidae. In a few cases the sex pheromones of parasitic Hymenoptera have also been identified. The sex pheromone of an ichneumonid *Itoplectis conquisitor* was identified as neral and geranial (Robacker and Hendry 1977), and that of another ichneumonid *Syndipnus rubiginosus* was identified as ethyl (Z)-9-hexadecanoate (Eller *et al.* 1984). Other than the biological interest on the sex pheromones of parasitic insects, pheromone traps can be practically used to evaluate and monitor a parasitic population (Lewis *et al.* 1971).

CHAPTER THREE

3 MORPHOLOGY OF SELECTED POPULATIONS IN THE ***COTESIA FLAVIPES*** COMPLEX

3.1 Introduction

The genus *Cotesia* belongs to the largest subfamily of Braconidae, the Microgastrinae, with over 1300 described species and an estimated world fauna of about 2500 species (Gauld and Bolton 1988). Traditionally, the majority of microgastrines were placed in the genus *Apanteles* including the C. *flavipes* complex. However, Mason (1981) rearranged the classification and transferred the C. *flavipes* complex from *Apanteles* to *Cotesia.* The genus *Cotesia* is one of the largest and contains over 300 described species. Species in the *C. flavipes* complex can easily be distinguished from other *Cotesia* species by their dorso-ventrally flattened shape and the absence of carinae on the propodeum. The purpose of this morphological study was to review characters used by previous workers using specimens from various localities and investigate the amount of intra- and interspecific variation within and between the populations of the species in the complex with the aim of finding discrete characters for the identification of specific populations or species.

3.2 MATERIALS AND METHODS

3.2.1 Insects

Insect populations in Culture

Colonies of the three C. *flavipes* complex species were initiated at ICIPE during 1991-92. The Pakistan station of the International Institute of Biological Control provided the parent material from north and south Pakistan for the C. *flavipes* colonies. *Cotesia chilonis* was obtained from Niigata in Japan *via* Texas A&M University in the U.S.A. The *C. sesamiae* colonies were initiated with field collected individuals from the Coast and Western Provinces of Kenya. The Thailand and Rio Grande Valley (Texas) populations of *C. flavipes* were from cultures maintained at the biocontrol laboratories of Texas A&M University. *Cotesia glomerata* L which is morphologically distinct from C. *flavipes* complex, was imported from the Netherlands and frozen in liquid nitrogen on emergence and used for the studies where an outgroup comparison was necessary.

Dead Insect Specimens

In addition to the above material, dead insect specimens were obtained from the Natural History Museum, London, UK and donations from individual collectors from various countries where the *C. flavipes* complex is reported to occur. Table 3.1 below is a summary of all the populations of the three species that were studied, their localities and host records.

Table 3.1 Summary of the populations of *C. flavipes* complex studied, their localities, host records, date and name of collector.

SPP.	LOCALITY	HOST RECORD	DATE & COLLECTOR/ DETECTOR
Cf	PAPUA NEW GUINEA. Madang Provance, Ramu Sugar Estate	*Sesamia grisescens*	18.vi.91, B. Rutu
Cf	NORTH PAKISTAN. Rawalpindi,	*Chilo partellus*	v.90 A.I.Mohyuddin
Cf	SOUTH PAKISTAN. Sindh	*Chilo partellus*	v.92 A.I Mohyuddin
Cf	PAKISTAN. Karachi	*Chilo partellus*	iii.94 Inayatullah
Cs	KENYA. Mombasa Homa Bay	*Chilo partellus* *Sesamia cat a mist is* *Busseola fusca* *Chilo orichalcociliellus*	vi.92 K. Ogedah vii 93 J. Okello
Cc	JAPAN. Niigata.	*Diatraea* sp (in culture)	
Cf	THAILAND. Kampaengsaen Nakhon Pathom	*Chilo infuscatellus,* *Chilo sacchariphagus,* *Sesamia inferens.* *Diatraea saccharalis* (in culture)	6-24.i.92. Sausa
Cf	USA. Texas. Rio Grande Valley	*Diatraea saccharalis*	In culture since 1985
Cc	JAPAN. Matsue, Shimane	*Chilo Psuppressalis*	xi. 1990 HE

[1] International Institute of Entomology

SPP.	LOCALITY	HOST RECORD	DATE & COLLECTOR/ DETECTOR
Cs	SOUTH AFRICA. Transvaal, Delmas	*Busseola fusca*	3.iv.91 R. Kfir
Cs	ZAMBIA. Mount Makulu, Research station	*Busseola fusca.*	1992-93 C. Mugoya
Cs	TANZANIA. Zanzibar Kizimbani	stem borer	23.V.86 H.R. Feijen
Cc	CHINA. ?Jiangsu	*Chilo supressa/is.*	1992 HE
Cs	NIGERIA NCRI Moor plantation	*S. ca la mist is* on *P. purpureum*	4.vi.76 Ogunwolu
Cc	NEW BRITAIN Keravat.	*Sesamia grisescens,*	1954 Din M-M
Cc	MALAYSIA. Jatu Serong	Lepidoptera larvae	18.iv.1929 Pagden
Cf	JAPAN-PAKISTAN Voucher specimens introd. to Pakistan from Japan.	*Sesamia* sp *Chilo partellus*	1964 C.I.B.C.
Cs	MALAWI Makoka	*Chilo partellus*	13.ix.92 G. Phiri
Cf	MAURITIUS Belle vue Maurel (sugar estate)	*Chilo sacchariphagus*	4.iii.94 R. Potting

Cf = *Cotesia flavipes* Cs = *Cotesia sesamiae* Cc = *Cotesia chilonis*

3.2.2 Techniques used to observe the various morphological features of the adults and immature stages.

The dissecting and compound microscopes, and scanning electron microscope were used to observe the morphological features.

Scanning Electron Microscopy

Whole adult male and female wasps were dehydrated by placing them in 70%, 80% and 100% ethanol for 10 minutes each and then transferring them to acetone before critical point drying (Balzers CPD 030 critical point dryer). The dry specimens were mounted on copper stubs with double sided tape. Stubs were sputter coated with a layer of gold-palladium using a Fine Coat ion sputter (JFC-1100). The specimens were observed on a Jeol Stereoscan (JSM-T1000).

Light microscopy

Colour

Inter- and intraspecific colour variation were visually compared and recorded

Characters on the mesosoma and metasoma

The following characters were scored from sixteen populations that occur worldwide (see figure 3.1 and plate 3.2 A-D for the morphological features).

1. The scuto-scutellar-sulcus; straight = 0.5, Strongly curved = 1, Slightly curved = 0.75.
2. Number of hairs on the scutellum.
3. Rugosity on the propodeum; basal half smooth = 0.5, > than half sculptured = 1.
4. Carapace on first gastral tergite and median field of second gastral tergites; fully sculptured = 1, nearly smooth = 0.5.

Preparation of the male and female genitalia

The method used for genitalia preparation was a modification of the method used for the preparation of male genitalia of *Telenomus* species (Hymenoptera: Scelionidae) (Polaszek and Kimani, 1990).

The gaster of a card mounted male *Cotesia* specimen was removed under a dissecting microscope and placed in a pyrex vial containing 0.5ml of 10% potassium hydroxide. The vial was appropriately labelled and placed in a heating block at 100° C for 10 minutes. This macerated all the tissues in the

gaster, leaving the sclerotised parts intact. The potassium hydroxide and the gaster were then transferred into an excavated glass block and the liquid removed. Glacial acetic acid (20//l) was added to the specimen and left for 3 minutes to neutralise the potassium hydroxide. To wash the specimen, the acid was removed and 0.5ml of distilled water added and left for 10 minutes. To dehydrate the specimen, an equal volume of 70% ethanol was added and left for 10 minutes. The liquid was removed and a further 0.5ml of 70% ethanol was added and left for 10 minutes. The gaster was then transferred to 80% ethanol for 5 minutes and then to absolute ethanol for 2 minutes. The ethanol was removed and 20-30/vl drops of clove oil were added and left for at least 10 minutes. The gaster was then transferred from the glass block to a drop of Canada balsam on a clean slide. The genitalia were excised using two entomological pins (No. 0), transferred to a fresh drop of Canada balsam on the same slide, and a 6mm cover slip placed gently on them.

Preparation and observation of whole larvae and mouth parts of the immature stages.

The methods used were modified from O'Donnell (1987) and Drake (1966). Suitable hosts were exposed to mated *Cotesia* females, that is, *Sesamia calamistis* to C. *sesamiae, Chilo partellus* to C. *flavipes* and *C. chilonis.* Host larvae were fed on artificial diet (Ochieng *et at.* 1985) and dissected after

every 24 hours from oviposition until cocoon formation.

Preparation of whole larvae

Larvae were dissected from their hosts in a 1.0 g/100ml solution of sodium chloride and preserved in a few drops of 70% ethanol on a cavity slide. The specimens were macerated on cavity blocks using a few drops of 10% potassium hydroxide and placed on a heating block at 50° C for about 30 minutes. The potassium hydroxide was then removed and a drop of acetic acid added. This was removed and a few drops of distilled water added. The larva was then mounted on a cavity slide containing a drop of Hoyers medium (Borror and Delong, 1970) for temporary mounts. Eggs and first instar larvae were mounted without macerating since they are normally transparent.

Preparation of the mouthparts

Mouthparts were prepared from third instar larvae. Each specimen was placed in a drop of 70% ethanol on a cavity slide. The head was cut off and placed in a cavity dish containing 3 drops of Potassium hydroxide and heated as above and placed on a microscope slide. A drop of glacial acetic acid was added and then wiped with blotting paper. The specimen was washed by adding a drop of distilled water and blotting it dry. The head with visible

mouthparts was mounted in Hoyer's solution on a microscope slide for observation.

Observation of the cocoon masses

Cocoon masses of laboratory reared and field collected samples of *C. flavipes, C. sesamiae* and *C. chilonis* were visually observed compared and photographed.

Illustrations

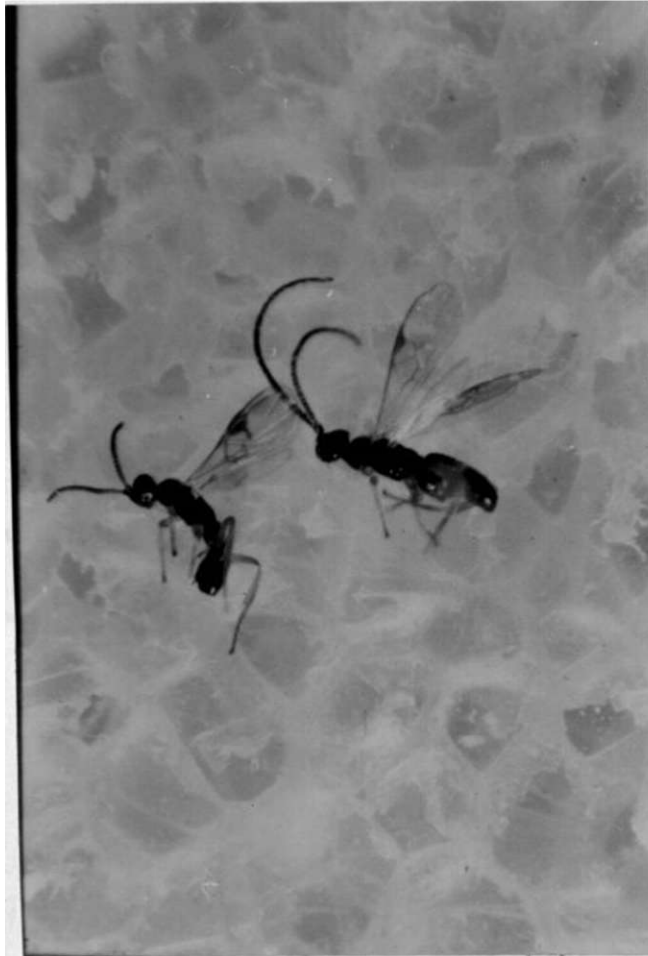
The mounted specimens were observed and illustrated using a compound microscope fitted with a Leitz camera lucida.

3.3 RESULTS

3.3.1 Examination of external morphological characters

The adult *C. flavipes* complex species measures about 2 mm with a wingspan of about 4 mm. The head, mesonotum and tergites of the metasoma are brown to dark brown and shiny. The wings are usually clear, the legs and antennae are yellowish brown to brown. The male antennae are about twice as long as the female antennae. A picture of male and female specimens of *C. flavipes* is presented in Plate 3.1.

The dorsal habitus of a typical adult female showing the external morphological features of a female of the *Cotesia flavipes* complex that were used in this study is presented in figure 3.1.



t 1.0 mm ı

Plate 3.1. Male (longer antennae) and female (shorter antennae) specimens of *C. flavipes* showing the colour, antennae and other external morphological features typical of species in the *C. flavipes* complex.

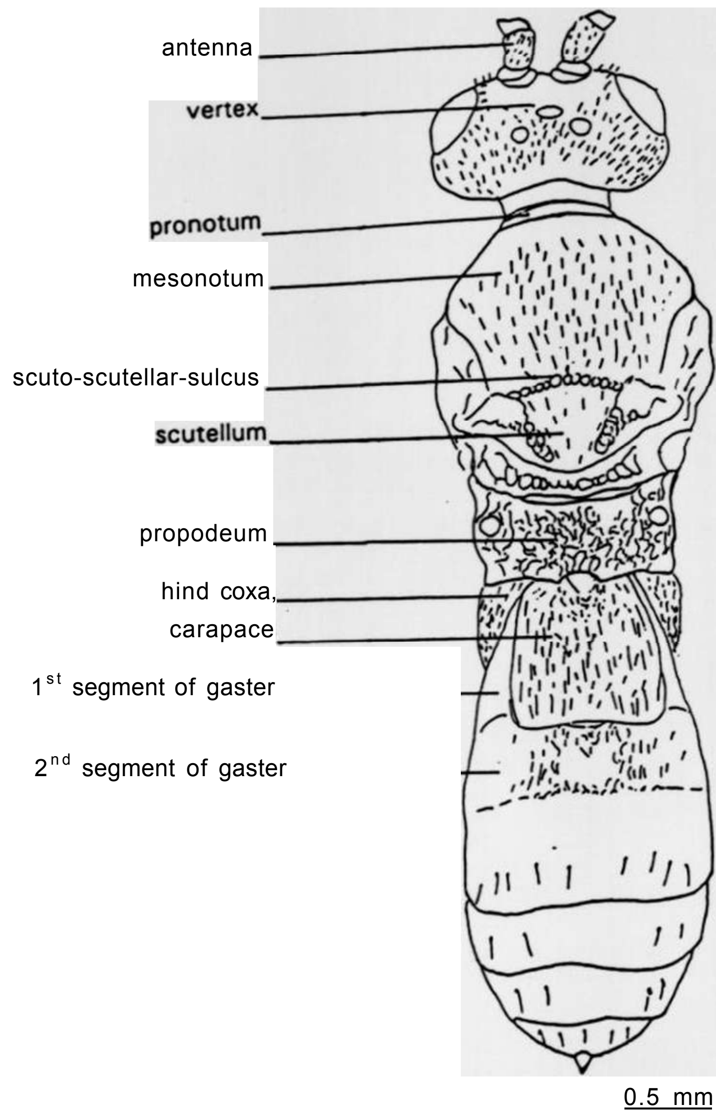


Fig. 3.1 Dorsal habitus of a typical female *C. flavipes* complex showing the morphological features on head, mesonotum and metanotum that were used in this study.

Colour

Examination of several specimens from various localities indicated that the colour separation suggested by various authors is not species specific and varies even between species from the same locality. For example *C. sesamiae* from the coast province of Kenya occurs in two colour forms; one with black hind coxae, a black mesosoma and translucent sooty wings with greyish black antennae and another with pale yellow hind coxae, brown mesosoma with yellow antennae and clear transparent wings. As well as being variable, colour was also found to be difficult to quantify.

Head

The ratio of width and length of the face was proposed by Rao and Nagaraja (1967) to distinguish *C. flavipes* from C. *chilonis.* Sigwalt and Pointel (1980) used several face measurements to characterise/diagnose the three species. The length and width of the face, the distance from tentorial pit to base of eye and the length and width of the first and last flagella segments were measured (chapter 4) and used for morphometric studies.

Mesosoma and Metasoma

Occurrence of the above mentioned character states are summarised in table 3.2.

Plate 3.2 Scanning Electron Microscope photographs showing the mesosoma and the first three segments of gaster (see fig. 3.1) of:

(A) *C. chilonis* showing a relatively straight scuto-scutellar sulcus (sss), and fully rugose propodeum and 1st and 2nd tergites of the gaster.

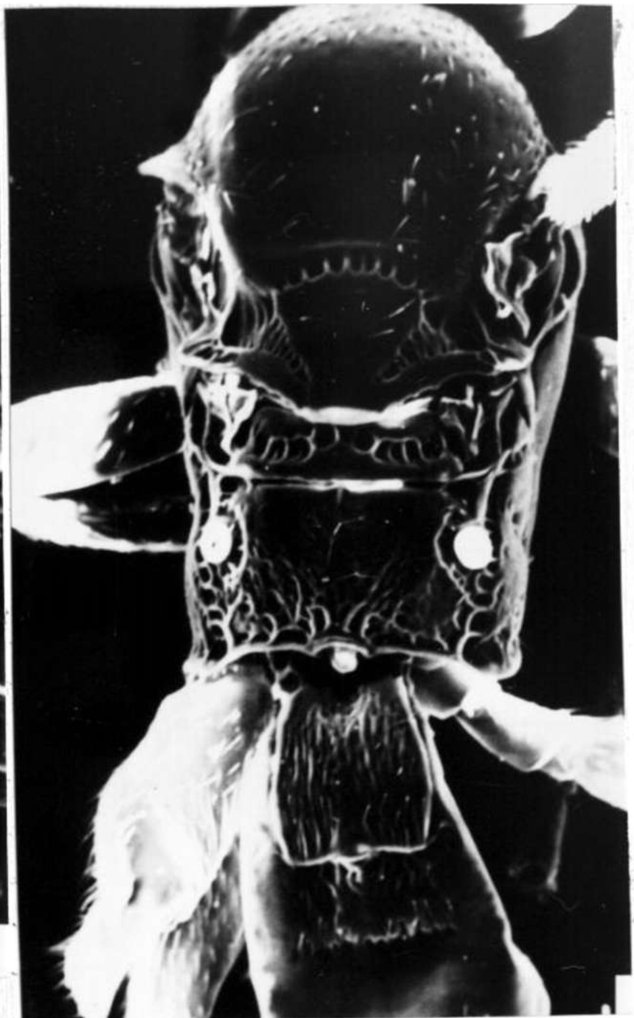
(B) *C. sesamiae* with slightly curved sss, and smooth basal half of propodeum.

(C) *C. flavipes* from Pakistan.

(D) *C. flavipes* from Thailand with characteristic sculpture on the propodeum and relatively smooth first and second segments of gaster.

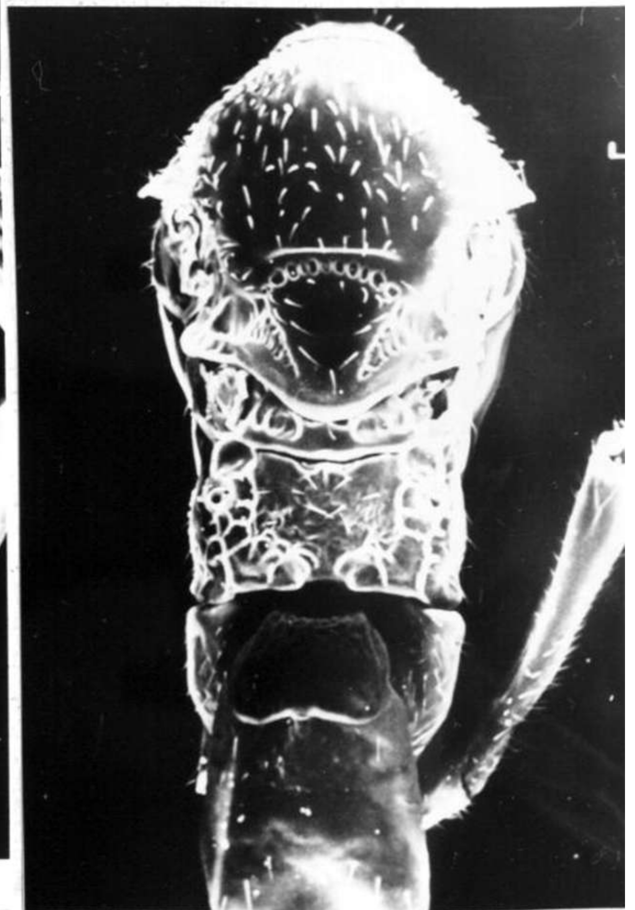
Magnification X90

a



b

c



d

Table 3.2 Results of examination of five morphological character states of specimens of the *C. flavipes* complex from sixteen populations.

sample[8]	Scuto-scutellar-sulcus	No. of hairs on scutellum	Rugosity on propodeum[b]	Sculpture on 1st tergite	Sculpture on 2nd tergite
C. flavipes					
PapuaNG[1]	1	14-25	0.5-1	0.5	0.5
NPakis[2]	1	14-15	1	1	1
Thailand	1	10-16	1	0.5	0.5
Texas	1	14-17	1	1	1
Jap-Pak[3]	1	14-15	0.5-1	1	0.5-1
C. sesamiae					
Malawi	1	13-17	0.5	1	0.75
Kenya	0.75	16-19	0.5	1	0.75
Nigeria	1	14-15	0.5	1	0.75
SAfrica	0.75	15-18	0.5	1	0.75
Zambia	0.75	13-16	0.5	1	0.75
T(Zanzibar)	0.75	10-15	0.5	1	0.75
C. chilonis					
N Britain	1	9 +	1	1	1
Malaysia	1	14-17	1	1	1
China	0.5	24-27	1	1	1
Japan	0.5	21-28	1	1	1
Japan	0.5	24-31	1	1	1

'See chapter 3 for details on localities and hosts. [1] Papua New Guinea [2] North Pakistan [3] voucher specimens of the introduction from Japan to Pakistan
specimens from PapuaNG & Jak-Pak had rugosity ranging from 0.5 to 1.

Key: Scuto-scutellar sulcus
- 0.5 = straight
- 0.75 = slightly curved
- 1 = curved

Number of hairs on the scutellum

Rugosity in the scutellum
- 0.5 = basal half smooth
- 1 = more than half of the propodeum rugose

First and second gastral tergites
- 1 = fully sculptured
- 0.75 = less sculptured
- 0.5 = almost smooth

3.3.2 Male genitalia

Drawings of the male genitalia of the three species and the intraspecific variants are presented in figures 3.3 A-H. In general, the male genitalia of all populations of *C. flavipes* were slender and elongated while those of *C. chilonis* and *C. sesamiae* were robust and short (about half the length of *C. flavipes'* male genitalia). The various parts of the male genitalia are shown in figure 3.2.

Basal ring (phallobase)

The basal ring of *C. flavipes* varied between the populations and even within the populations. That of *C. sesamiae* was consistent and as in figure 3.3 A-C. *C. chilonis* differed slightly from *C. sesamiae* in having the posterior margin straight (figure 3.3 D) and not concave as in *C. sesamiae* .

Parameres

There were no distinct differences between the parameres of the three species.

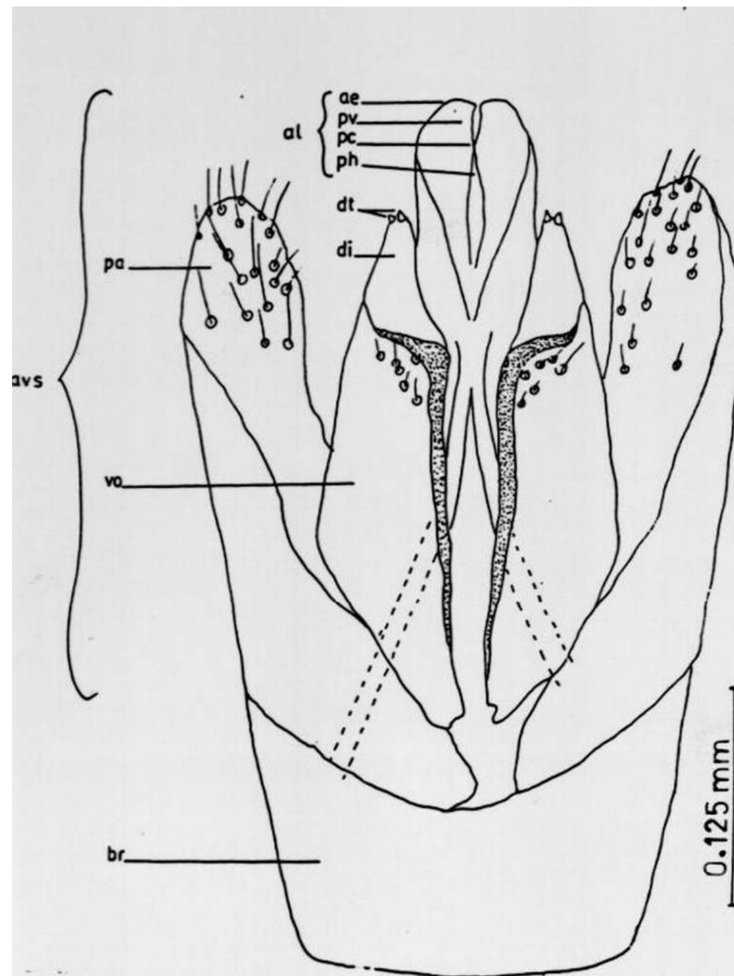


Fig. 3.2 Male genitalia of *Cotesia flavipes* (Rawalpindi, Pakistan population) to show nomenclature of various parts; ae aedeagus, al aedeagal lobe, br basal ring, vo volsella, di digitus *(plural* = digiti), dt digital teeth, avs aedeago-volsella shaft, pa paramere, ph phallotreme, pc penial crest pv penis valve.

Fig. 3.3 Camera Lucida drawings of male genitalia of specimens from various localities showing inter- and intraspecific variations:

(A) C. *sesamiae* from Malawi,

(B) *C. sesamiae* from Zambia

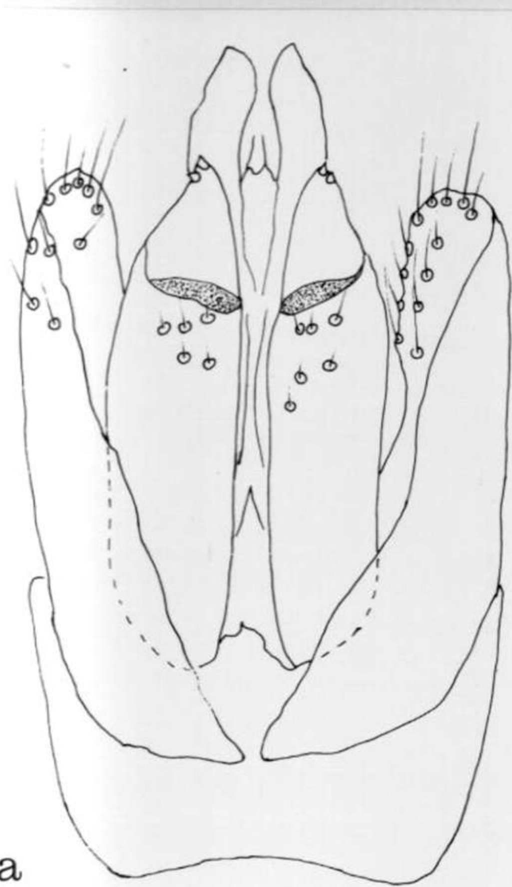
(C) *C. sesamiae* from Kenya

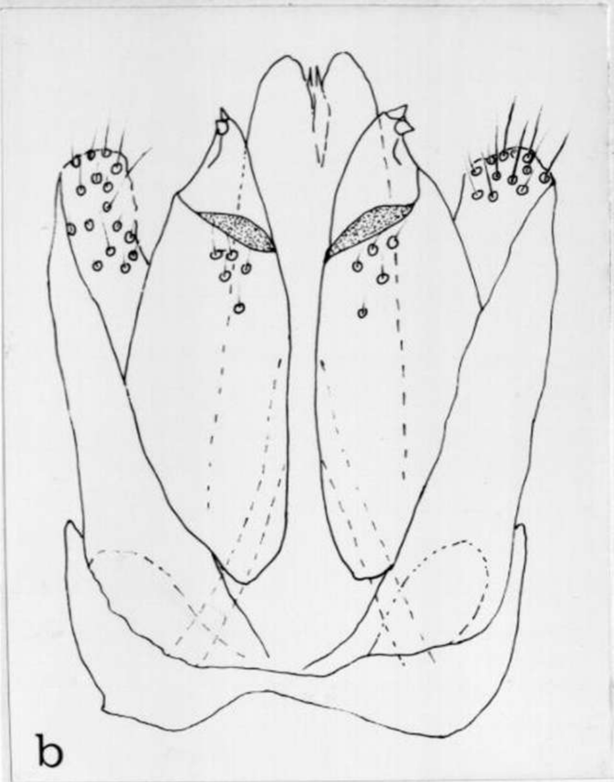
(D) *C. chi/onis* from Japan

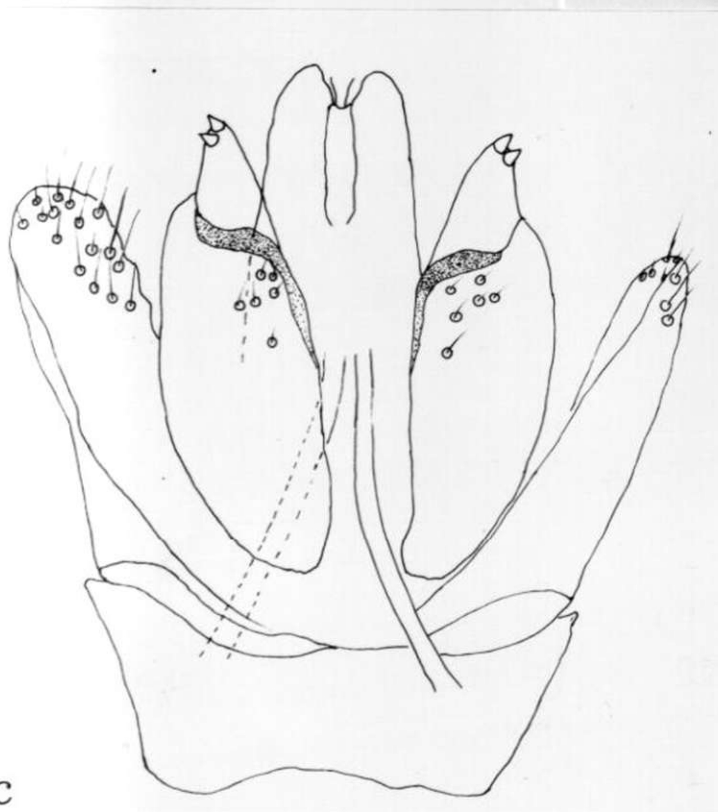
(E) *C. flavipes* from Pakistan (Sindh and Rawalpindi)

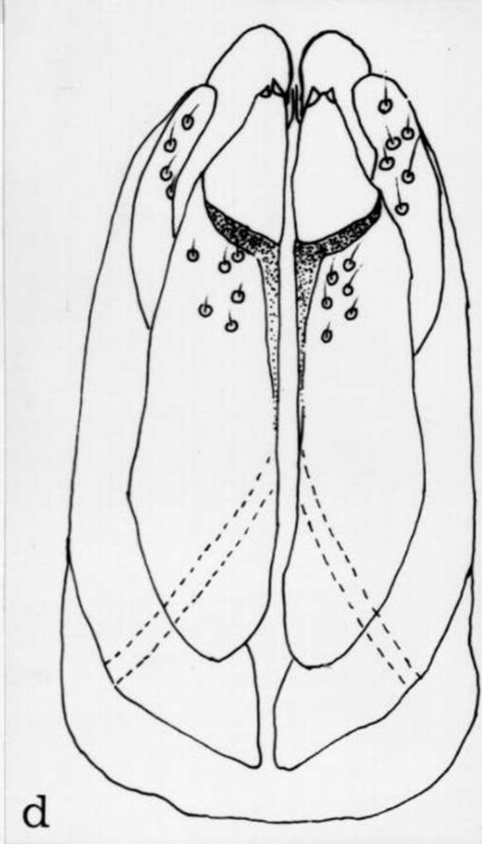
(F) ***C. flavipes*** from Pakistan (Karachi)

G & H) *C. flavipes* from Mauritius.

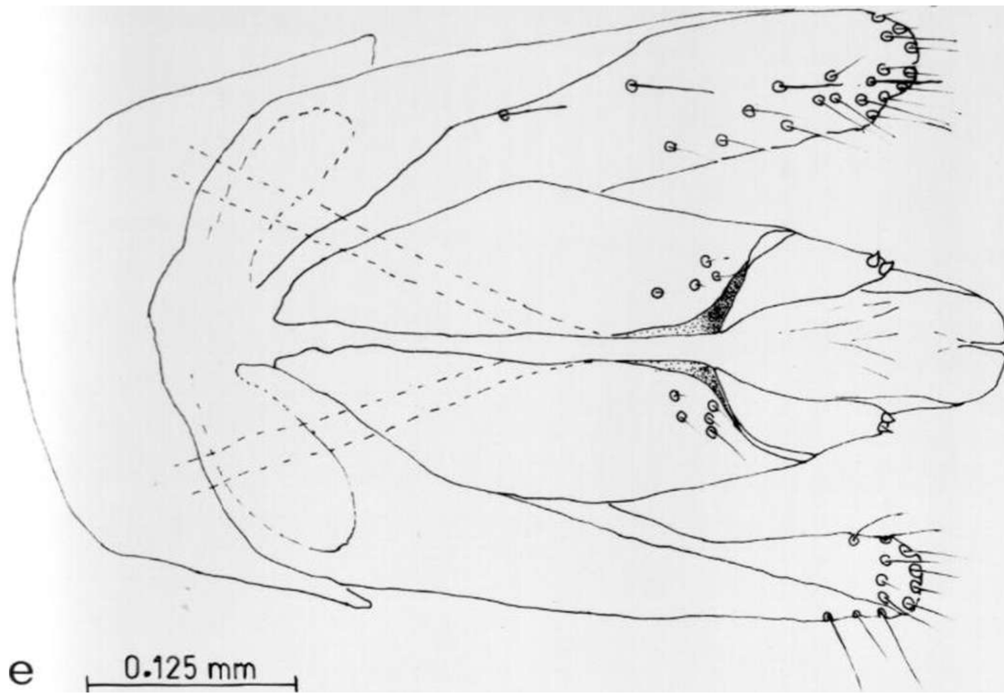


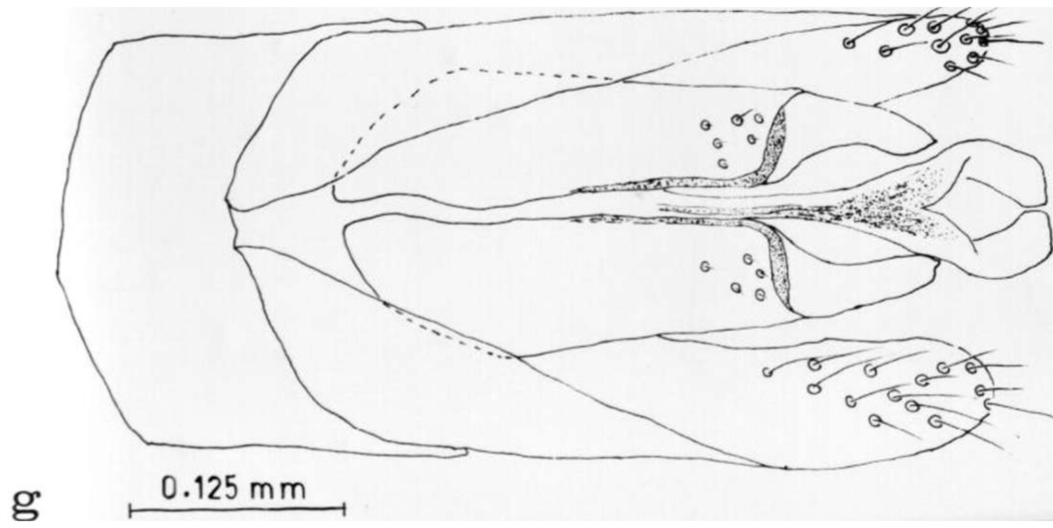






0.125 mm


e
0.125 mm


g
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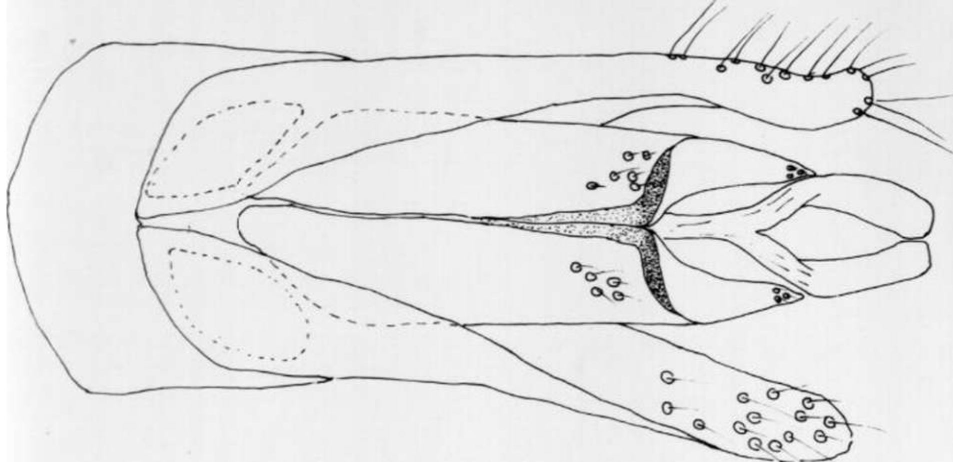
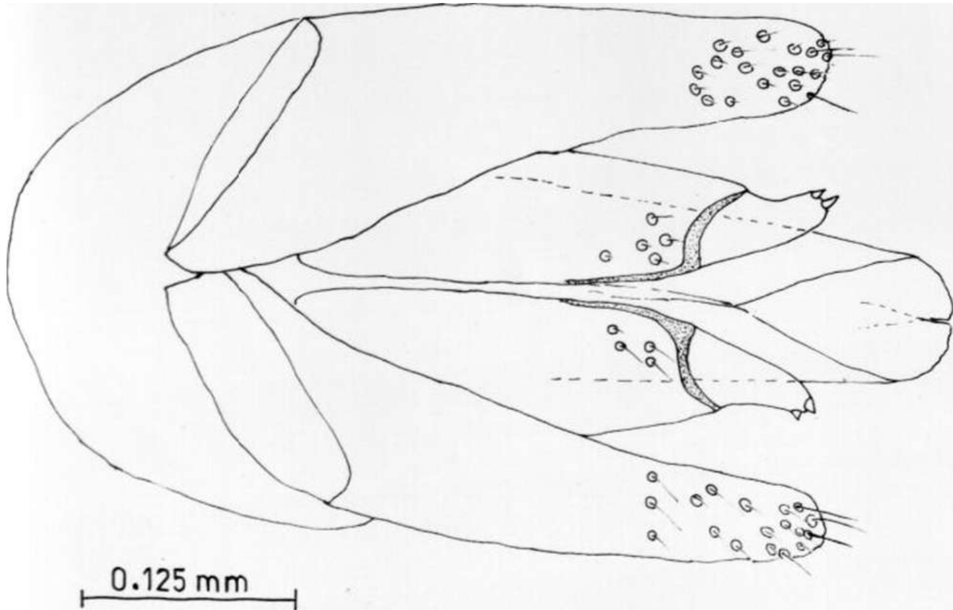


0.125 mm



Volsella

Sigwalt and Pointel (1980) showed that in ***Cotesia g/omerata*** the basivolsellar is cylindrical while in the ***C. flavipes*** complex it forms an open triangle. This was the case in all the populations except the Mauritius population of ***C. flavipes*** where the basivolsellar was more cylindrical than triangular (figure 3.3 H-l). They also showed that the interior edge of the digiti in ***C. glomerata*** were parallel and had three digital teeth while those of ***C. flavipes*** complex were diverging and had two digital teeth. This was observed in all the populations except the Mauritius population where the proximal lateral margins of the digiti tended to converge and had no digital teeth.

Aedeagal lobe

The general shape of the aedeagal lobe separated C. ***sesamiae/chilonis*** populations from those of C. ***flavipes*** although there were variations within the two groups. The apex of the aedeagus was truncate (figure 3.3 E) or slightly curved (figure 3.3 F) in ***C. flavipes*** with a median groove which terminated at the same level as the penial valves, the penial crest terminated just before the apex. This applied to all the populations of ***C. flavipes*** except the Mauritius population (figures G-H| where the phallotreme was enlarged and the penial valves had assumed a curved shape. In ***C. chilonis*** and ***C. sesamiae,*** with the

exception of the population from Malawi, the penial valves and the penial crest extended a little beyond the median groove, bifurcating at the apex. In the Malawi population (figure 3.3 A) the penial valves extended well beyond the penial crest and were slightly pointed at the tip.

3.3.3 Female genitalia

A drawing of the female genitalia of ***C. flavipes*** is presented in figure 3.4. No differences were observed in all the populations studied. However the total length of the ovipositor was measured in the morphometries studies (chapter 4).

3.3.4 Immature Stages

The egg stage, the three larval instars, and mouthparts of ***C. flavipes*** complex are presented in figures 3.4 A-E and 3.5 A-C. No morphological differences were found between the eggs or larvae except for the larval mouthparts. The hypostoma was more slender in ***C. flavipes*** than in ***C. sesamiae*** and ***C. chilonis,*** and the pleurostoma more developed in ***C. chilonis*** and ***C. sesamiae*** than in ***C. flavipes.*** There were two small labial setae in ***C. flavipes*** and ***C. sesamiae*** (figure 3.5 A & C) but not in ***C. chilonis.*** The posterior mandibular articulation was triangular in ***C. flavipes*** (fig. 3.5 A), slightly so in C. ***chilonis*** (fig 3.5 C) and almost undeveloped in ***C. sesamiae*** (fig 3.5 B).

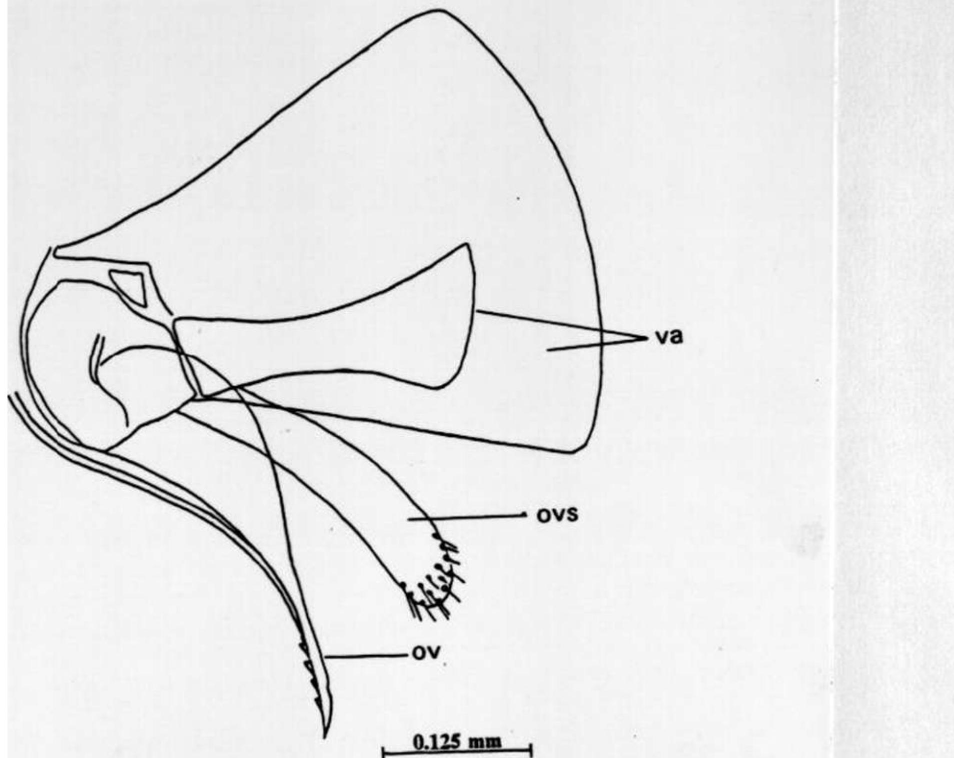


Fig. 3.4. Lateral view of female genitalia of *C. flavipes.*

va = valvifers, ovs = ovipositor sheath, ov = ovipositor

Fig. 3.5 Camera lucida drawings of immature stages of:

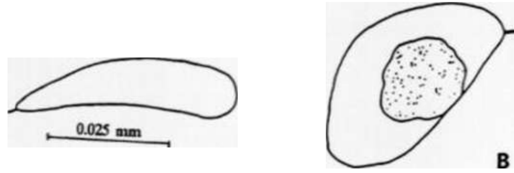
(A) one day old egg

(B) 3 days old egg

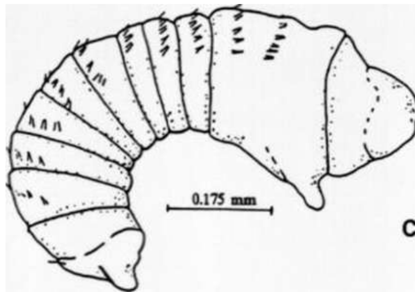
(C) 4-5 days old 1st instar larva (0.68-0.72mm in length)

(D) 7-10 day old 2nd larva (0.94-1.5mm in length)

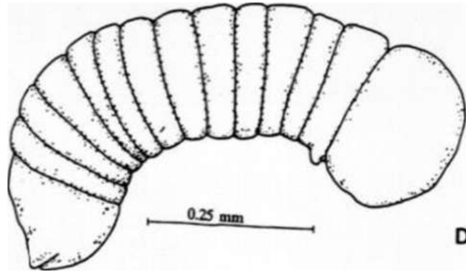
(E) 11-12 day old full grown 3rd instar larva (2.0mm in length)



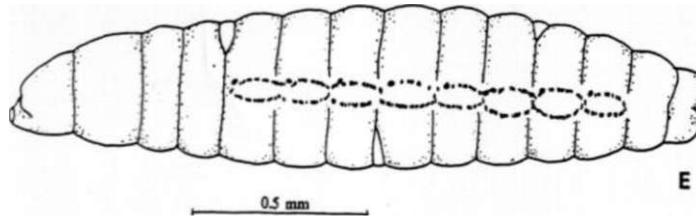
0.025 mm
B



0.175 mm
C



0.25 mm
D



E
0.5 mm

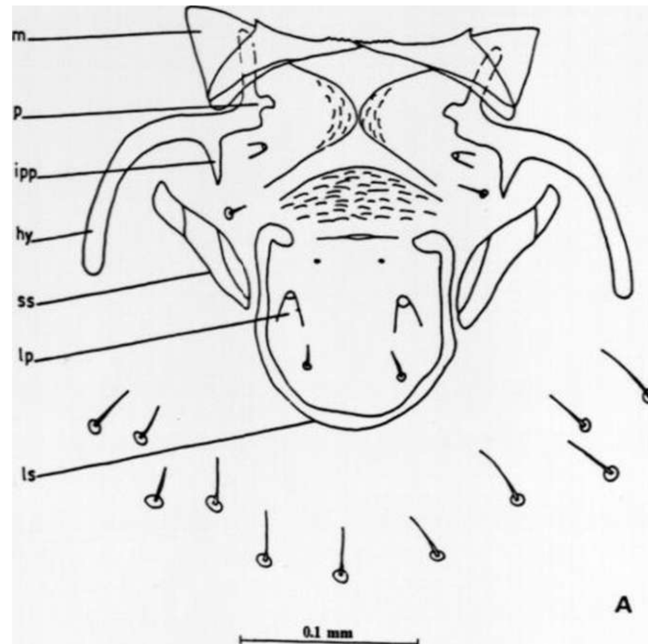
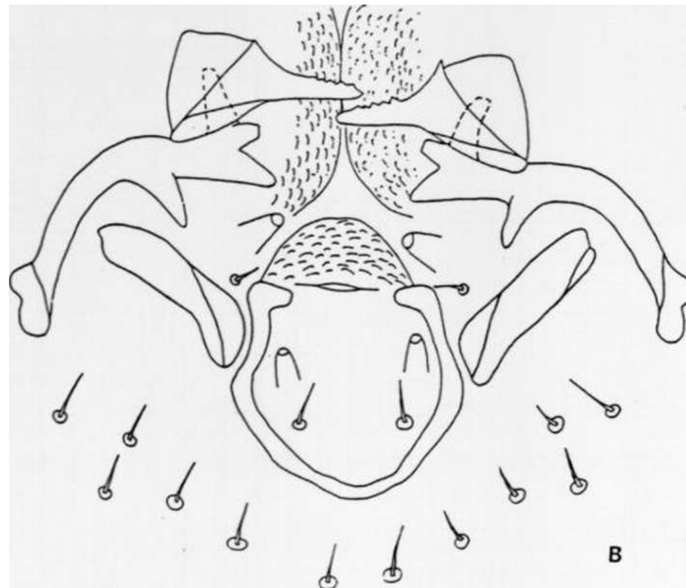
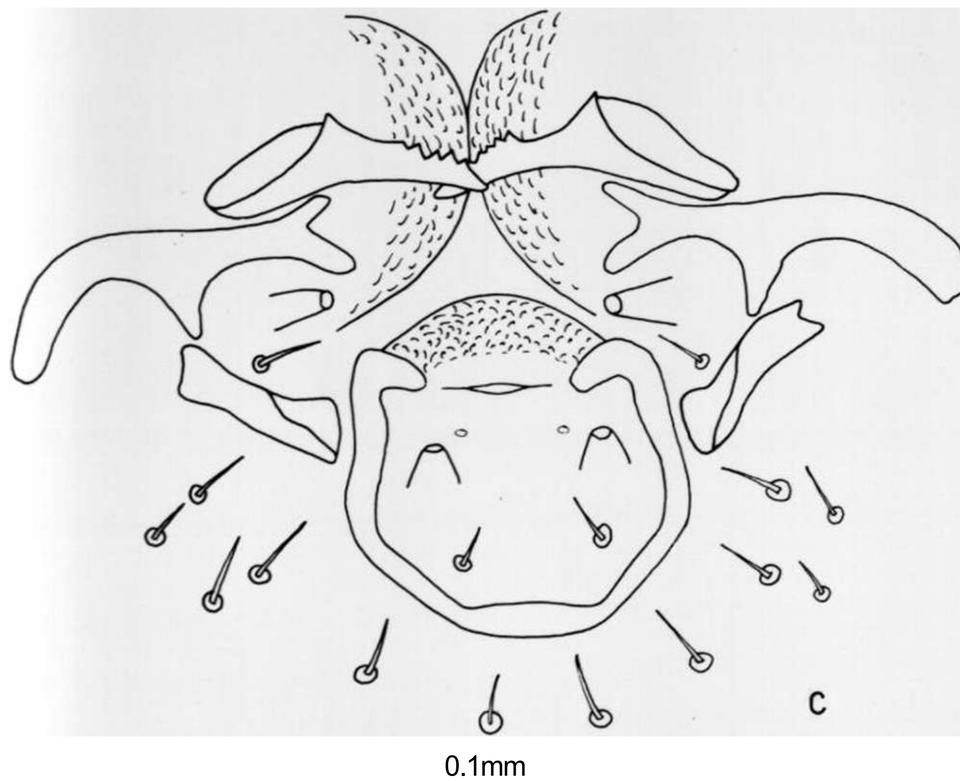


Fig. 3.6A Sclerotised parts of 3rd instar larval mouthparts of ***C. flavipes ;*** m mandible, p pleurostoma, ipp inferior pleurostomal process, hy hypostoma, ss stipital sclerite, lp labial palp, ls labial sclerite.



0.1 mm

Fig. 3.6B Sclerotised parts of 3rd instar larval mouthparts of ***C chi/onis.***



0.1mm

Fig. 3.6C Sclerotised parts of 3rd instar larval mouthparts of ***C. sesamiae.***

Observation of the cocoon masses

Mature third instar larvae emerge from the host (plate 3.3) and spin cocoons for metamorphosis to take place. The cocoons of ***C. flavipes*** species (plate 3.4) were more closely packed and smaller in size. The silk threads formed a network over the cocoons such that individual cocoons could not be easily located or separated. Those of ***C. sesamiae*** (plate 3.5) were loosely arranged and individual cocoons were spun neatly. ***Cotesia chiionis*** (plate 3.6) was intermediate between ***C. flavipes*** and ***C. sesamiae.***

Plate 3.3 Mature third instar larvae (***Cotesia f/avipes)*** emerging from host larva ***(Chi/o parte/lus)***

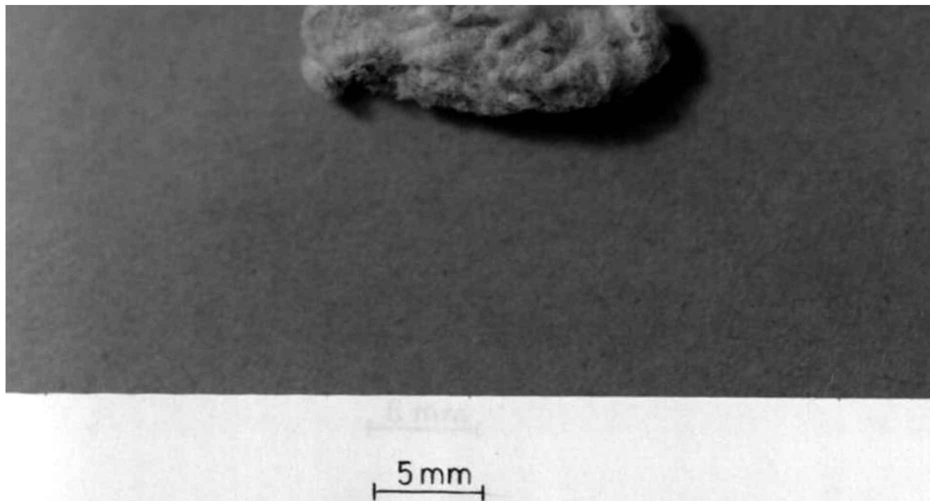


Plate 3.4 Cocoon mass of ***C. flavipes*** (North Pakistan population)

Plate 3.5 Cocoon mass of C. ***sesamiae*** (Kenya coast population)

6 nnm

Plate 3.6 Cocoon masses of C. *chilonis* (Japan, Niigata population)

3.4 DISCUSSION

Several workers have proposed various morphological characters to separate the species, primarily based on colouration, sculpturing, and male genitalia (Wilkinson 1932; Rao & Nagaraja 1967; Nagaraja 1971; Alam *et al.* 1972; Sigwalt & Pointel 1980). With the exception of male genitalia, these characters have not proven to be completely reliable (Polaszek & Walker 1991). Based on the male genitalia, Polaszek & Walker (1991) separated the species in the *C. flavipes* complex into two morphospecies: the *C. sesamiae/C. chilonis* subcomplex and *C. flavipes* and emphasized that further studies were necessary before any formal decisions concerning the taxonomy of the species could be made.

The external morphological characters examined showed a high degree of intraspecific variation, particularly in colour, the number of setae and surface sculpture (Table 3.1). However, a few characters could be used to distinguish between the three groups. The scuto-scutellar sulcus was straight in all the specimens of *C. chilonis,* and curved for the other two species. There were more than 20 hairs on the scutellum of *C. chilonis,* and less than 20 in the other species except the population from Malaysia. The propodeum was less rugose in the basal half in *C. sesamiae,* and almost fully rugose in the other two species, with the exception of the population from Papua New Guinea. The

overall shape of the male genitalia was valid for separating the populations of ***C. flavipes*** from the other two species. The Papua New Guinea population could only be separated from the Africa population by male genitalia. The character of the male genitalia varies slightly within and between populations of ***C. flavipes*** studied. This hitherto undocumented intraspecific variation within ***C. flavipes*** has been detected, with the male genitalia of the Mauritius population differing in various aspects. The placement of the Mauritius population in ***C. flavipes*** needs further investigation to verify whether this is yet another intraspecific variant of the character or an interspecific one. The male genitalia of ***C. chilonis*** and ***C. sesamiae*** could not be reliably separated.

Wilkinson (1928) used the prominence of the face to distinguish between ***C. flavipes*** and ***C. chilonis.*** He used the terms "face prominent" for ***C. flavipes*** and "face not especially prominent" for ***C. chilonis.*** Sigwalt and Pointel (1980) used the frontal protrudance just before the ocelli and referred to it as weakly convex in ***C. sesamiae,*** more accentuated in ***C. flavipes*** and practically convex in ***C. chilonis.*** Clearly, these characters and descriptions are too subjective to be of much use in distinguishing the three species, especially if one does not have reference material. In addition the characters varied intraspecifically as well as interspecifically. The frontal pubescence noted by Wilkinson (1928) and in ***C. sesamiae*** by Ullyett (1935) and used by Sigwalt and Pointel (1980) who stated that the pubescence appeared finer and more dence in ***C. chilonis***

than in ***C. sesamiae*** and even more in ***C. flavipes,*** could not be reliably used to separate the species. ***C. chilonis*** appeared to be generally more pubescent than the other two species but this character was too subjective to be of much use.

The larval morphology of ***C. chilonis*** and ***C. flavipes*** were compared by Drake (1966). Ullyett (1935) described ***C. sesamiae*** and included larval morphology in the description. The larval morphology of the three species had not been compared. There were no major differences in the larval morphology of the three species except the mouthparts. The position of the hypostoma (figure 3.5 A-C) suggested by Drake (1966) could not be reliably used as the position of the mandibles seemed to alter slightly depending on the slide mounts. However the two small labial setae found only in ***C. flavipes*** and C. ***sesamiae*** (figure 3.5 A & C) could be used to distinguish ***C. chilonis*** from the other two. Diagnosis of larvae is important and would be preferred since one does not have to rear the stemborer larvae and wait till the parasitoids emerge. Taxonomic characters such as allozymes could be used for the diagnosis of immature stages. The arrangement of cocoons of ***C. flavipes*** and ***C. sesamiae*** was used by Mohyuddin (1971) to distinguish between the two species. However like other external morphological characters, there were variations within and between species and ***C. chilonis*** could not be reliably separated from the other two species. In addition the character was subjective, difficult to quantify and unreliable since the arrangement of the cocoons also depended on

whether the host larva was stationary or mobile when parasitoid larvae emerged. However the character could be used in combination with others to separate ***Cotesia flavipes*** from ***Cotesia sesamiae***

Clearly the use of morphological characters alone is not sufficient to separate species in this complex, and should be supplemented by other techniques as described in the chapters that follow.

CHAPTER FOUR

4 MORPHOMETRICS

4.1 Introduction

The purpose of this study was to determine whether morphometries could be used to separate worldwide populations of the ***Cotesia flavipes*** complex into discrete groups. The similarities between the species and the apparent intraspecific variation within the complex prompted this study. In this study only females were examined. This made it possible to study the species complex without the previous groupings by male genitalia.

Prior to this study, no morphometric investigations had been conducted on the ***C. flavipes*** species complex. Moreover previous morphological comparisons among the species were restricted to a small number of specimens from a few localities, and therefore may have failed to detect intraspecific variation (Polaszek and Walker 1991; Sigwaltand Pointel 1980). Morphometric methods were therefore used to characterize size and shape differences between the populations at several localities.

4.2 MATERIALS AND METHODS

The majority of the specimens examined were from the Natural History Museum, London. Populations consisted of specimens originally collected from different geographical locations. Populations with missing data or with less than seven specimens were not analysed using multivariate methods. Sixteen characters were measured from specimens from the following localities: Thailand, Papua New Guinea, north and south Pakistan, Kenya, Japan (Niigata and Matsue shimane), South Africa, Zambia, Tanzania, USA (Texas) and China (see table 3.1 and figure 2.1)

4.2.1 Choice of characters

Characters were selected on the basis of their historical value for separating other Microgastrinae species. The following distances (mm) (figure 4.1) were measured using an image analysis system (videoplan) fitted with a video camera which sends the image of a specimen from a compound microscope to a frame-grabber board in a microcomputer.

Fig. 4.1 Landmarks (letters a to p) and distances (connecting lines) used to characterize size and shape differences of the antenna, head, thorax, hind leg and ovipositor for the three species, ***C. flavipes, C. sesamiae*** and ***C. chilonis***

a. flagellar segment 15: length (up middle)

b. flagellar segment 1 5: breadth (half way across)

c. flagellar segment 1: length (up middle)

d. flagellar segment 1: breadth (half way across)

e. tentorial pit to base of eye

f. a line across the face from the eyes through the antennal sockets

g. middle of the apical margin of the clypeus to a line across base of antennal sockets

h. length of the scuto-scutellar sulcus

i. middle of scuto-scutellar sulcus to the apex of the the scutellum

j. mid line between anterior and posterior margin of the propodeum

k. line between spiracles of propodeum

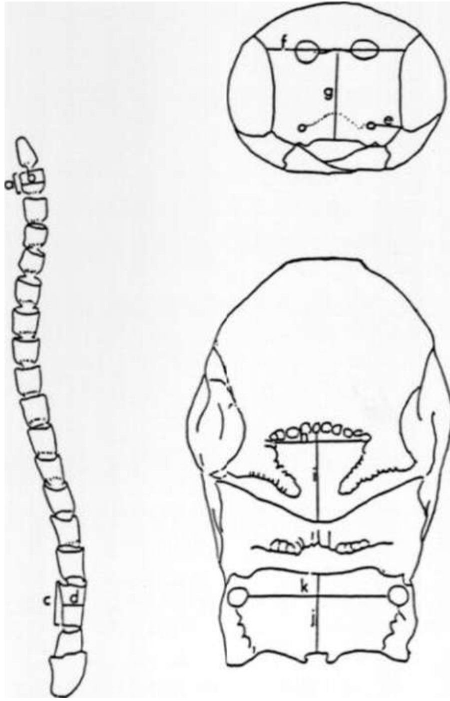
l. hind coxa, outer edge (longest side)

m. length of hind tibia on outer edge

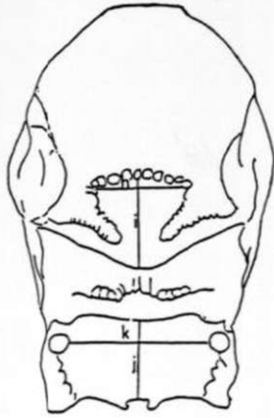
n. length of first segment of tarsus

o. length of the inner spine on apex of hind tibia

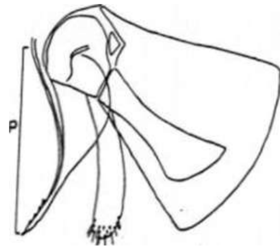
p. length of ovipositor



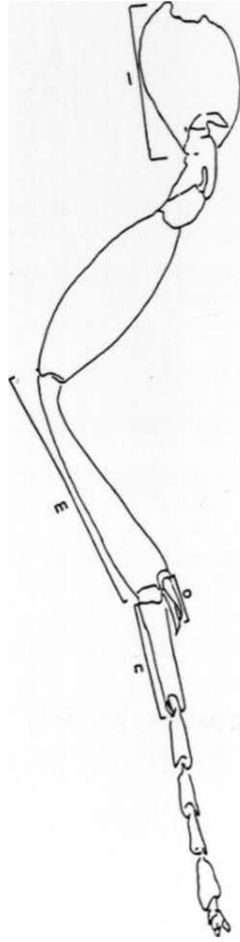
a
b
c
d



f
g
e
h
i
k
j



p



l
m
o
n

4.2.2 Method used to clear and macerate the specimens

Specimens were macerated in boiling potassium hydroxide for 5 minutes and then placed in a cavity dish containing hydrogen peroxide and ammonia (1:1) for two minutes. The specimens were next placed in concentrated acetic acid for about one minute to neutralize the alkaline solution. The samples were then dehydrated in 95% alcohol for three minutes and placed in clove oil. All the body parts to be measured were isolated and mounted in Canada balsam using cavity slides. The head, thorax, and abdomen were mounted in the cavity and the remaining body parts on the flat part of the slide. The body parts were mounted on a small drop of Canada balsam and positioned evenly for measurement. The slide was dried in an oven at 50°C for about three weeks to immobilize the body and then the cavity flooded with Canada balsam before placing cover slips on the slides.

4.2.3 Data Analysis

The measurements of the sixteen characters were analysed using principal component and canonical variate analyses. The analyses were performed using the Statistical Analysis System software (PROC PRINCOM & PROC DISCRIM, SAS version 6.04, SAS Institute 1988). Univariate and bivariate normality were tested for each of the sixteen variables.

The symbols used in the analyses were as follows: "s" = populations from sub-saharan Africa, "c" = populations from China and Japan, and "f" = populations from Pakistan, India, Thailand, Papua New Guinea and Texas, U.S.A. (see table 3.1 for specific localities and figure 2.1). Results of the univariate statistics are presented in table 4.1.

Principal components analysis (PCA) was performed on the variance-covariance matrix computed from logarithms (base 10) of the measurements. PCA was performed to determine the distribution of the twelve populations without the ***a priori*** constraint of assigning them to a particular species.

Canonical variates analysis (CVA) was used to evaluate the sixteen variables for discrimination between species with the three putative species treated as class variables. The data matrix was analysed using PROC DISCRIM using the canonical option (SAS version 6.04). Canonical variate analysis is a discriminant method, which presupposes that the data will fall into a given number of preassigned groups and then maximises inter-group covariance and minimises within-group variation. This analysis has particular value where taxonomic groups are very close or overlap (Sneath and Sokal 1973). The analysis provides the latent roots, percentage discrimination and centroids for each canonical axis. The latent roots provide a measure of the discriminatory power associated with each canonical variate. The first canonical variate,

which is the best discriminating axis attempts to maximise the distance between the centroids of all the groups. The second canonical variate provides maximal discrimination, subject to the constraint that it is orthogonal to the first and so on. The number of canonical variates that can be contructed are equivalent to the number of class variables minus one. The first two canonical variate axes account for a large proportion of the variation among groups. The distance between any two centroids in canonical variate space is termed Mahalanobis distance, usually presented in the squared form.

The distance between any two centroids in canonical space is termed Mahalanobis distance, usually presented in the squared form, D^2. Mahalanobis distance providesthe best mutivariate measure of the relative distance between groups, taking proper account of the variation within each of them. Mahalanobis distance was used in this study to determine the relative distances between the three species groups.

Table 4.1. Univariate statistics for variables used in morphometric analyses of the allopatric populations of species in the ***Cotesia flavipes*** complex.

Variable	mean	Std	N	Range	Prob <'
a	0.05	0.01	95	0.04-0.07	0.38
b	0.05	0.01	95	0.04-0.07	0.68
c	0.09	0.01	95	0.06-0.11	0.03
d	0.05	0.07	95	0.04-0.07	0.39
e	0.06	0.01	94	0.04-0.09	0.93
f	0.29	0.02	94	0.22-0.33	0.43
g	0.19	0.02	95	0.13-0.23	0.42
h	0.18	0.02	95	0.14-0.22	0.38
i	0.16	0.01	95	0.13-0.20	0.03
j	0.17	0.02	95	0.13-0.30	0.00
k	0.28	0.03	95	0.15-0.35	0.03
1	0.33	0.03	95	0.22-0.40	0.00
m	0.56	0.05	94	0.46-0.67	0.26
n	0.26	0.03	94	0.13-0.35	0.50
0	0.11	0.02	95	0.08-0.26	0.00
P	0.40	0.03	94	0.28-0.46	0.04

[1] Shapiro-Wilk statistic

4.3 RESULTS

4.3.1 Principal Components Analysis

The eigenvalues of the covariance matrix and eigenvectors of sixteen principal components are presented in table 4.2. Plots of individual specimens on the first and second principal components and the second and third principal components are shown in figures 4.2 & 4.3.

The first two principal components accounted for 53.8% of the variance. The third and fourth principal components accounted for 8.5% and 7.1%, respectively, but did not contribute to a clear segregation of the species. The remaining principal components accounted for smaller proportions of the variance ranging from 4.9% to 0.5% for the fifth and sixteenth principal components, respectively. The first eigenvector had approximatetly equal loadings on all variables except the length of the first flagella segment, measurement "c". The second eigenvector had high positive loadings on measurements "e", "f" and "g" and a high negative loading on measurement "c".

Table 4.2 Eigenvalues and weights for the three principal components, computed from the covariance matrix of the log-transformed data.

Variable	PCI	PCII	PCII
Eigenvalue	0.014	0.004	0.003
Proportion	0.412	0.127	0.085
log a	0.24	-0.02	0.02
log b	0.12	-0.02	0.14
log c	0.43	-0.33	-0.02
log d	0.23	-0.14	0.65
log e	0.03	0.69	0.37
log f	0.11	0.29	-0.03
log g	0.14	0.38	-0.19
log h	0.19	0.13	-0.15
log i	0.17	0.15	0.10
log j	0.28	-0.02	0.10
log k	0.30	-0.15	0.18
logl	0.31	0.01	0.14
log m	0.27	0.03	-0.10
log n	0.30	0.05	-0.49
log o	0.36	0.10	-0.18
log p	0.20	-0.13	-0.12

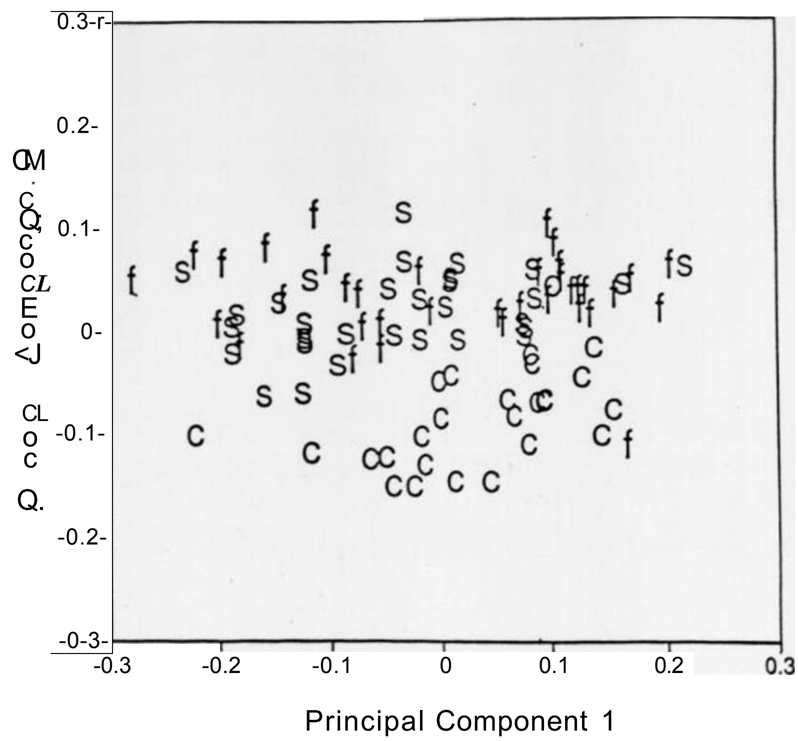


Fig. 4.2 Data for all the measurements plotted on the first two principal components. The first two principal components accounted for 53.8% of the sample variance, s = ***C. sesamiae*** f = ***C. flavipes,*** c = C. ***chilonis.***

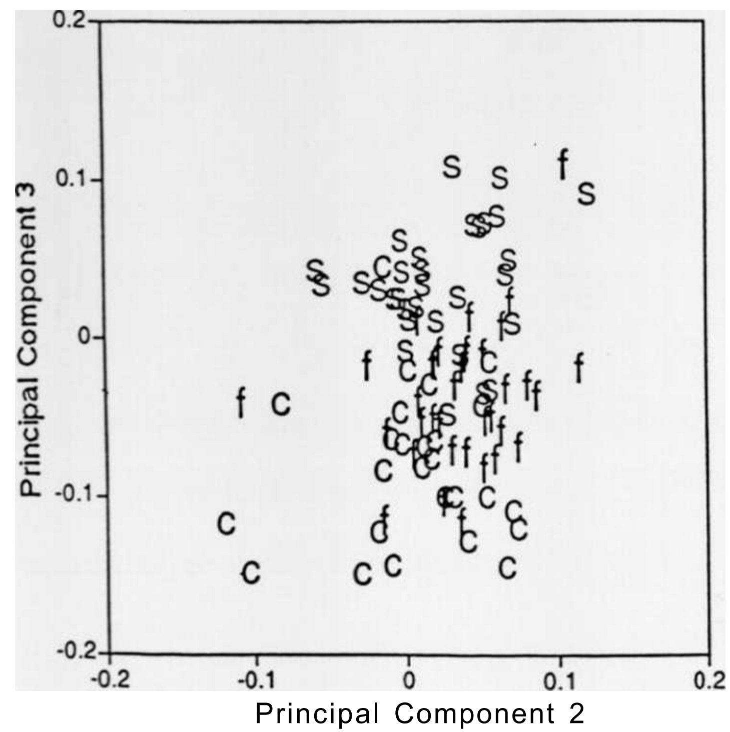


Fig. 4.3 Data for all the measurements plotted on the the 2nd and 3rd principal components. The third principal component accounted for 8.5% of the sample variance, s = C. ***sesamiae*** f = ***C. flavipes, c = C. chi/onis.***

4.3.2 Canonical Variate Analysis

Observations of the three species projected on the first and second canonical variates are shown in Figure 4.4. The first canonical variate accounted for 59% of the between-groups sample variance and essentially discriminated between ***C. chilonis*** and C. ***flavipes.*** The second canonical variate represented 41 % of the between group sample variance and discriminated between ***C. sesamiae*** and the other two species. The raw, standardized, total canonical structure coefficients and the Mahalanobis distances are presented in tables 4.3 & 4.4 respectively. Canonical variables are constructed to maximally discriminate between pre-assigned groups. A distinct separation of the three species on the two canonical variates axis was evident. The Mahalanobis squared distances (D^2) between the three species (table 4.4) showed that the distances between the species were nearly equal.

To test the performance of the discriminant functions (canonical variates) a test population was removed from the original eleven populations and projected on the first two canonical variates constructed using the other ten. Classification of the test population using the discriminant functions developed from the other ten populations assigned all individuals to their previous group.

Table 4.3. Standardized, raw and total canonical structure coefficients (CS) for the canonical variates analysis on the standardized data of the various populations of ***Cotesia flavipes*** complex species.

	CVI coefficients			CVII coefficients		
Var	Raw	Standardized	Total CS	Raw	Standardized	Total CS
a	-26.60	-0.14	0.00	-9.80	-0.05	-0.19
b	-36.52	-0.19	-0.27	47.43	0.24	0.16
c	-9.88	-0.12	-0.14	-91.69	-0.15	-0.56
d	-22.67	-0.14	-0.24	5.38	0.03	-0.03
e	32.72	0.25	0.36	17.98	0.14	0.39
f	32.82	0.62	0.55	22.37	0.42	0.23
g	5.39	0.12	0.30	0.01	0.02	0.18
h	66.76	1.04	0.59	-30.36	0.47	-0.06
i	-3.71	-0.05	0.05	78.74	1.04	0.32
j	-12.47	-0.21	-0.19	2.27	0.04	-0.18
k	-64.84	-1.86	-0.51	-21.27	-0.61	-0.18
1	-15.81	-0.51	-0.09	26.43	0.85	-0.03
m	14.40	0.66	0.13	14.22	0.65	-0.22
n	23.39	0.70	0.44	-39.00	-1.17	-0.45
0	11.40	0.16	0.14	40.24	0.58	-0.09
P	8.69	0.26	0.03	-34.68	-1.03	-0.63

Table 4.4. Canonical discriminant analysis; Mahalanobis squared distances of the three clusters representing the three species of the C. *flavipes* complex.

Species	C. *chilonis*	*C. flavipes*	*C. sesamiae*
C. chilonis	0.0		
C. flavipes	35.4	0.0	
C. *sesamiae*	28.0	28.1	0.0

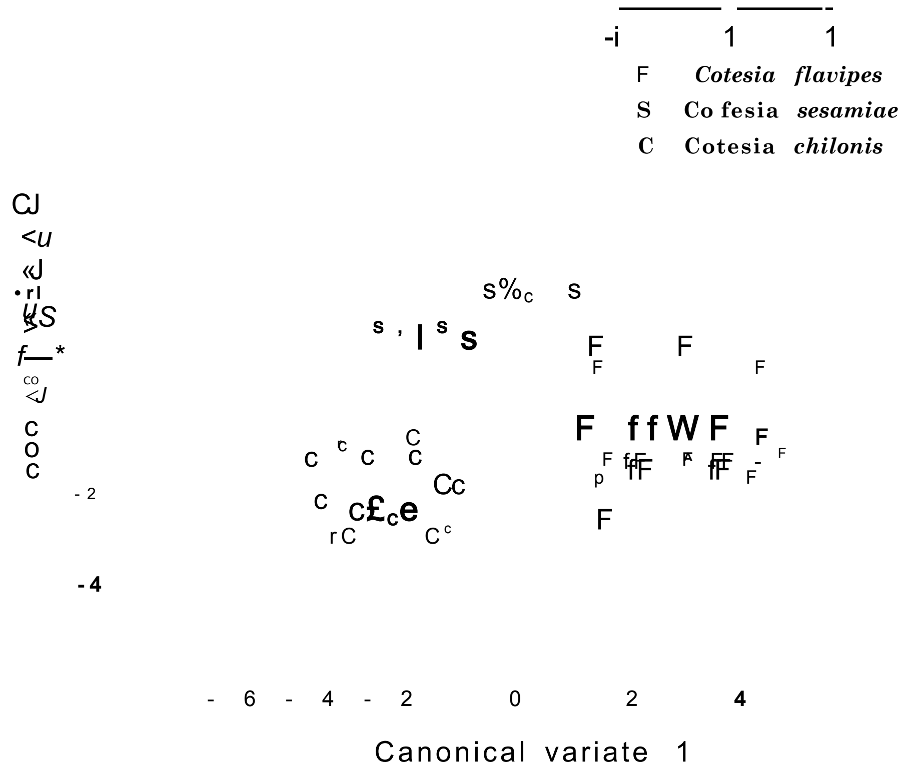


Fig. 4.4 Data for all the measurements plotted on the first and second canonical variates. The first canonical variate represents 59% of total sample variance, the second canonical variate represents 41 %. s = C. ***sesamiae*** f = C. ***flavipes,*** c = ***C. chilonis.***

4.4 DISCUSSION

A projection of the individual specimens on the first and second principal components resulted in two clusters of C. ***chilonis*** and C. ***flavipes/C. sesamiae.*** The clusters were distinct with respect to the second principal component only. The weights for the variables of the first principal component were all positive and the points were distributed along the axis. It can therefore be assumed that the location of points on the first principal component was strongly affected by overall size of the specimens (Marcus 1990). If location on the first principal component represented overall size, then differences on the second and subsequent principal components represented shape differences between the species. The relative magnitudes of weights provided an indication of the relative contribution of a variable to the location of a point on the principal component. In this case, measurements of the distances between the tentorial pits to base of the eye and the length and width of the face respectively, were shorter in C. ***chilonis*** than in the other two species (measurement "e", "f'Y'g", Figure 4.1). The decreased length of these characters in ***C. chilonis*** altered the shape of the face and distinguished this group from the rest, with the exception of a few outliers. Other characters that had relatively high magnitudes on the second principal component were the length of the first flagella segment ("c") and the width of the scutellum ("h"). The above results also showed that it was not possible to separate the species in the complex by size and or shape of the various characters alone.

The canonical variate analysis clearly indicated that there were three distinct groups in the ***C. flavipes*** species complex. As noted above, the covariation in traits distinguished the three taxa. No single measurement provided a reliable criterion for identification in all cases. Discriminant analysis produced a single linear transformation of the original variables that optimally discriminated between the predefined groups, and provided a statistical method to place unknown specimens in known groups. Classification of the test population using discriminant functions developed from the ten populations assigned all individuals to their previous group, demonstrating the value of the set of measurements in classifying unknown groups.

Although the three groups existed in some of the literature as three distinct species, the characters used by previous authors could not be used to separate the species with complete certainty. The results of the morphometric analysis support the earlier finding that C. ***flavipes*** is a distinct group, easily distinguished by examination of the male genitalia (Rao and Nagaraja 1967; Sigwalt and Pointel 1980). However the use of characters such as setae and shape of the face, rugosity of hind coxa, and male genitalia by Nagaraja (1971) and Sigwalt and Pointel (1980) to separate C. ***chilonis*** from ***C. sesamiae*** is not supported by the results of this study. These characters have been found to vary within and between the taxa (Polaszek and Walker 1991). However, considering the canonical variate analysis and morphological differences found so far in this study, the two species have been maintained as distinct taxa.

CHAPTER FIVE

5 CUTICULAR COMPONENTS PATTERN ANALYSIS

5.1 Introduction

Cuticular hydrocarbons are components of the superficial layer of lipid which occurs in the epicuticle. This lipid is shed with the exuvium at ecdysis (de Renobales and Blomquist 1983) and during each moult, fresh lipid is synthesized by a new generation of oenocytes which arise from the hypodermis at the time of mitosis (Wigglesworth 1988). Epicuticular lipids vary in composition, though nearly all contain aliphatic hydrocarbons. The experimental procedure for obtaining and examining these hydrocarbons usually involves (a) extraction of lipid from the cuticle with an organic solvent, (b) separation of the hydrocarbons by column chromatography and (c) analysis by gas chromatography and identification by combined gas chromatography-mass spectrometry (GC-MS) (Lockey 1988). Three main hydrocarbon classes that have been identified in hydrocarbon mixtures are: n-alkanes, alkenes and methylalkanes. Nearly all examined insect species have mixtures of n-alkanes. Hydrocarbon composition may vary between species and between the males, females and instars of a species (Lockey 1991). The aim of this work was to study the pattern of cuticular components in the ***Cotesia flavipes*** parasitoid complex and see whether the pattern could be used as a character to distinguish between the three putative species.

5.2 MATERIALS AND METHODS

5.2.1 Insects

Laboratory cultures of ***C. flavipes*** from North Pakistan, ***C. sesamiae*** from the Coast Province of Kenya and ***C. chilonis*** from Japan were examined in this study.

5.2.2 Sample Preparation

Parasitoids were killed at -20° C and immediately analysed or stored at the same temperature for later analysis. Specimens were brought to room temperature (22° C) before extraction of the cuticular components.

Single ***Cotesia*** specimens were placed in $2.5cm^3$ pyrex tubes sealed at one end. Redistilled pentane (25/yl) containing an internal standard (n-nonadecane) in the ratio of 10:1 was added as a solvent. After 5 minutes, the extract was removed and concentrated to 5/yl. 1//l of the extract was injected into a Hewlett Packard 5890A Gas Chromatograph (HP GC) connected to a Hewlett Packard 3393A computing integrator for plotting and integrating the chromatographic signals.

5.2.3 Gas Chromatography

The Gas Chromatograph was fitted with a 50m Hewlett Packard fused silica capillary column crosslinked with methyl silicone gum. The column had an internal diameter of 0.32mm and 0.17/vm film thickness. The chromatograph was fitted with a flame ionization detector (FID). The carrier gas was nitrogen.

Programming Conditions of HP GC for the ***Cotesia flavipes*** complex.

Initial temperature	100°C
Maximum temp.	280°C
Temp, rise	15°C/min
Initial time	4 min
Final time	20 min
Attenuation	2[1]
Chart speed	0.5 cm/min.
Area reject	100[2]

Attenuation determines the amplitude of the signal required to produce full-scale printhead deflection. Attenuation settings are in powers of 2 with ATT 2^a = 0 representing a full scale deflection voltage of approximately 1mV. Full scale in this context is the distance between plot position 100, approximately 71mm. Attenuation is chosen to make the smallest peaks of interest large enough to be readily visible in the plotted chromatogram. Attenuation affects only the presentation of the plotted signal on the printer/plotter and does not affect the amplitudeof the signal or data applied to the HP 3393A.

[2]Area reject is the area count value below which peaks will not be reported or stored in the processed peak file. This is programmed as "minimum area reject" where minimum area is an integer from 0 to 2147483647 and representing counts in 1/8uV/secs.

A chromatographic "blank run" was performed every day before analysing samples. The profile from the blank run was subtracted from sample profiles to remove baseline drift (usually caused by column bleed).

5.2.4 Data Recording and Analysis

A total of forty insects were analysed from each population. The integrated peak areas, which were assumed to be proportional to the concentrations of compounds with different retention times, were the raw data on which statistical analyses were based. The areas of all the major peaks (14) were recorded using the standard peak as a reference point. The peak areas of each of the fourteen peaks were standardized by coverting them into percentages of the area of the internal standard. Analysis of variance and Student-Newman-Keuls multiple comparison test (PROC GLM, SAS institute, 1988) were performed for each peak in the three species to test whether there was a significance difference between the mean peak areas of the species. A stepwise selection procedure (PROC STEPDISC, SAS Institute, 1988) was performed to identify the peaks that could best discriminate between the three species. Out of the fourteen peaks, seven were selected: peaks 2, 3, 4, 5, 6, 10 and 11 (figure 5.1). Univariate statistics were performed on the selected peaks.

Principal Component and Canonical Discriminant Analysis were then performed (PROC PRINCOMP and PROC CANDISC, SAS Institute, 1988).

5.3 RESULTS

5.3.1 Qualitative Analysis

Qualitative analysis of the profiles obtained from each of the three parasitoid species did not show differences in terms of presence or absence of peaks. However some mean peak areas differed significantly between the species. Chromatograms of cuticular components of; ***C. flavipes*** Cf, ***C. sesamiae*** (Cs), and ***C. chilonis*** (Cc) are presented in figure 5.1. A summary of the standardized mean areas of each peak in a species and the student-Newman-Keuls groupings are presented in table 5.1. Peaks two, three, and five were significantly different in C. ***sesamiae.*** Peak nine and eleven were significantly different in C. ***chilonis.***

5.3.2 Quantitative Analysis

The univariate statistics for comparing group mean hydrocarbon concentrations and test statistic for the null hypothesis that the input data were a random sample from a normal distribution constructed for the seven peaks is presented in table 5.2. The eigenvalues of the covariance matrix and the eigenvectors of the seven principal components are summarised in table 5.3. The eigenvalues indicate that the first two principal components provide a good

summary of the data, the two components account for 94% of the variance. Subsequent components contribute less than 6%. The first eigenvector has high positive loading on peak 5 (0.98) while the second eigenvector has a similar loading (0.97) on peak 6. The raw, total sample standardized canonical coefficients, the total canonical structure and the Mahalanobis distances are summarised in tables 5.4 & 5.5. Plots of the first and second principal components and canonical variates are presented in figures 5.2 & 5.3. No distinct separations were obtained using either principal components analysis or canonical variates. The Mahalanobis distance between the groups (table 5.5) indicated that ***C. flavipes*** was closer to ***C. chi/onis*** than to ***C. sesamiae.*** However, all ***C. sesamiae*** specimens fell in their appropriate class using the linear discriminant funtion developed from the discriminant analysis.

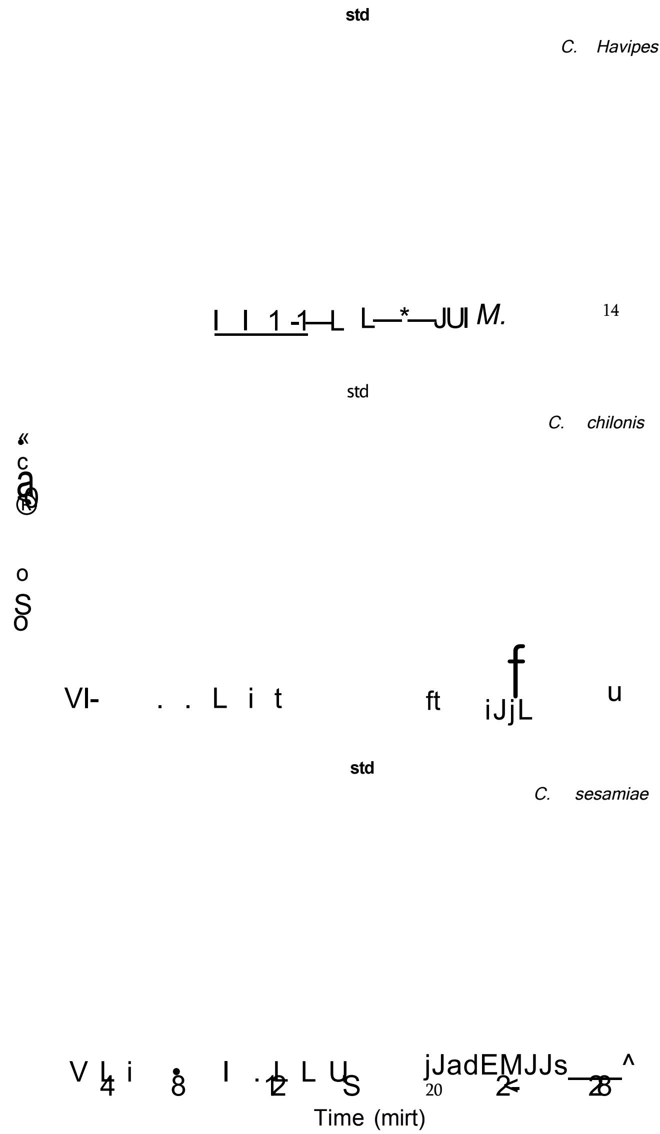


Fig. 5.1 Chromatograms of cuticular components of single specimens of ***C. flavipes, C. chilonis*** and ***C. sesamiae.*** (Column length = 50 crosslinked with methyl silicone gum, internal diameter = 0.32mm and 0.17//m film thickness).

Table 5.1. Means of peaks and Student-Newman-Keuls (SNK) multiple comparison groupings.

	Means, std & SNK groupings		
Peaks	*C. flavipes*	*C. sesamiae*	*C. chilonis*
Pk 1	0.0911 ± 0.16 a	0.0320 ± 0.10 a	0.1046 ± 0.07 a
Pk 2	0.4104 ± 0.26 a	0.0802 ± 0.05 b	0.48691 ± 0.34 a
Pk 3	0.0715 ± 0.05 a	0.0152 ± 0.01 b	0.0943 ± 0.07 a
Pk 4	0.0288 ± 0.02 a	0.0180 ± 0.03 a	0.0608 ± 0.02 a
Pk 5	2.4020 ± 1.61 a	0.3440 ± 0.29 b	3.0920 ± 2.31 a
Pk 6	0.6440 ± 0.67 a	0.3140 ± 0.30 a	0.5910 ± 0.61 a
Pk 7	0.0170 ± 0.01 a	0.0231 ± 0.02 a	0.0739 ± 0.26 a
Pk 8	0.0281 ± 0.02 a	0.0184 ± 0.02 a	0.0265 ± 0.02 a
Pk 9	0.0130 ± 0.02 b	0.0374 ± 0.08 b	0.1190 ± 0.16 a
Pk 10	0.2500 ±0.34 a	0.3740 ± 0.41 a	0.2810 ± 0.67 a
Pk 11	0.0629 ± 0.07 a	0.0732 ± 0.07 a	0.2340 ± 0.01 b

Table 5.2 Univariate statistics for variables used in mutivariate analyses of cuticular components of the three species in the *C. flavipes* complex.

Variable	Mean	Std	N	Range
Peak 2	0.313	0.30	101	0.020-1.64
Peak 3	0.065	0.06	84	0.003-0.33
Peak 4	0.035	0.09	87	0.004-0.82
Peak 5	1.860	1.97	101	0.040-11.02
Peak 6	0.510	0.55	98	0.013-3.79
Peak 10	0.310	0.48	98	0.014-3.62
Peak 11	0.054	0.06	84	0.007-0.30

Table 5.3 Eigenvalues and weights for the two principal components, computed for the covariance matrix of the standardized peak areas.

Variable	PCI	PCII
Eigenvalue	4.263	0.315
Proportion	0.882	0.065
Peak 2	0.150	0.010
Peak 3	0.030	-.005
Peak 4	0.010	0.002
Peak 5	0.980	-.119
Peak 6	0.110	0.972
Peak 10	0.040	0.197
Peak 11	0.002	0.031

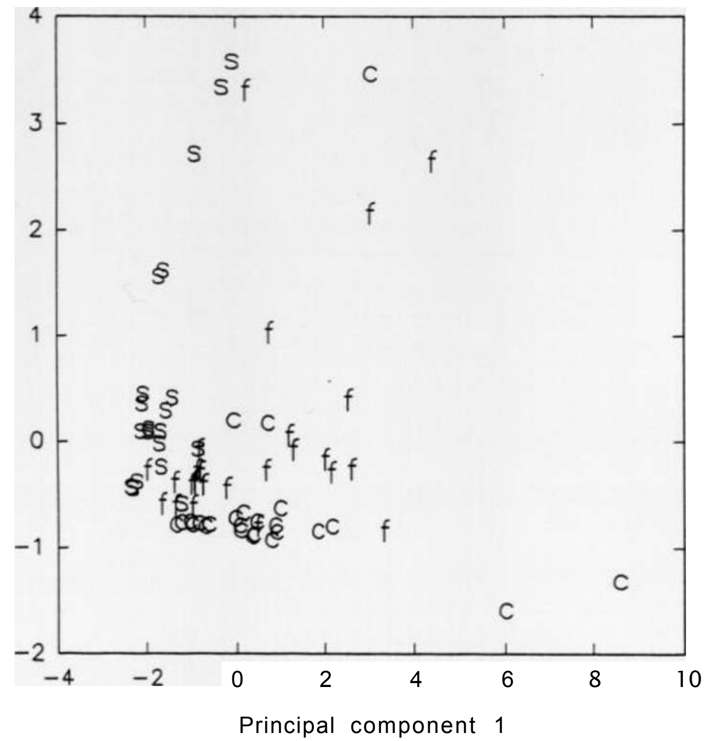


Fig 5.2 Data for all the measurements plotted on the first and second principal components, s = C. ***sesamiae c = C. chilonis*** f = ***C. flavipes***

Table 5.4. Standardized, raw and total canonical structure coefficients (CS) for the canonical variates analysis on the standardized data of the three species of the ***Cotesia flavipes*** complex.

	CVI coefficients			CVII coeffients		
Var	Raw	Standardized	Total CS	Raw	Standardized	Total CS
Pk2	8.23	2.56	0.79	-12.46	-3.88	-0.16
Pk3	2.74	0.16	0.76	1.14	**0.06**	-0.04
Pk4	-50.95	-0.95	0.51	-10.26	-0.19	-0.16
Pk5	-0.32	-0.64	0.78	1.93	3.92	0.04
Pk6	0.28	0.17	0.29	-0.51	-0.30	-0.42
Pk10	-0.31	-0.16	-0.04	1.28	0.65	0.09
Pk11	-10.39	-0.66	-0.40	-6.04	-0.38	-0.54

Table 5.5. Canonical discriminant analysis; Mahalanobis squared distances of the clusters representing the three species of the ***C. flavipes*** complex.

Species	C. ***chilonis***	***C. flavipes***	***C. sesamiae***
C. chilonis	0.00		
C. flavipes	1.31	0.00	
C. sesamiae	7.29	4.35	0.00

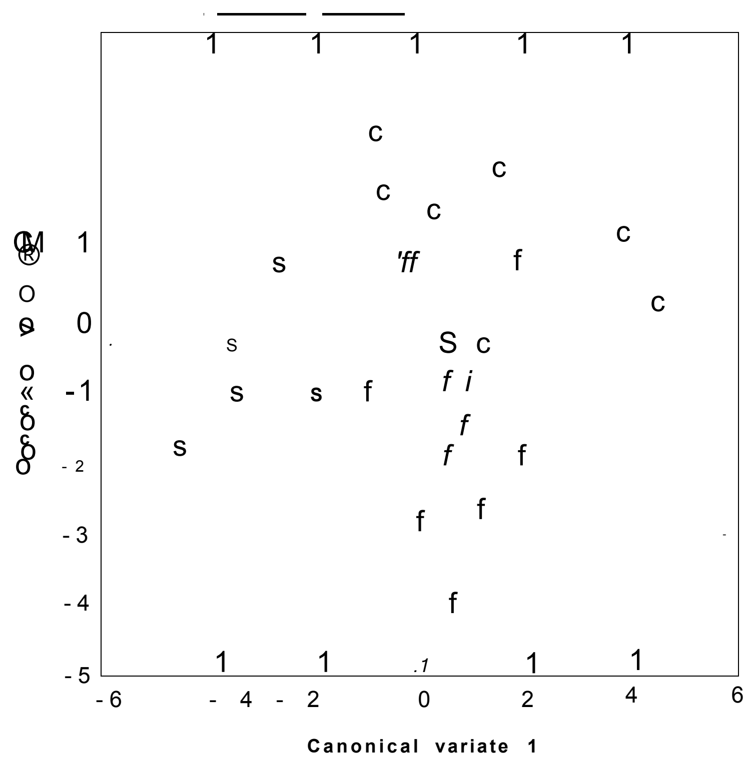


Fig. 5.3 Data for all the measurements plotted on the first and second canonical variates.

6.4 DISCUSSION

The pattern of cuticular components of the three species of the ***C. flavipes*** complex do not appear to be species specific except for the concentrations of some compouds (table 5.1) and therefore can not be used to separate the species. Principle component and canonical variates analyses did not separate the three species into any distinct groupings. However ***C. sesamiae*** individuals tended to cluster together with very few outliers with respect to the first principal component. The Mahalanobis distance between ***C. flavipes*** and C. ***chilonis*** was 1.31 and that between C. ***sesamiae*** and C. ***chilonis*** was 7.29. ***C. chilonis*** and C. ***flavipes*** were reared on ***Chilo partellus*** while C. ***sesamiae*** was reared on ***Sesamia calamistis.*** It is possible that the cuticular components of these parasitoids are influenced by their lepidopteran hosts. Insects synthesize cuticular hydrocarbons by an elongation-decarboxylation pathway in which malonyl derivatives are successfully incorporated to form a long-chain fatty acid, which on decarboxylation yields a hydrocarbon with one carbon less (Blomquist and Dillwith 1985). Since fatty acids serve as precursors in hydrocarbon biosynthesis (Blomquist ***et al.*** 1987) variations in the fatty acid composition of a host insect might affect the hydrocarbon pattern of parasitoids emerging from that host. Espelie and Brown (1990) studied the cuticular hydrocarbons of species which interact on four trophic levels: host plant (Apple), herbivore (codling moth), Hymenoptera

parasitoid (*Ascogaster quadridentata* Wesmael) and hyperparasitoid (*Perilampus fulvicornis* Ashmead), and found that there was a strong similarity in the surface chemistry of the species in all the trophic levels. The endoparasitoid, *A. quadridentata,* obtains all of its nutrients from its host. Each n-alkane present in the cuticle of codling moth larvae was also found in *A. quadridentata* adults. Kudon and Berisford (1981) found that the fatty acid profiles of several hymenopterous parasitoids were very similar to those of the host bark beetles from which they were reared. Although the various cuticular components in the *C. flavipes* complex were not identified, the qualitative and multivariate analyses demonstrated that the pattern of cuticular components in the three species was not distinct and therefore could not be used to separate the species. The surface chemistry may have been influenced by the lepidopterous hosts *Chilo partellus* and *Sesamia calamistis.* The pattern of cuticular components of *C. partellus* and *C. orichalcociliellus* were analysed by Kioko (1994) but the compounds were not identified. Multivariate analyses conducted using the percentage peak areas of the two species indicated that laboratory reared larvae (on artificial diet) were distinct (formed a single cluster) from the field collected larvae even when they were of the same species. Identification of the compounds found in the hosts and parasitoids would determine whether the surface chemistry of the parasitoids is affected by the host environment. Therefore further investigations including the analysis and identification the compounds in both the host and parasitoid are necessary to

clarify use and importance of cuticular components pattern analysis parasitoid systematics.

CHAPTER SIX

6 ALLOZYME ELECTROPHORESIS

6.1 Introduction

Protein electrophoresis, the migration of proteins under the influence of an electrical field, is among the most cost efficient methods of investigating genetic phenomena at the molecular level (Murphy ***et at.*** 1990). Using enzymatic proteins, numerous investigations have focused on enzyme efficiency, estimating and understanding genetic variability in natural populations, gene flow, hybridization, recognition of species boundaries, and phylogenetic relationships.

Two general forms of protein data can be gathered simultaneously using electrophoretic methods. One is derived from isozymes, which are all functionally similar forms of enzymes, including all polymers of subunits produced by different gene loci or by alleles at the same locus (Markert and Moller 1959). The other data set consists of allozymes, a subset of isozymes, which are variants of polypeptides representing different allelic alternatives of the gene locus. Both forms of data are important in molecular systematics, and both involve proteins that can be separated on the basis of net charge and size (Murphy ***et at.*** 1990). In this study, allozyme analysis was conducted using

starch gel electrophoresis and Iso-electric focusing (IEF) using the PhastSystem™ (Pharmacia, Uppsala Sweden)

Isoelectric focusing is an electrophoretic method which separates protein in a stable pH gradient established between two electrodes by a mixture of soluble carrier ampholytes with pH increasing from anode to cathode. Proteins migrate within the gradient to their respective isoeletric points (pi) and stops to form a sharp concentrated band where their charge is zero, because positive and negative charges of the molecules cancel, at the iso-electric point, movement ceases in the electric field (Olsson ***et al.*** 1988). A major problem encountered with electrophorectic data is the fact that the majority of amino acid subsititutions are undetected under standard electrophoretic conditions (Menken 1989). Isoecletric focusing with immobilised pH gradients is an equilibrium technique in which the effects of diffusion are overcome. It enables the separation of proteins up to 0.003 of a pH unit (Cossu and Righetti 1987). The PhastSystem™ is ideal for analysing small specimens like ***Cotesia*** species since only minute quantities are required. It is also convenient for routine taxonomy since it is fast (it takes only 30 minutes to run the gel) and one does not need to prepare the polyacrylamide gels which often involve handling toxic chemicals. The aim of the IEF work was to find out whether the allozymes could be resolved using the PhastSystem and also to confirm the banding patterns observed using starch gel electrophoresis by isoelectric focusing for genetic and phylogenetic analyses.

6.2 MATERIALS AND METHODS

6.2.1 Insects

For protein analysis, fresh or freshly frozen insect specimens were required, therefore this study was limited to the following populations: ***C. flavipes*** from south and north Pakistan, ***C. chilonis*** from Niigata in Japan C. ***sesamiae*** colonies from the coast and western provinces of Kenya. In addition, the Thailand and Rio Grande Valley (Texas) populations of ***C. flavipes*** from cultures maintained at the biocontrol laboratories of Texas A&M University were imported and stored in liquid nitrogen. An outgroup ***(Cotesia glomerata)*** was imported from Netherlands and also stored in liquid nitrogen.

6.2.2 Enzyme and buffer systems

Fourteen enzyme systems were examined in this study. The enzyme systems, enzyme classification (E.C.) numbers (IUPAC, 1973) and the specific gel electrode buffer systems are presented in table 6.1.

Table 6.1 Enzyme systems analyzed, enzyme classification (E.C) number (IUPAC,1973) and the gel-electrode buffer systems used in the analysis. See text for description of the buffer systems.

Enzyme system	E.C. No.	Buffer system
aDL-glycerophosphate dehydrogenase(aDL-GPDH)	1.1.1.8	TC
Mannose phosphate Isomarase(MPI)	5.3.1.8.	CA8
Hexokinase(HK)	2.7.1.1	CA8
Malic enzyme(ME)	1.1.1.40	CA8
Isocitric dehydrogenase(IDH)	1.1.1.42	CA8
Malate dehydrogenase(MDH)	1.1.1.37	CA8
Triose phosphate Isomerase(TPI)	5.3.1.1	CA8
Aconitase(ACO)	4.2.1.3	CA8
Phosphogluco mutase(PGM)	2.7.5.1	TM
Esterase(EST)	3.1.1.1	CA8
Glucose phosphate isomerase(GPI)	5.3.1.9	TC
Aldolase(ALD)	4.1.2.13	CA8
Sorbitol dehydrogenase(SDH)	1.1.1.14	CA8
6-Phoshogluconic acid dehydrogenase(6-PGDH)	1.1.1.1	CA8

The following buffer systems were used:

1. CA-8 buffer system: Gel buffer: 0.074 M Tris and 0.009 M citric acid, pH adjusted to 8.55. Electrode buffer: 1.37 M Tris and 0.314 M citric acid, pH adjusted to 8.15, (cathode tray buffer was diluted 1:4 with distilled water and anode tray 1:5) (Steiner and Joslyn 1979).

2. Tris maleate (TM) pH 7.4 for both gel and electrode buffers: 0.1 M maleic acid, 0.013 EDTA and 0.01 M $MgCl_2$- The gel buffer was diluted 1:10.

3. Tris- Citrate (TC) buffer: 0.22 M Tris and 0.08 M citric acid. Electrode buffer was adjusted to pH 6.3 and gel buffer to pH 6.7. The gel buffer was diluted 1:14 before use (Pasteur ***et at.*** 1988).

6.2.3 Sample extraction and starch gel electrophoresis

The insects were killed by deep freezing then individually homogenized in 20ul of 0.1 M Tris- HCl, pH 7.4 buffer on ice. Cotton threads 5mm long, previously boiled for five minutes and dried, were soaked in the crude homogenate and then loaded into the wells of a 7.6% thin starch gel (Sigma S-4501). The electrophoresis was carried out on a flat bed apparatus (FBE 3,000, Pharmacia, Sweden). Temperature was maintained at 4.3°C using a

hetofrig cooler (Heto Bikerod, Denmark). Sponge wicks were used to create a flow of current from the cathode buffer, through the gel, to the anode buffer. Current was supplied at a constant 250 V for 2[1]/* hours. Gels were stained immediately for specific enzymes using methods outlined by Harris and Hopkinson (1978) and Pasteur ***et a/.,*** (1988) with some modifications. The modified recipes are given in Appendix 1.

6.2.4 Iso electric focusing

Horinzontal Isoelectric focusing was performed using the flat bed of the PhastSystem™ (Pharmacia LKB, Biotechnology AB, Uppsala) for the above populations and stained for the same enzymes. Pre-cast PhastGel IEF pH 3-9 were used.

Sample extraction

Single specimens were homogenized in 20/yl of Triton X-100 extraction buffer (2% Triton X-100 & 20% glycerol) and centrifuged at 3000 rpm for 10 minutes. *2fj* of the supernatant was placed on a PhastSystem sample template, then using a phastsystem sample applicator, the samples were loaded on a polyacrylamide pre-cast phastgel (pH 3-9). The following programme was used to run the samples on the PhastSystem:

Programming for the PhastSystem™

Programme for the IEF of ***Cotesia*** species using the PhastSystem. (adopted from the PhastSystem™ Operating Manual, 1988)

Step	Stage	Voltage	Current MA	Power W	Temp °C	Vh
1	Prefocusing	2000	2	3.5	15	75
2	Sample appli.	200	2	3.5	15	15
3	Focussing	2000	5	3.5	15	410

Staining Procedure of the IEF pre-cast gels

The gels were stained immediately for specifc enzymes using the modified histochemical stains summarised in appendix 1.

6.2.5 Data recording and analysis

Gels were drawn, or photographed immediately after staining or in the case of pre-cast IEF gels, air dried after fixing with methanol: water: acetic acid (5:5:1). In scoring the different presumptive loci and alleles, "1" was arbitrarily assigned to the most anodal locus and "a" the most anodal band. All other numerals and alphabetic letters corresponded to approximations of their relative electrophoretic mobility.

6.2.6 Genetic variability/divergence

Allele frequencies were analysed with the computer software package BIOSYS-1 (Swofford and Selander 1981). Allozyme variability was illustrated by estimates of allele frequencies, average polymorphism level (P; a locus was defined as polymorphic when the frequency of the most common allele was lower than 0.99), mean number of alleles per locus, and expected heterozygosities per locus across all loci (Nei 1975). Contigency chi-square tests, based on the observed numbers of each allele at each locus, were used to compare allele frequencies among the different populations. Divergencies between populations were described by computing Roger's genetic distances (Rogers 1972) and analysing with the Neighbour-joining (Saitou and Nei 1987) method of PHYLIP version 3.53c from the genetic distances, using outgroup rooting.

6.2.7 Phylogenetic Analysis

Phylogenies for seven populations of ***C. flavipes*** complex and an outgroup C. ***glomerata*** were inferred by applying a cladistic systematic approach. The allozyme data was transformed into character state data. The stepmatrix of transformed allozyme data was used to construct minimum length phylogenetic trees using the branch and bound algorithm of PAUP (Phylogenetic Analysis Using Parsimony, version 3.1.1., Swofford, 1990). Optimization of character-state was by accelerated transformation (ACCTRAN). Electromorphs found in both the ingroup and the outgroup were considered primitive.

6.3 RESULTS

6.3.1 Diagnostic Loci

The main objective in this study was to identify diagnostic loci that could be used qualitatively to differentiate between the species of the ***C. flavipes*** complex. Five loci (GPI, EST, 6PGDH, HK, SDH), had fixed electromorph mobilities for the various populations in the complex and ***C. glomerata.*** Photographs of zymograms and corresponding schematic representations of the five diagnostic loci are presented in Plates 6.1 A-E. A combination of any two of the above loci could be used to identify the species in the complex.

6.3.2 Iso-electric focusing

The banding patterns obtained from isoelectric focusing were similar to those of the starch gel but the resolution was greater. However two enzymes (ALD & MDH) could not be resolved using IEF.

Plate 6.1A Photographandschematicpresentationofzymogram from starch gel electrophoresis of glucose phosphate isomerase. Abbreviations on the schematic representations stand for:

CG = C. ***glome rata,***

CSW = ***C. sesamiae*** from western Kenya,

CSC = ***C. sesamiae*** from coast province of Kenya,

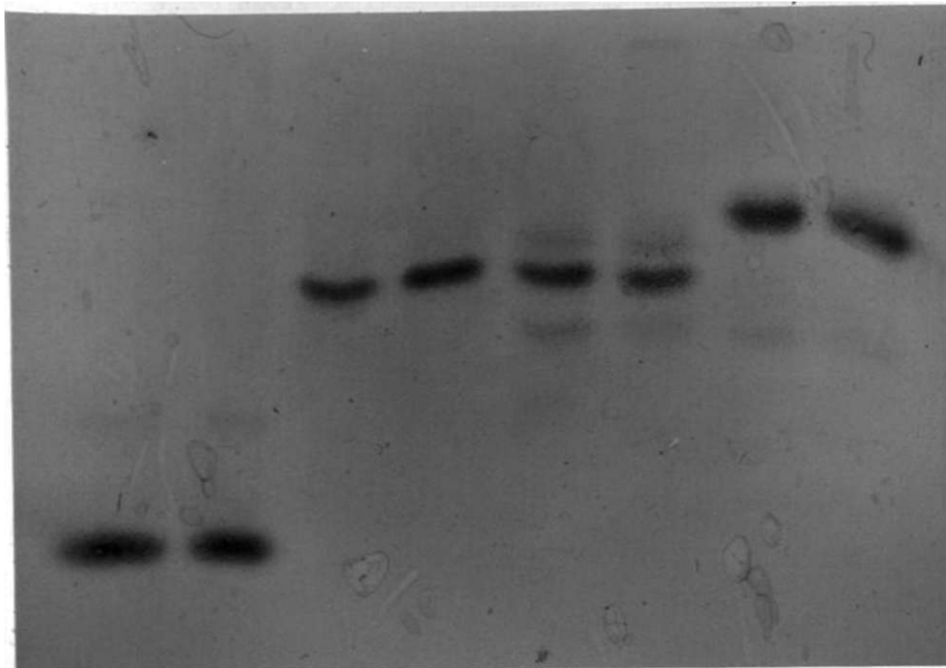
CC = ***C. chiton is,***

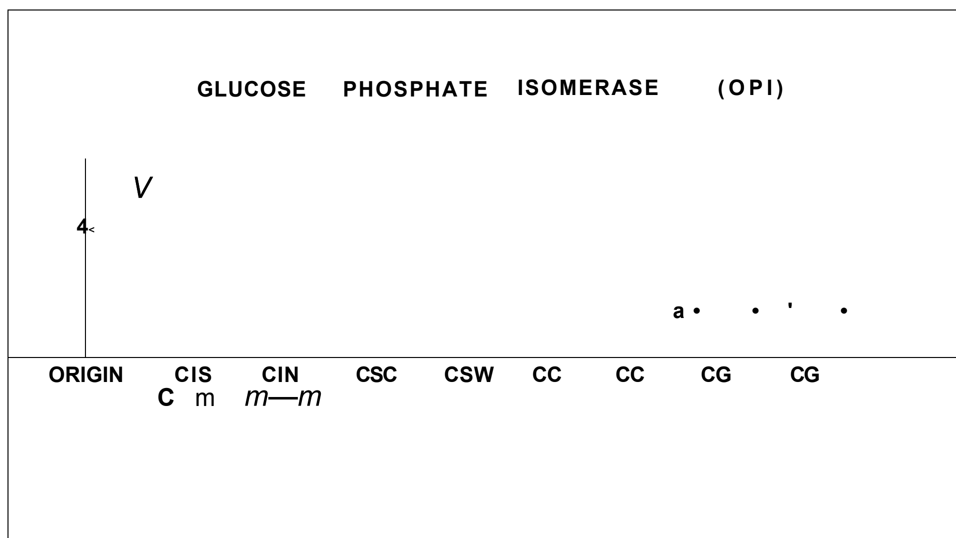
CfN = ***C. flavipes*** from north Pakistan,

CfS = ***C. flavipes*** from south Pakistan,

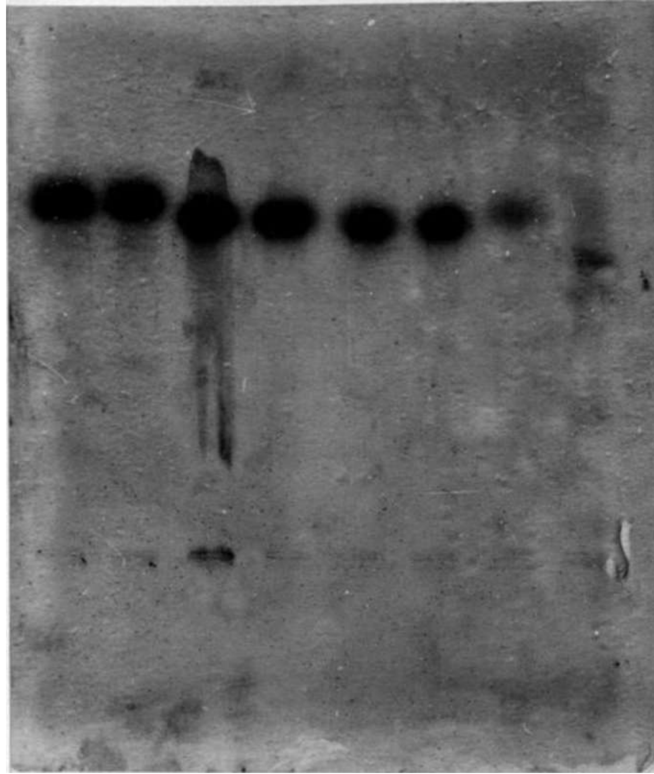
Thai = ***C. flavipes*** from Thailand,

RGV = ***C. flavipes*** from Rio Grande Valley, Texas USA.





GLUCOSE PHOSPHATE ISOMERASE (OPI)
ORIGIN
CIS
CIN
CSC
CSW
CC
CC
CG
CG



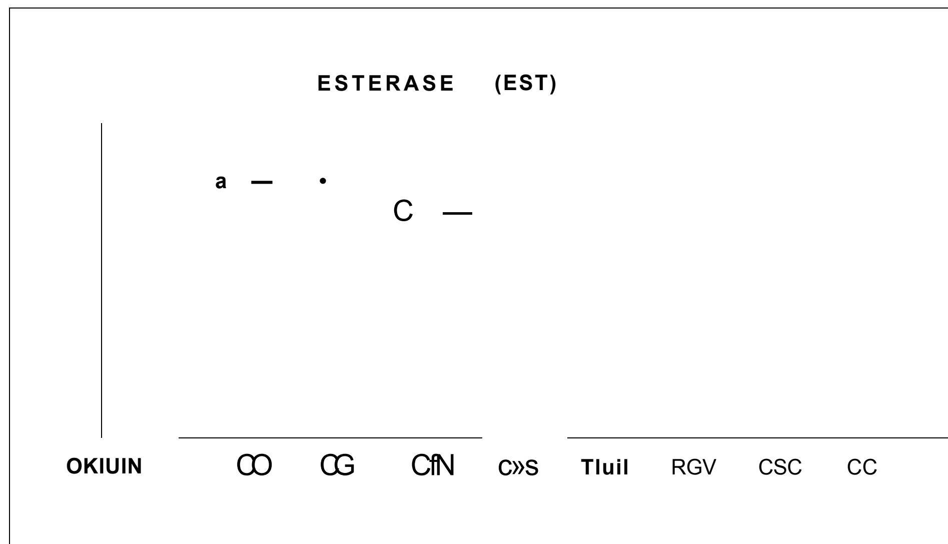
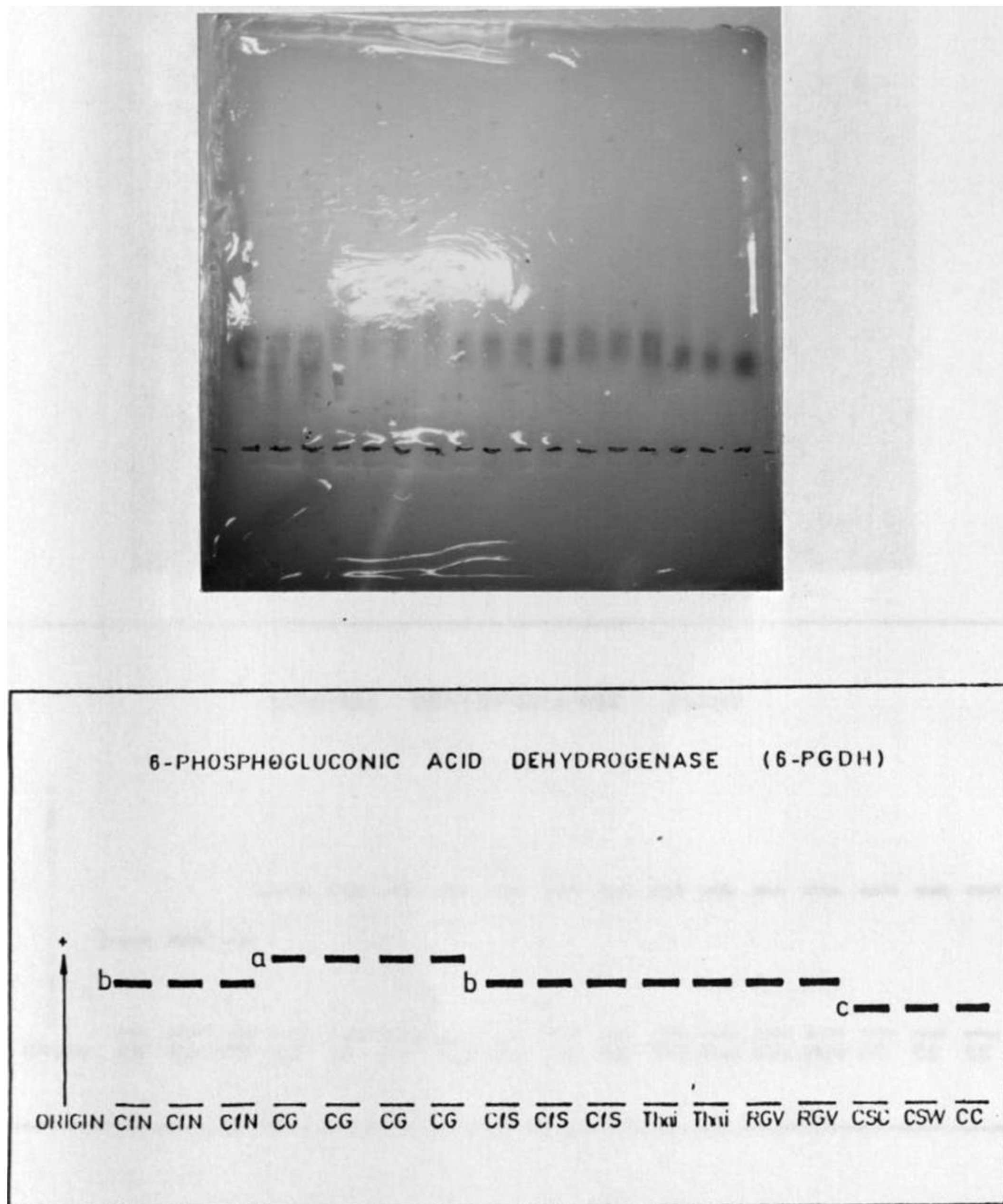
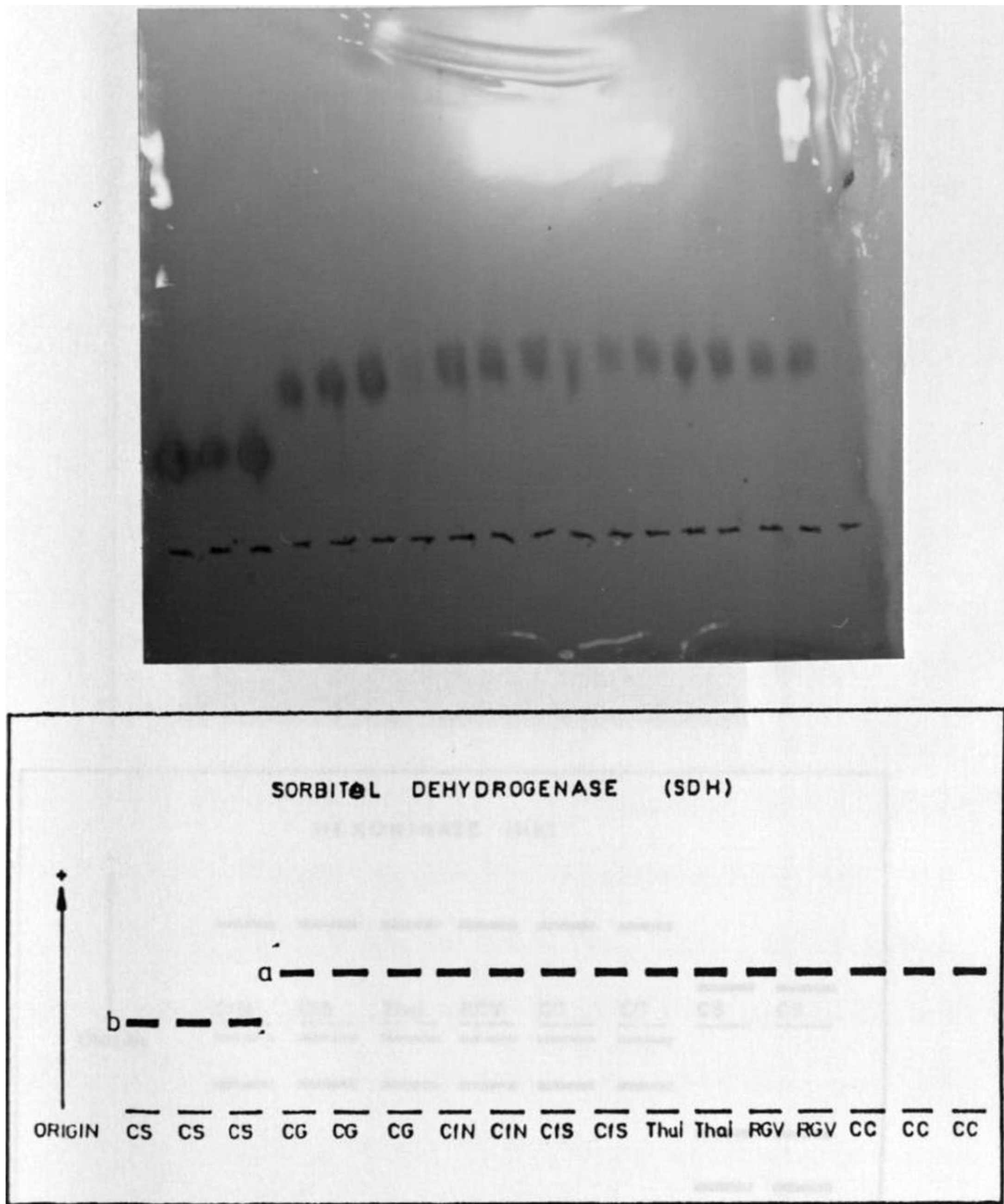


Plate 6.1B Photograph and schematic presentation of zymogram from Iso-Electric Focusing of esterase.

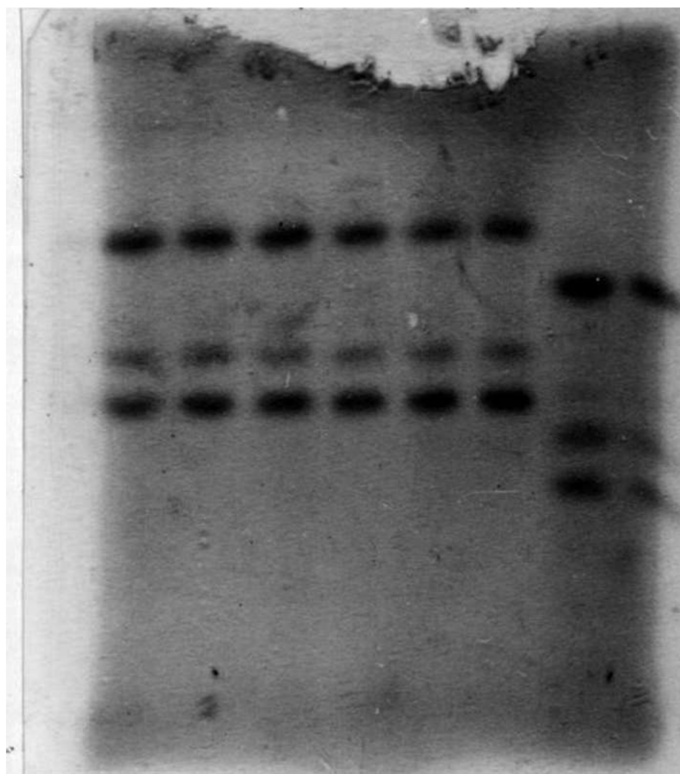
/



6.1C Photograph and schematic presentation of zymogram from starch gel electrophoresis of 6-phosphogluconic acid dehydrogenase.



6.1D Photograph and schematic presentation of zymogram from starch gel electrophoresis of sorbital dehydrogenase.



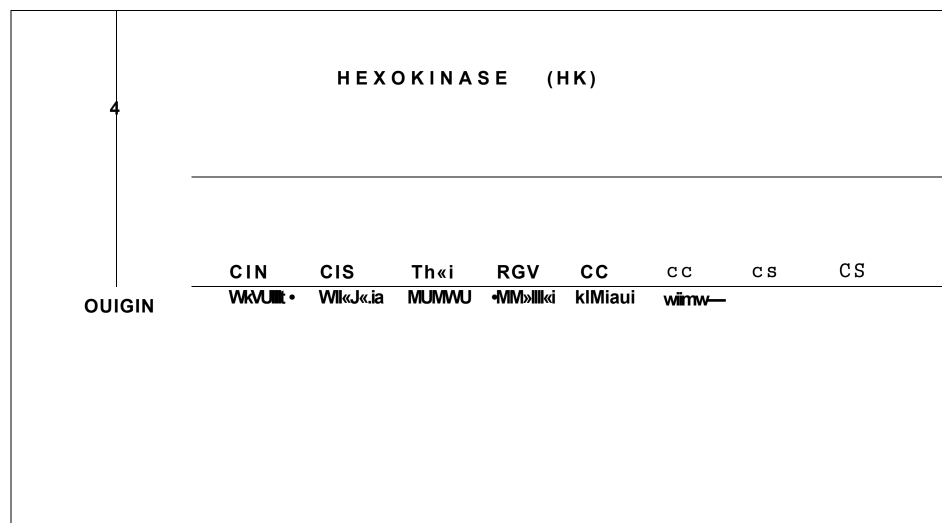


Plate 6.1E Photograph and schematic presentation of zymogram from Iso-Electric Focusing of hexokinase.

6.3.3 Genetic variability/divergence

The gene products of 22 loci were detected, 17 loci yielded interpretable results and 14 loci were used for all subsequent analyses. Allele frequencies for all loci observed among the populations are presented in table 6.2.

Four loci (MDH-2, ME-3, PGM-1, IDH-3) were monomorphic for the same allele in all the populations of ***Cotesia flavipes*** (Cf), ***Cotesia sesamiae*** (Cs) and ***Cotes/a chilonis*** (Cc). Being phylogenetically uninformative, these three were discarded from the Biosys-1 and PAUP analyses. Five loci (IDH-1, IDH-2, IDH-3, MPI-1, and MPI-2) were polymorphic for most of the populations but not all, these were also not used in the analyses because the number of alleles and loci had not be confirmed by breeding experiments. Fourteen loci (GPI, ME-1, ME-2, MDH-1, ALD-1, HEX-1, HEX-2, 6PGDH, TPI-1, TPI-2, ACO-1, EST-1, SDH-1, oDL-GPDH-1) showed at least some variation in some of the populations and were used for the analyses. The proportion of polymorphic loci averaged 7.7% (Median 5.9% range 0.0% to 23.5% for the 8 populations studied. Gene diversity or expected heterozygosity (H_{exp}) averaged 0.016 (Median 0.008) (table 6.3). Contigency chi-square test analysis of all the alleles in each locus in the populations of ***C. flavipes*** complex were highly significant indicating important geographic variation in allele frequencies in the allopatric populations (P = 0.00000).

The matrix of Roger's (1972) genetic distances founded on the 38 allozymes detected on the whole (eight populations) of population are presented in table 6.3. The Neighbour-joining (Saitou and Nei 1987) dendrogram of Roger's (1972) genetic distances is presented in Figure 6.2. The first dichotomy isolated ***C. chilonis*** from ***C. flavipes*** and ***C. sesamiae*** (d = 0.172). The second dichotomy from root isolated ***C. sesamiae*** from the ***C. flavipes.*** The Thailand population of ***C. flavipes*** was the first to be isolated from the rest of the ***C. flavipes.***

Table 6.2 Allele frequencies observed among seven populations of ***C. flavipes*** Complex and an outgroup (C. ***glomerata)***

Population*

Locus	Cg	CfN	CfS	RGV	Thai	CsC	CsW	Cc
GPI-1								
(N)	19	168	20	32	30	106	22	118
A	1.000	.000	.000	.000	.000	.000	.000	.000
B	.000	.000	.000	.000	.000	1.000	1.000	1.000
C	.000	1.000	1.000	1.000	1.000	.000	.000	.000
MDH-1								
(N)	9	23	16	19	19	19	8	18
A	.000	.804	.875	1.000	.000	.053	.000	.000
B	1.000	.196	.125	.000	.895	.947	1.000	1.000
C	.000	.000	.000	.000	.000	.000	.000	.000
D	.000	.000	.000	.000	.105	.000	.000	.000
MDH-2								
(N)	9	23	16	19	19	19	8	18
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
IDH-1								
(N)	9	52	47	6	6	48	34	44
A	.000	.139	.000	1.000	.000	.000	.000	.000
B	.000	.861	1.000	.000	1.000	1.000	1.000	.639
C	.000	.000	.000	.000	.000	.000	.000	.361
IDH-2								
(N)	9	52	47	6	6	48	34	44
A	1.000	.000	.000	1.000	.000	.000	.000	.000
B	.000	.000	.000	.000	1.000	0.000	0.000	0.000
IDH-3								
(N)	9	52	47	6	6	48	6	44
(A)	1.000	1.000	1.000	1.000	1.000	0.000	1.000	1.000
MPI-1								
(N)	9	11	11	9	6	10	4	14
(A)	.000	.833	.526	.000	.154	.000	.000	.000
(B)	.000	.167	.474	.176	.385	.333	.000	.261
(C)	.000	.000	.000	.588	.385	.667	1.000	.000
(D)	.000	.000	.000	.000	.076	.000	.000	.565
(E)	.000	.000	.000	.236	.000	.000	.000	.174
(F)	1.000	.000	.000	.000	.000	.000	.000	.000
ME-1								
(N)	9	9	9	9	9	9	9	7
A	.000	1.000	1.000	1.000	1.000	1.000	1.000	.000
B	1.000	.000	.000	.000	.000	.000	.000	1.000

ME-2								
(N)	9	9	9	9	9	9	9	9
A	.000	. I11	.111	1.000	.000	.000	.000	.000
B	1.000	.889	.889	.000	1.000	1.000	1.000	1.000
ME-3								
(N)					9	9	9	9
A		000	1.000	1.000	1.000	1.000	1.000	1.000
HEX—1								
(N)	9	32	32	32	33	33	9	32
A	1.000	000	.000	.000	.000	.000	.000	.000
B	.000	000	1.000	1.000	1.000	.000	.000	1.000
C	.000	000	.000	.000	.000	1.000	1.000	.000
HEX-2								
(N)	9	32	32	32	32	33	9	32
A	1.000	000	.000	.000	.000	.000	.000	.000
B	.000	000	1.000	1.000	1.000	.000	.000	1.000
C	.000	000	.000	.000	.000	1.000	1.000	.000
6PGDH								
(N)	9	38	38	15	15	35	9	35
A	.000	053	.053	.000	.000	.000	.000	.000
B	1.000	947	.947	1.000	1.000	.000	.000	.000
C	.000	000	.000	.000	.000	1.000	1.000	1.000
TPI-1								
N)	9	9	9	9	100	9	9	9
A	.000	1.000	1.000	1.000	.000	1.000	1.000	.000
B	.000	.000	.000	.000	.000	.000	.000	1.000
C	1.000	.000	.000	.000	.000	.000	.000	.000
D	.000	.000	.000	.000	1.000	.000	.000	.000
TPI-2								
(N)	9	9	9	9	9	9	9	9
A	1.000	1.000	.000	.000	.000	.000	.000	.000
B	.000	.000	1.000	1.000	1.000	1.000	1.000	1.000
PGM-1								
(N)	6	39	28	33	33	28	9	32
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ACO-1								
(N)	9	15	9	9	9	12	29	12
A	.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	1.000	.000	.000	.000	.000	.000	.000	.000
EST-1								
(N)	9	15	9	9	9	18	9	12
A	1.000	.000	.000	.000	.000	.000	.000	.000
B	.000	1.000	1.000	1.000	1.000	1.000	1.000	.000
C	.000	.000	.000	.000	.000	.000	.000	1.000
ALD-1								
(N)		9	9	9 9	9		9 9	9
A	1.000	.000	.000	.000	.000	.000	.000	.000
B	.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

SDH-1								
(N)	9	9	9	9	9	9	9	10
A	1.000	1.000	1.000	1.000	1.000	.000	.000	1.000
B	.000	.000	.000	.000	.000	1.000	1.000	.000
L-GPDH-1								
(N)	5	100	100	9	100	9	100	100
A	.400	.000	.000	.000	.000	.000	.000	.000
B	.600	.000	.000	1.000	.000	1.000	.000	.000
C	.000	1.000	0.111	.000	.000	.000	.000	.000
D	.000	.000	.889	.000	1.000	.000	1.000	1.000

*Cg = *Cotesia glomerata,* CfN = *Cotesia flavipes* from north Pakistan, CfS = *Cotesia flavipes* from south Pakistan, RGV = *Cotesia flavipes* from Rio Grande Valley, Thai = C. *flavipes* from Thailand, CsC = *Cotesia sesamiae* from the Coast province of Kenya, CsW = C. *sesamiae* from western Kenya, Cc = *Cotesia chilonis* from Japan.

Table 6.3 Genetic distances and similarities among seven populations of ***C. flavipes*** and an outgroup C. ***glomerata*** (Rogers 1972). Distances are above the diagonal; similarities are below. Average heterozygosities are on diagonal.

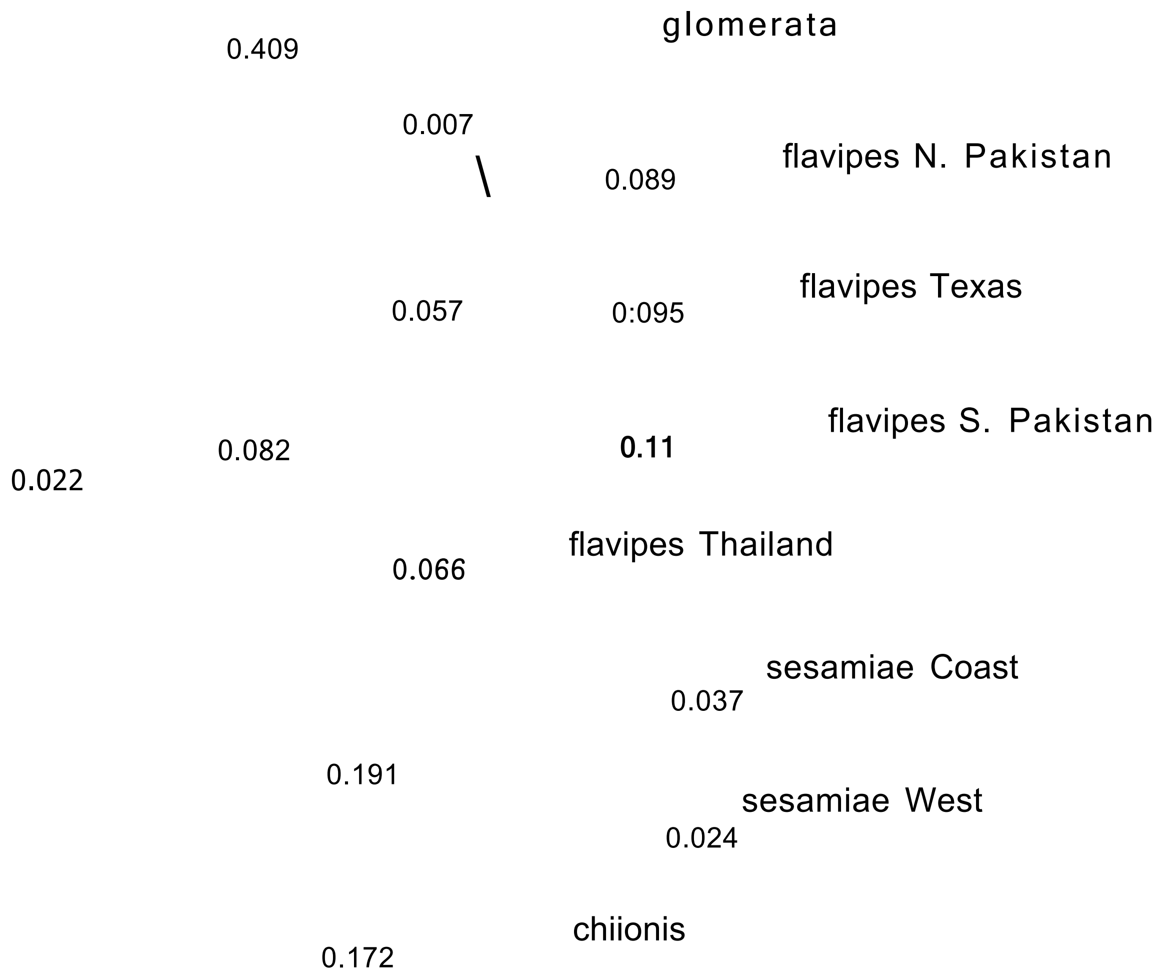
Popn*	Cg	CfN	CfS	RGV	Thai	CsC	CsW	CcJ
Cg	.031	.579	.638	.671	.587	.674	.698	.581
CfN	.421	.037	.115	.185	.231	.461	.464	.464
CfS	.362	.885	.044	.119	.124	.403	.357	.357
RGV	.329	.815	.881	.000	.232	.409	.471	.471
Thai	.413	.769	.876	.768	.011	.417	.359	.300
CsC	.326	.539	.597	.591	.583	.006	.062	.415
CsW	.302	.536	.643	.529	.641	.938	.000	.353
CcJ	.419	.536	.643	.529	.700	.585	.647	.000

*Cg = ***Cotesia glomerata,*** CfN = ***Cotesia flavipes*** from north Pakistan, CfS = ***Cotesia flavipes*** from south Pakistan, RGV = ***Cotesia flavipes*** from Rio Grande Valley, Thai = C. ***flavipes*** from Thailand, CsC = ***Cotesia sesamiae*** from the Coast province of Kenya, CsW = C. ***sesamiae*** from western Kenya, CcJ = ***Cotesia chilonis*** from Japan.

6.3.4 Phylogeny

The PAUP branch and bound procedure generated one most parsimonious tree topology with a total branch length of 53 steps. The most parsimonious phylogenetic hypothesis derivable from the genetic data using PAUP for eight populations of ***C. flavipes*** complex species is presented in figure 6.3. All character state changes are indicated on the respective branches in figure 6.3. Eleven alleles were identified as primitive based on their presence in both the ingroup (C. ***flavipes*** complex) and the outgroup (C. ***glomerata).***

Neighbour-joining dendrogram of Rogers (1972i genetic distances depicting the general relationships of species in the ***C. flavipes*** complex.



Neighbor-Joining Dendrogram of Roger's Genetic Distances

Fig. 6.3 Cladogram of ***C. flavipes*** complex using ***C. glomerata*** as an outgroup. Changes in allelic states are indicated along each stem (eg $EST^{b,c}$ = a change from electromorph b to electromorph c at a locus EST).

C. glomerata **Netherlands**

C. flavipes **North Pakistan**

C. flavipes **South Pakistan**

C. flavipes **Texas U.S.A**

C. flavipes **Thailand**

C. sesamiae **West Kenya**

C. *sesamiae* **Coast Kenya**

C. chilonis **Japan**

6.4 DISCUSSION

Allozymes are useful as characters for identification and discrimination of taxa at various levels of divergence and for description of patterns and processes such as population structure, breeding systems, gene flow, hybridisation and speciation. Nearly all animal and plant species harbour vast amounts of allozyme variation (Graur 1985), thus providing an almost inexhaustible supply of characters for species identification and recognition of distinct populations. The genetic basis of allozyme variation is generally of a simple Mendelian nature and can either be readily determined or be reasonably inferred. A major problem encountered with electrophoretic data is that the majority of single amino-acid substitutions are undetected under standard electrophoretic conditions (Menken 1989). Many allozyme bands are, effectively, populations of identically charged electromorphs (King and Ohta 1975). The technique of isoecletric focusing with immobilised pH gradients enables the separation of proteins up to 0.003 of a pH unit (Cossu and Righetti 1987). The observed low genetic variability among the populations of C. ***flavipes*** complex (mean H = 0.016) could therefore not be attributed to the electromorph problem. The Hymenoptera, with the exception of sawflies (Sheppard and Heydon 1986; Woods and Guttman 1987), exhibit levels of variation significantly lower than those of other insect groups. Graur (1985) compiled average heterozygosity for several species in the insect orders

Orthoptera, Hemiptera, Coleoptera, Lepidoptera, Diptera, and thirty species of Hymenoptera and concluded that the Hymenoptera are genetically less variable than most species of insects. He found the mean heterozygosity (H_{exp}) for haplodiploid Hymenoptera of 30 species to be (0.036 ± 0.004). He suggested that haplodiploidy may contribute to the low genetic diversity in Hymenoptera, other factors being sociality via its influence on reducing the effective population size and the amount of neutral polymorphism that can be sustained by a species. Haplodiploids may be less genetically variable than diploddiploids because selection eliminates exposed deleterious alleles in haploid males (Suomalainen 1962). In this study, the observed low heterozygosity could also be attributed to the fact that most of the polymorphic loci were not analysed. According to studies by Powell (1971) and Levinton (1973), a positive correlation is expected between a species genetic variation and the degree of its environmental diversity. The average heterozygosity of species in this complex would therefore be expected to be high considering the wide host range and geographic distribution.

Although reproductive isolation can be achieved by a few changes that do not result in morphological differences or genetic divergence in the set of commonly studied protein loci, most species still differ between one another in their allozyme patterns (Menken and Ulenberg 1987). If sympatric taxa are found to be fixed for different alleles at even a single locus, complete isolation,

and thus specific status can be assumed (Menken 1989). The status of allopatric taxa is not easily established. However, nearly all conspecific populations have Nei's genetic identity (Nei 1987) above 0.85, with the great majority above 0.95 (Menken 1989). Nei's genetic identity for the Thailand/North Pakistan populations was 0.784 and Thailand/Rio Grande Valley was 0.769, compared to 0.890 for populations from North Pakistan and / or South Pakistan. These figures suggest that the Thailand population is different from other populations, which has also been corroborated by biological studies (J.W. Smith, Jr, pers. comm.), morphological and phylogenetic analysis.

The allozyme data indicate that ***C. chilonis*** and ***C. sesamiae,*** which have similar male genitalia (Polaszek and Walker 1991) and partial mating compatibility (chapter eight), are distinct species (I = 0.587, as compared to the two populations of ***C. sesamiae,*** CsC/CsW, I = 0.941). The genetic Identity of ***C. chilonis/TUaWand*** population of ***C. flavipes*** was 0.704 and that of ***C. chi/onis/Souxh*** Pakistan was 0.655, which suggest that ***C. chilonis*** is not genetically any closer to C. ***sesamiae*** than it is to ***C. flavipes.*** Esterase, hexokinase,sorbitaldehydrogenaseand 6-phosphogluconicacid dehydrogenase alleles distinguished the two species.

6.4.1 Phylogeny

The most parsimonious cladogram is presented in Figure 6.3. All character state changes are indicated on the respective branches of the tree. Most of the principle branch lengths are supported by at least two or more synapomorphies which indicates that the hypothesized phylogeny is fairly well substantiated. Contrary to earlier morphological taxonomic work, parsimony analysis of the electrophoretic data excluded the Thailand population from the monophyletic clade of ***C. flavipes.*** This species was also dissimilar from other members of the C. ***flavipes*** in external morphology (chapter three), life history (J.W. Smith, Jr, pers. comm.) and allozyme composition (table 6.4). It is therefore concluded that the Thailand population may not belong to the ***C. flavipes*** group. Its taxonomic placement awaits DNA analysis of all the strains in the species group. The electrophoretic data, in combination with reciprocal mating data and genitalic morphology support the monophyletic status of C. ***chilonis*** and C. ***sesamiae.*** However this relationship, is not supported by the Neighbor-joining dendrogram of Rogers genetic distances (figure 6.2). This tree topology departed substantially from the the parsimony analysis (figure 6.3) in excluding C. ***chilonis*** from the monophyletic clade of ***C. chilonis*** and ***C. sesamiae.*** The principle of the neighbour-joining method is to find pairs of operational taxonomic units OTUs (= Neighbours) that minimize the total branch length at each stage of clustering of OTUs starting with a starlike tree. Saitou

and Nei (1987) studied the efficiency of this method in obtaining the correct tree in comparison with that of five other tree-making methods: the unweighted pair group method of analysis (UPGMA) (Sokal and Sneath 1963), Farris's method (Farris 1972), Sattath and Tversky's method (Sattath and Tversky 1977), Li's method (Li 1981) and modified Farris's method (Tateno *et at.* 1982). They found that the Neighbour-joining method and Sattath and Tversky's method were more efficient in obtaining the correct topology. Unlike the standard algorithm for minimum-evolution trees, the Neighbour-joining method minimizes the sum of branch lengths at each stage of clustering of OTUs. Therefore, the final tree produced may not be the minimum-evolution tree among all possible trees. However, Saitou and Nei (1987) stated that the real minimum-evolution tree is not necessarily the true tree. Saitou and Nei (1986) showed that the minimum-evolution or maximum parsimony tree often has erroneous topology and that the maximum parsimony method of tree making is not always the best in recovering the true topology. However, in this study, the topology suggested by minimum-evolution analysis (fig. 6.3) is supported by other data and seems to be the best hypothesis of the evolution of species in the *C. flavipes* complex at the moment.

CHAPTER SEVEN

7 RANDOM AMPLIFIED POLYMORPHIC DNA - POLYMERASE CHAIN REACTION (RAPD-PCR)

7.1 Introduction

Low allozyme variability is notable throughout the Hymenoptera (Graur 1985), and has been attributed to deleterious and lethal recessives being exposed directly to selection in the haploid male. The small amounts of genetic variation revealed by allozyme electrophoresis limits the use of this technique in separating the host specific strains known to occur in the C. *flavipes* complex (Mohyuddin 1971; Shami and Mohyuddin 1990). Phylogenetic analysis of allozyme data indicated that C. *flavipes* was not a monophyletic group (Chapter 6). RAPD-PCR (Random Amplified Polymorphic DNA - Polymerase Chain Reaction) is a technique that uses a ten or twenty oligonucleotide primer of random sequence but with a minimum guanine-cytocine content of 50% to amplify complementary regions of the genome. A low annealing temperature (35-50) is used. Low annealing temperatures allow the primer to anneal to arbitrary regions of the genome which may not be fully complementary to the primer. A low annealing temperature is maintained throughout all the cycles. In order for amplification to occur, the primer must

anneal on the complementary strands of the template DNA and the 3' ends of the annealed primers must face each other. The requirement that the 3' ends of the annealed primers face one another suggests that annealing sites are exact or similar inverted repeats. The observation that single substitutions, especially in the 3' end of the primer, can change amplified banding patterns (Williams ***et at.*** 1990) implies that annealing must be precise. This method has been used in the identification of sub-species and cryptic species, and has applications in population genetics and genetic fingerprinting. The advantages of this technique are that it does not require cloning or DNA sequence information for primer design, it does not employ radioactive markers, and genomic polymorphisms can be visualised within 24 hours from extraction of genomic DNA.

Host specific strains have been reported in ***C. flavipes*** complex (Mohyuddin 1971; Shami and Mohyuddin 1990) but can not been separated using the characters that have been investigated so far. Phylogenetic analysis also indicated that allopatric populations of ***C. flavipes*** were not monophyiotic A number of different papers have now been published employing the RAPD PCR method (Williams ***et. at.*** 1990) as a new approach to identifying DNA polymorphisms and analysing genomes and populations of various insect species. Most of these papers have dealt with questions relating to genetic variation in insect populations including studies of mosquitoes (Kambhampati

et. al. 1992), aphids (1992) and Whiteflies (Perring ***et. al.*** 1993). RAPD PCR was used in this study to investigate whether specific banding patterns could be obtained for the populations that were shown to be different using allozymes and mophology data. These patterns would then be used to identify the respective populations or strains.

7.2 MATERIALS AND METHODS

All the populations of the *C. flavipes* complex examined by **aiio**/yn** electrophoresis (chapter 6) were analysed using RAPD-PCR. In **addition** •• hybrids of *C. sesamiae* males and *C. chilonis* females (see chapter 8 .-..<• included in the study for qualitative analysis only.

7.2.1 DNA extraction

All water used in the following protocols, including that used lor • .»»ir buffers, was sterilised by autoclaving to minimize chances for contam n<it with non-target DNA that could produce artifacts during PCR. **T.** • . genomic DNA was extracted from individual male (except the hyt)' t: < '-<•< ••• using the following protocol: individual insects were placed m a 50 . microfuge tube and chilled by dipping the tube in liquid Nitrogen at • i R'»° : The insect was then ground using a sealed pippette tip m 60 of homogenizing buffer (10mM Tris-HCl, pH 8, 1mM EDTA, 1% Nonidet. 100 /jg/ml proteinase K). The pestle was discarded after use. The mix- incubated overnight in a 50° C water bath. The DNA was then extract: v equal volume of phenol chloroform and precipitated with ice-cold ab«;<> ethanol. Quantification of the DNA was accomplished by measuring the OD$_{;40}$ (optical density) using a spectrophotometer and calculating the concf v.v <

of each sample. The sample was then diluted accordingly to ••• <• " • M DNAsamples were of the same concentration using autoclaved do t to »«»tikc<3 deionised water.

7.2.2 RAPD-PCR protocols

Reaction Mixture

PCR reactions were done in a total volume of 20 conu • m$_U$; c' 10X reaction buffer (Promega Biotech Inc.) (50mM KCl. 10- M • 9.0) 1.5mM $MgCl_2$, 0.01% gelatin (w/v) and 1.0% Triton X 100 O V • 10mM $MgCl_2$, 2 of 200/ym dNTPs (Promega Biotech I- < 1. • ' . • ***O.Sp!p*** of ***Taq*** polymerase (Perkin-Elmer Cetus), 25ng ol ; ••••«- • DNA. The reaction mixture was made up to 20 ***p*** by add n g 1 <> ***i>*** Each reaction mixture was overlaid with two drops of nmipnii <> " quality) to prevent sample evaporation.

Primers

A set of 20 random decamer (Kit B) primers were Obtn nod'm- Or->- Technologies (Alameda, CA). The kit contained twenty CJ• " sequences and each tube contained 15 *pq* of primer.

T-,e primers (7 out of 20) that gave amplified PCR proch.i is i>t!»d

0PB-01, 5'-GTTTCGCTCC-3'; OPB-05. 5-TGCGCCCTTI 3 .

0PB-06, 5'-TGCTCTGCCC-3'; OPB-10, 5'-CTGCTGG(,a t

OPB-12, 5'-CCTTGACGCA-3'; OPB-17, 5'AGGGAAi ,a , 3

OPB-18, 5'-CCACAGCAGT-3'.

PCR Amplification

Amplifications were performed in a progran ,it) <• v , i *• < ..•, laboratory Prod. Inc. Model 50) using the following u— ;>.'•.»tu»» t r t i ^ initial3 minutes at 94° C followed by 35 cycles of i11 d r » i » t v , * < 1 minute. Once the template DNA had been denatured •• . m • sn^t m*» lowered to a level that allowed the primers to anneal :2 •nn»»Nngi 3V C *«• 1 min, with a ramp time of 0.2 minutes at 35° C i3« primer had annealed the temperature had to be raised io *> \m— »»* polymerase to add nucleotides to the 3' end of the pr- <• •< ° r to the template. The extension temperature was 72° C for 2 ~ C for 5 minutes to complete the extension. The PCR prfx? • v.p- « tt>* - at 4° C until the time of retrieval.

7.2.3 Gel electrophoresis

The PCR products (5 /yl) plus 1 of 6X dye ibro" • • * wm electrophoresed on 1.2% TBE agarose gels at 80 V lo? 2 ; • p o^e a 1kb ladder DNA size marker (Bethesda Research LaboMt , I t etnidium bromide. The gels were visualised and p'• • • i .»•«•'. aftertherunona302 nmultravioletlighttransillumir .ro' i »• mry a negative control containing all of the reaction comp«f •• DNA, was processed.

7.2.4 Data Analysis

Qualitative and cluster analyses were conductor 1 relationships of the populations. Qualitative analysis was t . • and comparison of all the bands amplified from each ;> ; > < ***** analysis was conducted using PAUP's Bootstrap r >••• >•*-•<» m *< bound search with 1000 replications. The western pop i ' i M< • * *sesamiae* and hybrid (which is not a naturally occur* q .• . excluded from the cluster analysis. Homology of bands w " * * base pairs was assumed for the purposes of this anaiy* " • r : 1 • ! scored for the seven primers listed above and a presence r " data matrix constructed.

7.3 RESULTS

7.3.1 Qualitative

The banding patterns obtained using the d . • .

A-H. Distinct banding patterns of the four spec es C c

C. sesamiae and *C. chilonis* can be seen especia y J> t -

no specific banding patterns for populations wr- in « **«

partem of the outgroup C. *glomerata* was d>s* • •

flavipes complex. Lanes 1-8 contained DNA 1m ;•• • i

of the populations of *C. flavipes* complex, lane 9 > •

from *C. glomerata.* There were distinct size df..

the two group in all cases. RAPD-PCR could xh« -»-'o «- u u . j i o # «

C. *glomerata* from the *C. flavipes* complex Uw ' . I*

populations of *C. flavipes,* lanes 3 and 6 c o n t a i ' : «U on-, o«C

are 4 contained *C. chilonis* and lane 7 contained v • ' • '1

C. *chilonis.* The amplified DNA fragments of the * yt • • ' * *

of *C. sesamiae* and *C. chilonis* in all the primers w *•*>

o ate 7.1 A lanes 1 & 2 of C. *flavipes* had s.rr ar t>a-"U ^ *

sesam/ae had some shared bands and differed • <-"

7-1B lanes 1 & 8 of *C. flavipes* were similar. 3 & -

chilonis respectively differed by lane 3 having two e i f a

7.1C only ***C. sesamiae*** and ***C. chilonis*** and their hybrid could be amp •

plate 7.1 G (OPB-12) only the DNA of the two (lane 3 & 6) popul.itkm % o* i

sesamiae could be amplified, the two had similar bands.

7.3.2 Cluster Analysis

The dendrogram obtained from PAUP's Bootstrap analys s •» ;>-.–..- •«-

in figure 8.1. The tree topology differed substantially from thai ot •> <

In all the replications C. ***chilonis*** and ***C. sesamiae*** shared .i conmxm • («jc

implying a close genetic relationship. The Thailand population of C. . *%

appeared to be the most distant in terms of genetic relationship s > < <• i '.* .vr •

the least similarity.

Plate 7.1 A-H. Gel photographs of single primer PCR on tota genomic DNA using random primers. Numbers on ge photographs refer to the loading sequence: (0) control (1) Cf from Thailand, (2) Cf from north Pakistan, (3) Cs from coast province of Kenya, (4) Cc from Japan (5) Cf from Texas, USA, (6) Cs frorr western Kenya, (7) hybrid of Cs6 x Cc$, (8) Cf from south Pakistan, (9) C. ***glomerata,*** (m) 1 kb ladder size marker, (gels ran at 80V for *2Vi* hours).

(A) OPB-7

(B) OPB-17

(C) OPB-5

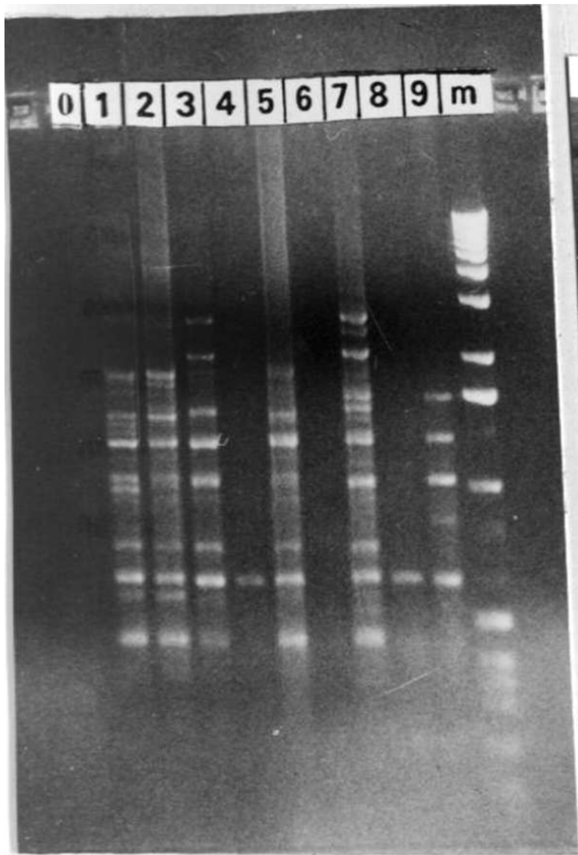
(D) OPB-1

(E) OPB-10

(F) OPB-6

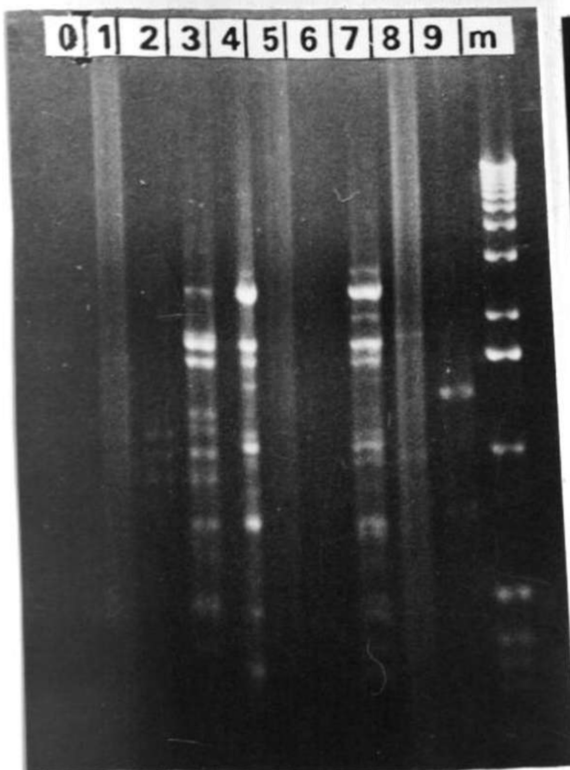
(G) OPB-1 2

(H) OPB-1 8

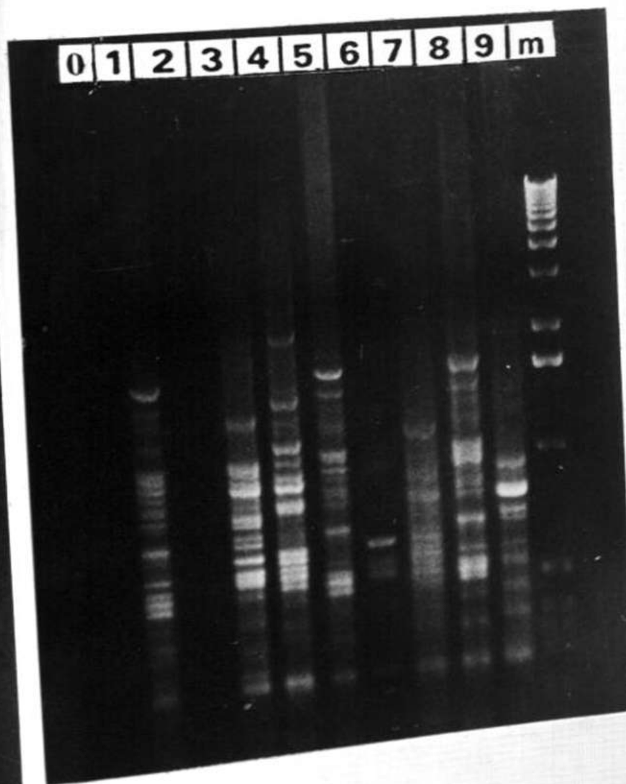


a

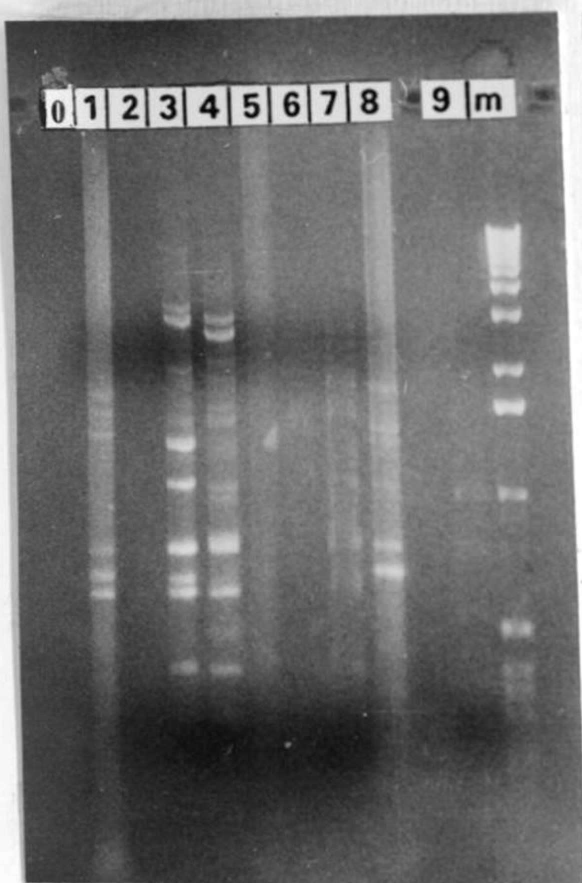
b



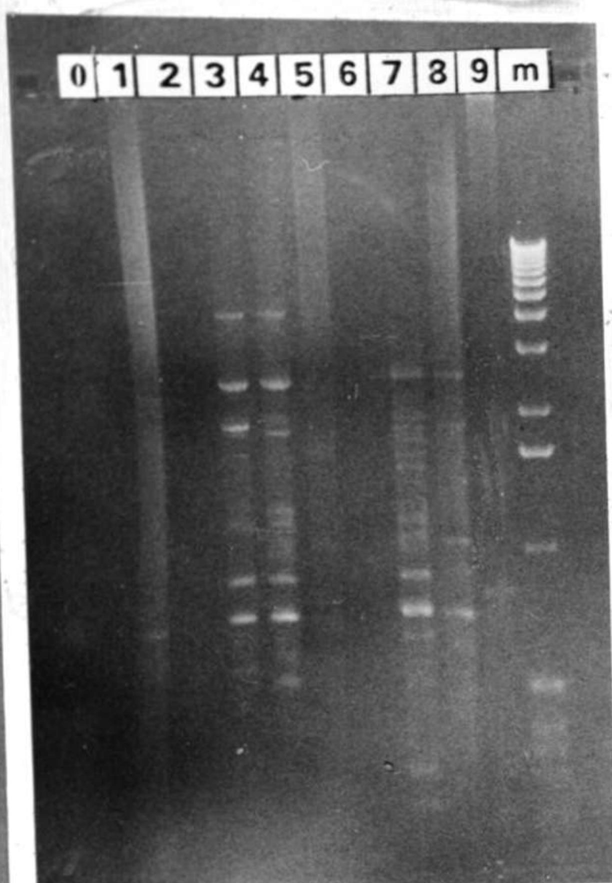
c



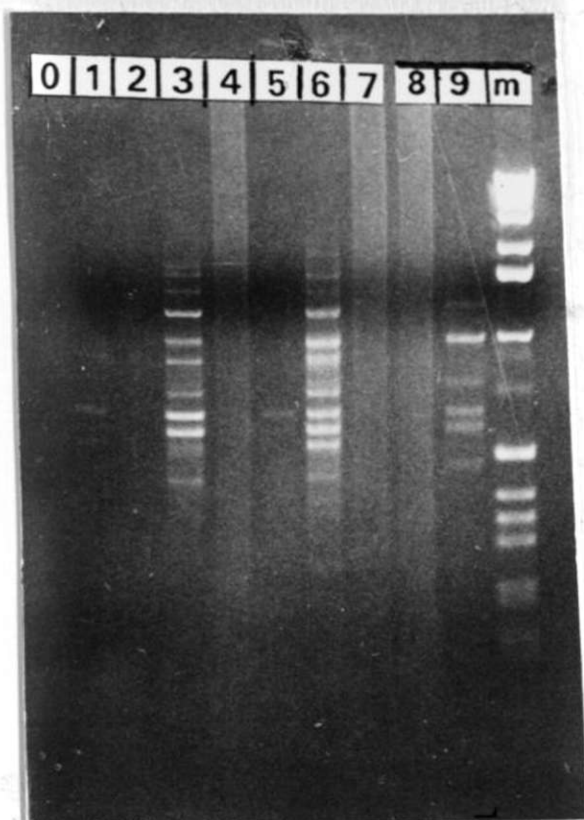
d



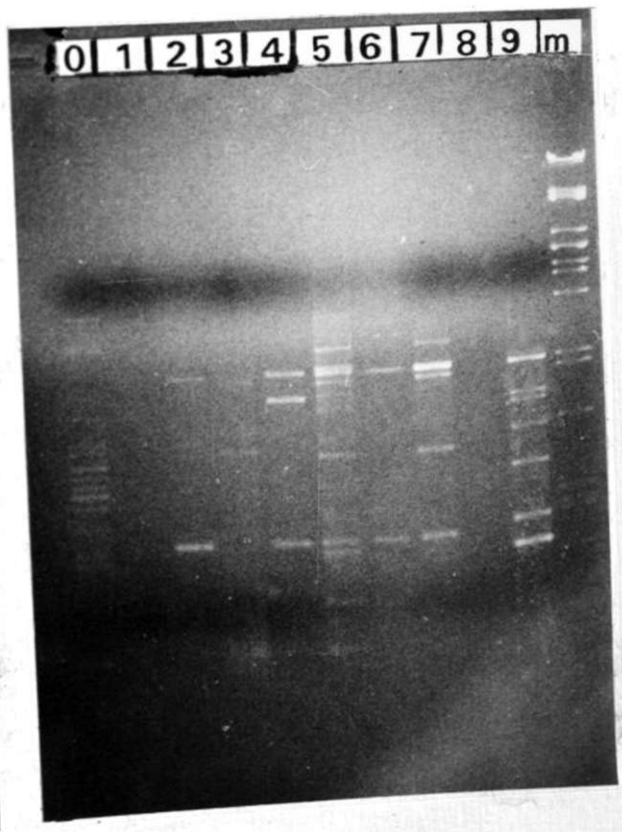
e



f



g



h

Fig. 7.1. Dendrogram of the PAUP analysis of PCR products depicting the relationship of ***C. flavipes*** complex and an outgroup ***C. glomerata,*** values above intervals represent minimum and maximum branch lengths under all models of state change. Values below intervals represent percent of bootstrap replications in which the illustrated clade was found.

C. g/omerata Netherlands

C. flavipes **S. Pakistan**

4-1 0

64%

C. chilonis **J^pan**

22-25

4-1 2

100%

C. sesamiae **Kenya Coast**

63%

9-1 9

AC. flavipes **Thailand**

C. flavipes **Texas**

7-1 1

65%

-C. flavipes **N. Pakistan**

7.4 DISCUSSION

This study demonstrates that short primers of arbitrary nucleotide secjence can be used to reproducibly amplify segments of genomic DNA from doseiy related *Cotesia* parasitoid species. Williams *et a/.* (1990) stated that - A.PD assay may in some instance detect single base changes in genomic DNA ch causes a complete change in the pattern of amplified DNA fragments. Polymorphisms occur as the presence or absence of a specific fragment among idividuals. By inference, a single base change in the genome may prevent £modification by introducing a mismatch in the primer, other sources of polvmorphisms may include deletions of a priming site, insertions that render zt ming sites too distant to support amplification, or insertions that change the size of a DNA segment. RAPD-PCR was used to amplify DNA in the eight Dcpulations of C. *flavipes* complex and one population of C. *g/omerata* L. The above primers gave patterns that could be used to identify species in the complex. Although some bands of the same size could be found in more than one species, for the most part the overall patterns for each species were distinctive. Qualitative analysis did not reveal any differences between populations within species. Host specific strains within the C. *flavipes* complex ~3ve been reported by Mohyuddin (1971) and Shami and Mohyuddin (1990).

Parsimony analysis of RAPD markers was used to test whether the cer ved phylogeny was congruent with that derived from allozymes data :t~ap:er 6). The tree topology differed substantially from the tree topologies ten ved from allozymes in chapter six. The *C. flavipes* population from Sindh "i south Pakistan clustered with the *C. sesamiae/chi/onis* group instead of the "est of the *C. flavipes* populations. However the monophyletic clade of C. *sesamiae* and *C. chiionis* was maintained in all the replications which indicates that the two are very closely related. Kambhampati *et at.* (1991) used cluster ana'ysis of RAPD markers to compare the phylogeny with those derived from ^orohological data, isozymes and mtDNA, and found that species belonging to t~e same subgroup did not cluster together. Black IV (1993) stated that shared resence of bands among species does not reveal "true" phylogenetic relationships among the taxa because comigrating bands may have no sequence similarity and also, homologous regions might not be amplified in both of the species to be compared and even if they were, might vary in size.

CHAPTER EIGHT

8 MATING BEHAVIOUR, RECIPROCAL CROSSING AND VOLATILE PHEROMONES.

8.1 Introduction

Species may be defined as natural populations that are reproductively soiated from one another and follow distinct and independent evolutionary Dcths Diehl and Bush 1984). Hybridization tests may therefore be considered as D-ov ding ultimate proof of the specific status of closely related populations. Reproductive isolation may be due to the visual and or tactile elaborate and orotracted behavioural interaction between males and females of particular species before insemination takes place, incompatibility of heterogamic sperm and egg (gametic isolation), inviability of hybrid zygotes or sterility of hybrid 32j:s. Sexual isolation ascribable to the existence of various behavioural barr ers, is in itself a valuable and relatively easy biosystematic criterion. Natural selection should favour females who are able to determine male species centity early in courtship sequences rather than late, since both courtship and copjlation are costly and dangerous for a female (Alexander and Otte 1967). Crossing experiments, observations on mating behaviour and responses to 'Olat e pheromones were used to analyse reproductive isolation in the C. *'avipes* species complex, and to gain insight into the taxonomic relationships among the species.

-2 MATERIALS AND METHODS

8.2.1 Insects

he north Pakistan population of *C. flavipes, C. chilonis* from Japan 'lais^e in Shimane) and *C. sesamiae* from the coastal province of Kenya, all r cj!:jre at ICIPE were used for this study.

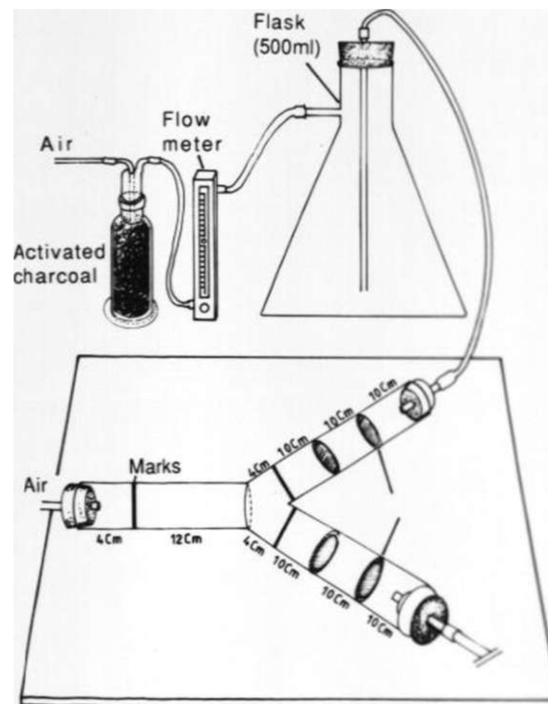
8.2.2 Mating behaviour

individual cocoons were separated from three to four cocoon masses for each species and isolated in vials to avoid mating on emergence. Vials were s-ooped with cotton wool and placed in an incubator at 28°C and 70% RH until r~ergence. Preliminary observations in small (0.6 cm diameter x 3 cm) and i'ce 2.5 cm x 7.5 cm) vials and in petri dishes (9 cm diameter x 1.5 cm) were ""ace to determine the type of containers to use in later trials. Mating trials .-.ere a so conducted for isolated pairs and in aggregation. The small vials were osed *or all the subsequent experiments because it was observed that mating Dccjrred more rapidly in the small vials. After emergence, single male and a,e pairs of each possible inter- and intraspecific combination were placed r. tne small glass vials. Courtship and mating behaviour were observed and *-e amount of time spent *in copula* was recorded. When copulation occurred,

es were dissected to check for the presence or absence of sperm packets spermathecae. The experiment was replicated three times with ten pairs " each replicate. Observations were made at room temperature under artificial cht.

3.2.3 Interspecific crosses

Hybridisation experiments were conducted using the same methods as lescibed above. Each pair of each cross was observed individually and tho Defriav our and copulation time were recorded. After copulation, each female «as provided with a suitable host. Prelimnary studies showed that fourth "Star arvae of *Chilo partellus* were suitable for development of ***C. chilonis*** and C. *flavipes,* and fourth-instar larvae of ***Sesamia calamistis*** Hampson 'Lepidoptera: Noctuidae) were suitable for ***C. sesamiae.*** The females were :~ allowed to oviposit once. Hosts were reared individually on artificial diet Dchieng *et a/.,* 1985) until the parasitoid larvae emerged from the host and spun cocoons. After cocoon formation, the host cadaver was dissected and any parasitoid larvae that had died inside the host were counted. Parasitoids :hat failed to emerge from cocoons were included in the total progeny count. The cocoons were isolated, adults were sexed and the females used in cac<cross experiments when the progeny included females.



A schematic representation of the Y-tube olfactometer

Pig. 8.1 A schematic representation of the Y-tube olfactometer

After every trial (15 individuals) the Y-tube was washed and blow-dried to remove the previous odour. This experiment was replicated five times to observe a total of 75 individuals for each combination. A two-step analysis was conducted. First, the proportions of individuals moving out of the olfactometer stem to either arm of the olfactometer were arcsine square root-transformed and compared between treatments within a species, and between species within a treatment using analysis of variance followed by Student-Newman-Keuls multiple comparison test. Second, the number of individuals that moved to the arm containing the odour source were compared to the number that moved to the control arm using a G-test of independence. The null hypothesis was that an equal number of individuals would move to each arm.

Interspecific bioassays were conducted by placing five live conspecific virgin females in one arm of the olfactometer and releasing singly 30 virgin males of one of the other two species. Control trials were included in which 30 conspecific males were released, but both arms of the olfactometer were empty. As in the intraspecific bioassays, the first choice of each individual was recorded, and if an individual had not entered one of the arms after five minutes it was recorded as undecided. The number of individuals that moved into the arms of the olfactometer were compared to the number that moved when both of the olfactometer arms were empty with a G-test of independence. The null hypothesis was that the number responding would not be different when the

one olfactometer arm contained females of a different species and when both olfactometer arms were empty.

8.2.6 Pheromone bioassays in field cages

Bioassays were conducted in large field cages to determine whether *C. flavipes* males could be caught in female-baited traps. This experiment was only conducted with *C. flavipes* because the insects were readily available in large numbers. Also the possibility of using pheromone traps to monitor colonization and dispersal of this species after field releases was of particular interest to the biological control project under which this study was conducted. Traps were made from sheets of aluminium foil (40 x 20 mm) coated with tangle-trap (Tanglefoot Company, Michigan, USA) on one side. The foil was then folded at an angle of approximately 90° to form a tent with the sticky side inside and hung from the centre of the fold. Virgin *C. flavipes* females were enclosed in a fine wire mesh cylinder (2 cm diameter x 5 cm) and hung 1.5 cm beneath the folded foil. One pheromone trap was placed at the centre of a 2.5 m x 2.5 m x 2 m field cage enclosing maize plants. A control trap without females was placed in an adjacent cage. Virgin C. *flavipes* males (40) were released in both cages and the entraped males were counted after 24 hours. The experiment was replicated eleven times. The number of males caught in traps baited with virgin females and traps with no females were compared using Wilcoxon's signed rank test.

8.3 RESULTS

8.3.1 Mating behaviour

No differences were observed in the mating behaviour of the three species. When pairs of males and females were joined in petri dishes or large vials the males performed various behaviours including antennal searching, antennal waving, antennal vibration, antennal contact with females, wing fanning and pursuit of mates for up to three hours without mating. The male attempted to mount several times, but the female did not remain still, a condition that appears to be important before the male can successfully mount. However, in the small vials the males managed to mount after exhibiting prolonged courtship behaviour. In aggregates of at least 59:2d and in the presence of the pupal cocoons, mating occurred within a few seconds without prolonged courtship behaviour. The males approached the females fanning their wings and vibrating the antennae. Females appeared to remain fairly motionless, occasionally grooming themselves. Once a virgin female was contacted, the male rapidly fanned its wings and increased walking activity. The male further pursued the female from the rear until the female slowed down and the male was able to mount. Antennal stroking was often exchanged by the pair prior to mounting and was continued by the male until copulation was completed. The male secured his position on the back of the female, facing the same direction, bent the hind region of his abdomen

downwards to fuse with the female's genital organs, and commenced copulation. Copulation lasted between two and ninety seconds, with a mean of 15 seconds. Contact between the pair was usually terminated when the female walked away. Thereafter, the female remained motionless for a few seconds before she resumed activity. In conspecific mating, the females Typically mated only once. Mated females did not appear as attractive as unmated females, and even when pursued by a male, mated females refused to mate by walking away from the pursuing male or vibrating their wings and ifting the abdomen, especially when mounting was attempted. Males mounted and mated with very weak and dying females, and were also attracted to a crushed female abdomen and attempted to mate with the debris.

The sequence of events leading to copulation is shown in figure 8.2. It should be emphasized that each step did not necessarily lead to the next. Repetitions of steps or behaviours were common and at times some steps in a sequence were omitted.

8.3.2 Reciprocal crosses

Interspecific crosses between the three species were conducted to determine whether wing fanning, mounting, copulation, sperm transfer and fertilisation would occur.

Fig. 8.2. Behavioural sequence of mating in species of *C. flavipes* complex.

MATING BEHAVIOUR

PHEROMONAL STIMULATION

MALE ANTENNAL WAVIN G/ SE ARCHIN G

WING FANNING
ANTENNAL VIBRATION

4

PURSUIT

FEMALE NON-RECEPTIVE

MALE MOUNTS FEMALE

1

ANTENNAL CONTACT

FEMALE NON-RECEPTIVE

J

FEMALE WALKS OFF

FEMALE QUIESCENT

I

COPULATION

/

FEMALE WALKS OFF

Since *Cotesia* species are arrhenotokous, sperm transfer followed by an all male progeny indicated that the spermatozoa and eggs were not compatible. A summary of the reciprocal crosses and the rearing data are shown in tables 0.1 and 8.2. Hybrid female progeny resulting from the cross of *C. sesamiae* males and *C. chilonis* females were reproductively compatible with males of both aarental lines, although the total number of progeny was significantly lower when the male was *C. sesamiae* ($t = 13.6$ $df = 47$ $P<0.0001$) (table 8.3). The ratios of males to females resulting from these crosses were similar to the ratios found for the homogametic crosses.

8.3.3 Sperm transfer

Dissection of females to check for sperm packets in the spermathecae showed that every copulation that lasted for at least nine seconds resulted in nsemination. The females in the interspecific cross appeared active and wealthy suggesting that no injury was experienced by the females during copulation. However, some females mated several times. Those that mated more than three times (maximum of eight) appeared sluggish and did not live to oviposit.

aole 8.1. A summary of the behaviour exhibited by the inter- and

"itraspecific crosses of the *Cotesia flavipes* complex.

		Female		
Male	Response[1]	*C. sesamiae* 9	C. *flavipes* 9	C. *chilonis* 9
C. sesamiae 6	WF	+	0	+
	M	+	0	+
	C	+	0	+
	ST	+	0	+
	FP	+	0	+
C. flavipes 6	WF	+	+	+
	M	+	+	+
	C	+	+	+
	ST	+	+	+
	FP	0	+	0
C. chilonis 6	WF	+	+	+
	M	+	+	+
	C	0	0	+
	ST	0	0	+
	FP	0	0	+

WF = wing fanning, M = mounting, C = copulation, ST = sperm transfer,
=P = female progeny + = positive 0 = negative

~afc!e 8.2. Mean number of males, females, total progeny per female and sex e: os from inter- and intraspecific crosses among the species of the *Cotesia fa/ipes* complex.

-"3SS	N	Total progeny/9	Males ± SD	Females ± SD	Sex ratio F:M
Cs<5 x Cs9	27	31.9 ± 8.4	11.4 ± 8.2	20.5 ± 8.5	**1.8:1**
C&3 x Cs?	18	20.8±9.7	20.8 ± 9.7		
Cf<5 x Cs9	14	23.7 ± 21.3	23.7 ± 21.3		
Cc& x Cc9	30	44.6 ± 6.8	11.2 ± 4.8	33.4 ± 8.8	3:1
ZsS x Cc9	24	19.4 ± 7.7	10.0 ± 8.7	9.4 ± 7.67	1:1.07
ZsS x Cc9	43	11.4 + 6.4	11.4 ± 6.4		
Cf<J x Cc9	13	16.2 ± 10.9	16.2 ± 10.9		
ZfS x Cf9	30	48.2 ± 10.5	12.7 ± 8.2	35.5 ± 12.8	**2.8:1**
Z&a x Cf9	18	19.1 ±9.5	19.1 ± 9.5		
Cc6 x Cf9	28	24.3± 11.2	24.3 ± 11.2		

the 67 crosses of Csd x Cc9, 24 broods included males and females while 43 were all **T**«ies.

":D:e 8.3. Mean number of males, females, total progeny, and sex ratios from zackcrosses of hybrid Csd x Cc9 females to males from parental species.

	N	Total prog./9	Mean 6 ± SD	Mean 9 ± SD	Sex ratio F:M
"+yb9 x C c d	30	27.9 ± 6.9	7.9 ± 5.9	20.4 ± 6.12	2.6:1
-tyb$ x *Cs6*	20	4.8 ± 3.8	1.0 ± 1.0	3.8 ± 3.63	3.8:1

2-3.4 Fecundity on first oviposition

Dissections of parasitised hosts three days after oviposition revealed that number of eggs laid was 44.6 ± 4.3, 44.4 ± 3.7, and 43.5 ± 2.9 for C. "a ••pes, *C. chilonis* and *C. sesamiae* respectively, and did not differ between :->e Three species (F = 0.93; df = 2, 102; P = 0.40).

3.3.5 Intraspecific attraction

The percentages of individuals moving out of the olfactometer stem in ~e ntraspecific bioassays are presented in table 8.4. Attraction of individuals to the opposite sexes (treatments 1 and 2) was higher for *C. flavipes* than attraction to the same sex. In *C. chilonis,* attraction to the same or opposite sexes was not different, although there appeared to be a tendency towards a - :ier response to the opposite sex. For *C. sesamiae,* the response was very •veak in all combinations, and lowest in the male to male treatment. In comparing between the species, *C. flavipes* clearly exhibited the strongest esponse to conspecific individuals of the opposite sex.

The number of individuals that moved to the odour source arm and the empty arm of the olfactometer are presented in table 8.5. The only responses nat were significantly different from the expectation of random movement *.e-e for *C. flavipes* males to females, and *C. flavipes* females to males.

"able 8.4. Percentage of individuals of ***C. flavipes, C. chilonis,*** and C. ***sesamiae*** moving to either arm of the olfactometer in response to conspecific ~>dividuals of the same or opposite sex[1].

	Combination			
Species	<y to 9	9 to *6*	*6* to *6*	9 to 9
C. flavipes	89.3 A a	93.3 A a	22.7 A c	53.3 A b
C. ***chilonis***	62.7 B a	54.7 B a	36.0 A a	37.3 A a
C. sesamiae	16.0 C a	26.7 C a	2.7 B b	13.3 B a

\4earts followed by the same upper case letter in the same column are not : gnificantly different. Means followed by the same lower case letter in the same row are not significanly different. (Student-Newman-Keuls multiple :cr-iparison test, P<0.05).

sbe 8.5 Intraspecific movement to the source arm and control arm of a Y-r.be in response to conspecific individuals of the same or opposite and same sex[1].

Species	TRT[2]	Positive response	Negative response	G-statistic
C. flavipes	1	48	19	12.98 *
	2	49	21	11.52 *
	3	9	8	0.06 NS
	4	20	20	0.0 NS
C. chiionis	1	26	21	0.53 NS
	2	20	21	0.02 NS
	3	15	12	0.33 NS
	4	11	17	1.27 NS
C *sesamiae*	1	8	4	1.36 NS
	2	12	8	0.81 NS
	3	0	2	
	4	6	4	0.38 NS

Total number of individuals tested in all cases was 75. * = significant at ^<0.005, NS = not significant at *P*< 0.05. [2]1 = male to female, 2 = female u -rale, 3 = male to male, 4 = female to female

£ 3.6 Interspecifc attraction

Results of the interspecific response in the Y-tube olfactometer are rsented in table 8.6. The response of *C.chilonis* males to odours emitted by T r *avipes* and *C.sesamiae* females was greater than the response when no ode jr source was present in the olfactometer. *Cotesia flavipes* did not respond : e strongly to pheromones emitted by the other species than to an empty =cTometer. The response of *C.sesamiae* to odours emitted by *C.chilonis* - ~ ales was stronger than the response when there was no odour source, but :ie "esponse to *C. flavipes* female was not different.

3 3.7 Bioassays in field cages with *C. flavipes*

Very few of the *C. flavipes* males released in the field cages were caught in the sticky traps baited with virgin conspecific females, or in the traps no females (table 8.7). However, more males were caught in the female-ed traps (17) than in the control traps (3) (Wilcoxon's signed-rank test; ***~ = 8,*** T = 0, ct<0.005) .

"able 8.6. Interspecific response of *C. flavipes* complex in a V-tube •acto meter[1].

Stimaius •rfSUS espondent[2]	Number of insects that moved to either arm.	Number of insects that did not move. (undecided)	G-statistic
Cd? to no stimulus	12	18	
Cc<J to Cf9	10	20	0.28 NS
Cc<J to Cs9	12	18	0.0 NS
Cf<J to no stimulus	13	17	
Cfd to Cc9	7	23	2.65 NS
Cff to Cs9	9	21	1.12 NS
Csc to no stimulus	0	30	
Cs<J to Cc9	8	22	4.35 *
Csd to Cf9	3	27	0.20 NS

Total number of individuals tested in all cases was 30 [2]Cf = C. *flavipes,* Cc = C. *chilonis,* Cs = C. *sesamiae,* * = significant at P<0.05

"anle 8.7. Number of ***Cotesia flavipes*** males caught in pheromone traps baited *ith live virgin female ***C. flavipes***[1].

fceplicate number	Number caught in female baited trap	Number caught in control trap
1	1	1
2	4	0
3	2	1
4	1	0
5	1	0
6	2	1
7	0	0
8	1	0
9	0	0
10	2	0
11	3	0
Totals	17	3

:orty virgin males released in all cages.

i4 DISCUSSION

The mating behaviour of *C. flavipes* complex males and females is aar-oarable to other braconid parasitoids. Kamano *et at.* (1989) reported a ar sequence of events in *Ascogaster reticulatus* (Hym.: Braconidae). They "ound that male antennation was elicited by a pheromone emitted by the e~~ales. Obara and Kitano (1974) found that wing fanning in *C. g/omerata* L. was elicited by a female-produced sex pheromone, but mounting and «pjlatory response were elicited by a combination of visual stimuli and the sex re^romone (Kitano, 1975). Kajita and Drake (1969) made similar observations and conclusions on the mating behaviour of *C. chilonis* and *C. flavipes,* and "cted that mating occurred readily when 2 or 3 males were placed with 6 to 5 -emales. When a greater number of males were supplied, the aggressive Dehaviour of males interrupted most attempts at copulation.

When close to the pheromone source, wing fanning may be used by -a es to emit sound signals (Decker, 1988). The sound produced by a fanning *.asonia vitripennis* (Hym.: Pteromalidae) male seemed to contribute to the ducement of receptivity in virgin females (Van den Assem and Putters, 1980). . nc fanning may also elicit increased pheromone release in the females Spangler, 1987). Courtship and mating behaviour was observed in all the -rre'specific crosses, even when copulation did not occur. Females in

rerspecific crosses appeared to exhibit the same behaviour as those in the onspecific crosses indicating that when placed in close proximity, the species ~ t'-.e *C. flavipes* complex recognised each other as potential mates.

Cotesia species exhibit arrhenotoky, therefore, the production of F1 5Tia es indicates fusion of gametes in a cross. Clutch size from the first : - position of homogametic crosses was approximately equal to the subsequent :ʳood size. The average brood size from the interspecific crosses was lower :ian the values from intraspecific crosses, which was probably due to mortality eggs that fused with incompatible sperms; the union of gametes produced : ethal lesion. The interspecific cross between *C. sesamiae* males and *C. :~ .'on/s* females resulted in viable female progeny in 36% of the crosses. The • I'd females were compatible with males of both parental species, although a hgher egg mortality must have occured when the male parent was C. *sessmiae.* One way compatibility has been reported in *Trichogramma* species :or-!plexes (Pinto *eta/.,* 1991; Nagarkatti and Fazaluddin, 1973; Oatman *eta/.,* 1970). Rao and Debach (1969) reported partial reproductive isolation among "ʳee allopatric sibling forms of *Aphytis /ingnanensis* (Hym.: Aphelinidae), and coreluded that gene flow between two of the three forms could occur oerimentally, but was unlikely to occur in nature because the populations •vere allopatric. Similarly, *C. sesamiae* is indigenous to Africa, and hence T:graphically isolated from *C. chilonis* which has only been reported in Japan

rid China. The two species are very similar morphologically and are not rDTipletely reproductively isolated. Geographic isolation could explain why ?*oi_Tion has not proceeded towards reproductive isolating mechanisms. -: /.ever, during classical biological control programmes, these species may be "oduced into each other's areas of endemnism, and since there are no irezygotic isolating mechanisms, mating could occur indiscriminately.

Males of C. *flavipes* mated with females of *C. chilonis* and *C. sesamiae* a"*d sperm is transferred but fusion of the gametes was not expressed in : ocjction of female progeny. The "mated" females then proceeded to oviposit -on-viable eggs. Indiscriminate interspecific mating is speculated to have contributed to some failures in classical biocontrol programmes (Jeff Waage, :e-sonnal communication). However, many gregarious species mate with their ;brgs (Waage, 1989). Thus, the probability of interspecific mating may be - e-nely low. Arakaki and Ganaha (1986) observed that male *Cotesia flavipes* ;~erging from cocoon masses placed in sugarcane stems remained on the cersurface of leaves near the cocoon mass from which they had emerged, 2nd ~ated with their siblings.

What evolutionary significance should be attributed to the one way atibility between *C. sesamiae* and *C. chilonis?* The partial compatibility .-sen the two species is not sufficient evidence to support an assumption

'.a: they are very closely related. Cracraft (1989) stated that the ability to 'eoreed is a primitive trait, known to transcend well defined species :o^daries and does not, in itself, establish evolutionary units or their crrponents. The male genitalia of ***C. sesamiae*** and ***C. chiionis*** are "onoholoqically indistinguishable, and therefore cannot be used to separate all "ie species in ***C. flavipes*** complex.

The pheromone studies indicated that ***C. flavipes*** responded to volatile :do_rs from conspecific individuals of the opposite sex in a Y-tube : -actometer. Wing fanning was exhibited by 72 out of the 75 ***C. flavipes*** es in reponse to virgin conspecific females, indicating that they perceived = SDecific pheromone from the females. Males also responded towards mated — e'es, suggesting that mated females also released a volatile pheromone. ~~e ack of response between ***C. flavipes*** individuals of the same sex suggests there may be two pheromones; one emitted by females and perceived by -nafces, and one emitted by males and perceived by females. The lack of e specific attraction between ***C. flavipes*** males and females of the other two es in the olfactometer provides further evidence that interspecific mating 3 un ikely to occur in nature.

There was no significant intraspecific response in the olfactometer in C. ***zesamiae*** and ***C. chilonis*** (table 8.4 & 8.5). Only two C. ***sesamiae*** males wing

•cried in the olfactometer, although during the mating trials (table 8.1), wing -=n ing behaviour was observed in this species. Most of the males flew about -asnazardly in the tube; a characteristic that was not observed in ***C. flavipes.*** **~~e** number of undecided individuals in ***C. sesamiae*** was greater than the .nbers moving to either of the arms of the olfactometer. ***Cotesia sesamiae*** do not appear to respond to volatile conspecific odours. One possible olanation is that the odours from these species are not highly volatile, and :an only be perceived at a very short distance. Alternatively, ***C. sesamiae*** may _tse other systems for mate recognition. However, during interspecific trials i3\e 8.6), ***C. sesamiae*** males responded to volatile odours emitted by ***C. oris*** females. This may suggest that mating between these two species could occur in nature.

The catches of conspecific males in female-baited traps suggest that :"-sibling mating may occur in nature. Moreover, there may be a potential for ne use of pheromones in ***C. flavipes*** monitoring. A trapping method could z'cvide a means of monitoring the presence of ***C. flavipes*** without destructive : ant sampling and rearing of collected stem borers. The low trap catches in "9 Dioassays could be due to trap design, or reflect a limited range of activity y the ***C. flavipes*** pheromone.

CHAPTER NINE

2 GENERAL DISCUSSION

Previous workers proposed various morphological characters to separate ~ species in the C. *flavipes* complex, primarily based on colouration, culpturing, and male genitalia (Wilkinson 1932; Rao and Nagaraja 1967; •ajaraja 1971; Alam *et al.* 1972; Sigwalt and Pointel 1980). Using the male :sn talia, Polaszek and Walker (1991) separated the species in the C. *flavipes* ~olex into two morphospecies; the *C. sesamiae/C. chilonis* subcomplex and *~'a<*ipes.* No valid characters were found that could distinguish accurately •veen *C. sesamiae* and C. *chilonis.*

"he absence of clear diagnostic characters may have confounded past -:s to assess the impact of introductions of the *C. flavipes* complex. A : rical control programme against *Chilo partellus* Swinhoe (Lep: Pyralidae) East Africa made releases of C. *flavipes* from 1968-72. The identification ecovered *Cotesia* spp. was based on the colouration of the hind coxae and rte'nae, and the density of hairs on the antennae (Mohyuddin 1971). It is : Die that the biocontrol agent may have established but remained unoticed - -o lack of proper diagnostic characters. In Mauritius, assessment of 5s tization by *Cotesia flavipes* and *Cotesia sesamiae on Chilo sacchariphagus*

:oje^r and *Sesamia* ***calamistis*** Hampson (Lep: Noctuidae) respectively was based n :he same morphological characters (Rajabalee and Govendasamy 1988). ->ese characters are now known to be highly variable and unreliable for separating ***C. flavipes*** and ***C. sesamiae*** (Sigwalt and Pointel 1980; Polaszek and •Vai<er 1991). In South Africa, where ***C. flavipes*** was released against C. *:<3rtefJus.* recoveries were recorded from both C. ***parte/lus*** and ***Busseola fusca*** *-j* ***er*** <Lep. Noctuidae) (Skoroszewski and Van Hamburg 1987). However, 'oryuddin (1971) reported that ***B. fusca*** was not a suitable host for ***C.*** ***-a .*** *^es,* and this has been corroborated by recent work in our laboratory (Ngi-Sorg *et at.* 1994), suggesting that there may have been misidentifications.

Cotesia ***sesamiae*** and ***Cotesia chilonis*** are similar and based on -orphological features, they would pass for a single species if it were not for **t** - ion-overlapping distributions and differences in host records, (Polaszek arxj Walker 1991). ***Cotesia chilonis*** (Matsumura) is an important parasite of u*x ***o*** *suppressalis* (Walker) in Japan, while ***C. sesamiae*** is a widespread parasite *Susseo/a fusca,* ***Sesamia*** spp. and ***Chilo*** species in Africa. Morphological rjdies by Nagaraja (1971), Sigwalt and Pointel (1980) and Polaszek and •atker (1991) have shown only slight variation in the thoracic terga and the -alia, which the authors agree, are subject to intraspecific variation. -s dering the wide geographical separation between the African and Asian ; _ ations of the ***chilonis/sesamiae*** sub-complex, and the widespread presence

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of C. *flavipes* in between, further studies combining the findings of the ~orphological features with other taxonomic characters were essential before =ny formal taxonomic decisions could be made regarding the status of the soec es in the ***C. flavipes*** complex.

Mohyuddin (1971) stated that it was likely that because of its nscontinuous distribution and conflicting host records, ***Cotesia flavipes*** may in ~act comprise a number of strains, sub-species or sibling species. Reciprocal crosses of different populations of ***C. flavipes*** from different localities (Shami "990) did not show hybrid sterility. Mohyuddin, ***et al*** (1981) suggested that rotypes may also exist that are morphologically identical and sexually cor-oatible, but prefer different habitats. Ngi-Song ***et al.*** (1994) found that a rcouiation of ***C. sesamiae*** from the Coast Province of Kenya was encapsulated :v ***B. fusca*** while a population of the same species from western Kenya was -3- This means that there are host adapted parasitoid populations within the ;oec»es. Since these are important biological control agents, it is crucial that - - ght (host adapted) population is identified for the stemborer in question. ***.as*** thus necessary to use other techniques and characters that were so far t > Dlored to study the biosystematics of this complex and if possible come . . th characters that could distinguish not only the species but also the host specific strains.

Comparative studies have shown that morphological change and "olecular divergence are quite independent, responding to different 2vo utionary pressures and following different rules (Wilson *et at.* 1977). In general, studies that incorporate both molecular and morphological data will rrovide much better descriptions and interpretations of biological diversity than lose that focus on just one approach. However some systematic problems car only be addressed by morphological data and others by molecular data - s i 987). In this study, morphological, biochemical and biological data .•>ere used. A summary of the finding of each study is presented below.

Morphology: Several morphological character states were scored and ?va uated for their use in the diagnosis of the species. The overall shape of the -.aie genitalia separated the complex into two groups, *C. flavipes* and C. *issamiae/chiionis.* Intraspecific variations within these two groups were noted. The male genitalia of populations of *C. flavipes* from Mauritius differed sjSstantially from the typical genitalia of populations from Pakistan, while the e genitalia of *C. sesamiae* from Malawi differed from the rest of the C. *sesamiae* populations by having the penial valves and the penial crest extended s I ttle beyond the median groove. Other intra- and interspecific variations that •vere observed were; rugosity of the propodeum, where the populations of *C. larvipes* were fully rugose except for the Papua New Guinea population where ~ie oasal half of propodeum was smooth. Another character that varied - --aspecifically was the relatively smooth carapace in the Thailand population

" *C. flavipes.* One character that can be used to separate ***C. chilonis*** from C. •*esamiae* and which has proved to be consistent in all the populations examined is the scuto-scutellar sulcus which is straight in ***C. chilonis*** and -jrved in ***C. flavipes*** and ***C. sesamiae.*** Nevertheless a combination of three or "T>oʳe characters suggested in chapter three could be used to identify each species with some degree of certainty.

Morphometries: studies conducted on 16 characters from different ropuiations of the ***C. flavipes*** complex from several areas of the world indicated canonical discriminant functions developed from a few of the populations the complex could be used to classify new specimens into their respective species. Principal component analysis separated ***C. chilonis*** from the rest with "aspect to the second principal component, with measurements on the face "aving relatively high magnitudes. This implies that the shape of the face of C. *ch^onis* differs from the other species. Canonical variates analysis indicated ~at there were at least three groups in the population analysed with the ~~ =i and population forming a sub-set of the IndoAustralian-Neotropics cluster. Thailand population differed morphologically from the other ***C. flavipes*** lobulations by having a smooth carapace and slightly distinct propodeum.

Biochemical characters: The gene products of 22 loci were detected, "ve loci were diagnostic and could be used to consistently separate

- -lations believed to represent the three species. Genetic distance analyses - ng allele frequencies suggested that the three taxa are closely related when ~oared to an outgroup, ***C. g/omerata.*** Phylogenetic studies on fourteen r. -r.e systems and Random Amplified Polymorphic DNA Polymerase Chain r=c-Jon (RAPD-PCR) suggest that ***C. sesamiae*** and C. ***chilonis*** are a ~~ j~cphyletic group while allopatric populations of ***C. flavipes*** are polyphyletic. st adapted strains could not be detected using the diagnostic loci but results genetic distances and similarities (table 6.3) showed a range of variations .veen populations within a species.

Three main hydrocarbon classes that have been identified mixtures are: a ones, alkenes and methylalkanes. Nearly all examined insect species have ~ >:ures of n-alkanes. Gas liquid chromatography of cuticular hydrocarbons -c .ved that C. ***flavipes*** and C. ***chilonis*** are more similar to each other than to " *sesamiae.* However, the two parasitoids were reared on the same host, and cular hydrocarbons may be influenced by the host environment. The :s~ern of cuticular components cannot therefore be reliably used to separate zec es in the ***C. flavipes*** complex. Further investigations to determine the cent ty of compounds in the hosts and parasitoids are necessary. This would "t 2 to determine whether cuticular components of parasitoids are species specific.

Preliminary results using RAPD-PCR showed specific banding patterns for rsch of the three species. However the banding patterns did not reveal any ""raspecific polymorphism. Nevertheless this study indicated that it is possible : extract and amplify DNA from these individuals.

Biological characters: Reciprocal crosses were made between all possible :: Tibinations of the three species to test whether the three species are J cgically valid. Interspecific mating was observed between all combinations, ***: JZ*** fertilization only occurred between males of ***C. sesamiae*** and females of ***C.*** *' •lonis.* The offspring of this cross were viable and produced both male and -~a e progeny, indicating that ***C. sesamiae*** and ***C. chiionis*** are not completely -i ʳoductively isolated. The hybrid females were compatible with their male rentals. Pheromone studies conducted using males and females of all the zecies showed that C. ***flavipes*** males and females responded to odours — "ed by conspecific members of the opposite sex in a Y-tube olfactometer, -c sating that attraction is mediated by volatile compounds. Female baited ^ps in large field cages were able to attract and entrap conspecific males, rgesting that pheromones may have potential for use in field monitoring of *; flavipes. C.* ***sesamiae*** did not respond to conspecifics in the Y-tube ;:tometer. This could be the difference between the two species and may that *C.* ***sesamiae*** sib-mates in the field while C. ***flavipes*** may also mate -eʳ individuals. This and the fact that ***C. flavipes and C. sesamiae*** individuals

ite indiscriminately in the laboratory, need further investigation under field iroitions. That parasitoids may mate indiscriminately in the field and waste •--rir off-spring has been speculated to be one of the reasons for some failures " classical biological control (Waage, personnal communication)

Summary of the discussion

The male genitalia of ***C. sesamiae*** and ***C. chilonis*** is inseparable and this ~as ed some workers (Polaszek and Walker 1991) to suggest that the two are roseiy related. This has been corroborated by the one way cross between C. *sesamiae* male and ***C. chilonis*** female. But the biochemical analysis indicates ~at they are separate entities no closer to each other than to ***C. flavipes.*** Morphological and biochemical data indicates that ***C. flavipes*** is not -onophyletic. The male genitalia, and surface sculpture varies within the scec es. Host specific strains within ***C. flavipes*** have been reported by several /•or<ers (Mohyuddin 1971, Shami and Mohyuddin 1990). Preliminary results **Tom** RAPD-PCR of genomic DNA did not show variations in banding patterns .vtnin the species. Other techniques, for instance, RFLP of mitochondrial DNA and other rapidly evolving nucleic acids could be used to find differences within :ie species which could then be used to develop strain specific probes for quick : agnosis of the strains and species.

All the above taxonomic characters and techniques, have contributed a eat deal in the understanding of the *C. flavipes* complex and to the :csvstematics of parasitoids in general. The use of one character in such :csely related and morphologically similar species can result into misleading "ornrvation on the biosystematics of the species/or strain. An incorrect •jxc-omic interpretation can lead to failures or substantial delays in effecting : logical control and eventually wrong assessment of the biological control ?f^orT. For routine taxonomic work, all the techniques except examination of morphological features are laborious, time consuming and expensive. However, -e choice of techniques and characters will depend on the group in question, • rce some systematic problems can only be addressed by specific data.

Conclusions

Morphological studies showed that male genitalia of the *C. flavipes* population from Mauritius (Belle vue Maurel sugar estate) was different from other populations of the same species. Other notable differences on the male genitalia were observed on the *C. sesamiae* population from Malawi. The Thailand population of *C. flavipes* had relatively less sculpture on the carapace than other populations of the same species. This is evidence that there are differences between allopatric populations in the *C. flavipes* complex.

Morphometries and allozyme electrophoresis can be used to identify the three species in the *C. flavipes* complex.

Reciprocal crosses indicate that there are no close range pre-zygotic isolating mechanisms among the species in the *C. flavipes* complex; results of the Y-tube olfactometer did not show interspecific attraction.

C. *sesamiae* and *C. chilonis* are not completely reproductively isolated.

Volatile pheromone studies show that there may be potential for the use of pheromones to monitoring the establishment and dispersal of *C. flavipes.*

Cladistic analysis of the allozyme data indicated that allopatric populations of *C. flavipes* may not be monophyletic.

Further studies should be conducted to develop diagnostic DNA/RNA probes for quick diagnosis of the species and host strains in the *C. fJavipes* complex.

These studies indicate that the species in the complex consist of allopatric populations that differ in various aspects from each other

including morphology, biochemical and host specificity.

Further investigations should be conducted using allozyme electrophoresis and/ DNA analysis to clarify the position of the Mauritius population in ***C. flavipes*** complex and also to determine whether populations from the Indo-Australian region are indeed the same species.

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4 Esterase

Sodium phosphate buffer	15 ml
o-Naphylacetate (0.3% in acetone)	0.5 ml
^-IMaphylacetate (0.3% in acetone)	0.5 ml

Incubate with gel for 15-20 minutes
Add 1 5mg GBC salt shake until bands appear

5 Malic enzyme

Tris A buffer	3 ml
Distilled water	3 ml
Malic acid	0.5 ml
NADP	0.5 ml
$MgCl_2$	1.0 ml
MTT	0.5 ml
PMS	0.5 ml

6 **Isocitric** dehydrogenase

Isocitric acid	30 mg
NADP	0.5 ml
$MgCl_2$	2.0 ml
MTT	0.5 ml
PMS	0.5 ml
Tris A buffer	3 ml
Distilled water	3 ml

7 **Malate** dehydrogenase

Malic acid	3 ml
Tris A buffer	3 ml
Distilled water	3 ml
NAD	0.5 ml
$MgCl_2$	1 ml
MTT	0.5 ml
PMS	0.5 ml

4 Esterase

Sodium phosphate buffer	15 ml
a-Naphylacetate (0.3% in acetone)	0.5 ml
£-Naphylacetate (0.3% in acetone)	0.5 ml

Incubate with gel for 15-20 minutes
Add 15mg GBC salt shake until bands appear

5 Malic enzyme

Tris A buffer	3 ml
Distilled water	3 ml
Malic acid	0.5 ml
NADP	0.5 ml
$MgCl_2$	1.0 ml
MTT	0.5 ml
PMS	0.5 ml

6 Isocitric dehydrogenase

Isocitric acid	30 mg
NADP	0.5 ml
$MgCl_2$	2.0 ml
MTT	0.5 ml
PMS	0.5 ml
Tris A buffer	3 ml
Distilled water	3 ml

7 **Malate** dehydrogenase

Malic acid	3 ml
Tris A buffer	3 ml
Distilled water	3 ml
NAD	0.5 ml
$MgCl_2$	1 ml
MTT	0.5 ml
PMS	0.5 ml

8 6-phoshogluconic acid dehydrogenase

Tris A buffer	6 ml
6-phosphogluconic acid	10 mg
NADP	0.5ml
$MgCl_2$	1 ml
MTT	0.5 ml
PMS	0.5 ml

9 Triose phosphate Isomerase

Tris A buffer	4 ml
o-Glycerophosphate	130mg
Sodium pyruvate	44mg
MAD	0.5 ml
©-Glycerophosphate Dehydrogenase	4ul
Lactate Dehydrogenase	4ul
Incubate for 2hrs at 37°c stop reaction with	Cone.
HCL to pH 2. adjust to pH 8 before use.	
G-3PDH	10 ul
Sodium Arsenate	10 mg
MTT	0.5 ml
PMS	0.5 ml

10 Phosphogluco mutase

Tris A buffer	3 ml
Distilled water	3 ml
G6PD {100mg/ml)	20/J\
G-I-P (20mg/ml)	10 mg
$MgCl_2$	1 ml
NADP	0.5 ml
MTT	0.5 ml
PMS	0.5 ml

1 1 Glucose phosphate isomerase

Tris A buffer	3 ml
Distilled water	3 ml
Fructose-6-phosphate	10 mg
$MgCl_2$	2 ml
NADP	0.5 ml
PMS	0.5 ml
MTT	0.5 ml
G6PD	10 A'1

1 2 Aldolase

Tris A buffer	25 ml
Fructose-1,6-diphosphate	0.1 g
NAD(10mg/ml)	2ml
Sodium arsenate	0.06 g
G-3-PDH	50 fj\
MTT	0.75 ml
PMS	0-25 ml

1 3 Aconitase

Tris A buffer	6 ml
cis-aconitic	25 mg
$Mgcl_2$ (0.1 m)	1 ml
NADP(IOmg/ml)	0.5ml
MTT (5mg/ml)	∘-5 ml
PMS (5mg/ml)	∘-5 ml

14 Sorbitol dehydrogenase

Tris A buffer	6 ml
Sorbitol	250mg
$Mgcl_2$	0.5ml
NAD	0.5ml
NBT	0.5ml
PMS	0.5ml
MTT	0.5ml