

**THE PHYLOGENETIC AND PHYLOGEOGRAPHIC RELATIONSHIPS OF  
*PEDIOBIUS FURVUS* (HYMENOPTERA: EULOPHIDAE) POPULATIONS  
IN WEST AFRICA.**

**IBRAHIM MANGHA OKEKE SHAMIE**

**DOCTOR OF PHILOSOPHY, 2004**

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**THE PHYLOGENETIC AND PHYLOGEOGRAPHIC RELATIONSHIPS OF  
*PEDIOBIUS FURVUS* (HYMENOPTERA: EULOPHIDAE) POPULATIONS  
IN WEST AFRICA.**

**BY**

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**DIPLOMA IN INSECT TAXONOMY (DISTINCTION)  
MASTER OF PHILISOPHY  
CARDIFF UNIVERSITY**

**THESIS SUBMITTED FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY  
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## **DEDICATION**

**This thesis is dedicated to the memory of my parents**

## ABSTRACT

In spite of introduction of new improved and disease resistant cultivars of maize to Africa by the International Institute for Tropical Agriculture (IITA) high quality maize production remains hindered by insect pests, notably lepidopterous stem borers. Field trials using classical biological control methods are currently under way. These pests may be naturally kept in balance by cryptic parasitoid wasps, one of which is the topic of this thesis. *Pediobius furvus* Gahan (Hymenoptera: Eulophidae) is a gregarious pupal parasitoid of *Sesamia calamistis*, *Chilo partellus* and *Busseola fusca*. The targeted application of this parasitoid is a potentially important element of an integrated pest management strategy for African maize stem-borer control, yet we know very little about its biology, especially its population structure and diversity with respect to geography and host-relationship.

Six populations were sampled across Africa. Experiments were carried out to examine morphological differentiation among the populations, to ask whether geographic variation supports their biological identity, and to investigate genetic variation and phylogenetic structure within and among populations. Thirteen morphological characters were examined on 120 females. Morphological characters measurements were used as descriptive data and were analysed using morphometric techniques. Results obtained based on differences among populations due to differences between individuals within populations placed populations in three groups and recognised one of the groups as separate.

Mate recognition experiments were carried out with three UK species of *Pediobius eubius* complex (*Pediobius deschampsiae* Dawah, *P. phalaridis* Dawah and *P. calamagrostis* Dawah, and their *Tetramesa* hosts (Hymenoptera: Eurytomidae) as a model for test of different species compatibility. No female offspring was recovered from 2-species mating in all tests, whereas females were recovered from single-species mating. Field and laboratory tests results showed that the three species were reproductively incompatible in spite of living in sympatry. Behavioural isolation was due to courtship failure between male and female of different species.

Molecular data were analysed from the same six African populations of *P. furvus*. Phylogenetic relationships among populations were explored using DNA sequence data from the nuclear large ribosomal subunit (28S) and the mitochondrial cytochrome *b* genes. Phylogenetic analyses at, above and beneath the putative species levels were carried out using minimum evolution model for both 28S rDNA and cytochrome *b*. An absence of large scale, but trend towards smaller-scale geographic structure was found using analysis of molecular variance of putative cytochrome *b* sequences. However, the sequences were found to be homologous.

## CRYNODEB

Mae'r Sefydliad Rhyngwladol dros Amaeth Trofannol (*International Institute for Tropical Agriculture, IITA*) wedi llwyddo datblygu a thyfu cyltiferau o india corn sy'n wrthiannol i nifer o wahanol afiechydon ar gyfandir Yr Affrig. Er hyn, erys cynaeafau toreithiog o gnydau safonol yn darged i drychfilod plaol, yn arbennig y mwynwyr coes lepidopteraidd. Yn gyfredol, disgwylir canlyniadau treialon maes sy'n defnyddio rheoli biolegol clasurol fel modd i leihau poblogaethau'r trychfilod plaol. Cedwir y plâu dan reolaeth trwy ddefnyddio gwenyn parasitig, cryptig; un ohonynt yw gwrthrych y traethawd hwn. Trychfilyn parasitoid gregaraidd sy'n ymosod ar bwpa *Sesamia calamistis*, *Chilo partellus* a *Busseola fusca* yw *Pediobius furvus* Gahan (Hymenoptera: Eulophidae). Mae potensial defnyddio'r trychfilyn parasitoid hwn mewn rhaglen o reoli cyfannol ar boblogaethau'r mwynwr coes india corn Africanaidd yn fawr, ond ychydig iawn a wyddom am ei fioleg, yn arbennig ei strwythur poblogaethol a'i amrywiaeth daearyddol a rhyngweithiol.

Samplwyd chwe phoblogaeth ar draws Yr Affrig. Trwy ddulliau arbrofol archwiliwyd gwahaniaethau morffolegol ymysg poblogaethau, i ddarganfod a ydyw amrywiaeth daearyddol yn cydreddeg â nodweddion biolegol, ynghyd ag amrywiaeth genetegol a threfn esblygiadol oddi-mewn ac ymysg poblogaethau. Archwiliwyd 13 nodwedd ar 120 o drychfilod benywaidd. Defnyddiwyd nodweddion morffolegol fel data disgrifiadol a dadansoddiwyd y rhain gan ddulliau morffometrig. Dengys y canlyniadau y gellir gwahaniaethu'r poblogaethau, ar sail gwahaniaethau ymysg unigolion oddi mewn i boblogaethau, i dri grŵp. Adnabyddwyd un grŵp fel grŵp arwahan.

Defnyddiwyd arbrofion adnabod-cymar ar dair rhywogaeth Brydeinig o *Pediobius* (*Pediobius deschampsiae* Dawah, *P. phalaridis* Dawah a *P. calamagrostis* Dawah) ynghyd â'u gwestywr *Tetramesa* (Hymenoptera: Eurytomidae). Trwy ddefnyddio'r rhain fel modelau bu'n bosibl archwilio cyfaddasrwydd gwahanol rywogaethau. Tra ymddangosodd had benywaidd o ganlyniad i gyplysu un rhywogaeth, ni welwyd yr un benyw mewn cyplysiadau dwy rywogaethol. Dengys arbrofion maes a labordy bod y tair rhywogaeth yn hollol anghymarus, er iddynt gydcynefino. Deillia'r arwahanrwydd ymddygiadol o feddiant carwriaeth rhwng gwryw a benyw gwahanol rywogaethau.

Dadansoddiwyd data moleciwlaidd o chwe phoblogaeth Africanaidd o *P. furvus*. Archwiliwyd perthnasedd esblygiadol ymysg y poblogaethau trwy ddefnyddio data olyniaeth DNA o'r is-uned ribosomal gnewyllol (28S) a genynnau Sytocrom *b* mitochondriaidd. Defnyddiwyd is-fodelau esblygiadol ar 28S rDNA a Sytocrom *b* ar gyfer dadansoddiad esblygiadol ar lefel rhywogaeth, uwch ac islaw. Yn absenoldeb unrhyw batrwm ar raddfa uwch, darganfuwyd patrwm daearyddol ar raddfa is wrth edrych ar amrywiaeth moleciwlaidd olyniaethau dybiedig Sytocrom *b*. Darganfuwyd bod yr olyniaeth yn gwbl gyfatebol.

## DECLARATION

**This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.**

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**CHAPTER ONE**  
**GENERAL INTRODUCTION**

## **1.1 Background**

West Africa falls into two zones in terms of vegetation, (i) tropical rainforest, with dense surface covered by shrubs, ferns and trees, with an average annual rainfall of about 1200mm; and (ii) savannah grassland with annual rainfall between 500-850mm. However, the climate throughout the region is almost uniform with two main seasons, the rainy season (May – September) and dry season (October - April).

### **1.1.1 Main food crops**

Cereals are the main food crops in West Africa. Maize is the most important and staple food for about 60% of the population in the region. The crop is easy to manage. It can grow well in a wide range of agroecological zones and its production has been intensified in recent years by the introduction of new varieties. These factors make the crop available all the year round within the sub-region. In spite of this, most maize is grown by small-scale farmers, sometimes in the backyards or small plots. According to a FAO report in 2000, Africa produces only 7% of the world's maize with an average yield of 1300kg per hectare compared to 8600kg per hectare in the USA. Rice and sorghum are also popular in the region with mechanisation of rice cultivation in some countries (Heuer *et al.*, 2003, Harsch, 2004).

### **1.1.2 Pest problems**

Improved maize varieties that are disease resistant have been developed and introduced by the International Institute for Tropical Agriculture (IITA)

to increase maize production. These new varieties are recorded to yield twice as much as their parent traditional varieties. However, the main constraints with the cultivation of maize crop, often resulting to food shortage in the region are insect pests such as stemborers and leaf defoliators. The most prominent among insect pests that frequently attack these crops are the stem-borers and leaf-miners belonging to the Lepidoptera and Diptera. Perhaps the stemborers in the lepidopteran families are the most destructive of cereal crops in the region, infesting and damaging stems from seedling stage through crop maturity and harvest (see Polaszek *et al.*, 1998).

The Lepidoptera and Diptera contain very important stem-boring and leaf mining species. Those that infest maize and sorghum include the African maize stalk borer *Busseola fusca* Fuller, the African pink borer *Sesamia calamistis* Hampson and *Sesamia* sp. (Lepidoptera: Noctuidae) and the spotted stem borer *Chilo partellus* Swinhoe (Lepidoptera: Pyralidae); whilst rice is infested by the African white borer *Maliarpha separatella* Ragonot, the African sugarcane borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae) and the African rice gall midge *Orseolia oryzivora* Harris and Gagné (Diptera: Cecidomyiidae). Adults of these insects are large, particularly the moths, measuring between 14-17mm body lengths with wingspans of over 30mm. The borers attack the crops at least from two weeks of germination and throughout the plants' lives. These insects have at least three generations in a year, and a single female could lay between 150-400 eggs per generation depending on the

species and season. For example, *Busseola fusca* has been recorded laying between 400-1000 eggs. These insects lay their eggs in masses on leaf surfaces, on the undersides of leaves or inside leaf sheaths depending on the species and the host plant. After hatching the young larvae bore into the leaf sheaths and start feeding on the tissues, and eventually enter the stems. Some stem borer species attack the stems just above the first node, while others attack stem internodes and continue feeding upwards. When maize is attacked, stem borer larvae start feeding within leaf sheaths then on the stems and migrate to maize cobs and continue feeding on the grains until fully grown. The first generation of adults normally appear in the field between April and July upon hatching from the stubbles and ready to re-infest. Oviposition takes place in June or July, and pupation and adult emergence in September. The second generation occurs between August and October. With the exception of *B. fusca*, the emerging adults form the population of the third generation which survive on wild grasses, sorghum or millet. The third generation larvae of *B. fusca* enter into diapause with the onset of the dry season and complete their development in the following months (Ofomata *et al.*, 2000; Chinwada and Overholt, 2001). Larvae have a prolonged feeding life span before pupation, between 18 to 193 days depending on the species, host plant, ecology, season and temperature. Because of the length of time they spend feeding inside the plant, stem borer larvae cause considerable damage to crops. The dipteran *Orseolia oryzivora* Harris and Gagné looks superficially like a large mosquito but causes serious

damage to rice plants especially grown on lowlands. Damage is caused when the young larvae bore into rice tillers and eat away the inside causing death of immature grains of damaged tillers which turn to greyish, hence the name 'white heads'.

### 1.1.3 Other important crop pests

The cassava mealybug *Phenacoccus manihoti* Matile-Ferrero (Homoptera: Pseudococcidae) and the cassava green mite *Mononychellus tanajoa* Bondar (Acari: Tetranychidae) were accidentally introduced in Africa from South America, the genetic ancestral home of their cassava host *Manihot esculenta* Crantz (Euphobiaceae) (see Herren and Neuenschwander, 1991). Until the early 1990s these pests threatened the lives and livelihood of more than 200 million people in sub-Saharan Africa when IITA-Biological Control Centre for Africa made a breakthrough in their control. IITA carried out intensive research for species that limits their populations in their original habitat. Two exotic species, the hymenopteran parasitoid *Apoanagyrus lopezi* De Santis (Hymenoptera: Encyrtidae) and the phytoseiid *Typhlodromalus aripo* DeLeon (Acari: Phytoseiidae) were identified and introduced. They successfully controlled the two pests *Phenacoccus manihoti* and *Mononychellus tanajoa* respectively. Previously, *A. lopezi* was unknown as a natural parasitoid of *P. manihoti*. *A. lopezi* was successfully differentiated from its sister species *A. diversicornis* Howard based on structural morphology and sexual dimorphism such as antennal colour differences. Because of these

successes in the containment of these two destructive pests of cassava in Africa, attempts are now being made by IITA-Biological Control Centre for Africa in Cotonou, Benin on identifying indigenous parasitic species to develop a classical biological control strategy to contain some of the pests such as maize stemborers and the like throughout sub-Sahara Africa. Field and laboratory studies have shown that some chalcidoid species are natural parasitoids of some of the stem borers. A good example are the species of *Pediobius* Walker (Hymenoptera: Eulophidae) which are frequently recovered as a natural control agent for some of the African stem borer species notably *S. calamistis*, *C. partellus*, *B. fusca* and *E. saccharina* (Jordan, 1966; Polaszek *et al.*, 1998). These include Sarhan and Quicke (1990) who have recently identified a braconid parasitoid in *Mesobraconoides* gen. n (Hymenoptera: Braconidae) as natural control agent of the African white stem borer *M. separatella*, a very serious insect pest of rice in West Africa.

## 1.2 The Eulophidae

The first study of a eulophid was made by Westwood (1828) when he proposed the name *Eulophina* to represent the family based upon the oldest chalcidoid genus *Eulophus* Mueller. Until recently, and because of morphological similarity the family included a wide range of other chalcidoids. Some of them separated in later years have been given family rankings and are known today as Aphelinidae, Tetracampidae and Elasmidae (Burks, 1979). However, the Elasmidae whose species are morphologically closely related to

the present Eulophidae has recently been down-graded to subfamily level within Eulophidae (Yoshimoto, 1984; Gauthier *et al.*, 2000).

The Eulophidae has undergone several systematic reviews and is one of the largest chalcidoid families. There is still controversy among workers over the number of genera and species. For example, Hayat (1988) noted that the family comprised about 300 genera and about 3000 species; Gibson (1985) recognised 540 genera with over 3900 nominal species distributed worldwide, whilst Noyes (1998) mentioned 283 genera and 3977 described species. For the African eulophids, Prinsloo (1980) mentioned that the species are poorly known but contained species of economic importance. There is also disagreement as to the number of subfamilies. Bouček (1964) and Prinsloo (1980) recognised five subfamilies (Eulophinae, Elachertinae, Euderinae, Entedontinae and Tetrastichinae) whilst Gibson *et al.* (1997) recognised only four (Eulophinae, Tetrastichinae, Euderinae and Entedontinae). The subfamilies themselves have been reviewed occasionally. For example, among the Eulophinae, Miller (1970) produced a key to the genera of Eulophini which he treated as a subfamily for the North American species with reviews of the genera *Pnigalio* Schrank and *Sympiesis* Förster. Other reviews have been made by Yoshimoto (1976) for the genus *Di cladocerus* Westwood; Gordh and Hendrickson (1979) reviewed the genus *Diglyphus* Walker; and Gumovsky (1999) reviewed the genus *Entedon* Dalman, recognising 11 species and described four species as new (*Entedon angorensis* sp. n., *E. jozsefi* sp. n., *E.*

*marusiki* sp. n., and *E. levadae* sp.). The genera *Zagrammosoma* Ashmead and *Hyssopus* Girault have been reviewed by Gordh 1978, and the genus *Elachertus* Spinola by Schauff (1985). Kerrich (1973) and Bouček (1976) made taxonomic studies on some African species of *Pediobius* of economic interest. Quite recently, Schauff (1998) reviewed the genera of chalcidoid parasitoids and included some eulophid species associated with parasitisation of the citrus leaf-miners. The large number of species is probably one of the factors that have deterred many taxonomists from studying the group.

Eulophids are mostly small insects measuring between 0.4 and 6.0mm in length. Morphologically, they can be recognised by their metallic lustre, short antennae with 5-10 flagellomeres (females with 2-4 flagellomeres with a clava of 3 or fewer flagellomeres) and four tarsal segments with protibial spur. The mesosoma (i.e. thoracic region including the propodeum) is usually subtriangular in shape with conspicuous prepectus, the mesoscutum with or without a pair of notauli; each stemming, when present, from either sides of the base of the pronotum or transscutal articulation, a scutellum with/without carinae; a propodeum with or without carinae. Other recognisable characters include a characteristic forewing vein break, distribution of hairs on the forewing; separation of gaster by a strong and distinctive constriction with a long transverse petiole (Gibson *et al.*, 1997).

### 1.2.1 Their significance

Eulophids are well represented throughout the world, and like many other chalcidoids they are biologically very diverse. The majority of eulophids have been recorded as primary or secondary parasitoids of many phytophagous Lepidoptera, Diptera, Coleoptera, Homoptera, Thysanoptera, and other Hymenoptera species; as well as egg parasites of many Arachnids. A large number of species in these orders are major crop pests.

### 1.2.2 Parasitic diversity

Diverse parasitism is observed in almost all the subfamilies. There are endo- and ecto-parasitic species; egg, larval, or pupal parasitoids; solitary or gregarious; hyperparasitic or facultative hyperparasitic; idiobionts or koinobionts; monophagous or polyphagous forms. For example, the genus *Melittobia* Walker includes species parasitic on aculeate Hymenoptera (bees and social wasps) such as *M. digitata* Dahms, a pupal parasitoid of the honeybee *Apis mellifera* Linnaeus (Hymenoptera: Apidae), and endo-hyperparasites such as *Tetrastichus blepyri* Ashmead a hyperparasitoid of several Encyrtidae on the mealybug *Pseudococcus* (Cooperband and Vinson, 2000). The majority species of *Euplectrus* Westwood are gregarious ectoparasitoids of lepidopteran larvae (e.g. *Euplectrus melanocephalus* Girault), larval ectoparasitoids of the fruit-piercing moth genus *Eudocima* Billberg (Noctuidae) (e.g. *E. salaminia* Cramer, *E. Fullonia* Clerck and *E. maternal* Linnaeus (Jones and Sands, 1999)). Many *Aprostocetus* Westwood species are

gall-mite predators as well as parasitoids of gall midges (e.g. *Aprostocetus microscopicus* Rodani parasitoid of the gall midges *Orseolia oryzivora* Harris and Gagne and *O. bonzii* Harris (Diptera: Cecidomyiidae)) (Harris *et al.*, 1999). Larvae of many thrips are parasitised by many species of the genera *Ceranisus* Walker, *Goetheana* Girault, and *Aprostocetus* Westwood. For example, the thrips *Ceratothripoides claratris* Shumsher, *Frankliniella occidentalis* Pergrande and *Thrips tabaci* Linderman (Thysanoptera: Thripidae) are parasitized by *Ceranisus menes* Walker, *C. americensis* Girault and *Goetheana shakespearei* Girault, respectively (see Lacasa *et al.*, 1996). In a recent review of the parasitoids of the whiteflies *Bemisia* Gennadius (Homoptera: Aleyrodidae), LaSalle and Schauff (1994) placed all eulophid parasitoids under the tribe Euderomphalini. However, not all eulophids are parasites; some are phytophagous gall-formers.

### 1.2.3 Reproductive strategies

Bivoltinism has been observed in some species with generations synchronous with those of their hosts, and sexual dimorphism is pronounced among many species (LaSalle, 1994). Species of Entedontinae in which *Pediobius* Walker belongs reproduce bisexually and in most species females dominate, e.g. 2 females:1 male in *Pediobius metallicus* Nees, and 3:1 in *Pediobius benefica* Gahan (Salt, 1931). Eulophids also have one of the highest recorded reproductive capacity, e.g. one female *Melittobia acasta* Walker was recorded with 1086 eggs (Clausen, 1940) and *Pediobius furvus* Gahan with 560

eggs (Jordan, 1966). These qualities make this group of parasitoids a subject of interest in search of potential biological control agents for classical introduction in Africa.

#### 1.2.4 Morphological diversity

In recent decades, interest in parasitic Hymenoptera has increased because of continual demand for biological methods for pest control in many Integrated Pest Management systems targeting different kinds of organisms. In the identification of suitable natural enemy species, the main problem is the existence of cryptic species complexes (species which are different biologically or genetically, but morphologically similar) within groups (Claridge *et al.*, 1997). Many examples of cryptic species complexes have been recorded. Within parasitic Chalcidoidea (Hymenoptera) this phenomenon has long been illustrated by the *Eurytoma rosae* Nees aggregate, *E. appendigaster* Swederus species-group, *E. mayri* Ashmead (Eurytomidae) (Claridge and Askew, 1960) and the *Pediobius eubius*- complex (Eulophidae) (Peck, 1963). The latter which is the nucleus of this thesis, has been observed to comprise many morphologically similar species. As a result previous workers on the group had separated them into several species-groups e.g. *Pediobius nigratarsis* Thomson, *P. cassidae* Erdős, *P. planiventris* Thomson, *P. alaspharus* Walker, and *P. brachycerus* Thomson species-groups (Bouček, 1965; Burks, 1971). The African species have not been placed in any of these species groups and their taxonomic position therefore remains uncertain.

### 1.3 The Genus *Pediobius* Walker

#### 1.3.1 Taxonomic position of *Pediobius*

*Pediobius* belongs to the subfamily Entedontinae. This subfamily is a very large group and members can be recognised by the presence of a pair of strong setae on the scutellum, two setae on the submarginal vein of forewing, and forewing venation distinctly interrupted at the base of the parastigma (Gibson *et al.*, 1997). Taylor (1937) made exclusive studies on the biology of the subfamily and a few species. Like many other insect groups, *Pediobius* Walker has gone through many changes in taxonomic ranking. The name *Pediobius* was first proposed by Walker (1846) in his description of some unidentified Chalcidoidea. However, Förster (1856) proposed the name *Pleurotropis* for the genus and presented a key to the species, which was essentially relied on, and all subsequently erected species continued to be known under that name for several decades. Ashmead (1904) in his studies of the species disagreed with this generic determination, however, he recognised it as a subgenus of *Entedon* Dalman and designated *Entedon imbrues* Walker as type species. Waterston (1915) description of the genus laid the foundation for a later study of some of the African species by Kerrich (1973). Subsequent keys to the species were produced with descriptions of new species. Masi (1940) produced a key and included those species already described by Waterston with an addition of three new species. His descriptions and species erection were based on artistic drawings of morphological characters, which might have been misleading in some cases. Other keys produced include those species

described by Risbec (1958), which are based mainly on male morphology alone, and some of the descriptions were made from a single individual male. New species erected remained known under the name *Pleurotropis* until Ferrière (1953) who established *Pediobius* Walker as the senior synonym. Until then many generic synonyms were given based on the number of species continued to be described under the genus.

### 1.3.2 Economic importance of *Pediobius* species

Among the chalcidoids, the first classical biological control introduction of a parasitoid was a member of *Pediobius*. *P. epigonus* Walker was introduced in New Zealand in 1894 for the control of the hessian fly, *Phytophaga destructor* Say, in wheat. *Pediobius* species are cosmopolitan in distribution with over 200 species recorded worldwide. The largest number of species is believed to be found in the tropics and subtropical regions (Kerrich, 1973). Twenty species are recorded in Britain alone (Fitton *et al.*, 1978). Some of the European species are associated with parasitism of: (a) grass stem-boring *Tetramesa* Walker species (Hymenoptera: Eurytomidae) (Claridge, 1987), (b) leaf-mining species of *Phyllonorycter* Hübner and *Lithocolletis*, (Lepidoptera: Gracillariidae) found in oaks and birch galls e.g. *Pediobius alcaeus* Walker and *P. saulius* Walker (Shaw and Askew, 1999); (c) gall-forming cynipids on oaks and birches, and parasitoids of the European corn borer *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae), (d) *P. facialis* Walker, a larval and pupal parasitoid of the citrus leaf-miner *Phyllonorycter millierella* Staudinger (Diptera:

Gracillariidae) and the strawberry leafroller *Ancylis comptana* Frölich (Lepidoptera: Tortricidae) (Schauff, 1999). The African species have been found parasitising larvae and pupae of stem-boring Lepidopterans such as the African rice ‘whitehead’ *Maliarpha separatella* Ragonot (Pyralidae), *Chilo partellus* Swinhoe (Pyralidae), the pink borer *Sesamia calamistis* Hampson (Noctuidae), the corn stalk borer *Busseola fusca* Fuller (Noctuidae) and the African sugarcane borer *Eldana saccharina* Walker (Pyralidae), and leaf mining dipterans associated with cereal crops (rice, maize, millet and sorghum), citrus and coffee (Duale and Okwakpam, 1997).

Elsewhere *Pediobius* species have also been recorded as parasitoids of stem boring and leaf mining dipteran and lepidopteran species e.g. the polyphagous *Pediobius acantha* Walker on the pea leafminer *Liriomyza huidobrensis* Blanchard, and on *Chromatomyia* spp. (Diptera: Agromyzidae) in Australia (Stolz and Blümel, 1998). *P. foveolatus* Crawford is used as a biological control agent for the egg-plant spotted beetle *Henosepilachna vigintioctopunctata* Fabricius (Coleoptera: Coccinellidae) in India (Rajendran and Copalan, 1997); and *Pediobius orientalis* for the control of the diamondback moth *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae) in cabbage fields in Japan (Noda *et al.*, 1996).

In North America and Japan, *P. foveolatus* has become the most popular parasitoid species among gardeners and vegetable production centres (Saitoh and Matsumoto, 2000). The Mexican bean beetle *Epilachna varivestis* Mulsant

(Coleoptera: Coccinellidae), commonly known as MBB, is a major insect pest of beans in North America particularly in the state of New Jersey. Both adults and larvae eat the undersides of bean leaves causing lacing of the foliage resulting in high levels of defoliation significantly reducing bean yields. An estimated 87,000 acres of soybean are in the area susceptible to MBB and if 25% of this is infested, control cost could mount up to £192,000, which is now saved by the introduction of *P. foveolatus*. The parasitoid is reared *en mass* for effective control of MBB. Imported from India, this non-over-wintering wasp has a high searching ability, and when released in the fields will seek out and destroy MBB larvae (Saitoh and Matsumoto, 2000). In the USA in particular where it has been in use since 1970s, *P. foveolatus* is a major contributor to the biocontrol network of beneficial insects, and is regularly available for worldwide distribution; whilst in Japan the parasitoid is integrated into the pest management programmes in the regions of Yamanashi and Nagano.

Other species of economic importance include *Pediobius furvus* Gahan, a gregarious pupal parasitoid for the control of the Mexican rice borer *Eoreuma loftini* Dyar (Lepidoptera: Pyralidae) in the states of Florida and Washington (Overholt and Smith, 1989). In the Philippines, the parasitoid is reared for field augmentation for the control of another coccinellid beetle *Epilachna philippinensis* Dieke on solanaceous plants (Chiu and Moore, 1993). In Latin America, *P. furvus* collected from Trinidad and Tobago is used in the containment of three West African races of *Chilo* species accidentally

introduced in Bolivia. In Africa, *P. furvus* parasitizes several lepidopteran stem-borers such as *Busseola fusca* Fuller, *Sesamia calamistis* Hampson, *Chilo partellus* Swinhoe, *C. orichalcociliellus* Strand (Pyralidae) and the sugarcane borer *Eldana saccharina* Walker (Ogol *et al.*, 1998). In Europe the populations of the European corn borer *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae) are kept in balance by their natural parasitoid *P. facialis* Giraud though this species is not yet introduced in a classical biological control programme. Other beneficial species recently discovered include: (i) *P. bruchicida* Rondani, a parasitoid of the rice leaf folder *Cnaphalocrocis melinalis* Guenee (Lepidoptera: Noctuidae) in India (Manisegaran *et al.*, 1997); (ii) *P. saulius* parasite of horse chestnut leafminer *Cameraria ohriodella* Deschka and Dimic (Lepidoptera: Gracillariidae), *Phyllonorycter millierella* Staudinger and *P. lanthanella* Schrank (Lepidoptera: Micropterigidae).

### 1.3.3 New records

New species have continued to emerge recently with descriptions based on morphological characters and host-associations. For example, Sheng and Li (1992) described and determined *Pediobius fujiangensis* as new from India, whilst Ahlstrom and McDonald (1993) determined *P. smithi* sp. n. reared from eggs of the longhorned grasshopper *Scudderia curvicauda* De Geer (Orthoptera: Tettigoniidae) in northern California. In Japan four new species have recently been identified as parasitoids of crop insect pests: *Pediobius narangae* sp. n. reared from the pupae of the leafroller *Naranga aenescens* Moore (Lepidoptera:

Noctuidae), *Pediobius polychrosis* Sheng and Wang from *Polychrosis cunninghamiacola* Liu and Pai (Lepidoptera: Tortricidae), *P. grisea* Sheng and Wang from the chrysomelid beetle *Galerucella grisea* Joannis (Chrysomelidae), and *P. sinensis* Sheng and Wang from *Plusia agnata* Staudinger (Lepidoptera: Noctuidae) (Sheng and Wang, 1994). Other new records include *P. erdosei* Villar from the cynipid *Andricus coriaceus* Mayr (Hymenoptera) in India (Pujade and Miller, 1995); *Pediobius* sp. reared from the pupae of the uzifly *Blepharipa zebina* Walker (Diptera: Tachinidae) (Singh and Sinha, 1995); *Pediobius viggianii* Khan and *Pediobius ni* Peck as larval parasitoids of the lepidopterans *Nymphula depunctalis* Guenee (Pyrilidae) and *Trichoplusia ni* Hübner (Noctuidae), respectively; *P. cydiae* Khan, a pupal parasitoid of the moth *Cydia cratica* (Tortricidae); *P. salicifolii* Khan from the leafminer *Japanagromyza salicifolii* Spencer (Diptera: Agromyzidae) (Khan, 1996); and *P. puertoricensis* Schauff, a parasitoid of the citrus leafminer *Phyllocnistis citrella* Stainton (Diptera: Gracillariidae) (Schauff, 1998). In Bulgaria a new *Pediobius* species has recently been described as an egg parasitoid of the pine processionary moth *Thaumetopoea pityocampa* Denis and Schiffermüller (Lepidoptera: Thaumetopoeidae), and the polyphagous *P. pyrgo* Walker has also recently been recorded from pupae of the satin moth *Stilpnotia salicis* Linnaeus (Lepidoptera: Lymantriidae). New records of *Pediobius* species as parasitoid of economic pests are overwhelming, and species names have been repeated, and synonyms of one species are still used. A typical

example is the African species *P. furvus*; the name is sometimes interchanged with *P. furvum* in North America (see Schauff, 1985). The taxonomic position as well as phylogenetic relationships between the populations of *P. furvus* in Africa therefore needs scrutiny.

#### 1.3.4 Taxonomic problems

Among insect parasitoids, there are many recorded instances where within a genus genetic diversification has occurred with little morphological differentiation making taxonomic separation and systematics of the species very difficult. For example, the taxonomic status of the populations of the *eubius* Walker complex in Britain has proved very problematic where morphology alone has been used. Based on the structure of the female gaster, and food plant association, Graham (1963) and Bouček (1965) recognised and separated *Pediobius* into four species-groups. Dawah (1988) combined morphological characters, behavioural studies (mating behaviour) and electrophoretic analysis to describe six new species in Britain. However, Graham (1963) and Dawah (1988) believed that several species might be involved in the *P. eubius* complex.

#### 1.3.5 New descriptive characters

In recent studies, new useful taxonomic characters for separation of eulophids and identification of *Pediobius* have been revealed e.g. the shape of the propleural plates and the prosternum, the presence or absence of a transverse frontofacial suture, the notauli reaching or not reaching the

mesoscutum transscutal articulation, the presence or absence of a propodeal carina, the length of postmarginal vein of forewing, distribution of bristles on marginal vein, shape and structure of female antennal segments, and structure of the ovipositor. A full list is given in Chapter 3.

In the tropics and semi-arid regions, new species continued to be identified based upon morphology, hosts and host-food plant association (Sheng and Li, 1992). Some of the new species may have been named due to misidentification, or are synonymous. The frequently encountered species in most parasitoid studies is *P. furvus*. However, the *P. furvus* in Africa might consist of several distinct species, host races, or other variants of one species due to sympatric or allopatric speciation. Because of this, Kerrich (1973) decided not to deal with the African species in isolation, and therefore dealt with them together with the Asian forms. The *P. furvus* species complex remains in a state of confusion today.

### 1.3.6 Why resolve the West Africa species?

*Pediobius* species worldwide are mostly parasitoids of economically important pest species of food crops and forestry. This has been shown by several field as well as laboratory studies. Since its designation by Walker 1846, many revisions of the genus have been made. The Palearctic species have been recently revised three times, the latest being by Bouček (1988). The latest review of the Japanese and Korean species had been made by Kamijo (1986). Other reviews include those of the Indian subregion by Khan and

Shafee (1982); and the North American species by Peck (1985). In spite of the discovery of their usefulness as being relatively recent, the species in these regions have been propagated and incorporated in classical biological control programmes e.g. *Pediobius foveolatus* Crawford, *P. parvulus* Ferrière and *P. furvus* previously identified on the African continent.

The first study of the African *Pediobius* was made by Waterston (1915), followed by Mohyuddin and Greathead (1970) who described them as potential biological control agents of many graminaceous stem-borers. Kerrich (1969, 1973) revised the tropical and subtropical species with a description of 15 African species and further suggested a closer look at the system associated with cereal stemborers hence, the species are very diverse in their choice of hosts. Recent studies of parasitic species of cereal stemborers in Africa have consistently identified *Pediobius furvus* as the persistent natural enemy of *S. calamistis* Hampson, *B. fusca* Fuller, *E. saccharina* Walker and *C. partellus* Swinhoe (Ogol *et al.*, 1998) in Africa, and *C. partellus* Swinhoe in North America (Schauff, 1985). In a West Africa Rice Development Association/International Rice Research Institute (WARDA/IRRI) joint report, Jordan (1966) maintained that the eulophid parasite *P. furvus* was a potential candidate for cereal stemborer control, notably *Chilo*, *Busseola* and *Sesamia* species. *P. furvus* has also been reported in other regions as a parasitoid of the sugarcane stem borer *S. grisescens* Walker in Papua New Guinea; and the Mexican rice borer *Eoreuma loftini* Dyar in USA. Therefore,

this parasitic diversity demands both taxonomic and biogeographic investigation for a phylogenetic relationship of *P. furvus* system associated with cereal stemborers in Africa where the species status has yet remained unresolved.

#### 1.4 Species and Speciation

There has been much debate over the definition of species, and how species are recognised. From a taxonomist viewpoint, species are taxonomic entities and can be considered the building blocks of systematics. They are recognised as morphologically and genetically distinct. Their recognition as real units in nature is not new because they form the bridge between evolution of populations and evolution of taxonomic diversity due to speciation (Futuyma, 1997). Speciation is defined as the evolutionary process by which species multiply and is one of many theoretical debates yet to be resolved amongst biologists. Often, a species is recognised and defined by phenetic characters. Phenotypic characters of bones, beak, feathers, antennae, wings etc have been used to distinguish individuals from others. Therefore any phenotypic character may be useful in the practical recognition of species. However, phenotypic characters often vary between individuals of a population at any one place, and between populations living in different places. When a population of organisms diverge and becomes isolated for a considerable period, a new species may emerge (Eldredge and Cracraft, 1980). However, diverging species are problematic and as a consequence of this

several species concepts have been proposed. There are at least 22 proposed species concepts which are today in use by various disciplines of biology. Mayden (1997) divided these into primary and secondary hierarchical groups, some of which are listed below (For a full list see Appendix I).

**(a) Agamospecies concept (ASC)**

This refers to all taxa that are uniparental and multiply via asexual reproduction. Agamospecies reproduce by hybridisation, often with no fertilisation (Ghiselin, 1984). Where species complexes are involved, they may be facultative or obligate apomicts and may be diagnosed morphologically or by allele frequencies (Mayden, 1997).

**(b) Biological Species Concept (BSC)**

BSC defines species as a group of actually or potentially interbreeding natural populations that are reproductively isolated from other such groups (Dobzhansky, 1935; Mayr, 1942). This refers to a single population of interbreeding individuals in a different geographic location and morphologically uniform.

**(c) Cohesion Species Concept (CSC)**

CSC describes species as the most inclusive population of individuals having the potential for phenotypic cohesion through intrinsic cohesion mechanisms (Templeton, 1989).

**(d) Ecological Species Concept (ESC)**

ESC defines species as a lineage (or closely related lineages) that occupies an adaptive zone minimally different from that of any other lineage in its range and which evolves separately from all lineages outside its range (Van Valen, 1976).

**(e) Evolutionary Species Concept (ESc)**

This concept defines species as a single lineage of populations of organisms that maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate (Wiley, 1978).

**(f) Internodal Species Concept (ISC)**

ISC recognises species as individual organisms that are conspecific by virtue of their common membership in a part of the genealogical network between two permanent splitting events or between a permanent split and an extinction event (Kornet, 1993).

**(g) Morphological Species Concept (MSC)**

MSC defines species as the smallest groups of organisms that are distinguishable by distinctive morphological characters from other groups. Where similar or closely related species are involved, morphological distinctiveness serves as the only evidence for separation of such groups (see Cronquist, 1978).

**(h) Phylogenetic Species Concept (PSC)**

PSC recognises species as an irreducible cluster of organisms that is diagnostically distinct from other such clusters, and within which there is a parental pattern of ancestry and descent (Cracraft, 1989); or the smallest monophyletic group of common ancestry (de Queiroz and Donoghue, 1990).

**(i) Recognition species concept (RSC)**

RSC defines species as the most inclusive population of individual biparental organisms that share a common fertilisation system (Paterson, 1985).

**1.4.1 Biological Species Concept (BSC)**

The most common species concept among taxonomists has been the Biological Species Concept. Biological species share common morphological characters and inbuilt behaviours such as specific mate recognition systems (SMRS) (Claridge, 1987). SMRS are biological characters and are species-specific that thus maintain reproductive barriers between different species that share a common geographic location. Such species have been recognised as sympatric species (Paterson, 1985). Sympatric species are capable of encountering each other without gene exchange (Futuyma, 1997). Most biological species are defined based on morphological differences that provide diagnostic characters for distinguishing them. Morphological differences may be due to ecological adaptations, or genetic traits. Such species have often been observed as biologically dissimilar and referred to as sibling species

(Claridge, 1988). However, careful re-examination of such species has often revealed species diversity. Research have shown that different groups of organisms have a propensity to produce sibling species that are defined as groups of closely related species, morphologically indistinguishable, live sympatrically but are reproductively isolated (Coyne, 1992). Reproductive isolation may therefore produce genetically inherited differences in closely related and often morphologically similar species. The chances of sufficient gene exchange between populations of organisms can be greatly reduced by physical factors such as unfavourable habitat, climate, topography or water. Such species have been recognised as allopatric species. Allopatric speciation is defined as the evolution of reproductive barriers in populations that are prevented from exchanging genes by geographic barriers (Mayr, 1963). For example, two geographically separate species may be morphologically different but their genetic status may be questionable. However, Futuyma (1997) states that geographically located populations of most species often differ genetically. For example, from systematic studies of geographic variation within species, the species of the leopard frogs previously considered as variants of *Rana pipiens* Schreber (Ranidae) were found to consist of 27 distinct species (see Hillis, 1988). This example is also applicable to *P. fuvvus* populations from Africa, Latin America, North America, and Asia hence they are separated by physical factors. *P. fuvvus* in these regions parasitizes lepidopterous maize stem borers such as species of *Chilo* and *Sesemia*.

### **1.4.2 Species limitation in BSC**

For decades in entomology, morphological differences, host association, ecological differences and behavioural characteristics have been used as species delimiting factors (e.g. Graham, 1959; Bouček, 1965, 1976; Burks, 1971; Peck, 1985). Recent studies have demonstrated that for populations to be considered as separate species a certain level of phylogenetic corroboration is useful. Some morphologically different organisms previously thought as different species have been found to represent a cluster of close relatives that derived from a recent common ancestor but can only be characterised by genetic polymorphism (see Eldredge and Cracraft, 1980). The Biological Species Concept views populations that evolve in a very narrow interval of time (Futuyma, 1997). This implies that a simple correlative evolution of changes in morphology within a population is insufficient to account for phylogenetic diversity (Cracraft, 1990).

### **1.4.3 Phylogenetic Species Concept (PSC)**

The 'Phylogenetic Species Concept' on which some molecular techniques are based has increasingly become popular especially amongst cladists (e.g. Nunn and Cracraft, 1996). Molecular techniques have been applied in a wide range of genetic studies. For example, analysis of gene sequence data revealed genetic variation among closely related species, monogamous taxa, and identified different evolutionary events in bird-feather ornamentation (see Cuervo and Møller, 1999). The brachypterous scarabaeid in New Zealand poses

some biogeographic and evolutionary questions to systematists. Using sequence data from the mitochondrial Cytochrome Oxidase II (COII) gene and phylogeny reconstruction separated the beetle species of *Prodontria* Broun genus into 14 flightless species of which the data supported conspecificity of two morphologically similar species - *P. modesta* Broun and *P. bicolorata* Given (Coleoptera: Scarabaeidae) (Emerson and Wallis, 1995). McPheron and Han (1997) used results of analysis of mitochondrial gene sequences to separate and recognise three distinct species of the fly *Rhagoletis* Loew (Diptera: Tephritidae) formerly considered a single species based on morphology. The recognition of the parrot genus *Parotia* as different from other morphologically similar genera *Ptiloris* and *Lophorina* Pennant (Paradisaeidae) was made from analysis of gene sequence data (see Nunn and Cracraft, 1996). Other similar recent studies include the systematics and estimation of population relatedness of the species of *Ophraella* Wilcox (Coleoptera: Chrysomelidae) from a collection of sequence data from the 16S rDNA and COI (Funk *et al.*, 1995); the use of sequence data to detect divergence of insect orders, and hierarchical analysis (see Liu and Beckenbach, 1992). The emphasis of the concept is on the phylogenetic lineage of an organism and not its interbreeding ability or reproductive isolation defines it as a species (Futuyma, 1997).

This present study tests this concept and its applications, in conjunction with alternative definitions such as the Biological Species Concept and

Morphological Species Concept to elucidate the genetic and systematics status within and among *P. furvus* populations.

### 1.5 Aim:

The overall objective of this thesis is to investigate phylogeographic and genetic structure of *Pediobius furvus* populations associated with parasitisation of lepidopteran stemborers of maize in Africa using multivariate techniques such as morphometric and molecular analyses. Reproductive compatibility tests will be carried out with species of the *P. eubius* complex associated with parasitisation of grass-feeding eurytomids in Britain as a model study for the African *Pediobius*. The aims of this study were threefold:

- [i] To study genetic variation and phylogenetic structure within and between *P. furvus* Gahan populations in Africa.
- [ii] To correlate this with the results of morphological variation between populations.
- [iii] To investigate behavioural and morphological differences between the most studied *Pediobius* species in UK which live sympatrically.

## 1.6 References

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**CHAPTER TWO**  
**GENERAL MATERIALS AND METHODS**

## 2.1 Introduction

Many studies on Chalcidoidea classification, carried out by taxonomists mostly, involve rearing as the source of obtaining specimens. Rearing results can therefore be very informative as they can provide accurate biodiversity data of an organism such as its geographic location, climatic tolerance, trophic level and community structure, host utilisation, host range and preference, number of developmental stages, longevity, fecundity, species-specific behaviour and morphological variability. Rearing has frequently been used to determine a large number of species, population relatedness and differences. In this study I have collected *Pediobius* samples from various sites in the United Kingdom and West and East Africa. *Pediobius* species collected in the UK were used to investigate their mate selection system, whilst trying to establish population relatedness of *Pediobius furvus* (Hymenoptera: Eulophidae) collected in six countries in Africa. Samples were collected at various locations and different ecologies as described below.

## 2.2 Sample sites in the U. K (Fig. 2.1)

### 2.2.1 Caerphilly (NGR: ST 155852)

This site is located about 12 km from Cardiff. Collection of grass stems was carried out on the hillsides where grasses and shrubs intermix, providing a large collection of grass species comprising *Deschampsia* Rispengräser, *Dactylis* L. and *Calamagrostis* L. (grass species commonly infested by phytophagous Hymenoptera).

### **2.2.2 Cosmeston Park (NGR: ST 174613)**

Cosmeston Park Nature Reserve is situated about 8 km south of Cardiff. The area is generally moist encouraging the growth of many wide grasses including *Deschampsia*, *Elymus* L., and *Phalaris* L. which tend to dominate. *Pediobius* and *Tetramesa* species were collected here in large numbers for morphological study and mate recognition experiments.

### **2.2.3 Merthyr Mawr Dunes, West Glamorgan (NGR: SS 860773)**

This site is located east of Porthcawl and is characterised by an extensive area of sand dunes. Collections of grasses including *Deschampsia* and *Dactylis* species for dissecting were made at this site.

### **2.2.4 Kenfig Pool Nature Reserve (NGR: ST 794816)**

Kenfig Pool Nature Reserve is a large area of sand dunes managed by Bridgend County Borough Council. The site covers an area of 1,100 acres extending from Swansea to the mouth of the Ogmore river. For most of the growing season, the dunes are covered by a wide variety of wild grasses and shrubs. In the wet areas near the river Ogmore, are found *Calamagrostis* L., *Deschampsia* Rispengräser, *Phalaris* L. and many other wild grasses. Some of the plots for mate recognition experiment were set at this site.

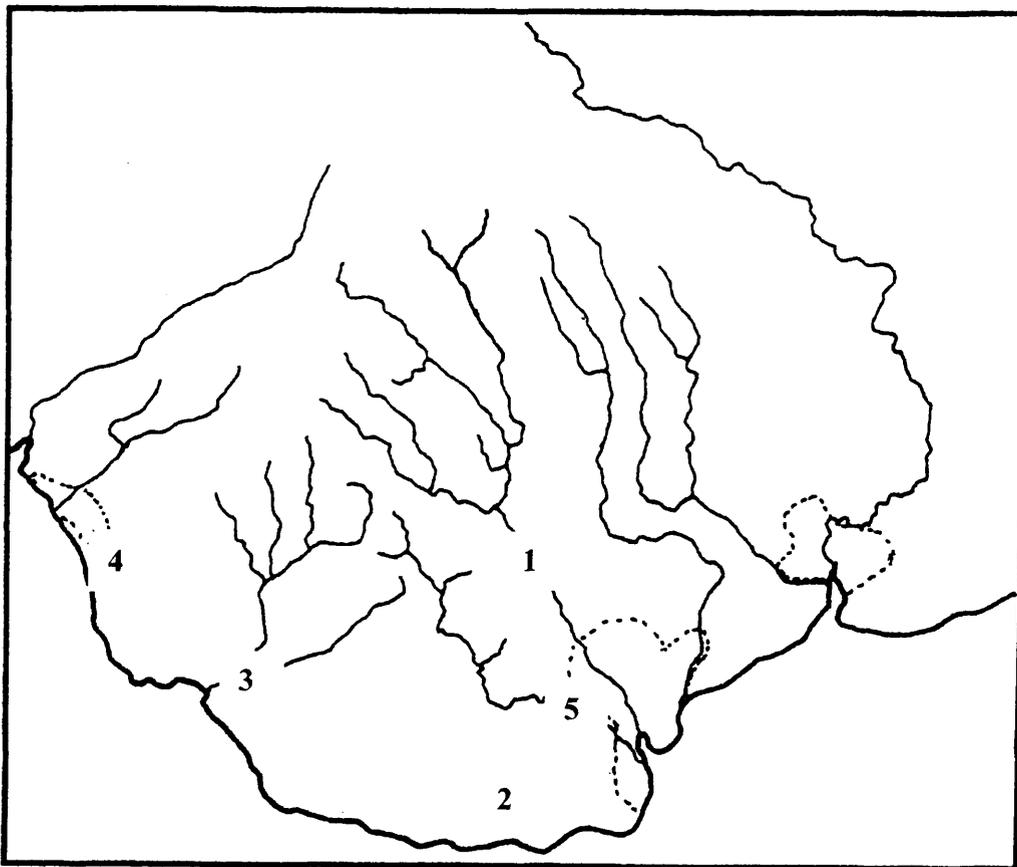
### **2.2.5 Fairwater Nature Reserve (NGR: ST 133788)**

Fairwater Nature Reserve comprises a large area of undisturbed shrubs and tall trees. Wild grasses are mostly found in the swampy area which gets very wet and waterlogged during winter months. Wild grass species grow in

patches; some patches dominated by species of *Phalaris* and *Deschampsia*.

Part of mate recognition experiment was carried at this site.

**Fig. 2.1:** Map of South Wales showing areas infested grasses were collected for rearing of *Pediobius* and *Tetramesa* species. Mating tests in the field were carried out at the two pre-selected distance locations: Kenfig Pool and Fairwater Nature Reserves sites.



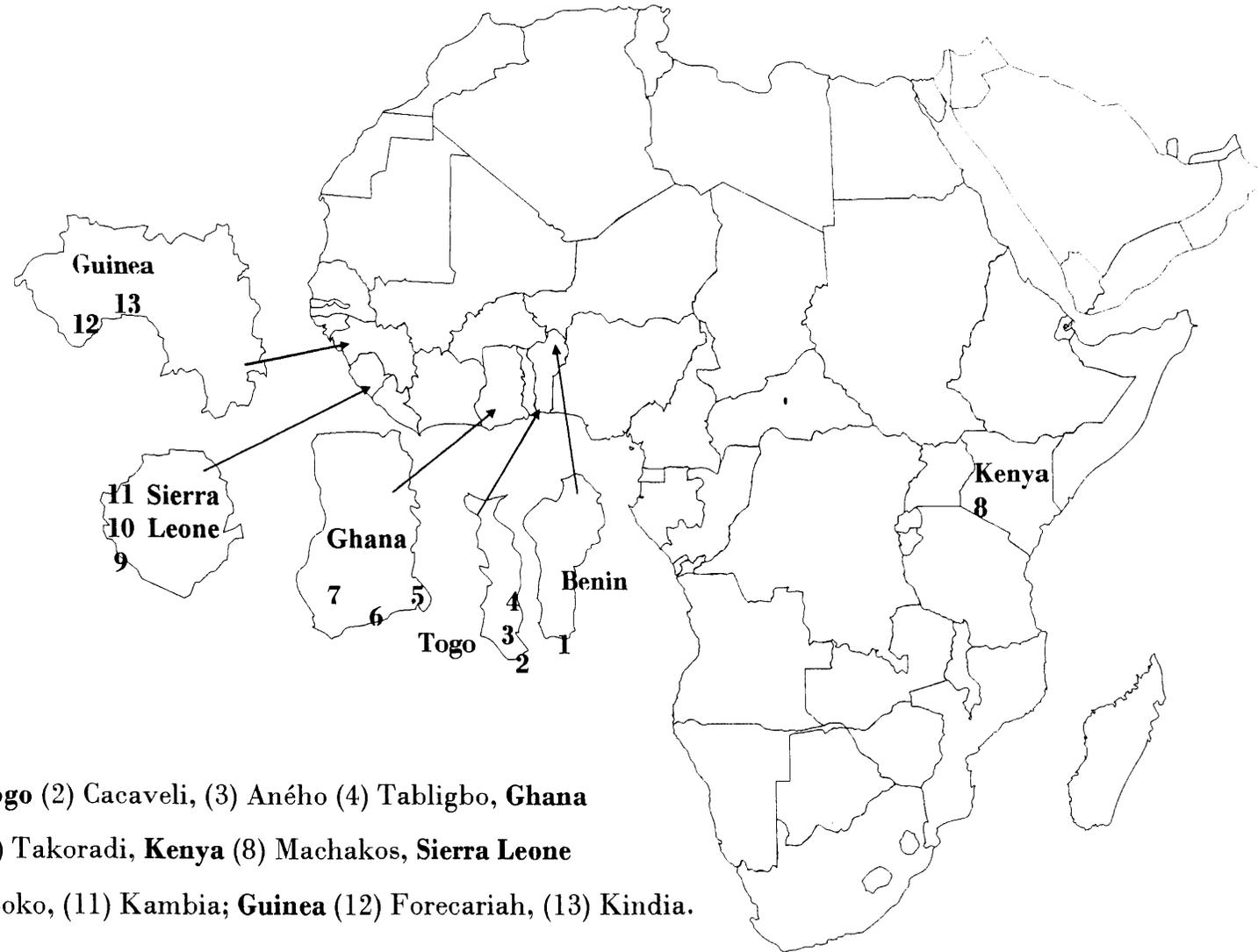
### Legend

1. Caerphilly mountains Nature Reserve
2. Cosmeston lake Nature Reserve
3. Merthyr Mawr Dunes Nature Reserve
4. Kenfig Pool Nature Reserve
5. Fairwater Nature Reserve

### **2.3 Sampling sites in Africa**

Sampling was carried out in six countries (Fig. 2.2). In each country, many localities were visited and stems and infested maize cobs collected. Only those sites which provided hosts and parasitoids have been included.

**Fig. 2.2:** Map of Africa showing countries and sampling sites of *Pediobius furvus* used for morphometric and molecular analyses.



**Country and sites**

**Benin** (1) Cotonou, **Togo** (2) Cacaveli, (3) Aného (4) Tabligbo, **Ghana** (5) Tema (6) Accra (7) Takoradi, **Kenya** (8) Machakos, **Sierra Leone** (9) Lungi, (10) Port Loko, (11) Kambia; **Guinea** (12) Forecariah, (13) Kindia.

## 2.4 Methodologies

### 2.4.1 Sampling protocol (West & East Africa)

#### 2.4.1.1 Collecting rearing materials

Obtaining rearing materials involved collecting of infested grasses for hosts and parasitoid larvae. For *P. furvus* and its hosts, plant samples were collected in West Africa during the dry season (September to January) when maize crop was available in the field and about to be harvested or already harvested. During this period, maize stem borers have reached full larval stage, or pre-pupal or pupal stage. Plant stems were collected from large maize fields, mixed-crop fields or small plots. Only infested stems were collected and placed in straw basket for examination. Plants collected from each locality were placed separately for dissecting. Stems were cut and split opened to collect larvae, pupae or mummies (inactive parasitized larva/pupa). Maize cobs were also split for the presence of larvae or pupae. In some of the fields Malaise traps were set to capture flying wasps (Fig. 2.3). Wasps caught in Malaise traps were kept for identification and museum use only as their hosts could not be identified.

1-3 day old pupae were isolated in individual Petri dishes where they were exposed to *Pediobius* wasps reared from the same sample for oviposition. A 24 hr period was normally allowed for oviposition. Parasitised pupae were kept in vials for emergence. To confirm whether superparasitism occurs in *Pediobius*'s host utilisation strategy, single host pupae were exposed to several parasitoid females some from different locations for oviposition. Pupae were then kept separately for emergence. Upon emergence, adults were left for 5 days to mature before they are killed and identified. Adults from such rearing were kept for museum use.

#### **2.4.1.4: Rearing of herbivores and parasitoids (UK samples)**

Two simultaneous rearings were carried out, one in the field and the other in a greenhouse. These rearings were conducted to carry out mating systems experiments of UK *Pediobius* species associated with grass-infesting eurytomids (see Chapter 3). Large wooden cages (110cm x 65cm x 65cm) covered in white voile mesh were used in the field and in greenhouse cultures. In the field pre-selected hosts food-plants were isolated *in situ* and cages placed over them to prevent natural infestation either by hosts *Tetramesa*, other phytophagous insects, or their parasitoids. In greenhouse cultures grass stands were grown in large-sized cone-type flower pots with perforated bottoms. Black polythene sheets were placed underneath to retain moisture. Three *Poaceae* species *Phalaris aurindinacea* L., *Deschampsia caespitosa* L. and *Calamagrostis epigejos* L. were used as natural host plants for the following

grass-infesting *Tetramesa* Walker species: *T. deschampsiae* Dawah, *T. phalaridis* Dawah and *T. calamagrostidis* Dawah, and their parasitoid species *Pediobius deschampsiae* Dawah, *P. calamagrostidis* Dawah and *P. phalaridis* Dawah (Hymenoptera: Eurytomidae).

#### 2.4.2 Mate recognition experiments

Using three species of UK *Pediobius* parasitising different grass-infesting *Tetramesa* species (Hymenoptera: Eurytomidae) experiments were carried out to establish whether mating is selective or random. The tests were necessary because in the wild, the hosts food-plants grow sympatrically and therefore their parasitoids also live sympatrically, but no reproductive isolation has been investigated between these species. To carry out the experiments, small plots of grass stems were selected. Grasses in the plots were then infested with their natural *Tetramesa* hosts. Males and females of *Tetramesa* species were released to mate and for females to oviposit and infest healthy growing grasses. After a known number of days following infestation, *Pediobius* females used in the two mating patterns were released to oviposit into their *Tetramesa*-infested grass stems. To obtain mated *Pediobius* females, mature pupae were collected from infested grass stems and reared until adult emergence. Upon emergence, males and females from different species were set for mating in the laboratory in small-sized glass vials used as mating chambers. To these chambers females were transferred first to saturate their species-specific sex hormones. Small amounts of CO<sub>2</sub> were released into the chambers to anaesthetise the wasps.

Wasps were marked with pigment ink for easy identification of females before and after mating sessions. A 24 hr mating period was normally allowed and mated females were later released to parasitise their natural *Tetramesa* hosts both in the field and in greenhouse cultures. At the end of the experiments, grass stems were dissected and examined for the presence of *Pediobius* larvae. Larvae were reared to adults and males and females were recorded. The results were used to determine species isolation or reproductive compatibility. These UK *Pediobius* species were used as a model for future study of the African species.

#### 2.4.3 Morphometric analysis

Preliminary examinations of specimens were carried out with a high-power dissecting microscope. 20 wasps, 5 from a single mummy were selected per sample for analysis. Specimens were washed in 100% alcohol, dried and mounted on glass slides for measuring. Two software programmes, Montage and Aequitas1A were used to visualise and measure morphological characters, respectively. Measurements were taken in  $\mu\text{m}$  and stored on Excel 2003 spreadsheet for analysis. Two statistical analyses were carried out to compare results. Principal Component Analysis (PCA) procedures were carried out using character measurements to separate groups of individuals based on differences in their body measurements. PCA procedure calculates and rotates highly correlated variables to new positions to identify the number of significant factors known as principal components. These components were

then plotted against each other to separate and detect populations' demarcation. PCA procedures were calculated in Minitab package. Characters that contributed the most based on their Eigenvalues were revealed in PCA procedures. The second analysis, Discriminant Functions Analysis (DFA) or Canonical Discriminant functions was carried out in SPSS procedure. DFA is used to investigate independent variable mean differences between groups formed by the dependent variable. The technique requires prior designation of comparative groups. In this study, the aim was to investigate which characters discriminate between groups and percent variation between populations, and to assess the importance of each variable in population separation. The six populations were divided into three groups based on their geographic proximity, i.e. Benin, Togo and Ghana in one group, Sierra Leone and Guinea in another, and Kenya in a separate group. The two analyses were used to see if geographic differences support their separation. In both procedures (PCA and DFA), the products showed differences among *Pediobius* within the subregions and variables that contributed the most in detecting individual or population differences. Detailed annotations of each method, their usefulness and disadvantages in separating populations are given in Chapter 6.

#### **2.4.4 Scanning Electron Microscope (SEM)**

For this technique, specimens examined included *Pediobius* collected in Africa, Asia and North America (USA). Samples from other areas were

included because records showed that some *Pediobius* species from Africa e.g. *Pediobius furvus* and *P. parvulus* Ferrière were originally collected in west Africa then exported to various countries in Asia and countries in the Pacific Ocean such as Papua New Guinea by the Commonwealth Institute of Biological Control (now known as CABI) for the control of stem borers and leaf miners on sugarcane and coconut, respectively (see Taylor, 1937; Simmonds, 1972). Morphological characters were examined from electron-scanned micrographs of female wasps. The electron-scanned microscope produces 3-dimensional photographs of each image with a wide range of resolutions and magnifications. The JOEL 5200 SEM was used for this purpose. Selected specimens were cleaned in 100% ethanol, dried and mounted on stubs with the aid of a dissecting microscope. Specimens were then gold-sputtered to enhance resolution during scanning. Body sections examined included head capsule, mesosoma, antennae, hind legs and gaster. Electron micrographs were then examined and compared for character differences. Morphological character differences were used to review previous *Pediobius* identification keys with inclusion of new and synonymised characters (Chapter 3).

#### **2.4.5 Molecular phylogenetics**

Molecular techniques have become the cutting-edge in providing fundamental understanding of nature of genetic variability of many organisms. Pilot studies were carried out to test some of the techniques used by previous workers. Specimens collected in Wales as well as those from Africa

and Iran were used for those initial tests. For samples collected in Wales, live adults were placed in Eppendorf tubes and deep frozen at  $-70^{\circ}\text{C}$  5 days after emergence and remained frozen until required for DNA extraction. Samples from Africa and Iran were kept in EDTA and 90% ethanol respectively and frozen for at least three months, or until required for homogenisation. At the completion of pilot studies, one of the techniques, analysis of mitochondrial DNA was used in the final analysis to establish genetic differences and similarities between *Pediobius furvus* populations in Africa. Primers were tested for DNA amplification and to establish those that reveal heterogeneity. Techniques such as those used in obtaining good quality DNA through to revealing genetic differences between individuals and among populations are described below.

#### **2.4.5.1 DNA extraction techniques**

Because of the small size of the wasps, three DNA quantification protocols (Phenol-chloroform, Livak Buffer and QIAamp DNA) were preliminary carried out to determine which method works best for the wasps. These methods have proved successful for extracting DNA from small insects such as parasitic wasps (see Simon *et al.*, 1994, Gauthier *et al.*, 2000). Wasps were homogenised in Eppendorf tubes containing buffers supplied by product manufacturers. All various steps on DNA extractions of various techniques were strictly followed or sometimes optimised where necessary.

#### 2.4.5.2 Amplification of DNA

For DNA amplification from West African samples, a series of primers with thermocycling optimisation for the following gene regions 12S rRNA, ITS2, 28S D2, cytochrome b, COI and COII were tested because these have been shown to be very successful in amplifying within some insect orders including Hymenoptera (Simon *et al.*, 1994). All the PCR mixtures were prepared in batches. Upon completion, samples were then checked on agarose gels, and amplified using PCR techniques on ABI machine. Based on the selected primers, PCR products were purified in Geneclean and sequenced (Chapter 5).

#### 2.4.5.3 Sequencer analysis

Sequences and bases were aligned using Sequencher ver. 3.1.2. Both sequences of each individual wasp were initially assembled before alignment. This involved deletion/insertion of bases so that both forward and reverse sequences of each individual wasp correlate. Differences in the bases are indicated by black dots and none-base characters, e.g. Ns or Ms. These were replaced on both sequences with correct bases that correlate with particular sequence codes. During the editing, ill-defined ends of most sequence electropherograms were removed. Bases were further assembled to form consensus contigs and these were compared against each other to detect haplotypes. Haplotypes are formed where differences in the bases of individuals or groups of individuals differ in their sequences.

#### 2.4.5.4 Phylogeny analysis

Only two genes regions were amplified, the nuclear gene 28S and the mitochondrial gene cytochrome *b*. Initial analyses of cytochrome *b* sequences were carried out using a software programme designed by Templeton *et al.* (1992). The software Templeton Crandall Sing (TCS) is effective in inferring population level genealogies when divergences are low and when data are small (Gerber and Templeton, 1996). In TCS procedure, sequences are separated into haplotypes based on the similarity in their bases and frequencies within the samples are then calculated. From cytochrome *b* sequences, five haplotypes and their samples were revealed. There was a problem in the haplotypes as they could not produce viable amino acids. As a result, one of the haplotypes, BN1 was used as representative of *P. furvus* and cytochrome *b* sequences of other invertebrates that were found very similar were imported from GenBank using BLAST in an NCBI web search. A phenogram was constructed from imported sequences of *Sycosapter* species with five *Pediobius* haplotypes in Molecular Evolution and Genetic Analysis (MEGA) software procedure using the Kimura 2-parameter model based on the Minimum evolution method.

Sequences of 28S rDNA gene of other Hymenoptera species were imported from GenBank using BLAST search and aligned with 28S gene sequence of *P. furvus*. A phenogram was also constructed with bootstrap values to reveal the degree of variation or similarity between the African

*Pediobius furvus* and other Hymenoptera species including *Pediobius* species from elsewhere.

#### **2.4.5.5 Statistical analysis**

Haplotype and sample distribution were investigated by means of an Analysis of Molecular Variance (AMOVA) in Arlequin ver. 2.0. Populations were analysed and compared to detect their genetic structure differences, and were grouped according to their proximity and were plotted against each other. Only regional variation was revealed.

## 2.5 References

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**CHAPTER THREE****MORPHOLOGICAL CHARACTERISATION OF *PEDIOBIUS* SPECIES USING  
THE SCANNING ELECTRON MICROSCOPE**

### 3.1 Introduction

Most studies of Chalcidoidea classification and species description are based on morphological differences, some of which may be clear-cut, others only slight and not very clear. However, these differences form the main source of taxonomic characters. For example, within the Eulophidae, Walker (1846) first described *Pediobius* based on the differences in morphological characters from other chalcidoids. When differences are only slight, then determination becomes a matter of opinion. For instance, because of similarity in morphology, Ashmead (1904) placed *Pediobius* as a subgenus of *Entedon* Dalman (Hymenoptera: Eulophidae). Graham (1959) first separated the European species of *Pediobius* and placed *Pediobius eubius* Walker as a complex comprising of many species that were eventually separated by Dawah (1987, 1988). For the African species of *Pediobius*, the first attempt of their description was made by Waterston (1915) by comparing drawings of wasps' morphological features. Similar studies were also made by Masi (1940) who included some of Waterston's (1915) species. Kerrich (1969, 1973) described 20 species based on morphological variation.

To reveal morphological character differences in many small organisms such as in Chalcidoidea, the scanning electron microscope (SEM) has been and still is of great value for taxonomy and many other research disciplines of difference groups of organisms e.g. Jervis (1992). Using other techniques in combination with electron-scanned micrographs, Jervis (1992) examined and

described 21 species of the pipunculid genus *Chalarus* (Diptera: Pipunculidae) and determined 11 new species. Another example is a critical reappraisal of electron-scanned micrographs to reveal mouthparts structural differences in order to assign mouthpart functionality, feeding techniques and source of food of flora-feeding parasitoids (see Jervis, 1998). Basibuyuk and Quicke (1999) investigated both external and internal morphology of multiporous plate sensilla throughout the Hymenoptera order using SEM micrographs. Serrao (2000) has used the results of the SEM to confirm the usefulness of morphological characters as powerful tools in the study of insect phylogeny.

Morphological characters of many chalcidoids including *Pediobius* species have been used to hypothesise phylogenetic implications (see Gauthier *et al.*, 2000). Difficulty in morphological characterisation in species description and separation only arise when there are no new characters available to effect differentiation. In the present study, attempts were made to reveal morphological differences between *P. furvus* populations from Africa based on examination results of SEM micrographs of specimens.

### 3.2 Materials and Methods

Separation of *Pediobius* populations has been made in previous studies, sometimes with few specimens using a variety of techniques to reveal morphological character states in species descriptions. In the present study, 61 specimens were examined (all females). Specimens examined include six populations of *P. furvus* Gahan reared from pupae of the maize stemborer

*Sesamia calamistis* collected in five countries in West Africa, and one population from Kenya in East Africa. West African specimens were selected from fresh field collected samples. Method of collection is as described in Chapter 2. Dry specimens from Kenya were supplied by the IITA-Biological Control Centre Laboratory in Cotonou. For morphological comparison only, other *Pediobius* specimens were also examined but their micrographs are not included. These included fresh field collections of UK *Pediobius* species associated with the eurytomid species of *Tetramesa* (Hymenoptera) from the grass species of *Deschampsia* and *Phalaris*; and museum specimens supplied by Texas Agricultural University in the USA and Taiwan.

### 3.2.1 Characters examined

Morphological characters previously found useful in population discrimination in *Pediobius* were examined, including new characters recently revealed in the phylogenetic study of Eulophidae (see Gauthier *et al.*, 2000). These include characters of the antenna, head capsule, pronotum in ventral view, mesoscutum in dorsal view, legs, propodeum and gaster. In all, 28 characters were initially examined but only 15 were studied. Characters examined are listed in Table 3.1, and diagrammatic representations of these characters were drawn from *P. furvus* from Benin collection (Fig. 3.1). The number of specimens examined from each sample is given in Table 3.2). A high power dissecting microscope was used for stub mounting and drawings, whilst the pre-set scanning electron microscope (SEM) (JOEL 5200LV) was

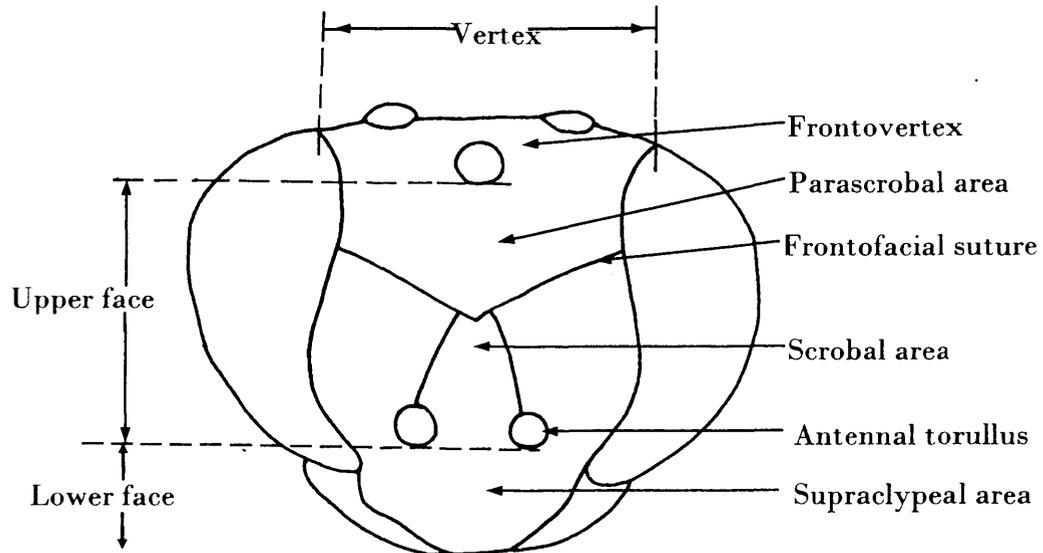
used to scan materials. Kerrich (1973) key to the African *Pediobius* was reviewed, and modified with current terminologies where necessary following Gibson (1985) and Gibson *et al.* (1997). The descriptions and key were used to check and confirm species status of all six *Pediobius* populations reared from pupae of the maize stemborer *Sesamia calamistis*.

**Table 3.1: List of characters examined on *Pediobius* species (*P. furvus*, *P. phalaridis*, *P. foveolatus*)**

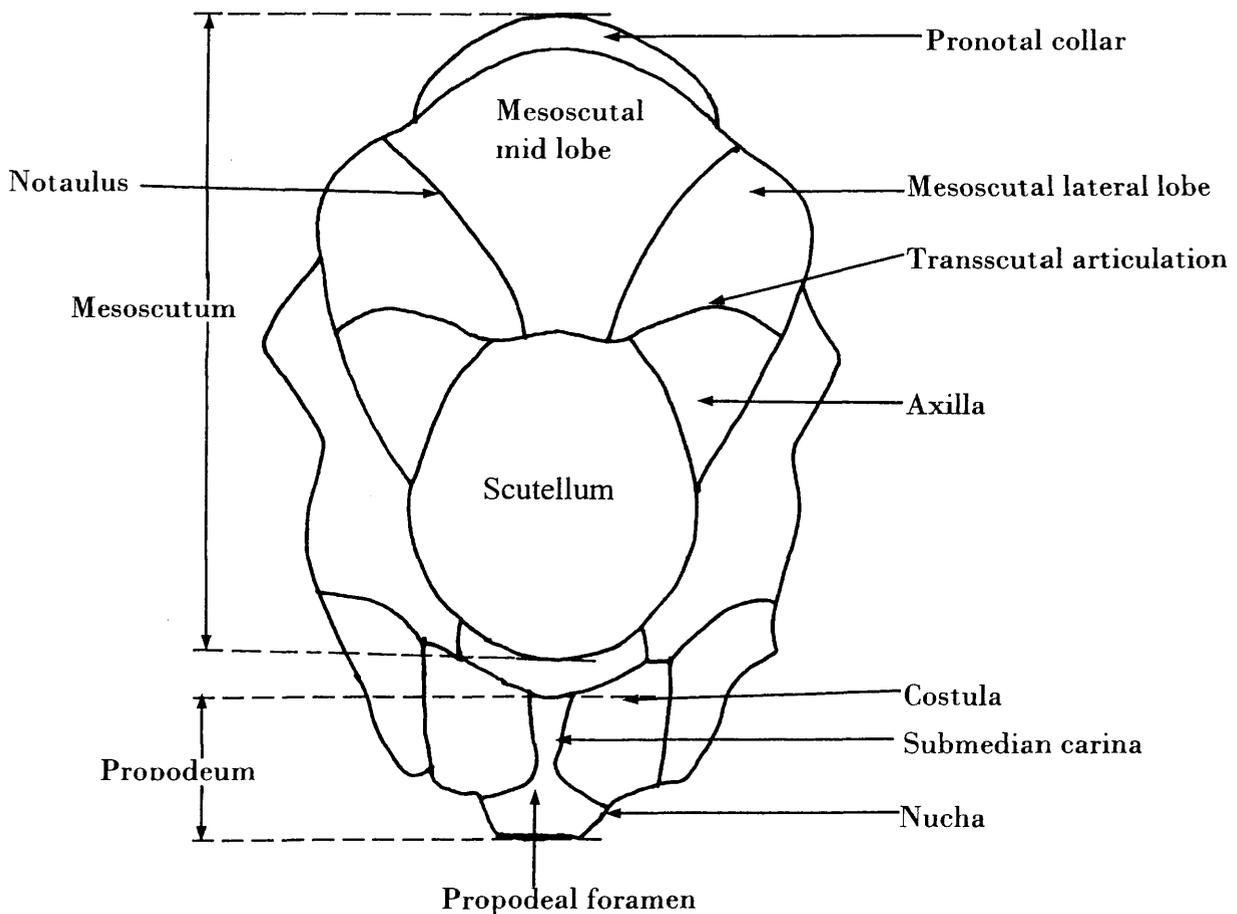
- |   |  |
|---|--|
| (1) Antennal torullus level                         | (16) Sculpture of vertex and between ocelli      |
| (2) Axillae sculpture                               | (17) Sculpture of clypeus                        |
| (3) Distribution of setae on gaster                 | (18) Sculpture of petiolar segment               |
| (4) Form of propodeal lateral and submedian carinae | (19) Shape and length of first abdominal tergite |
| (5) Form of propodeal                               | (20) Shape and length of hind tibial spur        |
| (6) Gaster shape and sculpture                      | (21) Shape and length of scape                   |
| (7) Length of first metatarsus segment              | (22) Shape and length of clava                   |
| (8) Length of mesoscutal notauli                    | (23) Shape of propodeal foramen                  |
| (9) Mesoscutal transscutal articulation             | (24) Structure of mandibles                      |
| (10) Nucha type                                     | (25) Structure of prosternum plates              |
| (11) Number of anellus                              | (26) Structure and length of pedicel             |
| (12) Overall sculpture of mesosoma                  | (27) Structure and sculpture of propleura plates |
| (13) Sculpture of parascrobal area                  | (28) Type of frontofacial suture                 |
| (14) Sculpture of scutellum                         |  |
| (15) Sculpture of supraclypeal area                 |  |

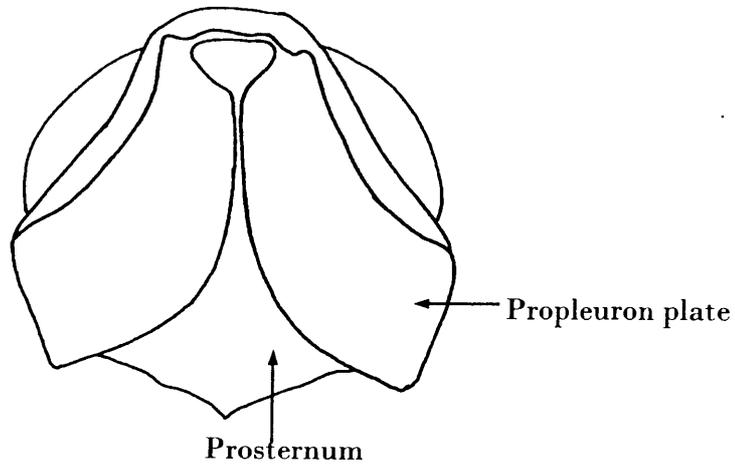
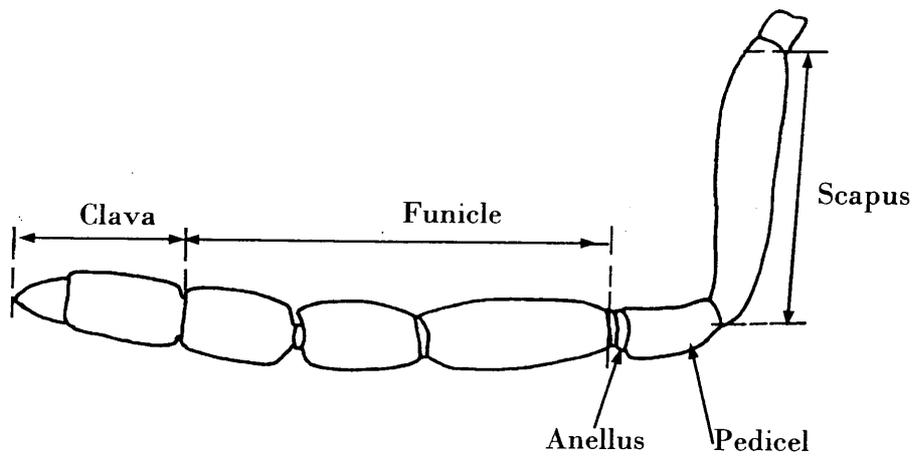
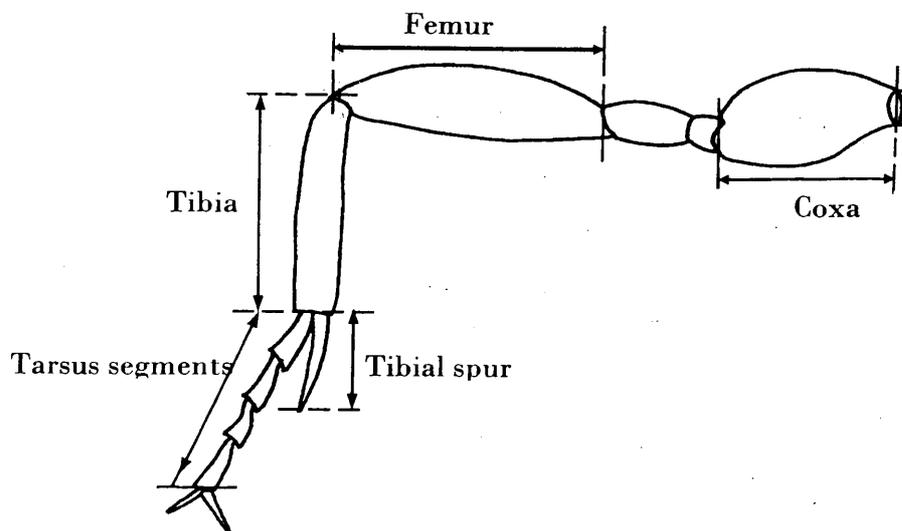
**Fig. 3.1:** Diagrammatic representation of female *Pediobius furvus* wasp showing morphological characters examined.

**Fig. 3.1.1:** Head capsule in frontofacial view



**Fig. 3.1.2:** Mesosoma in dorsal view



**Fig. 3.1.3: Pronotum in ventral view****Fig. 3.1.4: Female antenna in lateral view****Fig. 3.1.5: Hind leg in lateral view**

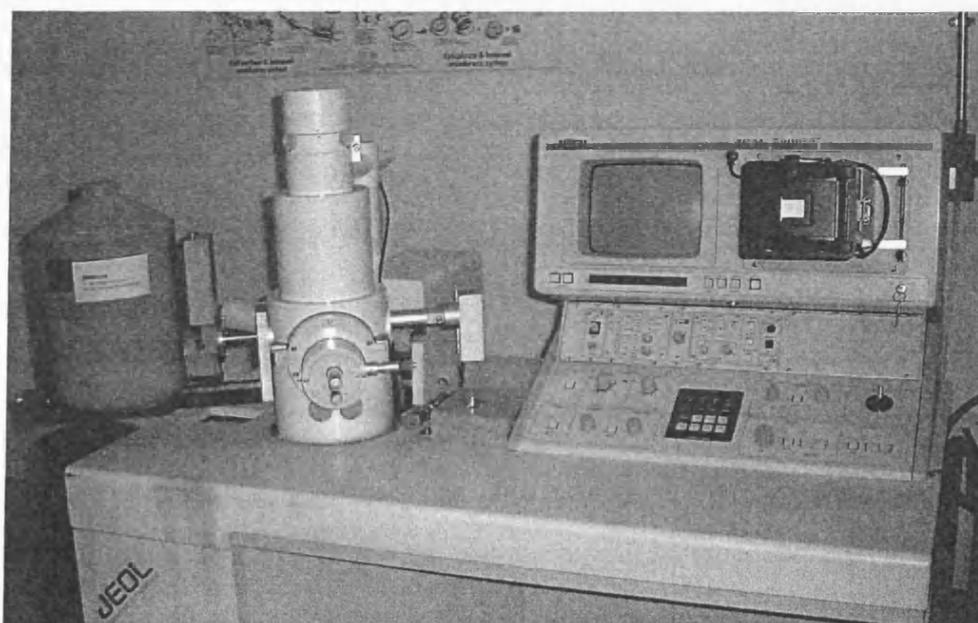
**Table 3.2:** Number of material examined, collection date, locality and author.

- (1) *Pediobius furvus* Gahan reared from larvae of *Sesamia calamistis*: **BENIN**, Cotonou, 4♀, 20: x: 2001, (SHAMIE); *P. furvus*, same, **BENIN**, Cacaveli, 4♀, 2: xi: 2001, same, Aného, 4♀, 5: xi: 2001, same, Tabligbo, 4♀, 8: xi: 2001 (SHAMIE).
- (2) *P. furvus* Gahan, same, **KENYA**, Machakos, 4♀, 12: iv: 2000, (OVERHOLT).
- (3) *P. furvus*, reared from larvae of *S. calamistis*: **GHANA**, Accra, 2♀, 2: xi: 2001; same, Tema, 2♀, 7: xi: 2001; same, Takoradi, 2♀, 8: xi: 2001 (SHAMIE).
- (4) *P. furvus* reared from *S. calamistis*: Republic of **GUINEA**, Forecariah, 4♀, 18: xi: 2001; same, Kindia, 4♀, 20: xi: 2001 (SHAMIE); *P. furvus* reared from larvae of *S. calamistis*, **SIERRA LEONE**, Lungi, 4♀, 25: xi: 2001, Port Loko; same, 4♀, 27: xi: 2001; same, Kambia, 4♀, 2: xii: 2001 (SHAMIE).
- (5) *P. phalaridis* Dawah reared from stems of *Phalaris aurandinacia*, **WALES**, Kenfig Dunes, 2♀, 22: ix: 2000 (SHAMIE); *P. deschampsiae* Dawah reared from stems of *Deschampsia* .
- (6) *P. foveolatus* Crawford, Texas, **USA**; 2♀, 20: xi: 1993 (SCHAUFF); *P. tarsalis*, **TAIWAN**, 2♀, 20: xi: 1997 (CHIU and MOORE).

### 3.2.2 Specimen preparation and examination

Both fresh and dry specimens were first washed in 100% ethanol and dried on strips of filter paper. After drying, specimens were further cleaned, using the tip of a fine brush dipped in 100% ethanol. With the aid of a dissecting microscope, body sections were separated. Sections were then glued on aluminium mounting stubs and treated in gold-sputter for 2 minutes to achieve high quality resolutions for even the smallest specimens. After the gold-sputtering process specimens were photographed on the SEM (Fig. 3.2), using various resolutions based on the size of the specimen. Scanned micrographs were studied. Characters and obvious body features such as antennal segments, propleuron and prosternum plates were measured with a digital calliper. After scanning, micrographs were carefully examined for morphological differences.

**Fig. 3.2:** Scanning Electron Microscope equipment used in the investigation.



### 3.3 Results

All scanned micrographs were stored on TIF filing system and printed on laser printer, and examined for character differences. Kerrich's (1973) key to the African species was reviewed and modified, and old terminologies replaced by those in current use that are shown in bold (Table 3.3). The key confirmed that all the six populations reared from the maize stemborer *Sesamia calamistis* belong to a single species, *Pediobius furvus* Gahan, as previously identified based on morphological characters and host association. Morphological characters of heads in frontal aspect, mesosoma in dorsal view, and those of the gasters were very similar and difficult to distinguish between populations. However, distinct differences were found between the six populations and these include the shape of scape, pedicel, funicle segments and distribution of setae (Fig 3.3.1), and the structure and sculpture of prosternum plates on the ventral side of pronotum (Fig. 3.3.2). A very obvious difference was recorded between specimens from West Africa and those from Kenya is the shape and length of hind tibial spur. All West African specimens examined have a similar hind tibial spur, strong and long, extending beyond the tip of the first tarsal segment; whereas the hind tibial spur of the Kenya population is short and does not extend to tip of corresponding tarsal segment (see Fig. 3.3.3). Body colourations are the same in all populations although differences are found in the antennal segments colour patterns. Benin and Togo populations have similar antennal colour patterns with the scape and pedicel

bluish-green, whilst the flagella segments are all pale yellowish-green. Ghana and Kenya have all antennal segments coloured greenish-blue, while Sierra Leone and Guinea populations have all segments yellowish-green to pale yellow. The differences, especially character ratios, are described in detail (section 3.3.1), and where populations have similar characters, these are indicated. Digital colour plates of body sections and that of a mature larva representing Benin population are shown in Fig. 3.4.

Unused specimens from Texas including those from Taiwan collections, and micrographs were returned to Texas University, whilst stub-mounted specimens, UK samples, and some of African collections are deposited in the National Museum and Galleries of Wales in Cardiff. The rest of the African collections are deposited in the Entomological reference collection, Department of Zoology, University of Sierra Leone in West Africa for future use by taxonomy students and WAFRINET Entomologists.

**Table 3.3:** Key to the females of some of the African species of *Pediobius*. This is an updated and improved of the key by Kerrich (1973) as a result of this study. Synonyms of *Pediobius* have been replaced and differences are highlighted in bold.

- 1 Frontovortex reticulation distinct or moderately distinct and more or less regular.....2
- Frontovortex reticulation rather weak to indistinct, or regular .....7
- 2 Bristles on head and thorax mostly black, relatively short and stout; eyes strongly but very sparsely hairy; sculpture on frontovortex, mesoscutum and scutellum remarkably similar, reticulate, usually a little coarser on mesoscutum than on frontovortex and a little coarser on scutellum than on mesoscutum; **propodeal submedian carinae** about parallel; antennal scape in greater part pale testaceous; femora and tibiae pale testaceous, with only slight metallic darkening on the femora. ....***ropalidiae*** Risbec (p. 68-72)
- Bristles on head and thorax paler; eye pilosity very different; sculpture on mesoscutum and scutellum markedly dissimilar; propodeal submedian carinae closer at base and more or less divergent at apex; antennal scape, femora and tibiae in greater part strongly metallic blue-green.....3
- 3 Propodeum and pronotal collar covered with fine reticulation, not shining; mesoscutum and scutellum dull, sculpture distinct .....***rhyssonotus*** Waterston (p. 120)
- Propodeum and pronotal collar shining, less reticulate and very fine; mesoscutum and scutellum more or less shining, the sculpture less close .....4
- 4 Very small wasps about 1.5mm in length, eyes strongly convergent below and extending far below level of toruli, third funicular segment much broader than long; petiolar segment over 1½ times as long as its greatest breadth, gaster oval and rounded at apex and densely hairy.....***taylori*** Kerrich (p. 121)

- Mostly larger wasps, eyes not extending far below level of toruli, third funicular segment about quadrate or longer than broad; gaster pointed toward apex .....5
- 5** Frontovortex narrow, eyes very densely hairy; hind tibial spur long, nearly reaching apex of second tarsal segment.....*angustifrons* Kerrich (p. 122)
- Frontovortex broad, eyes sparsely hairy; hind tibial spur not extending beyond apex of corresponding metatarsus ..... 6
- 6** Frontovortex broader than long, ocelli arranged in about acute triangle; eyes densely hairy; petiolar segment transverse; gaster elongate and pointed, the first large tergite less than half its length; fourth segment of mid and hind tarsi slightly darkened; wasps associated with Lepidoptera.....*neavei* Waterston (p. 123)
- Head short and broad, frontovortex about one-half times broader than long; eyes densely hairy; petiolar segment longer than broad; gaster less elongate and pointed, the first large tergite about half its length; fourth segment of mid and hind tarsi well darkened; parasitoid of Epilachninae.....*foveolatus* Crawford (p. 124)
- 7** Scutellum bearing about ten strong setae on either side; mesoscutal setae stout and numerous; sixteen strong setae arising behind raised margin of pronotal collar.....*setigerus* Kerrich (p. 125)
- Scutellum bearing two pairs of bristles; setae on mesoscutum not so numerous; pronotum with six setae arising from behind anterior margin of pronotal collar.....8
- 8** Head as seen from above rounded before and behind eyes, occiput narrow and sloping backward; petiolar segment strongly produced forward at sides; gaster very elongate, first large tergite bulbous, the first five strongly

overlapping their opposite sides beneath; legs exceptionally slender, hind femur five times as long as its greatest breadth; hind tibial spur short, about three-fifths the length of corresponding metatarsus.....*acraconae* Kerrich (p. 126)

- Head not rounded as above; gastral tergites overlapping their opposite sides beneath; legs not exceptionally slender.....9

9 Head very broad, about three-quarters times breadth of its median length; scutellum rather strongly convex .....10

- Head not broad, and scutellum flatten.....11

10 Sides of upper face reticulate; sculpture of mesoscutum and scutellum reticulation distinct, gaster gradually pointed and very broad and rounded at apex, the first large tergite relatively large; eyes with pilosity sparse.....*aspidomorphae* Girault (p. 128)

- Head shining all over, without distinct sculpture; sculpture of mesoscutum and scutellum dissimilar, scutellum reticulation very coarse; gaster pointed at apex, eyes not distinctly hairy; antennal scape pale.....*marjoriae* Kerrich (p. 131)

11 Pronotal lateral angles prominent, pronotal collar strongly contracted behind its sharp anterior margin; notaular pits with deep longitudinal impression, very sharp, anterior margin with strong seta near inner corner, scutellum with very distinct longitudinal striate extending almost to apex..... *coffeicola* Ferriere (p. 132)

- Pronotum with distinct lateral angles, sides of pronotal collar moderately narrowed; notaular pits bearing the main seta behind the middle .....2

12 Clypeal area broad and flat; mandibles elongate, sickle-shaped, with the lower tooth much longer and stronger than the upper; notaular pits rather

shallow but very conspicuous, anterior margin sharp; notauli striae converging before reaching the **transscutal articulation**....*clinognathus* Waterston (p. 345)

- Clypeal area not so broad and flat; mandibles not elongate; notaular pits more widely separated..... **13**

**13** Frontoververtex delimited from upper face by a sharp transverse carina; propodeal submedian carinae markedly convergent up to middle then diverge; fore wing basalis bearing several hairs arranged in two rows; mesoscutum with a developed median longitudinal impression.....*arcuatus* Kerrich (p. 135)

- Frontoververtex not delimited from upper face by a sharp carina, the head rounded; propodeal submedian carinae sub-parallel, and markedly divergent from near base; fore wing basalis bearing a single row of hairs.....**14**

**14** Scutellum strongly elongate-reticulate at sides with a median band of moderate breadth, very distinctly reticulate.....**15**

- Scutellum with sculpture otherwise, either more uniform, sometimes narrowly weaker in mid line, or having a median band shining, smooth or with sculpture indistinct.....**17**

**15** Frontoververtex broader than its median length; first funicle segment broad in side view, about one and three-quarters times length of its greatest breadth; propodeal microsculpture obvious between the submedian carinae.....*homoeus* Water. (p.345)

- Frontoververtex over 1½ times broader than its median length; first funicle segment, in side view, not more than about one and a third times length of its greatest breadth; propodeum without obvious microsculpture..... **16**

**16** Mesoscutum finely reticulate throughout, notaular pits shallow and indistinct; pronotum and frontoververtex having similar in reticulation;

- frontovortex blue-green, and thorax dull green; Lepidoptera egg-parasitoid.....*anastati* Crawford (p. 137)
- Mesoscutum with longitudinal striate reticulation, notably between the notaular pits; pronotal collar more shining, with very fine reticulation; propodeum strongly convex; frontovortex with **bronze** ridges; hemipteran egg-parasitoid.....*africanus* Waterston (p. 44)
- 17** Scutellum flat dorsally .....**18**
- Scutellum convex .....**21**
- 18** Scutellum almost regularly sculptured throughout, and less strongly so in mid line .....**19**
- Scutellum with a smooth median band.....**20**
- 19** Scutellum rather coarsely longitudinally striate, occipital margin sharp; clypeus with a pair of lateral teeth; pronotal collar almost straight-sided; first large tergite less than half length of gaster; hind tibial spur reaching well beyond apex of corresponding metatarsus; femora and tibiae testaceous, with only slight metallic colouring on hind femora.....*praeveniens* Kerrich (p. 138)
- Scutellum finely and densely longitudinally reticulate to striate-reticulate; occipital margin sharp to below middle of eye; clypeus broad and truncate at apex; sides of pronotal collar sharply contracted, finely but very distinctly reticulate behind; first large tergite about three-fifths length of gaster; hind tibial spur not reaching apex of corresponding metatarsus; legs except tarsi almost entirely dark blue-green.....*modestus* Masi (p. 139)
- 20** Frontovortex microsculpture reticulation before the ocelli quite distinct; pronotal neck elongate, collar with very distinct longitudinal striate-reticulation; petiolar segment with longitudinal ridges above; first large tergite about half gaster length; found on cotton..... *hirtellus* Masi (p. 141)

- Frontovortex sculpture distinct before the ocelli; pronotal neck rounded at sides and slightly narrowed; petiolar segment with distinct longitudinal ridges above; first large tergite about two-thirds length of gaster; parasitoid of lepidopterous stalk-borers in Sudan to Zimbabwe and to Sierra Leone in West Africa (Fig. 5.5).....*furvus* Gahan (p. 142)
  
- 21** Very small wasps, a little more than 1mm in length; legs before tarsi in greater part dull ochreous, with only light metallic colouring; funicular segments quadrate or broader than long; nucha small, well rounded at apex; mostly dark; egg parasitoid .....*telenomi* Crawford (p. 143)
  
- Larger wasps; legs before tarsi wholly metallic; nucha more or less emarginate at apex..... **22**
  
- 22** Length about 1.2 mm, **mesosoma** blackish brown; median band of scutellum narrow, having longitudinal striate sculpture; gaster relatively short; fore wings extending far beyond apex of gaster; frontovortex partly reticulate; first funicle segment longer than broad.....**23**
  
- Larger wasps, mostly about 1.5 mm in length, mesosoma blue-green; median band of scutellum broad, gaster elongate-ovate; fore wings not extending far beyond apex of gaster; frontovortex reticulate, third funicular segment distinctly longer than broad in side view.....**25**
  
- 23** Head markedly broader than **mesosoma**, strongly transverse; propodeum distinctly convex; eyes not hairy; eggs parasitoid..*africanus* Waterston (p. 44)
  
- Head not transverse, ocelli arranged in an acute triangle; propodeum slightly convex; eyes evidently hairy; not eggs-parasitoid.....**24**
  
- 24** Middle of mesoscutal midlobe finely and regularly reticulate; scutellum more convex; antennal scape less slender, about four times length of its greatest breadth; eyes distinctly hairy.....*parvulus* Ferrière (p. 182)

- Mesoscutal midlobe coarsely elongate-reticulate; scutellum less convex; antennal scape slender, about six times length of its greatest breadth; eyes distinctly hairy.....*vignae* (Risbec) (p. 51-53)
- 25** Frontovortex reticulation quite distinct throughout; antennal funicle not evidently broadened in side view, third segment distinctly longer than broad; sculpture of mesoscutum distinct.....*afronigripes* Kerrich (p. 147)
- Frontovortex more or less shining, its sculpture hardly distinct; antennal funicle evidently broadened in side view, third funicular segment broader than long; mesoscutum weakly sculptured.....**26**
- 26** Head less sharply narrowed behind eyes; eyes extremely sparsely hairy; propodeal nucha rather deeply emarginate at apex; petiolar segment little broader at base than long .....*vigintiquinque* Kerrich (p. 148)
- Head very sharply narrowed behind eyes; eyes less finely and sparsely hairy, propodeal having nucha weakly emarginate at apex; petiolar segment much broader at base than long .....**27**
- 27** Frontovortex about twice the dorsal breadth of eye; third funicle segment in side view little broader than long; mesoscutum shining, notaular pit nearly reaching transscutal articulation.....*amaurocoelus* Waterston (p. 149)
- Frontovortex about 1.5 times the dorsal breadth of eye; third funicular segment much broader than long in side view; mesoscutum dull; notaular pit not reaching transscutal articulation.....*dipterae* Risbec (p. 66-67)

### 3.3.1 Detail analysis of *P. furvus* populations (Fig. 3.2)

These descriptions are based on characters revealed in the present study from examination of individual specimens (see Subsection 3.2.1). No head and gaster structure and sculpture differences were found between populations, however, differences and similarities found on the antennae, pronotum and hind legs. Each population is therefore described separately indicating where similarity occurs.

#### 3.3.1.1 Benin

##### (i) Antennal

Scape slender and cylindrical, 5.1 times as long as its maximum median breadth, 3.2 times as long as length of pedicel; pedicel very slender and sparsely hairy; funicle and clava dense with fine long hairs; 1<sup>st</sup> funicle segment slender and long, 4.1 times as long as broad, and 1.6 and 2.0 times as long as 2<sup>nd</sup> and 3<sup>rd</sup> segments respectively; 2<sup>nd</sup> segment parallel sided and petiolate at apex; 3<sup>rd</sup> segment petiolate at base and truncate at apex; clava not as broad as 3<sup>rd</sup> segment, and pointed at apex; densely hairy (Fig. 3.3.1a).

##### (ii) Pronotum

Propleuron plates tightly but not fused anteriorly, very broad, fine scale-like sculpture; prosternum narrow 1.6 times as broad as long, and raised in middle, smooth, posterior ending in a sharp point in middle (Fig. 3.3.2a).

**(iii) Hind leg**

Tibial spur straight and about 8.0 times as long as its basal breadth, and 1.3 times as long as its corresponding metatarsus segment; metatarsus segments dense with fine long hairs (Fig. 3.2.3).

**3.3.1.2 Togo****(i) Antennae**

Same as Benin (Fig. 3.3.1a).

**(ii) Pronotum**

Propleuron plates fused at anterior end, finely scale-like sculpture; prosternum plate smooth, raised in middle, posterior end with 3 protrusions, the middle longer than those on either side (d).

**(iii) Hind leg**

Same as Benin (Fig. 3.3.3).

**3.3.1.3 Kenya****(i) Antennae**

Scape slender and long, 4.6 times as long as its maximum breadth, 2.3 times as long as pedicel, tapering at base, and sparse with fine hairs; pedicel cone-like, as long as 1<sup>st</sup> funicle segment, its posterior end about 2 times as broad as its base; funicle dense with fine hairs, 1<sup>st</sup> funicle segment longer than broad, and swollen in middle; 2<sup>nd</sup> and 3<sup>rd</sup> segments about equal, parallel sided, 1.3 times as long as broad; clava 2.2 times as long as its greatest breadth (Fig. 3.3.1b).

**(ii) Pronotum**

Propleuron plates fused at anterior end, posterior ends curve inward and pointed at apex, plain and smooth; prosternum 1.8 times as wide as long, smooth, posterior margin smooth with a single sharp point in middle (Fig. 3.3.2e).

**(iii) Hind leg**

Metatarsus slender and dense with fine short hairs; tibial spur short not extending to apex of corresponding metatarsus segment, 1<sup>st</sup> metatarsus segments 1.3 times as long as spur (Fig. 3.3.4).

**3.3.1.4 Ghana****(i) Antennae**

Scape 4.1 times as long as its maximum breadth, almost cylindrical in shape, sparse with fine hairs; pedicel cone-like, 2.3 times as long as its maximum breadth, and smooth; funicle segments all petiolate at base, dense with short hairs, 1<sup>st</sup> funicle segment slender, 2.1 times as long as its median breadth, 2<sup>nd</sup> segment 1.4 times its median breadth, and 3<sup>rd</sup> funicle segments about quadrate, 1.1 times as longer as broad, and clearly petiolate at base; clava 2.1 times as long as broad (Fig. 3.3.1c).

**(ii) Pronotum**

Same as Togo (Fig. 3.3.2b)

**(iii) Hind leg**

Same as Benin (Fig. 3.3.3).

### **3.3.1.5 Sierra Leone**

#### **(i) Antennae**

Scape about 3.4 times as long as its median breadth, distinctly swollen in middle, tapering at ends, and sparsely hairy on dorsum, and smooth ventrally; flagellum dense with fine long hairs; pedicel cone-like, its apex twice its basal width; funicle slender, all segments petiolate, 1<sup>st</sup> segment 2.7 times as long as its median breadth, and 1.3 times as long as 3<sup>rd</sup> segment; clava 2.2 times as long as its swollen median width, and 1.6 times as long as 3<sup>rd</sup> funicle segment, and pointed at apex (Fig. 3.3.1d).

#### **(ii) Pronotum**

Propleuron plates fused at anterior end; plain and smooth; prosternum plain, smooth, posterior margin irregular, almost truncate, exposed area 1.7 times longer than wide as long (Fig. 3.3.2d).

#### **(iii) Hind leg**

Same as Benin (Fig. 3.3.3).

### **3.3.1.6 Guinea**

#### **(i) Antennae**

Same as Sierra Leone (Fig. 3.3.1d).

#### **(ii) Pronotum**

Propleuron plates not fused, strongly scaled about three quarter, and smooth at apex; prosternum strongly scaled, raised in middle, its lateral

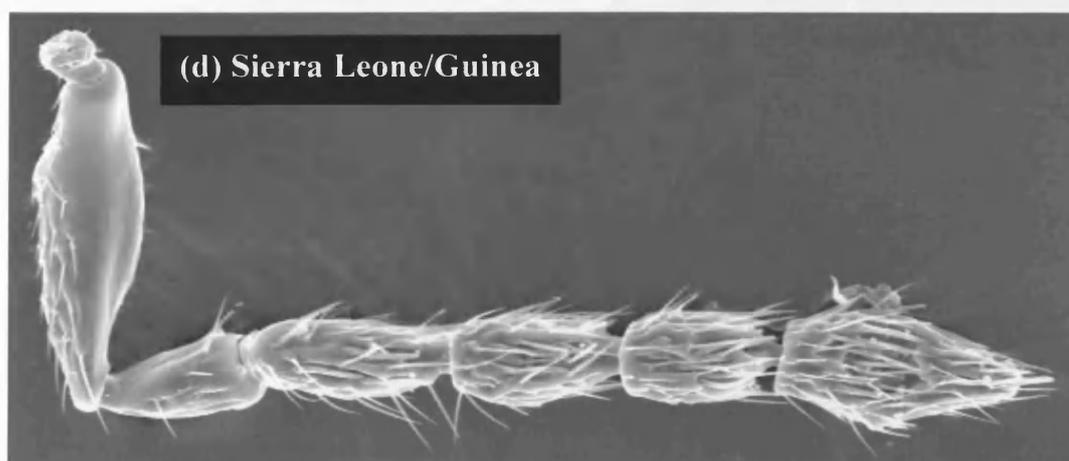
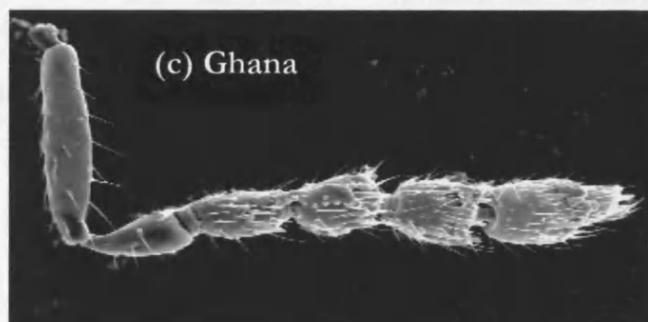
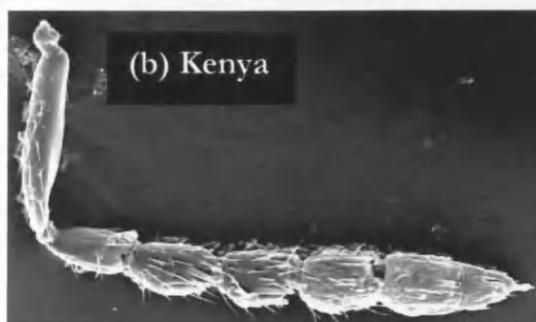
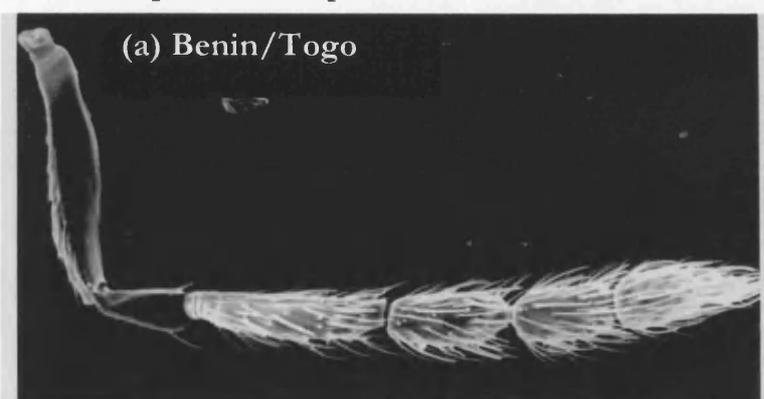
posterior margin straight with a small sharp point in middle; broad, about 2.4 times wide than long (Fig. 3.3.2e).

**(iii) Hind leg**

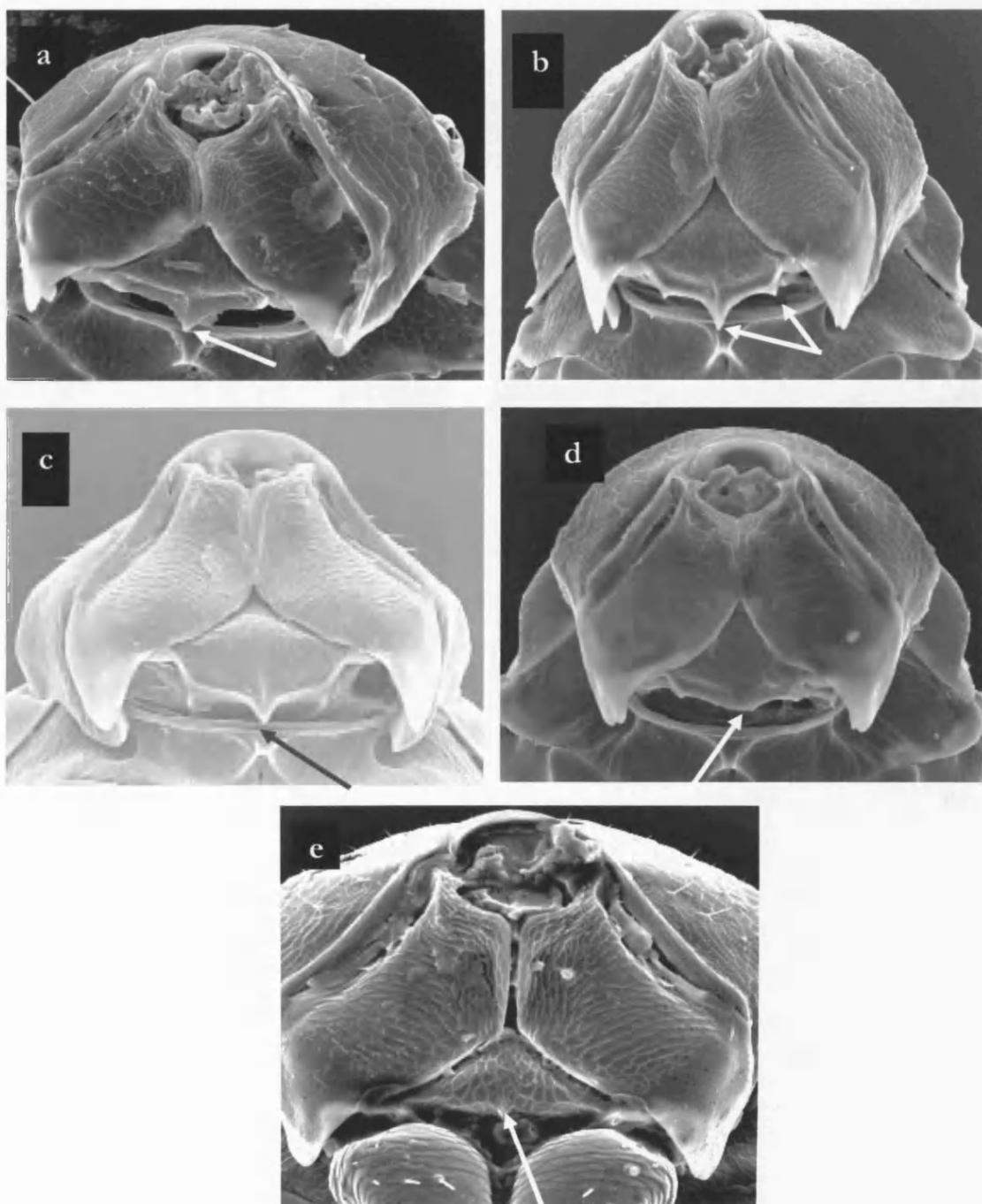
Same as Sierra Leone (Fig. 3.3.3).

**Fig. 3.3.1:** Female antennal character differences found between populations.

**(a) Benin/Togo:-** Funicle segments dense with long setae, first funicle segment slender and twice as long as 2<sup>nd</sup> and 3<sup>rd</sup> segments, 2<sup>nd</sup> segment distinctly petiolate at both ends, clavus with long dense setae; **(b) Kenya:-** Scape cylindrical and smooth ventrally, funicle sparse with short setae, pedicel as long as 1<sup>st</sup> funicle segment, 1<sup>st</sup> segment swollen in middle, 2<sup>nd</sup> and 3<sup>rd</sup> parallel sided and truncate; **(c) Ghana:-** Scape cylindrical, stout and sparse with short thin setae, funicle sparse with thin setae, 1<sup>st</sup> segment 1.5 times longer than 2<sup>nd</sup> and 3<sup>rd</sup>; **(d) Sierra Leone/Guinea:-** Scape stout and swollen in middle, smooth ventrally and dense with fine setae on the dorsal, funicle long, petiolate and dense with long thin setae, 3<sup>rd</sup> segment shorter than 1<sup>st</sup> and 2<sup>nd</sup> and distinctly petiolate anteriorly, clavus dense with thin setae, swollen in middle and pointed at apex.



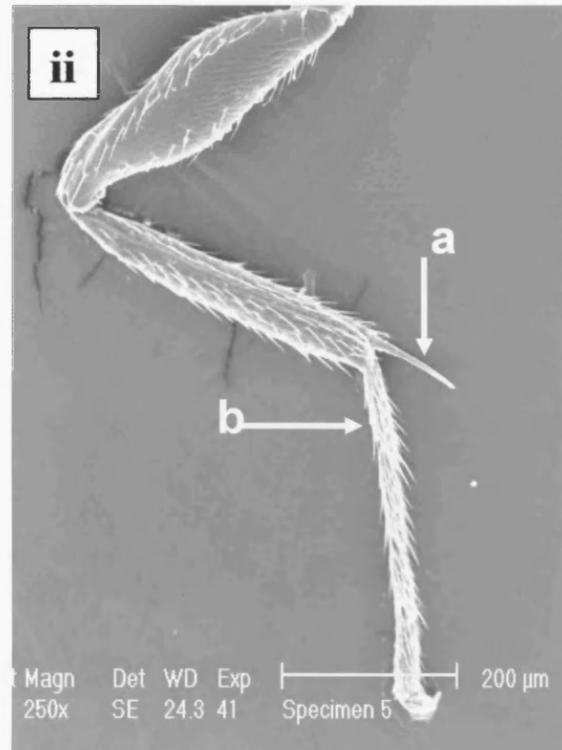
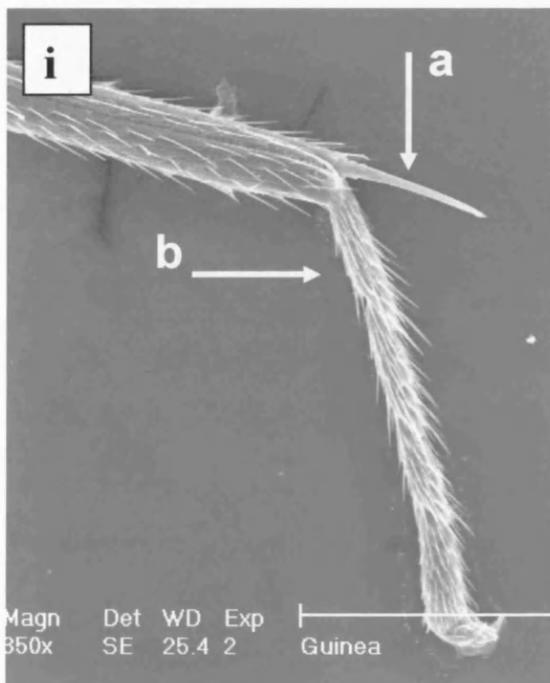
**Fig. 3.3.2:** Pronotum in ventral view showing propleuron and prosternum plates differences between populations. **(a) Benin:-** Propleuron plates fused anteriorly, smooth scales, prosternum raised in the middle with a sharp point (arrow); **(b) Ghana/Togo:-** Propleuron plates fused anteriorly, smooth and pointed at apex, prosternum plate raised in middle ending with 3 sharp points (shown by arrow); **(c) Kenya:-** Propleuron plates fused anteriorly and sharply curved at apex, prosternum with short, smooth setae and pointed in the middle; **(d) Sierra Leone:-** Propleuron plates fused, smooth, no scales, prosternum plate very smooth, no scales, ending in irregular edge (arrow); **(e) Guinea:-** Propleuron plates not fused, strongly scaled, prosternum plate broad and scaly, edge regular with an inconspicuous point in the middle (arrow).



**Fig 3.3.3: Hind legs characteristics showing hind tibial spur**

(i) *P. furtvus* (all West African populations):-hind tibial spur (a) longer than corresponding tarsal segment (b) (arrows), tibia with strong setae, densely hairy tarsal segments.

(ii) *P. furtvus* (Kenyan population): hind tibial spur (a) shorter than corresponding tarsal segment (b) (arrows), tibial with dense short setae, tarsal segments densely hairy.



**Fig. 3.4: Colour plate of Africa *Pediobius furvus* collected in Benin.**

**(a) Head:-** Frontoververtex smooth and strongly raised between ocelli, parascrobal area depressed in anterior and raised posteriorly, scaled; frontofacial suture protruding into scrobal area, upper facial area strongly scaled; supraclypeal area smooth and depressed; antennal sockets levels with lower line of eyes; **(b) Mesosoma:-** Mesoscutal mid lobe and scutellum striated mesoscutal lateral lobes and axillae with strong setae, propodeal submedian carinae slightly divergent outward; **(c) Gaster:-** First large gastral segment about two-thirds of length of abdomen, very smooth and shiny, other segments telescopic and sparse with short setae; **(d) Larva:-** Mature larva, smooth and shining, brownish, swollen in middle and tapers at both ends.

**(a) Head in frontal view**



**(b) Mesosoma in dorsal view**



**(c) Gaster in lateral view**



**(d) Full-fed larva in ventral view**



**Picture: Courtesy of Dr. G. Goergen, Entomologist and Curator, IITA-Benin**

### 3.4 Discussion

Results from micrographs examination show that out of 15 characters studied only eight represented discriminating features between populations. Amongst populations, there are significant differences in the structures and forms of female antennae. These differences include the shape of the scapus, pedicel, first funicular segment, clavus and distribution of hairs. Of these characters, the most obvious discriminating are the scape, first funicular and clavus segments (Fig. 3.3.1). Using these characters, the populations can be separated into four groups:(1) Benin and Togo, (2) Kenya and (3) Ghana as individual groups, and (4) Sierra Leone and Guinea in their own separate group.

Distinct differences are also shown in the shapes and sculptures of the propleura and prosternum plates on the ventral side of the pronotum (Fig. 3.3.2). The structure of the prosternum makes the most differences between populations. This character also regrouped the various populations as follows: Benin in one group, Ghana and Togo in another, and Kenya, Sierra Leone and Guinea in individual groups. All west African populations are separated from Kenya in the pro-hind tibial spur length which is strong and long extending beyond tip of corresponding metatarsus segment, whilst Kenya population has weak and short protibial spur, clearly not extending to tip of corresponding metatarsus segment (Fig. 3.3.3).

The results of this part of *Pediobius* investigation suggests that the populations can be separated into four groups based on morphology, and this is supported by their geographic locations (Chapter 2, Fig. 2.2). Only when characters are used independently are clear differentiations between populations revealed. However, the difference in the length of pro-hind tibial spur does demonstrate a clear-cut between West Africa and Kenya populations (see Fig. 3.3.3). These differences may have been due to environmental and geographic adaptations, suggesting that the Kenyan population is different from all the West African populations. However, because of these subtle differences, molecular phylogenetic analyses were carried out to reveal any phylogenetic relationships that support the differences between these host-associated populations (see Chapter 5). Molecular data analysis offers opportunity to test the validity of morphological characters currently used in placing *P. furvus* system into different groups.

### 3.5 References

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**CHAPTER FOUR**  
**DIFFERENTIATION AMONG *PEDIOBIUS EUBIUS* POPULATIONS IN**  
**MATE RECOGNITION**

#### 4.1 Introduction

Mating behaviour in many organisms involves courtship, copulation and post-copulatory behaviours. In insects, these behaviours are a means of advertising reproductive state, species identity and possibly gene quality. Males are usually ready to mate with any female, whereas, receptive females do not normally allow copulation until a male induces readiness to copulate by means of its courtship cues (van den Assem, 1996). Successful copulation occurs when a receptive female accepts a male's courting patterns. While some of the courtship cues, particularly within Chalcidoidea, may be common, species-specific cues have been observed and mate choice experiments have been made to establish the reproductive isolating mechanism that prevents gene flow between species (Khasimuddin and DeBach, 1975). In spite of these communalities, behavioural plasticity in courtship and mating processes has long been observed in Hymenoptera e.g. in *Muscidifurax zarapter* Kogan and Legner (Pteromalidae) (see van den Assem and Povel, 1973, van den Assem, 1985) and in *Nasonia vitripennis* (van den Assem and Jachmann, 1999) and *Coccophagus gurneyi* Compere (Aphelinidae) (Parkes and Walter, 2001). Mate choice experiments to study pre- and post-copulatory mating behaviour have been used to separate a species populations. For example, Boake *et al.* (1997) used mate selection experiments to demonstrate reproductive isolation between the species of the Hawaiian fruit fly *Drosophila heteroneura* and *D. silvestris*; and van den Assem (1996) used mate choice experiments to separate

populations of *Muscidifurax*, *Nasonia* and *Spalangia* (Pteromalidae). For this study, mate choice experiment is carried out to establish whether there is gene flow between sympatric species of *Pediobius*. Lack of gamete exchange among species prevents interbreeding between species.

#### 4.1.1 Mate recognition signals

Males and females of many insect species are known to exchange distinctive signals or mating songs to identify their species and potential mates. Mayr (1997) noted that species-specific songs and call notes are easy species discriminatory components. For example, the planthopper populations of *Nilaparvata* (Homoptera: Delphacidae) have been found to use specific mating songs to locate potential mating partners. These signals or mating calls have been variously used successfully in the field as well as in laboratory experiments in planthopper population differentiation (see Claridge, 1985; De Winter, 1992). In one such study, Claridge (1985) found out that sympatric populations of cicadas can be clearly separated based on the differences in their acoustic signals. Combined laboratory and field experiments have shown that pulse repetition of both male and female calls of planthoppers are essential elements of species differences and possibly responsible largely for observed levels of reproductive isolation (Claridge, 1988). Such differences may comprise characteristics male and female calls, which sometimes apparently differ in the rates of repetition of pulses within the signals. Paterson (1985) describes these as components of 'the specific mate recognition



system' (SMRS) now referred to as the recognition species concept (RSC) (Mayden, 1997). Hunt (2001) concluded that in the planthopper *Graminella nigrifrons* Forbes recognising female's vibrational signals increases a male's mating success when involved in a scramble competition for access to a responding female.

#### 4.12 Morphological differences

Mating as a component of sexual reproduction involves the existence of functional and morphological differences between partners, each playing a different role to the tune of the other in a probable sequence. For example, within the *Apis* genus (Hymenoptera: Apidae) differences in mating behaviour have been used to detect underlying morphological character differences between drones of two populations (see Parker *et al.*, 1972). Morphologically *Apis dorsata* Fabricius was found to have developed adhesive organs on the basitarsus used for attachment to the queen during mating, whereas *A. andreniformis* Smith and *A. floreae* Fabricius drones have lesser amounts of adhesive mucus and therefore depend mainly on the hind legs for copulatory connection to the queen (Boake *et al.*, 2000). These terminal organs are known to be absent in *A. cerana* Fabricius and *A. mellifera* L. (Koeniger and Koeniger, 1991). In chalcids differences have also been found between species of *Eurytoma* Illiger (Hymenoptera: Eurytomidae) such as in the length and shape of male antennae, directional position of antennal sensilla, shape and length of female gaster. Querino *et al.* (2002) used morphological differences in male

genital capsule of *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae) collected from 10 different Lepidoptera host species into four different host groups and the use of male genitalia differences in the separation of the mymarid wasp genus *Anagrus* into 13 species (Chiappini and Mazzoni, 2000) and the description of *Anagrus miriamae* sp. nov. reared from the eggs of the planthopper *Delphacodes sitarea* Lenicov & Teson (Delphacidae) based on the morphology of male genitalia (Triapitsyn and Virla, 2002). Phenotypic selection analysis has revealed that both direct and indirect selections have an effect on the size of morphological characters. For example, Boake *et al.* (1997) recognised head shape as species genetic trait involved in behavioural isolation between *Drosophila* species.

#### 4.1.3 Courtship differences

The sequence of courtship behaviours has been observed in many insect species and has often been found to be very complex between species, and its occurrence is assumed to ensure reproductive isolation (see Boake *et al.* 2000). In parasitic wasps, in particular, mating behaviours consist of a series of highly specific reciprocal-stimulus response sequences between the sexes which include attraction, orientation, wing vibration or wing fanning, antennation, head nodding, leg tapping, copulation and post-copulatory grooming (Matthew, 1975, Jarvis, 1979). Courtship displays consist of characteristic combinations of patterns which are repeated a variable number of times by individuals for the attention of the other. Results of observations of these

components have demonstrated that closely related species of parasitoids that are morphologically similar also differ markedly in their courtship sequences (see Jervis (1979; van den Assem and Werren, 1994). Jervis (1979) carried out mating behaviour experiments both in the field and in the laboratory of the dryinid wasp *Aphelopus melaleucus* Dalman (Hymenoptera: Dryinidae). Although the courtship sequences in *A. melaleucus* appeared relatively simple, Jervis observed that they were different from those of other species of Dryinidae previously studied, for example, *Anteon brachycerum* Dalman, *Dicondylus bicolor* Haliday and *Pseudogonatopus distinctus* Kieffer (see Waloff, 1974). Van den Assem (1996) observed diverse differences in courtship position of six species of four Hymenoptera families.

Probably the best studied chalcidoid families in terms of courtship and mating behaviours are the Pteromalidae and Eulophidae. Previous mating behaviour studies of these families include van de Assem (1996) on the populations of *Muscidifurax*, *Lariophagus*, *Nasonia*; *Spalangia* and *Achrysochroides* (Pteromalidae) and *Pediobius eubius* complex (Dawah, 1988). Differences in courtship behaviour have been used to separate two allopatric populations of *Nasonia* (Hymenoptera: Pteromalidae). The species of *Nasonia* are traditionally thought to mate around their host pupae before emerging out from the host pupa remains, in which case intraspecific mating may occur. However, recent results have shown this behaviour in three species, *N. giraulti* Darling, *N. vitripennis* and *N. longicornis* Darling in which mating

occurs within the host pupa or near their emergence exits (Drapeau and Werren, 1999). Drapeau and Werren (1999) called this behaviour 'within host mating' (WHM). WHM has been found to be a behavioural trait with strong effect on both level of inter-breeding and the level of interspecific mating. This behaviour observed in the two allopatric species *N. giraulti* and *N. longicornis* thus acts as a pretext to escape hybridisation with *N. vitripennis* that co-occurs micro-sympatrically (van den Assem and Jachmann, 1999). With *N. vitripennis*, a female may be sexually receptive while being courted and this is then followed by a specific signal which the courting male must react to promptly. The courting male is usually ignored if its reaction does not coincide with female's signals (Jachmann and van den Assem, 1993).

#### 4.1.4 Pheromones in mate selection

Mating behaviours are important cues in selecting the correct copulating partner by any organism. Whilst males are often ready to mate right away upon emergence, females seem to be more careful and selective. This is because particularly in Chalcidoidea, females are receptive only once in their life-time and therefore mating with a different species would have more severe consequences for a female than a male (Manning, 1965). Where differences occur in mating behaviour responses, this may cause a mating isolation between species. Behavioural isolation in mate selection has been implicated in the radiation of the highly studied Hawaiian fruitfly *Drosophila* (Diptera: Drosophilidae). This species has served as a model organism for the study of

genetics (see Coyne and Orr, 1997; Boake *et al.*, 2000). Among the species of the braconids *Eubazus* (Hymenoptera: Braconidae) no interaction was observed between males and females of two species, even when placed together. Kenis and Mills (1998) suggested that such behaviour might have been due to certain species specific isolating biological traits.

In most insects mating is preceded by chase in which the female attempts to escape from the courting male (see Jervis, 1979). Such behaviours have been associated with pheromones interactions. A pheromone is an infochemical that conveys information between members of the same species. Pheromones emitted by insects have long been perceived to be responsible for such chases are recognised as important cues in the mate selection process (Ward and Morton, 1991; Phelan, 1997). In a mating repertoire females have been proven to emit long-range chemical signals that attract potential mates. Males on the other hand produce short-range courtship pheromones, which are used by the female to discriminate species status between available males (Svennsson, 1996; Ayasse *et al.*, 2001). Courtship pheromones play a vital role in determining a male's mating success because their pheromone chemical components have to be understood, interpreted and recognised by a responding female to accept the male. Chemical signalling seems to be the most common method employed in sex attraction and recognition in Hymenoptera as well as in other insect orders (Hölldobler, 1984, Guerrieri *et al.*, 2001; Ayasse *et al.*, 2001). Research has shown that pheromone-mediated mate choice suggests a

positive correlation between olfactory attractiveness and dominance (Moore *et al.*, 1995), subsequent fertilisation success and offspring fitness (Lewis and Austad, 1994).

#### 4.1.5 Fertilisation

In laboratory or field experiments, cross-mating studies of species are measures to establish any evidence of successful transfer of gametes, which determines species reproductive compatibility or population isolation. Females of most parasitic Hymenoptera are facultatively parthenogenetic (haploidiploid) i.e. unfertilised eggs develop into males and fertilised ones into females. Females may not need mating for offspring reproduction because mating in haploidiploid organisms is only required when female offspring are to be produced (Soumalainen *et al.*, 1976). Successful mating and gamete transfer in some species of Hymenoptera is evidenced by the production of both male and female progenies in the process (with the exception of some species that exhibit thelytokous parthenogenesis, e.g. the egg endoparasitoids in the genus *Trichogramma* (Hymenoptera: Trichogrammatidae). Unsuccessful mating or sperm depleting in females are therefore constraints to producing all male broods (Godfray and Hardy, 1993). In the natural environment where a female wishes to produce both males and females the female wasp may face the choice between seeking hosts for oviposition before mating and producing males only, or searching for males with the possibility of producing both males and females after mating (Hardy, 1994). Even in such situation, fertile diploids are

only produced if there is fertilisation in accordance with the Biological Species Concept (Mayr, 2001).

#### 4.1.6 Species status

Previous studies have variously regarded the *Pediobius eubius* complex as three species (Graham, 1963). Dawah (1988) carried out mate choice experiments, olfactometer tests and protein electrophoresis analysis of the *eubius* species in Britain and identified nine species. However, he did not carry out similar tests between the morphologically similar species such as *Pediobius deschampsiae*, *P. phalaridis* and *P. calamagrostidis*. This chapter examines reproductive compatibility of three sympatric species of *Pediobius* associated with parasitism of grass-infesting *Tetramesa* in Britain.

#### 4.2 Hypothesis

Cross-mating experiments may be important in revealing genetic status of morphologically similar species that live sympatrically. If sympatric species are forced to mate with non-conspecifics, although they may court fertilisation may not occur resulting in the production of all-male offspring. To test this hypothesis, induce-mating experiments were carried out with three sympatric species of the *Pediobius eubius* complex associated with parasitisation of gall-forming phytophagous hymenopteran species of *Tetramesa* in the United Kingdom. Components of behavioural isolation, sexual selection and reproductive isolating mechanisms are discussed.

### 4.3 Methodology

Three wild grass species of the family Poaceae were used in the experiments, *Phalaris arundinacea* L., *Deschampsia caespitosa* L. and *Calamagrostis epigejos* L., their natural phytophagous hymenopteran species *Tetramesa phalaridis*, *T. deschampsiae* and *T. calamagrostidis*, and their respective parasitoids of the *Pediobius* species *P. phalaridis*, *P. deschampsiae* and *P. calamagrostidis*. For monitoring of mating activity, a digital video camera linked to a 14-inch mono television screen was used. Medium sized glass vials were used as mating chambers (Fig. 4.1). A low-intensity fluorescence light placed about 5cm above mating chambers provided a source of light. Plants used in the experiments were collected from two separate locations (Kenfig Dunes and Fairwater Nature Reserves) in South Wales. In the greenhouse, plants were raised in large-sized cone-type flower pots (30cm in diameter) with flat bottom, whilst in the field selected plant stands were used *in situ*. In both situations, large-sized wooden cages (110cm x 65cm x 65cm) covered in white voile were used to contain the plants. CO<sub>2</sub> and pigment ink were used to anaesthetise and mark wasps, respectively. All insects were reared from fully fed larvae obtained from dissected grass stems infested by their phytophagous hosts. This was important because the phytophagous *Tetramesa* species are attacked by many other hymenopteran parasitoids such as *Eurytoma*, *Sycophila* or *Chlorocythus*. A dissecting microscope was used to examine a wasp's morphology to confirm its species status.

**Fig. 4.1:** Mating chambers used in mating observation showing stems of grass species. *Tetramesa* infested grasses were used in each chamber as a resting substrate and mimicking natural conditions to prevent change in behaviour.



Two mating patterns were used; (a) direct cross-mating involving two species for any replicate, e.g. a male of one species cross-mated with females of another species; and (b) indirect mating also involving two species with a male of one species cross-mated with non-conspecific females plus its conspecific females. Females were given the chance to accept or reject males. Only a single male was normally introduced into a mating chamber containing females. This method was adopted to avoid sperm competition between available males, and to control the operational sex ratio, which is normally female-biased, and because male insects have the propensity to mate with many females (see Werren, 1980). Insects were coded as follow: *Pd* for *Pediobius deschampsiae*, *Pp* for *P. phalaridis*, and *Pc* for *P. calamagrostidis*.

### 4.3.1 Culture preparations

Experimental sites and greenhouse cultures were set up and prepared in the spring and summer (2002). In the field, selected plant stands were cut at ground level leaving just the rhizomes to allow new re-growth. This was to prevent using infested stems in the experiments. To ensure that infested stems were not used a few stems from each isolated plot were dissected and examined for the presence of *Tetramesa* or larvae of other stem-borers. Plant stands were then isolated by dropping culture cages over them with their peripherals sealed with soil to prevent insects from infesting emerging shoots (Fig. 4.2). Secluded plant stands were left until new shoots sprouted and mature enough for infestation with gall-forming *Tetramesa* species associated with each grass species. To establish the greenhouse culture, plants were dug out in the field and planted in flowerpots. Old stems were cut from each stand as in field experiments so that plants used were almost of the same age and devoid of infestation.

**Fig. 4.2a:** Experiment site at Fairwater Nature Reserve showing rearing cages dropped over selected plant stands in the field to prevent entry of other stemborers and parasitoids.



**Fig. 4.2b:** Experimental site at Kenfig Pool Nature Reserve; rearing cages dropped over selected plant stands to prevent entry of other stemborers and parasitoids. Mated females were released in these cages to oviposit on *Tetramesa* hosts.



### 4.3.2 Host rearing and infestation

To accumulate rearing material for use in the experiment, grass stems were collected from *Tetramesa* infested fields in the Brecon Beacons and Merthyr (Wales). Stems were dissected, and larvae identified and separated using the key in Dawah and Rothfritz (1996). Larval rearing techniques are described in Chapter 2. *Pediobius* larvae recovered from dissected stems were kept separately at 18°C to prevent premature emergence normally caused by high temperatures (above 22°C). *Tetramesa* larvae recovered were placed in gelatine capsules in an incubator set at 21-23°C and relative humidity (70-80%) for emergence that happened 15-25 days after dissection. Rearing insects in an incubator prevented desiccation of pupae. Upon emergence individuals of each species were placed in rearing vials and fed with aqueous solution (containing 10% honey) smeared on 5 cm of grass stems of their host plants. Insects were observed feeding on the honey while walking on the stems or searching for mates. A 24 hr mating period was allowed. At the end of the mating period, the three species of *Tetramesa* were released separately in cages containing their individual natural host-plants both in the field and in greenhouse cultures for oviposition. Release dates (September/October) were recorded and these coincided with the occurrence of adult wasps in the wild. This was confirmed by field sweeping in which *Tetramesa* adults were recovered. *Tetramesa* release dates were very useful in determining host larval-

instars suitability for *Pediobius* oviposition, whilst field emergence dates were useful in determining emergence of *Pediobius* in greenhouse cultures.

#### **4.3.3 Preparation for mating *Pediobius* species**

Using pupal structure and morphology that show distinct differences between the sexes, males and females were separated prior to emergence and placed in small-sized glass vials. Other sets of vials were also prepared and used as mating chambers. Inside each mating chamber fresh grass stems (10cm long) were placed to provide a resting surface for the wasps as well as mimicking natural field environment. To these chambers, females of each population were first transferred for at least one hour before transferring males. This was to prepare them for mating ahead of the males, because behavioural studies have shown that females of Chalcidoidea will first actively search for their host food-plants before they are willing to mate (Guertin *et al.*, 1996). Whilst in the chambers, females may saturate their species-specific pheromones that may act as stimuli for mate selection for mate-searching males (see Section 4.4.4).

#### **4.3.4 Mating protocols**

Prior to the tests, insects were standardised by selecting large females. Only virgin females were used because prior behavioural experience could have major influence on their subsequent interactions. Insects' age differences have also been observed as an important source of variation in courtship behaviour

and copulation (Tagawa *et al.*, 1985). Insects were grouped according to their emergence dates.

Three replicates were made for each test. For every male there were 5 females used in each replicate (Tables 4.1-2). Conspecific females were in the controls and these were normally marked with pinkish water-proof pigment ink on the mesosoma to enhance quick identification prior to release for oviposition. This was achieved by anaesthetising them with CO<sub>2</sub> slowly released into rearing tubes. Different identification materials such as nail polish have been used in previous mating experiments involving Chalcidoidea (Dawah, 1988). The advantages in the use of pigment ink are that it is odourless, light in weight, easily dried and non-sticky, and has no adverse effect either on the mating behaviour or choice and acceptance of partner by both sexes. All mating sessions were monitored for at least an hour using a 15 volts low-intensity florescence lamp as a source of light as high-intensity illumination may inhibit the performance and behaviour of insects in captivity (van den Assem, 1996). A 14-inch mono-screen television attached to a Panasonic CCD-TYRV66E (Hi8xR) video camera with an AVC 76W lens converter (for magnification and quality resolution) was used to monitor mating sessions. A full mating session of 24 hours was normally allowed so that there was ample time to mate or not to.

#### 4.3.4.1 Direct cross mating (DCM)

In this test males of one population were cross-mated with females of the other two species separately. In mating chambers containing females of *Pp* males of *Pd* were introduced for mating and vice versa. Other sets of cross mating were carried out with females of *Pc* and *Pd*. Males of *Pd* were introduced into chambers containing females of *Pc*, and the reverse for chambers containing females of *Pd*. With these tests no choice of females was offered to the males as there were only females of one population per chamber (Table 4.1).

#### 4.3.4.2 Indirect cross mating (ICM)

Tests were conducted to determine whether mating occurred randomly between males and females of *P. deschampsiae*, *P. calamagrostidis* and *P. phalaridis*. In indirect cross mating system an equal number of females of any two species were normally placed in each mating chamber per session (i.e. one conspecific male and a non conspecific). Four tests of three replicates each were carried out. In the first test, males of *Pd* were introduced into chambers containing females of *Pd* and *Pp* for mating, and the situation reversed for the second sets of mating. In the third sets of mating chambers containing females of *Pd* and *Pc*, males of *Pd* were introduced for mating, and the situation again reversed. In these tests, introduced males of one species were offered the opportunity to select their mating partners. Conspecific females were used as controls (Table 4.2).

**Table 4.1:** Patterns of direct cross mating. A single male of one species cross mated with five females of another species for each replicate. No choice of mating pairs was offered to such males, as well as the reverse. Females were then released to oviposit into grasses infested with their *Tetramesa* hosts.

Mating patterns	Inoculation		Infestation
	Parasitoid	Phytophage	Host plant
(i) 1♂ <i>Pd</i> × 5♀♀ <i>Pp</i>	5♀♀ <i>Pp</i>	<i>T. longicornis</i>	<i>Phalaris</i>
(ii) 1♂ <i>Pp</i> × 5♀♀ <i>Pd</i>	5♀♀ <i>Pd</i>	<i>T. petiolata</i>	<i>Deschampsia</i>
(iii) 1♂ <i>Pd</i> × 5♀♀ <i>Pc</i>	5♀♀ <i>Pc</i>	<i>T. calamagrostis</i>	<i>Calamagrostis</i>
(iv) 1♂ <i>Pc</i> × 5♀♀ <i>Pd</i>	5♀♀ <i>Pd</i>	<i>T. petiolata</i>	<i>Deschampsia</i>

#### Legend

*Pd* = *Pediobius deschampsiae*

*Pp* = *P. phalaridis*

*Pc* = *P. calamagrostidis*

*T* = *Tetramesa*

**Table 4.2:** Patterns of indirect cross-mating. For each replicate, ten females from two species were placed in mating chambers to mate with a single conspecific male of one of the females. The situation also reversed for males from other species. There is a choice of mating pairs offered to such males. Females were then released to oviposit into their *Tetramesa* hosts-infested grasses.

Mating patterns	Inoculation		Infested
	Parasitoid	Phytophage	Host plant
(i) 1♂ <i>Pd</i> × (5♀♀ <i>Pd</i> + 5♀♀ <i>Pp</i> ) -	5♀♀ <i>Pd</i>	<i>T. petiolata</i>	<i>Deschampsia</i>
	5♀♀ <i>Pp</i>	<i>T. longicornis</i>	<i>Phalaris</i>
(ii) 1♂ <i>Pp</i> × (5♀♀ <i>Pd</i> + 5♀♀ <i>Pp</i> ) -	5♀♀ <i>Pd</i>	<i>T. petiolata</i>	<i>Deschampsia</i>
	5♀♀ <i>Pp</i>	<i>T. longicornis</i>	<i>Phalaris</i>
(iii) 1♂ <i>Pd</i> × (5♀♀ <i>Pd</i> + 5♀♀ <i>Pc</i> ) -	5♀♀ <i>Pd</i>	<i>T. petiolata</i>	<i>Deschampsia</i>
	5♀♀ <i>Pc</i>	<i>T. calamagrostis</i>	<i>Calamagrostis</i>
(iv) 1♂ <i>Pc</i> × (5♀♀ <i>Pd</i> + 5♀♀ <i>Pc</i> ) -	5♀♀ <i>Pd</i>	<i>T. petiolata</i>	<i>Deschampsia</i>
	5♀♀ <i>Pc</i>	<i>T. calamagrostis</i>	<i>Calamagrostis</i>

#### Legend

*Pd* = *Pediobius deschampsiae*

*Pp* = *P. phalaridis*

*Pc* = *P. calamagrostidis*

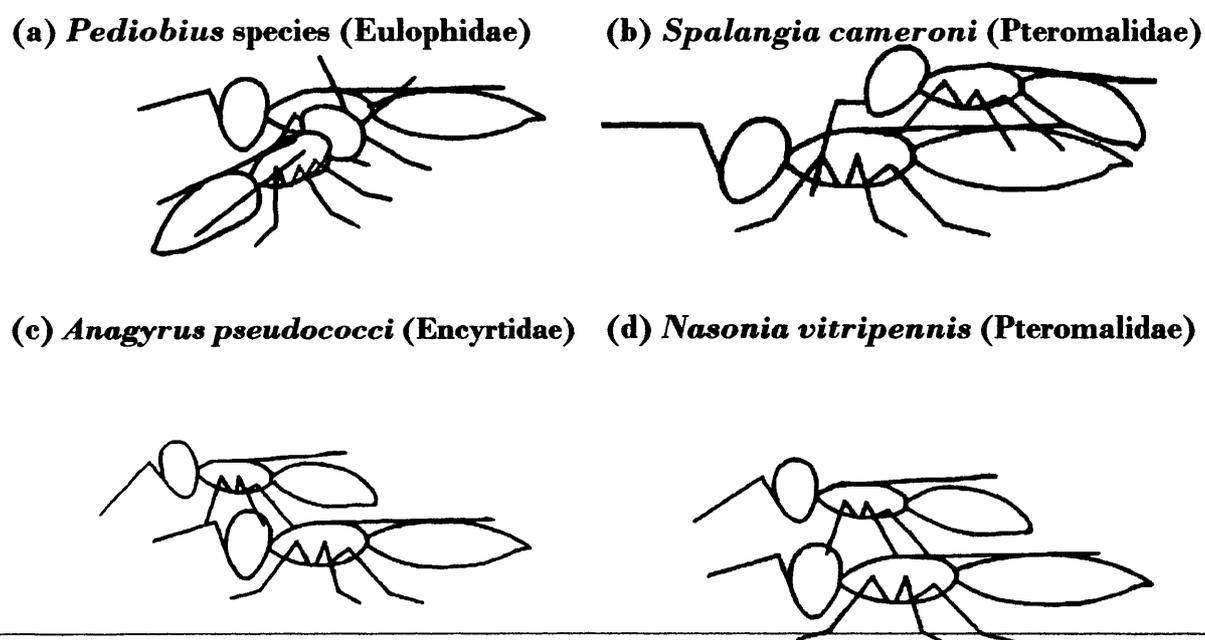
*T* = *Tetramesa*

#### 4.3.5 Mating observations

Several species-specific courtship positions displayed by parasitoids have been observed as being distinct between species as demonstrated in Fig. 4.3. In this experiment where non-conspecifics were placed for mating, introduced males were frequently quickly rejected by the females by moving away from advancing males. During the mating periods, few contacts were observed between males and females (non-conspecifics as well) lasting less than a minute

on average, but no mounting was recorded. Nonetheless, courting positions were assumed by all males used in the tests and these were typical of *Pediobius* species as illustrated in Fig. 4.3a. Other activities observed included wing fanning, antennal stroking and chasing by males which were usually brief.

**Fig. 4.3:** Diagrammatic representation of various courtship positions of parasitoid wasps (Hymenoptera: Chalcidoidea).



**Source:** Assem van den, J (1996): Mating behaviour. In: *Insect Natural Enemies: practical approaches to their study and evaluation* (Eds. M. Jervis and N. Kidd), *Chapman and Hall*, 163-221.

#### 4.3.6 Test for successful insemination

One conspecific and a non-conspecific female from each test were normally dissected in saline on cavity slides and examined under a high-powered light microscope with a x100 magnification. The procedure involved is the same as for extracting female or male genitalia for examination. The ovipositor was normally pulled out with a pair of forceps whilst gradually

massaging the abdomen with the head of a mounting pin. The spermatozoa are stored in the spermathecae until they are needed for egg fertilisation. Khasimuddin and DeBach (1975) stated that Hymenoptera sperm could live more than 10 minutes at room temperature after dissection. In spite of this, examinations were performed quickly and immediately after mating. During examination undulating movements within the spermatheca indicates sperm presence (Gullan and Cranston, 1999). However, during examination, such movements were not seen.

#### **4.3.7 Parasitoid release for oviposition**

After mating insects were normally anaesthetised for 25-30 sec by gradual release of CO<sub>2</sub> through a narrow rubber tube. Because wasps become motionless rapidly, this facilitates identification and easy removal with 100% accuracy, and lesser handling time of marked females. Mated females were released both in the field and greenhouse cultures for oviposition. Each *Pediobius* species was released only to oviposit into cages containing their natural *Tetramesa* host-infested grass species (Tables 4.1-2). To release the parasitoids, mating chambers were usually opened and dropped inside the cages so that wasps leave the vials undisturbed. Once releases were completed, plants in greenhouse cultures were maintained by regular watering to keep them moistened until the following autumn when they were harvested, dissected and examined for the presence of larvae. Those in the field were checked until when plants were ready for dissecting in autumn. Males used in

the tests were killed in  $-70^{\circ}\text{C}$  freezer, then mounted on card points and preserved for future use.

#### **4.3.8 Rearing offspring larvae and examination of adults**

In nature, larvae of *Tetramesa* and their parasitoid *Pediobius* species are usually fully matured by the autumn (Boucek, 1965, Peck, 1985, Dawah, 1988). At this time grass stems were cut below the first node and dissected. Larvae recovered were examined under a high-power dissecting microscope and identified to genus level, separated and placed in individual gelatine capsules. The capsules in plastic containers were then placed in an outdoor insectary where they were regularly checked until pupation. Sexes were identified by the shapes and structure of the pupae which show clear differences. These were kept and reared separately for emergence. Upon emergence adults were further examined under a dissecting microscope for confirmation using keys in Dawah and Rothfritz (1996).

#### **4.4 Results**

Offspring numbers recovered, with numbers of both males and females from all mating patterns, are given in Tables 4.3-4.

##### **4.4.1 Direct cross mating**

Females had only one option in these studies (either to mate with a non-conspecifics or do not mate at all). Only males were recovered, all of which are offsprings of the female species (see Table 4.3). This was confirmed by comparing results of adult morphological characters of those reared from the

tests and those from a previous identification. The results indicate that there was reproductive barrier between non-conspecifics used in these tests.

#### 4.4.2 Indirect cross mating

In mating sessions involving both conspecifics and non-conspecifics two distinct results were obtained. Tests of reciprocal mating (i.e. optional mating either with conspecifics or non-conspecifics) produced both male and female offspring (Table 4.4). However, only in replicates involving conspecifics did female offspring emerged. The results revealed that the non-conspecific females used in the tests were incompatible with the introduced males where only male offsprings emerged.

**Table 4.3:** Results of direct cross-mating showing progeny recovered from mating occurring between non-conspecifics. Progenies recovered from mating involving only two separate species in which no choice was given to males seeking mating partners. Results show no progeny recovered from mating occurring between pairs of two different species.

Mating pattern	Replicate	Progenies				
		♀♀	♂♂	<i>Pp</i>	<i>Pd</i>	<i>Pc</i>
(i) 1♂ <i>Pd</i> × 5♀♀ <i>Pp</i>	1	0	1	1	0	0
	2	0	1	1	0	0
	3	0	2	2	0	0
(ii) 1♂ <i>Pp</i> × 5♀♀ <i>Pd</i>	1	0	2	0	2	0
	2	0	1	0	1	0
	3	0	2	0	2	0
(iii) 1♂ <i>Pd</i> × 5♀♀ <i>Pc</i>	1	0	0	0	0	0
	2	0	1	0	0	1
	3	0	1	0	0	1
(iv) 1♂ <i>Pc</i> × 5♀♀ <i>Pd</i>	1	0	0	0	0	0
	2	0	1	0	1	0
	3	0	1	0	1	0

**Table 4.4:** Results of indirect cross-mating showing offsprings recovered from mating between males and combines conspecific and non-conspecific females.

Mating pattern	Replicate	Progenies					
		<i>P. deschampsiae</i>		<i>P. phalaridis</i>		<i>P. calamagrostis</i>	
		♀♀	♂♂	♀♀	♂♂	♀♀	♂♂
(i) 1♂ <i>Pd</i> × (5♀♀ <i>Pd</i> + 5♀♀ <i>Pp</i> )	1	0	2	0	2	0	0
	2	1	1	0	2	0	0
	3	1	1	0	1	0	0
(ii) 1♂ <i>Pp</i> × (5♀♀ <i>Pd</i> + 5♀♀ <i>Pp</i> )	1	0	0	2	2	0	0
	2	0	1	0	1	0	0
	3	0	0	0	3	0	0
(iii) 1♂ <i>Pd</i> × (5♀♀ <i>Pd</i> + 5♀♀ <i>Pc</i> )	1	3	2	0	0	0	2
	2	0	2	0	0	0	1
	3	2	1	0	0	0	1
(iv) 1♂ <i>Pc</i> × (5♀♀ <i>Pd</i> + 5♀♀ <i>Pc</i> )	1	0	2	0	0	0	1
	2	0	0	0	0	1	2
	3	0	1	0	0	1	1

#### 4.5 Discussion

Three *Pediobius* species {*P. phalaridis* (*Pp*), *P. deschampsiae* (*Pd*), *P. calamagrostidis* (*Pc*)} associated with parasitisation of separate grass-feeding eurytomid species: *Tetramesa phalaridis*, *T. deschampsia*, *T. calamagrostis* respectively, were used in mate recognition experiments as a model for the African *Pediobius* species. All *Pediobius* species used in the study are solitary ecto-parasitoids. The hosts *Tetramesa* species lay their eggs inside young grass stems which they drill with their ovipositor. Hosts grass species are usually found growing together or side by side.

The objective of the study was to establish whether there is either gene flow between sympatric species of *Pediobius* or they are reproductively

isolated. From direct cross-mating where non-conspecifics were mated, 0 females and 13 males were recovered, whilst indirect mating yielded both sexes, 13 females and 7 males.

Full results are shown in Tables 4.3 and 4.4. Statistical analyses were not possible due to the small number of offspring produced.

The lack of females offspring and small numbers of males from non-conspecifics mating pattern may have been as a result of one or a combination of the following: (i) unsuccessful insemination, (ii) insemination but no fertilisation, (iii) fertilisation but no development, (iv) not enough host larvae for oviposition, (v) unsuitable host-larval stage for *Pediobius* oviposition, or (vi) wrong time of parasitoid release for oviposition.

The male courtship repertoire in some Hymenoptera has been demonstrated as species-specific, which involves a number of useful features for species recognition, and these include visual displays such as leg tapping, antennation, wing vibration, genitalia and mouthparts display, ritualised dancing, other tactical stimulation, attraction, recognition and post-copulatory grooming (van den Assem, 1975; Matthews, 1975; Jervis, 1979). The following factors may have had an effect in the mate recognition system.

#### **4.5.1 Mate selection barriers**

The behaviour of insects whilst in captivity, such as in laboratory experiments has been shown to differ from that in the field. In the field mate locating behaviours have been observed in many insect species. Two forms of

mate waiting aggregations have been recognised: (i) lekking polygyny and, (ii) searching polygyny. In the former, males aggregate to wait for females. For example, in the mating behaviour of males of *Aphelopus melaleucus* Dalman (Hymenoptera: Dryinidae) that aggregate in the canopies of trees in large numbers and wait for females (Jervis, 1979), whilst the sandfly *Lutzomyia longipalpis* Lutz and Neiva (Diptera: Psychodidae) (Jones and Hamilton, 1998) and the prairie mole cricket *Gryllotalpa major* Saussure (Orthoptera: Gryllotalpidae) aggregate in burrows from which they make their mating calls to attract the females. In the latter approach, males move in numbers searching for females (Ide and Kondoh, 2000).

#### 4.5.2 Reaction to pheromones

A relatively recent study has reported that environmental differences may modify both male pheromone production and female mate choice (Clark *et al.*, 1997). In the laboratory where conditions differ from those in the field, both male and female mating behaviour strategies could be disrupted resulting in a change of mate selection system (Ide and Kondoh, 2000). All insects used in this study were reared and mated in laboratory cultures, which may have affected male pheromone production and quality, as well as female receptivity. Male mating success may have also been influenced by the mix-mating systems and the type of signals produced by the females, which may have not been recognised by the males, due to pheromone communication barrier between non-conspecifics. Females are attracted to a male by the type and chemical

structure of pheromone dispersed because sex pheromones play an important role in species isolation (Ward and Morton, 1991). Studies conducted on parasitoid Hymenoptera have shown that mate selection relies on the perception of chemicals (sex pheromones) from the opposite sex, and these chemicals have long been recognised as important cues in sexual selection in insects. Heimpel *et al.* (1997) conducted mating system experiments to confirm reproductive isolation and genetic variation between two species of *Bracon* Ashmead (Hymenoptera: Braconidae). Heimpel *et al.* first considered the braconid population that attack the noctuid moth larvae *Heliothis virescens* Fabricius (Lepidoptera) in Barbados as a strain of *B. hebetor* Say, a parasitoid of *Plodia interpunctella* Hübner infesting store grains in Barbados and USA. Although they parasitise different hosts, and live allopatrically, these two species were found morphologically indistinguishable. However, from the results of cross-mating experiments, they found out that the two parasitoids were reproductively isolated, and their result was further confirmed from sequence analysis of the mitochondrial 16S rRNA gene data.

#### 4.5.3 Behavioural differences

In eulophids copulatory behaviours have been observed and described as consistent within a population and used as reliable characteristics for separating sibling species (see Matthew, 1975, Bryan, 1980). Characteristic wing fanning by males, for example, has been used to separate species of the pteromalid wasp *Lariophagus distinguendus* Förster (Kogan and Legner, 1970).

Askew (1968) suggests that chalcid male wings may be more important for use in courtship than in dispersal. The importance of male wing fanning in pheromone dispersal was first investigated by Miller and Tsao (1974) in the mating behaviour of *Nasonia vitripennis*. In the tests, Miller and Tsao removed the wings from the males and set them for mating with conspecific females. They found that offspring from females paired with wingless males were all males. They concluded that production of all-male offspring could have implications on either (i) the females not recognising wingless males because of their new morphology, or (ii) the males' inability to disperse their sex pheromone in the absence of wings to attract the females. In another behavioural study of *Lariophagus* species, Ruther *et al.* (2000) described male wing-fanning as component of female-derived sex pheromone because they detected that wing fanning was only activated when the males become close to the females between 0-5mm.

#### 4.5.4 Sexual dimorphism

Morphological and structural differences have also been described as very useful tools in species mate recognition system particularly in parasitoid Hymenoptera that are sexually dimorphic. For example, differences in antennal structure, segmentation, length, and distribution of setae, shape and structure of the abdomen. During the precopulatory phase, for example, males of the ichneumonid wasp *Pimpla turionellae* L. perform antennal strokes on the female's antennae. The frequencies of the strokes become very rapid only when

a female becomes receptive and ready to copulate. When examined after copulation using the SEM, a glue-like substance was normally found on the antennae of conspecific males (Bin *et al.*, 1999). This glue substance was not found on antennae of non-conspecific males that were rejected by females when paired for mating. They concluded that the stroking was correlated with female receptiveness. Amongst *Pediobius* species used in this study, there are distinct morphological differences such as distribution of setae and length and shape of female gaster. These differences may have influenced mate selection system and recognition among the different species.

#### 4.5.5 Mate recognition signals

Successful copulation may depend on mate recognition and acceptance, which are strongly influenced by the types and mate selection characteristics such as vibrational signals. Sivinski and Webb (1989) found that signals produced by courting males of three species of braconid as they approach females differ in frequency, duration and intervals between sounds. Another example is the successful separation of the tree frog species *Litoria electrica* Ingram and Corben (Anura: Hylidae) from its close relative *Litoria rubella* Gray based on morphological differences and mating calls (Ingram and Corben, 1990).

Gwynne and Jamieson (1998) acknowledged the importance of sexual dimorphisms as aspects of mate selection and reproductive barriers in insect mating systems. Where sex dimorphisms are clearly differentiated between

species, this would influence mate selection process because peculiar characteristics found on functional parts such as on the abdomen, legs, antennae, wings and mouthparts may not be recognised for male acceptance by a female. Because of the differences in morphology sexual antagonistic behaviours become prevalent as females/males could not recognise their partner, a behaviour that plays a key role in the evolution of mating systems and many reproductive traits in insects (see Arnqvist and Nilsson, 2000). In his review of sexual selection acting on signals and receiving mechanisms in leafhopper species, Hunt (2001) revealed that sexual competition among males of *Graminella nigrifrons* involves differences in their vibrational signals, and that females of *Enchenopa binotata* Say (Homoptera: Membracidae) discriminate among males based on characteristics of such signals. Therefore, mate selection acting on signal mechanism may result to competitive interactions amongst males and females preferences.

#### 4.5.7 Sex allocation

In parasitoid Hymenoptera a rare phenomenon in reproductive behavioural pattern has also been recently described. Previously, species with this phenomenon now recognised to have a 'dual developmental pathways' have been described as sibling complexes (Parkes and Walter, 2001). Males and females of a population with this rare phenomenon develop separately. For example, the heteronomous aphelinid parasitoid *Coccophagus gurneyi* Compere (Hymenoptera) where females are diploid and develop internally within their

mealybug host *Pseudococcus calceolariae* Maskell (Homoptera: Pseudococcidae) whilst males are haploid and hyperparasitic, and develop on primary parasitoid larvae within the mealybug. This alternate host relationship and sex allocation was revealed through mating behaviour experiments in which oviposition was dictated by two factors, (i) whether the host mealybug is parasitised, and (ii) the size of the parasitoid it contains (Parkes and Walter, 2001). Parkes and Walter (2001) found out that male eggs were deposited if the mealybugs were small or if the parasitoid in it was small, and the reverse for the female. In an earlier study, Walter (1993) carried out mating behaviour experiments between two sympatric species of *Coccophagus battletti* Distant and *C. lutescens* Distant. He observed protracted interactions between male and female of the two species, especially with post-copulatory behaviour, and concluded that both are reproductively isolated. Although there were interactions between males and females of different *Pediobius* species used in this study, the results showed that no fertilisation occurred and therefore no progeny.

#### 4.5.8 Conclusion

The three *Pediobius* species studied here occur sympatrically in nature, and on different hosts. The absence of female progeny in the F1 generation where non-conspecifics were cross-mated could have been as a result of the following: (a) males sex pheromones not recognised by non conspecific females, (b) *Pediobius calamagrostidis* male may not recognise *P. phalaridis* morphologically because a female of *P. phalaridis* has a longer abdomen than a

female of *P. calamagrostidis*, (c) mate searching strategy may have also been a factor in the failure for males securing females for fertilisation, because if two species have different mate finding strategies, then it will be very unlikely for a successful insemination to occur, (d) courtship differences causing females of one species not to recognise non-conspecific males, or (e) genetic isolation. Morphological characters found different between species used in this study are the shape and length of the female abdomen of all three species of *Pediobius*. These morphological differences may have contributed in behavioural differences during mating sessions between species.

Studies involving female insects mating preferences and factors responsible for behavioural differences between different species have been carried by many workers (Allen *et al.*, 1994; Heimpel *et al.*, 1997; Jones and Hamilton, 1998). However, what is crucial is to identify the underlying mechanisms associated with reproductive isolation between sympatric species in an attempt to understand the evolutionary process involve in insect mating systems (e.g. Wells and Henry, 1995). Mating tests in this study produced no evidence to suspect that the three UK *Pediobius* species represent a single taxon. This study may be used as a model in investigating cross-species mating of African *Pediobius* species associated with parasitisation of lepidopteran pests of cereal crops in order to establish their reproductive compatibility, an important factor in considering potential candidates for use in a classical biological control programme.

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**CHAPTER FIVE****PHYLOGENETIC ANALYSIS OF *PEDIOBIUS FURVUS* POPULATIONS**

## 5.1 Introduction

In most classical biological control programmes of insect pests, exotic parasitic Hymenoptera species are the most commonly used. A few such examples are (i) the successful control of the hispid beetle species *Promecotheca reichei* Baly in Fiji and *P. cumingi* Baly in Sri Lanka by the eulophid parasitoid *Pediobius parvulus* Férrière (see Fernando, 1972) and (ii) the control of the Mexican bean beetle *Epilachna varivestis* and the eggplant spotted beetle *Henosepilachna vigintioctopunctata* in USA and India, respectively by *Pediobius foveolatus* Crawford (Saitoh and Matsumoto, 2000, Chiu and Moore, 1993). In Africa, the most successful neotropical hymenopteran parasitoid among several natural enemies used to combat the homopteran cassava mealybug *Phenacoccus manihoti* Matile-Ferrero was *Apoanagyrus (Epidinocarsis) lopezi* De Santis (Encyrtidae) (see Herren and Neuenschwander, 1991; Neuenschwander, 2001). However, before being released into their new environment and thereafter, the natural enemy species (parasitoid) of any given pest should be identified and be identifiable in order to monitor their establishment and spread, and to determine their relative abundance. To achieve this many workers have relied previously on traditional morphological characterisation, host association and behaviours as sources of information for species identification and delimitation (see Bouček 1965; Kamijo 1986; Graham, 1990). Whilst these traditional techniques remain popular among taxonomists molecular distinction between species is potentially more accurate

and sensitive than those traditional methods. This is because evidence has been shown that different natural populations of parasitoids with distinct adaptations may exist with no or very little morphological or behavioural divergence. Such organisms have frequently been referred to as biological species (see Claridge and den Hollander, 1983; Diehl and Bush, 1984; Claridge and Morgan, 1993; Perring, 1995; Lin and Ritland, 1997; Pinto *et al.*, 1997; Hoy *et al.*, 2000).

Biological species have been commonly demonstrated and defined as natural populations that are reproductively isolated from one another (Mayr, 1942). However, reproductive isolation often correlates with genetic differences in populations of closely related and morphologically similar species, a phenomenon which may maintain their biological characters as a result of lack of gene flow between them (Claridge, 1988). For example, within panmictic populations, careful studies of insect biotypes such as parasitoids of aphid species have been found often with the existence of distinct clones in the parthenogenetic forms, and genetic distinctiveness and reproductive isolation in sexual ones (Loxdale *et al.*, 1983). Successful separation of such biotypes has only been possible with the application and use of molecular techniques to reveal genetic character differences between individuals (Taylor *et al.*, 1997).

Many workers have reviewed and discussed the applications of molecular techniques; their suitability, reliability and methods of data interpretation (e.g. Loxdale and Lushai, 1998; Caterino and Cho, 2000). Cook

(1996) provides a very useful beginners guide to molecular markers for entomologists. Simon *et al.* (1994) compiled a list of primers that is still widely used for the amplification of genes in the mitochondrial genome and provide a tool for assessing phylogenetic usefulness of specific genes.

The rapid evolution of molecular biology and its techniques have provided fundamental understanding of the nature of genetic material of individual organisms and methods of investigating such genetic variability (Awise, 1994). Among the plethora of conventional molecular techniques now used are protein (allozyme) electrophoresis analysis and many DNA-based techniques such as restriction fragment length polymorphism (RFLP), single and multilocus DNA fingerprinting (minisatellite), mitochondrial DNA (mtDNA) and sequencing, randomly amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP). The applications of these techniques provide new sources of genetic markers that in most instances allow individual organisms to be identified either as homozygote or heterozygote at given loci. Those techniques are now widespread among geneticists and gaining popularity among taxonomists.

### **5.1.1 Polymerase Chain Reaction (PCR)**

As an *in vitro* technique, PCR allows the linear enzymatic amplification of a specific DNA region that lies between two regions of a known sequence (Innes *et al.*, 1990). It is a cycling process that involves heating, denaturing, and annealing during which the target DNA is replicated in each cycle.

Reaction components include buffers, magnesium chloride ( $\text{MgCl}_2$ ), a pair of synthetic oligonucleotides or primers, dNTPs and *Taq* polymerase. PCR-based approaches permit increased direct access to the phylogenetic information content of DNA sequences from both nuclear and cytoplasmic genes, coupled with appropriate methods for sequence determination and interpretation of haplotypes (see Kocher *et al.*, 1989; Clark, 1990; Ruano *et al.*, 1990). The development of PCR and its advantages has revolutionised the importance of molecular biology in systematics because with PCR, only minute quantities of DNA are required for amplification and, these have been used to reveal useful sets of individual characters for the estimation of phylogenies of organisms (Arnheim *et al.*, 1990). The technique involves extraction of DNA, amplification and electrophoretic separation of the molecules on a gel so that polymorphism can be detected, either by reading sequences directly or by using restriction enzymes to produce fragments of DNA which indirectly quantifies sequence variation. In this section of the thesis, PCR-based techniques were carried out to investigate relationships of six populations of *Pediobius furvus* in Africa by analysis of mitochondrial DNA sequences.

## 5.2 Materials and Methods

Several pilot studies were carried out in this study. *Pediobius* species and other chalcidoid species including *Spalangia* and *Nasonia* (Pteromalidae) were used in the pilot study for DNA extraction (Table 5.1). A known number from each species was used in each extraction method and results compared.

Three DNA extraction methods previously used for insects were initially used for DNA extraction due to the paucity of specimens and these are described below. All extractions by each method were carried out according to manufacturer's recommendations. At the end the method that yielded the best quality and larger amount of DNA was used for DNA extraction in *Pediobius* and analysed.

**Table 5.1:** Chalcidoid species used in the pilot extractions of DNA

1. <i>Pediobius brachypodium</i>	Eulophidae	10. <i>Nasonia</i> sp.	Pteromalidae
2. <i>P. phalaridis</i>	Eulophidae	11. <i>P. furvus</i> (Kenya)	Eulophidae
3. <i>P. festucae</i>	Eulophidae	12. <i>P. furvus</i> (Benin)	Eulophidae
4. <i>P. deschampsiae</i>	Eulophidae	13. <i>P. furvus</i> (Ghana)	Eulophidae
5. <i>Tetramesa</i> sp.	Eulophidae	14. <i>P. furvus</i> (Togo)	Eulophidae
6. <i>P. dactylis</i>	Eulophidae	15. <i>P. furvus</i> (Guinea)	Eulophidae
7. <i>P. calamagrostidis</i>	Eulophidae	16. <i>P. furvus</i> (S. Leone)	Eulophidae
8. <i>P. phleum</i>	Eulophidae	17. <i>P. furvus</i> (Benin)	Eulophidae
9. <i>Eurytoma mayri</i>	Eurytomidae	18. <i>Spalangia cameroni</i>	Pteromalidae

The following extraction methods were used in pilot studies.

### 5.2.1 Phenol-chloroform method

Sample preparation in Eppendorf tubes was carried out in a laminar flow hood to prevent contamination. 0.4g SDS was added to 20ml of phenol-chloroform extraction buffer (Tris HCL+EDTA) in a universal test tube to make a 2% SDS solution and mixed well by shaking. Sterilised Eppendorf tubes were labelled. 100µl of extraction buffer was eluted into each tube containing a single individual and then homogenised. After grinding, 2µl of

10mg/ml proteinase-K was added into each tube, and incubated at 55°C for 2 to 3 hrs, or at 37°C overnight.

An equal volume Phenol chloroform (Phenol: Chloroform: IAA in a ratio 24:24:2) was added to each tube, and tubes agitated for about 5 minutes until the solution was an emulsion. This was followed by centrifugation at 13000 rpm for 15 minutes. The supernatant was then removed discarding the bottom phenol layer. After transferring of supernatant into new Eppendorf tubes, an equal volume of chloroform (chloroform: IAA, 24:1) was then added to remove the phenol chloroform and further extract the DNA. Tubes were again agitated until the extraction emulsified. Tubes were centrifuged at 13000 rpm for another 15 minutes. The supernatant was again removed. Two volumes of 100 % ice-cold ethanol plus 1.3µl of sodium acetate were added to the supernatant and tubes were placed in freezer set at -70°C for 15 minutes. Tubes were again centrifuged at maximum speed (1300 rpm) for 15 minutes, followed by removal of ethanol from tubes leaving the DNA pellets, followed by the addition of 70% ethanol. With a P200 pipette, ethanol was completely removed from each tube leaving the pellets. Tubes were vacuum-dried for complete removal of ethanol. 50µl of TE buffer was then added into tubes to re-suspend the DNA at room temperature. 20µl of sterile water (nqH<sub>2</sub>O) and 0.4 RNase were then added at room temperature for 10 minutes and placed in warm bath incubator at 37°C for two hours. DNA extractions were run immediately in agarose gels or stored at -20°C.

### **5.2.2 Livak DNA extraction**

50  $\mu$ l of pre-heated Livak grinding buffer (5M NaCl, 0.5M EDTA, 20% SDS, Tris Base, Sucrose) was added to each sample, and samples were homogenised by grinding followed by the addition of 20 $\mu$ l of Proteinase-K. A separate pestle was used per tube to prevent contamination. Tubes were placed in a 65°C bath for 30 minutes. After incubation, tubes were briefly micro centrifuged at 13000 rpm and 14 $\mu$ l 8M K-acetate was added, mixed by inversion and then incubated on ice for 30 minutes. Tubes were then centrifuged at 13000 rpm for 15 minutes. The supernatant was then transferred into new tubes which were centrifuged for another 15 minutes. This was repeated until pellets (protein and other cell debris) were no longer seen. 200 $\mu$ l ice-cold 100% ethanol was then added and tubes placed at -20°C for 2 hr or, for overnight. Samples were centrifuged at 13000 rpm for 5 mins and ethanol was removed. Tubes were centrifuged in Eppendorf dryer for 10 mins at room temperature. Pellets were then re-suspended in 20 $\mu$ l of TE buffer and incubated (in warm bath) at 65°C for 10 minutes. After incubation pellets were briefly centrifuged and vacuum-dried at room temperature for 10 minutes. The DNA concentrate was then stored in a -20°C freezer, or run on an agarose gel immediately.

### **5.2.3 QIAamp DNA extraction protocol**

Alcohol-preserved samples deep frozen at -70°C were extracted in this way following manufacturer's instructions. Individual wasps were placed in

Eppendorf tubes with 180µl of buffer ATL and homogenised using a pestle. 20µl of Proteinase-K was added to the tube and mixed by pulse-vortexing and tubes were incubated on a rocking-platform in a 56°C hybridisation oven for 3-5 hrs. Incubated tubes were normally briefly centrifuged and 200µl of AL buffer was added and mixed by pulse-vortexing for 15 seconds, followed by 10-min incubation at 70°C. A white precipitate normally formed on addition of the AL buffer. 200µl of 100% ethanol was then added to the samples, which were mixed by vortexing for about 15 secs and then transferred into 'spin' columns containing hydrophobic filters. Columns were centrifuged at 8000 rpm for 1 min. Discarding previous collection tubes containing the filtrate, spin columns were transferred into new collection tubes and 500 µl of buffer AW1 added followed by 1 min centrifugation at 8000 rpm. Spin columns were again transferred into new collection tubes. 500µl of buffer AW2 was then added to each column and centrifuged at 13000 rpm for 3 min. To eliminate any chance of possible carryover of buffer AW2, columns were transferred into new collection tubes and centrifuged at maximum speed (13000 rpm) for 1 min. Spin columns were then transferred into 1.5ml Eppendorf tubes in which the DNA was re-suspended in 200µl of buffer AE by incubation for 1 min at room temperature. A final centrifugation at 8000 rpm for 1 min was done to collect the DNA. Final collection tubes containing the DNA were stored at -20°C. With this extraction method, three DNA concentrations were normally obtained. Stored DNA was then amplified using general insect primers and

products were visualised under ultra violet transilluminator on 1.5% agarose gel stained with ethidium bromide. The latter was used in the final analysis because of its provided reliable extraction of high quality DNA in a larger volume. Samples investigated in the study are coded according to localities as listed in Table 5.2.

**Table 5.2:** *Pediobius furvus* populations investigated in the study.

Origin	Locality	Sample code	No. of samples
Benin	Cotonou	BN	24
Togo	Cacaveli	TC	6
	Aneho	TA	4
	Tabligbo	TT	10
Ghana	Tema	GT	6
	Accra	GA	4
	Takoradi	GK	10
Kenya	Machakos	KM	9
Sierra Leone	Lungi	SL	6
	Port Loko	SP	6
	Kambia	SK	9
Guinea	Forecariah	GF	9
	Kindia	GN	12

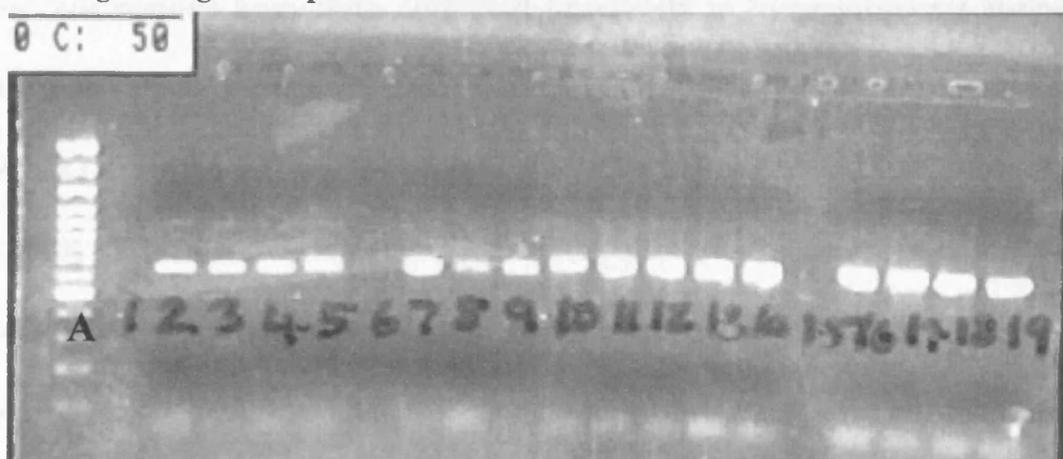
#### 5.2.4 PCR protocols

##### 5.2.4.1 Checking gel (Agarose)

The quality of DNA fragments was normally checked on agarose gels prepared by mixing required amount in 1xTBE and heated in microwave for 2 mins. To enable fragment visualisation, ethidium bromide was added to agarose after heating and left to set in casting trays. Supernatants were individually loaded into gel lanes and electrophoresis was performed at a

constant 90 volts for 1 hr and 10 minutes. When electrophoresis was completed gels were removed from casting trays and placed on a UV transilluminator for DNA visualisation. The size of DNA amplicons was estimated by comparing with a standard Lambda ( $\lambda$ ) ladder. Gels that show clear bands were photographed and kept as reference (Fig. 5.1).

**Fig. 5.1:** QIAamp DNA extraction test results of various Chalcidoid wasps on agarose gel compared with standard lambda ladder.



#### Legend

A. Lambda ladder, 1. Control, 2. *Pediobius brachypodium*, 3. *P. phalaridis*, 4. *P. festucae*, 5. *P. deschampsiae*, 6. *Tetramesa* sp., 7. *P. dactylis*, 8. *P. calamagrostidis*, 9. *P. phleum*, 10. *Eurytoma mayri*, 11. *Spalangia cameroni*, 12. *Nasonia* sp., 13 *P. furvus* (Kenya), 14 *P. furvus* (Benin), 15 *Tetramesa petiolata*, 16 *P. furvus* (Togo), 17 *P. furvus* (Ghana), 18 *P. furvus* (Guinea), 19 *P. furvus* (Sierra Leone).

#### 5.2.4.2 DNA amplification

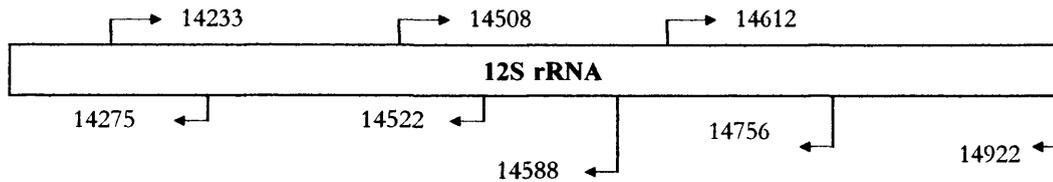
Nuclear and mitochondrial DNA (mtDNA) which consists of many major coding regions requires specific primers for amplification. As a result, several primer pairs were tested for the amplification of the mitochondrial genome and nuclear genes of insects. These included primers already used in recent studies for DNA amplification of a wide range of organisms including

the Hymenoptera (see Liu and Beckenbach, 1992; Simon *et al.*, 1994, Arias and Sheppard, 1996; Taylor *et al.*, 1997; Gauthier *et al.*, 2000; Babcock *et al.*, 2001; Chang *et al.*, 2001; Whitfield *et al.*, 2002). Pilot tests were carried out for the amplification of the following gene regions, the nuclear genes 28S gene, Internal Transcribe Subunit1 (ITS1), 12S ribosomal DNA, and the mitochondrial cytochrome oxidase I, cytochrome oxidase II and cytochrome *b*. These gene regions have been amplified previously in hymenopterous species such as in *Trichogramma* (Trichogrammatidae), *Encarsia* (Encyrtidae) and *braconids* (Braconidae) (see Campbell *et al.*, 1993; Babcock *et al.*, 2001; Chang *et al.*, 2001, Whitfield *et al.*, 2002). Tests were first carried out for the amplification of the D2 region of 28S (= 560 bp) since this had recently been amplified on 87 species of Eulophidae including European species of *Pediobius* and their sister group, the Elasmidae, which showed a high level of length and sequence polymorphism (see Gauthier *et al.*, 2000). Gauthier *et al.* (2000) PCR protocols for the amplification of 28S were followed. Amplifications were normally carried out in a 25 $\mu$ l total volume containing 2.5 mM *Taq* DNA polymerase, 1.5 mMol MgCl<sub>2</sub>, 12.5 nMol dNTPs, and 20 pmol primers. Amplification was achieved on few samples that showed no variation between Kenya, Togo and Ghana samples.

Initially, suggested PCR reactions and thermocycling programmes were tested and when these failed to produce good result, reactions were optimised by altering the proportion of reaction cocktail ingredients and thermocycling

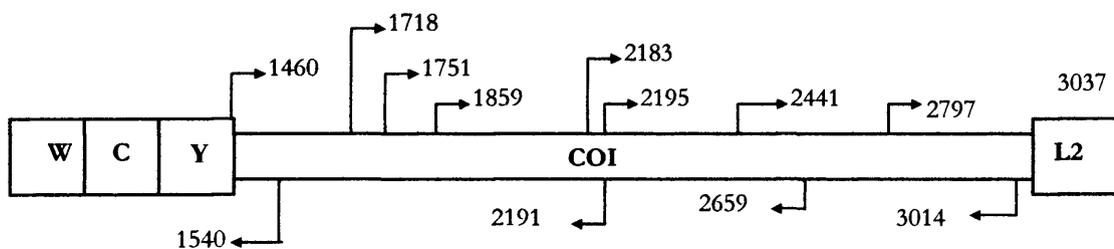
programmes. The same samples were always used for testing all the genes with great precautions to prevent contamination. A multiplex master mix containing 5 $\mu$ l of each primer, 90 $\mu$ l H<sub>2</sub>O sigma, and 12.5 $\mu$ l of Multiplex kit was also used for optimisation. PCR products were cleaned using QIAQuick PCR purification kit and Geneclean PCR Turbo kits respectively, the sequences did not reveal any variations between individual sequences. Reactions were also optimised when bands were weak or doubled by increasing the amounts of MgCl<sub>2</sub> and *Taq* polymerase thereby decreasing the amount of sigma H<sub>2</sub>O in a 25 $\mu$ l master mix. As the amount of DNA extractions were running low, further tests were carried out for cytochrome *b* with optimisation of MgCl<sub>2</sub> and *Taq* polymerase (e.g. 2.5 $\mu$ l Buffer, 2.0 $\mu$ l MgCl<sub>2</sub>, 2.0 $\mu$ l dNTPs, 0.25 $\mu$ l of each primer (CB1/CB2), 0.2 $\mu$ l *Taq* polymerase, 5.0 $\mu$ l DNA and 10.8 $\mu$ l H<sub>2</sub>O sigma amplified Cytochrome *b* gene region. PCR conditions for the above were 15 mins at 95°C initial denaturation, 35 cycles of 94°C denaturation (30 secs), 50°C annealing (90 secs), 72°C (90 secs) extension, and 72°C (10 mins) final extension. The latter primer pair CB-J-10933 and CB-N-11367 (alias CB1/CB2) of Simon *et al.* (1994) amplified more than 60% of *Pediobius* tested. Primer pairs tested, their sequences and some of the regions of insect mitochondrial and nuclear genome tested for amplification are shown in Figs. 5.2.1-5.

**Fig. 5.2.1:** Primers tested for DNA amplification and their positions along the rRNA region in insects.



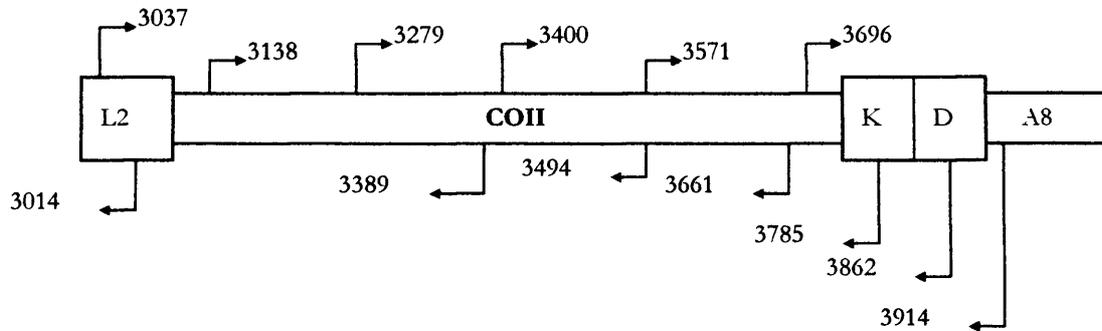
Oligonucleotides	Direction	Sequences
SR-N-14588 (alias 12 Sair)	Forward	5'-AAACTAGGATTAGATACCCTATTAT-3'
SR-N-14756 (alias 12 Sh)	Reverse	5'-GACAAATATTCGTCGCCAGCAGT-3'

**Fig. 5.2.2:** Primers tested for DNA amplification and their positions along the Cytochrome Oxidase I (Adapted from Simon *et al.*, 1994).



Oligonucleotides	Direction	Sequences
C1-J-1751 (alias Ron)	Forward	5'-GGATCACCTGATATAGCATTCCC-3'
C1-N-2191 (alias Nancy)	Reverse	5'-CCCGGTAAAATTTAAAATATAAACTTC-3'
C1-J-2183 (alias Jerry)	Forward	5'-CAACATTTATTTTGATTTTTTGG-3'
TL2-N-3014 (alias Pat)	Reverse	5'-TCCAATGCACTAATCTGCCATATTA-3'
C1-J-2441 (alias Dick)	Forward	5'-CCAACAGGATTTAAAATTTTTAGATGATTAGC-3'
C1-N-2659 (alias Milal)	Reverse	5'-GCTAATCCAGTGAATAATGG-3'

**Fig. 5.2.3:** Primers tested for DNA amplification and their positions along the Cytochrome Oxidase II (Adapted from Simon *et al.*, 1994).

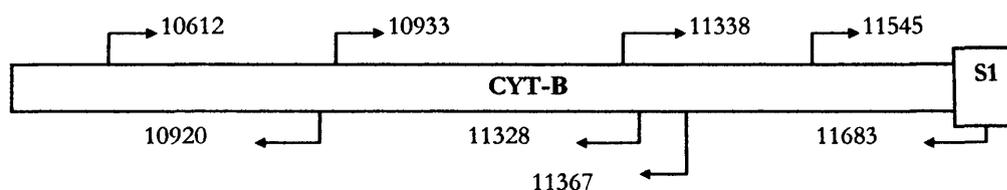


Oligonucleotides	Direction	Sequences
C2-J-3279 (alias A171)	Forward	5'-CGCTGAAATTATTTGAAC-3'
C2-N-3661 (alias Barbara)	Reverse	5'-ATTTCTGAACATTGACCA-3'
TL2-J-3037 (alias A-tLeu)	Forward	5'-ATGGCAGATTAGTGCAATGG-3'
A3389 (alias Marylin)	Reverse	5'-TCATAAGTTCARTATCATTG-3'

**Fig. 5.2.4:** Primers tested for amplification of 28S rDNA gene region in insects.

Oligonucleotides	Direction	Sequences
D2-28SrDNA	Forward	5'-TGTTGCTTGATAGTGCAG-3'
D2-28SrDNA	Reverse	5'-ATTCGTGTTTCAAGACGGG-3'

**Fig. 5.2.5:** Primers tested for DNA amplification and their positions along the Cytochrome *b* region (Adapted from Simon *et al.*, 1994)



Oligonucleotides	Direction	Sequences
CB-J-10933 (alias CB1)	Forward	5'-TATGTACTACCATGAGGACAAATATC-3'
CB-N-11367 (alias CB2)	Reverse	5'-ATTACACCTCCTAATTTATTAGGAAT-3'

#### 5.2.4.3 GeneClean

This is the process of removing PCR products (inhibitors/impurities) from samples prior to sequencing using the GeneClean Turbo for PCR kit (BIO 101). Only samples amplified with primer pair CB1/CB2 were used. 20µl of PCR product was normally transferred to a 1.5 ml Eppendorf tube to which 100µl of GeneClean Turbo salt was added. Tubes were vortexed and then centrifuged for 5 secs. From Eppendorf tubes the samples were transferred into GeneClean Turbo filter cartridges placed in catch tubes, and the tubes were centrifuged at 13000 rpm for 5 secs. 500µl of GeneClean Turbo Wash was then added to each sample and centrifuged at 13000 rpm for 5 secs. After centrifugation, catch tubes were emptied and cartridges replaced into them. To each, 500µl of GeneClean Turbo Wash was added and centrifuged at 13000 rpm for 5 secs. Catch tubes were again emptied and cartridges replaced into catch tubes and centrifuged at 13000 rpm for 4 mins. After centrifugation,

filter columns were transferred into new GeneClean catch tubes with a removable lid. 30 $\mu$ l of GeneClean Turbo elution solution was added into each tube and incubated for 5 mins at room temperature. Tubes were again centrifuged at 13000 rpm for 30 secs, cartridges were removed and discarded, catch tubes were labelled and pellets were stored in freezer (-20°C).

#### **5.2.4.4 Better Buffer protocol**

The Better Buffer was prepared using the following ingredients, 3 $\mu$ l of mixture of 2.5 $\mu$ l better buffer plus 0.5 $\mu$ l of sequence kit, and 1.5 $\mu$ l primer at a concentration of 1.6 pmol/ $\mu$ l and the mixture was placed in freezer. Two primers (forward and reverse) were used in this protocol. For each sample two PCR strips were prepared and labelled. 1.5 $\mu$ l of one primer was first added into the forward PCR reaction and the other into the reverse. This was followed by the addition of 3 $\mu$ l of sequencing kit, and DNA added to the strips. The PCR was run using Better Buffer thermocycling programme stored on PCR machine. At the completion of thermocycling, samples were ready for sequencing.

#### **5.2.4.5 Cleaning of PCR products using Isopropanol protocol**

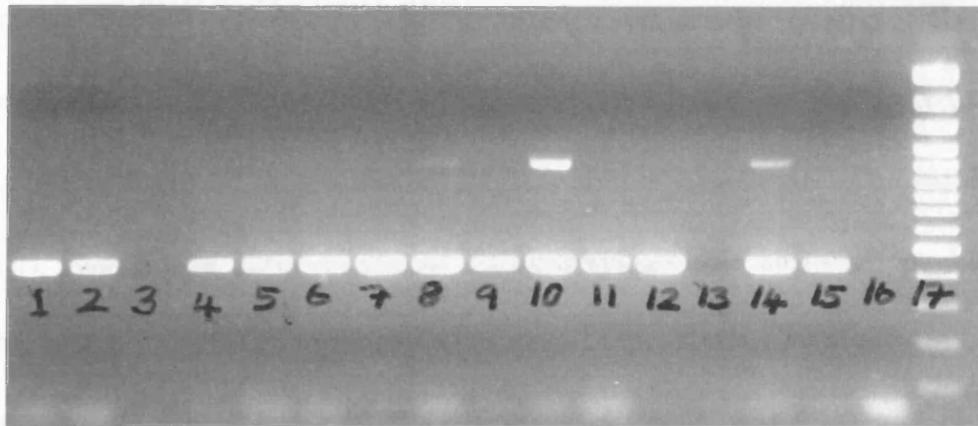
Samples from Better Buffer PCR were used in this protocol. 10 $\mu$ l of each PCR was transferred into a 0.5ml Eppendorf tube, and 90 $\mu$ l of 100% isopropanol mixture was added and vortexed briefly, and precipitated at room temperature for 10 mins. Samples were then centrifuged at 13000rpm for 30 mins. After centrifugation, supernatant was removed from each tube using a

suction pump. 150µl of 70% isopropanol was then added to each tube and mixed by gentle tapping followed by centrifugation at maximum speed (13000 rpm) for 12 mins. The supernatant was then removed and tubes containing pellets vacuum-dried for 10-15 mins and stored in freezer at -20°C and ready for sequencing.

#### 5.2.4.6 Sequencing

All PCR samples that produced the required single target band (e. g. Fig. 5.3) were sequenced using an Applied Biosystems 3100 semi-automated sequencer, following manufacturer's instructions. Samples to be sequenced were first defrosted and then centrifuged at 13000 rpm for 1 min. Prior to sequencing, 15µl of Hi-di formamide was added to the pellets, vortexed for 20 secs and centrifuged, then transferred to plates and denatured for 3 mins at 90°C. 3µl of sequencing dye plus 1.35µl of each primer were added to each tube, and 2.8µl of DNA template was added, and the sequencer set to run overnight.

**Fig. 5.3:** Amplification of *Pediobius* using the Cytochrome b primers CB-J-10933 and CB-N-11367 compared with lambda ladder.



#### Legend

1) KM7 = Machakos, 2) KM8 = (Machakos (Kenya); 3) SP1 = Port Loko, 4) SK2 = Kambia (Sierra Leone); 5) GT3 = Tema, 6) GT4 = Tema (Ghana); 7) TC3 = Cacaveli (Togo); 8) GA2 = Accra, 9) GA1 = Accra, 10) GK1 = Takoradi, 11) GK2 = Takoradi (Ghana); 12) GK 5 = Kindia (Guinea); 13) BN1 = Cotonou, 14) BN11= Cotonou (Benin); 15) TT1 = Tabligbo (Togo); 16) Control strip, 17) Lambda ladder.

#### 5.2.5 Sequence editing

In total, 51 samples were successfully sequenced for the Cytochrome *b* gene region with an average of 372 bp in length. Both forward and reverse sequences for each individual were then assembled automatically to form a sequence contig using Sequencher. Editing of sequences was made when bases between forward and reverse sequences of a sample did not match at certain points. Where substitutions were confirmed indicating differences between individuals within populations, these were cross-checked with the original sequences for confirmation and re-sequenced if necessary.

### 5.2.5.1 Sequence alignment and phylogeny construction

After editing all the sequences were assembled together using the programme Sequencher ver. 3.1.2 to form a consensus contig. Having confirmed with original sequences the longest sequences were brought to the size of the shortest at both 5' and 3' ends before any analysis. The resulting consensus contig was then analysed using phylogenetic software packages, Templeton *et al.*'s (1992) software TCS ver. 1.0.3, Molecular Evolution and Genetic Analysis (MEGA ver. 2.0) (Kumar *et al.*, 2001) and PAUP, (Swofford, 1998).

TCS procedure enables estimation of genealogical relationships among genes at population level using parsimony (Clement *et al.*, 2000). The procedure has been shown to be very useful in revealing ancestral haplotypes that other phylogenetic approach (e.g. Neighbour-joining) assume no longer exist in the populations (see Watterson and Guess, 1997); and also where there is paucity of data with many invariable characters (Clement *et al.*, 2000) as is found with samples used in the present study. During analysis all the sequences are automatically collapsed into haplotypes and the frequencies of the haplotypes in the samples were then calculated based on pairwise comparison from an absolute distance matrix (Templeton *et al.*, 1992). These frequencies were used to estimate haplotypes among populations (see Castelloe and Templeton, 1994).

### 5.2.5.2 Statistical analysis

The nature of distribution of haplotype frequencies of cytochrome *b* sequences (Table 5.3) were investigated by means of an Analysis of Molecular Variance (AMOVA) using Arlequin ver. 2.0 (Schneider *et al.*, 2000). This was done to compare populations statistically to detect genetic structure differences among groups and among populations, and whether the differences (if any) are due to differences in geographic location. The AMOVA procedure hierarchically partitions molecular variance in the DNA sequence data into *apriori* defined groups. To achieve this, sampled area was divided into groups defined according to geographic and regional proximity as follow: Guinea and Sierra Leone (west West Africa) (WWA), Benin, Ghana and Togo (mid West Africa) (MWA) and Kenya (EA) and analysed. For a two-group analysis, WWA populations were grouped with MWA populations together and compared to Kenya population. As a means of control, WWA populations were also grouped with Kenya population and compared to MWA populations. The extent of regional distribution of genetic heterogeneity was investigated by partitioning the data into their six respective originating countries. Statistical significance of the resulting  $F_{CT}$  values was assessed by permutation tests of 10000 iterations each.

## 5.3 Results

### 5.3.1 DNA extraction

High quality DNA from dried, as well as alcohol-preserved, *Pediobius* samples and other hymenopterans used in this study were obtained from QIAamp DNA extraction protocol. Two batches of high quality DNA extractions were obtained for each individual wasp either from -70°C deep-frozen or in 100% ethanol preserved or dry samples and these were suitable for amplification. Livak and Phenol Chloroform DNA extraction methods also worked but did not yield high quality DNA from some hymenopteran samples.

### 5.3.2 Amplification and sequence data

The nuclear genes 28S rDNA amplified only some samples from the *Pediobius furvus* set. No amplification was achieved for samples from Sierra Leone, Guinea and Kenya, and no variation was revealed among samples that were amplified from Benin, Togo and Ghana. However, one of the amplified samples BN1 was used as a representative of *P. furvus* and compared with 28S rDNA sequences of other species of Hymenoptera in GenBank that are used as out-groups and these included *Pediobius alcaeus* (UK), *P. alcaeus* (Ukraine), *Pediobius* sp. (UK), *P. metallicus* (Canary Island) *P. brachycerus* (Spain), *Systasis* sp. (Canary Islands) and *Platynocheilus* sp. (Tunisia). To effect alignment, the original sequence size of *P. furvus* (605 bp long) was trimmed to match with the sequences of out-group species to a total of 459 bp. Variable sites were revealed between out-group species and *P. furvus* (Table 5.4).

Complete sequences for cytochrome *b* gene were amplified with a maximum base length of 412bp for 45 samples. Small differences were found between samples and these were all substitutions at positions 47, 129, 213 and 318 and all were transitions (i.e. 3 A-G, 1 T-C).

### 5.3.3 Phylogenetic analysis

#### 5.3.3.1 Cytochrome *b*

From a TCS-based parsimony network analysis of cytochrome *b* sequences of *Pediobius*, 5 haplotypes- BN1, BN18, BN20, TA1 and KM9 were revealed (Fig. 5.4). The analysis revealed BN1 and BN20 the dominant haplotypes as both constitute the largest numbers of specimens 18 and 15, respectively, whilst haplotype KM9 comprised only one. The various haplotypes and their frequencies distributed in different geographic localities revealed correct placement of all samples according to their original localities.

#### 5.3.3.2 28S rDNA gene

Phenogram reconstruction from 28S rDNA gene sequences of *P. furvus* and other hymenopteran species used as out-groups was carried out using the Kimura 2-parameter model in MEGA procedure based on minimum evolution method (Fig. 5.5).

### 5.3.4 Amino acid translation

No cytochrome *b* sequences were found in GenBank for eulophid species. However, partial cytochrome *b* sequences that were very similar to *P.*

*furvus* sequences were found for *Sycoscapter* sp. 405 (No. AJ298409) with 474bp and *Sycoscapter* sp. 399 (No. AJ298411) with 454bp, which aligned using the BLAST algorithm in an NCBI web-based search (see Altschul *et al.*, 1997). The two hymenopterans are gall wasps of the Agaonidae family associated with *Ficus* species (see Lopez-Vaamonde *et al.*, 2001). Sections of the sequences of both species were very similar to sequences of *Pediobius* haplotype BN1 with each species showing 93% similarity. However, in spite of these regions of similarity, it was observed that cytochrome *b* sequence alignments for the agaonids were split into 3 fragments with the 1<sup>st</sup> complete 120bp followed by an unidentified section, then a 2<sup>nd</sup> with 70bp, then another unidentified section, and the last 60bp; all of which were supposed to be together as one. The discrepancies in the nature of the sequences causing BLAST not to recognise the fragmented sections posed two intriguing questions: (i) are the sequences of the two species non-continuous, or, (ii) is *P. furvus* sequence not a mitochondrial sequence but a non-coding fragmented sequence such as might be found in a nuclear copy? These were not nuclear copies as BLAST revealed alignment positions of haplotype BN1 with a shorter base length (412bp) within the longer base length sequences (474bp and 454bp) of *Sycoscapter* sp. 405 and *Sycoscapter* sp. 399, respectively (see Fig. 5.6). These aligned well together with all *P. furvus* haplotypes in Sequencher ver. 3.1.2 using invertebrate mitochondrial code, and a complete amino acid translation was achieved (Fig. 5.7). Sequences were then analysed in MEGA

ver. 2.0 and a phenogram with bootstrap values was then constructed using Kimura 2-parameter model based on minimum evolution method (Fig. 5.8).

### 5.3.5 Group comparison

Group comparison based on allele frequencies showed that there is variation within populations as well as among populations. Only the proportion of variation present among groups (measured as FCT) is reported which is indicative of group-level phylogeographic structuring (Tables 5.5.1-4). Groups vary by region as shown by  $F_{ST}$  where  $P > 0.05$  (WA/EA = 0.06243, WWA/MWA+EA = 0.06676, WWA/MWA/EA = 0.2827).

## 5.4 Discussion

The initial taxonomic hypothesis of the populations of *Pediobius* species associated with parasitisation of maize stem borers across Africa was that all are different species parasitizing a single host, or perhaps, genetically some local adaptation, reflected in genetic differentiation due to restricted gene-flow might have taken place as a result of the differences in geographic and climatic patterns of its ecological zone. In the light of this, populations were sampled from 13 localities of 6 populations across Africa from Kenya in the East to Sierra Leone in the West and were investigated to reveal whether all the populations are genetically differentiated or comprise a single demographic unit. Analysis for the mitochondrial gene cytochrome *b* revealed 5 haplotypes with their frequency distribution (Fig. 5.4).

#### 5.4.1 Sequence comparison

Sequences of the nuclear gene 28S rDNA did not show any variation between samples. However, sequences of 28S rDNA of five *Pediobius* species (*P. alcaeus* (UK), *P. alcaeus* (Ukraine), *Pediobius* sp. (UK), *P. brachycerus* (Spain)) and two other Hymenoptera species (*Systasis* (Canary Island) and *Platynocheilus* (Tunisia) imported from GenBank aligned fairly well with 28S rDNA sequences of *P. furvus* and revealed differences with all other Hymenoptera species.

Cytochrome *b* sequences of other Hymenoptera species (e.g. *Sycosapter* sp. 405 and *Sycosapter* sp. 399) that were found to be very similar to *P. furvus* sequences were imported from GenBank and these aligned well with the sequences of *Pediobius* haplotypes and yielded a complete amino acid translation (Fig. 5.7).

#### 5.4.2 Phylogenetic inference

Sequence data of cytochrome *b* analysed to establish phylogenetic relationships among the 6 populations collected in Africa using a TCS-based parsimony network analysis for the 5 haplotypes (BN1, BN18, BN20, TA1 and KM9) correctly placed all samples in their different geographic localities which confirmed that they are somehow different genetically at least at population level.

In a previous study, Gauthier *et al.* (2000) used two sets of primer pairs to amplify the 28S rDNA D2 subunit, which proved most informative at

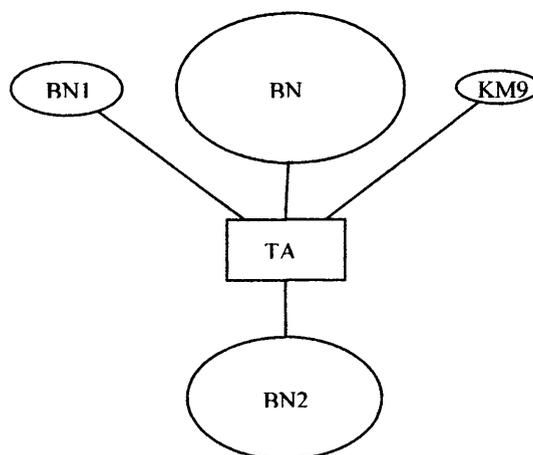
family level in eulophids. In the present study, 28S rDNA gene did not reveal any differences between the few specimens that amplified. However, the combination of primers CB-J-10933 and CB-N-11367 spanned almost the whole of the presumed cytochrome *b* gene region revealing genealogical relationships among individuals within *Pediobius* populations. Geographic differences within the region *Pediobius* was sampled and TCS-based genealogical relationships reflected in this result indicate that gene flow between populations is very low, and that the destructive selection keeping populations of one species apart is strong enough to maintain at least a minimum level of genetic differences between *Pediobius* populations in the subregion. Phenogram based on gene sequence data may represent evolutionary steps that interrelates the various haplotypes. Samples within haplotypes are correctly distributed throughout the whole sampled area according to their original localities (Fig. 5.4) suggesting that the West African populations of *Pediobius* may be clusters of genotypes of one species and the Kenya population may be described as a different species altogether (see Fig. 5.8).

**Table 5.3:** *Pediobius* haplotype frequency distribution from cytochrome b gene sequences.

Country	Locality	Haplotypes					Total
		BN1	BN18	BN20	TA1	KM9	
Guinea	Kindia	-	2	1	2		5
	Forecariah	-		3	1		4
Sierra Leone	Kambia	-	1	1	-	-	2
	Port Loko	4	-	1	-	-	5
Ghana	Accra	1	-	-	1		2
	Tema	1	-	-	1	-	2
Togo	Tabligbo	-	-	1	-	-	1
	Cacaveli	-	-	1	-	-	1
	Aného	2	-	1	1	-	4
Benin	Cotonou	9	2	1	-	-	12
Kenya	Machakos	1	-	5	-	1	7
	<b>Total</b>	<b>18</b>	<b>5</b>	<b>15</b>	<b>6</b>	<b>1</b>	<b>45</b>



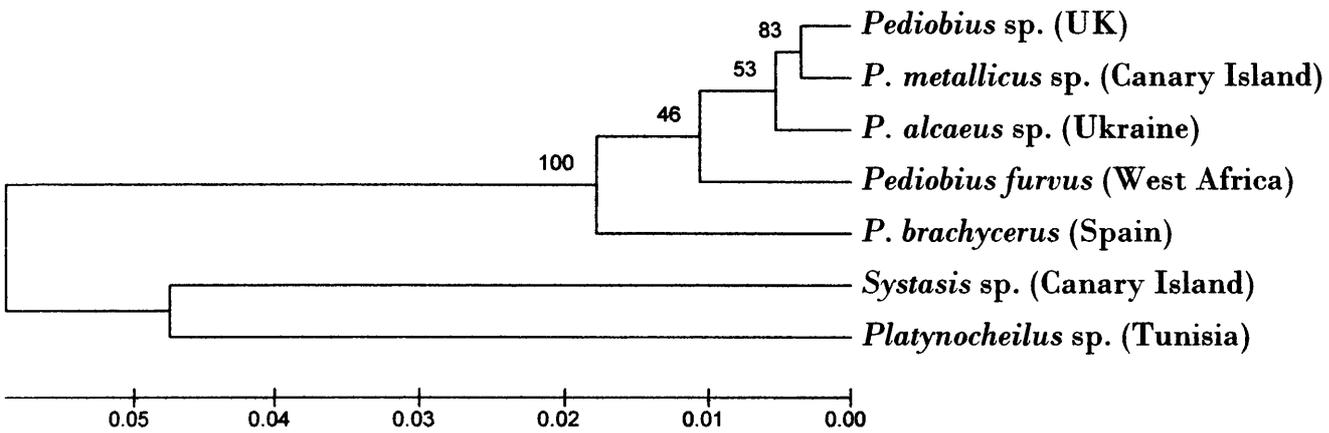
**Fig. 5.4:** Haplotype network from cytochrome b gene fragments uncovered by the present study as estimation of genealogical relationship among the populations, showing specimens distribution.



### Legend

Haplotype	Samples	Original locality	New placement	Country
<b>BN1</b>	BN1, BN2, BN3, BN4, BN8, BN9	Cotonou	Same	Benin
	BN12, BN13, BN16	Aného	Same	Togo
	TA6, TA8	Accra	Same	Ghana
	GA1	Tema	Same	Ghana
	GT4	Machakos	Same	Kenya
	KM7	Port Loko	Same	S. Leone
	SP1, SP2, SP4, SP5			
<b>BN18</b>	BN1, BN21	Cotonou	Same	Benin
	SK7	Kambia	Same	S. Leone
	GF8, GN11	Kindia	Same	Guinea
<b>BN20</b>	BN20	Cotonou	Same	Benin
	KM1, KM2, KM3, KM5, KM6	Machakos	Same	Kenya
	TA3	Aného	Same	Togo
	TC6	Cacaveli	Same	Togo
	TT1	Tabligbo	Same	Togo
	GF4, GF6, GF9	Forecariah	Same	Guinea
	GK2	Kindia	Same	Guinea
	SP5	Port Loko	Same	S. Leone
	SK5	Kambia	Same	S. Leone
<b>KM9</b>	KM9	Machakos	Same	Kenya
<b>TA1</b>	TA1	Aného	Same	Togo
	GK2, GK3	Kindia	Same	Guinea
	GF3	Forecariah	Same	Guinea
	GA3	Accra	Same	Ghana
	GT1	Tema	Same	Ghana

**Fig. 5.5:** Phenogram showing bootstrap values constructed from 28S gene sequence of *P. furvus* and other hymenopterans as out-groups using Kimura 2-parameter model based on Minimum evolution method.







*Sycoscapter* 405 -----T--T-----C-----C--  
 -----  
*Sycoscapter* 399 -----T--T-----C--  
 -----  
 BN1 -----  
 TA1 -----  
 -----  
 BN1 -----  
 -----  
 BN2 -----  
 -----  
 KM9 -----  
 -----

ATAATGCAACATTAAATCGATTTTATTCATTCCATTTTATTTTACCTTTAT  
 N N A T L N R F Y S F H F I L P F I

*Sycoscapter* 405 -----T---T-A--T--T-----T-A--GAGACA--T---  
 - - - L - L I - - - - - E T - -  
*Sycoscapter* 399 -----T---T-A--T--T-----T-A--GA-ACA--T---  
 - - - L - L I - - - - - E T - -  
 BN1 -----G-----  
 -----  
 TA1 -----  
 -----  
 BN1 -----  
 -----  
 BN2 -----  
 -----  
 KM9 -----  
 -----

TATTTTATTTATAGTTATTATACACTTAATATTTCTTCATAGATATGGATCA  
 I L F M V I M H L M F L H S Y G S

*Sycoscapter* 405

A-T-----T-A-----T-----T--T-----  
T - - - - L - - - - - - - - - - - - - - -

*Sycoscapter* 399

A-T-----T-A-----T-----T--T-----  
T - - - - L - - - - - - - - - - - - - - -

BN1

-----

TA1

-----

BN1

-----

BN2

-----

KM9

-----

TCAAATCCAA TAGGAATTAACAGAAATATAAAATAAAATCCCATTTAATCCTT  
S N P M G I N S N M N K I P F N P

*Sycoscapter* 405

-T-A-A-----T-AC-A-----T--T-----CT--GT--A-  
- Y T - - - L L - - I - L - L S - I

*Sycoscapter* 399

-T-A-A-----T-AC-A-----T--T-----T--GT--A-  
- Y T - - - L L - - I - L - F S - I

BN1

-----

TA1

-----

BN1

-----

BN2

-----T-----

KM9

-----

ACTTTTCAATTAAAGATATTATCGGATTTATAATTATAATTTCTATATTACT  
Y F S I K D I I G F M I M I S M L L

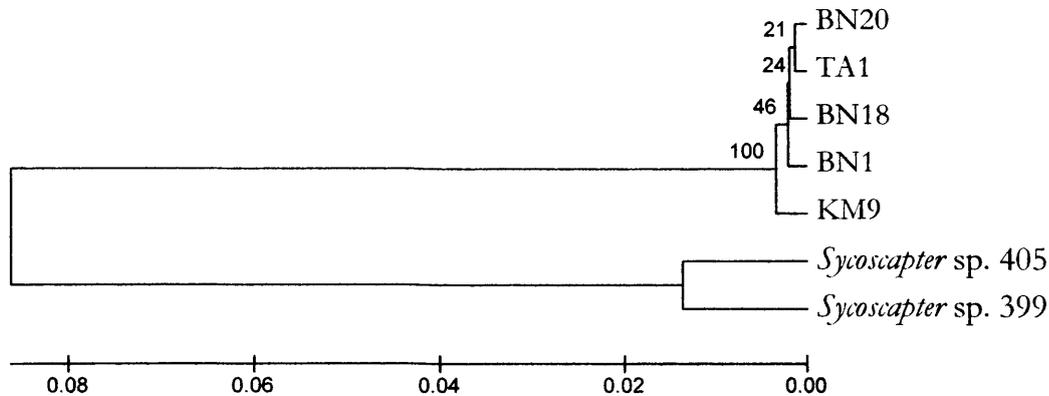
*Sycoscapter* 405 -----TG-T-TC-----T-----C-T--T--T-----  
 - - C F - - - - - L - - - - -  
*Sycoscapter* 399 -----TG-T-CC-----T-----C--C-T--T--T-----  
 - - C F - - - - - L - - - - -  
 BN1 -----  
 TA1 -----  
 BN1 -----  
 - -----  
 BN2 -----  
 KM9 -----T-----  
 - - - - - - - - - - - - - - - - -

TTTAATTAATATATTTAAACCCCTTATATAATATCAGACCCAGAAAATTTTAAT  
 L I N M L N P Y M M S D P E N F N

*Sycoscapter* 405 -----T-----T-----T-G-----  
 - - - - - - - - - - - - - - - - -  
*Sycoscapter* 399 -----T-----T-----C-----T-----  
 - - - - - - - - - - - - - - - - -  
 BN1 -----  
 TA1 -----  
 BN1 -----  
 - -----  
 BN2 -----  
 KM9 -----  
 - - - - - - - - - - - - - - - - -

ATAGCAAACCTCAATAATTACACCTATTTCATATTCAACCAGAATGATACT  
 M A N S M I T P I H I Q P E W Y

**Fig. 5.8:** Phenogram with bootstrap values constructed from Cytochrome b gene sequences of *Pediobius haplotypes* and other species of Hymenoptera used as out-groups using Kimura 2-parameter model based on Minimum evolution method.



**Table 5.5: AMOVA results - Conventional *F*-Statistics from haplotype frequencies**

**Table 5.5.1: Two-group analysis - West Africa vs. East Africa (WA/EA)**

Source of variation	Sum of squares	Variance components	Percentage of variation
Among groups	0.990	0.02335	6.24
Among populations within groups	4.441	0.06346	16.97
Within populations	8.902	0.28715	76.79
<b>Total</b>	<b>14.333</b>	<b>0.37396</b>	

**Table 5.5.2: Two-group analysis – West West Africa vs. Mid West Africa**

<b>Source of variation</b>	<b>Sum of squares</b>	<b>Variance components</b>	<b>Percentage of variation</b>
Among groups	0.262	-0.02317	-6.68
Among populations within groups	5.170	0.08301	23.92
Within populations	8.902	0.28715	82.75
<b>Total</b>	<b>14.333</b>	<b>0.34700</b>	

**Table 5.5.3: Three-group analysis - WWA vs. MWA vs. EA**

<b>Source of variation</b>	<b>Sum of squares</b>	<b>Variance components</b>	<b>Percentage of variation</b>
Among groups	1.595	0.01021	2.83
Among populations within groups	3.836	0.06375	17.65
Within populations	8.902	0.28715	79.52
<b>Total</b>	<b>14.333</b>	<b>0.36111</b>	

**Table 5.5.4: All six populations analysis**

<b>Source of variation</b>	<b>Sum of squares</b>	<b>Variance components</b>	<b>Percentage of variation</b>
Among groups	3.851	0.06097	16.89
Among populations within groups	1.581	0.01286	3.56
Within populations	8.902	0.28715	79.55
<b>Total</b>	<b>14.333</b>	<b>0.36099</b>	

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**CHAPTER SIX****POPULATION DIFFERENTIATION OF *PEDIOBIUS* SPECIES USING  
MORPHOMETRIC TECHNIQUES**

## 6.1 Introduction

*Pediobius* has a worldwide distribution being found in both tropical and subtropical regions. It has been recognised as a common parasitoid of pyralids and noctuids associated with major graminaceous crops. Descriptions and species separation are based on morphological character differences and host associations. However, population separation based on morphology and host association is not reliable because in Africa alone *Pediobius* has been reared from more than nine different host species; and there is evidence of morphological obscurity within these populations (see Kerrich, 1969, 1973; Bouček, 1976; Peck, 1985). Are these populations' variants of one species parasitizing various hosts, or are they different species altogether? This chapter discusses the use of qualitative and quantitative taxonomic approaches and methods to determine the boundaries within and between the populations of *Pediobius* species that attack *Sesamia calamistis* Hampson, *Busseola fusca* Fuller (Lepidoptera: Noctuidae) and other lepidopterans associated with Gramineae in Africa.

In biology, morphology is defined as the study of the configuration or structure of living organisms. These structural features may be heritable and may be ancient or recently derived traits (morphological adaptation to make them fit in a particular environment) or phenotypic plasticity. Morphology provides the primary source of taxonomic characters in most groups (Wiley, 1981). As a result, taxonomists have frequently used morphological variation

as primary parameters in the differentiation and separation of many natural populations of organisms and many species have been identified based on the results of these studies. In most instances, morphologically diagnostic characters are not available or refined enough to separate very closely related species e.g. eurytomids. Chalcidoids display a wide range of morphological variability such that without prior knowledge it is virtually impossible to make specified determination on morphological characters alone (Claridge *et al.*, 1997). Some factors that contribute to insects' morphological diversity may include:

#### **6.1.1 Effects of biological factors**

Various biological factors such as variation in developmental stages, differences in the number of larval instars, different foods, individual growth rates, etc have also complicated effective use of morphological characters in population differentiation (Atchley and Martin, 1971). This may be the case, for example, with *P. furvus* in Africa, despite some differentiating characters that may be clear-cut. It is also necessary to use many characters to separate *Pediobius* populations because several of these characters are intergraded, a phenomenon observed among populations of many Hymenopteran species of which *Pediobius* is no exception (Danforth, 1989). In the study of Chalcidoids, no single character has ever been found to have the absolute value in separating different populations of a species (see Gibson *et al.*, 1997).

Many studies of *Pediobius* already carried out on population differentiation and even species differences have been based on colouration, body size, number of antennal segments, differences in body sculpturing, distribution of hairs, setae, sensilla, and wing venation, host association and host food plants (Kerrich, 1973, Dawah, 1988). When colour was used to distinguish individuals, the most frequently described characters are the colour of frontovertex, upper face, propodeum, different colouration of antennal segments, and gastral tergites. The colour patterns of these characters are usually not reliable as they are subject to much environmental variation.

#### **6.1.2 Environmental factors**

With developmental polymorphism and ecological effects, there is also the possibility for colour misidentification by individual taxonomists. Such examples include the description of the tarsi segments as pale testaceous, antennae and gaster as shining with metallic reflections. These have been described differently in other studies (Kamijo, 1986). Body size, antennal length and number of segments have also been used to separate populations. These also have their ambiguities as they vary considerably depending on geographic location, seasonal influences, size of host and number of individuals emerging from a single host pupa. The number of larval instars may also differ among different generations at different temperatures. All these factors make the use of morphological characters for population differentiation very difficult.

### 6.1.3 Morphometrics in systematics

The computation and application techniques to reveal underlying differentiation based on shape and size differences using numeric statistics becomes a necessary option as a result of the aforementioned problems. This name morphometrics is derived from the Greek words 'morpho' and 'metrien' meaning 'form' and 'measure', respectively. The application of morphometric methods was proposed in the early 1960s by taxonomists who argued that taxonomy and systematics should be based on the use of multivariate statistical analysis of morphological characters as opposed to the use of underlying evolutionary or biological information (see Sokal and Crovello, 1970). The use of morphometrics had come from an earlier concept that if two animals have similar mean mandible measurements, they are closely related; if they have rather different measurements, then they are distant from each other (McKechnie *et al.*, 1975). Since then a variety of factors to measure have been suggested, e.g. environmental effects, temperature, gene frequencies, etc, and these have been used in multivariate analysis as distance measures and many populations have been separated based on such multivariate statistics (see Rinderer *et al.*, 1990).

Morphometric techniques involve measuring, and comparing sizes of different characters for quantitative and qualitative interpretation of organisms' morphological diversities. Morphometric analysis has become the cutting-edge in morphological analysis in a wide variety of organisms,

including plants, and has been found useful in differentiating population variation between closely related or sibling species. Typical examples include population differentiation of sibling species of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (McNamee and Dytham, 1993), population differentiation between some biotypes of brown planthoppers, *Nilaparvata lugens* Stål (Homoptera: Delphacidae) (Claridge *et al.*, 1984), intraspecific variability among populations of the staphylinid beetle *Erichsonius tuberculatus* Uhlig and Masch (Coleoptera) (Masch and Plötner, 1993), morphological variation between populations of *Hydroporous glabriusculus* Aube (Coleoptera: Dystiscidae) (Bilton, 1993), and geographical variation between allopatric populations of *Rhinocyllus coniscus* Frölich (Coleoptera: Curculionidae) (Klein and Seitz, 1994).

Using multivariate statistics, various populations of *Apis* (Hymenoptera: Apidae) were grouped into different geographical variants based on the results of morphometric character analysis. Bermejo-Orantes and Garcia-Fernandez (1995) used seven morphometric characters on 12 populations of *Apis mellifera iberica* Goetze to place them into three biometric groups in the South of the Iberian Peninsula. In a similar study, Sylvester *et al.* (1998) explored 58 morphometric characters to separate *A. cerana* Fabricius sampled from 120 colonies in 44 locations into four groups. Morphometric analysis can provide information on changes in an organism's evolutionary

associations, and between ecological and morphological traits in particular clades based upon morphological size differences.

#### **6.1.4 Size as diagnostic trait**

In an organism's the ecogeography, size has been recognised as a character trait and fundamental in population separation, although early studies stated that variation in size is predominantly the result of environmentally induced growth differences (Jolicouer and Mosimann, 1960). However, when different populations of one species under the same geographical conditions have reached maximum growth at the same stage, size is then recognised as a hereditary and diagnostic trait (Atchley, 1983). This phenomenon compromises the value of the use of morphometric data of insects in particular, which retain their actual body form and size when maximum growth is reached. As a result, raw data of character sizes are used during multivariate statistical procedures as descriptive variables to reveal morphospecies.

#### **6.1.5 Morphometrics in populations differentiation**

Multivariate statistical techniques used to analyse morphological character measurements in population differentiation include: Principal Component Analysis, Discriminant Functional Analysis and geometric morphometrics. Elmes (1978) examined 12 morphometric measurements of antennal segments, mandible sizes, maximum thoracic width, diameter of head capsule, transverse section of petiolar segment, and breadth of frons of a

population of *Myrmica sabuleti* Meinert to separate and describe a new parasitic species *Myrmica hirsuta* Elmes which is closely related to *M. sabuleti* (Hymenoptera: Formicidae). A miniature queen which had never been described and the identification of an analogous male were highlighted. Chimimba *et al.* (1999) investigated species diversity of the rodent family Muridae recorded from eastern Zimbabwe using multivariate analysis in conjunction with comparative morphology and protein electrophoresis analysis to describe five new species. Multivariate morphometrics have been used to determine growth effects, and discriminate closely related species of the crested newt *Triturus cristatus* Laurenti and *T. carnifex* Laurenti (Caudata: Salamandridae) and their hybrids (Brede *et al.*, 2000). Morphometric techniques have also been used to interpret diversity in morphology as genetically derived traits such as the beak size of finches *Geospiza fortis* Nahrung (Fringillidae) (Keller *et al.*, 2001).

#### **6.1.6 Morphometrics in Hymenoptera systematics**

In Hymenoptera alone, there have been a series of studies involving the application of morphometric techniques on population differentiation. By comparing morphometric data based on the Jaccard coefficient and cluster analysis, Sujii *et al.* (1996) were able to evaluate 21 parasitoid species as potential candidates for use as biological control agents. Punglerl (1986) used a variety of different morphometric techniques to examine the propodeum, chaetotaxy of the female genitalia, antennal segmentation and costulae on the

first gastral tergite to separate and recognise three species of aphid parasitoids in the genus *Aphidius* (Hymenoptera: Braconidae) formerly recognised as a single species. Although morphological measurements have been used to separate and define species of *Pediobius* Walker, those measurements have neither been presented to date nor transformed as categorical data for any statistical analysis to support their claim. The measurements have only been used and compared in their raw forms. Their primary hypothesis was based on size differences in morphological character. These differences are usually not constant among individuals within a population and, as a result, very little is still known in terms of the usefulness and discriminating power of these character measurements in delimiting population differences within *Pediobius*.

The application of morphometric techniques has yielded varying degrees of success as an indirect method of determining developmental differences in the study of many insect groups (Schmidt and Lauer, 1977). Because of its popularity of use among taxonomists, randomisation tests have been introduced in morphometric analysis to determine if the average of several observations is different from a constant. This method is important where there is reason to suspect that the populations of an organism are different at least morphologically. Morphometric analysis using multivariate techniques could therefore be a very useful tool in the investigation of the extent of phenotypic variation within and between different *Pediobius* populations. I have attempted to evaluate the usefulness of multivariate

techniques in morphometric analysis as diagnostic tools and to establish the contributing power of using measurements as descriptive characters in population differentiation. In this study, multivariate statistical analyses were performed to transform character measurements to categorical data to reveal the primary character-limiting factors that defined such populations and host associated groups of *Pediobius furvus* from Africa.

## 6.2 Methodology

Specimens used in the analyses were collected in six countries of Africa. An outline of collection sites shown in Fig 2.1 and are coded below. All collection sites were maize fields or small maize plots. Sampling was carried out in both pre- and post-harvest fields with evidence of stemborer damage to crops.

**Country codes:** Benin – B, Togo – T, Ghana - Gh, Kenya - K , Sierra Leone – S, and Guinea – Gu. These countries codes are used to represent each *Pediobius* population throughout this chapter.

### 6.2.1 Sampling protocol

Maize stems were cut about 30cm from ground level. These stems were later dissected and any larvae or pupae of stem borers recovered were reared in laboratory cultures in individual petri dishes until the emergence of adults. Mummies were kept separately according to locality. Five days after adult emergence, a known number of chalcidoid wasps were collected and killed in

95% alcohol and left to dry. Upon drying specimens were removed and placed separately, whilst other Hymenopteran parasitoids reared in the collection were mounted on card points for correct identification and preserved for museum collections.

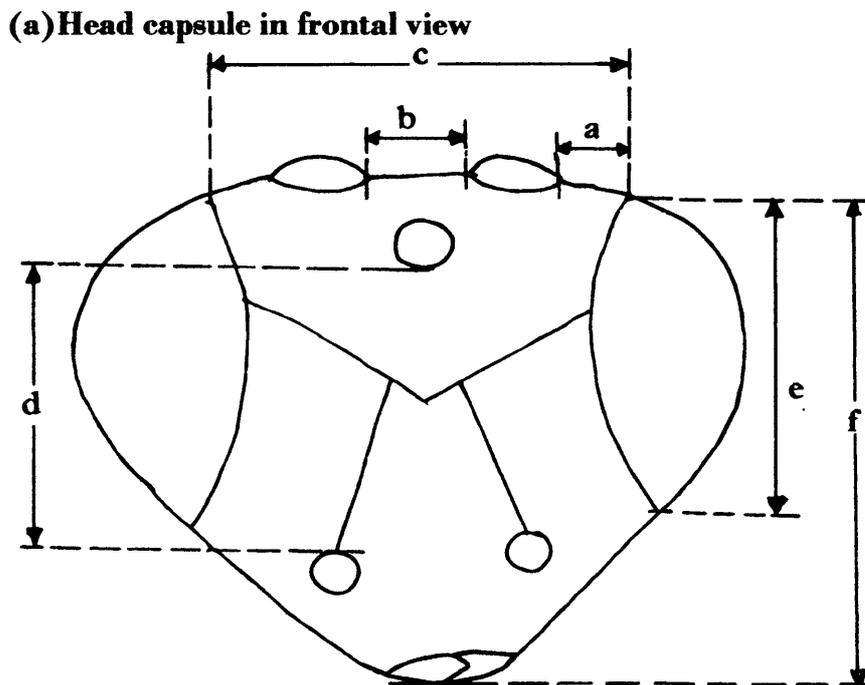
### 6.2.2 Specimen preparation

*Pediobius* specimens were washed in pre-heated KOH for at least 5 minutes and rinsed in 70% alcohol to reduce the greenish or bluish colourations, which might have had an adverse effect on the image quality and visualisation under microscope. Upon drying, these were also mounted on cards to facilitate handling during dissection. For examination under a microscope, dissections were mounted on clear glass slides to enhance a 3-dimensional view to obtain accurate measurements of morphological characters. Morphometric parameters used in this study were selected from a list of morphological characters commonly used in taxonomic studies of Chalcidoidea, and those traditional characters previously used to describe and separate *Pediobius* species (see Dawah, 1988). Other characters (not mentioned in previous studies) revealed upon microscope examination, which were thought to be of potential use, were also included. Terminology used for characters followed Dawah (1988) and Dawah *et al.* (2002). Because of the prevalence of arrhenotoky in most chalcids, and to prevent bias, only female wasps were used for morphometric measurements and further analysis. In some samples, no males were recovered.

### 6.2.3 Measuring procedure

Measurements were taken from randomly selected specimens from each population and locality. Selected characters were measured between landmark points. Characters measured are shown in Fig. 6.1.

**Fig. 6.1:** Characters measured on individual wasp for analysis.



#### Characters measured

**a = OOL** - distance between posterior ocellus and upper level of eye.

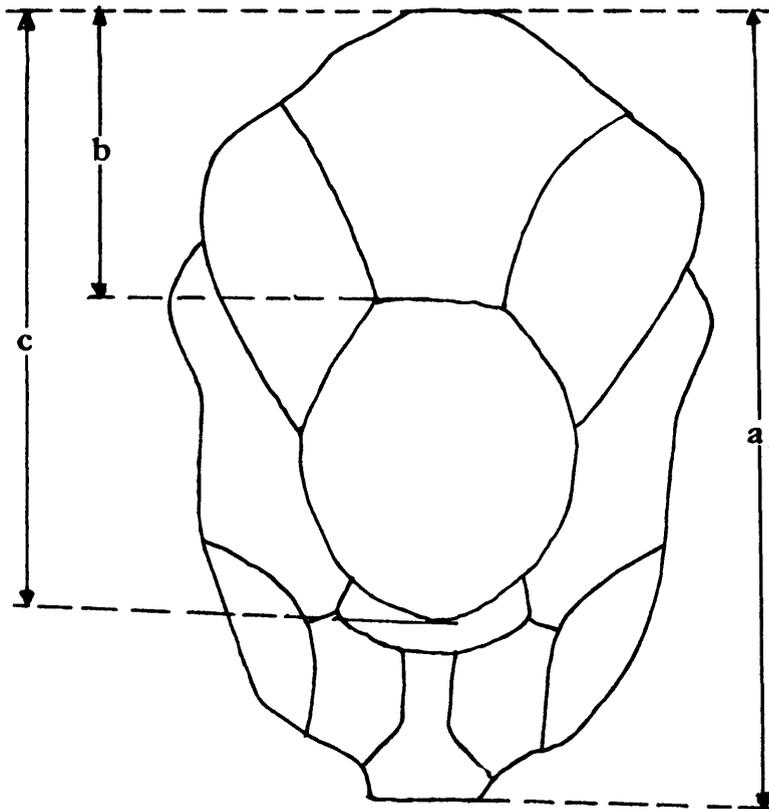
**b = POL** - distance between posterior ocelli.

**c = BE L** - distance between upper level of eyes.

**d = TEL** - distance between lower level of anterior ocellus and upper level of antennal socket.

**e = DEL** - distance between lower and upper levels of compound eye.

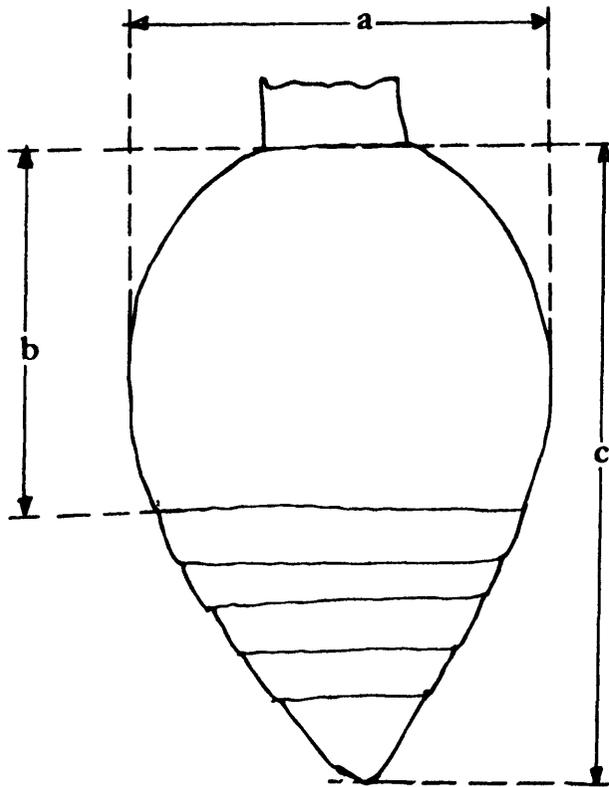
**f = VET** - distance between lower level of mouth parts and vertex.

**(a) Mesosoma in dorsal view****Characters measured**

**a = MEL - Length of mesosoma**

**b = MLM - Distance between transcutal articulation and anterior end of mesosoma.**

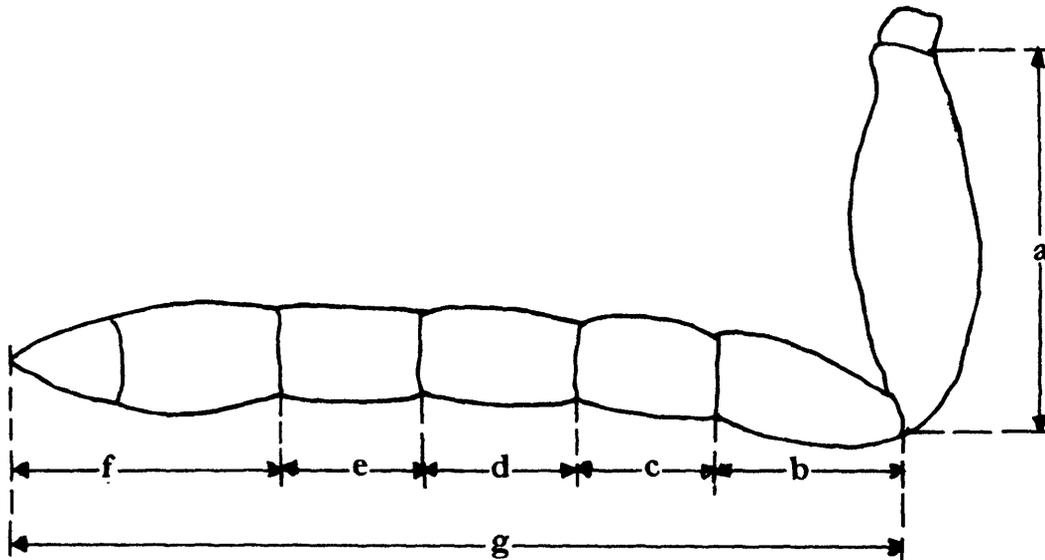
**c = MES - Distance between posterior end of scutellum and anterior end of mesosoma.**

**(f) Female gaster****Characters measured**

$a = \text{GAW}$  – maximum width of abdomen

$b = \text{FGT}$  – length of first large tergite

$c = \text{GAL}$  - length of abdomen

**(c) Female antenna in lateral view****Characters measured**

*a* = SCP - length of scape

*b* = PED - length of pedicel

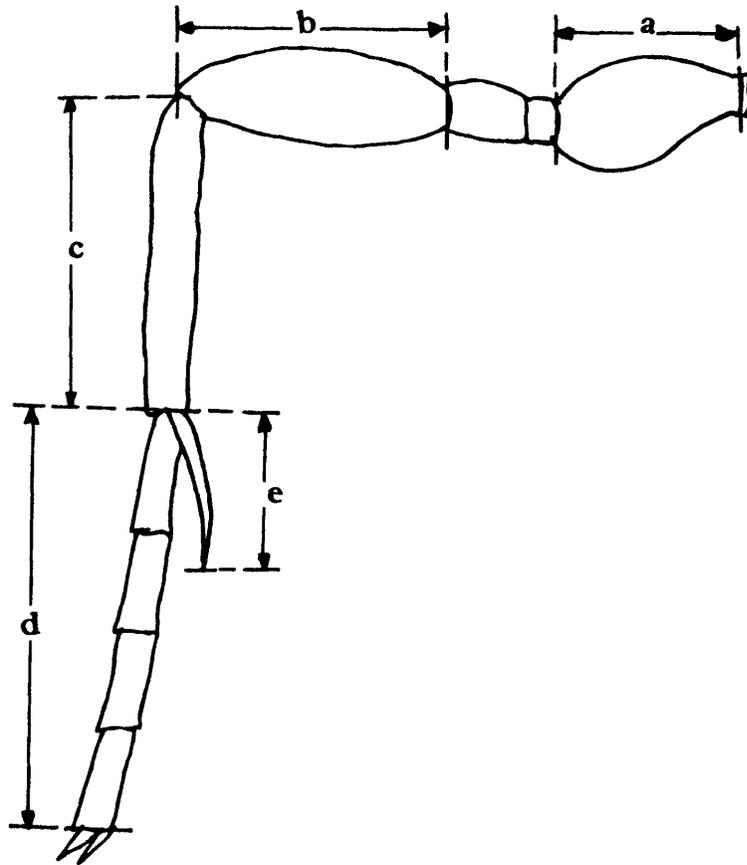
*c* = FU1 - length of first funicle segment

*d* = FU2 - length of second funicle segment

*e* = FU3 - length of third funicle segment

*f* = CLB - length of clava

*g* = FLA - length of flagellum

**(d) Hind leg in lateral view****Characters measured**

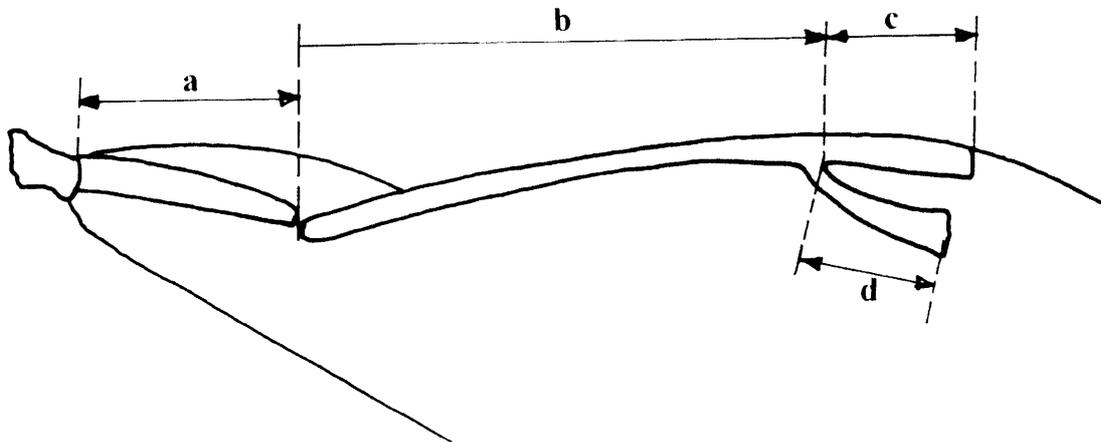
**a = COX- length of hind coxa**

**b = FEM- length of hind femur**

**c = TIB - length of hind tibia**

**d = TAR - length of tarsal segments**

**e = SPR - length of hind tibial spur**

**(e) Diagrammatic representation of fore wing****Characters measured**

**a = SUV** – length of submarginal vein (distance from wing base to vein break).

**b = MV** – length of marginal vein (distance from vein break to stigmal joint).

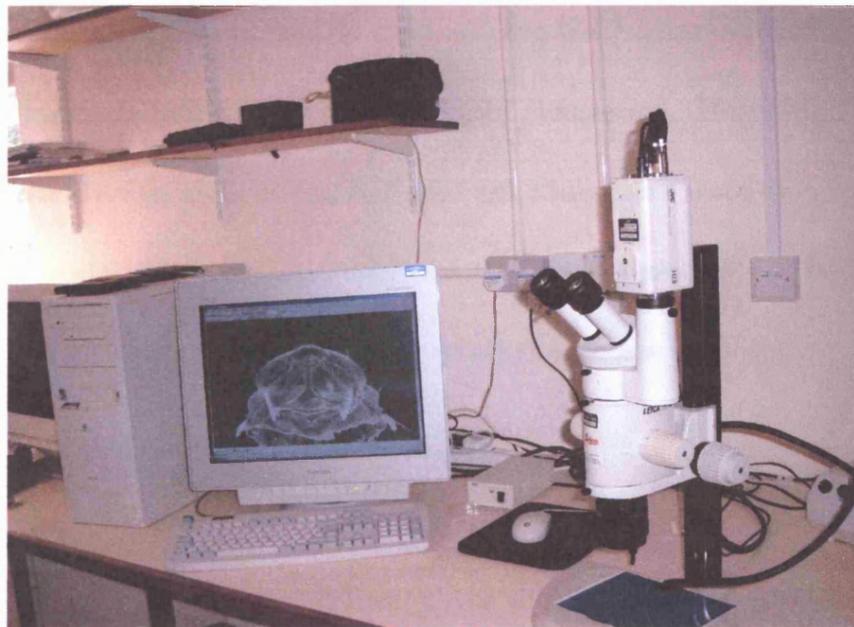
**c = PMV** – length of postmarginal vein (distance from stigmal joint to vein visibility).

**d = STV** – length of stigmal vein.

#### 6.2.4 Devices used in measuring

Equipment for morphometric measurements included a high-power microscope and a digital camera. The high-power dissecting microscope was pre-set to magnify to 100% of original image size. Attached over the microscope was a Sony digital camera equipped with a Lucida to aid image focussing. The camera was linked to a Pentium III computer with Aequitas 1A and Montage application programmes. The Montage programme captures and analyses the images for visualisation while Aequitas 1A is a digital application for measuring objects in a variety of unit measures (Fig. 6.2).

**Fig. 6.2:** Morphometric measuring equipment (microscope, digital camera, computer and image capture and measuring softwares)



Slide-mounted samples were viewed under the microscope and photos taken when an image was well focused on the screen. This way even the smallest but potentially useful characters were measured. The objective was to

capture enough detailed information for measurements of well-defined forms and structures, and be able to reproduce a general outline of such structures. Due to the minute size of some of the specimens, and to avoid bias, obscured characters were avoided. Geometrical measurements of landmark points of characters that allowed the use of short distance measurements and fewer characters were used. These parameters used as descriptive variables were decoded and transformed into categorical data, or decoded, standardised and transformed for further analysis. A total of 28 characters were initially selected and these were measured on 120 individuals from 6 populations in 13 localities for preliminary analysis. Measurements were made in micro-metres ( $\mu\text{m}$ ) so that the smallest characters were included in the preliminary analysis. Some problems were encountered in determining the beginning and end point when taking measurements on curved-surfaced characters. However, to obtain accurate results during measuring, such curved characters were only measured at meeting points of imaginary diagonal/vertical lines, which should be uniform on all samples; otherwise, such curved characters were ignored.

#### **6.2.5 Results of consistency of measurements**

Each day measurements were carried out; a few specimens were normally selected and measured at least three times to test the operator's hand movements, and eyesight consistency and accuracy. Once landmark points had been chosen, measurements were continued. A total of 15 individuals were used for these tests and data analysed using analysis of variance (ANOVA).

The results showed no significance differences between the sets of measurements (Table 6.1). The software-measuring device (Aequitas 1A) was also calibrated once and measurements stored as reference file, which was used for all the samples throughout. When taking measurements one character was selected at a time and measured on all samples. Measurements were used as independent descriptive variables and analysed for the separation of individuals and between populations.

**Table 6.1:** Consistency test result ( $P>0.05$ ) indicating that there was no significant difference on hand movements and sight in obtaining measurements on different characters.

	<b>Sum of squares</b>	<b>df</b>	<b>Mean square</b>	<b>F</b>	<b>Sig.</b>
Between groups	10.655	8	1.332	0.436	0.897
Within groups	339.345	111	3.057		
Total	350.000	119			

### 6.2.6 Choice of methods and data analysis

Morphometric data analysis was carried out using a variety of application techniques. Previous workers have used the following methods [either in a preliminary analysis or in the final analysis, or both], and below are descriptions, usefulness and problems in the use of some of the techniques.

### 6.2.7 Multiple analysis of variance (MANOVA)

A one-way analysis of variance (ANOVA) is the most commonly used statistical method for morphometric analysis amongst taxonomists who have

tried to prove the usefulness of character measurements statistically. The key point in ANOVA is the  $F$ -test of differences of groups of means, i.e. testing if the means of the groups formed by the values of independent variables are different enough not to have occurred by chance (Lin and Butler, 1990). The ANOVA method is more appropriate when a single character or variable is used for the test of homogeneity of variance. For example, Bilton (1993) used a one-way analysis to determine morphological differences between the populations of the diving beetle *Hydroporus glabriusculus* Aube (Coleoptera: Dystiscidae). However, measurement evaluation of a single character is very unlikely to produce reliable or unambiguous results. Secondly, when ANOVA is used, if the  $F$ -test shows that the independent variable is related to the dependent variables or other subsets, then a multiple comparison test Multiple Analysis of Variance (MANOVA) becomes necessary to evaluate the importance of such independent variables.

A MANOVA examines the main and interaction effects of categorical variables on multiple dependent interval variables. MANOVA has become popular in the study of variation within and between populations of one species (e.g. Anez *et al.*, 1997; Azidah *et al.*, 2000; Yang and Chen, 2004). MANOVA can also test for differences in the centroid of means for various categories of the dependents and identify the independent variables which differentiate a set of dependent variables the most. MANOVA used in this

study include (i) Principal Component Analysis (PCA), and (ii) Discriminant Function Analysis (DFA) also known as Canonical Discriminant Function.

### 6.2.8 Principal component analysis (PCA)

Principal component analysis is a method of ordination in which components are extracted from a matrix of similarities or differences of a data set of variables between attributes. In the process, PCA reduces the dimensionality of the data that are correlated by rotating the original data to new positions such that the maximum variabilities are projected onto the axes known as principal components. Principal components are extracted in descending values such that the first principal component explains the greatest amount of variation; whereas the second principal component defines the next largest amount of variation and so on. PCA procedure has recently been used in population discrimination using body size measurements of insects. For example, Kjaerandsen (2004) on the populations of the afrotropical genus of *Dhatrichia* (Trichoptera: Hydroptilidae) in which PCA revealed good separation between the sexes based on the number and shape of segments and eye size. Sharma *et al.* (2003) used morphological measurements of stem hypanthium, leaf shape and petal lengths to distinguish the species of groundnuts resistant to insect infestation; and Anez *et al* (1997) used principal component scores to separate the species of Phlebotomine sand flies (Diptera: Psychodidae).

### 6.2.9 Discriminant function analysis (DFA)

Discriminant function analysis is a multivariate method for demonstrating the significance and nature of the differences between naturally occurring groups. The objective of the analysis is concerned with the problem of seeing whether it is possible to separate different groups on the basis of given measurements. DFA therefore (i) investigates which variables discriminate between groups or groups of individuals; (ii) determines the percentage of variation in the group as explained by the variable measurements, and (iii) assesses the relative importance of each variable in classifying the groups based on the values of the discriminant function. Discriminant functions are results of a linear combination of discriminating independent variables. The Eigenvalue of each discriminant function reflects the ratio of importance and the percentage of variance of the discriminant function. During the process DFA uses the principles of the Wilks' lambda to test if the discriminant model is significant enough to classify the groups. Lambda values are measured inversely (see Krusinska and Liebhart, 1989). Variables with lambda values near zero denote high discrimination between groups.

### 6.2 10 Analysis

PCA and DFA have been proved very useful for numerical taxonomic studies based on the morphometric characteristics of different populations (see Tabachnick and Fidell, 1989; Addison *et al.*, 2003; Brown and Freitas, 2000).

They were therefore used in this study to analyse individual *Pediobius*, and populations to detect variables that contribute in discriminating individuals and separate populations.

From previous studies, we can see that PCA is very useful in investigating correlations between different morphological character measurements to distinguish between individuals or groups of individuals. In this present study, six populations of *P. furvus* collected in Africa were studied. For this study, collection sites were divided into three subregions and coded as follow: west West Africa (Sierra Leone and Guinea); mid West Africa (Benin, Togo and Ghana), and East Africa (Kenya). These were analysed for similarities and differences based on their morphological character measurements. Two separate analyses were carried out. In the first analysis, all six populations were plotted together to see if PCA could separate them based on different character measurements. Further analyses were carried out to separate populations from each subregion except for K. Based upon the first results obtained, populations of B, T and Gh and S and Gu were then analysed separately.

## **6.3 Results**

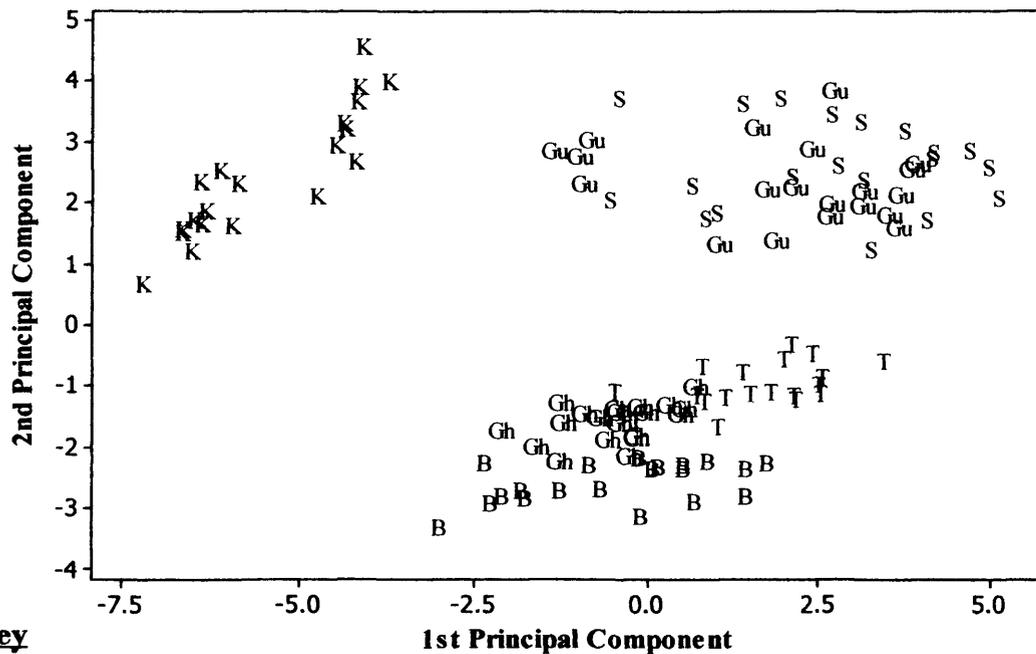
### **6.3.1 Principal component analysis**

In following PCA procedure, plotting all six populations using the scores of PC1 and PC2 from standardised variables confirmed three main groups (Fig. 6.3). There is clear demarcation between all populations within the West

African sub-groups i.e. S and Gu and B, T and Gh populations, and K can be seen distinctly separated from all other groups. The plot also clearly showed strong clustering of B, T and Gh populations. Despite the separation, some individuals within groups are widely placed from other group members e.g. K, S and Gu populations. Characters influenced in the separation of the groups are shown in Table 6.2. K population is separated by the following characters: the length of submarginal vein, post marginal vein, and length of abdomen; S and Gu group separated by the third funicle segment, pedicel and flagellum lengths, whilst B, T and Gh group by the length of thorax, abdomen width, and length of tibial spur. Based on the output of this plot, K population that showed clear separation from all others was excluded from the next analysis.

Plotting the scores of PC2 vs. PC3 improved the separation of B and T populations (Fig. 6.4). However, plotting PC1 vs. PC2 for B, T and Gh populations indicates the existence of three distinct populations (Fig. 6.5). This shows that the first two components are robust in the separation of B, T and Gh populations. Plotting independent variables on the same axes clearly showed which characters contributed the most in population separation (see Table 6.4). Populations B and Gh are associated with PED, FMV, FEM, MES, GAL and MEL, while population T influenced by POL, FLA, CLB, BEL, FUL and PMV. Plotting of S and Gu populations resulted in producing of scattered individuals, did not cluster as a single group (Fig. 6.6).

**Fig. 6.3:** Scatter plots of six populations of *Pediobius furvus* with respect to PC1 (32.3%) vs. PC2 (18.1%) based on standardised variables.



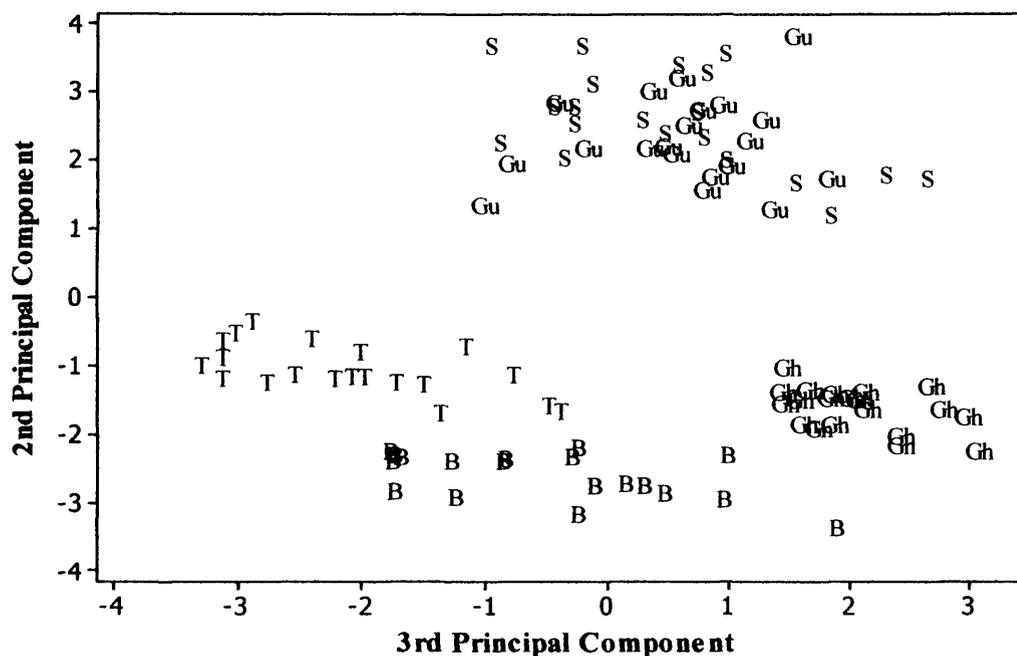
**Key**

- B = Benin
- Gh = Ghana
- Gu = Guinea
- K = Kenya
- S = Sierra Leone
- T = Togo

**Table 6.2:** Characters that influenced the separation of the different groups based on PC1 (32.3%) and PC2 (18.1%).

1st Component	2nd Component
MEL - 0.209	PED - 0.409
TEL - 0.189	FLA - 0.377
MES - 0.209	FU3 - 0.372
FEM - 0.156	SUV - 0.217
TIB - 0.211	PMV - 0.189

**Fig. 6.4:** Scatter plot of *Pediobius* populations from five countries of West Africa (Benin, Togo, Ghana, Sierra Leone and Guinea) extracted from calculations of PC2 (15.7%) vs. PC3 (8.5%) based on standardised variables.



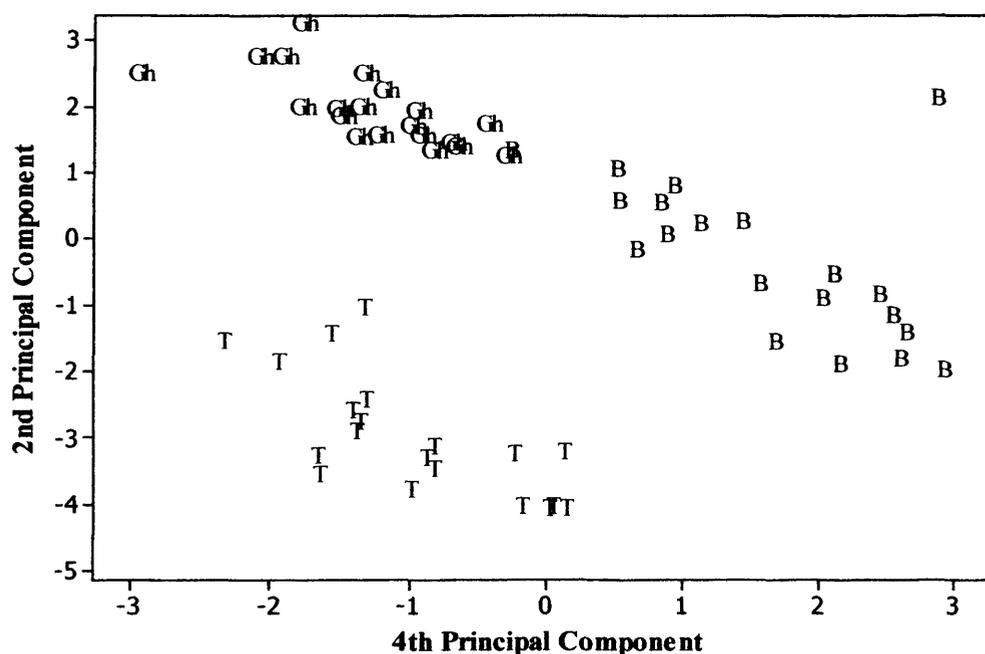
**Key**

- B = Benin
- Gh = Ghana
- Gu = Guinea
- S = Sierra Leone
- T = Togo

**Table 6.3:** Variable contribution in principal component calculation in separating S and Gu populations, Gh and B and T into groups based on PC2 (15.7%) vs. PC3 (8.5%) (Fig. 6.4).

2 <sup>nd</sup> Principal component	3 <sup>rd</sup> Principal component
GAL .303	FGT -.382
SUV .293	TIB -.220
PMV .265	COX -.210
BEL .254	MEL -.192
FU3 .228	SPR -.186
PED .222	FEM -.167
FLA .197	VET -.163

**Fig. 6.5:** Scatter plot of *Pediobius* from B, T and Gh populations from PC2 (15.7%) vs. PC4 (8.2%) based on standardised variables.



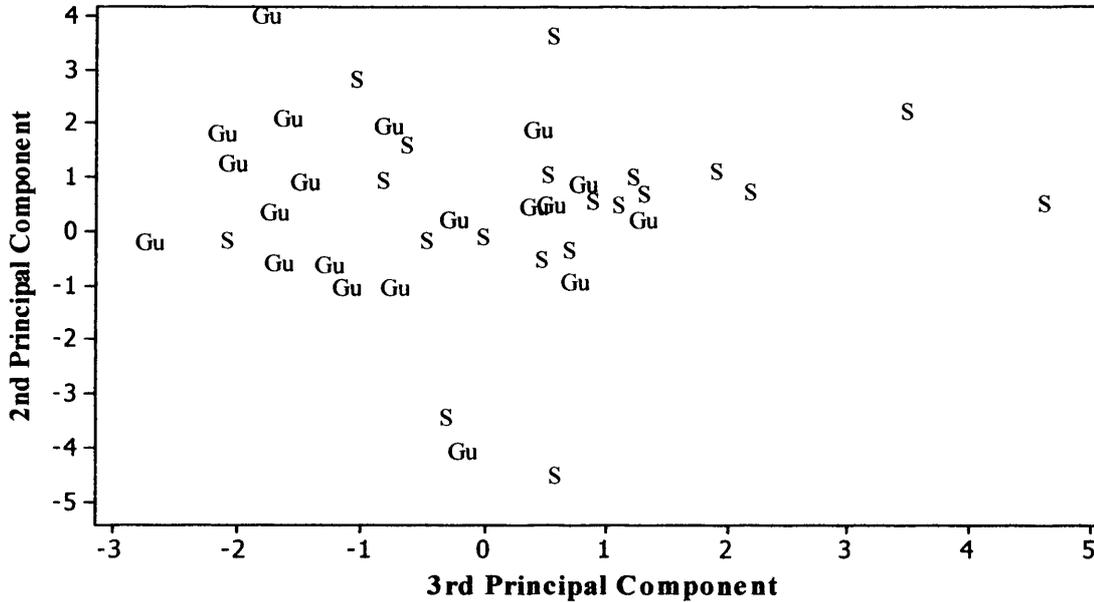
**Key**

B = Benin  
Gh = Ghana  
T = Togo

**Table 6.4:** Variables that contributed the most in separating B, T and Gh populations (Fig. 6.5) into three separate groups based on PC2 (15.7%) vs. PC4 (8.2%) calculations.

2 <sup>nd</sup> Component	4 <sup>th</sup> Component
FMV 0.366	FLA 0.303
FEM 0.372	FUI 0.300
MES 0.332	PMV 0.303
MEL 0.28	CLB 0.271
GAL 0.292	POL 0.239
PED 0.368	BEL 0.236

**Fig. 6.6:** Scatter plot of *Pediobius* from S and Gu populations extracted from PC2 (12.2%) vs. PC3 (9.4%) based on standardised variables.



### Key

Gu = Guinea

S = Sierra Leone

### 6.3.2 Discriminant function analysis

Discriminant function analyses were calculated using unstandardised variables to evaluate the capacity of morphometric measurements in reclassifying groups and to see if the six populations could be separated on the basis of character measurements. The analyses were also carried out to investigate the contribution of each character in the separation of the groups or individual populations. As with PCA, all specimens from all localities were first plotted together in DFA procedure. Results obtained indicate that only eight morphological variables were very effective with significant contribution

in the extraction of five discriminant components (Table 6.5). The values of each character demonstrate its influence in the qualitative description of individual or groups of individuals. Most of the variation lies in DF1 and DF2 (85.1% and 9.0%) respectively as shown by Eigenvalues and Wilks' lambda (Table 6.5-6). Plotting DF1 vs. DF2 using categorical variables, all the specimens from all six populations were separated into three groups (Fig. 6.7). In the plot the K population can be seen distinctly separated as a single group as previously shown in PCA analysis (Fig. 6.3); whilst populations S and Gu heavily clustered with few individual scatter from the cluster, as well as B, T and Gh populations clustered with few individuals widely separated from each other and from their group cluster. K population was excluded in the next analyses.

Further analyses carried out to separate other groups were made by exploring discriminant functions. Based on the values of variables used in the calculations (see Table 6.8) in functions scores (Table 6.9), only functions 1 and 2 yielded a three-group plot separating Gh in a single cluster leaving B and T populations as one group (see Fig. 6.8). Gh population was therefore excluded in the next analysis. In spite of this, no further improvement was achieved in separating populations S from Gu, and B from T to form individual clusters (see Fig. 6.9). Although individuals were scattered about in the plot, most were placed around their group centroid. We can see that group separation is explained by percent correct reclassification (Table 6.10). Although other sets

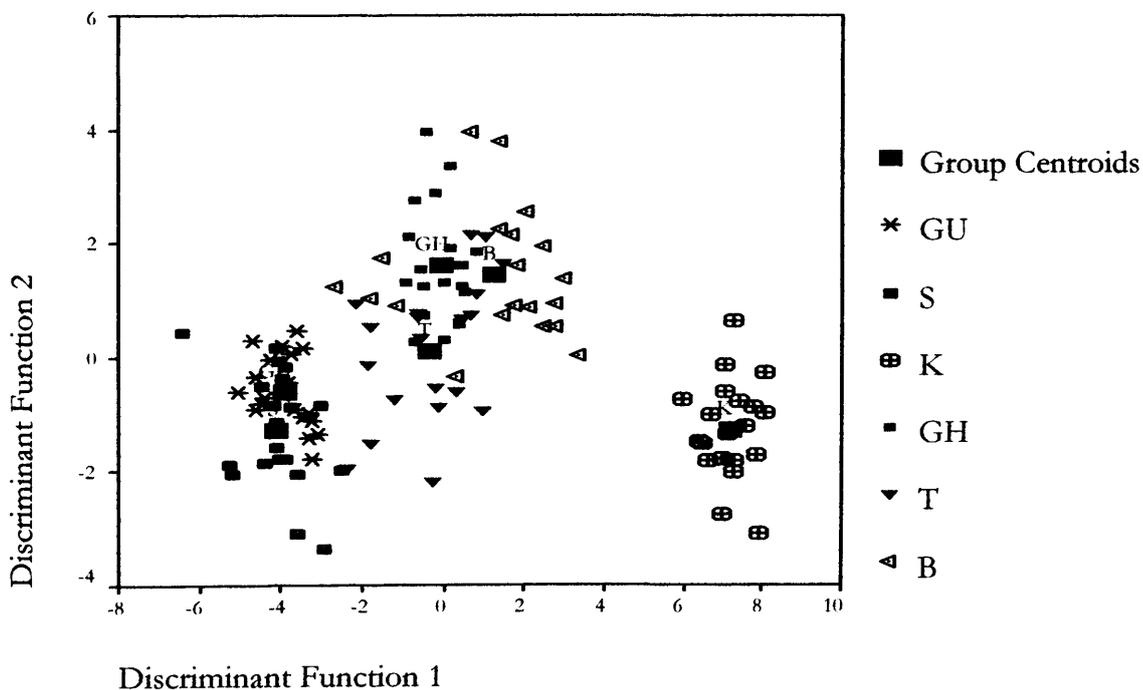
of discriminant functions were explored, no further separation was achieved between populations S and Gu, and between B and T. However, group centroid of B and T populations clearly demonstrate that they are individual populations, whereas S and Gu centroid distinctly overlap (Fig. 6.9).

**Table 6.5:** Eigenvectors and percent variance of canonical discriminant analysis to separate individual specimens and populations or groups of populations (see Fig 6.7).

Function	Eigenvalue	Lambda	% Variance	Sig.
1	14.714	0.011	85.1	.000*
2	1.552	0.175	9.0	.000*
3	0.784	0.447	4.5	.000*
4	0.148	0.798	0.9	.000*
5	0.092	0.916	0.5	.000*

\* indicates significance less than 0.001

**Fig. 6.7:** Scatter plots from canonical discriminant analysis of unstandardised variables of six *P. furvus* populations collected in Africa based on DF1 (85.1%) and DF2 (9.0%) using stepwise selection method.



**Table 6.6:** Variables and their effective values in analysing populations from all localities and their contribution in each Discriminant function (see Fig. 6.7).

Wilks' Lambda			Discriminant function matrix				
Variables	Lambda	Sig.	1	2	3	4	5
SCP	.097	.000*	.786	.075	-.444	.248	.196
MEL	.057	.000*	.229	.503	.201	-.306	-.229
OOL	.035	.000*	.225	.304	.588	.098	.457
FLA	.027	.000*	.188	.110	.636	-.368	.174
FU1	.021	.000*	.124	.201	.457	.021	.387
BEL	.017	.000*	.080	.317	.216	-.399	.671
GAW	.014	.000*	.015	-.264	-.154	-.079	-.003
POL	.011	.000*	.066	.210	.385	.223	-.387

\* indicates significance less than .001

**Table 6.7:** Six populations of *Pediobius* from Africa with a total of 82.5% percent correct classification based on Stepwise and Squared Euclidean distance methods calculated from unstandardised variables. Correct reclassification of each population lies in the diagonal e.g. K = 100%.

Population	B	T	Gh	K	S	Gu
B	80	5	15	0	0	0
T	20	70	0	0	10	0
Gh	0	5	95	0	0	0
K	0	0	0	100	0	0
S	0	0	0	0	70	30
Gu	0	0	0	0	20	80

**Table 6.8:** Variables used in discriminating S and Gu and B, T and Gh populations based on lambda value and their contributions in each Discriminant function.

Wilks' Lambda			Discriminant function matrix			
Variables	Lambda	Sig.	1	2	3	4
POL	.050	.000*	.128	.304	.209	-.139
DEL	.061	.000*	.191	.264	-.321	.096
FEM	.073	.000*	.330	.181	-.084	.402
FGT	.088	.000*	.047	.023	.202	.005
FLA	.106	.000*	-.193	.381	-.156	.220
MLM	.129	.000*	.150	.074	.449	.739
VET	.152	.000*	.346	.254	.226	-.249
MEL	.196	.000*	.560	.292	.264	.374
PED	.421	.000*	-.339	.722	-.145	-.064

\* indicates significance less than .001

**Table 6.9:** Discriminant function calculated in five-population analysis. DF1 (54.2%) and DF (35.0%) were used in the separation of Gh population from B and T of B, T and Gh group and populations S and Gu as one group.

Function	Eigenvalue	Lambda	% Variance	Sig.
1	3.027	.050	54.2	.000*
2	1.956	.202	35.0	.000*
3	0.454	.597	8.1	.000*
4	0.152	.868	2.7	.042**

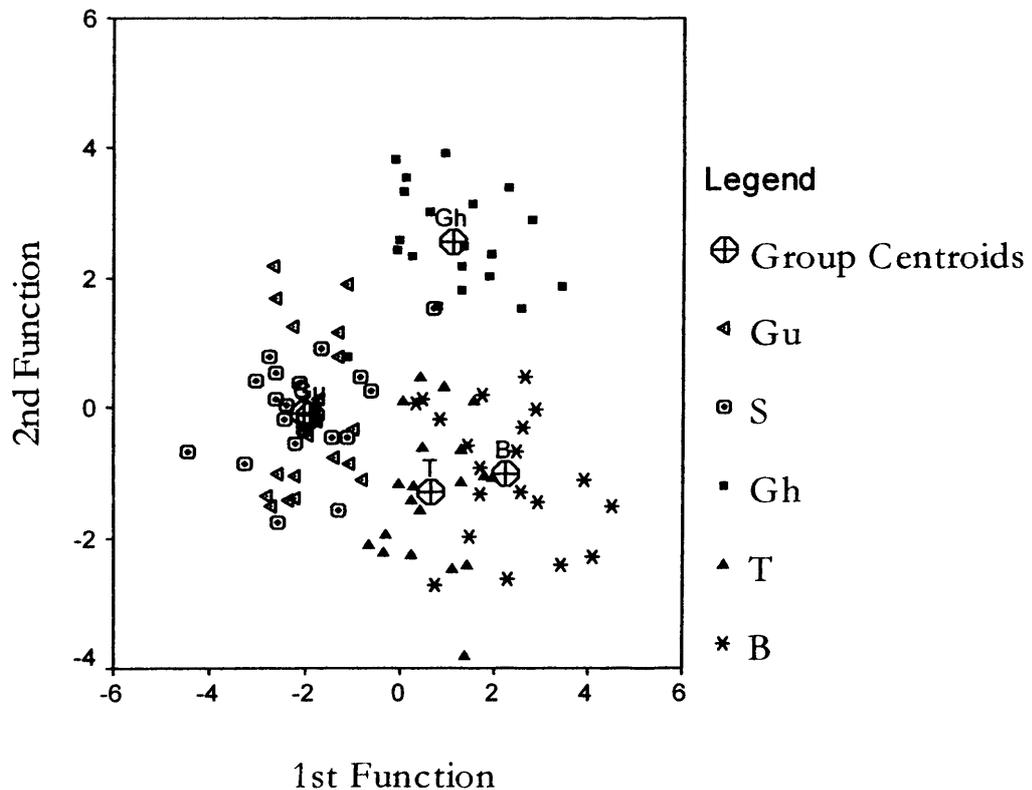
\* indicates significance less than .001 (P< 0.001)

\*\* indicates significance less than .05 (P< 0.05)

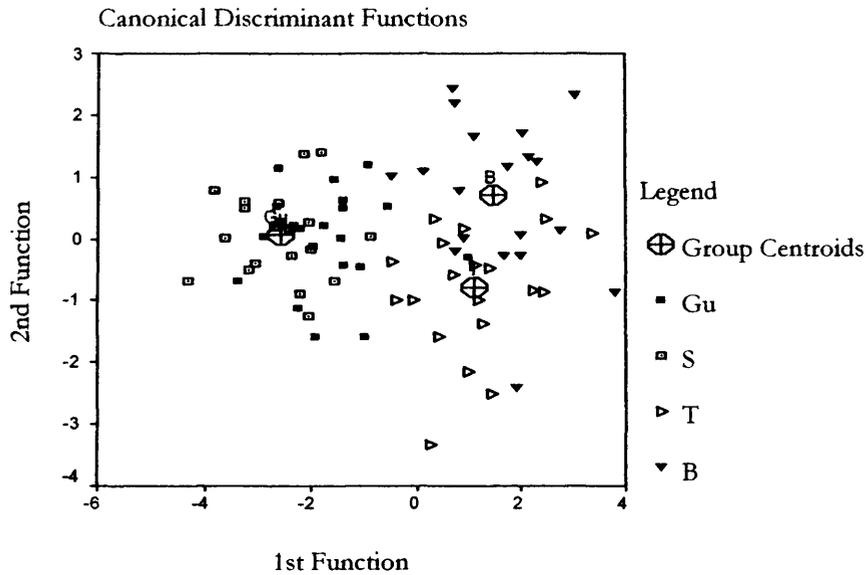
**Table 6.10:** Reclassification of S and Gu and B, T and Gh populations based on Squared Euclidean distance method calculated from unstandardised variables. 89.0% of all populations correctly reclassified with individual population % classification in the diagonal.

Population	B	T	Gh	S	Gu
B	80	20	0	0	0
T	5	90	5	0	0
Gh	0	0	95	0	5
S	0	0	5	95	0
Gu	0	0	0	10	85

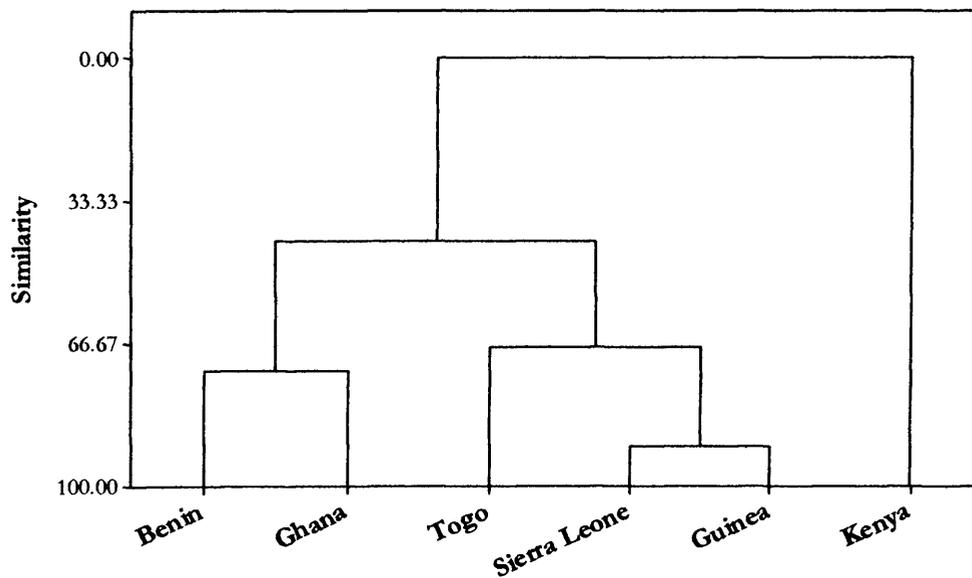
**Fig. 6.8:** Scatter plots from Discriminant Functions analysis of unstandardised variables of five *Pediobius furvus* populations collected in west Africa based on DF1 (76.7%) and DF2 (23.3%) using stepwise selection method.



**Fig. 6.9:** Scatter plots from Discriminant Function analysis of unstandardised variables of four *P. furvus* populations collected in west Africa based on DF1 (76.7%) and DF2 (23.3%) using stepwise selection method.



**Fig 6.10:** Populations similarity dendrogram extracted from complete linkage and Squared Euclidean Distance measure based on standardised morphological character variables of six populations of *Pediobius* from Africa.



#### 6.4 Discussion

The results of both analyses carried out in this study indicate that PCA and DFA are robust in discriminating individuals as well as populations (Figs. 6.3-4, 6.7-8). In both analyses, K population was distinctly separated from all other groups as well as from all other individuals, a result that reflects its geographic location. The result also confirmed that morphologically Gh population differs based on morphometric body measurements from its closest neighbours as shown in two separate analyses results, whilst populations S and Gu were inseparable in both analyses (Figs.6.4-6, 6.8-9). The main variables that contributed the most in the first principal component (32.3%) are MEL, TEL, MES, FEM and TIB; and PED, FLA, FU3, SUV and PMV in the second principal component (18.1%) for the separation of populations from all localities into three groups that placed K as separate from all other groups (Fig. 6.3). Separation of population Gh from B and T as well as placing the S and Gu populations as one separate group was achieved by plotting the second principal component versus the third principal component (Fig. 6.4). The main variables that contributed the most in PC2 (18.1%) are FMV, FEM, MES, MEL, GAL and PED; and FLA, FU1, PMV, CLB, POL and BEL in PC4 (8.5%). Individual group separation of B, T and Gh populations improved when PC2 (15.7%) was plotted against PC4 (8.2%) from calculations of the following variables, FEM, PED, FMV, MES, GAL and MEL in the second principal component and FLA, PMV, FU1, CLB, POL and BEL in the fourth principal component (Fig.

6.5; Table 6.4). Separation of S and Gu populations was not achieved even when all possible pairs of principal components were explored (Fig. 6.6).

In Discriminant analysis procedure, most of the variance was identified in the first two discriminant functions, DF1 (85.1%) and DF2 (9.0%) (see Table 6.5). The main contributing variables include SCP, MEL, OOL, FLA, FU1, BEL, GAW and POL (Table 6.6) and these effectively separated all six populations into three groups with K in a separate group (Fig. 6.7). By plotting individuals and populations using the scores of DF1 and DF2, all graphs showed that both analyses are effective in the separation of populations at identical positions. DFA procedure was robust in the analyses as 82.5% of all individuals were correctly reclassified in their original predicted groups, and phenotypic relationships among individuals within populations are demonstrated in Appendix II. Hierarchical analysis by population revealed six separate populations in four clusters (Fig. 6.10). Previous studies have shown that significant correlations between qualitative characters either positively or negatively are important for morphological identification of populations of a taxon (Collins *et al.*, 1994).

Insects have been found to increase in size in correlation to the mean annual temperature of their geographic range, but decrease in relation to the length of the dry season. Variation in body size using morphometric techniques has been proven associated with evolutionary changes in some species of Hymenoptera (see Singh *et al.*, 1990). However, geneticists have

argued that phenetic classification based on morphology may group unrelated forms into paraphyletic or even polyphyletic taxa, and sexual dimorphisms can affect morphological characters (Mallet, 2001). In the present study all analyses were unaffected by sexual dimorphisms as only female specimens were used. Other studies have shown that variation may occur between distinct populations of a species if they are geographically isolated and there is no area of overlap.

*Pediobius* pre-historical distribution could have severe implications on morphological variability between WWA and MWA populations *Pediobius*, hence the two groups were effectively separated in both PCA and DFA carried out in this study. Records showed that *P. furvus* was first identified as a parasitoid of maize and other cereal stemborers in Sierra Leone, and then transported to other areas within the region and within the British Commonwealth as natural control agent (see Taylor, 1937; Jordan, 1966). Population variation may occur in a taxon due to geographic variation and other forces during the period in time, a phenomenon found widespread in most insect species (see Mayr, 1963). Naturally there is geographic separation between West African and East African populations of *Pediobius*. Studies have also shown that phenotypic characters often vary between individuals of a population at any one place, and between populations living in different places. This phenomenon has also been demonstrated in this study (see Appendix II). K population can therefore be recognised as allopatric and this has been shown in all analyses (Figs. 6.3,

6.7, 6.10, Appendix II). The results of PCA and DFA demonstrate that distinct populations of a species could be effectively separated using morphological character measurements. The analyses also revealed a wide differentiation of morphological characters exist between K and all West African populations of *P. furvus*.

## 6.5 References

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**CHAPTER SEVEN**

**GENERAL DISCUSSION AND CONCLUSION**

## 7.1 Introduction

Distinct geographic populations differ in their adaptive traits and often are genetically different (Unruh *et al.*, 1986). Genetic variation within a species is an inevitable consequence of geographical variation of the environment each population occupy (Mayr, 1963). Comparison of such populations rarely provide enough evidence about the causes of the differentiation (Berry & Peters, 1977). However, causes of variation could be inferred from genealogical differences based on genetic and behavioural analyses. The objectives of the present study were to investigate genetic variation and phylogenetic structure within and between *Pediobius furvus* populations in Africa, to correlate this with the results of morphological variation between populations, investigate whether geographic variation supports population differentiation and to investigate reproductive compatibility among the most studied sympatric species of the *P. eubius* complex associated with grass-feeding eurytomid species in the United Kingdom.

Taxonomists have frequently used morphological differences in a wide range of species delimitation and determination. This is because many morphological characters have been found to be clear-cut between species, and morphological characters seemed the most readily available information in most cases. Historical records have shown that the African *P. furvus* was

exported to other tropical and subtropical regions within the British Commonwealth for biological control of sugarcane stemborers (see Taylor 1937; Kuniata and Sweet, 1994). Research has demonstrated that differences between populations of a species are often related to differences in the ecological resources they exploit and geographic variation (Mayr, 1963; van Valen, 1976). Morphological character studies on eulophids have revealed that the protibial spur appears bifid in some species (see LaSalle and Schauff, 1994). Differences such as in their sexual behaviours are contributing factors in mate selection systems. Sexual dimorphism has long been found very common among Hymenoptera species and studies have been made on the female reproductive system (e.g. Copland and King, 1971; 1972; Copland *et al.*, 1973). Quicke *et al.* (1994) compared ovipositor structures of many Hymenoptera species and found two different apomorphic states of the ovipositor stylets on all female specimens they examined. Geographically distinct populations often differ in their adaptive traits and differences in morphology are important characters that have been revealed to prevent hybridisation among different species thereby maintaining genetic isolation (see Unruh *et al.*, 1986). The populations of *Pediobius* species used in this study were collected from three sub-regions in Africa. Therefore, if the difference in protibial spur among *P. furvus* in Africa is sexual dimorphism then the Kenyan population may be reproductively isolated from those in West Africa. Morphological character

differences as found in the Kenyan population of *Pediobius* may also suggest population divergence. The question now is, does geographic isolation of a population result in change in genetic structure as might be with the Kenyan population of *Pediobius*?

When populations of a species become geographically separated for a considerable period of time, they may diverge and become new species, and may not recognise each other as one species when they meet again, such as in a ring species. The extreme forms of a ring species do not interbreed in the region of overlap, and could provide important evidence for evolution due to intra-specific differences (Ridley, 1996). However, if closely related species share the same geographic range and do not interbreed, certain isolating mechanisms must be responsible for keeping them apart. Mating behaviours investigated in three *Pediobius* species (*P. deschampsiae*, *P. phalaridis*, *P. calamagrostidis*) associated with parasitisation of grass-feeding *Tetramesa* species in UK as a model for the African *Pediobius* have demonstrated this. In some species of Hymenoptera female multiple mating with more than one phenotype has been reported (Fjerdingstad *et al.*, 2003; Cheng *et al.*, 2004; Khanh *et al.*, 2004) and monoandrous and polyandrous aggregation have been linked with solitary and gregarious parasitoid wasps, respectively (Hardy, 1994). As a result it was assumed that males and females of different species will not mate even in close

proximity resulting in producing all male progeny because of reproductive barrier between them. The dearth of females in the F1 generation may perhaps be due to the following factors: (i) sex pheromones not recognised by females from different species as females may discriminate among potential male mates by means of the sex pheromones that they emit which play an important role during the complex sequences of courtship behaviour (Ide and Kondoh, 2000; Ayasse *et al.*, 2001; Romani *et al.*, 2003) (ii) sexual dimorphism that is common between species of Hymenoptera (e.g. female antennal lobe and gaster shape, male genitalia and antennal glands) may inhibit recognition by a different species (Bin *et al.*, 1999; Isidoro *et al.*, 1999; Hansson and Anton, 2000; Guerrieri *et al.*, 2001; Chiappini and Mazzoni, 2000; Pienaar and Greeff, 2003), (iii) incompatible mate searching strategy by different species not recognised by the other (van den Assem and Povel, 1973), and (iv) different mating preferences and the genetic control of behavioural differences (van den Assem and Werren, 1994; Beukeboom and van den Assem, 2001; Khanh *et al.*, 2004).

Careful studies of insect pest biotypes have often found distinct clones in parthenogenetic forms, and genetic distinctiveness and reproductive isolation in sexual ones. However, separation of some insect biotypes may only be possible with the application of molecular techniques to elucidate the genetic characteristics of each individual (Loxdale *et al.*, 1983; Taylor *et al.*, 1997). For

example, Haymer and McInnis (1997) used a variety of primers to detect level of population differences of the Mediterranean fruitfly *Ceratitis capitata* by identifying the regions of the genome that were essentially monomorphic in flies. *Pediobius* species studied in these mate recognition experiments are all solitary ecto-parasitoids of different grass-feeding *Tetramesa* species. Previous studies have found that they are specific in their parasitisation strategies. On the other hand, *P. furvus* from Africa is a gregarious pupal parasitoid especially of lepidopterous maize stem borers, with a high fecundity rate (Jordan, 1966, Overholt and Smith, 1989; Duale and Okwakpam, 1997; Ogol *et al.*, 1998; Chinwada and Overholt, 2001). In the present study more than 200 adults were recovered from a single host, and previous studies recorded between 250-400 from a single mummy. However, mating with more than one phenotype by the African *Pediobius* species might not be possible due to 'within host mating' system in which males emerge first and wait around the exit hole in the pupa to mate with subsequent emerging females (see Overholt and Smith, 1989). This pattern of mating behaviour has been noted in other Hymenoptera species e.g. *Nasonia giraulti* Darling and *N. longicornis* Darling and is believed to prevent hybridisation with other closely related species (Drapeau and Werren, 1999). Mating mechanisms are components that prevent gene exchange between species because in insects, conspecifics and sexual partners are identified and located via sexual and aggregation pheromones, respectively (see Hansson and

Anton, 2000). However, Khanh *et al.* (2004) state that the pteromalid *Anisopteromalus calandrae* can perform multiple mating in the simultaneous presence of several males, and a female mated with males of different phenotypes produces offspring of both phenotypes throughout its reproductive life. Other studies have also recorded similar multiple mating system, e.g. in *Cephalonomia tarsalis* Ashmead (Hymenoptera: Bethyridae) (Cheng *et al.*, 2004) and *Lasius niger* (see Antolin *et al.*, 2004). But van den Assem and Bruijn (1977) state that multiple mating in the pteromalid *N. vitripennis* diminishes the rate of fertilised offspring. Therefore, if behavioural isolation between species can evolve as a consequence of sexual selection among species, then traits that are both sexually selected and used as criteria for species recognition by females should be identifiable (Boake *et al.*, 1997).

The three *Pediobius* population groups, the east African, the mid West Africa and the west West African populations are widely separated geographically. Are these populations reproductively isolated or morphologically different due to geographic isolation; or are they genetically different? Sequence data analysis of cytochrome *b* gene only supported phylogenetic differences between all the West African and the east populations. In a previous study, morphological characters and genetic data based on allozymes and mitochondrial sequence analyses have been used to confirm the

identity of a cryptic species and other closely related Hymenoptera species (see Pinto *et al.*, 1992). Separation of eulophids from their closely related sister species the Elasmidae has been made based on morphology and nuclear gene sequence data (Gauthier *et al.*, 2000). Habitat characteristics in conjunction with morphology have been found useful in populations' differentiation. Typical examples have been revealed between parasitic body lice *Haffneria* sp. (Phthiraptera: Philopteridae) and mammals, and the obligate mutualism between the fig wasp species e.g. *Sycoscapter* species and the *Ficus* plant species (Moraceae) (Lopez-Vaamonde *et al.*, 2001).

*Pediobius* species investigated in this study is a gregarious pupal parasitoid with more than one female ovipositing in a single host. Offspring emerging from a multiple-parasitised host may differ from one another based on the genetic identity of the adult parent. Members of different species are reproductively isolated, and often dramatically differ in morphology. Such differences may involve one or several genes (Lin & Ritland, 1997). Species differences that involve many genes might involve a major gene, while differences involving a single gene might not involve a major gene (Orr, 2001). Molecular analysis of morphological differences has been used to support this hypothesis. For example, in two sibling species of *Drosophila simulans* and *D. sechellia*. Results of genetic mapping experiments revealed that the absence of

hairs in first-instar larva of *D. sechellia* is caused by a single gene (see Sucena & Stern, 2000). In most phylogenetic inference of many Hymenoptera species, the use of partial sequences of the ribosomal RNA (rRNA) from both nuclear and mitochondria highly conserved genes (e.g. 28S RNA gene, 12S and 16S subunits, Cytochrome *b*) have become very popular (e.g. Gauthier *et al.*, 2000; Campbell *et al.*, 1993; Lopez-Vaamonde *et al.*, 2001). Phylogenetic structure of *P. furvus* haplotypes was inferred from 28S rDNA and cytochrome *b* gene sequence alignment with partial sequences of *Sycoscapter* species of Lopez-Vaamonde *et al.* (2001) imported from GenBank. Cytochrome *b* gene sequence analysis revealed that the haplotype KM9 (Kenya population) differ from all the West Africa populations (Fig. 5.8).

When a population migrates to new locations for generations, genetic differentiation from the original may occur due to geographic variation and habitat resource (Dodd, 1989). This might be the case with the Kenya population as well as other populations of *P. furvus* now known to parasitize different host species, e.g. in USA (Overholt and Smith, 1989) and in Papua New Guinea (see Kuniata and Sweet, 1994). Mayr (1969) suggested that much geographical variation is clinal. Clines are the products of two conflicting forces; selection, which would make every population uniquely adapted to its local environment, and gene flow, which would tend to make all populations of

a species identical. Several factors have been suggested to cause enzyme variation in insects. For example, in many populations, allele frequency changes with age or seasonal variation. Therefore, analysing enzymes in insects of varying age minimises such variation (Berry *et al.*, 1987).

## 7.2 Conclusion

*Pediobius*, as with many other chalcidoid species are made up of populations that exploit a variety of environments, which are contributing factors of natural selection. The results of all analyses carried out in this study clearly separated Kenyan population from all others in each first calculation, and the GH population was equally separated on the same level in Discriminant function analysis (Figs 6.3-4, 6.7-8. Some taxonomists have argued that variation in size is a result of environmental induced differences. For example, Bookstein (1989) argued that it is difficult to define the exact meaning of 'size' and 'shape'. These may be considered in a multivariate sense as general factors present among the relative lengths of morphological distance measurements (Humphries *et al.*, 1981). Gillham and Claridge (1994) also argued that although Discriminant function analysis on distance measurements can maximise the morphological variation found among populations, it does not address the question of partitioning size and shape within population. However, in this study, the results suggest that the Kenyan population showed

distinctive morphological difference from all others. The variation among populations was tested using analyses of Principal component (PCA) and Discriminant functions (DFA). Distance measurements of body characters were used to quantify morphological variation of *P. furvus* populations. The results are being supported by the result of cytochrome *b* sequence analysis of all populations which also placed the Kenyan population as separate (Fig. 5.9; 6.3, 6.7). Separation of the Kenyan population is also supported by the result of stepwise Squared Euclidean distance analysis which calculated the Kenyan population with 100% correct reclassification with an overall 82.5% for all populations (Table 6.7). The analysis revealed strong morphometric plasticity between morphological characters and *Pediobius* populations. The six populations studied show high morphological character similarity, and both mitochondrial cytochrome *b* gene sequence data and morphometric analyses revealed that the KM9 (Kenyan population) is different from all the West Africa populations of *P. furvus* (Figs. 5.9; 6.3 & 6.7). Although population separation inferred from mitochondrial DNA genes sequence data is more reliable than morphological distinctiveness, however, in this present study both analyses revealed similar results, indicating that effective use of morphometric data could provide a better understanding of morphological differences in population separation.

### 7.3 References

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**Appendix I: Various species concepts in practice**

1. **Agamospecies (ASC)**
2. **Biological species Concept (BSC)**
3. **Cohesion Species Concept (CSC)**
4. **Cladistic Species Concept (ClSC)**
5. **Composite Species Concept (CpSC)**
6. **Ecological Species Concept (EcSC)**
7. **Evolutionary Significant unit (ESU)**
8. **Evolutionary Species Concept (ESC)**
9. **Genealogical Concordance Concept (GCC)**
10. **Genetic Species Concept (GSP)**
11. **Genotypic Cluster Definition (GCD)**
12. **Hennigian Species Concept (HSC)**
13. **Internodal Species Concept (ISC)**
14. **Morphological Species Concept (MSC)**
15. **Non-dimensional Species Concept NDSC)**
16. **Phenetic Species Concept (PhSC)**
17. **Phylogenetic Species Concept (PSC)**
  - a. **Diagnosable Version**
  - b. **Monophyly Version**
  - c. **Diagnosable and Monophyly Version**

**18. Polythetic Species Concept (PtSC)**

**19. Recognition Species Concept (RSC)**

**20. Reproductive Competition Concept (RCC)**

**21. Successional Species Concept (SSC)**

**22. Taxonomic Species Concept (TSC)**

Appendix II: Morphology-based dendrogram of 120 individuals of six *Pediobius* populations using average group linkage method based on Squared Euclidean Distance procedure from rescaled variable.

