MORPHOMETRIC AND MOLECULAR COMPARISONS OF TWO ISOLATED POPULATIONS OF THE DESERT LOCUST, *SCHISTOCERCA GREGARIA*, (ORTHOPTERA: ACRIDIDAE)

BY

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To my best Teacher ..
Prof. A. Hassali ...

Thanks for all you did to me ...

Your Son
Hatem Mohamed
2/8/96

DECLARATION

I hereby declare that the work embodied in this thesis is a result of my own investigations during the three years research undertaken under supervision at the International Center of Insect Physiology and Ecology, Nairobi, Kenya, and has not been submitted before for any degree in any other Univeristy.

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DEDICATION

** This thesis is dedicated to my dear parents for love, prayers, encouragement and support throughout my whole life.*

** To my dear lovable wife, Eman for the sacrifies she made and the constant prayers she offered througout my study period.*

** To my children, Mohammed and Karim for the many hours they had to spend without their daddy around, which they never understood but bore so courageously*

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ABBREVIATIONS

A	Adenosine
ATP	Adenosine 5'-triphosphate
Amp	Ampicillin
bp	base pair
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
C	Cytosine
conc	Concentration
CsCl	Caesium chloride
D^2	Mahalonobis distence
dATP	2'-deoxyadenosine 5'-triphosphate
ddATP	2',3'-dideoxyadenosine triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddCTP	2',3'-dideoxycytidine triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
ddGTP	2',3'-dideoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate

ddTTP	2',3'-dideoxythymidine triphosphate
DTT	1,4-dithio-L-threitol
EDTA	Ethylenediaminetetra-acetic acid
EtBr	Ethidium bromide
G	Guanine
hr(s)	hour(s)
IPTG	Isopropyl-ß-D-thiogalactopyranoside
kb	kilobase pairs
kDa	Kilo-dalton
kg	Kilograme
km	kilometers
l	liter
LB	Luria-Bertani broth
min	minute(s)
M_r	Relative molecular mass
OD_{260}	Optical density at 260 nm
PAGE	Polyacrylamide gel electrophoresis
s	second(s)
SDS	Sodium dodecyl sulfate
T	Thymine
TBE	Tris-borate-EDTA

TEMED	N,N,N',N'-tetraethylethlenediamine
Tris	Tris (hydromethyl) aminomethane
U	units of enzyme activity
UV	ultraviolet (light)
v/v	volume/volume
w/v	weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
a	alpha
ß	beta
m	micro

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بسم الله الرحمن الرحيم

"وما أوتيتم من العلم إلا قليلا"

صدق الله العظيم

SUMMARY

In Africa, there are two well known subspecies of the desert locust: the sahara subspecies, *Schistocerca gregaria gregaria* (Forskal) and the southern west subspecies, *Schistocerca gregaria flaviventris* (Burmeister). The sahara desert area, which is invaded by the *S. g. gregaria* is divided into three main regions: the north central region, south central and western regions. These populations are connected by migration circuits of swarms and gene flow occurs continuously between the populations. The invasion area of *S. g. flaviventris* covers an area of about 860,000 Km^2. It occurs in Namibia, South Africa, Botswana, Angola and Ascension Islands. It is known that the desert area of north Africa was in contact with that of southern Africa via an arid corridor across central Africa. Successive reduction in the aridity have isolated *S. g. flaviventris* population from *S. g. gregaria* when the corridor became filled with woodland and savanna unsuited to the locusts to breed. The objective of the present study was to evaluate the effect of the geographical barrier on the two desert locust populations, *Schistocerca g. gregaria* and *Schistocerca g. flaviventris*. The research focused on the degree of similarity or differences in: (a) the morphometrics by using the univariate and multivariate analyses;

(b) biochemical studies on the cuticular protein by using two dimensional gel electrophoresis technique and (c) genomic studies by using Random Amplified Polymorphic DNA (RAPD)-Polymerase Chain Reaction (PCR) technique. The findings are:

1. Morphometric comparisons by using univariate and multivariate discriminant analyses

Three different measurements (E, F, C) and two ratios (E/F, F/C), were selected for comparison of the morphometrics of the locust populations. The analysis of data was performed to examine the morphological relationships between the different populations with respect to their location only and to examine the relationships between the different forms (males and females) from the four different populations, with respect to their locations.

Univariate analysis technique using ANOVA test was performed for the four locust populations. The means of the different measured characters and the two ratios, indicate significant differences between the southern population and the other three northern populations. The mean values of E, F, C and for E/F ratio were lower for the southern population compared to the three northern populations. The coefficients of variances

(C.V.), were significantly different for all the measured characters. The highest C.V. was for the elytron character (80%) and the lowest was for the E/F ratio (33.41%). This indicates that, the four populations are highly variable in the length of the elytron, followed by those of the F and C measurements.

Multivariate analysis of variance using Wilk's Lambda and Hoteling-Lawley trace test, indicate that the four locust populations were significantly different ($P< 0.0001$). The first characteristic root, for the three measured characters (E, F, C), was 4.95 and the corresponding canonical variate explained 97.42% of the variability. This indicates that samples of both northern and southern populations differ considerably in a systematic way. The first characteristic root for the two ratios, was 5.07 and the corresponding canonical variate was 99.8%. The respective canonical correlations were highly significant ($P< 0.0001$).

Locusts representing different locations were shown in a canonical discriminant space for the three measured characters and for the two ratios. The southern population was represented in group 1 while the three northern populations were grouped together in group 2.

The Mahalanobis distances (D^2) between the centroids of the different populations for the three measured characters and for the two ratios were significantly different ($P< 0.0001$). The distances recorded between the southern and the northern

populations were higher than the distances between the three northern populations.

Standardized canonical coefficients for the three characters and for the two ratios are given. Weights for characters are listed for the first and the second canonical variate. The characters with the greater weights are more effective in population discrimination.

In the univariate analysis, means of the variable characters for different individuals representing the four populations were significantly different. The mean values of the southern males and females show that the former have smaller E, F, C values than the latter, but the means values for the E/F and F/C ratios were approximately the same. The coefficients of variances indicated significant differences between the different sexes for the three measured characters and the two ratios ($P< 0.0001$). The highest C.V. was for the length of the femur (40.18%) and the length of the elytron (38.49%), while the lowest C.V. was the E/F ratio (33.49%).

Multivariate analysis indicates that the two forms of the four population are significantly different from each other ($P< 0.0001$). The first characteristic root for the three measured characters, was 9.05 and the corresponding canonical variate accounted for 75.21% of the variability. The first characteristic root for the two ratios, was 5.16 and the

corresponding canonical variate accounted for 98.9% of the variability. The respective canonical correlations were highly significant ($P < 0.0001$).

Different sexes of the four different populations are shown in a canonical discriminant space for the three measured characters. There are differences in the morphology within the southern population. The females are grouped in group 1 while the males are grouped in group 2. The three northern populations (males and females) are grouped in a separate group 3. The canonical discriminant space for the two ratios, shows that the females and the males of the southern population have similar ratios (group 1) and they are separated from the northern population (group 2).

The Mahalanobis distance between the centroids of the males and females of the four populations showed that the separation between the southern males and females is smaller compared to those between the southern and the northern individuals. Thus the southern males and females are more morphologically closer to each other than to either sex of the northern locusts. Also, the two sexes of the northern populations are more or less similar in their morphological features.

2. Biochemical studies on the cuticular protein by using two dimensional gel electrophoresis technique

Differences in the protein composition of the cuticle of the two sexually mature locust populations (northern and southern), were demonstrated by the two-dimensional gel electrophoretic resolution of the abdominal protein. That of the northern, *S. g. gregaria*, males was resolved into six major bands, while that of the southern, *S. g. flaviventris*, males was resolved into three major bands only. Bands number 1, 2 and 3 were recorded in the two gels of the populations at the same molecular weight ranging from 21.5 to 31.0 kDa. On the other hand, bands number 4 and 5 at high molecular weights ranged from 70 to 97.4 kDa and band number 6 at low molecular weight (~ 20 kDa), were recorded only in the northern males. *S. g. gregaria*, females gave five major bands. These bands showed to be correlated with the bands from the northern males. Bands number 1, 2, 3, 4 and 5 were recognized in the two gels at the same molecular weights. However, band number 6 was recognized only in the northern males. Separation of the abdominal extracts of southern female locust, gave two major protein bands. Bands number 2 and 3 were recorded also in the northern females at the same molecular weights. Band number 1 was not recorded in the southern females as it shown in

the northern females. On the other hand, the protein profile of the southern females resembled that of the southern males except for the absence of low molecular weight (~ 25 kDa) protein (number 1) which is characteristic to the southern males only.

Thus, the major differences between the northern and southern abdominal protein compositions result from the presence or absence of bands number 4 and 5 corresponding to 70- 97.4 kDa, respectively.

3. Genomic analysis using Random Amplified Polymorphic DNA- PCR technique

Twenty primers were screened with the pool genomic DNA of both males and females of the two populations. Sixteen primers (A1, A3, A4, A5, A6, A7, A8, A9, A10, A11, A13, A14, A15, A16, A17 and A19), were examined and excluded from further screening because they produced homologous patterns as well as smeared or faint bands and over bright bands for any DNA templates.

Amplification product with four primers A2, A12, A18, A20, produced monomorphic and polymorphic patterns with the DNA pool samples. The fragments which differentiated the southern from northern locusts were scored at low molecular weights (100- 600 bp). However, three primers, A12, A18 and A20, failed to give

reproducible patterns with the different individuals and pools of the tested populations.

Primer A2, which produced diagnostic polymorphic patterns with the DNA pooled samples, was subjected to a second round of evaluation with individuals (5 males and 5 females) and pooled samples from the two sets of locusts. In the reaction, the amplified patterns were consistent and reproducible for any given DNA template. A distinct band (~ 600 base pairs), clearly visualized on Ethidium bromide agarose gel, was produced with template DNA derived from both males and females of the *S. g. flaviventris*. This band was not apparent in any of the RAPD-PCR products of templates derived from males and females of *S. g. gregaria*.

Southern blots of EcoRI digested genomic DNA of *S. g. gregaria* and *S. g. flaviventris*, were probed with the cloned 600 bp product of the southern males and females locusts. The results obtained from both hybridized membranes for individuals and pooled samples for males and females, were found to hybridize strongly to all males and females and pool DNA samples prepared from the southern locusts. This result revealed that sequences are highly repeated in the genome of the *flaviventris* population but not to the *gregaria* population.

The differences in the morphological, biochemical and genetic characters recognized between the south African locust population, *S. g. flaviventris*, and the northern population *S. g. gregaria* are due to the differences in the environmental factors and the genetic constitutions. The north African locust populations, *S. g. gregaria*, and the south African locust population *S. g. flaviventris* do not meet in nature, that is, the gene flow between them is prevented. This and our results suggests that the two populations have diverged into two different species. *S. g. flaviventris* represents an evolutionary dichotomy of the genus *Schistocerca* in the African continent.

Multivariate analysis of the morphometrics, two dimensional gel electrophoresis and RAPD-PCR techniques represent powerful tools in the differentiation of the locust populations, as in other insects.

Primers A2, A12, A18, A20 displayed specific amplified bands with the genomic DNA obtained from the southern locust *S. g. flaviventris*, which are not recognized by that from the northern *S. g. gregaria*. These primers are thus useful as genetic markers for differentiation between the northern and the southern locusts.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

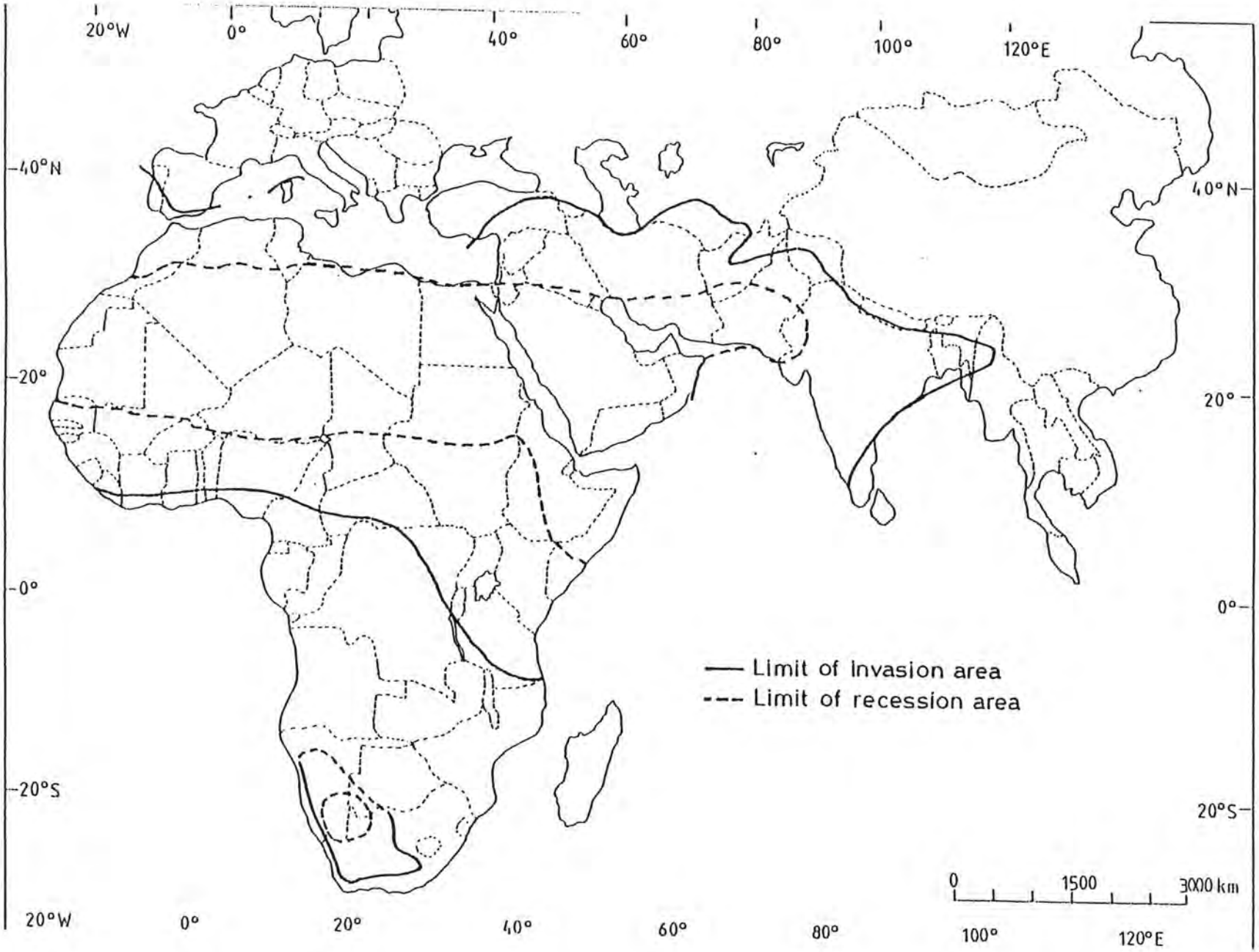
Since the biblical times it has been known that locust swarms cause great damage to grassland, agricultural crops and various other plants. These losses are not confined to Africa, India or Asia minor, which are the invasion territories of the desert locust, *Schistocerca gregaria*, but also affected other areas of the earth infested by other species of locusts. The desert locust is not a significant pest in its solitarious phase, in the recession areas, but becomes a major pest when gregarized and form swarms stimulated by the rain and improved vegetation (Nolte, 1974).

In Africa, the desert locust is one of four Acridid species of economic importance. The extent of its vast invasion area became appreciated only in the 1930 when Sir Boris Uvarov organized the systematic collection and analysis of its records and published a series of accounts of its 1926-1934 plague (Jago, 1970).

In Africa, there are two well known subspecies of the desert locust: the Sahara subspecies, *Schistocerca gregaria gregaria* (Forskal) and the southern west subspecies, *Schistocerca gregaria flaviventris* (Burmeister) (Meinzingen, 1993).

The Sahara desert area, which is invaded by the *S. g. gregaria* is divided into three main regions: the north central region, south central and western regions (Figure 1.1). The northern populations are connected by migration circuits of swarms or gene flow between the populations (Waloff, 1976). The invasion area of *S. g. flaviventris* covers an area of about 860,000 Km^2. It occurs in Namibia, South Africa, Botswana, Angola and Ascension Islands (Fig. 1.1). *S. g. flaviventris* is recognized as a subspecies separated from the north African populations in the degree of phase changes in the colouration (Nolte, 1965; Botha, 1967; Waloff, 1976; Harvey, 1981; Waloff and Pedgley, 1968). It is known that the desert area of north Africa was in contact with that of southern Africa via an arid corridor across central Africa. Successive reduction in the aridity could have isolated *S. g. flaviventris* population from *S. g. gregaria* when the corridor became filled with woodland and savanna unsuited to the locusts to breed (VanZinder-Bakker, 1976; Jago *et al.*, 1979)

Figure 1. 1 Invasion and recession area of the desert locust, *Schistocerca gregaria*, in the African continent



Subspecies in grasshoppers, in general, are usually a kind of averaging of the tiny inter-population differences with names attached only to what are presumably populations of insects carrying large numbers of integrated enzyme polymorphs, or a few polymorphs with dramatic externally observable features (Descamps, 1970). For future systematic work in the Acridoidea, it has been suggested that in addition to the criteria based on classical morphology, differences in internal organ morphology, genotype, karyotype, biochemical analysis should be taken into account (Jago, 1970).

Mayr (1963) pointed out that the use of trinomials does not always indicate the application of the modern species concept. Bolivar (1914) was one of the earliest to use trinomials, but leaves doubt the application of the trinomials, are applied to geographical races or to units previously defined by the term variety. The taxonomic problem is where to start drawing lines between geographical races, what nomenclature to use and how to indicate to outsiders, on what bases a certain taxa was raised. White (1968) mentioned that in groups where there are many cases of sibling species, subspecies and species complexes, it does not seem possible to carry out alpha-taxonomy (dealing with the description of species and arrangement in a comprehensive genera) in a fruitful manner and it would be more fruitful to proceed at

the beta level (dealing with species relationship) and the level of gamma-taxonomy (dealing with intra-specific variation).

The objective of the present study was to evaluate the effect of the geographical barrier on the two desert locust populations, *Schistocerca g. gregaria* and *Schistocerca g. flaviventris*. What has been the extent of the genetic divergence between the two populations? What is the degree of speciation between them ?

To answer the above questions, the research focused on :

1. The degree of similarity or differences in the morphometrics by using the univariate and multivariate analyses.
2. Biochemical studies on the cuticular protein by using two dimensional gel electrophoresis.
3. Genomic studies by using Random Amplified Polymorphic DNA (RAPD)-Polymerase Chain Reaction (PCR) technique.

1.2 LITERATURE REVIEW

1.2.1 MORPHOMETRIC COMPARISON USING UNIVARIATE AND MULTIVARIATE ANALYSES

The body size of an animal, of which morphometrics are a numerical expression, is dependent upon genetically transmitted characters and the modifying influence of the environment on them (Stower *et al.*, 1960). Morphometric methods are powerful research tools when used in the context of sound biological knowledge. Morphometrics allows one to summarize morphological data numerically and graphically, to express and test hypothetical relationships in many dimensions. There was a common opinion that the variance of size of organisms has a smaller genetic component than the variance of shape, hence more attention has been given to shape in systematic and evolutionary studies (Daly, 1985). On the other hand, Atchley (1983) has argued that size deserves more emphasis. Body size is heritable and is obviously a fundamental consideration in the ecogeography of an organism (Gould and Johanston, 1972). Many physiological processes are size related (Platt and Silvert, 1981). Studies on the geographic variation of *Apis mellifera* (Gadbin *et al.*, 1979), *Musca domestica* (Bryant, 1977), *Musca autumnalis* (Bryant and

Turner, 1978) and chromosomal races of morabine grasshoppers (Atchley and Hensleigh, 1974) indicate that size is the most important source of variation.

Qualitative characters are usually favored for describing and delimiting higher taxa of insects. The measurements of the wings of species in various genera and families of calyptrate Diptera, was found to be useful in assessing phenetic relationships. In this regard, canonical variate analysis has been used for ordination and cluster analysis of the pair-wise distance statistics for comparison between taxa. The results indicated that the wings of the flies are subject to strong stabilizing selection and exhibit moderately conservative changes among the taxa studied (Brown, 1979).

Plowright and Stephen (1973) chose 38 variables from the forewings of 60 species of the bee *Bombus* and 13 species of the closely related cuckoo bee *Psithyrus* in an effort to elucidate the generic relationships of the bumble bees and their parasites. Numerical taxonomic and canonical variate analyses yielded groupings of species of *Bombus* consistent with known subgenera and a single group for *Psithyrus*, thus supporting a monophyletic origin of the parasites.

The results of morphometrics analyses have been used by taxonomists to justify synonymy as well the recognition of new

taxa at the species level. Hogue (1982) examined 15 characters of male midges of the genus *Bibiocephala* in North America. Univariate and multivariate analyses indicated that no subsets clearly and consistently justified more than one of the four nominate species. Butler (1982) noticed two periods of adult emergence in a population of *Chironomus sp.* in Alaska. Univariate and bivariate analyses revealed two new species that were distinguishable as larvae, pupae, and male and female adults.

Distinct differences between proximate populations have been demonstrated by morphometric methods. Doyen and Slobodchikoff (1984) found size differences in microgeographic races of a coastal dune beetle within distances up to 500 m from the highest tide. Cornuet *et al.*,(1978) were able to distinguish between populations of honey bees in France by discriminant analysis of six characters. A partial discrimination was obtained between colonies of the same ecotype only 8 Km apart; complete discrimination was obtained between colonies of different ecotypes 120 Km apart.

Historical changes in present distributions of some insects have been reconstructed by morphometric comparisons. Foottit and Mackauer (1980) made a multivariate analysis of 21-33 characters of the European balsam woolly aphid that was introduced into

eastern North America several times before 1900. The divergence into three distinct populations was traced in the context of the aphid's parthenogenetic reproduction and colonization history.

Janson (1980) was able to reconstruct the history of postglacial distribution of the water boatman *Arctocorisa carinata* in northern Europe by a multivariate analysis of continuous and meristic characters. Isolated populations in previously glaciated areas could be recognized by their morphometric affinities to southern populations.

Kimani (1995) used the canonical discriminant analysis and the principle component analysis to separate the parasite *Cotesia flavipes* complex (Hymenoptera) into discrete groups. The canonical variate analysis indicated that there were three distinct groups in the *C. flavipes* species complex.

Morphometrics was first used in locust studies by Uvarov (1921). He employed pairs of measurements compounded into ratios to investigate differences between the supposed species *danica* and *migratoria* in the genus *locusta*. He showed that these were extreme polymorphs, which he termed phases, of the same species, the morphometric ratios providing a measure of the differences between the extremes of a continuous range of morphological variation.

In studies on the desert locust, *Schistocerca gregaria*, Maxwell-Darling (1934) gave a table of the morphometric data, including those for E: F (length of the elytron to length of the femur) ratios of seven populations showing different types of behaviour. Kennedy (1939) using the related morphometrics and behaviour of populations which he had observed, compared them with Maxwell-Darlings table and found marked divergences from the suggested associations. Rao (1942) used a number of ratios, including E: F, and divided their total ranges of values into three parts characteristic of the phases *solitaria, transiens* and *gregaria*, respectively.

Dirsh (1953) investigated a large number of body measurements and their ratios for adults of the desert locust, collected from a series of localities on different occasions, and referred to as being of phase *solitaria*, as well as for adults from swarms of phase *gregaria*. He concluded from a consideration of the means and ranges of different ratios that the best practical separation between the two extreme phases was given by the F: C (length of the femur to width of the head capsule) ratio. A sequel to the work of Dirsh (1953) was the graphical method proposed by Rungs (1954) taking into account both F: C and E: F ratios to differentiate between the phases of *S. gregaria*. Roonwal (1949) and Roonwal and Nag (1951) set up morphometric

limits for the phases *solitaria* and *gregaria* of the desert locust. They compared the measurements and the ratios of the characters E, F, C, P (length of pronotum at the keel), H (height of pronotum), and M (width of the pronotum at constriction), as well as sexual dimorphism and eye-strip number, made on samples taken from scattered populations in Mekran (Baluchistan) during 1935-6 and from swarms in India during the periods 1889-91 and 1930-1. There was no recorded evidence for the population histories before sampling. Misra *et al.*, (1952) using E, F, C and P, applied the method of discriminant function analysis to differentiate the phase status of various populations. The characters F, C, and P were all found to discriminate, but in varying degrees, C being the best. In the case of females, F also was a reliable discriminant. They pointed out that the sample of phase *gregaria* was possibly heterogeneous because the adults were collected from different localities in different seasons.

Albreeht and Blackith (1957) and Blackith (1957) used a modification of a method of analysis related to that employed by Misra *et al.*, (1952) in order to study phase status in *Nomadacris septemfasciata* (Serv.) and *Schistocerca gregaria* and also polymorphism in some Australian Acrididae. The form of multivariate analysis employed by Albreeht and Blackith (1957)

has distinct advantages over the previous single-character and ratio comparisons in that it makes much fuller use of all the morphometric data. It is more sensitive and can provide a concise geometrical representation of the relationships of morphometrics with various recorded environmental factors.

Stower *et al.*, (1960) used E, F, C measurements and the E: F and F:C ratios for 16 populations of the desert locust *S. gregaria*. They concluded that morphometrics may be used for the measurements of phase and phase change, only if the environmental factors under which particular populations developed are also taken into account.

Symmons (1968) described the derivation of a compound shape measure for discriminating between populations of the desert locust, *S. gregaria*. Three characters (E, F, C) were measured from twelve locust populations and multivariate discriminant analysis was conducted. He has argued that, the first canonical variate is a measure of phase. Deng *et al.*, (1996) used three parameters (E, F, C) and two ratios (E/F and F/C) for phase differentiation of the desert locust *S. gregaria*.

1.2.2 SEPARATION OF THE ABDOMINAL CUTICULAR PROTEINS BY TWO DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

Insect cuticle is an extracellular layer, consisting mainly of proteins and chitin, which surrounds the whole insect. The cuticle functions both as skeleton and as a barrier towards the environment, and it is also responsible for the colouration of most insects, either by pigments in the cuticle or by submicroscopic structures (Neville, 1975; Hepburn, 1976). The ultrastructural organization of cuticle follows a general plan in all insect groups: long filaments of chitin are embedded in a matrix of protein, and most differences between cuticles are due to differences in the proteinaceous matrices and the way they are modified during maturation (Andersen *et al.*, 1986).

The cuticle consists of an outer thin epicuticle, containing lipids and proteins and often known to lack chitin, and a thicker procuticle, consisting mainly of chitin and protein (Silvert, 1985). The outermost part of the procuticle is deposited during the pharate period preceding ecdysis. Cuticular proteins deposited during this period are called either pharate proteins or pre-ecdysial proteins. The post-ecdysial proteins are formed in the inner part of the procuticle, the endocuticle. The

epicuticular proteins tend to be inextractable, and quantitatively they constitute a very minor part of the total cuticular proteins. The pre-ecdysial part of the procuticle is in some regions stabilized by the process of sclerotization, whereby the cuticle is hardened and the proteins are made inextractable. In other cuticular regions, the pre-ecdysial proteins remain unmodified, and the cuticle stays soft and flexible (Andersen *et al.*, 1995).

Proteins which can be extracted from pieces of cuticle may not be represented for the total mixture of proteins secreted, and the properties reported for proteins purified from various cuticles may not give a complete picture (Anderson and Barrett, 1971; Hackman, 1974).

Insect cuticles are often classified as either soft and plaint or hard and stiff materials. Much of the stiffness of cuticles is caused by sclerotization, a secondary modification of the cuticular proteins due to incorporation of oxidation products of catecholamine derivatives, but some of the stiffness is also inherent and apparently related to the pronounced hydrophobic character of the proteins in these cuticles (Hillerton and Vincent, 1993). The stiff proteins tend to have alkaline (high pH) isoelectric points, so they will be positively charged at physiological pH-values. The soft and pliable cuticles are

weakly sclerotized, and their proteins tend to be hydrophilic and to have acidic (low pH) isoelectric points (Vincent, 1975).

There is still some uncertainty with regard to where the cuticular proteins are synthesized, whether they originate somewhere else in the body and are taken up from the hemolymph by the epidermis and transported into the cuticle, or whether they synthesized in the epidermal cells. The close similarity between some of the proteins extracted from the hemolymph and the protein of the epidermal cells, has been shown by gel electrophoresis (Koeppe and Gilbert, 1973; Phillips and Loughton, 1976).

Cuticular proteins can be extracted with a number of solvents which affect different intermolecular types of chemical bonds. Solvents generally used for extraction are: distilled water; weak solutions of neutral salts, assumed to break electrostatic and van der Waals interactions between macromolecules; strong solutions of urea, assumed to break hydrogen bonds; and dilute sodium hydroxide, assumed to break stronger bonds, such as Schiff's bases and susceptible peptide bonds (Andersen, 1979).

Gel electrophoresis of proteins, is by far, the most widely used technique in insect molecular systematics. The technique relies on the fact that the identical proteins migrate the same distance under the electrical force applied to an electrophoretic gel, while nonidentical proteins usually migrate different

distances (Berlocher, 1984). Gel electrophoresis has proven useful at all three important systematic tasks: species discrimination, species identification, and hierarchical classification (Avise, 1974; Bush and Kitto, 1978). The amount of genetic variation within and between the natural populations is central to understanding the evolutionary processes. However, most of the early attempts to assess genetic variability were based on the analysis of segregation patterns in progeny of particular crosses, and they suffered from the inherent bias that only genes that exhibited variation could be recognized (Lewontin, 1974).

In the mid 1960s, one dimensional electrophoretic technique was applied to the problem of quantifying genetic variability and divergence in protein products of individual loci (Aquardo and Avise, 1981). This new technique could sample an array of proteins of about 20-50 polymorphic and monomorphic loci. The proteins assayed in conventional electrophoretic surveys may also be biased toward a more variable class of gene products (Leigh Brown and Langly, 1979).

Two dimensional gel electrophoresis (2-D), as introduced by O'Farrell in 1975, has allowed examination of a substantially increased number of protein-encoding loci. In 2-D technique, a mixture of denatured proteins is separated first on the basis of charge by isoelectric focusing, and then on the basis of

molecular weight by SDS slab gel electrophoresis. The technique is capable of separating as many as 1100 peptides from crude cell extracts on a single gel. The ability of this technique to resolve most protein variation resulting from charge-change is well documented as is the typical Mendelian behavior of the variants examined (Berlocher, 1984).

Two-dimensional techniques have been more widely used than one-dimensional gel electrophoresis to assay membrane and other structural proteins in addition to some water-soluble, largely enzymatic, proteins (Leigh Brown and Langley, 1979; Racine and Langley, 1980). Recent applications of 2-D to the analysis of intrapopulation variation have revealed substantially less genetic heterozygosity than had been estimated by single gel electrophoresis (SGE) (Liegh Brown and langley, 1979; McConkey *et al.*, 1979; Walton *et al.*, 1979; Racine and Langley, 1980). Aquadro and Avise (1981) used 2-D technique to compare an average of 189 polypeptides between six species of wild mice representing levels of evolutionary divergence ranging from different subspecies to different families. The magnitude of protein divergence estimated by 2-D was, on the average, only about one-half that predicted by SGE. This discrepancy may result from differences in sensitivities between the techniques or

differences in the mean level of variation and divergence between the sets of loci assayed by the two methods.

The level of heterozygosity in a wild population of the house mouse, *Mus musculus*, was estimated by means of 2-D gel electrophoresis for the whole kidney proteins. The observed level (2%) was substantially below the level detected by starch gel electrophoresis (Racine and Langley, 1980). This result was also similar to the results observed in the *Drosophila* populations (Leigh Brown and Langley, 1979).

The cuticular proteins of the migratory locust, *Locusta migratoria*, have been extracted from different parts of the body and analyzed by 2-D technique (Andersen *et al.*, 1986). An abrupt change in protein pattern is seen immediately after ecdysis, and the protein extracted from cuticle of pharate nymphs or newly emerged adults are different from the proteins extracted from the cuticle of mature nymphs or mature adults, respectively (Andersen and Hojrup, 1987). One- or two-dimensional gel electrophoresis of proteins extracted from abdominal cuticle of adult *Locusta* indicated that only quantitative changes occur in the protein pattern, and also pre-ecdysial cuticle dissected out two days before expected adult ecdysis gave the same protein pattern as cuticle obtained during ecdysis (Andersen *et al.*, 1995).

The pre-ecdysial (pharate) cuticle from adult locusts contain about 80% protein. About 8% of the protein cannot be extracted with 6 M urea. The inextractable fraction is presumably covalently linked to the chitin fraction. The major part of the proteins can be extracted with acidic (<pH 4) or alkaline (>pH 10) solutions, whereas only little protein is extracted at pH-values between 7 and 10 at 20° C (Andersen *et al.*, 1986).

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

Molecular systematics encompasses three main areas of studies: population structure studies (geographic variation, mating systems, heterozygosity, and individual relatedness), identification of species boundaries (including hybridization) and estimation of phylogenies. The genetic structure of a population is perhaps the most fundamental piece of information for a species that requires management (Hills and Mortiz, 1990).

In the past few years there has been a remarkable increase in the application of the DNA analysis to problems in population genetics and systematics. Population genetics deals with genetic changes that occur within populations and evolution is a process of change in the genetic makeup of populations (Li and Graur, 1991). Analysis of DNA has several significant advantages; the genotype rather than the phenotype is assayed, one or more sequence appropriate to inheritance can be made; DNA can be prepared from small amounts of tissue, is relatively stable and the structure of DNA being simple linear sequences of only four nucleotide bases (Post *et al.*, 1992).

There are several approaches of assaying DNA variation in the molecular genetics. Restriction Fragment Length Polymorphism

(RFLP) has become widely used for the construction of genetic maps of many species and for mapping of genetic traits (Ganal *et al.*, 1990; Tanksley *et al.*, 1989). The assay is usually practiced with species-specific probes; it requires DNA blotting and hybridization of several micrograms of DNA, which has been digested with a restriction endonuclease. Radioactive probes are usually used with each probe detecting one or more genetic loci that share sequence homology. Each allele at a locus is identified as a mobility variant of an endonuclease restriction fragment. The RFLP requires well equipped laboratories and is relatively laborious (Rafalski *et al.*, 1991).

Recently, two groups independently and simultaneously described a novel polymorphism assay. Williams *et al.*, (1990) described genetic mapping application and called the new method the RAPD assay, for Random Amplified Polymorphic DNA. Another group, Welsh and McClelland (1990) focused on genome fingerprinting and called their assay Arbitrarily- Primed PCR (AP-PCR). Both of these assays are based upon the observation that a single, short oligodeoxynucleotide (10 bp) of randomly chosen DNA sequence, with at least 50% GC (Ganidine-Cystine) content are preferred. Only 15-25 nanograms of genomic DNA templates are needed. The amplification is preformed in the presence of dNTPs as substrate and catalyse by *Taq* polymerase

enzyme. In order for amplification to occur, low annealing temperature allow the primers to anneal to the arbitrary regions of the genome which are complementary to the primers. The primers annealing sites must be separated at a distance of no greater than 3000 bp as this is the maximum size that can be amplified with routine PCR. The observation that single substitutions, especially in the 3' end of the primer, can change amplified banding pattern, implies that annealing in RAPD-PCR must be precise (Williams *et al.*, 1991a).

The reaction products are separated on standard agarose gels and visualized with Ehidium bromide staining. The nature of the fragments that are amplified, is highly dependent on the primer and the genome sequences. DNA amplification with random sequence primers is a highly sensitive method for discovering polymorphism randomly distributed throughout the genome. A primer usually amplifies several bands, each originating from a different genomic location. The multiplex nature of the RAPD assay results in increased analytical throughput (Black, 1993).

Absence of a fragment presumably occurs because amplification cannot proceed on DNA strands from either of the homologous chromosomes in an individual. This can occur through point mutation at one or both primer annealing sites on a DNA strand, inversions surrounding a site or insertion that separate

the annealing sites at a greater distance than can be amplified (Rafalski *et al.*,1991).

Arbitrary primed PCR is not quantitative and consequently it is unknown whether individuals whose DNA yields a specific fragments are heterozygous (have one copy) or homozygous (two copies) for an amplifiable allele. Alleles at arbitrary primed loci therefore segregate as dominant markers (Williams *et al.*, 1990).

Williams *et al.*, (1991b) reported that more than 95% of the loci amplified in *Nurospora crassa* contained dominant alleles. Hunt and Page (1992) reported that 90% of alleles segregated as dominant markers in *Apis mallifera*. Black *et al.*,(1992) showed that RAPD-PCR could be used to identify polymorphism within and among greenbug (*Schizaphis graminum*) biotypes, an aphid species for rare allelic variation between biotypes was detected at only a single allozyme locus. Abid *et al.*, (1989) have found variation among *Acyrthosiphon pisum* and *Uroleucon ambrosiae* individuals from single plants and demonstrated polymorphism within two endoparasitic wasps, *Lysiphlebus testaceipes* and *Diaretiella rapae*.

Puterka *et al.*, (1993) used allozyme and RAPD-PCR markers to examine genetic relationships among populations of the Russian wheat aphid (*Diuraphis noxia*). Three of twenty allozyme loci

were polymorphic and cluster analysis with these markers was only able to separate thirty-five populations into five groups. In contrast, cluster analysis with RAPD-PCR polymorphism placed populations into the same five groups but further separated populations within each group. A large number of polymorphism has been detected in honey bees with RAPD-PCR (Hunt and Page, 1992).

Roehrdanz *et al.*, (1993) found intraspecific variation in two endoparasitic hymenoptera species and extensive intrspecific variation among closely related species. Roehrdanz and Flanders (1993) also found interaspecific variation in four coccinelid species. Perring *et al.*, (1993) were able to use arbitrary primers to identify variation within and among whitefly taxa in the genus *Bemesia*.

Chapco *et al.*, (1992) used RAPD-PCR markers to identify intraspecific variation within *Melanoplinae* and *Oedipodinae* (grasshopper) species. RAPD-PCR distinguished subspecies in *Aedes aegypti* (Ballinger-Crabtree *et al.*, 1992). Kambhampati *et al.*, (1993) used RAPD-PCR loci as species diagnostic markers in *Aedes scutellaris* group. Wilkerson *et al.*, (1993) used RAPD-PCR to distinguish the two cryptic species *Anopheles gambiae* and *An. arabiensis*. Apostol *et al.*, (1993) described a technique to use

RAPD alleles as fingerprinting markers in estimating the number of full sibling families at an oviposition site.

Hymer *et al.*, (1993) used RAPD-PCR to distinguish different populations of the Mediterranean fruit fly, *Ceratitis capitata*.

The populations used include strains which differ primarily in terms of geographical origin. In addition, strains that have been in a long term laboratory culture can also be distinguished from wild caught strains. Kimani (1995) used RAPD-PCR to differentiate the *Cotesia flavips* complex (Hymenoptra).

The advantages of these techniques (RAPD-PCR, AR-PCR) are that they do not require cloning or DNA sequence information for primer design, they do not employ labelling with radioactive markers, and genetic polymorphism can be visualized within 24 h from extraction of genomic DNA. This contrasts with techniques that employ Southern analysis (RFLP) in which the researcher must wait a minimum of 5-7 days to visualize polymorphism. The large number of bands amplified in individuals, permits their use in genetic fingerprinting, species diagnostics and differentiation of cryptic species (Black, 1993).

CHAPTER TWO

MORPHOMETRIC COMPARISONS USING UNIVARIATE AND MULTIVARIATE DISCRIMINANT ANALYSES

2.1 Introduction

Analysis of geographical variation within species continues to receive attention by taxonomists. Character differences between populations have traditionally been compared using univariate statistical procedures. Increasingly, more multivariate statistical studies have been used to examine geographical variations in insect populations (Gould and Johnston, 1972; Niles, 1973; Kamani, 1995).

The use of multivariate analysis in systematics has been formalized in several ways. It includes both canonical discriminant analysis and generalized distance, or the Mahalanobis distance (D^2). The discriminant analysis model, also known as the classification criterion, is based either on the individual within-group covariance matrices or the pooled covariance matrices (Zimmerman and Ludwing, 1974; Fargo *et al.*, 1986).

Canonical discriminant analysis is a dimension-reduction technique related to the principal component analysis and canonical correlation. Given two or more groups of observations, with measurements on several quantitative variables, canonical discriminant analysis derives the linear combination of the variables with the highest possible multiple correlation with the groups (Inayatullah *et al.*, 1987). The maximal multiple correlation is designated as the first canonical correlation. Canonical correlation is a generalization of correlation and regression that is applicable when the attributes of a single group of objects can be divided naturally into two sets. Canonical correlation calculates overall correlations between two sets of variables. Linear combinations within the first set of variables and within the second set are considered simultaneously and the linear combinations that maximize the correlation between the two sets are selected. The coefficients of the linear combinations are designated as the canonical coefficients or canonical weights. The variable defined by the linear combination is the first canonical variable or canonical component. The second canonical correlation is obtained by finding a linear combination uncorrelated with the first canonical variable, but having the highest multiple correlation with the groups (SAS Institute, 1988).

The relationship between the groups is measured by the Mahalanobis distances between their centroids (Mahalanobis, 1936; Rao, 1952). Small distances indicate close relationships between groups, whereas greater distance indicates more distant associations. Distance analysis determines how physiology, anatomy, behaviour, or ecology of one population contrasts with those of other populations (Pimentel, 1979).

The objective of this chapter was to use univariate and multivariate discriminant techniques to evaluate the differences between the northern and the southern African locust populations, and to quantify the extent of variation present between these populations.

2.2 Materials and Methods

2.2.1 Insects

Four different locust populations from different localities, were selected for the study. The north African gregarious populations, *Schistocerca g. gregaria*, were freshly collected from ICIPE field station at the Red Sea in the Sudan (f), and from Ethiopia (Ethiopian field population); (e), and the third was the Red Sea population (r), reared at the ICIPE insectary, Nairobi, which has been reared for twenty generations. The South African gregarious population (s), *Schistocerca gregaria flaviventris*, was collected by Dr. Dick Brown (Plant protection institute, Protoria) from the Kalahari desert in the Republic of South Africa. The males and the females of the different populations, were collected from the field as fledglings.

Insects were reared in the Insect and Animal Breading Unit (IABU) of ICIPE, according to the method described by Ochieng-Odero *et al.*,(1991). Gregarious locusts were maintained under crowded conditions (~ 200 per cage), the four locust populations were kept at 28-34^{0} C, 40-50% relative humidity and daily illumination of 12 hours. The diet consisted mainly of fresh sorghum plants and wheat brans. After two weeks from maturation,

twenty males and twenty females from the different populations were collected and subjected to morphometric measurements.

2.2.2 Choice of the characters and the measurements

Three characters were selected, the length of fore-wing (E), posterior femur (F) and the widest part of the head capsule (C) (Deng *et al.* 1996). The characters were measured using an electronic caliper (Trimos Sylvac Metrology Ltd, London, UK, range of 0-150 mm, accuracy $\pm$ 0.03 mm), according to the method described by Ochieng- Odero *et al.*, (1991). Two ratios (E/F, F/C) were also calculated and compared for the different populations.

2.2.3 Data analysis

The measurements were analyzed using One-way Analysis of variance (ANOVA) and Multivariate discriminant analysis of variance. The data analysis were performed using the Statistical Analysis System software (SAS version 6.04, SAS Institute, 1988).

2.3 Results

Three different measurements (E, F, C) (Fig. 2.1) and two ratios (E/F, F/C), were selected for comparison of the morphometrics of the locust populations. The analysis of data was performed to examine the morphological relationships between the different populations with respect to their location only and to examine the relationships between the different forms (males and females) from the four different populations, with respect to their locations (Appendix A).

A. Comparison with respect to location

Univariate analysis technique using ANOVA test was performed. The means of the different measured characters and the two ratios indicated significant differences between the southern population and the other three northern populations (Table 2.1). The means values of E, F, C and for E/F ratio (except F/C ratio), were lower for the southern population compared to the three northern populations.

The coefficients of variances (C.V.) were significantly different for all the measured characters (Table 2.2). The highest C.V. was for the elytron character (80%) and the lowest

Figure 2.1 Diagrammatic scheme of the three measurements of the desert locust, *Schistocerca gregaria*. E: length of elytron; F: length of femur; C: width of head capsule.

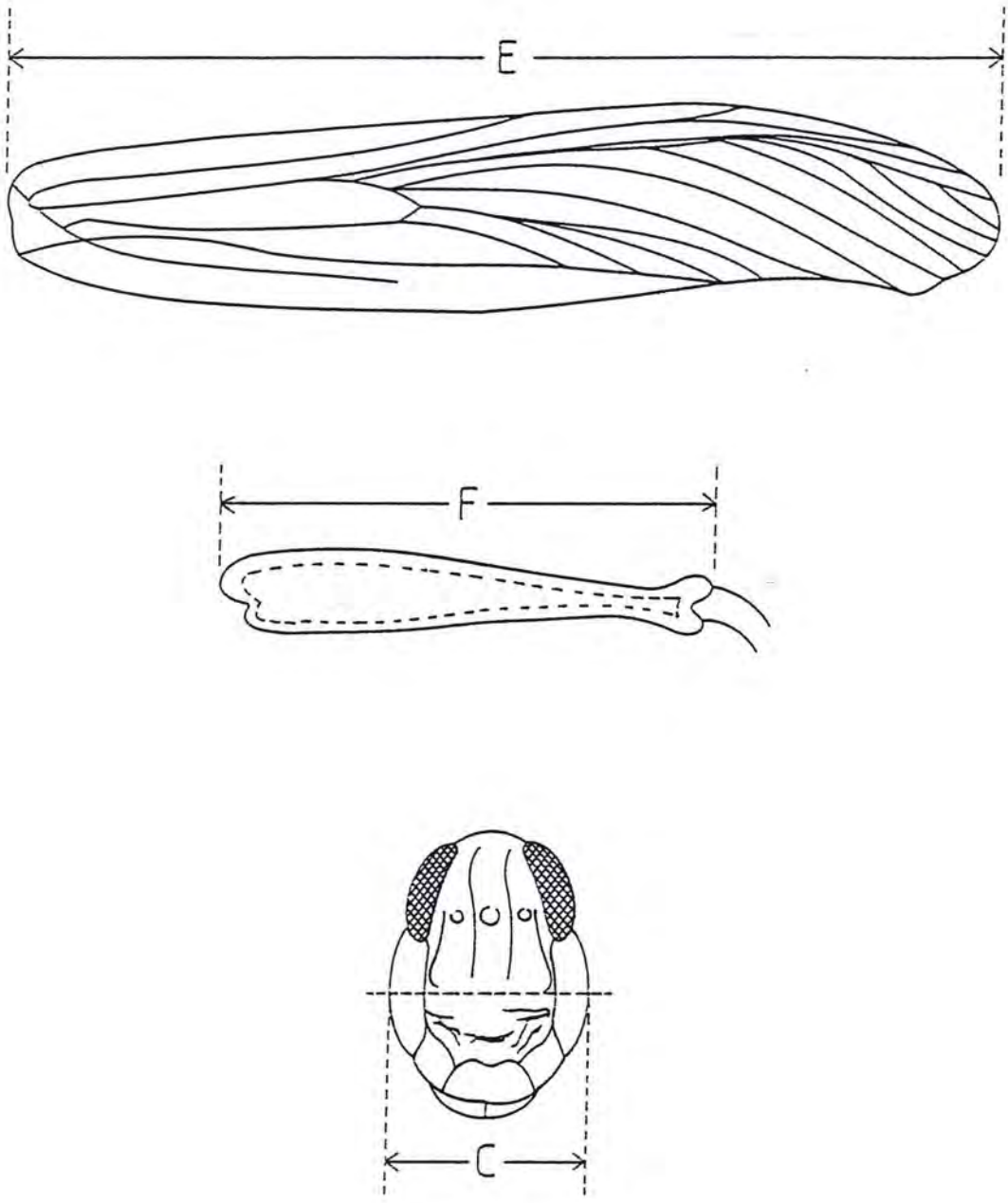


Table 2.1 Mean values and standard errors of different morphological characters for the four locust populations, *Schistocerca gregaria*

POPULATION/CHARACTER	E	F	C	E/F	F/C
f	58.03 ±0.79a	26.55 ±0.32a	7.40 ±0.09a	2.18 ±0.01b	3.58 ±0.02b
r	57.57 ±0.81a	26.20 ±0.35a	7.34 ±0.09a b	2.19 ±0.01b	3.57 ±0.02b
e	55.17 ±0.58b	26.02 ±0.27a	7.12 ±0.07b	2.24 ±0.01a	3.45 ±0.02c
s	52.87 ±0.70c	24.64 ±0.37b	6.24 ±0.08c	2.03 ±0.01c	4.16 ±0.02a

* Means followed by the same letter in a column are not significantly different (P = 0.001).

* E: elytron length F: femur length C: head capsule width.

* f: red sea population r: Ex-Addis population

* e: Ethiopian population s: Southern population.

Table 2.2 Coefficient of variations in characters of the desert locust populations, *Schistocerca gregaria*

VARIABLES	C.V. %	Pr > F
E	80.00%	0.001
F	78.80%	0.003
C	71.50%	0.001
E/F	33.41%	0.001
F/C	35.40%	0.001

* E: elytron length F: femur length C: head capsule width

was for the E/F ratio (33.41%). This indicated that, the four populations were highly variable in the length of the elytron, followed by variation in the F and C values.

Multivariate analysis of variance using Wilk's Lambda and Hoteling-Lawley trace test (Lindeman *et al.*, 1980) indicated that the four locust populations were significantly different ($P< 0.0001$). Discriminant analysis grouped the similar morphometric locusts in one group and the different morphometric locusts into other groups. In order to account for the relationships between the variables and to obtain a three dimensional representation, the E, F, C axes are inclined at angles depending on the correlations between them, and the sum of the squares of the distances between sample means is then D^2. The sample means also will form a cloud of points in three dimensions and in order to reduce the dimensions, the first canonical variate is calculated. The first canonical variate is thus the best single linear compound measure, based on values of E, F, C, E/F and F/C. The first characteristic root for the three measured characters (E, F, C) was 4.95 and the corresponding canonical variate explained 97.42% of the variability (Table 2.3). This indicates that samples of both northern and southern populations differed considerably in a systematic way. The second characteristic root was 0.13 with a

corresponding canonical variate that explained 2.57% of the variability. The first characteristic root for the two ratios was 5.07 and the corresponding canonical variate was 99.8% , while the second characteristic root was 0.01 and the corresponding canonical variate explained 0.2% of the variability (Table 2.4). The respective canonical correlations were highly significant ($P< 0.0001$).

Locusts representing different locations were shown in a canonical discriminant space for the three measured characters (Fig. 2.2) and for the two ratios in Fig. 2.3. Two different and well separated groups were obtained, are representing the northern populations (group 2) and the southern population (group 1).

The Mahalanobis distances (D^2) between the centroids of the different populations for the three measured characters are shown in Fig. 2.4 and for the two ratios in Fig. 2.5. The distances between the four different populations were significantly different ($P< 0.0001$). However, the distances between the southern and the northern populations were significantly higher than those between the three northern populations.

Standardized canonical coefficients for the three characters and for the two ratios are given in Table 2.5. Weights for characters were listed for the first and the second canonical variate. The characters with the greater weights are the most

Table 2.3 Characteristic roots and the propotions of the three measured characters for the four desert locust populations, *Schistocerca gregaria*

First characteristic root (Eigenvalue)	Proportion (%)
4.95	97.42
0.13	2.57

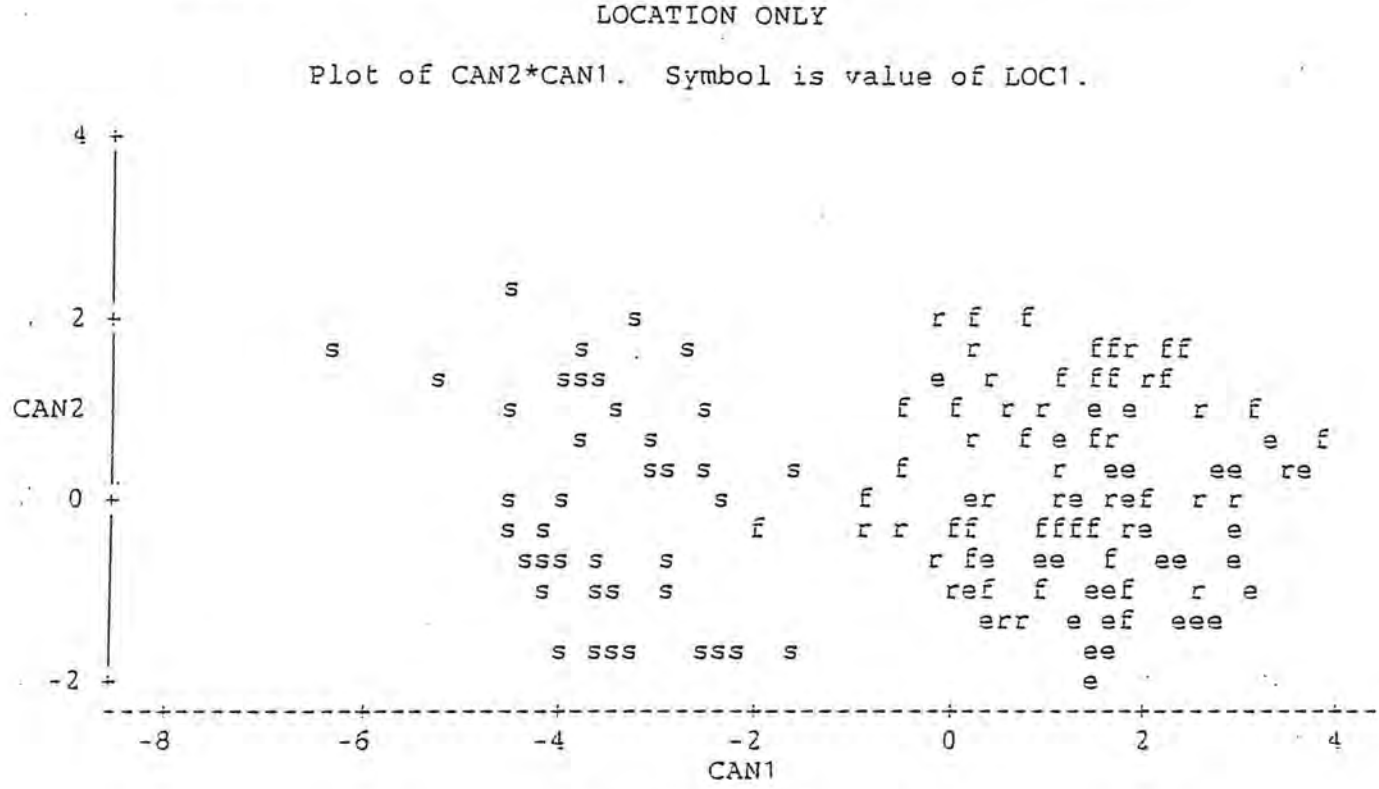
Table 2.4 Characteristic root and the proportions of the two ratios for the four different locust populations, *Schistocerca gregaria*

First characteristic root (Eigenvalue)	Propotion (%)
5.07	99.80
0.01	0.20

Table 2.5 Standardized canonical coefficient of the measured characters and ratios for the four desert locust populations, *Schistocerca gregaria*.

Characters	Can 1	Can 2
E	0.44	- 0.59
F	- 2.32	1.22
C	2.79	0.48
E/F	0.26	1.58
F/C	- 2.22	1.30

Figure 2.2 Plot representing the four different desert locust population along the two axes of canonical discriminant space for the three measured characters (E, F, C). f= Red sea field population; r= Red sea lab. population; e= Ethiopian population and s= Kalahari field population. Group 2= north African desert locust populations and Group 1= south African desert locust population.



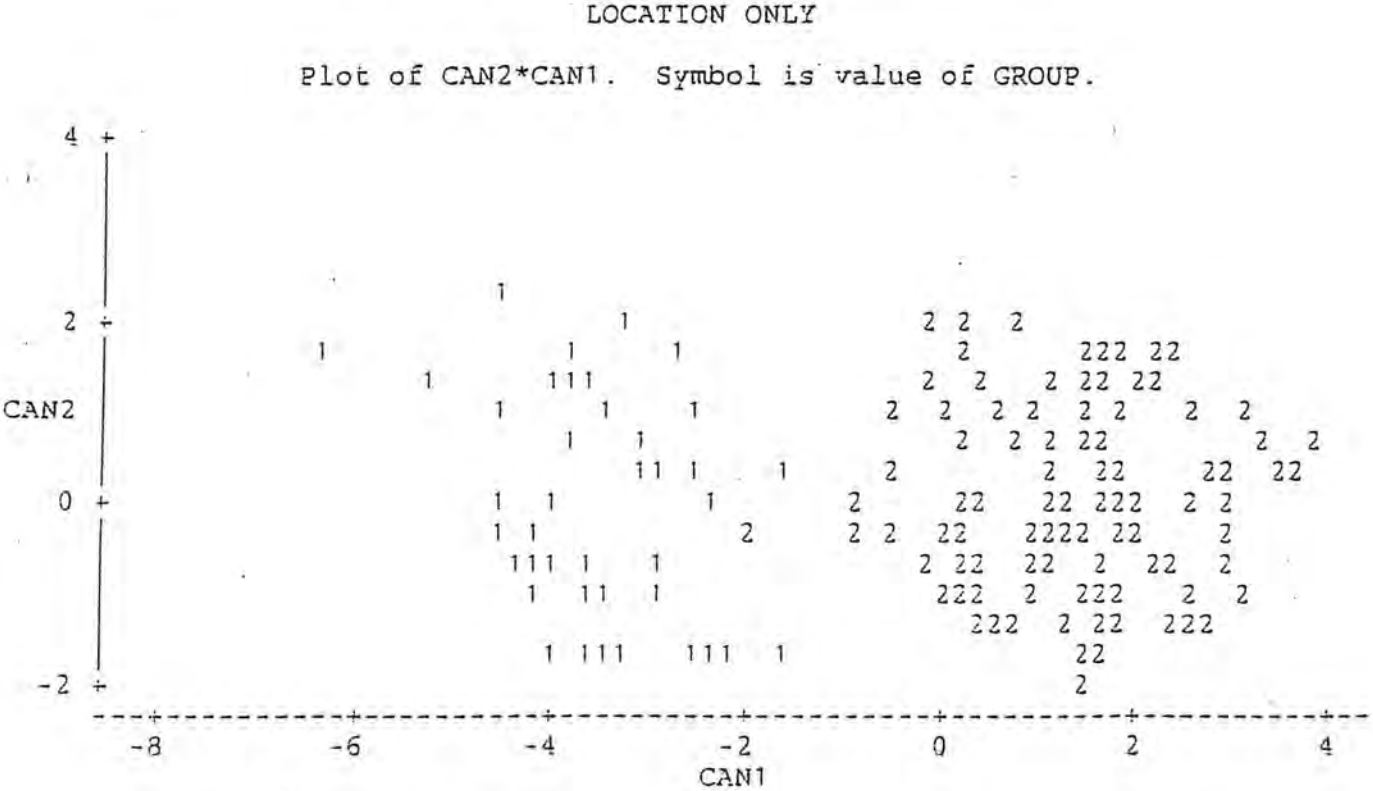
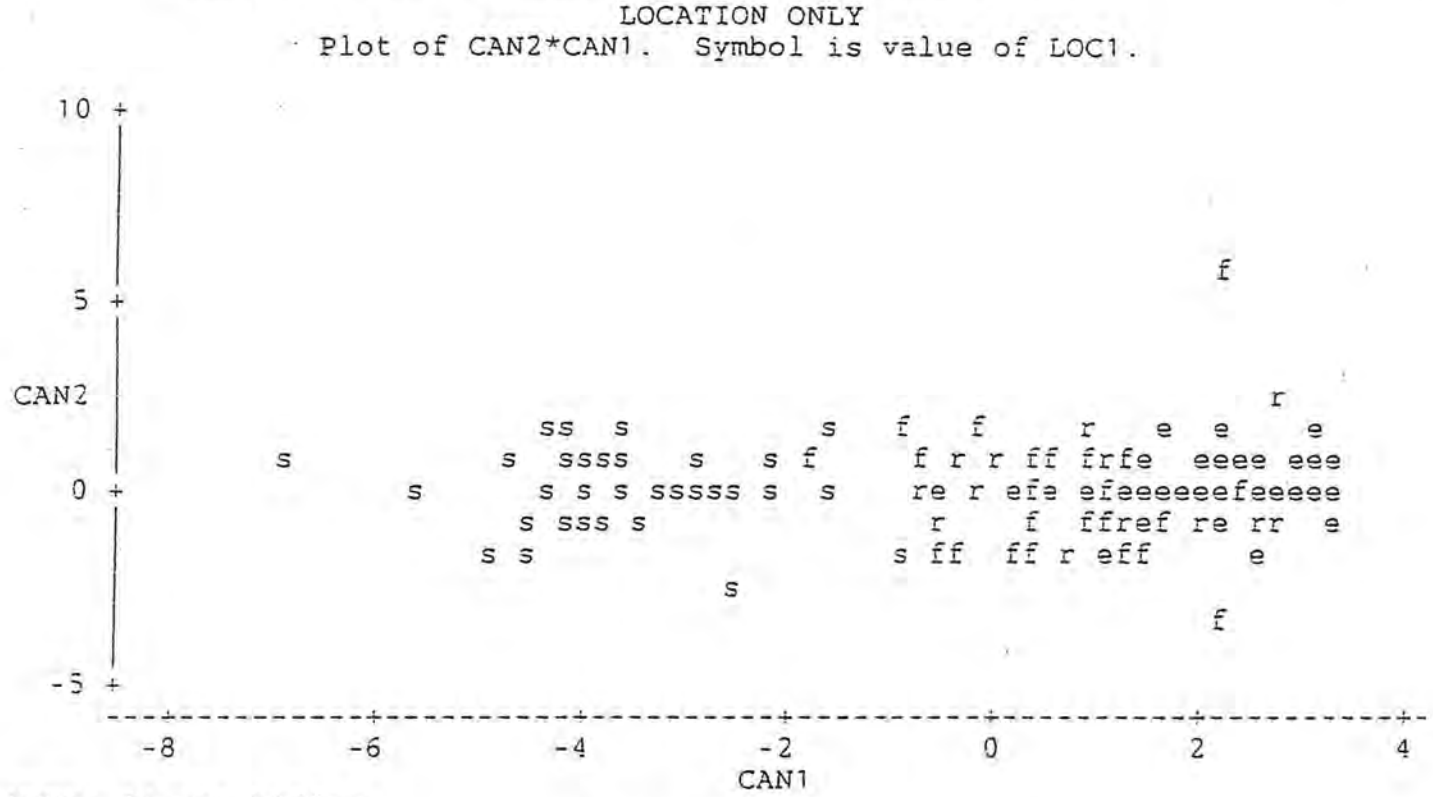


Figure 2.3 Plot representing the four different desert locust population along the two axes of canonical discriminant space for the two ratios (E/F, F/C). f= Red sea field population; r= Red sea lab. population; e= Ethiopian population and s= Kalahari field population. Group 2= north African desert locust populations and Group 1= south African desert locust population.



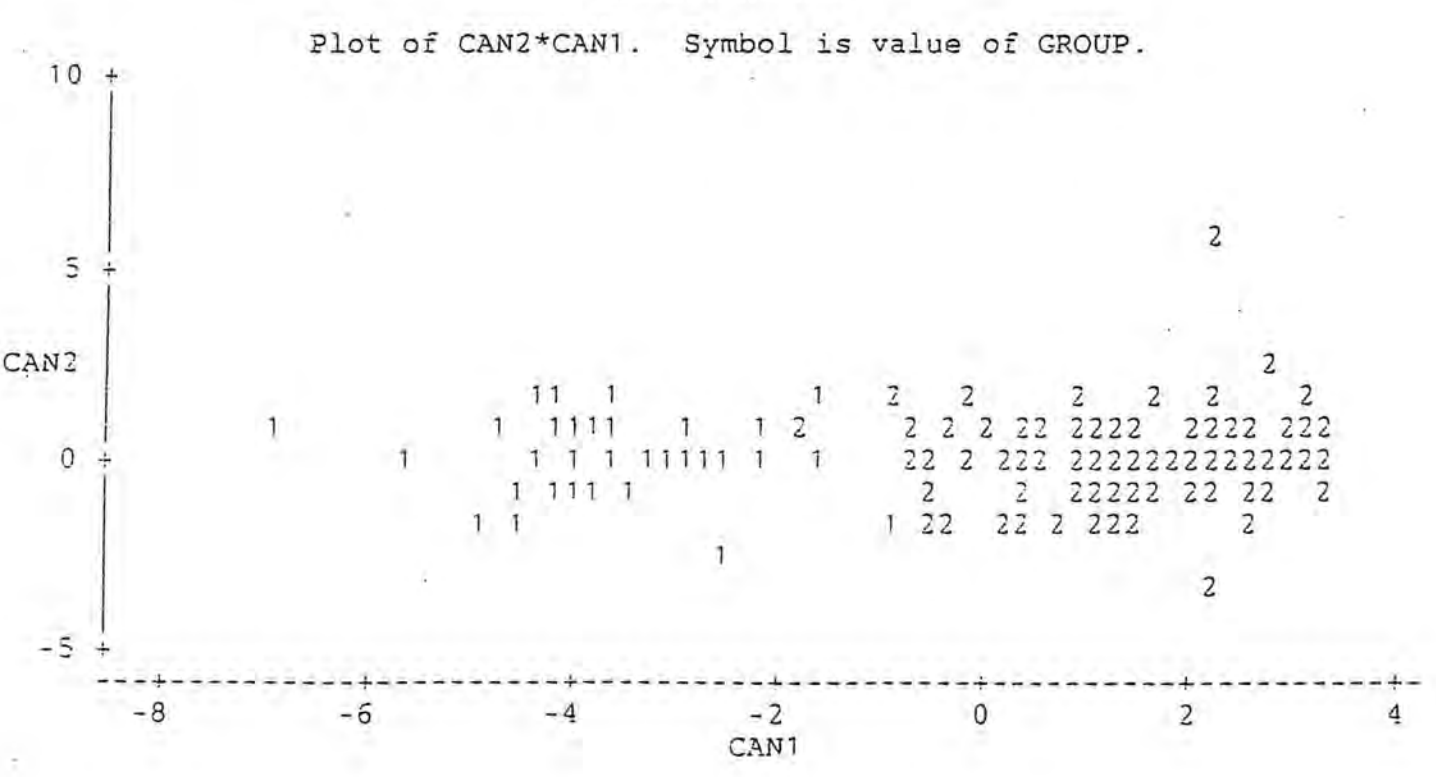


Figure 2.4 Mahalonobis distance relationships for the three measured characters (E, F, C) among the four different locust populations of, *Schistocerca gregaria*. f= Red sea field population; r= Red sea lab. population; e= Ethiopian population and s= Kalahari field population

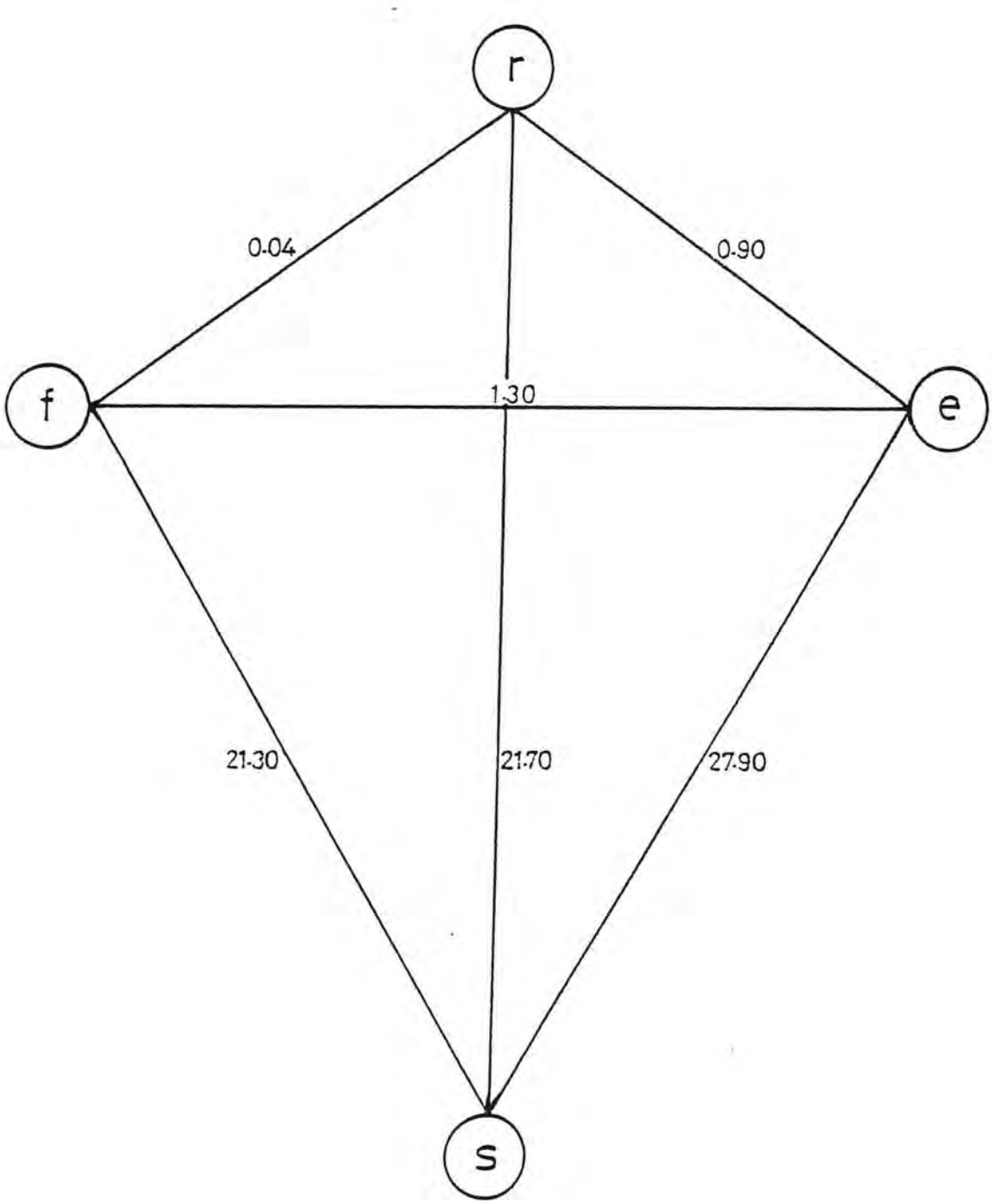
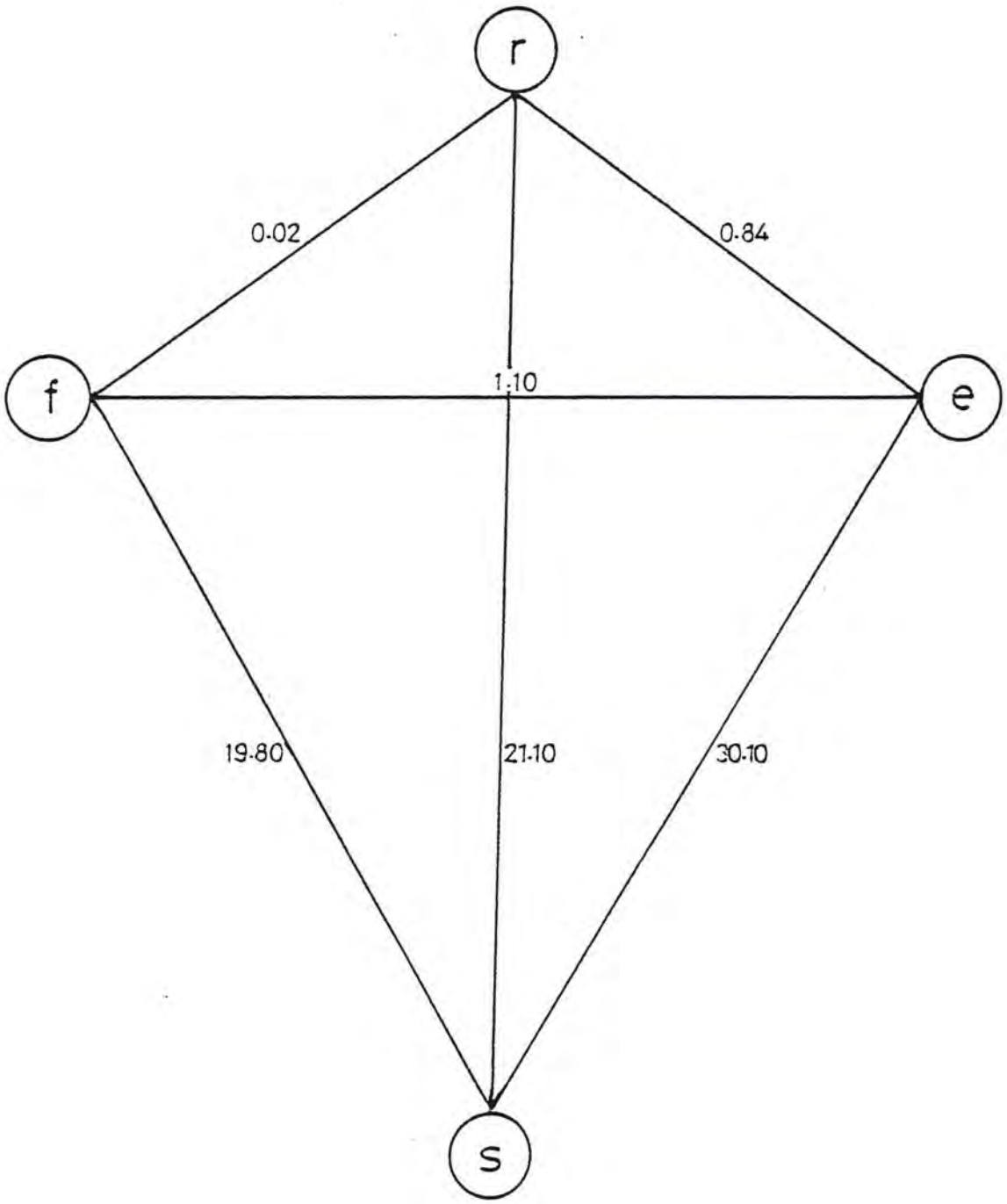


Figure 2.5 Mahalonobis distance relationship for the two ratios (E/F, F/C) among the four different locust populations of, *Schistocerca gregaria*. f= Red sea field population; r= Red sea lab. population; e= Ethiopian population and s= Kalahari field population



effective in population discrimination. The width of the head capsule (C) and the length of the elytron (E) shows higher weights in differentiating the four locust populations.

B. Comparisons with respect to location and sex

In the univariate analysis using the ANOVA test, means of the variable characters for the different individuals representing the four populations were significantly different (Table 2.6). The mean values of the southern males and females showed that the males had smaller values than the females for E, F, C measurements, but the mean values for the E/F and F/C ratios were approximately the same.

The coefficients of variances also indicated significant differences between the different forms for the three measured characters and the two ratios ($P< 0.0001$) (Table 2.7). The highest C.V. was for the length of the femur (40.18%) and the length of the elytron (38.49%), while the lowest C.V. was the E/F ratio (33.49%).

Multivariate analysis indicated that the two sexes of the four population were significantly different from each other ($P< 0.0001$). The first characteristic root for the three measured characters was 9.05 and the corresponding canonical

Table 2.6 Mean values and standard errors of the three characters for males and females from the four desert locust populations, *Schistocerca gregaria*

Form/ Character	E	F	C	E/F	F/C
r Male	53.61 ±0.39 c	24.64 ±0.18 c	6.95 ±0.08 a	2.17 ±0.01 c	3.54 ±0.03bc
r Female	61.45 ±0.63 a	27.75 ±0.37 a	7.72 ±0.08 b	2.21 ±0.02abc	3.59 ±0.03 b
s Male	48.57 ±0.46 c	23.89 ±0.23 d	5.77 ±0.04 c	2.03 ±0.02 d	4.13 ±0.03 a
s Female	56.97 ±0.37 b	28.04 ±0.27 a	6.68 ±0.06 f	2.03 ±0.01 d	4.19 ±0.03 a
e Male	52.03 ±0.36 d	23.33 ±0.18 d	6.76 ±0.05 fc	2.23 ±0.01 ab	3.45 ±0.02dc
e Female	58.31 ±0.46 b	25.94 ±0.28 b	7.48 ±0.06 c	2.24 ±0.02 a	3.46 ±0.03dc
f Male	53.81 ±0.75 c	24.71 ±0.19 c	6.90 ±0.04 dc	2.17 ±0.02 c	3.58 ±0.03 b
f Female	62.23 ±0.40 a	29.38 ±0.14 a	7.91 ±0.05 a	2.19 ±0.01 bc	3.59 ±0.03 b

* E: elytron length F: femur length C: head capsule width

* r: Ex-Addis population s: Southern population

* e: Ethiopian population f: Red sea population.

Table 2.7 Coefficient of variations in characters for males and females from the four desert locust populations, *Schistocerca gregaria*.

CHARACTERS	C.V. (%)	P > F
E	38.49	0.001
F	40.18	0.001
C	34.69	0.0001
E/F	33.49	0.0001
F/C	35.55	0.0001

* E: elytron length F: femur length C: head capsule width

variate explained 75.21% of the variability (Table 2.8). The second characteristic root was 2.95 with a corresponding canonical variate that explained 24.54% of the variability. The first characteristic root for the two ratios was 5.16 and the corresponding canonical variate explained 98.9% of the variability (Table 2.9). The second characteristic root was 0.06 and the canonical variate explained 1.1% of the variability. The respective canonical correlations were highly significant ($P < 0.0001$).

Different forms of the four different populations are shown in a canonical discriminant space for the three measured characters (Fig.2.6). There are differences in the morphology within the southern populations. The females are grouped in group 1 while the males are grouped in group 2. The three northern populations (males and females) are grouped in group 3. The canonical discriminant space for the two ratios showed that the females and the males of the southern populations have the same ratios (group 1) and that they are separated from the northern population (group 2) (Fig. 2.7).

The Mahalanobis distance between the centroids of the males and females of the four populations for the three measured characters and for the two ratios are shown in Table 2.10 and 2.11, respectively. The distances between the southern males and females are smaller compared to the distances between the

Table 2.8 Characteristic roots and proportion of the three measured characters for the males and the females from the four desert locust populations, *Schistocerca gregaria*.

Characteristic root (Eigenvalue)	Proportion (%)
9.05	75.21
2.95	24.54

Table 2.9 Characteristic roots and proportions of the two ratios for males and females from the four desert locust populations, *Schistocerca gregaria*

Characteristic root (Eigenvalue)	Proportion (%)
5.16	98.90
0.06	01.10

Table 2.10 Mahalanobis distances for the three measured characters between the centroids of the males and females from the four desert locust populations, *Schistocerca gregaria*

	r M	r F	s M	s F	e M	e F	f M	f F
r M	0	16.35	31.30	22.96	1.60	7.16	0.10	22.00
r F	16.35	0	77.76	32.73	25.42	3.26	16.55	0.63
s M	31.30	77.76	0	21.57	30.60	64.29	28.90	89.20
s F	22.96	32.73	21.57	0	32.33	34.71	20.66	37.82
e M	1.60	25.42	30.60	32.33	0	11.92	1.81	32.71
e F	7.16	3.26	64.29	34.71	11.92	0	7.70	5.94
f M	0.10	16.55	28.91	20.66	1.81	7.70	0	22.43
f F	22.00	0.63	89.20	37.82	32.71	5.94	22.43	0

* M: males F: females

* r: Ex-Addis poplation s: Southern population

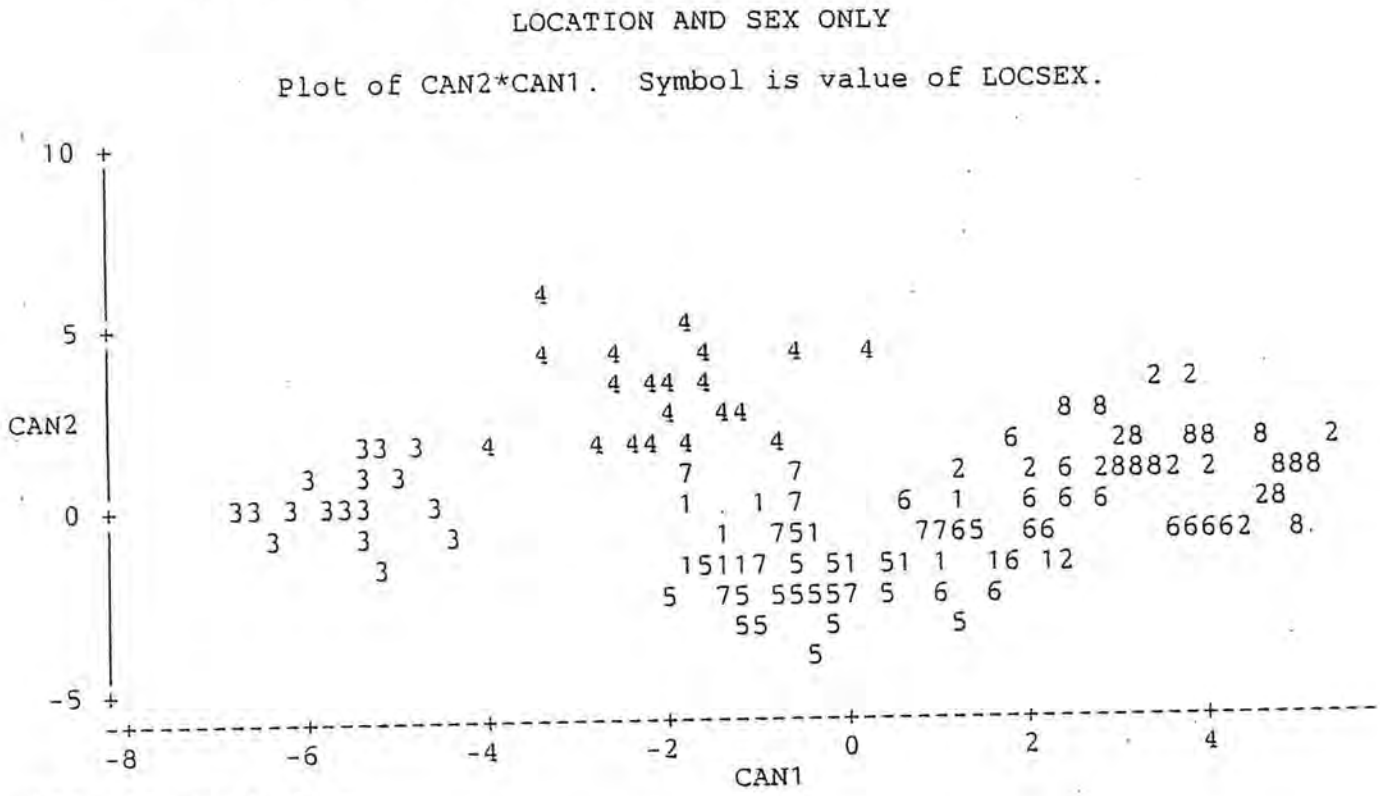
* e: Ethiopian population f: Red sea population

Table 2.11 Mahalanobis distance for the two ratios between the centroids of the males and females from the four desert locust populations, *Schistocerca gregaria*

	r M	r F	s M	s F	e M	e F	f M	f F
r M	0	0.63	19.91	24.07	0.83	1.10	0.08	0.24
r F	0.63	0	18.15	21.76	1.21	0.92	0.36	0.13
s M	19.92	18.15	0	0.24	27.81	27.18	17.89	17.55
s F	24.08	21.76	0.24	0	32.48	31.62	21.79	21.28
e M	0.83	1.22	27.81	32.48	0	0.13	1.15	1.18
e F	1.10	0.92	27.18	31.62	0.13	0	1.27	1.12
f M	0.08	0.36	17.89	21.77	1.15	1.27	0	0.63
f F	0.24	0.13	17.55	21.28	1.18	1.12	0.06	0

* M: males F: females
* r: Ex-Addis population s: Southern population
* e: Ethiopian population f: Red sea population

Figure 2.6 Plot representing the four different desert locust individuals along the two axes of canonical discriminant space for the three measured characters (E, F, C). 1, 2= Red sea lab. males and females; 3, 4= south African males and females; 5, 6= Ethiopian field males and females; 7, 8= Red sea field males and females. **Group 1**= south African desert locust females; **Group 2**= southern desert locust males; **Group 3**= Three north African desert locust males and females.



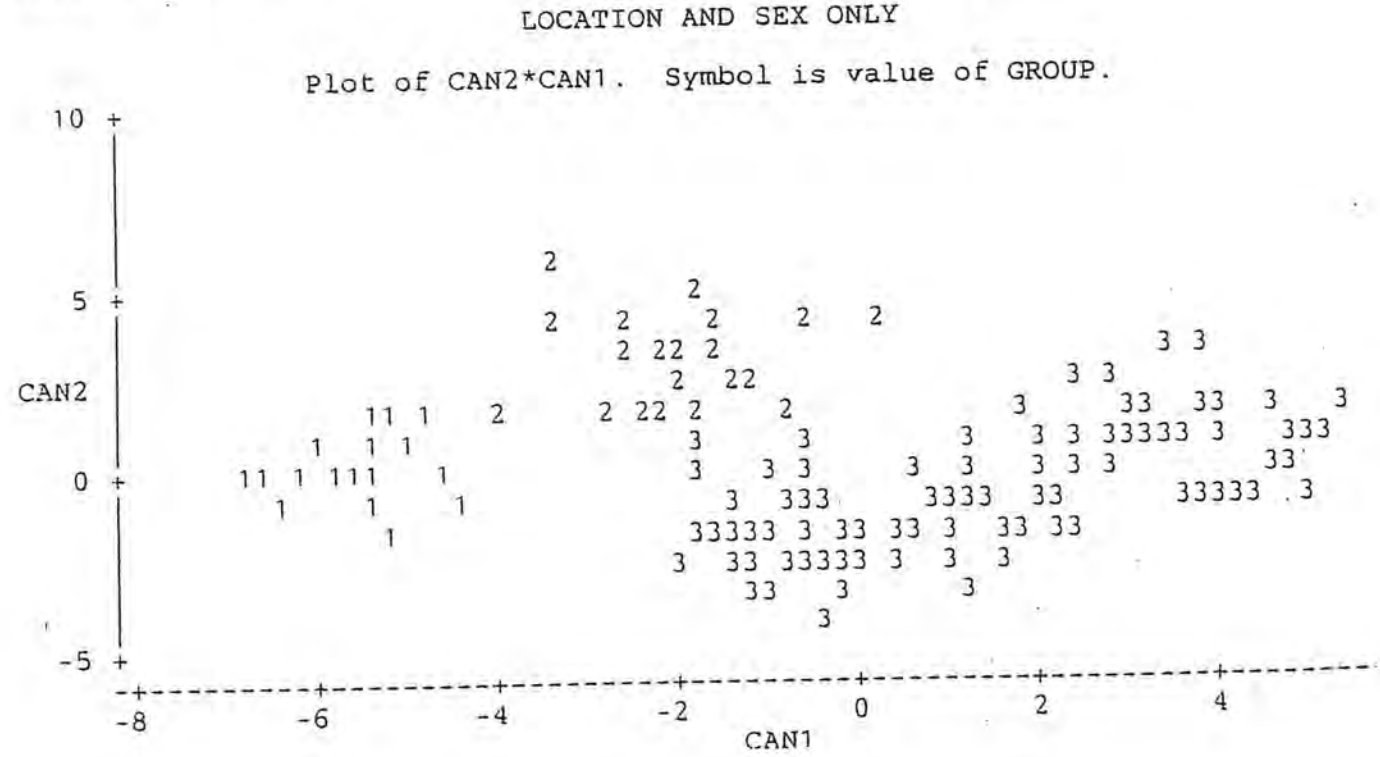
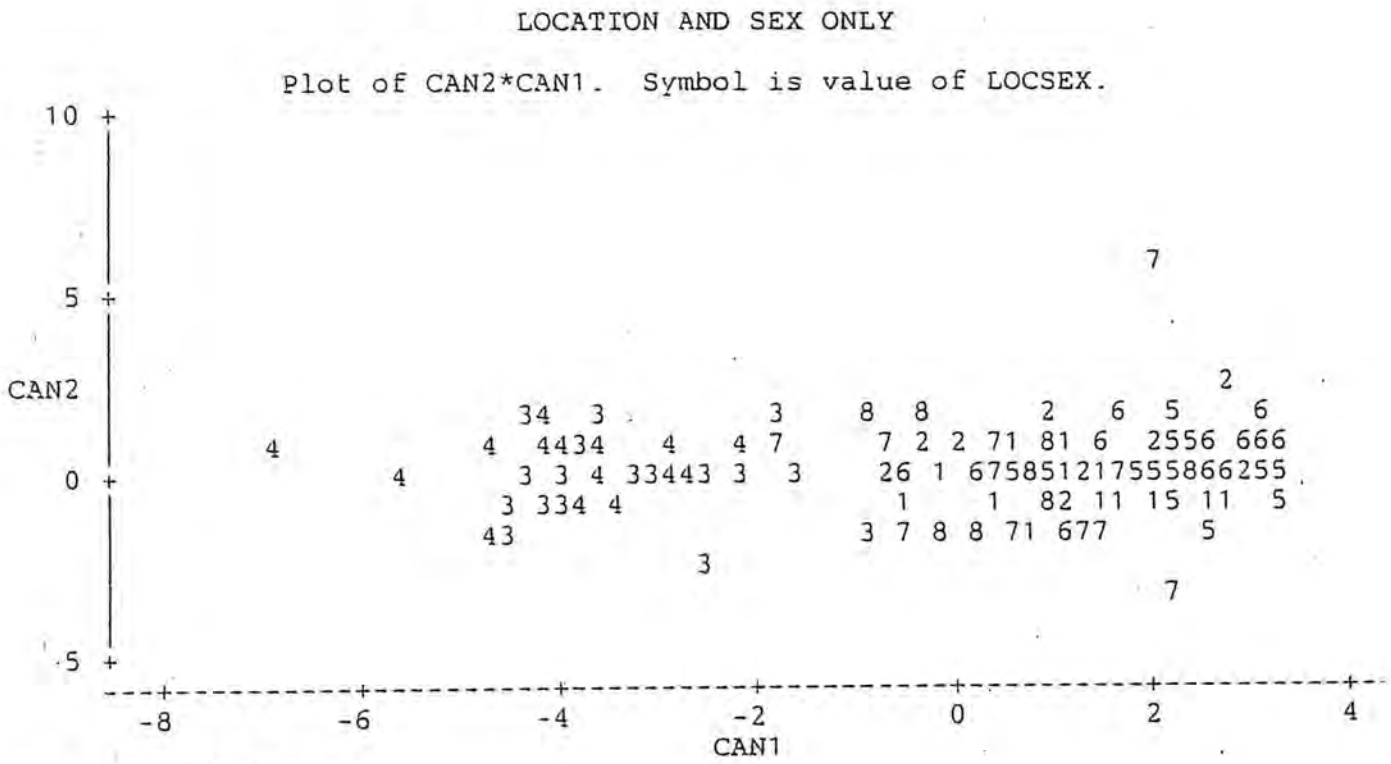
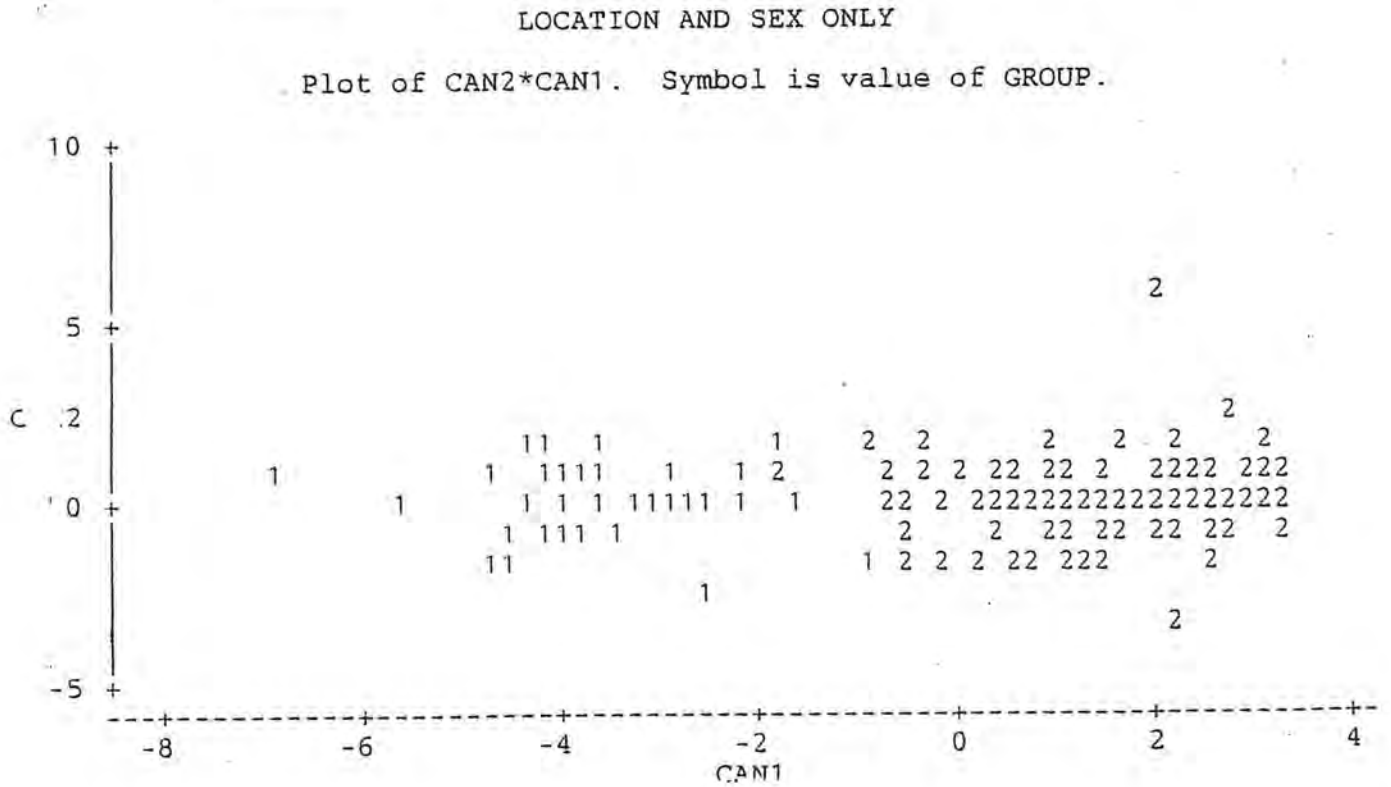


Figure 2.7 Plot representing the four different desert locust individuals along the two axes of canonical discriminant space for the two ratios (E/F, F/C). 1, 2= Red sea lab. males and females; 3, 4= south African males and females; 5, 6= Ethiopian field males and females; 7, 8= Red sea field males and females. Group 1= south African desert locust males and females; Group 2= Three northern desert locust males and females.





southern and the northern individuals. This indicates that the southern males and females are more morphologically closer to each other than to any form of the northern locusts. Also, the two sexes of the northern populations are more or less similar in their morphological features.

2.4 Discussion

Univariate analysis showed that the south African population has smaller mean values for the three measured characters (E, F, C). The coefficient of variations indicated that the elytron character represents the highest variation between the four locust populations. The coefficient of variations for the two ratios showed lower values than the three measured character values. This implies that the E, F, C characters are more important in the differentiation between the different populations than their ratios.

On the other hand, the three northern populations showed relatively similar mean values for their measured characters. This indicated that the three northern locust populations are closer to each other than to the southern counterparts.

The southern population showed some differences in the morphometrics between the males and the females. The means values of the males for the three characters were smaller than the means values for the females. This indicates that the southern males are shorter than southern females. On the other hand, the means values for the northern individuals were higher

than those of the southern populations. The northern males and females have relatively the same morphometrics.

The coefficient of variations obtained imply that the length of the femur (F) and the length of the elytron (E) can be used in differentiating between the different locust populations better than the E/F or F/C ratios.

Multivariate analysis was conducted to determine whether the four locust populations are different morphologically, and if they are, to classify the locust individuals into its appropriate group. The four locust populations were grouped into two distinct groups. The three northern populations were clustered into one group and the southern population was separated into another group. The multivariate analysis results have confirmed the univariate results and indicated that the three northern populations are more similar in their morphometrics and they are different from the southern population. The first canonical variate accounts for most of the morphological difference between the populations. Symmons (1968) used the first canonical variate based on the measurements of the three parameters (E, F, C) for samples from twelve *S. gregaria* populations. He concluded that the first canonical variate was a measure of phase in the desert locust populations.

The Mahalanobis distances between the southern and the northern populations were higher than those between the three northern populations. This implies that, the southern populations has diverged morphologically from the northern populations. On the other hand, the similarities in the morphometrics of the northern populations imply little tendency for divergence.

The Mahalanobis distances between the southern males and the southern females indicate that the differences within the population are smaller than between the populations. This shows that the males and the females are more similar to each other than to any of the northern individuals.

Metter and Pauken (1969) in their study of the striped frog in the Northwest United States, and Jackson (1973) on the distribution and population phenetics of the Florida scrub lizard, interpreted D^2 values as measures of changes in gene flow. Zimmerman and Ludwing (1974) used multivariate discriminant analysis to study the geographical variation in the aquatic beetle *Rhantus gutticollis*. The results also were interpreted as a reflection of gene flow. The short distance between the population implies close relation due to the flow of the genetic constitutions. Another interesting finding was that the isolated peripheral populations were those which tended to

show the greatest degree of differentiation. Any prolonged separation of these populations could lead to formation of new species. The longer Mahalanobis distances between the southern locust population to these between the three northern locust populations indicates that there is no gene flow between them. On the other hand, the distances between the three northern populations show the flow of the genetic constitutions within these populations.

The results indicate that the southern populations acquired different morphological characters due to the geographical isolation between the northern and the southern locusts. Changes in the morphological characters of many organisms are as a result of changes in the ecological and physiological traits. Morphology is the end product of physiological activity, initiated by the genome and modified by the environmental factors (Eastop, 1973).

Environmental factors which include density-dependent factors (associated with the number of individuals per unit area or unit volume of habitat) and density-independent factors (including the habitat and its micro-climate) (Stower *et al.*, 1960). The Namibia desert in southwest Africa displays reddish yellow colouration characteristic of that desert's soils, gravel, rocks, insects and other animals (Mayr, 1970). Changes in the

climatic factors and absence of gene flow between the northern and the southern African locust populations have led to changes in their morphological characters.

CHAPTER THREE

STUDIES OF CUTICULAR PROTEINS BY TWO DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

3.1 Introduction

Two-dimensional gel electrophoresis system that gives the best resolution for separating a complex mixture of protein. The method combines the techniques of isoelectrofocusing (IEF) in the presence of urea and a neutral detergent in the first dimension, and slab gel electrophoresis under denaturing conditions using sodium dodocyl sulfate (SDS) in the second dimension (Klose, 1975; O'Farell, 1975; Scheele, 1975; Iborra and Buhler, 1976). The separation makes the use of independent protein characteristics, One in the charge, which is reflected by the isoelectric point (pI), and the other is molecular weight, which determines the mobility of the SDS-protein complexes in polyacrylamide gels (Weber and Osborn, 1969).

O'Farell (1975) first demonstrated the great potential of such a technique when using isotopically labelled proteins by resolving more than a thousand polypeptides and detecting

components as minor as 0.001% or less of the total cellular protein. This method has been extensively applied in resolving proteins from both prokaryotic and eukaryotic organisms and can be used for the separation of many types of cellular proteins.

This chapter explained the use of the two-dimensional gel electrophoresis in the separation of the abdominal cuticular protein of the northern African locust *Shistocerca g. gregaria* and the southern African locust *S. g. flaviventris* as a basis of differentiating between the two population.

3.2 Materials and Methods

3.2.1 Chemicals

The laboratory chemicals used in this experiment were obtained from Sigma Chemical Co., (England); Pierce Chemical Co., (Rockford) and Pharmacia LKB, (Swisserland).

3.2.2 Insects

F1 gregarious males and females adults of the Red Sea population and the south western African population, were collected after two weeks of maturation and kept in -20° C before protein extraction.

3.2.3 Protein extraction

Abdominal cuticular proteins were extracted according to the method described by Andersen and Hojrup (1987). Frozen locusts were allowed to thaw. The abdominal cuticle was then peeled away from the still frozen interior. The cuticles were washed briefly in 1% potassium tetraborate. The epidermal cells and remnants of muscles were carefully scraped away. After another brief washing

in distilled water, the cuticular proteins were extracted in 6 M urea, 0.1% Trifluoroacetic acid or in 6 M urea, 25 mM imidazole, pH 6.8. Samples were centrifuged at 15,000 rpm in a microcentrifuge for 30 minutes. The supernatant was decanted in clean Eppendorf tubes. Estimation of protein concentration was carried out by the BCA protein assay method (according to the manufacture procedure). Equal amounts of protein (50 µg/µl) from twenty individuals of each sexes for both populations, were pooled in aliquotes of 0.5 ml and stored in -20° C until needed.

3.2.4 First Dimensional gels (Isoelectric focusing)

Standard glass tubes 1-1.5 mm internal diameter and 10-15 cm long were thoroughly cleaned in Chromic acid. The tubes were rinsed in water, placed in fresh potassium hydroxide in ethanol (0.4 g KOH in 20 ml Ethanol) for 20 minutes, rinsed thoroughly first with distilled water, then ethanol, and finally allowed to air dry.

The tube bottoms were sealed with three layers of parafilm and lined up vertically. The tubes were marked to the same point with a felt-tipped pen to ensure that gel lengths are uniform. Isoelectric focusing gel solution (10 ml) (Appendix B) was poured in 125 ml side arm flask. The gel solution was degassed by using

vacuum pump for 5 minutes with steering. NP-40 was then added, and 7 µl of TEMED and 10 µl of Ammonium persulfate (APS) were also added to the degassed solution. The solution was immediately loaded into the tubes by using a long, narrow gauge blunt end needle. To avoid trapping air bubbles, the tip of the needle was inserted to the bottom of the tube and withdrawn slowly as the acrylamide mixture runs in. The tubes were filled up to a specified mark (11.5 cm). The gel mixture was overlaid with 10 µl of H_2O and allowed to polymerize for at least 2 hours at room temerature.

The parafilm was then carefully removed by cutting rather than by pulling so as to avoid damaging the bottom of the gels. The tubes were then loaded into a standard gel chamber, the lower reservoir was filled with 10 mM Phosphoric acid (H_3PO_4). Air bubbles were removed from the bottom of the gel by gently tapping the tube. The solution above the gels was removed by aspiration.

The top of the gel then overlaid with 10 µl of lysis (overlay) buffer (Appendix B), 20 mM sodium hydroxide (NaOH) to fill the tubes. The upper chamber was then filled carefully with 20 mM NaOH, previously degassed, so as not to disturb the layers of solutions over the gel. The positive electrode was connected to the lower reservoir and the negative electrode to the upper reservoir. Gels were equilibrated as follows: 200 V for 15

minutes, 300 V for 30 minutes and finally 400 V for 60 minutes. During the prerun at each voltage, the current reading was constantly decreased (if the current was increasing, the electrode connection and the solutions in the reservoirs was checked). After the prerun the NaOH from the upper chamber was removed and discarded. The lysis buffer above the gels was aspirated, and the top of the gels was washed twice with 50 µl of distilled H_2O.

The protein samples (50 µg/ml) were conveniently loaded with a Gilson Pipetman or Hamilton syringe. After loading, 10 µl of overlay buffer was carefully added, the tubes were filled with 20 mM NaOH, the upper chamber was replenished, and the gels were run for 18 hrs at 400 volts.

To remove the gels from the tubes after IEF running, water was injected with a blunt thin needle between the gel and the glass tubes. Gels were collected in screw cap tubes containing 200 µl of equilibration buffer (Appendix B). They were allowed to equilibrate for 10-20 minutes and then frozen in -20° C freezer until preparation of the second dimension SDS slab gel.

3.2.5 Second dimension SDS-slab gel electrophoresis

The slab gel technique in use was readily adapted to the two dimensional system. Essentially the method and the notched plate system described by O'Farrell (1975) was used. The gel was formed between two uniform glass plates, each about 3 mm thick. One plate is a rectangular glass of 17 X 18 cm and the other plate is the same size with a notch approximately 2-cm deep and 14-cm wide. Spacers were made of perspex with 1.5 mm thick and 20 cm long. The glass plates were cleaned carefully with detergent followed by several rinses in water and a final wash with ethanol to eliminate any remaining grease that could affect polymerization.

Two glass plates and three spacers were mounted together, one spacer along the bottom and the other two running down the vertical sides of the plates, taking care that the vertical spacers were in close contact with the one at the bottom. The assembled plates were sealed by slightly grease the spacers with vaseline and hold together with strong clips.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was preformed as described by Laemmli (1970). The gradient gels (4-15%) (Appendix B), were casted by using gradient marker (BRL). The gel solution was degassed before adding TEMED

and freshly made ammonium persulfate (APS). Once TEMED and APS were added, the solution was carefully mixed and poured into the assembled vertical plates up to 2.5 cm from the top of the notched plate. The resolution gel was overlaid with H_2O or with water-saturated isobutanol. Gels were left to polymerize for at least 1 hr. The overlay isobutanol solution was then removed and the gel was rinsed and dried with 3 MM blotting paper before pouring the stacking gel solution. The stacking gel solution (3.13%) (Appendix B), was degassed before adding TEMED and APS. Stacking gel should be poured as far as the top of the notched plate and overlaid with water.

3.2.6 Application of the first dimension on top of the second dimension and running of the slab gel

Before the first gel was applied on top of the second, the stacking gel should was dried with a blotting paper. All the clamps were removed together with the bottom spacer and the plates were laid flat to facilitate the application of the first dimension gel. The first dimension gel was thawed, removed from the equilibrating buffer, and carefully laid on top of the plates avoiding any stretching. 3 ml of 1% melted agarose solution (Appendix B), was used to keep the first dimension gel in place.

Agarose was layered on with a Pasteur pipette from one end of the plate between the stacking and the first dimension gel. Any trapped air bubbles should be eliminated to avoid artifacts in the SDS gel. The gel was completely covered with the agarose solution and left to set for 3 to 5 minutes before the slab gel was clumped to the electrophoresis chamber (Pharmacia, Co.). The SDS size marker (Biolab, UK) was included on the wright side of the SDS gel to demonstrate the molecular weight. The size marker was ranged from high molecular range (97.4 kDa) to low molecular range (14.4 kDa). Running buffer (Appendix B), was added to the upper and lower tanks. Air bubbles from the bottom of the gel was removed to allow good contact with the buffer. This was done by using a syringe with a bent needle. Electrodes then connected and the gels were run overnight at constant current (9-11 mA). At the end of the run, the plates were removed from the apparatus and the protein was developed by using color silver stain (Gelcode) method.

3.2.7 Colour silver stain (Gelcode) method

The Gelcode method is a unique application of colour silver staining to the two dimension protein electrophoresis. It is designed to produce coloured protein spots in the gel matrix, and

was first described by Sammons *et al.*, (1984). The gel was fixed overnight (16 hrs) in 50% ethanol with 5% acetic acid solution. After fixation, the gel was washed four times with H_2O (1 hr each). Gel staining was carried for 1 hr with silver nitrate ($AgNO_3$). The excess of the stained was removed by quick rinse in distilled water for 10-20 seconds. The gel was developed by reducing the silver with formaldehyde (7.5 ml/l) in 0.75 N NaOH for 10 minutes. To enhance the colour of the gel, three changes (1 hr each) of sodium carbonate (Na_2CO_3) (7.5 g/l) were added to the gel after removing the silver reduction solution. The silver reduction and colour enhancement steps were preformed with gentle agitations and the reagent solutions was equilibrated to 25° C. After the colour stained image was stabilized (approximately 4 hours after the last Na_2CO_3 step), the gel was stored in an airtight bag with a small amount of 0.75% Na_2CO_3 without significant loss of stain intensity.

3.3 Results

Differences in the configuration of the cuticle of the two sexually mature locust populations, were demonstrated by the two-dimensional gel electrophoretic resolution of the abdominal proteins.

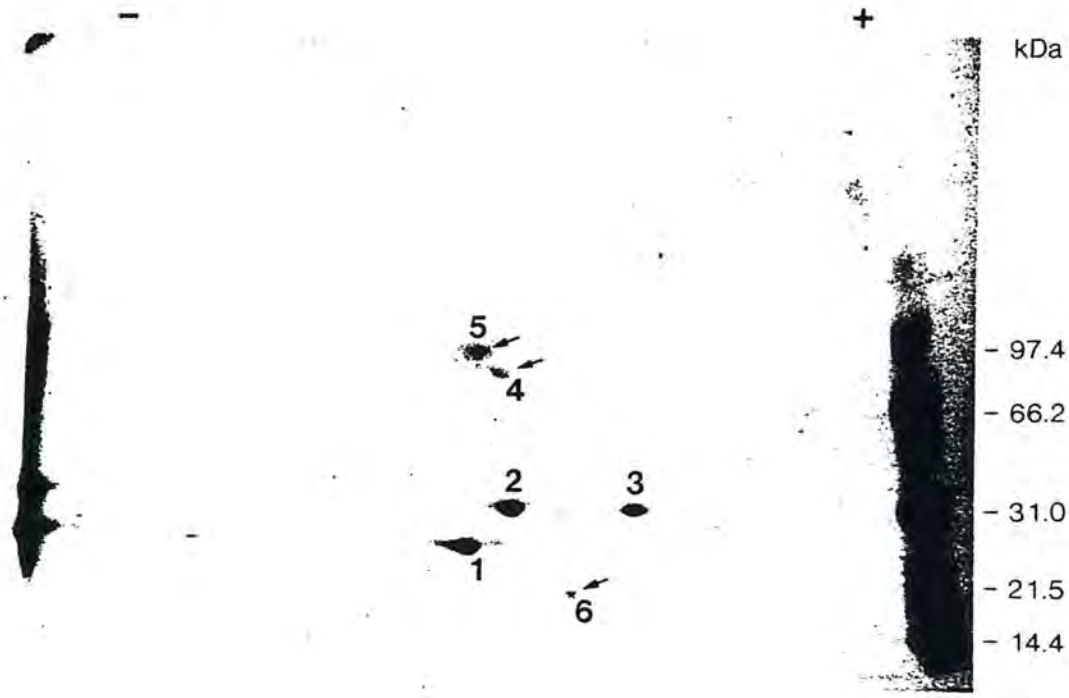
Figure 3.1 illustrates the resolution of the abdominal proteins of the northern males *S. g. gregaria*. The protein configuration was resolved into six major bands, while the configuration of the southern males *S. g. flaviventris* showed that, the abdominal protein was resolved into three major bands only (Fig. 3.2). Bands number 1, 2 and 3 were observed in the two gels at the same molecular weight (~ 21.5-31.0 kDa). On the other hand, bands number 4 and 5 at high molecular weight (~ 70 - 97.4 kDa) and band number 6 at low molecular weight (~ 20 kDa), were only observed in the northern males.

Resolution of the abdominal protein of the northern females *S. g. gregaria* showed five major bands (Fig. 3.3). These bands showed to be correlated with the bands from the northern males. Bands number 1, 2, 3, 4 and 5 were recognized in the two gels at the same molecular weights. While band number 6 was shown only in the northern males.

Abdominal protein separation of the southern female locust was resolved into two major protein bands (Fig 3.4). Bands number 2 and 3 were recorded also in the northern females at the same molecular weights. Band number 1 was not apparent in the southern females as it was in the northern females. On the other hand, the protein arrays of the southern females resembled that protein of the southern males except that band number 1 at low molecular weight (~ 25 kDa) was confined to the southern males only.

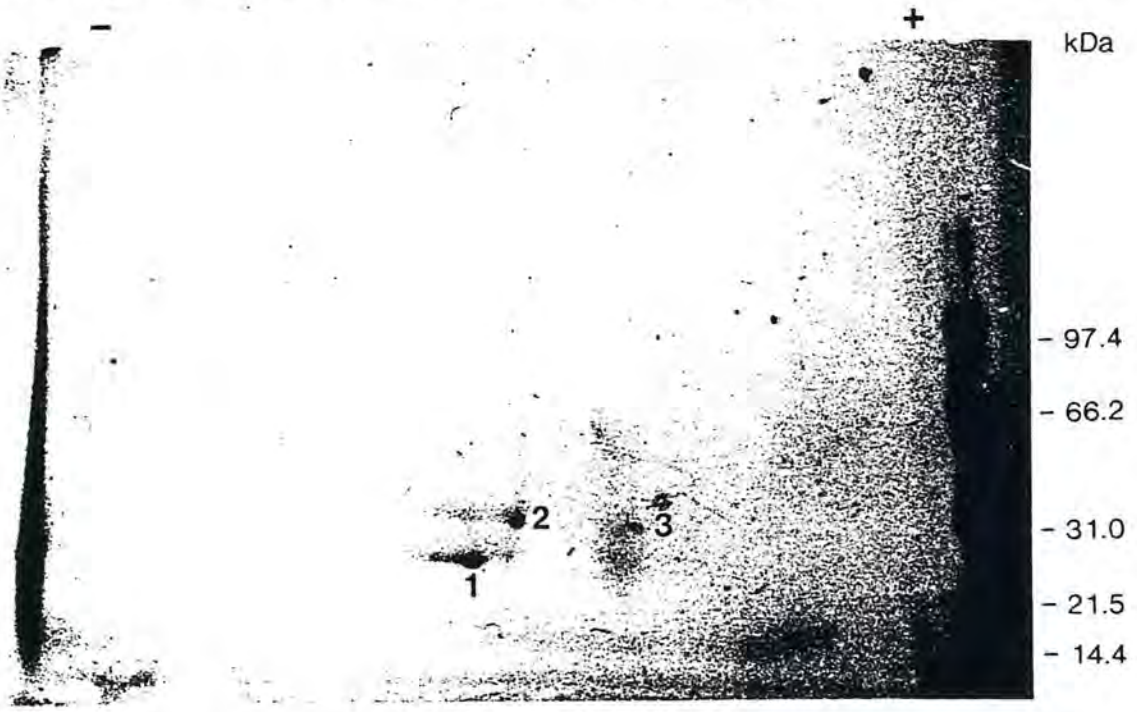
The major differences between the northern and southern abdominal protein patterens were bands number 4 and 5 at high molecular weight (~ 70-97.4 kDa).

Figure 3.1 Two-dimensional separation of proteins extracted from the abdominal cuticle of the north African males, *S. g. gregaria*. Isoelectric focusing between pH 3.5 and 11 for the first dimension and SDS-electrophoresis in the second dimension.



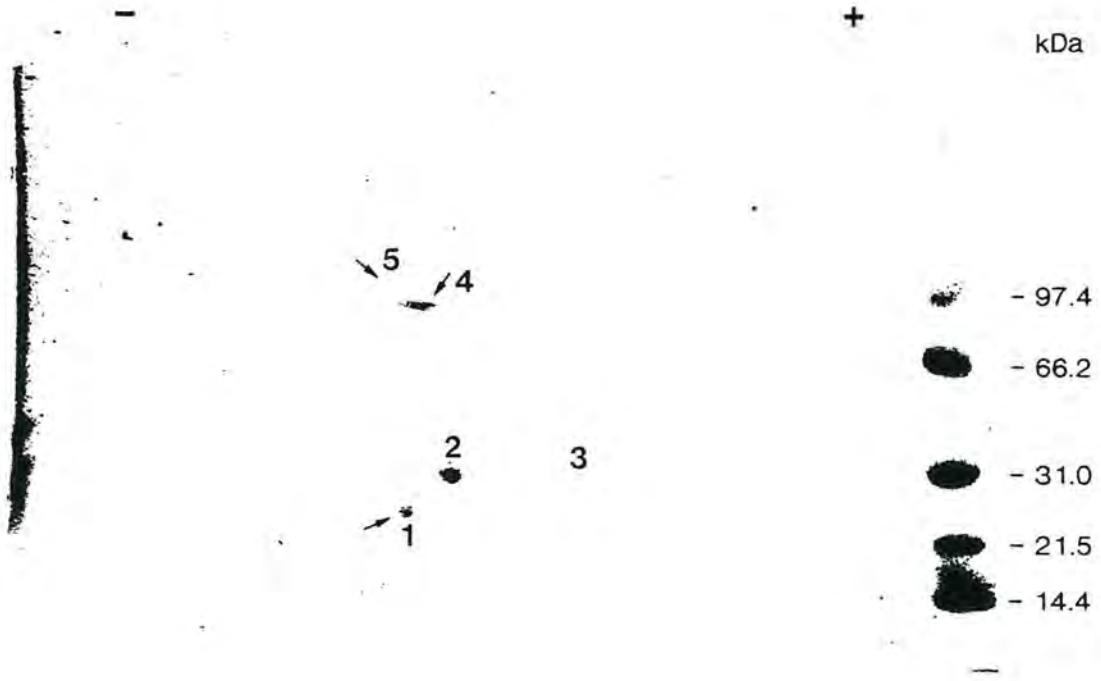
Arrows indicate the bands 4, 5 and 6 which are characteristic of the north African males.

Figure 3.2 Two-dimensional separation of proteins extracted from the abdominal cuticle of the south African males, *S. g. flaviventris*. Isoelectric focusing between pH 3.5 and 11 for the first dimension and SDS-electrophoresis in the second dimension.



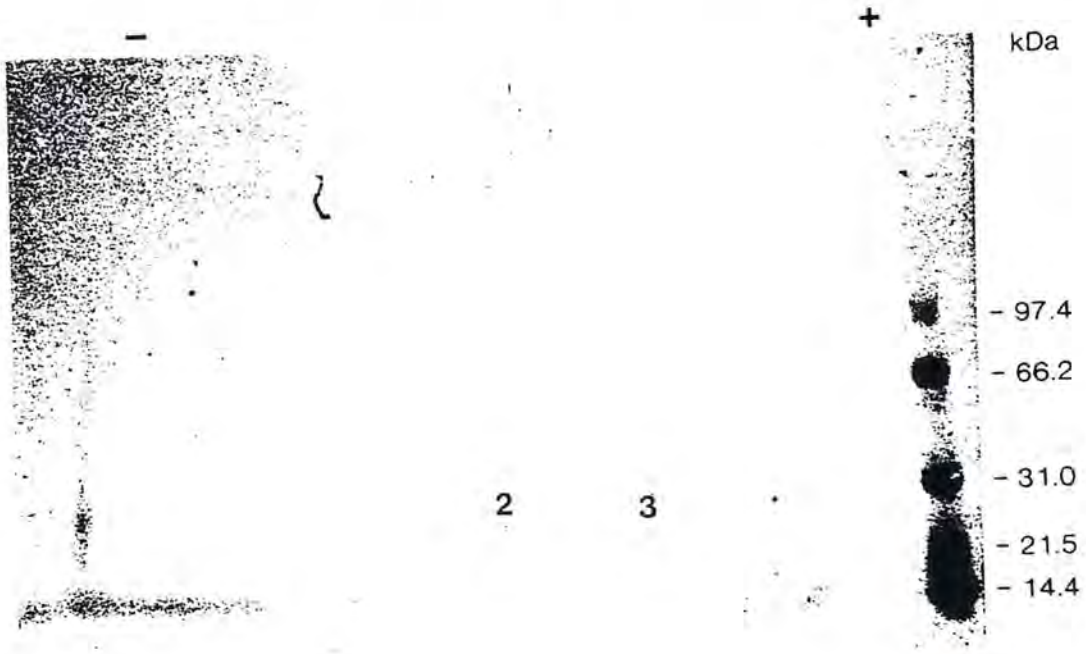
The bands 1, 2 and 3 appeared in the gel.

Figure 3.3 Two-dimensional separation of proteins extracted from the abdominal cuticle of the north African females, *S. g. gregaria*. Isoelectric focusing between pH 3.5 and 11 for the first dimension and SDS-electrophoresis in the second dimension.



Arrows indicate the bands 4 and 5 which are characteristic of the north African females.

Figure 3.4 Two-dimensional separation of proteins extracted from the abdominal cuticle of the south African females, *S. g. flaviventris*. Isoelectric focusing between pH 3.5 and 11 for the first dimension and SDS- electrophoresis in the second dimension.



The bands 2 and 3 appeared in the gel.

3.4. Discussion

The results presented in this chapter demonstrate differences in the patterns of the abdominal cuticular proteins extracted from the two locust populations. The results are based on the analysis of the protein by using 2-D gel electrophoresis.

Proteins are composed of amino acids joined together by covalent peptide bonds to form polypeptides. These sequences, or "primary structures", are genetically determined. Each of the twenty different amino acids has a unique side chain, characterized by its shape, size and charge. The side chains of five of these amino acids are either basic and this positively charged, or acidic and negatively charged. Charged side chains are responsible for the movement of the proteins through a matrix during electrophoresis. The net charge of each protein varies with pH; at a low pH the amino groups become positively charged, and at high pH the carboxyl groups become negatively charged. Most proteins have a point at which the effect of positive and negative charges are equal, the isoelectric point (pI). Isoelectric proteins do not move in an electrical field because they do not attract to neither (positive) anode nor the (negative) cathode (Murphy *et al.*, 1990).

The amino acid sequences of the proteins are changed by mutations in the encoding DNA locus. Such mutations may alter shape and the net charge as well as catalytic efficiency and stability of the protein (Show, 1965). Protein electrophoresis aims to reveal as many of these changes as possible. Thus if the banding patterns of any two populations are different, it is assumed that these differences are genetically based and heritable (Matson, 1984).

Two dimensional gel electrophoresis as introduced by O'Farell in 1975, has allowed the examination of a substantially increased number of protein-encoding loci. In 2-D gel electrophoresis, a mixture of denatured protein is separated first on the basis of charge by isoelectric focusing and then on the basis of molecular weight by SDS slab gel electrophoresis.

An advantage of utilizing 2-D techniques to estimate protein variation and differentiation is that at least some representatives of each of a broader array of protein classes can be monitored. Thus the 2-D technique may provide a more comprehensive and balanced view of variation at protein-encoding loci through the genome. The 2-D technique shares with unidimensional gel electrophoresis a general objectivity in scoring the spots, which are either the same or different according to the migration position (Aquadro and Avise, 1981).

Ohnishi *et al.*, (1981), compared protein differences between *Drosophila melanogaster* and *D. simulans* using 2-D and obtained differences in the protein patterns of the two species. However, the results were similar to those obtained by conventional electrophoresis. In a large study on rodents, Aquardo and Avise (1981) compared an average of 189 polypeptides between six species of wild mice representing levels of evolutionary divergence ranging from different subspecies to different families. The magnitude of protein divergence estimated by 2-D was on the average only about one-half that predicted by unidimensional gel electrophoresis. This may be attributed to differences in sensitivities between the techniques or differences in the mean level of variation and divergence between the sets of loci assayed by the two methods. Nonetheless, the ranking of genetic distances by 2-D was identical to that by unidimensional electrophoresis.

Leigh Brown and Langley (1979) had compared 10 species of *Drosophila* using α-glycerophosphate dehydrogenase identified on a 2-D gel. Several species showed slight differences in mobility in standard starch gel conditions but on two dimensional gels only major charge differences were detected. Carbamylation experiments however, showed that 2-D electrophoresis can resolve all substitutions in the primary structure of a protein which

result in a charge difference (Anderson and Hickman, 1979). Racine and Langley (1980) examined heterozygosity in a wild population of the house mouse, *Mus musculus*, by 2-D electrophoresis of whole kidney proteins, and the observed level of heterozygosity (2%) was lower than the level detected by starch gel electrophoresis.

Gel electrophoresis of the proteins has introduced a new term in the molecular systematics, this term "Electromorph" which describes a group of protein extracted from different individuals with identical electrophoretic mobility in a gel matrix. The results obtained by the 2-D electrophoresis for the two locust populations have showed some differences in the cuticular protein patterns of the two desert locusts. These differences are attributed to the differences in the charge of the amino acids side chains and the shape as well as the size of the molecules, which are encoded in the genomes. So we can conclude that, these two populations are not identical electromorphs. The differences in the cuticular protein array reflect substational divergences between the two populations.

CHAPTER FOUR

GENOMIC ANALYSIS USING RANDOM AMPLIFIED POLYMORPHIC DNA-POLYMERASE CHAIN REACTION (RAPD-PCR)

4.1 INTRODUCTION

Williams *et al.*, (1990) and Welsh and McClelland (1990) described a novel means of obtaining genetic markers, which is not dependent on a prior sequence information and which may be technically accessible to a wider range of molecular taxonomists. This technique, random amplified polymorphic DNA (RAPD) technique is PCR based. The PCR is an enzymatic chain reaction that leads to the amplification of specific DNA sequences from a given template. In addition to the template, single, short oligodexynucleotoides decamer primers with random sequences are used in the reaction to amplify patterns mostly from the repetitive units located at numerous locations throughout the genome. The amplification is performed in the presence of dNTPs as a substrate and catalyzed by *Taq* polymerase. The sequence complementary to the primer is doubled by repeating the cycle,

which consists of a series of temperature steps in which the DNA is denatured, the primer is annealed, and the polymerization of a specific sequence occurs. The newly synthesized sequence is available as a template in the next cycle; therefore, theoretically, a twofold increase of the template is achieved in each step. In theory, 30 cycles should yield an amplification of a specific sequence by a factor of $2^{30} = 1 \times 10^9$, as long as the substrate or the enzyme is not limiting (Landgraf and Wolfes, 1993). DNA segments amplified with RAPD-PCR are inherited in a Mendelian manner in many organisms (Williams *et al.*, 1990). The relative speed and ease with which these amplified regions (RAPD markers) can be produced, make them suitable for a number of applications, including species diagnosis, population differentiation and genetic studies (Gawel and Bartlett, 1993; Wilkerson *et al.*, 1993; Hymer *et al.*, 1993; Black, 1993; Favia *et al.*, 1993).

This chapter describes the use of RAPD-PCR to differentiate the two locust populations on genetic basis.

4.2 MATERIALS AND METHODS

4.2.1 Insects

F1 gregarious males and females adults of the Red Sea population and the southern populations were collected after two weeks of maturation and stored at -70^0 C prior to the extraction of the genomic DNA.

4.2.2 DNA extraction

High molecular-weight template genomic DNA samples were prepared from individual locusts. Template DNA isolation procedures were slightly modified from those described by Flook *et al.*, (1992). Individual locusts were placed in a small mortar filled with liquid nitrogen and triturated with a pestle. The homogenate was added to an Eppendorff tube containing 100 µl of homogenate buffer (Appendix C). The preparation was vortexed and incubated (65^0 C, 2 hrs). Prechilled 8 M Potassium acetate (15 µl) was added and mixed well by tapping the tubes. The tubes were incubated on ice for 45 minutes. The supernatant solution were extracted by centrifugation (15,000 rpm, 10 min) in a small Microcentrifuge. DNA extraction was carried out using Phenol:

Chloroform: Isoamyl Alcohol (25: 24: 1) (v/v) followed by Phenol: Chloroform (1: 1) (v/v) and finally Chloroform was added to the aqueous phase. DNA was recovered by precipitation with two volumes of ethanol followed by centrifugation (15,000 rpm, 5 min). Genomic DNA samples resuspended in 30 µl of double distilled water. For PCR reaction, DNA concentrations were estimated by measuring the OD_{260} (optical density) using Gene Quant- II spectrophotometer (Pharmacia Inc., Piscataway, N. J., USA). Samples were diluted by adding double distilled water to final concentration of 20 ng/µl. The diluted samples were aliquoted 20 µl in small Eppendorff tubes and stored at 4^0 C; there remained stable for several months. Pools of DNA were made by mixing equal volumes of DNA from twenty individuals of each sex for both populations.

4.2.3 RAPD-PCR reactions

RAPD-PCR reactions were performed in a total volume of 25 µl. Each reaction was assembled on ice and contained 2.5 µl of 10X PCR reaction buffer (Appendix C), 2.0 mM $MgCl_2$, 100 mM dNTPs (25 mM each) (Promega Biotech. Inc. USA); 5 picomole of a single decamer primer and 0.5 U Taq polymerase (prepared at the International Livestock Research Institute, Nairobi, Kenya). The

reaction mixture was added to 60 ng of genomic DNA. Autoclaved light mineral oil (30 µl) was layered on top of each reaction to prevent sample evaporation. Control tubes were also included in the reaction and contained the reaction mixture without any DNA templates. All reaction tubes, pipette, tips and water (double distilled in millipore-Q^{50} and filtered through a 0.2 µm filter prior to autoclaving), were irradiated with UV light to destroy possible contaminating surface DNA (Gawell and Bartlett, 1993). All reactions were assembled aseptically in a laminar flow hood.

4.2.4 Primers

A set of twenty random decamer (Kit A) primers were obtained from Operon Technologies (Alamada, CA, USA).

4.2.5 PCR Amplification

PTC-100 Programmable thermal controller (MJ Research Inc., Watertown, USA) was used for the reactions with the following cycles: The tubes were heated to 95^0 C for 5 minutes and then cycled 45 times using 94^0 C for 1 min., 33^0 C for 1 min. and 72^0 C for 2 min. A final extension was carried out at 72^0 C for 10 min. Upon completion of the amplification, samples were maintained at 4^0 C (Innis *et al.*, 1990).

Products from RAPD reactions were separated by electrophoresis at 2.7 V/cm in 2% agarose gels, 0.5X Tris-Borate electrophoresis buffer (TBE), pH 8.3, (Appendix C). DNA fragments were visualized by staining with Ethidium bromide (5 µg/ml) and photographed on a 310 nm UV transilluminator (Sambrook *et al.*, 1989).

4.2.6 Extraction and purification of 600 base pairs fragments. The 600 base pairs fragments were extracted and purified by using GeneClean II Kit (Bio 101 Inc., LaJolla, CA, USA). The PCR amplification products were separated by electrophoresis through low melting point (LMP) agarose (2%) (Sigma Chem., Dorset, UK), in 0.5X TBE buffer, pH 8.3. The gel was stained with Ethidium bromide after electrophoresis and the DNA bands were viewed under UV light (310 nm). The desired 600 bp bands from south westren *flaviventris* DNA individual (males and females) and pooled samples, were excised in as small a volume as possible using sterile scalpel blades. The gel slices were placed in 1.5 ml Eppendorff tubes. Four and half volume of 6 M NaI (Sodium Iodide) and half volume of TBE modifier (supplied in kit) were added to the volume of agarose slices. The tubes were incubated in 45^0 - 55^0 C water bath incubator for 5 minutes to dissolve the agarose. Glassmilk suspension (silica matrix in water) (5 µl) was added to the melted solution and the tubes were incubated for 5 minutes. Glassmilk/ DNA complex were centrifuged in a Microcentrifuge for approximately 5 seconds. Pellets were washed 3 times with NEW wash (Appendix C). Washed white pellets were resuspended in 25 µl of distilled water. The tubes were incubated at 45^0-55^0 C for 2-3 minutes. Centrifugation was carried for 30 seconds. The supernatant containing the eluted DNA was removed and placed in clean tubes. Approximately 80% or

more of the bound DNA was recovered in this step. A second elution was carried resulting in an additional 10-20% recovery of DNA.

4.2.7 Cloning of 600 bp fragments

The recovered DNA was then cloned in the pMOs Blue T-vector kit (Amersham, Little Chalfont, Buckinghamshire, UK). The concentration of DNA eluted from agarose were estimated by Gene Quant-II spectrophotometer (Pharmacia Inc., Piscataway, N. J., USA). For optimal cloning efficiencies the vector to insert ratio were in the range 1: 5 or 1: 10. For PCR insert to be cloned, the ligation reaction was prepared as followed: 1 µl 10X ligation buffer (supplied in kit), 0-5 µl 100 mM DTT (Dithiothreitol), 1.0 µl (50 ng/µl) pMoS vector, 2 µl (100 ng) amplified PCR insert, 0.5 µl T_4 DNA ligase (2-3 weiss units) and the mixture completed to 10 µl with nuclease free water. The reaction was incubated at 16^0 C for two hours.

4.2.8 Transformation of cloned vectors

pMOS Blue competent cells with the cloning kit (Amersham, Little Chalfont, Buckinghamshire, UK) were provided in 200 µl aliquotes. The cells were prechilled on ice (standard transformation requires 20 µl) and 1 µl of ligation mixture was added directly to the cells and stirred gently to mix. The mixture was incubated on ice for 30 minutes, and subjected to heat shock for 40 seconds in a 42° C water bath. The mixture tubes were placed on ice immediately for 2 minutes. Room temperature SOC media (80 µl) (Appendix C), was added to each tube and shaken at 200-250 cycles/minute in Environ-Shaker 3597-1 (Lab Line Instruments, Inc., Illinois, USA) for 1 hour at 37° C. LB agar plates (Appendix C), containing 50 µg/ml ampicillin and 15 µg/ml tetracycline , were prepared for the transformation. For blue and white colour screening of recombinant, X-gal/IPTG plates were prepared as follows: 35 µl of 50 mg/ml X-gal and 20 µl of 100 mM IPTG were spread on LB agar antibiotic plates. The plates were left to soak for 30 minutes prior to plating. 50 µl to 100 µl of each transformation were spread onto the agar plates. Plates were incubated in inverted position overnight at 37° C. White colonies (recombinant plasmids) were scored and picked by sterile tooth pick under Laminar sterile hoot. The

colonies were grown overnight in 3 ml SOC media containing 100 µg/ml Ampicillin at 37^0 C with vigorous shaking (250- 300 cycles/minutes) in a rotary shaker. Isolation and purification of the recombinant plasmids was carried by small scale DNA preparation.

4.2.9 Small scale DNA preparation (Miniprep)

Isolation and purification the plasmids were carried out by using Magic™ Minipreps DNA kit (Promega Biotech. Inc., USA). Cells from the 3 ml antibiotic culture, were harvested by centrifugation in a microcentrifuge for 30 seconds. The cell pellet was resuspended in 200 µl of cell resuspension solution (Appendix C) by vigorous vortexing. The resuspended cells were transferred to a Eppendorf tube. Cell lysis solution (200 µl) (Appendix C) was added and mixed by inverting the tubes several times. The cell suspension became clear immediately (if it did not, inversion of the tubes was continued until it cleared). Neutralization buffer (200 µl) (Appendix C), was added and mixed by inverting the tubes several times. Spinning was carried at top speed in a microcentifuge for 5 minutes. The supernatant was decanted to a new Eppendorf tube. 1 ml of DNA purification resin was mixed with the solution by inverting the tubes several times. Magic minicolumn was prepared by removing the plunger from a 3

ml disposable syringe and the syringe barrel was attached to the minicolumn extension. Resin/DNA mix then loaded into the syringe barrel and the slurry was pushed gently into the minicolumn. The syringe was detached from the minicolumn and the plunger also removed from the syringe; 2 ml of column wash solution (Appendix C) was pipetted in the syringe and gently pushed through the minicolumn. The minicolumn was then transferred to a new 1.5 Microcentifuge tube and centrifuged at top speed in a Microcentrifuge for 20 seconds to dry the resin. The minicolumn then transferred to a new tube and 50 µl of water or TE buffer (Appendix C), was applied. After 1 minute, the DNA was eluted by spinning the minicolumn at top speed in a microcentrifuge for 20 seconds. The recombinant plasmids were screened by double restriction endonuclease digestion. Digestion reaction was carried out in small tubes as follows: 1 µl (5 weiss units) HindIII and 1 µl(5 weiss units) EcoRI restriction enzymes (New England Biolabs, MA, USA), 1 µl of 10X reaction buffer (Appendix C), 2 µl purified plasmids and 5 µl of double distilled water.

The tubes were incubated for 1 hour in 37^0 C in water bath. After digestion, 5 µl of the mix was resolved in 1% agarose gel. The true recombinant (plasmid ligated with insert) were scored and stored in 4^0 C (Sambrook *et al.*, 1989).

4.2.10 Southern blotting of DNA to nylon membranes

Genomic DNA (10-12 µg) from individuals and pooled samples of both populations, were digested to completion with EcoRI (5 weiss units/ µl) restriction endonuclease and resolved in 1% agarose gel overnight at 1.5 V/cm. DNA was transferred to nylon membranes (Hybond N, Amersham, UK) by the method of Sambrook *et al.*, (1989). After electrophoresis the agarose gel was incubated in two volumes of 0.25 N HCl for 15 min at room temperature with gentle agitation, followed by a brief rinse in distilled water to partially hydrolyse the DNA by acid depurination. Denaturation of DNA was carried out with denaturation buffer (Appendix C) for 30 minutes followed by rinse in distilled water. DNA was neutralized for 30 minutes with neutralization buffer (Appendix C), followed by rinse in distilled water. Finally, the gel was soaked 5 minutes in 8X SSC transferring buffer (Appendix C). The gel was then placed on top of three layers of Whatman 3 MM filter paper (wetted with 8X SSC, and placed on top an inverted gel-casting tray in a plastic box such that the filter papers touched the base of the box, forming a wick), and overlaid with a nylon membrane and three layers of filter paper, all which had been pre-wetted in 8X SSC. A 5 cm-thick wad of dry tissue paper was placed on the gel/membrane/filter paper sandwich, followed by another glass plate and a 5 kg weight. Blotting was continued by

capillary transfer to the membrane, using 8X SSC buffer. For efficient transformation, blotting was carried overnight. DNA was fixed onto the membrane by either UV-crosslinking the membrane (254 nm) for 5 minutes or by backing in an oven at 80° C for 2 hours. Membranes were used for hybridization immediately, or stored until used at 4° C in a sealed plastic bag.

4.2.11 Hybridization assays

Hybridization and washing conditions were essentially carried out according to Sambrook *et al.*, (1989). The membrane was gently shaken in pre-hybridization solution (Appendix C) overnight at 65° C, while the probe (cloned vector) was being prepared. DNA probes were denatured by boiling for 5 min then snap cooled on ice and randomly primed by incorporation of [α- ^{32}P] dCTP using Random Primed DNA labelling kit (Boehringer Mannheim Biochemica, Mannheim, Germany). The labelled probe were added to the prehybridization solution and hybridization carried out overnight at 65° C water bath with shaking (200 cycle/ min). For radioactive blots, the filter were washed 3 times at room temperature (10 minutes each) in low stringency washing buffer (2.0X SSC; 0.1% SDS). Moderate (0.5X; 0.1% SDS) and high stringency (0.1X SSC; 0.1% SDS) washes were done at 65° C for 1 hour (as judged using a hand-held Geiger Counter). Finally, the

filter was wrapped in Saran wrap and subjected to autoradiography.

4.2.12 Autoradiography

Radioactive blots were exposed to Fuji 1 X- ray film (Fuji, Japan) in X- ray cassettes with a Cronex lightning Plus intensifying screen (Dupont, Delware, USA) overnight at -70^{0} C

4.2.13 DNA Nucleotide Sequencing

Nucleotide sequencing was performed by a modification of the dideoxy chain termination method of Sanger *et al.*, (1977) using Sequenase 2TM (a modified T7 DNA polymerase) from a Sequenase version 2.0 kit (US Biochemicals, Cleveland, USA). Double stranded DNA templates used in sequencing reactions were purified by Caesium Chloride density gradient centrifugation (Sambrook *et al.*, 1989) and digested with 1 µl of a 10 mg/ml Dnase-free RNase A for 30 minutes at 20^{0} C, followed by one Phenol: Chloroform (1: 1) extraction and Ethanol precipitation. 5 µg of template DNA was then diluted to 18 µl with H_2O, mixed with 2 µl of 2 N NaOH, and left at room temperature for 5 minutes. The alkaline denatured DNA was then precipitated by addition of

120 µl neutralizing/ precipitating solution (Appendix C). After 10 min incubation on ice, denatured DNA templates were spun down by centrifuged (20 min, 4^0 C) and the pellet washed gently with ice cold 70% Ethanol and vacuum-dried.

4.2.14 Priming, Labelling and Termination

For sequencing of the "Reverse Strand", U-19 mer (5' ACGTCGTGACTGGGAAAACCCT 3') was used as a primer, while T-7 promoter (5'TAATACGACTCACTAT 3') was used as the "Forward" sequencing primer. The vacuum-dried DNA templates were resuspended in 7 µl H_2O, 2 µl Sequenase buffer (supplied in kit) and 1 µl of appropriate primer (1 pmol/ml). Primers were annealed to the template by heating to 42^0 C for 5 min followed by slow cooling (15 min) to room temperature. To each 10 µl primer/ template complex were added: 1 µl 0.1 M DTT, 2 µl labelling mix (1.5 mM each of dCTP, dATP, dGTP and dTTP), 1.75 µl DMSO, 0.5 ml [α^{35}S] dATP (100 Ci/mmol; 10 Ci mg/ml) and 2 µl Sequenase 2TM (1/8 dilution in cold 10 mM Tris-Cl pH 7.5; 5 Mm DTT; 0.5 mg/ml BSA). Labelling reactions were mixed thoroughly and incubated at 20^0 C for 3 minutes. Four aliquots of 4.3 µl each were then transferred, and mixed well with four respective pre-warmed termination mixes, each containing a different ddNTP.

Tubes containing termination mix (2.75 µl) were prepared before starting the labelling reactions. Each termination mix consisted of a particular dNTP/ddNTP in a 80 mM/8mM ratio, 50 Mm NaCl and 10% DMSO. For each template there were four tubes: dATP/ddATP, dCTP/ddCTP, dGTP/ddGTP and dTTP/ddTTP. These tubes were pre-warmed at 45^0 C, one minute before addition of the labelling mix. Termination reactions were incubated at 45^0 C for 5 min before being stopped by the addition of 5 ml stop buffer (Appendix C). Reactions were either electrophoresed immediately on a denaturing sequencing gel, or stored at -20^0 C for up to two weeks.

4.2.15 sequencing gel preparation

The gel apparatus used for DNA sequencing was obtained from GIBCO (BRL), USA. Glass plates were washed and rinsed thoroughly with deionized water. Denaturing polyacrylamide gels (Appendix C) 6% was containing 7 M Urea (ICN Biomedicals, Inc., Cleveland, USA) were prepared by mixing 60 ml of gel mix with 600 µl of freshly prepared 10% Ammonium persulphate (Sigma) and 100 µl TEMED just before pouring. The gel mix was poured smoothly, avoiding bubbles, by using 60 ml syringes, after which the gel was allowed to polymerize for 30 min before use.

4.2.16 Electrophoresis

After assembly of the gel apparatus, the buffer tank was filled with 1 X TBE (Appendix C) that was pre-heated to 65^0 C, which eliminated the need for pre-running the gel to heat it up to the running temperature (40^0 - 45^0 C). Templates, were heat denatured in a 80^0 C water bath for 2 min immediately prior to loading, and snap cooled on ice. The wells were cleared from crystallizing Urea by blowing out with a buffer filled syringe, and samples were run at 96 W for approximately 60 min in 6 % gels (normal runs) or 92 W for 2.5- 4 hrs (extended runs). The gel temperature was kept constant at 40^0 to 45^0 C by means of a fan. After running, the top glass plate was lifted and a sheet of Whatmans filter paper was layered over the gel. The gel was "dry-lifted" from the glass plate, and dried onto the filter paper at 80^0 C for 30 min by means of a vacuum dryer coupled to a water trap and a vacuum pump, before being exposed to Fuji X-ray film overnight at -70^0 C.

4.3 RESULTS

4.3.1 RAPD- PCR Products

Twenty primers were screened with the pooled genomic DNA of both males and females of the two populations (Table 4.1). Each of the twenty primers produced multiple amplification products. RAPD reaction often produce patterns of bright bands together with fainter bands or faintly smeared regions in the gel. Complex patterns of faint bands and too bright patterns were so difficult to compare between the different samples. Primers which produced complex of homologous amplified fragments in both populations were considered as poor primers and not submitted for further diagnosis.

Sixteenth primers A1, A3, A4 (Figure 4.1), A5, A6 (Fig. 4.2), A7, A8, A9, A10, A11 (Fig.4.3), A13, A14, A15, A16, A17 (Fig. 4.4) and A19 (Fig. 4.5), were examined and excluded from further screening because they produced homologous patterns as well as smeared or faintly bands and over bright bands for any DNA templates.

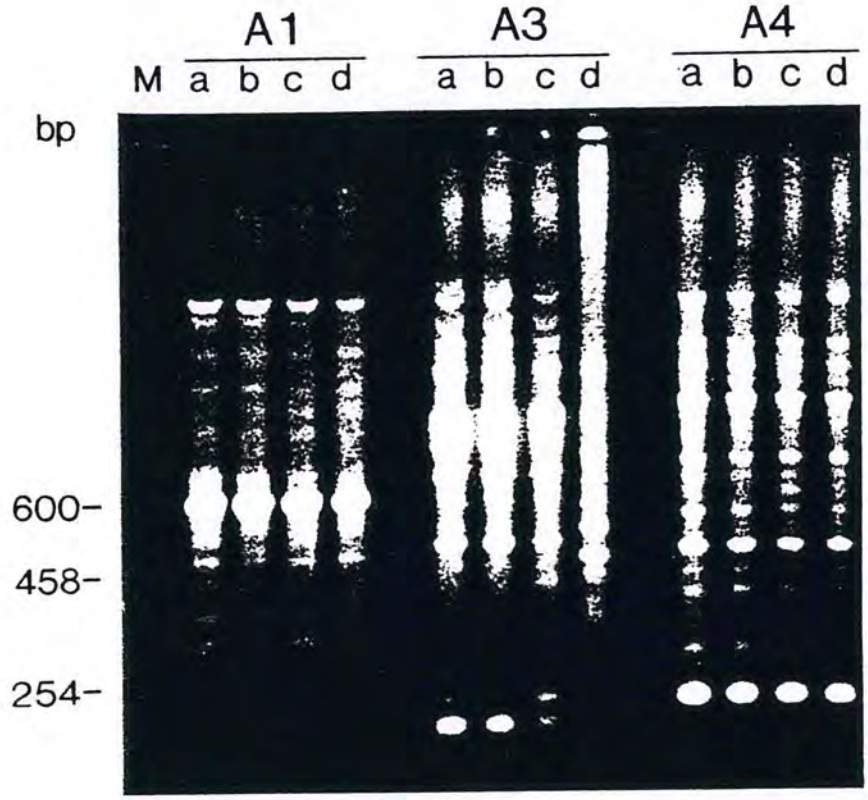
Amplification product with four primers A2, A12, A18, A20 (Fig. 4.6) produced monomorphic and polymorphic patterns with the pooled DNA samples. The fragments which differentiated the southern from northern locusts were scored at low molecular

Table 4.1 RAPD primers (Kit A) used in the differentiation of the desert locust populations, *Schistocerca gregaria*.

KIT A

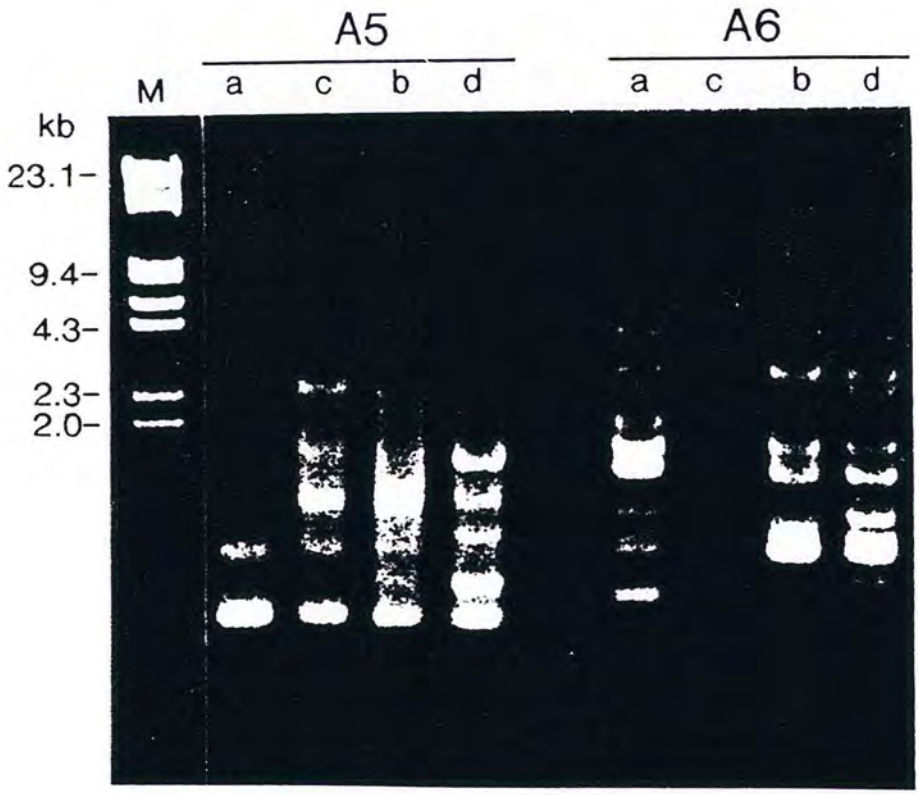
code	5′ to 3′	M.W.	pmoles	ug/tube
OPA-01	CAGGCCCTTC	2955	6014	18.0
OPA-02	TGCCGAGCTG	3035	5493	16.5
OPA-03	AGTCAGCCAC	2988	5192	15.5
OPA-04	AATCGGGCTG	3059	5088	15.5
OPA-05	AGGGGTCTTG	3090	5192	16.0
OPA-06	GGTCCCTGAC	2995	5742	17.0
OPA-07	GAAACGGGTG	3108	4625	14.5
OPA-08	GTGACGTAGG	3099	4892	15.0
OPA-09	GGGTAACGCC	3044	5158	15.5
OPA-10	GTGATCGCAG	3059	5088	15.5
OPA-11	CAATCGCCGT	2979	5531	16.5
OPA-12	TCGGCGATAG	3059	5088	15.5
OPA-13	CAGCACCCAC	2933	5493	16.0
OPA-14	TCTGTGCTGG	3041	5783	17.5
OPA-15	TTCCGAACCC	2939	5783	17.0
OPA-16	AGCCAGCGAA	3037	4710	14.5
OPA-17	GACCGCTTGT	3010	5654	17.0
OPA-18	AGGTGACCGT	3059	5088	15.5
OPA-19	CAAACGTCGG	3028	4988	15.0
OPA-20	GTTGCGATCC	3010	5654	17.0

Figure 4.1 RAPD amplification products of DNA pools from two locust populations with three primers (A1, A3, A4).



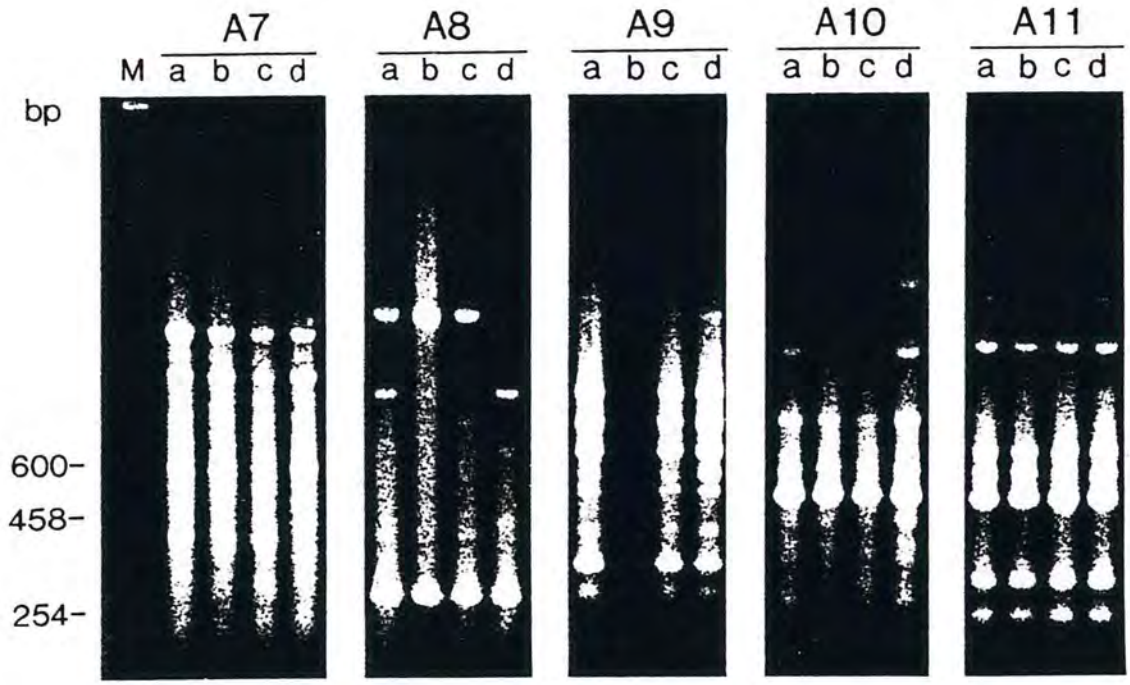
a= males *S. g. flaviventris*; b= males *S. g. gregaria*; c= females *S. g. flaviventris*; d= females *S. g. gregaria*; M= DNA size marker (Bluescript SK^+/ Hae III digest).

Figure 4.2 RAPD amplification products of DNA pools from two locust populations with two primers (A5, A6).



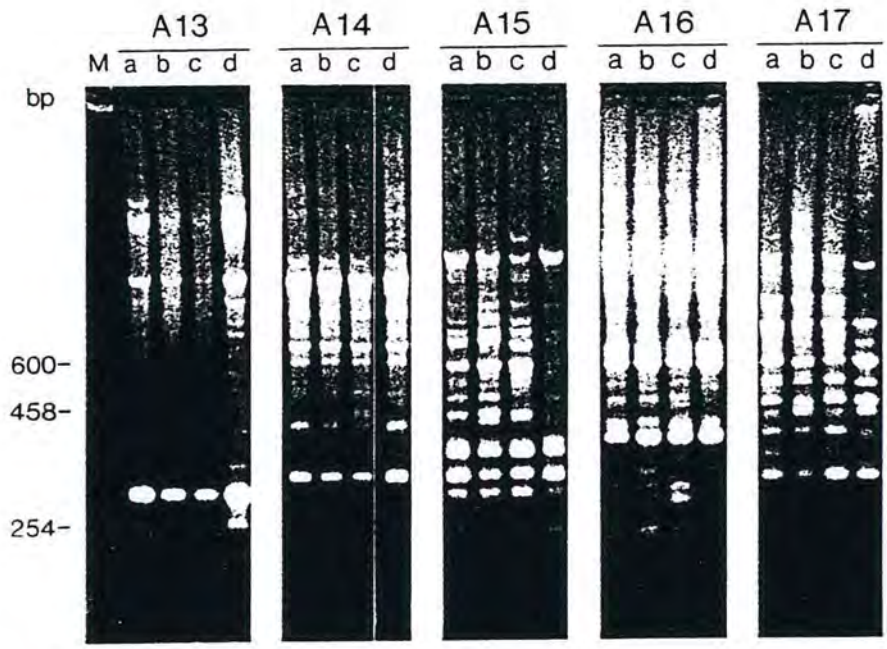
a= males *S. g. flaviventris;* b= males *S. g. gregaria;* c= females *S. g. flaviventris;* d= females *S. g. gregaria;* M= DNA size marker (Lamda DNA/ HindIII digest).

Figure 4.3 RAPD amplification products of DNA pools from two locust populations with five primers (A7, A8, A9, A10, A11).



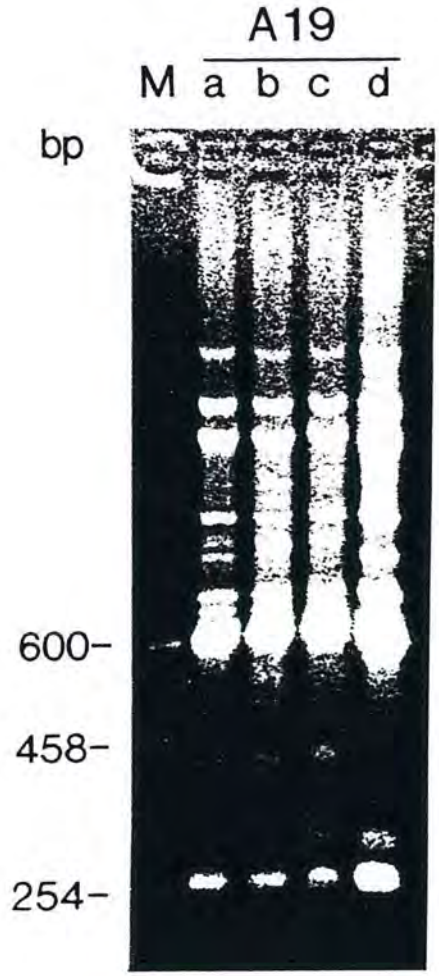
a= males *S. g. flaviventris*; b= males *S. g. gregaria*; c= females *S. g. flaviventris*; d= females *S. g. gregaria*; M= DNA size marker (Bluescript SK^{+}/ Hae III digest).

Figure 4.4 RAPD amplification products of DNA pools from two locust populations with five primers (A13, A14, A15, A16, A17).



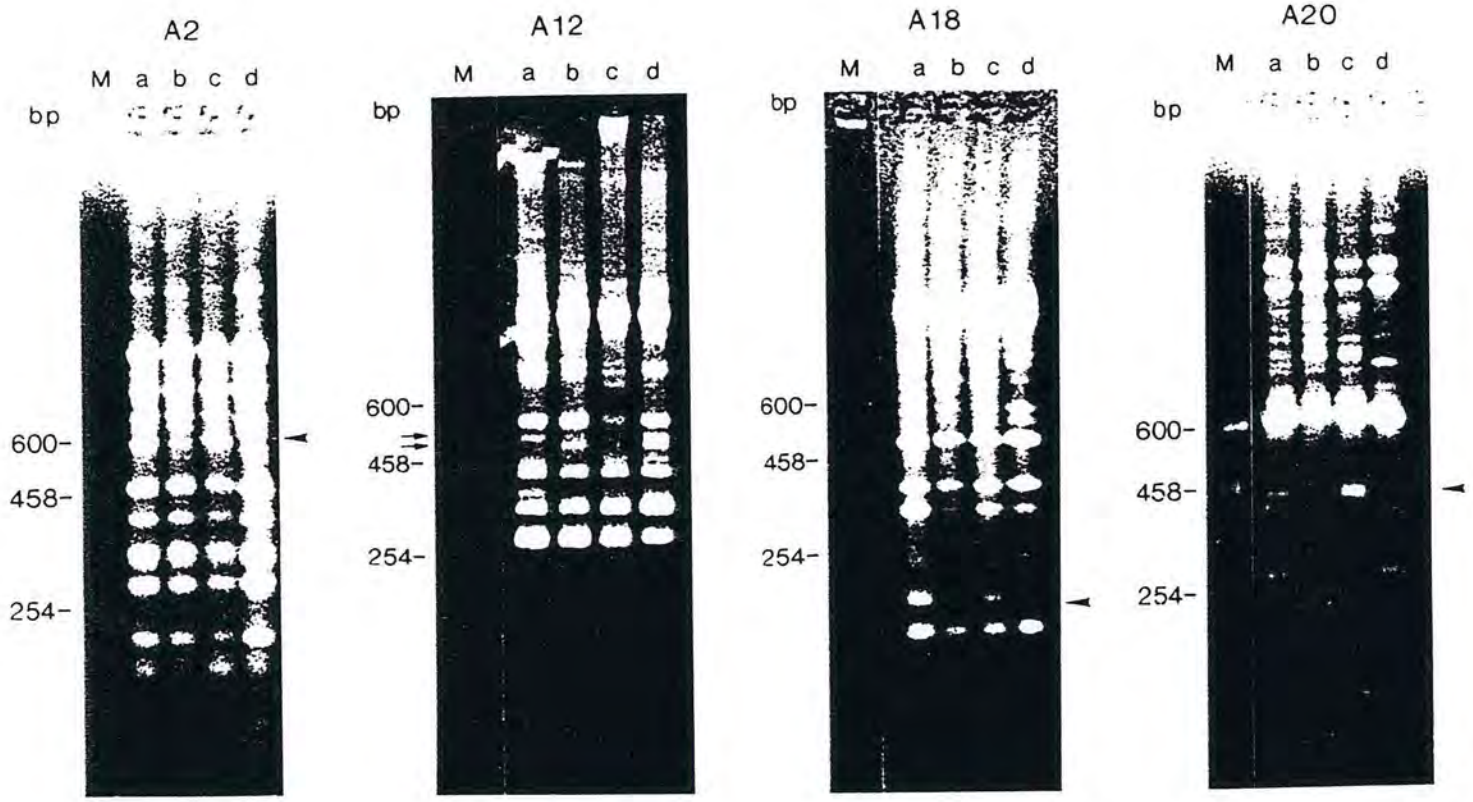
a= males *S. g. flaviventris;* b= males *S. g. gregaria;* c= females *S. g. flaviventris;* d= females *S. g. gregaria;* M= DNA size marker (Bluescript SK^+/ Hae III digest).

Figure 4.5 RAPD amplification products of DNA pools from two locust populations with primers A19.



a= males *S. g. flaviventris;* b= males *S. g. gregaria;* c= females *S. g. flaviventris;* d= females *S. gregaria;* M= DNA size marker (Bluescript SK^+/ Hae III digest).

Figure 4.6 RAPD amplification products of DNA pools from two locust populations with four primers (A2, A12, A18, A20).



The arrow indicate the polymorphic bands in the two locusts.

a= males *S. g.flaviventris;* b= males *S. g. gregaria;* c= females *S. g. flaviventris;* d= females *S. g. gregaria;* M= DNA size markers (Bluescript Sk+/ HaeIII digest).

weight. These primers were then submitted to a second round of screening to test their diagnostic ability and investigate the amount of genetic variability between the individuals. Three primers A12 (Fig. 4.7), A18 (Fig. 4.8) and A20 (Fig. 4.9) were failed to give reproducible patterns with the different DNA individuals (5 males and 5 females) as well as pooled samples of the tested populations.

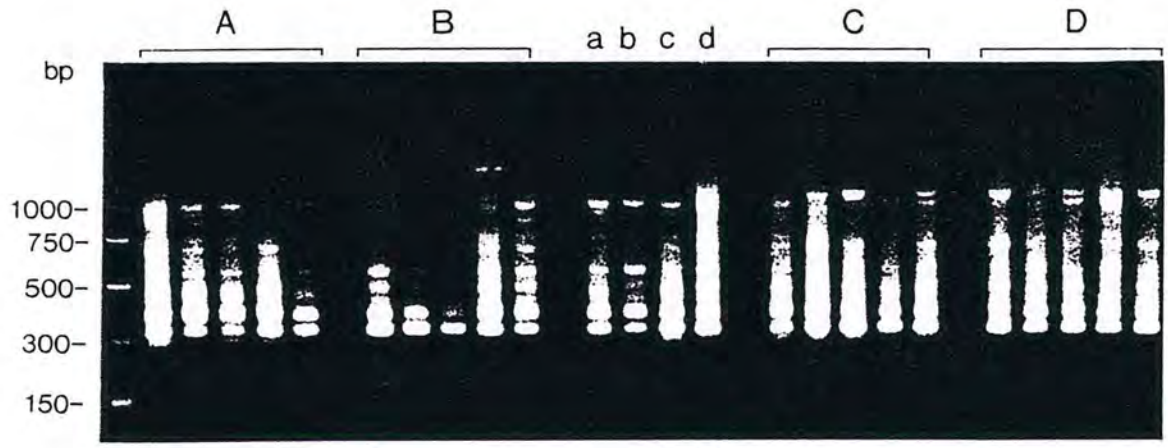
Primer A2 which produced diagnostic polymorphic patterns with the pooled samples (Fig. 4.10) was subjected to a second round with individuals (5 males and 5 females) and pooled samples from the two locusts. In the reaction, the amplified patterns were consistent and reproducible for any given DNA template.

A distinct band (~ 600 base pairs) clearly visualized on Ethidium bromide agarose gel was produced with template DNA derived from both males and females of the *S. g. flaviventris*. This band was not apparent in any of the RAPD-PCR products of templates derived from males and females of *S. g. gregaria*.

4.3.2 Southern blot analysis of genomic DNA

Southern blots of EcoRI digested genomic DNA of *S. g. gregaria* and *S. g. flaviventris* were probed with the cloned 600 bp product of the southern males and females locusts. The clones were screened with double restriction endonuclease

Figure 4.7 RAPD amplification products from DNA individuals and pools of the two locust populations with primer A12.



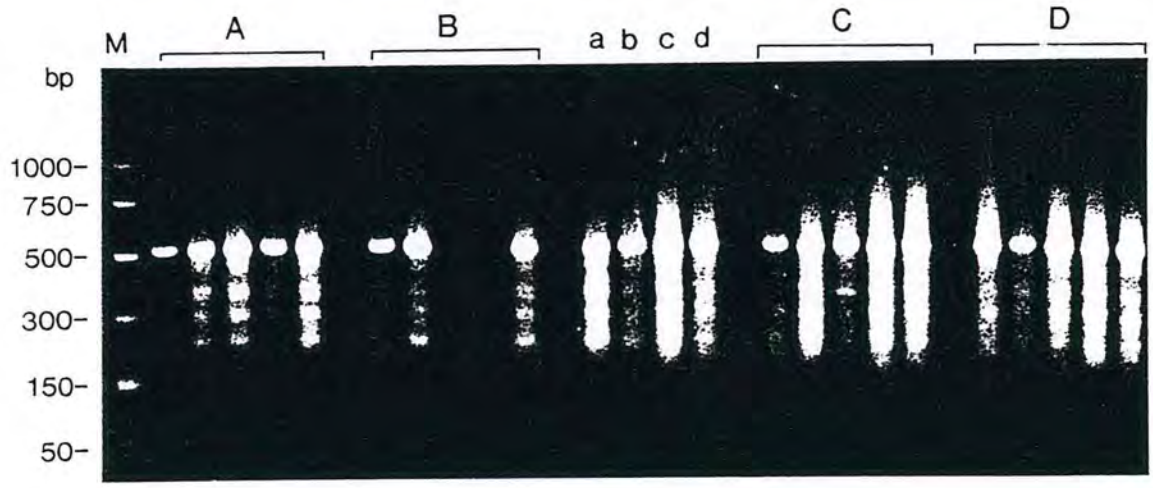
A,a= individuals and pools DNA of males *S. g. flaviventris;*

B,b= individuals and pools DNA of males *S. g. gregaria;*

C,c= individuals and pools DNA of females *S. g. flaviventris;*

D,d= individuals and pools DNA of females *S. g. gregaria;* M= DNA size marker (Promega)

Figure 4.8 RAPD amplification products from DNA individuals and pools of the two locust populations with primer A18.



A,a= individuals and pools DNA of males *S. g. flaviventris;*

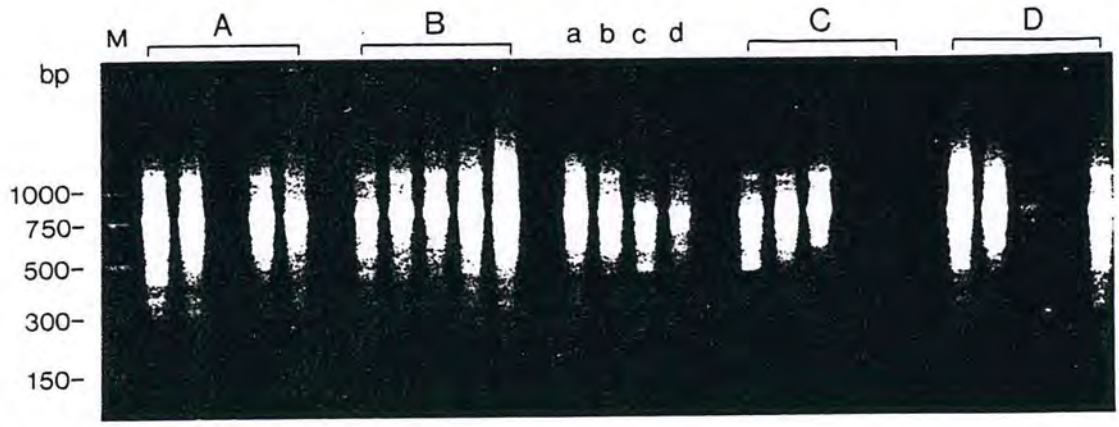
B,b= individuals and pools DNA of males *S. g. gregaria;*

C,c= individuals and pools DNA of females *S. g. flaviventris;*

D,d= individuals and pools DNA of females *S. g.gregaria;*

M= DNA size marker (Promega)

Figure 4.9 RAPD amplification products from DNA individuals and pools of the two locust populations with primer A20.



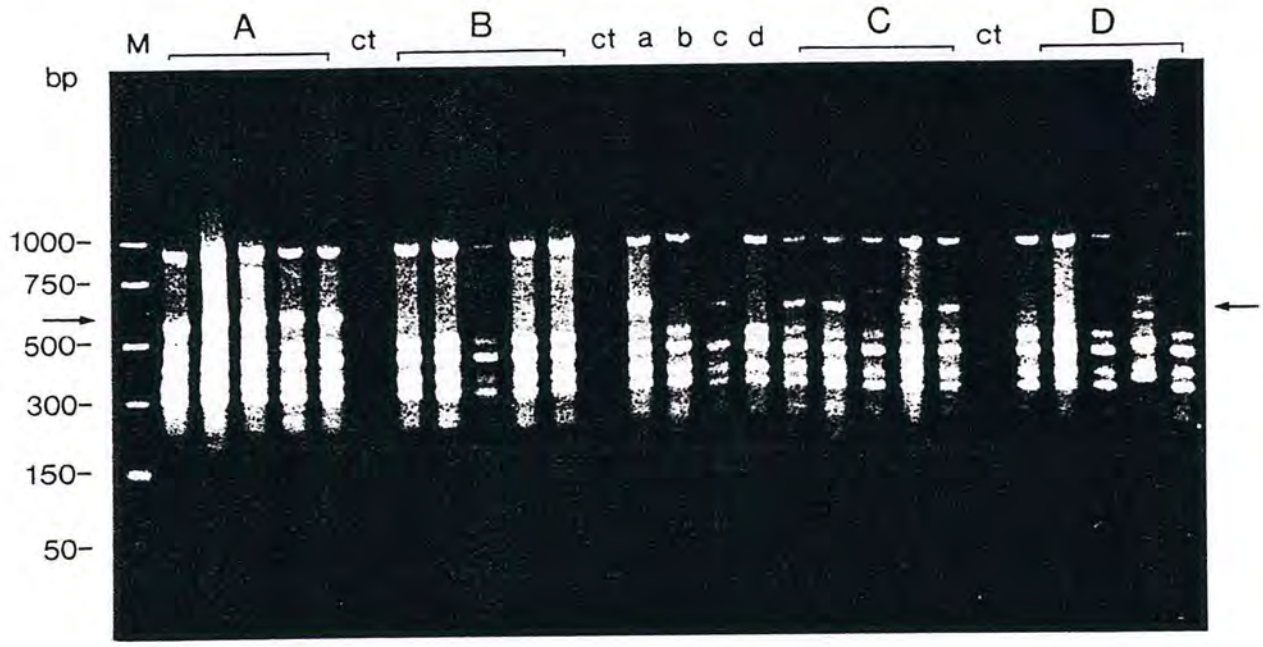
A,a= individuals and pools DNA of males *S. g. flaviventris;*

B,b= individuals and pools DNA of males *S. g. gregaria;*

C,c= individuals and pools DNA of females *S. g.flaviventris;*

D,d= individuals and pools DNA of females *S. g. gregaria;* M= DNA size marker (Promega)

Figure 4.10 RAPD amplification products from DNA individuals and pools of the two locust populations with primer A2.



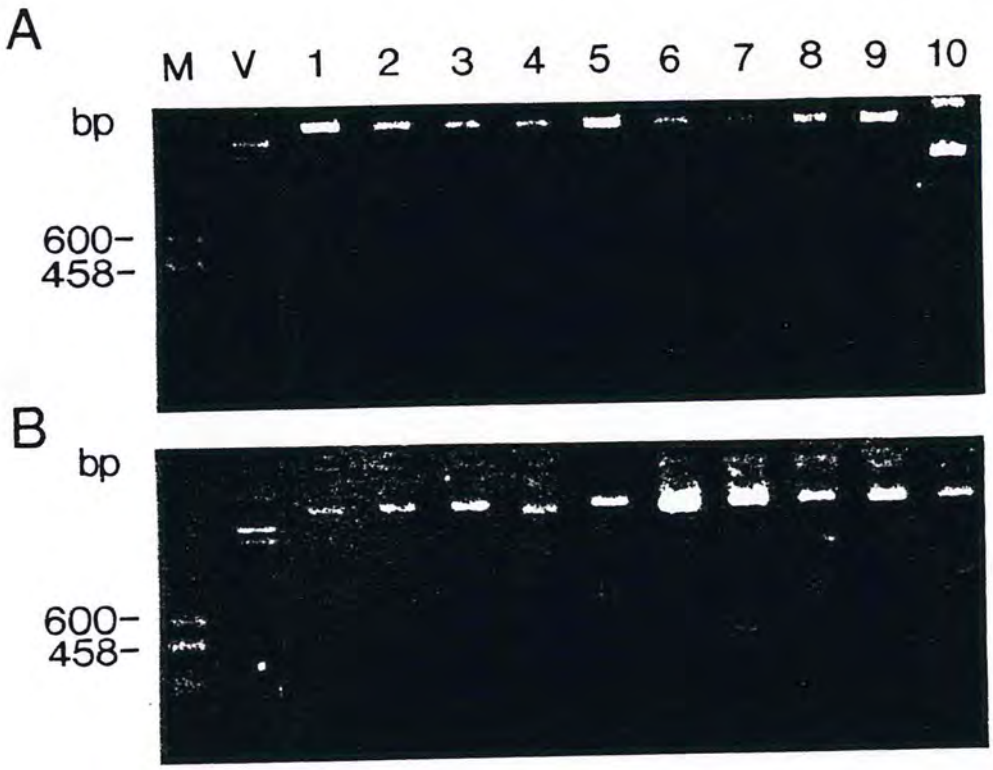
The arrow pointed to the 600 bp specific bands in the individuals and pools of the south locust, *S. g. flaviventris*. A,a= individuals and pools DNA of males *S. g. flaviventris;* B,b= individuals and pools DNA of males *S. g. gregaria;* C,c= individuals and pools DNA of females *S. g. flaviventris;* D,d= individuals and pools DNA of females *S. g. gregaria;* ct= control samples without DNA templates, M= DNA size marker (Promega)

digestion before used in the hybridization assays (Fig. 4.11). The results obtained from both hybridized membranes for DNA individuals and pooled samples for males (Fig. 4.12) and females (Fig. 4.13) were found to hybridize strongly to all males and females and pooled DNA samples prepared from the southern locusts. This result revealed that these sequences are highly repeated in the genome of the *flaviventris* population but not specific to the *gregaria* population.

4.3.3 Sequence of the 600 base pairs band amplified by A2 primer from *S. g. flaviventris*

The DNA sequence of the 600 bp clone was compared with the GeneBank data base. No homology was observed with the males (Fig. 4.14) and females (4.15) sequences. The DNA sequence of the two clones were submitted to the GeneBank, accession numbers: U60233 (male) and U60265 (female).

Figure 4.11 Recombinant plasmids after cut with HindIII and EcoRI restriction endonuclease enzymes to release the 600 bp DNA insert.

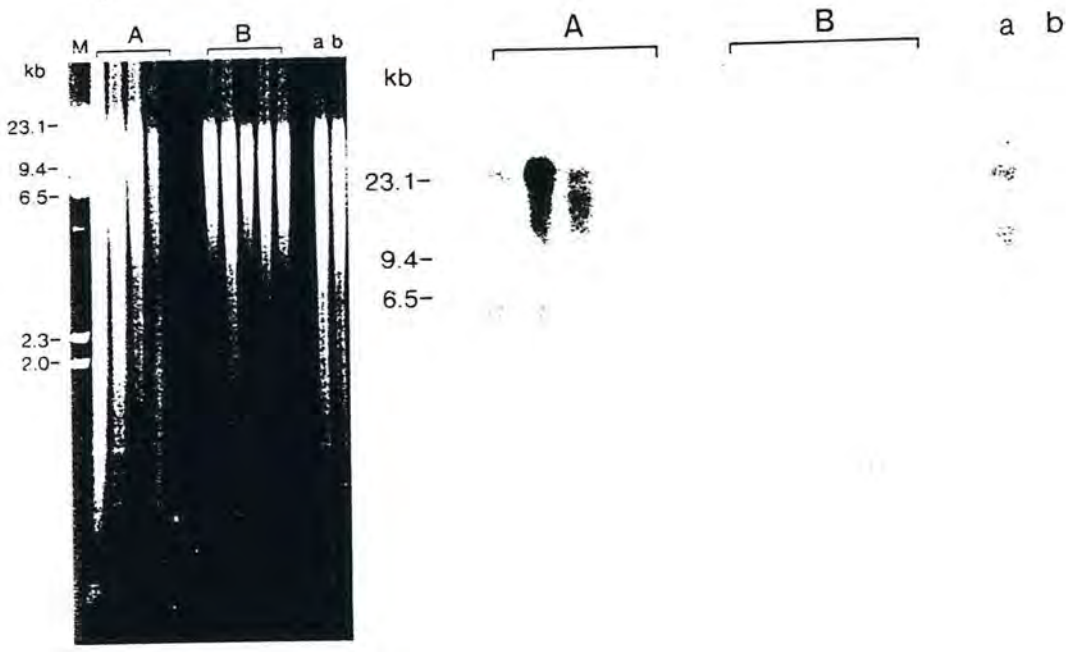


Panel A: southern male 600 bp insert at lanes 1 and 9.

Panel B: southern female 600 bp insert at lanes 5, 7, 9 and 10

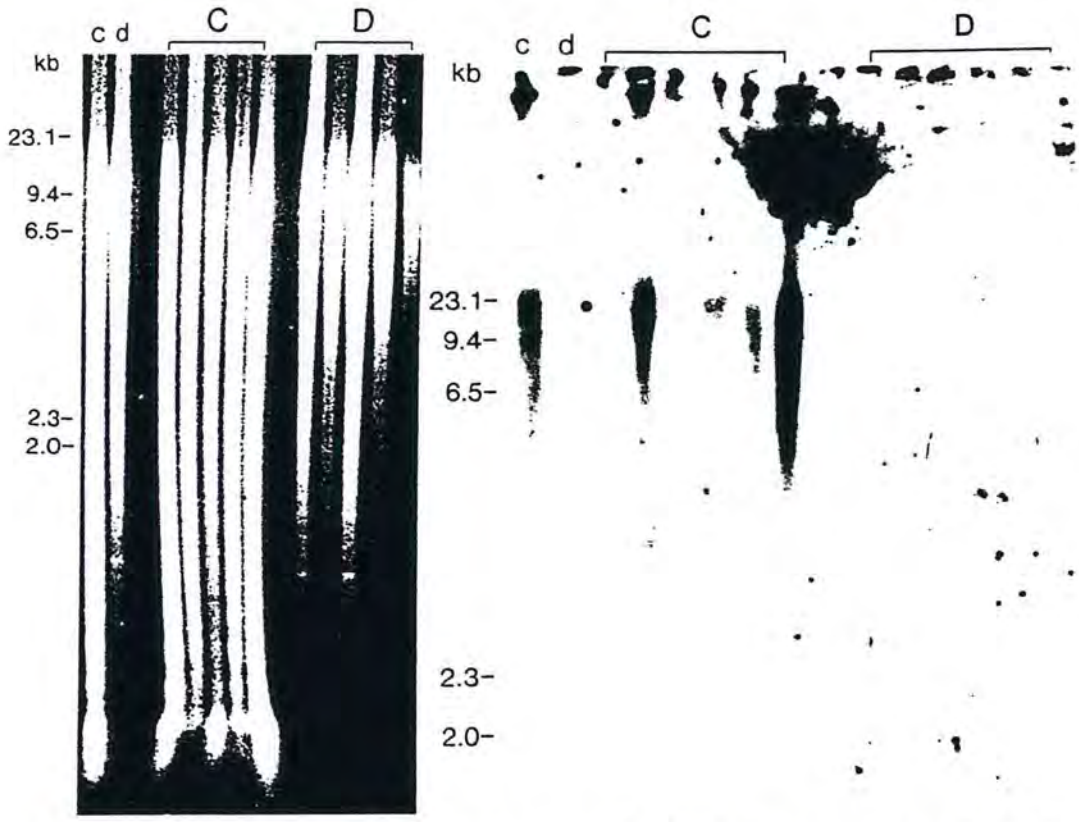
V= pMos vector (uncut); M= DNA size marker (Bluescript SK^{+}/ Hae III digest).

Figure 4.12 Autoradiograph following a Southern blot of genomic DNA of the north and south African locust males with the cloned 600 bp RAPD-PCR south male product.



The cloned product can be seen to hybridize to multiple amplifications of the males of *S. g. flaviventris* but not to the males *S. g. gregaria* males of *S. g. gregaria*. A,a= individuals and pools DNA of males *S. g. flaviventris;* B,b= individuals and pools DNA of males *S. g. gregaria*

Figure 4.13 Autoradiograph following a Southern blot of genomic DNA of the north and south African locust females with the cloned 600 bp RAPD-PCR south female



The cloned product can be seen to hybridize to multiple amplifications of the females of *S. g. flaviventris* but not any to the females of *S. g. gregaria*. C,c= individuals and pools DNA of females *S. g. flaviventris*; D,d= individuals and pools DNA of females *S. g. gregaria*.

Figure 4.14 Sequence of the 600 bp of the south African male desert locust, *S. g. flaviventris*.

```
        10         20         30         40         50         60
GATTTGCCGAGCTGGACCGTACCATTACTGCACAGCTGCACTAAAAGCTAAGGAAAAAGG

        70         80         90        100        110        120
CAAAACCTTAAGTAACCAACAGCACGAGGACTTTTTAGTTTCTCACCCACCCGAGTACTA

       130        140        150        160        170        180
ACGCCGCCCAACGCTGCCTAATTTCGGTGATCGGACATGAAAAGCGTCGTTGGCAAGGCA

       190        200        210        220        230        240
TACAAGTTGGCGAGAATGTTTCCTGACGCGCGGACGTCTTAGTCCGTGTACAGATCACTT

       250        260        270        280        290        300
GGGACTTGCCTGGTAGTCTTTCTCGTTGCCCTTTTGGGAAGCCACAACACAACGACCTAC

       310        320        330        340        350        360
GGAAACGTCCGTGAGAAGAGCTAAAACTCGGCACGGAAAAAATATGTTCCGCTATTTCTT

       370        380        390        400        410        420
TTCGACCGATCGGGCTATCGGACGTGCGACGCCTGCACTGCTGCGACTGTCGTCTCTAGT

       430        440        450        460        470        480
AAAATCTCCACGGCGTGTTTCTGCGTAATTTACTTGTTCTATAAGATCAAGGGAGTACGT

       490        500        510        520        530        540
TTCCTCAGAATGTTTAAAATGCATTGTCGGATGTGGGGTTCACACCCACGCCCCTTACAG

       550        560        570        580
TAACCAGAGCTTAAATCAGGCGACATACACAGCTCGGCAAATC
```

Figure 4.15 Sequence of the 600 bp of the south African female desert locust, *S. g. flaviventris*

```
        10         20         30         40         50         60
GATTTGCCGAGCTGTGTATGTCGCCAGATTTAAGCTCTGGTTACTGTAAGGGGCGTGGGT

        70         80         90        100        110        120
GTGAACCCCACATCTCACAATGCATTTTAAACATTCTGAGAAAACGTACTCCCTTGATCT

       130        140        150        160        170        180
TATAGAACAAGTAAATTACGCAGAAACACGCCGTGGAGATTTTACTAGAGACGACAGTCG

       190        200        210        220        230        240
CAGCAGTGCAGGCGTCGCACGTCCGATAGCCCGATCGGTCGAAAAGAAATAGCGGAACAT

       250        260        270        280        290        300
ATTTTTCTCGTGCCGAGTTTTAGCTCCTCTCACGGACGTTTCCGTAGGTCGTTGTGTTGT

       310        320        330        340        350        360
GGCTTCCCAAAAGGGCAACGAGAAAGACTACCAGGCAAGTCCCAAGTGATCTGTACACGG

       370        380        390        400        410        420
ACTAAGACGTCCGCACCTCAGGAAACATTCTCGCCAACTTGTATGCCTTGCCAACGACGC

       430        440        450        460        470        480
TTTTCATGTCCAATCACCGAAATTAGGCAGCGTTGGGCGGCGTTAGTACTCGGGTGGGTG

       490        500        510        520        530        540
AGAAACTAAAAAGTCCTCGTGCTGTTGGTTACTTAAGGTTTTGCCTTTTTCCTTAGCTTT

       550        560        570        580
TGGTGCAGCTGTGCGGTAATGGTACGTTCCAGCTCGGCAAATC
```

4.4 Discussion

Pools of genomic DNA representing two geographically isolated populations of the desert locust were amplified with short oligonucleotide primers. In previous studies for identification of markers of loci controlling disease resistance in plants (Michelmore *et al.*, 1991) and differentiation of two cattle species (Kamp and Teale, 1994), pools of genomic DNA were constructed from individuals of a given population. The rationale is that the constitution of such pools are randomly distributed for all genetic regions except those contributing to the selected phenotype. The RAPD fragments which appear to be unique to pooled templates would have high probability of being linked to the gene controlling the trait and highlighting consistent differences between the pools and masking variability common to pools. Wilkerson *et al.*, (1993) also used pool samples of genomic DNA from cryptic mosquito species to investigate the ability of fifty-seven primers to produce RAPD patterns.

Twenty primers were screened for their diagnostic ability to amplify the genomic DNA from the two population of locusts. Sixteenth primers were considered as poor primers and not used further because the amplified patterns were homologous and some were faint or too bright to score any difference in both populations.

Four of these primers, A2, A12, A18 and A20, at the first screening with the DNA pool templates, they were able to produce polymorphic fragments that distinguished southern from the northern locusts. One of a problem with the RAPD technique is the reproducibilty of the bands. Three primers, A12, A18 and A20, did not show consistent amplification patterns when used to amplify DNA from different individuals from both populations. This may be due to the sensitivity of the DNA templates to the amplification conditions.

Primer A2, which generated polymorphic fragments was examined also for population specificity in pooled and individual DNA samples of the two locust populations. The 600 bp RAPD-PCR product which was apparently found in all pooled and individuals (males, females) of *S. flaviventris* and not shown in the amplification patterns of the DNA templates of the *S. gregaria*.

In order to examine further the specificity of this 600 bp RAPD-PCR product produced by the A2 primer, the clones were used to probe Southern blot of EcoRI digested genomic DNAs of southern and northern individual and pooled samples. The results revealed that these sequences are highly restricted to the *flaviventris* males and females and not found in all of the *gregaria* individuals. This suggests that primer A2 detects priming sites polymorphism in southern population and that this priming sites is in repetitive DNA elements. The Southern blotting approach

proved simple, rapid and effective. It overcomes a number of problems potentially associated with the RAPD techniques. Dot blots can be used also for detecting the repetitive nature of the RAPD markers (Favia *et al.*, 1994; Teale *et al.*, 1994).

Regions of the genome that contain repetitive sequences or inverted terminal repeats may contain a great number of sites amenable to RAPD amplification than the coding sequence regions. Repetitive DNA can change by a variety of translocation mechanisms of the sequences on the chromosome and more important for systematics, the exact base can change by small deletions and insertions (point mutation). If two populations are isolated from one another they will slowly accumulate independent substitutions and their DNA sequences will diverge (Post *et al.*, 1992; Gawel and Bartlett, 1993).

The genomic difference in the repetitive units of the DNA of the two populations is attributed to the geographical barrier which prevented the genetic constitutions to flow between these locusts where the sexual matings is prohibited.

The major advantage of RAPD analysis over other means of examining polymorphism such as RFLP or microsatellite analysis is in the ease with which informative markers are found without prior sequence information (Kemp and Teale, 1994). This approach may have general use as a means of revealing DNA sequences that are characteristic of entire species and of genetically distinct

populations. Additionally, this method can be used in developing molecular markers in the Acrididae and other insects.

The results have demonstrated the use of RAPD-PCR to document differences in the DNA genomes of the southern *flaviventris* and northern *gregaria* populations of the African desert locusts.

CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSION

Systematics is regarded by some biologists as synonymous with taxonomy. But others, such as Blackwelder (1967), Mayr (1969) and Simpson (1961), regard it as the study of the diversity of organisms and their relationships. Systematics includes aspects of the study of evolution (the process by which the diversity of living organisms is produced) and taxonomy (the process by which this variation is arranged into a meaningful and useful order). Both are involved in the study of variation among organisms (Ferguson, 1980).

All organisms occur in groups of similar individuals with a number of features in common, such groups of recognizably similar individuals constitute ***populations***. Mayr (1970) defined the population as: ***the community of potentially interbreeding individuals at a given locality. All members of a local population share a single gene pool, and such a population may also be defined as a group of individuals so situated that any two of them have equal probability of mating with each other and producing offspring. The population, not the individuals***

comprising it, is the natural unit of evolution and is therefore the basic unit used in systematic studies.

The features of organisms that are used to establish relationships are called characters. This term, in general, refers to any attribute of a species that differentiates it from other species. The species may differ from other species not only in aspects of external morphology, but also in size, colour, internal structural, genetic characters, chemical constituents (particularly proteins), ecological requirements and behaviour (Ferguson, 1980).

Morphological characters are most useful for diagnostic purposes. Morphological variation may be classified in various ways, it may concern ***meristic*** characters that can be counted, like the number of scales and hairs; ***quantitative*** characters, that can be measured, like dimensions or weight; or ***qualitative*** characters, like the presence or absence of spots. The genetic bases for these classes of characters is the same (Mayer, 1970).

Multivariate analysis is an important branch of statistics because most sets of data collected by biologists are multivariate and it is less expensive and more powerful (Gauch and Kogan, 1984). Entomologists use multivariate analysis in a great variety of contexts. In order to understand the biological and evolutionary implications of multivariate techniques as a taxonomic tool, it is necessary to portray their relationships to

quantitative genetics. Quantitative genetics deals with the evolutionary aspects of correlated continuous phenotypic attributes that are assumed to be predominantly genetic (Sorensen and Sawyer, 1989). Mahalonobis distances can be interpreted as a reflection of extent of gene flow between different populations. A short distance indicates close relation due to the flow of genetic constitutions; longer distances reflect divergence in the gene flow.

The northern locust populations, *S. g. gregaria*, have small Mahalanobis distances due to the periodic mixing that tackes place as a result of swarming and resultant flow of the genetic make up between the individuals. The large Mahalanobis distances between the southern population and the northern locust populations indicate that there is no gene flow between these populations. This is no doubt because of the geographical barrier between these populations which prevent the genetic make up to flow and lead to morphometric changes.

The process of evolution depends on the occurrence of genetic variation. If DNA replication was always perfect, life could not have evolved and diversified. In general, genetic changes fall into one of the three categories: point mutations involving changes in a single coden; chromosomal aberrations in which a large section of the chromosome undergoes change, and change in chromosome number (Ferguson, 1980). RAPD-PCR technique

detects the repetitive sequences or the inverted terminal repeates in the genomic DNA. Repetitive DNA can change by small deletion and insertions (point mutations). The specific bands that were detected in the southern locust population, *S. g. flaviventris* by the four primers (A2, A12, A18, A20) are due to such point mutations in their chromosomes. If two populations are isolated, they will acquire point mutations and they will diverge genetically (Post *et al.*, 1992; Gawel and Bartlett, 1993).

A species in time and space is composed of numerous such local populations, each one intercommunicating and intergrading with the others (Mayr, 1942). Several species concepts have been advanced with regard to sexually reproducing organisms. Under the popular ***biological species concept (BSC)*** championed by Dobzhansky (1937), ***species are characterized as groups of actually or potentially interbreeding natural populations which are reproductively isolated from other groups.***

All populations of a species can be classified under one (or more) of the following three broad structural components of species: (1) series of gradually changing contiguous populations (clinal variation); (2) populations that are geographically separated from the main body of the species range (geographical isolates); (3) rather narrow belts, often with sharply increased

variability (hybrid belts), bordered on either side by stable and rather uniform groups of populations (Mayr, 1970).

The geographic speciation is the considered exclusive mode of speciation. The ***geographic speciation theory*** is one of the key theories of evolutionary biology (Avise, 1994). The theory states that, ***in sexually reproducing animals a new species develops when a population that is geographically isolated from the other populations of its parental species acquires, during this period of isolation, characters that promote or guarantee reproductive isolation after the external barriers break down*** (Mayr, 1942). Ferguson (1980) stated that populations of a species which are isolated from each others will acquire different random mutations and will diverge genetically. If they are isolated for long periods, they may acquire sufficient differences to be regarded as separate species. The desert area of northern Africa was in contact with that of southern Africa via an arid corridor across central Africa. Successive reduction in the aridity have isolated *S. g. flaviventris* population from the central *S. g. gregaria* (geographical barrier) (Jago *et al.*, 1979).

As soon as two populations are separated, they will drift in their genetic contents, for a number of reasons. The probability that the same mutations will occur in the two populations in the

same sequence is extreemly low. Each incorporated mutation changes the genetic background of the population and thus affects the selective value of all subsequent mutations. Furthermore, recombination will produce different genotypes in the two gene pools and thus, since the same gene may have different selective values in different genotypes, it will lead to gradual shifting of gene frequencies (Gould and Johnston, 1972). The selection pressure to which the two separated populations are exposed are not the same, since there are no two places on the face of the earth where the physical environment is identical. Every completely isolated population exists in a biotic environment that is different from any other, and this shift of the biotic environment adds another powerful selection pressure. Competition, predation, and other ecological interactions are apt to be entirely different in the new environment. These local conditions exert selection pressures reenforcing the steady change of gene contents and leading to the development of numerous new adjustments (Mayr, 1970).

The northern locust populations, *Schistocerca g. gregaria,* are considered as central populations for the genus *Schistocerca* (Waloff, 1976). The central populations of the species range are usually completely contiguous; they also show a relatively high population density (per unit area) and a greater individual variation than is the average for populations of the species

(Mayr, 1969). On the other hand, southern locust population, *S. g. flaviventris*, was peripherally isolated in the south western part of Africa (Meinzingen, 1993). Waloff (1986), reported that the southern locusts have not significantly swarmed or gregarized since 1935. This implies that the population density per unit area is small. Peripheral populations tend to have opposite values for the three characteristics of the central populations.

The genetic differences between the central and the peripheral populations can be explained as follows: the total amount of gene flow is reduced in peripheral populations and near the periphery gene flow becomes increasingly one-way outward (Mayr, 1963). Many of peripheral populations, particularly the more isolated ones, are established by a single fertilized female or a small group of founders that carry only a fraction of the total genetic variability of the species. Contiguous central populations, on the other hand, are in the midst of a stream of multidirectional gene flow and harbour at all times a large store of freshly added immigrant genes. Environmental conditions are marginal near the species border, selection is severe, and only a limited number of genotypes is able to survive these drastic conditions. Reduction of gene flow and increased selection pressure combined deplete the genetic variability of the peripheral populations. Central populations, being in the area ecologically most favorable for the species, tend to build up

large populations whose size is mainly controlled by density dependant factors (Mayr, 1970).

Finally, it can be concluded that:

1. Multivariate analysis of morphometrics, two dimensional gel electrophoresis and RAPD-PCR techniques have proven to be powerful tools in the differentiation of the locust populations as in other insect.
2. Primers A2, A12, A18, A20 displayed specific amplified bands with the genomic DNA obtained from the southern locust *S. g. flaviventris*, which not recognized in the northern *S. g. gregaria*.
3. The differences in the morphological, biochemical and genetical characters recognized in the southern locust population, *S. g. flaviventris*, are due to the differences in the

environmental factors and the genetic constitutions. The northern locust populations, *S. g. gregaria,* and the southern locust population, *S. g. flaviventris,* do not meet in nature, that is, the gene flow between them is prevented. This suggests the need to separate the two populations into two different species. *S. g. flaviventris* represent an evolutionary dichotomy of the genus *Schistocerca* in the African continent.

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Appendix A

SAS Univariate and Multivariate Discriminant Analysis programme

```
TITLE'CLUSTER ANALYSIS OF LOCUST MORPHOLOGY - ALL VARIABLES';
OPTIONS LS=78 PS=500;
DATA MORPH;
INFILE'MORPH6.PRN';
INPUT LOC$ SEX$ E F C LOC1$ LOCSEX$;
REF=E/F;
RFC=F/C;
TITLE'LOCATION ONLY';
PROC FASTCLUS DATA=MORPH OUT=CLUST MAXCLUSTERS=4;
VAR E F C;
RUN;
PROC SORT DATA=CLUST;
BY CLUSTER LOC1 DISTANCE;
* - IF YOU HAVE MORE THAN TWO VARIABLES, USE PROC CANDISC - *;
* - TO COMPUTE CANONICAL VARIABLES FOR PLOTTING THE CLUSTERS- *;
PROC CANDISC DATA=CLUST OUT=CANON DISTANCE ANOVA;
CLASS LOC1;
VAR E F C;
RUN;
OPTIONS LS=78 PS=30;
PROC PLOT DATA=CANON;
```

```
PLOT CAN2*CAN1=LOC1 CAN2*CAN1=CLUSTER;
RUN;
DATA NEW;
SET CANON;
IF LOC1='s' THEN GROUP=1; ELSE
IF (LOC1='f' OR LOC1='e' OR LOC1='r') THEN GROUP=2;
PROC PLOT DATA=NEW;
PLOT CAN2*CAN1=GROUP;
RUN;
PROC SORT DATA=NEW;
BY GROUP;
RUN;
PROC MEANS NOPRINT DATA=NEW;
BY GROUP;
VAR CAN1 CAN2; OUTPUT OUT=T1 MEAN=ME_CAN1 ME_CAN2;
RUN;
DATA T2;
SET T1;
L=ME_CAN2-ME_CAN1;
L2=L**2;
SUM_L2+L2;
D=SQRT(SUM_L2);
PROC PRINT DATA=T2;
VAR GROUP ME_CAN1 ME_CAN2 L L2 SUM_L2 D;
```

```
RUN;
OPTIONS LS=78 PS=500;
PROC GLM DATA=NEW;
CLASS LOC1 CLUSTER GROUP;
MODEL DISTANCE=LOC1 CLUSTER GROUP;
MEANS LOC1 CLUSTER GROUP / LSD LINES ALPHA=0.05;
MEANS LOC1 CLUSTER GROUP / LSD LINES ALPHA=0.10;
RUN;
PROC FASTCLUS DATA=MORPH OUT=CLUST MAXCLUSTERS=4;
VAR REF RFC;
RUN;
PROC SORT DATA=CLUST;
BY CLUSTER LOC1 DISTANCE;
* - IF YOU HAVE MORE THAN TWO VARIABLES, USE PROC CANDISC - *;
* - TO COMPUTE CANONICAL VARIABLES FOR PLOTTING THE CLUSTERS- *;
PROC CANDISC DATA=CLUST OUT=CANON DISTANCE ANOVA;
CLASS LOC1;
VAR REF RFC;
RUN;
OPTIONS LS=78 PS=30;
PROC PLOT DATA=CANON;
PLOT CAN2*CAN1=LOC1 CAN2*CAN1=CLUSTER;
RUN;
DATA NEW;
```

```
SET CANON;
IF LOC1='s' THEN GROUP=1; ELSE
IF (LOC1='f' OR LOC1='e' OR LOC1='r') THEN GROUP=2;
PROC PLOT DATA=NEW;
PLOT CAN2*CAN1=GROUP;
RUN;
PROC SORT DATA=NEW;
BY GROUP;
PROC MEANS NOPRINT DATA=NEW;
BY GROUP;
VAR CAN1 CAN2; OUTPUT OUT=T1 MEAN=ME_CAN1 ME_CAN2;
RUN;
DATA T2;
SET T1;
L=ME_CAN2-ME_CAN1;
L2=L**2;
SUM_L2+L2;
D=SQRT(SUM_L2);
PROC PRINT DATA=T2;
VAR GROUP ME_CAN1 ME_CAN2 L L2 SUM_L2 D;
RUN;
OPTIONS LS=78 PS=500;
PROC GLM DATA=NEW;
CLASS LOC1 CLUSTER GROUP;
```

```
MODEL DISTANCE=LOC1 CLUSTER GROUP;
MEANS LOC1 CLUSTER GROUP / LSD LINES ALPHA=0.05;
MEANS LOC1 CLUSTER GROUP / LSD LINES ALPHA=0.10;
RUN;
TITLE'LOCATION AND SEX ONLY';
PROC FASTCLUS DATA=MORPH OUT=CLUST MAXCLUSTERS=4;
VAR E F C;
RUN;
PROC SORT DATA=CLUST;
BY CLUSTER LOCSEX DISTANCE;
* - IF YOU HAVE MORE THAN TWO VARIABLES, USE PROC CANDISC - *;
* - TO COMPUTE CANONICAL VARIABLES FOR PLOTTING THE CLUSTERS- *;
PROC CANDISC DATA=CLUST OUT=CANON DISTANCE ANOVA;
CLASS LOCSEX;
VAR E F C;
RUN;
OPTIONS LS=78 PS=30;
PROC PLOT DATA=CANON;
PLOT CAN2*CAN1=LOCSEX CAN2*CAN1=CLUSTER;
RUN;
DATA NEW;
SET CANON;
IF LOCSEX=3 THEN GROUP=1; ELSE
IF LOCSEX=4 THEN GROUP=2; ELSE GROUP=3;
```

```
PROC PLOT DATA=NEW;
PLOT CAN2*CAN1=GROUP;
RUN;
PROC SORT DATA=NEW;
BY GROUP;
PROC MEANS NOPRINT DATA=NEW;
BY GROUP;
VAR CAN1 CAN2; OUTPUT OUT=T1 MEAN=ME_CAN1 ME_CAN2;
RUN;
DATA T2;
SET T1;
IF GROUP=1 OR GROUP=2;
L=ME_CAN2-ME_CAN1;
L2=L**2;
SUM_L2+L2;
D=SQRT(SUM_L2);
PROC PRINT DATA=T2;
VAR GROUP ME_CAN1 ME_CAN2 L L2 SUM_L2 D;
RUN;
DATA T2;
SET T1;
IF GROUP=1 OR GROUP=3;
L=ME_CAN2-ME_CAN1;
L2=L**2;
```

```
SUM_L2+L2;
D=SQRT(SUM_L2);
PROC PRINT DATA=T2;
VAR GROUP ME_CAN1 ME_CAN2 L L2 SUM_L2 D;
RUN;
DATA T2;
SET T1;
IF GROUP=2 OR GROUP=3;
L=ME_CAN2-ME_CAN1;
L2=L**2;
SUM_L2+L2;
D=SQRT(SUM_L2);
PROC PRINT DATA=T2;
VAR GROUP ME_CAN1 ME_CAN2 L L2 SUM_L2 D;
RUN;
OPTIONS LS=78 PS=500;
PROC GLM DATA=NEW;
CLASS LOCSEX CLUSTER GROUP;
MODEL DISTANCE=LOCSEX CLUSTER GROUP;
MEANS LOCSEX CLUSTER GROUP / LSD LINES ALPHA=0.05;
MEANS LOCSEX CLUSTER GROUP / LSD LINES ALPHA=0.10;
RUN;
PROC FASTCLUS DATA=MORPH OUT=CLUST MAXCLUSTERS=4;
VAR REF RFC;
```

```
RUN;
PROC SORT DATA=CLUST;
BY CLUSTER LOCSEX DISTANCE;
* - IF YOU HAVE MORE THAN TWO VARIABLES, USE PROC CANDISC - *;
* - TO COMPUTE CANONICAL VARIABLES FOR PLOTTING THE CLUSTERS- *;
PROC CANDISC DATA=CLUST OUT=CANON DISTANCE ANOVA;
CLASS LOCSEX;
VAR REF RFC;
RUN;
OPTIONS LS=78 PS=30;
PROC PLOT DATA=CANON;
PLOT CAN2*CAN1=LOCSEX CAN2*CAN1=CLUSTER;
RUN;
DATA NEW;
SET CANON;
IF (LOCSEX=3 OR LOCSEX=4) THEN GROUP=1; ELSE GROUP=2;
PROC PLOT DATA=NEW;
PLOT CAN2*CAN1=GROUP;
RUN;
PROC SORT DATA=NEW;
BY GROUP;
PROC MEANS NOPRINT DATA=NEW;
BY GROUP;
VAR CAN1 CAN2; OUTPUT OUT=T1 MEAN=ME_CAN1 ME_CAN2;
```

```
RUN;
DATA T2;
SET T1;
IF GROUP=1 THEN X1=ME_CAN1; Y1=ME_CAN2;
IF GROUP=2 THEN X2=ME_CAN1; Y2=ME_CAN2;
D=SQRT(((Y2-Y1)**2)+((X2-X1)**2));
DATA T2;
SET T1;
L=ME_CAN2-ME_CAN1;
L2=L**2;
SUM_L2+L2;
D=SQRT(SUM_L2);
PROC PRINT DATA=T2;
VAR GROUP ME_CAN1 ME_CAN2 L L2 SUM_L2 D;
RUN;
OPTIONS LS=78 PS=500;
PROC GLM DATA=NEW;
CLASS LOCSEX CLUSTER GROUP;
MODEL DISTANCE=LOCSEX CLUSTER GROUP;
MEANS LOCSEX CLUSTER GROUP / LSD LINES ALPHA=0.05;
MEANS LOCSEX CLUSTER GROUP / LSD LINES ALPHA=0.10;
RUN;
```

Appendix B

I. Stock solutions and buffers for the first isoelectrofoucing dimension

1. Lysis (overlay) buffer

9.8 M urea

2% w/v NP-40

2% v/v Ampholine (1.6%, pH 5-7; 0.4%, pH 3-10)

5% v/v ß-mercaptoethanol

The buffer may be stored frozen at -20° C for long periods in 0.5 ml aliquots. But do not continually freeze and thaw it. Use aliquot once and discard the remainder.

2. Isoelectric focusing gel

Urea	5.5 g
Acrylamide (stock)	1.33 ml
NP-40	2.0 ml
Distilled water	1.7 ml
Ampholines (pH 5-7)	0.6 ml
Ampholines (pH 3-10)	0.15 ml

3. 30% Acrylamide stock solution

28.38% w/v acrylamide

1.62% w/v N,N- methylene-bisacrylamide

All acrylamide solutions are light sensitive and should be stored in the dark bottles at 4° C. Acrylamide is a potent neurotoxic, so caution should be exercised.

4. NP-40 solution

10% w/v Nonidet-40 in water

5. Anode electrode solution

0.01 M Phosphoric acid

This should be made fresh from 1 M stock (63.64 ml phosphoric acid to 1 L distilled water).

6. Cathode electrode buffer

0.02 M sodium hydroxide

This should be made fresh from 1 M stock and degassed.

7. Equilibration buffer

0.06 M tris-HCl pH 6.8

2% w/v SDS

5% v/v ß-mercaptoethanol

10% v/v glycerol

8. Agarose solution

1% w/v agarose in equilibration buffer

9. Ammonium persulfate (APS)

10% w/v solution made up freshly and stored at 4° C

II. Stock solutions and buffers for the SDS- slab gel dimension

10. Acrylamide stock

Acrylamide	58 g
N,N bismethylene acrylamide	2 g

Make up to 200 ml in dark bottle and filtrate the solution.

11. 5X Separating buffer, pH 8.8

Tris	225.06 g
SDS	5.0 g

Adjust pH with HCl and make up to 1 L.

12. 10X stacking buffer, pH 6.8

Tris	75.60 g
SDS	5.0 g

Adjust the pH with HCl and make up to 500 ml

13. 4-15% SDS separating gel

	4%	15%
Acrylamide (stock)	2.4 ml	9.00 ml
5X separating buffer	4.6 ml	4.60 ml
Distilled water	10.8 ml	4.20 ml
10% SDS	180 µl	180 µl
TEMED	6.00 µl	6.00 µl
10%APS	130 µl	90 µl

14. 3.13% SDS stacking gel

Acrylamide (stock)	1.02 ml
10X stacking buffer	2.50 ml
Distilled water	6.10 ml
10% SDS	100 µl
TEMED	100 µl
APS	50 µl

15. SDS running buffer

Tris	30.28 g
Glycine	144.14 g
SDS	10.0 g

Make up to 1 L. and dilute 1:10 for running the gel.

Appendix C

1. EDTA (0.5 M, pH 8.0)

EDTA. $2H_2O$	168.1g
Distilled water	1000 ml

EDTA will dissolve when pH has been adjusted to 8.0

2. Enzymatic restriction buffer

Nacl	50 mM
Tris-HCl, pH 7.9	10 mM
Dithiothreitol (DTT)	10 mM

3. Ethidium bromide solution

A solution of 10 mg/ml were made in distilled water and stored in dark bottle.

Dissolve 0.1 g in 10 ml of distilled water. Shake well to dissolve.

4. Gel loading buffer (6X)

Bromophenol blue (0.25%)	0.25 g
Xylene cyanol FF (0.25%)	0.25 g
Ficoll (Type 400; Pharmacia) in water	15.0 g
Distilled water	100 ml

The solution were stored at room temperature.

5. Homogenate buffer

0.1 N Nacl

0.2 M Sucrose

0.1 M Tris-Hcl

0.05 M EDTA, pH 8.0

0.5% SDS

proteinase K 0.33 µg/ml

Prepare in 100 ml with deionized water

6. IPTG (Isopropylthio- ß- D- galactoside)

Make solution by dissolving 2 g of IPTG in 8 ml of distilled water. Adjust the volume to 10 ml with distilled water and sterilize by filtration through a 0.22 µ filter. Store in aliquotes at -20° C.

7. LB (Lúria-Bertaini) Agar Plates

Bacto-Treptone	10 g
Bacto- Yeast extract	5 g
NaCl	10 g
Agar	15 g

Adjust the pH 7.0 with 5 N NaOH. Autoclave. Allow the medium to cool to 55° C before adding the antibiotic reagents (50 µg/ml ampicillin and 15 µg/ml tetracycline). Pour 30-35 ml of medium into 100 mm petri dishes.

8. Miniprep buffers

A. Cell resuspension solution

Tris-HCl, pH 8.0	500 mM
EDTA	10 mM
R-Nase	100µg/ml

B. Cell lysis solution

0.2 M NaoH

1 % SDS

C. Neutralization solution

2.55 M Potassium acetate, pH 4.8

D. TE buffer

Tris-HCl, pH 7.4	1.0 mM
EDTA	1.0 mM

E. Column wash solution

NaCl	200 mM
Tris-HCl, pH7.5	20 mM
EDTA	5 mM

Dillute 1:1 with 95% ethanol

9. NEW wash solution

NaCl-Tris, EDTA, pH 8.0	14 ml
Distilled water	280 ml
Ethanol absolute	310 ml

10. PCR reaction buffer

KCl	500 mM
Tris-HCl, pH 8.0	100 mM
Triton X100	1.0%
dNTPS (25 mM dATP, 25 mM dTTP, 25 dCTP, dGTP)	2.0 mM

11. Sequencing Stock solutions

A. Neutralising/precipitating solution

5% 2 M Ammonium acetate, pH 7.5

95% Ethanol

B. Sequence buffer

Tris-HCl, pH 7.5	10 mM
DTT	5 mM
BSA (Bovine Serium Albumin)	0.5 mg/ml

C. Stop buffer

95% Formamide	
EDTA	29 mM
0.05% Bromophenol blue	
0.05% Xylene Cyand	

D. 40% Acrylamide

Bis-Acrylamide	2.00 g
Acrylamide	38.00 g
Water	100 ml

The mixture deionised by stirring overnight, followed by filtration and stored at 4° C in dark bottle.

E. 6% Acrylamide

Urea (Ultra pure)	42 g
40 % Acrylamide (stock)	15 ml
10 X TBE	10 ml

Warm up to dissolve, cool to 4° C

Add 10 % Ammonium Persulpate	600 µl
TEMED	100 µl

12. SOC media

Bacto-Tryptone	20 g
Bacto- Yeast extract	5 g
NaCl	0.5 g

Add 10 ml 250 mM KCl and 5 ml 2 M $MgCl_2$, Adjust the pH to 7.0 with 5 N NaOH (0.2 ml). Autoclave 20 min. at 15 Ib/Sq, after autoclaving, allow to cool to 60° C and add 20 ml of sterile 1 M glucose.

13. Southern blot solution

A. Denaturation buffer

10 M NaoH	20 ml
H20	1000 ml

B. Neutralization buffer

2 M Tris- HCl, pH 7.6	250 ml
5 M NaCl	300 ml
Water	1000 ml

C. 20 X SSC (stock)

3 M NaCl

0.3 M Na Citrate

Make up to 100 ml with distilled water.

D. Prehybridization solutions

20 X SSPE	6.25 ml
Denhardt's solution	1.25 ml
10% SDS	1.20 ml

Make up to 25 ml with H^2O

E. 20 X SSPE

3.6 M NaCl

0.2 M Sodium phosphate

0.02 M EDTA, pH 7.7

F. Denhardt's solution

2% BSA (Bovine Serum Albumin)

2% Ficoll

2% PUP (PolyvinylPyrrolidone)

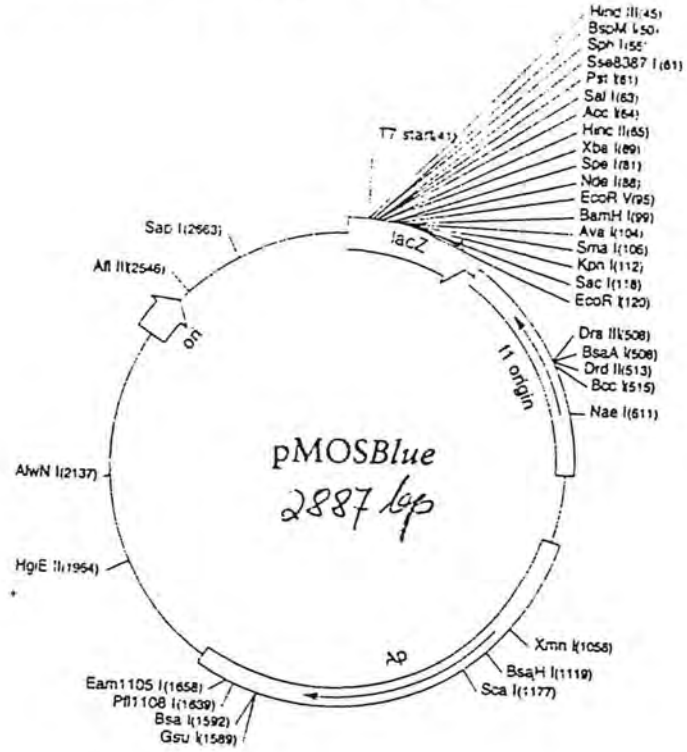
14. 10X TBE (Tris-Borate Buffer)(Stock)

Tris-HCl	108 g
Boric acid	55 g
0.5 M EDTA	9.3 g
Water	1000 ml

15. X-Gal (Bromo-4-chloro-3-indolyl-ß-d-galactoside)

Dissolve X-gal in dimethylformamide to make a 20 mg/ml solution. The tube should be wrapped in aluminum foil and stored in -20° C

pMOSBlue vector map



T7 promoter primer

Sal I
Sse8387 I *Hinc* II

lacZ start — T7 promoter — *Hind* III *Sph* I *Pst* I *Acc* I *Xba* I — *Spe* I

ATGACCATGATTACGCCAAGCTCTAATACGACTCACTATAGGGAAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCTACTAGT

Sma I
Ava I *Ban* II

Nde I *EcoR* V *BamH* I *Kpn* I *Sac* I *EcoR* I

CATATGGATATCGGATCCCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCT

T-vector cloning

U-19mer primer

...t...ggatT atcggat...
...atacCta Ttagcct...

pMOSBlue Multiple Cloning Region