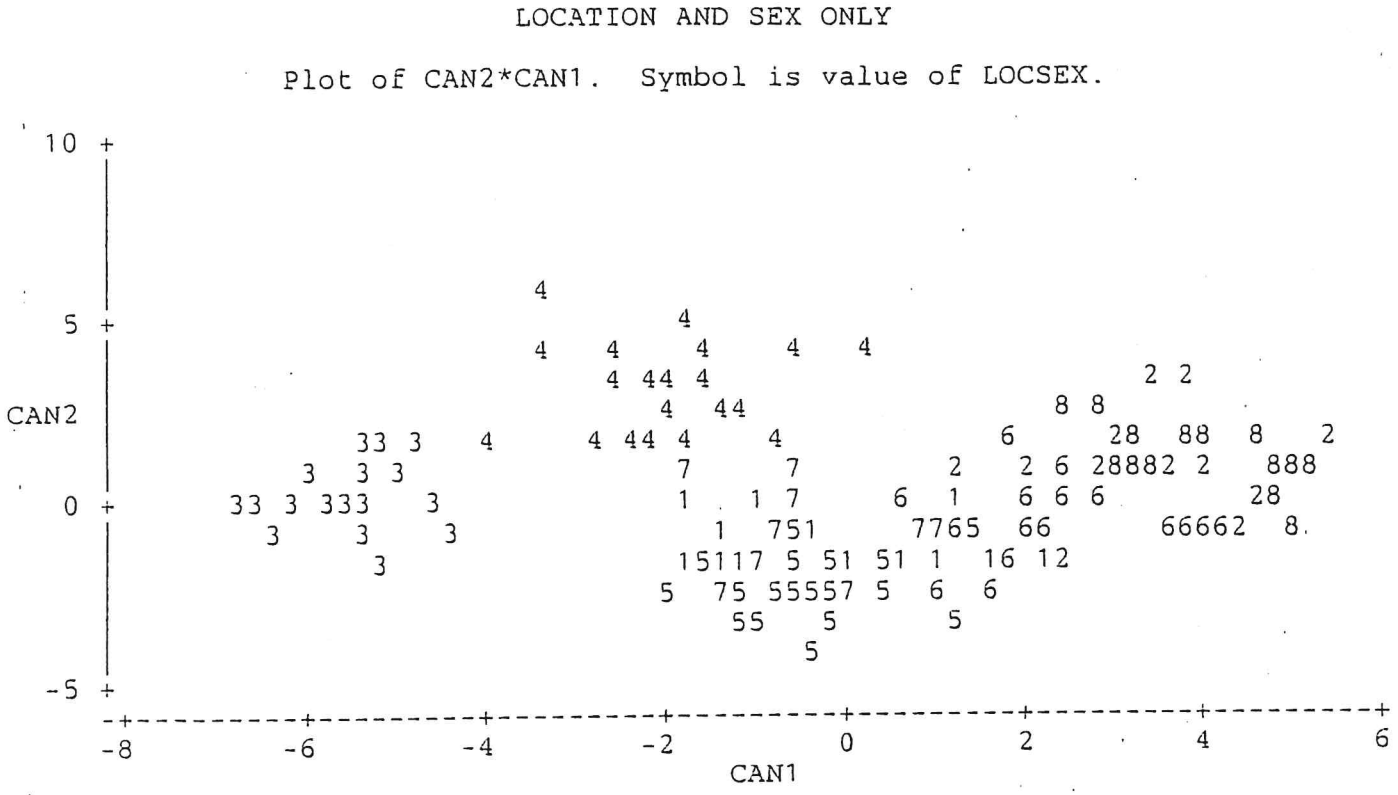
Figure 2.6 Plot representing the four different desert locust individuals along the two axes of canonical discriminant space for the three measured characters (E, F, C). 1, 2= Red sea lab. males and females; 3, 4= south African males and females; 5, 6= Ethiopian field males and females; 7, 8= Red sea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



NOTE: 34 obs hidden.

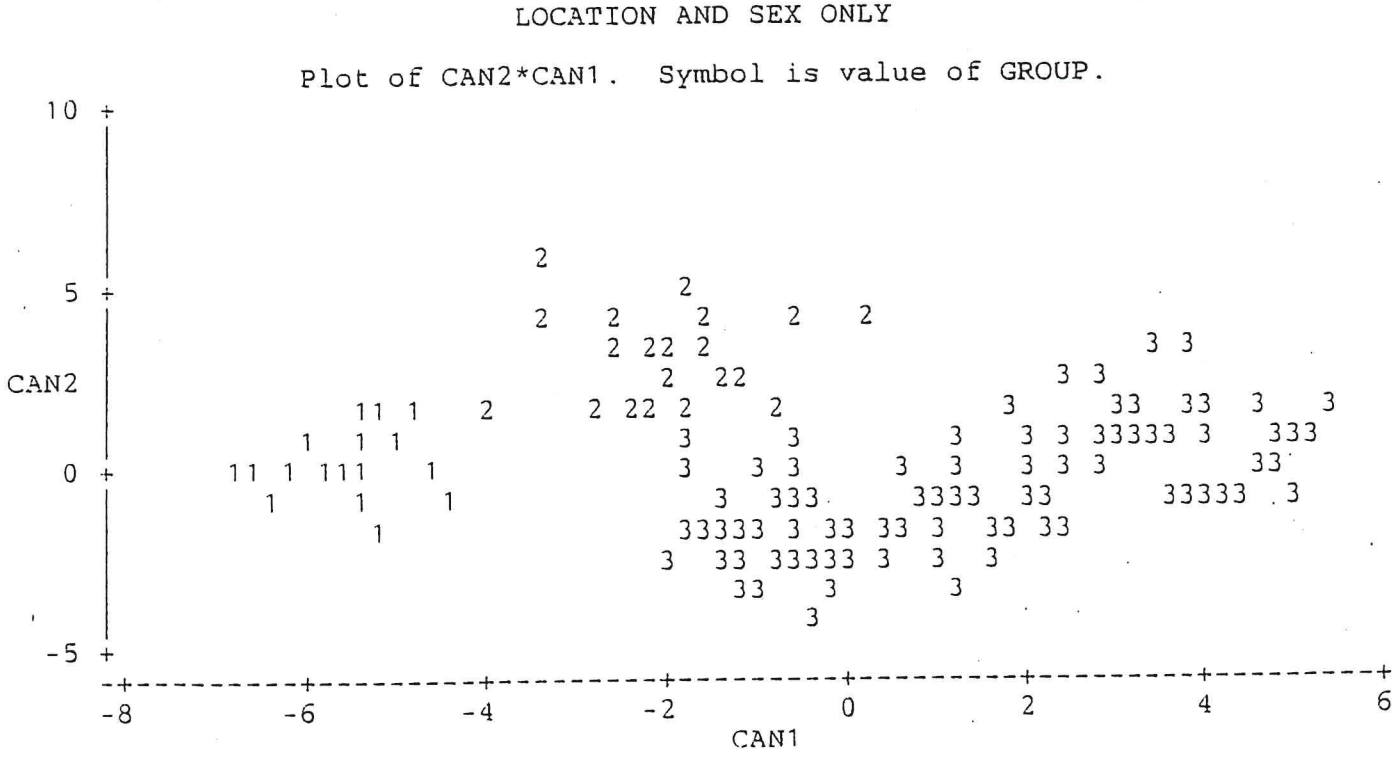
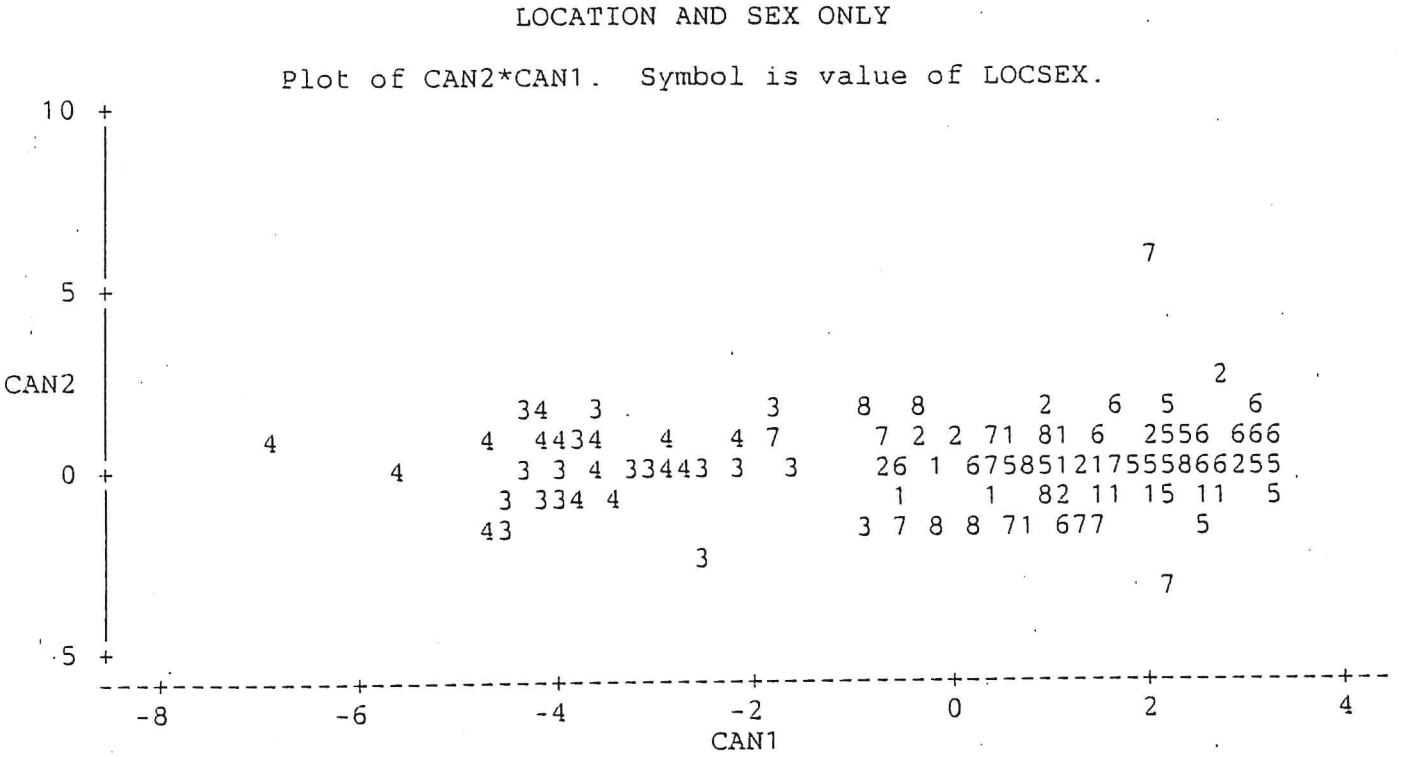
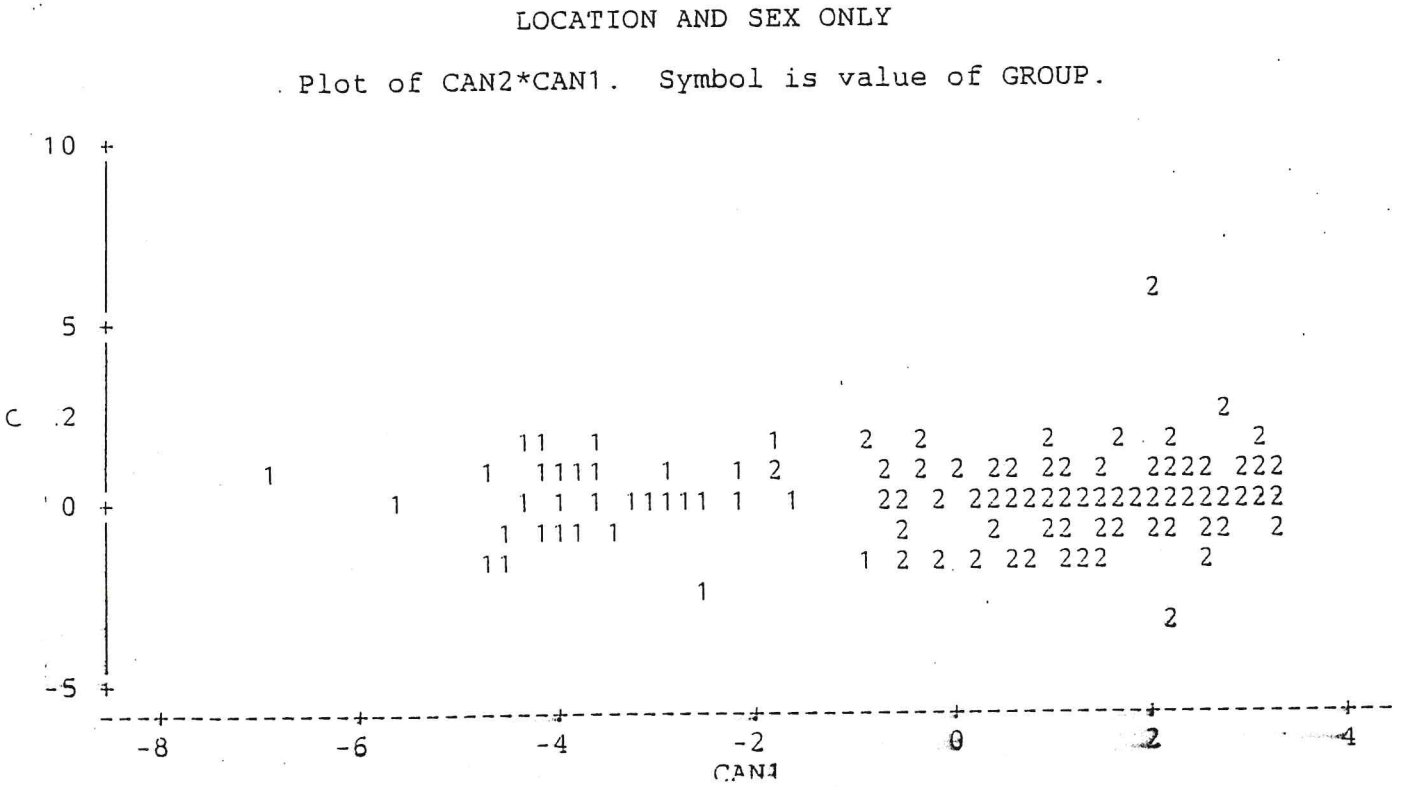


Figure 2.7 Plot representing the four different desert locust individuals along the two axes of canonical discriminant space for the two ratios (E/F, F/C). 1, 2= Red sea lab. males and females; 3, 4= south African males and females; 5, 6= Ethiopian field males and females; 7, 8= Red sea field males and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

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.

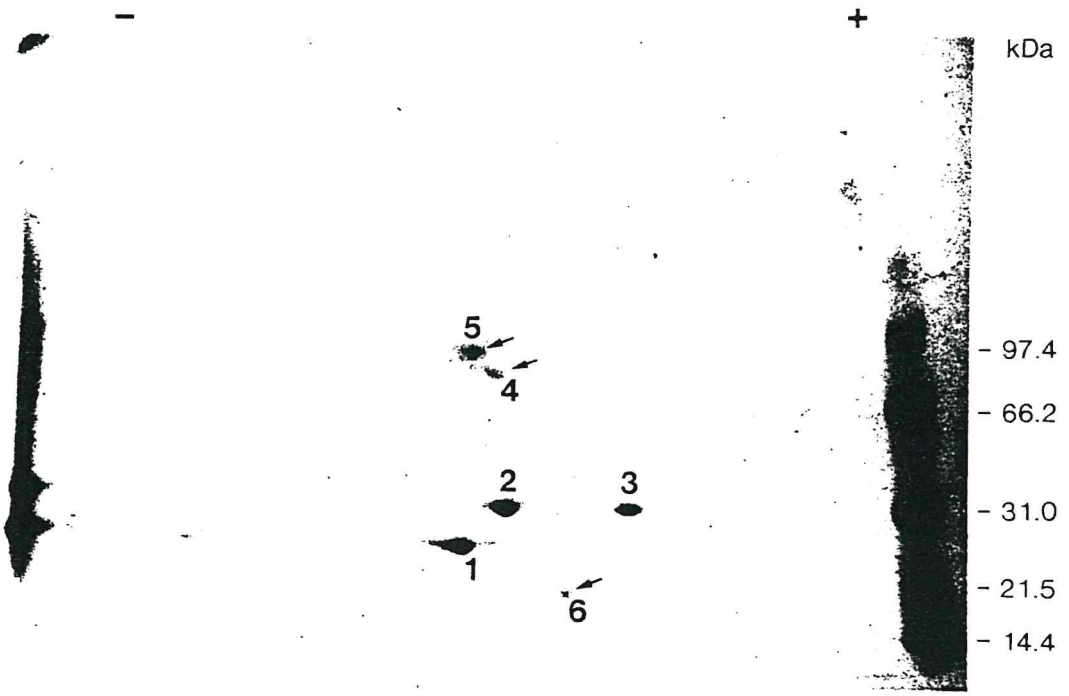
Figure 3.1 illustrates the resolution of the abdominal proteins of the northern males *S. g. gregaria*. The protein configuration was resolved into six major bands, while the configuration of the southern males *S. g. flaviventris* showed that, the abdominal protein was resolved into three major bands 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.

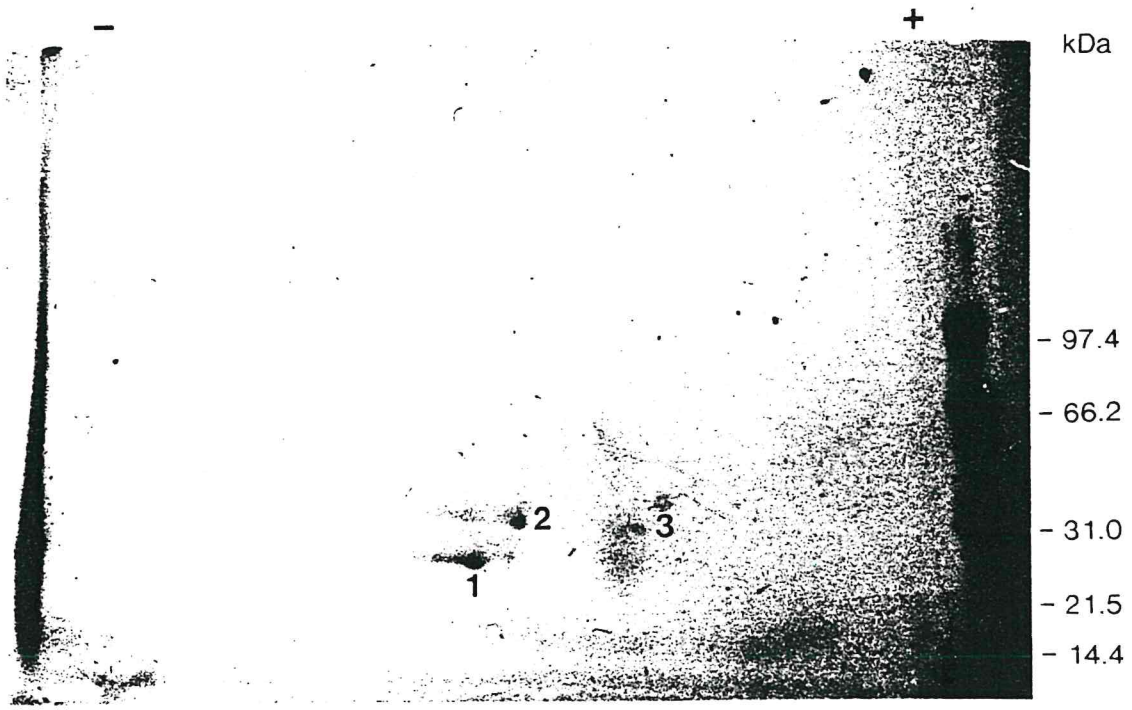
The major differences between the northern and southern abdominal protein patterens were bands number 4 and 5 at high molecular weight (~ 70-97.4 kDa).

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.



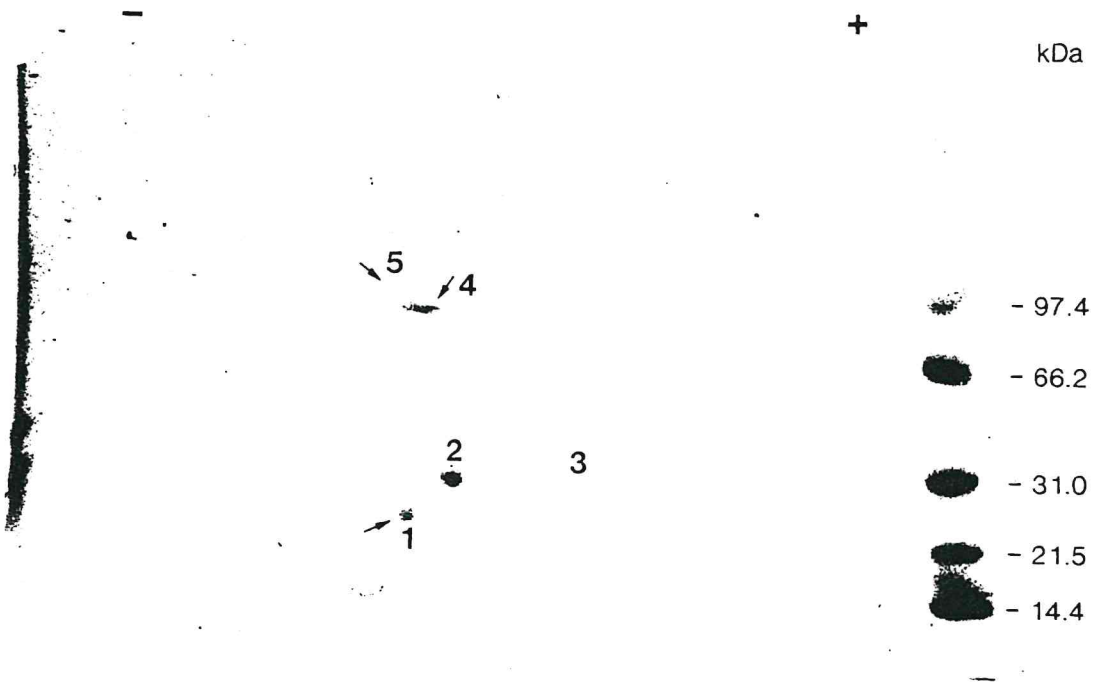
Arrows indicate the bands 4, 5 and 6 which are characteristic of the north African males.

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.



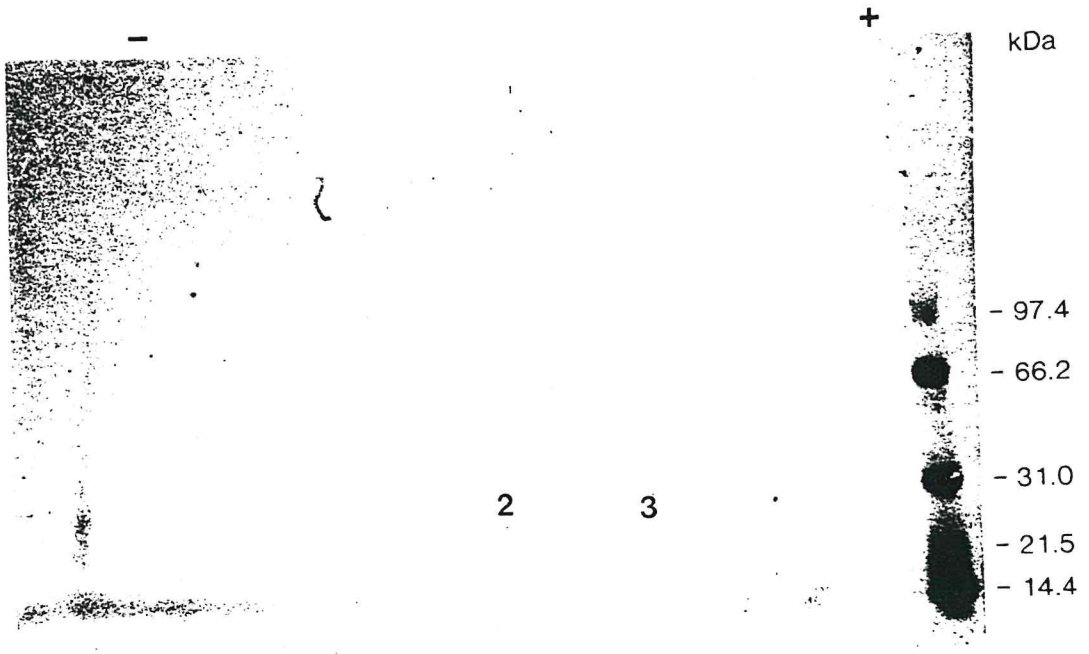
The bands 1, 2 and 3 appeared in the gel.

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).

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

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).

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° C for 5 minutes and then cycled 45 times using 94° C for 1 min., 33° C for 1 min. and 72° C for 2 min. A final extension was carried out at 72° C for 10 min. Upon completion of the amplification, samples were maintained at 4° 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.

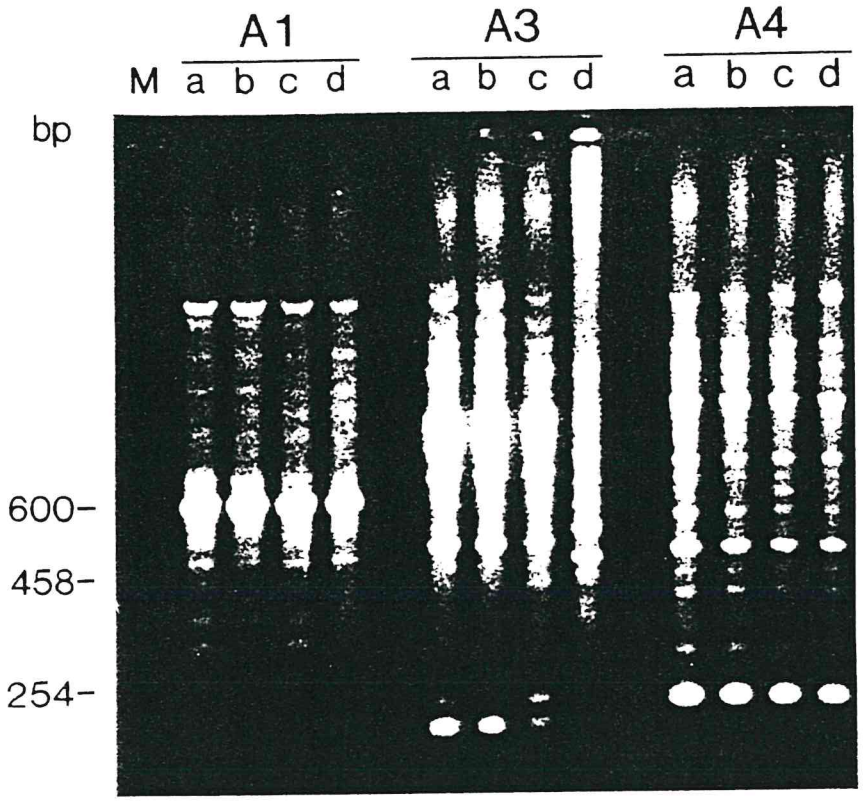
Amplification product with four primers A2, A12, A18, A20 (Fig. 4.6) produced monomorphic and polymorphic patterns with the pooled DNA samples. The fragments which differentiated the southern from northern locusts were scored at low molecular

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

KIT A

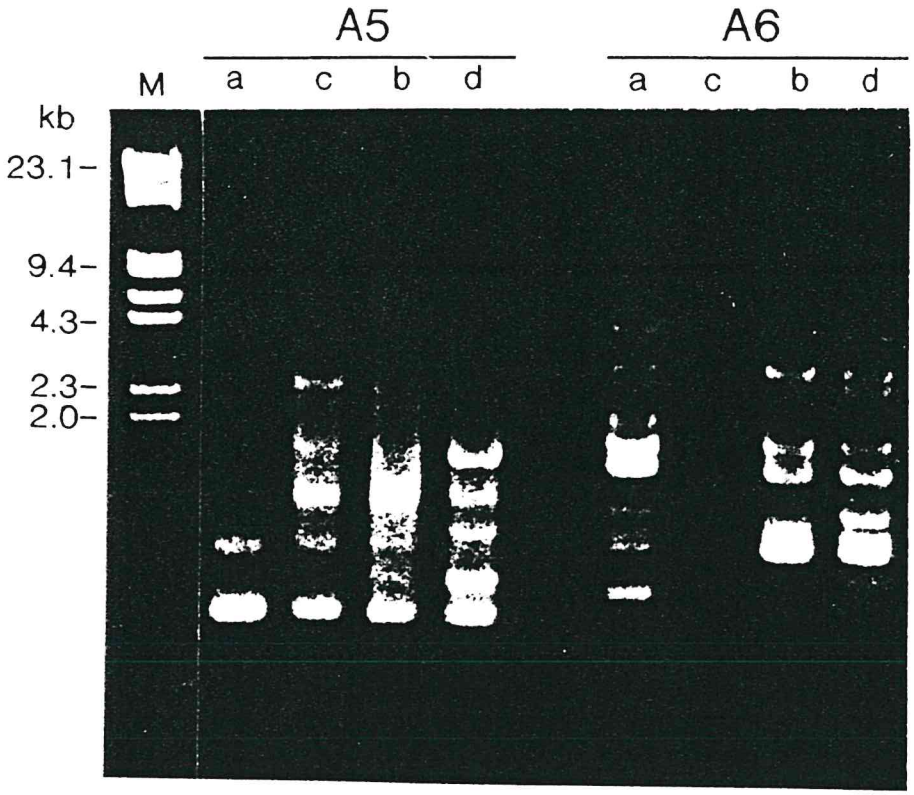
code	5' to 3'	M.W.	pmoles	ug/tube
OPA-01	CAGGCCCTTC	2955	6014	18.0
OPA-02	TGCCGAGCTG	3035	5493	16.5
OPA-03	AGTCAGCCAC	2988	5192	15.5
OPA-04	AATCGGGCTG	3059	5088	15.5
OPA-05	AGGGGTCTTG	3090	5192	16.0
OPA-06	GGTCCCTGAC	2995	5742	17.0
OPA-07	GAAACGGGTG	3108	4625	14.5
OPA-08	GTGACGTAGG	3099	4892	15.0
OPA-09	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).



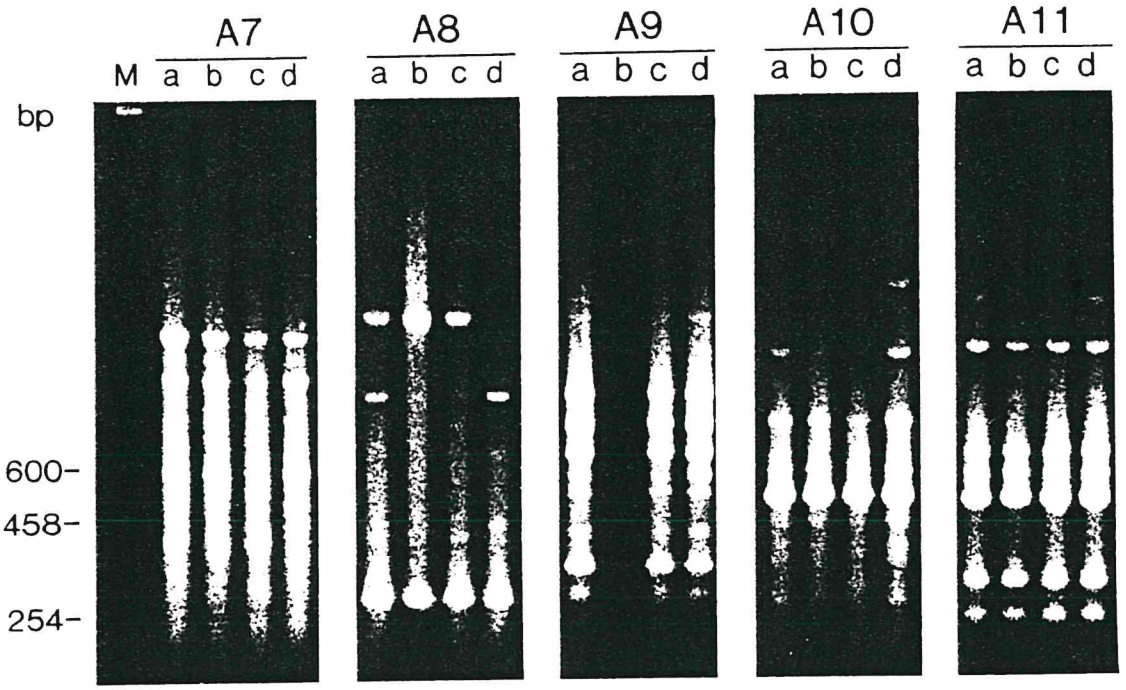
a= males *S. g. flaviventris;* b= males *S. g. gregaria;* c= females *S. g. flaviventris;* d= females *S. g. gregaria;* M= DNA size marker (Bluescript SK^+/ Hae III digest).

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



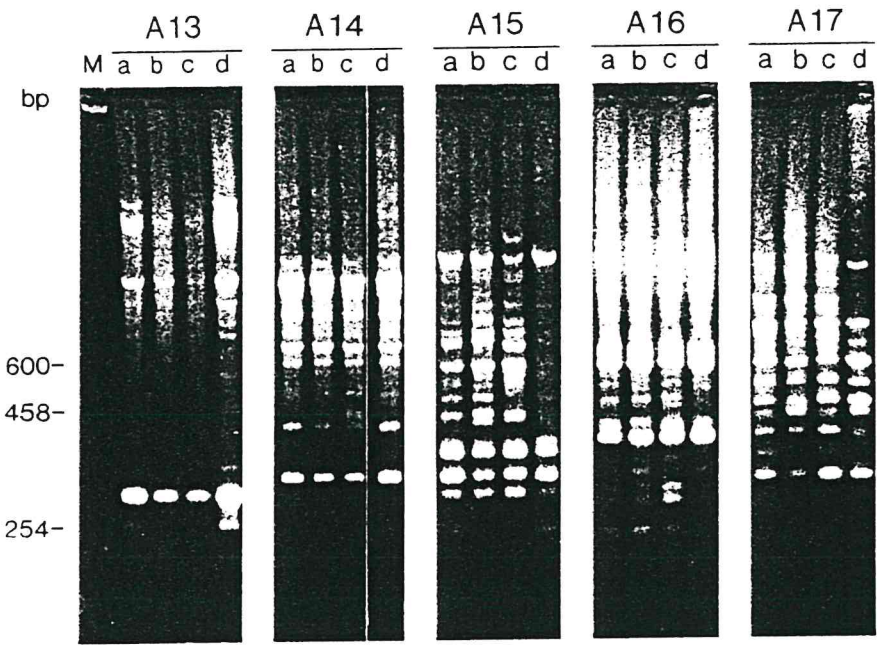
a= males *S. g. flaviventris;* b= males *S. g. gregaria;* c= females *S. g. flaviventris;* d= females *S. g. gregaria;* M= DNA size marker (Lamda DNA/ HindIII digest).

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



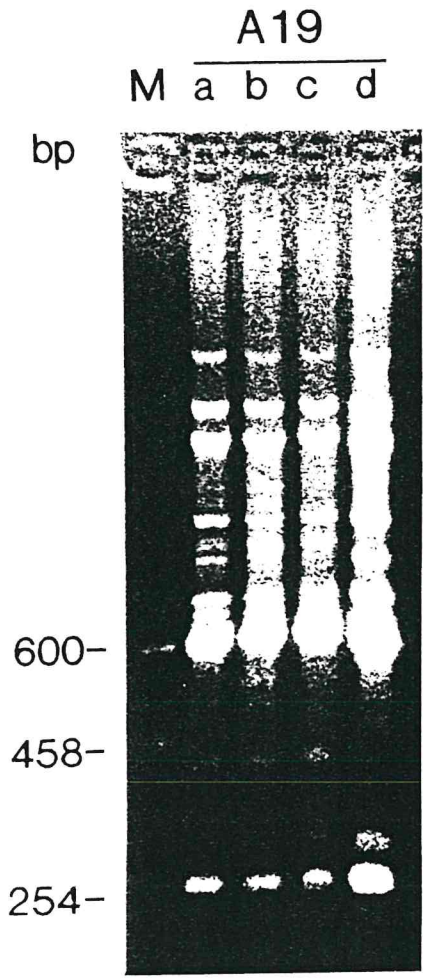
a= males *S. g. flaviventris*; b= males *S. g. gregaria*; c= females *S. g. flaviventris*; d= females *S. g. gregaria*; M= DNA size marker (Bluescript SK^+/ Hae III digest).

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



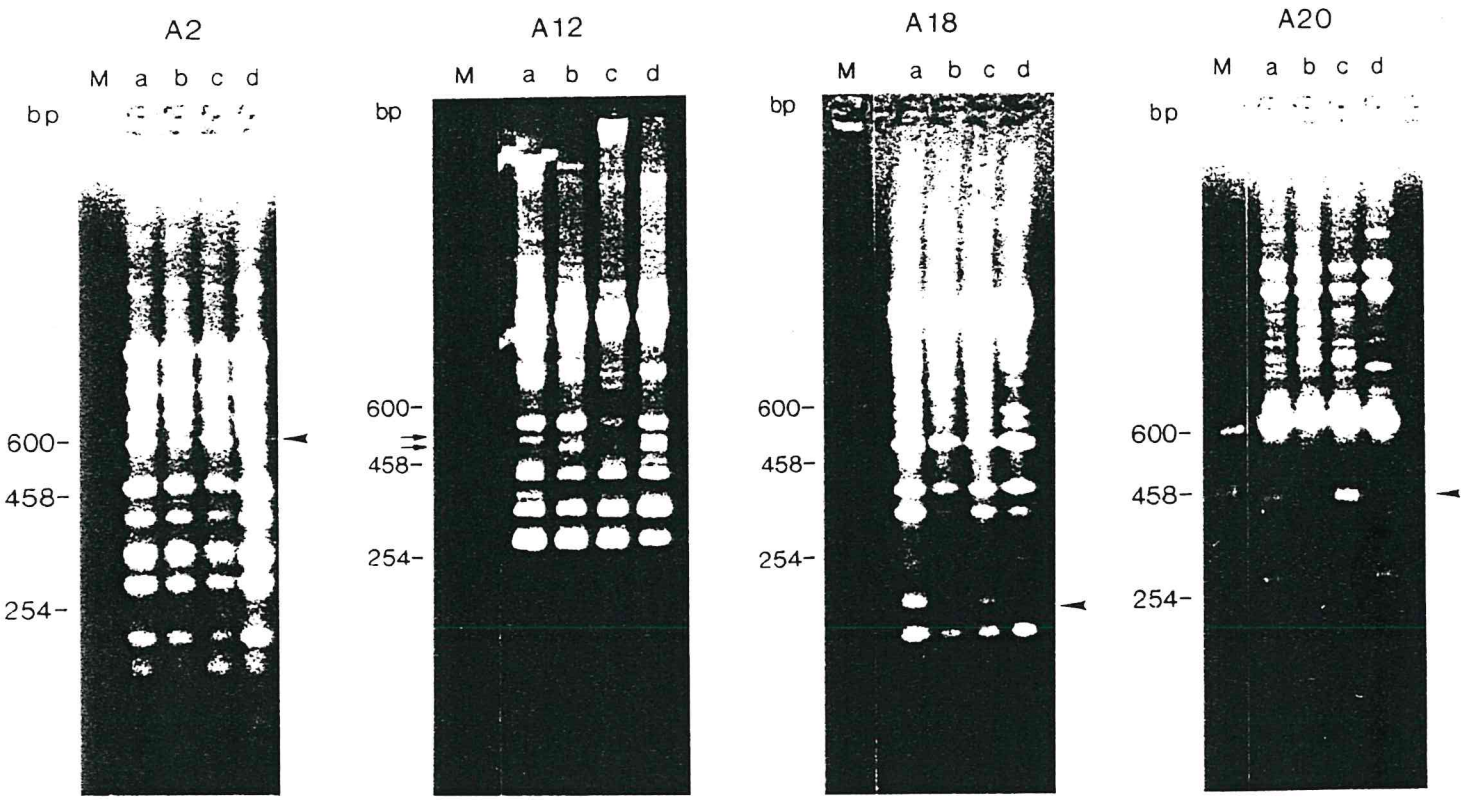
a= males *S. g. flaviventris;* b= males *S. g. gregaria;* c= females *S. g. flaviventris;* d= females *S. g. gregaria;* M= DNA size marker (Bluescript SK^+/ Hae III digest).

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.

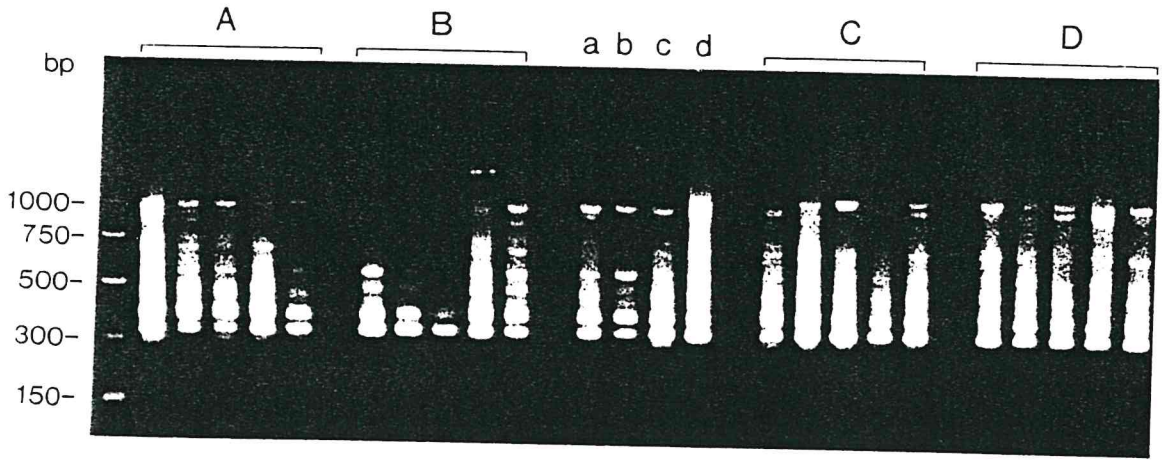
Primer A2 which produced diagnostic polymorphic patterns with the pooled samples (Fig. 4.10) was subjected to a second round with individuals (5 males and 5 females) and pooled samples from the two locusts. In the reaction, the amplified patterns were consistent and reproducible for any given DNA template.

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.



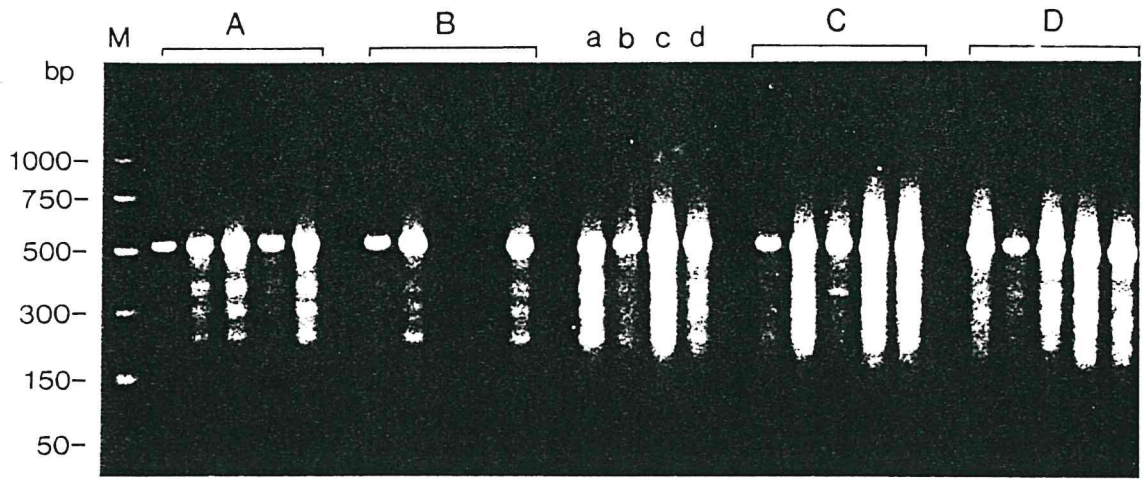
A,a= individuals and pools DNA of males *S. g. flaviventris;*

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;*

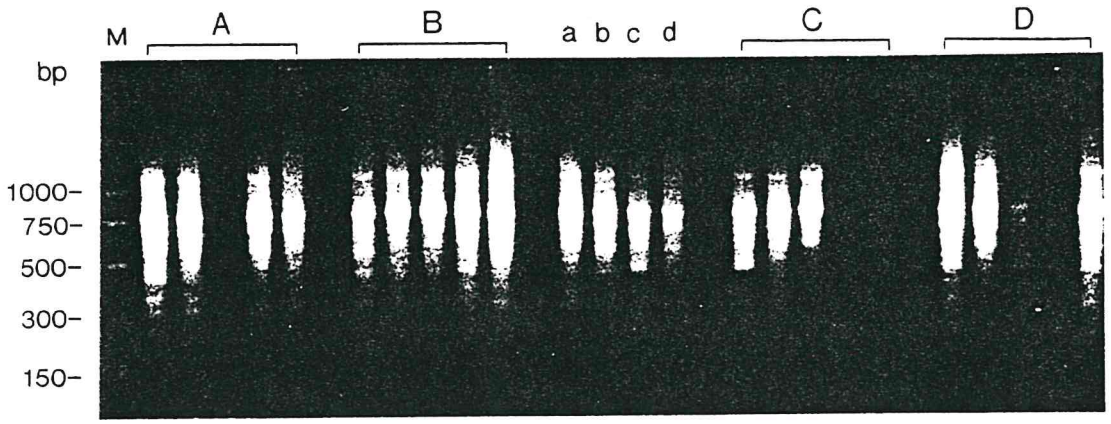
B,b= individuals and pools DNA of males *S. g. gregaria;*

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.



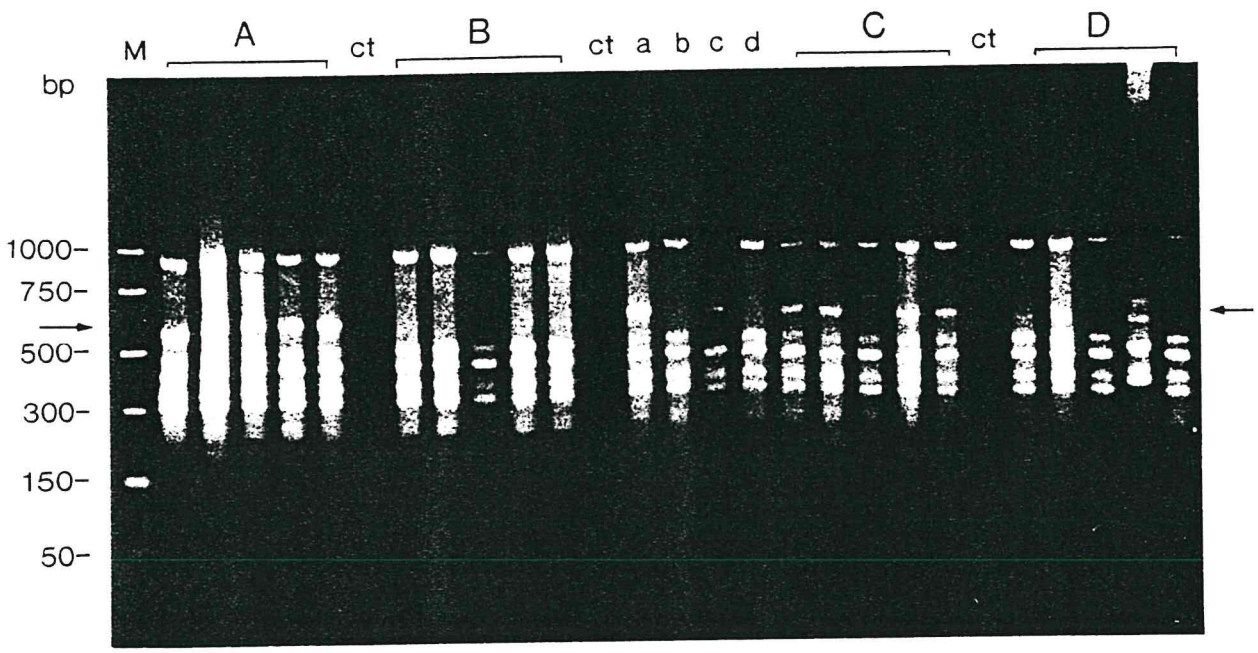
A,a= individuals and pools DNA of males *S. g. flaviventris;*

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.



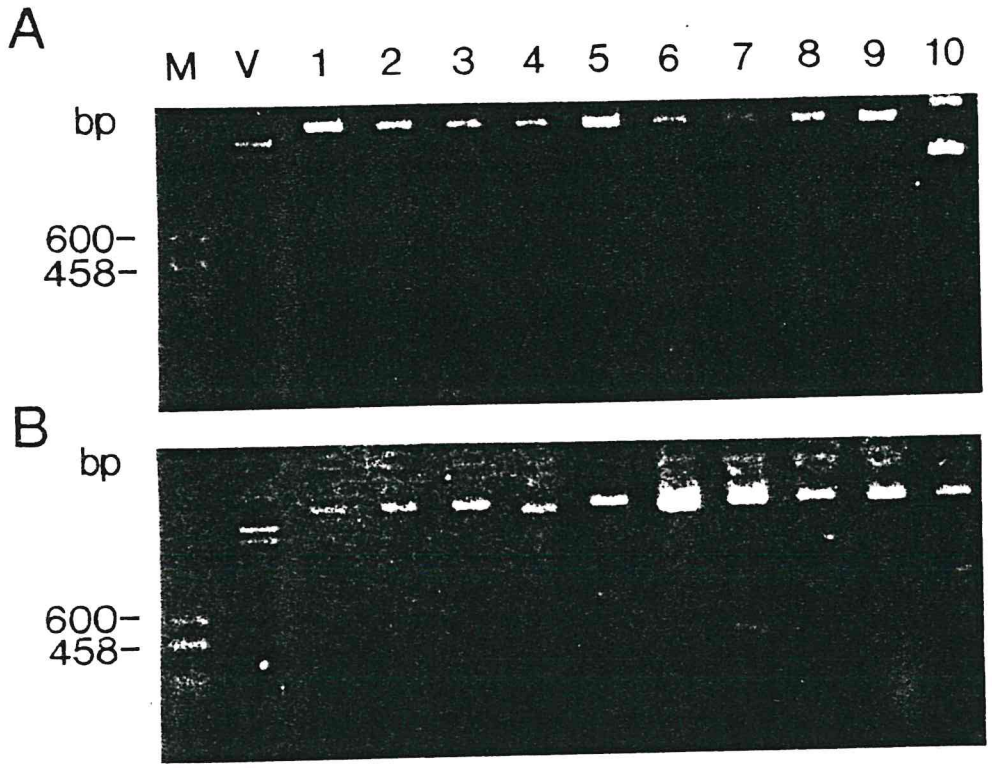
The arrow pointed to the 600 bp specific bands in the individuals and pools of the south locust, *S. g. flaviventris*. A,a= individuals and pools DNA of males *S. g. flaviventris;* B,b= individuals and pools DNA of males *S. g. gregaria;* C,c= individuals and pools DNA of females *S. g. flaviventris;* 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).