Chapter 8

CONCLUSION

Malaria infections are still being recorded in the malarious regions of South Africa, Northern Province, Mpumalanga and Kwazulu/Natal, where malaria control programmes have been in place since the 1950's. Two of the main African malaria vectors do not occur within these regions, namely *An. funestus* and *An. gambiae*. Other important species found are *An. arabiensis*, *An. quadriannulatus* and *An. merus* all belonging to the *An. gambiae* complex (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). *Anopheles arabiensis* is considered a major vector, but is found in very low numbers in these provinces. The other members of the *An. gambiae* complex are mainly zoophilic species, not considered to play a major role in malaria transmission in the region (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). *Anopheles vaneedeni* and *An. rivulorum* are found abundantly in these provinces. It has been shown that *An. vaneedeni* can be infected with *P. falciparum* under laboratory conditions (De Meillon *et al.*, 1977). *Anopheles rivulorum* was associated with malaria transmission in Tanzania during a study conducted by Wilkes *et al.* (1996).

8.1 MORPHOLOGICAL IDENTIFICATIONS

Transmission studies on members of the *An. funestus* group have been neglected owing to the difficulty in identifying individual members and the difficulty in colonizing and rearing these species under standard insectary conditions. Behavioural and biological studies on the group have also been neglected due to the above-mentioned reasons.

Morphologically identified *An. funestus* group specimens were collected in 1996 during the summer season and were sent to the SAIMR to determine if they were infected with *P. falciparum*. Results after ELISA analysis revealed a 0.18% infection rate in specimens collected at the end of 1996 and beginning of 1997. Specimens were preserved with desiccant and no species identification could be done at that stage. This motivated the need and urgency to develop a molecular-based method for rapid identification of members of the *An. funestus* group commonly found in South Africa.

The use of morphology to identify members of the *An. funestus* group is time consuming since the aquatic stages of the life cycle is long (21-41 days) and rearing larvae under insectary conditions is not very successful owing to high larval mortality. Egg morphology can be used to identify *An. leesoni*. Egg morphology can also be useful to identify *An. rivulorum* and *An. confusus*, but needs to be confirmed using larval morphology. The egg morphology of *An. brucei* and *An. fuscivenosus* remains undescribed (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).

Egg morphology cannot be used to identify *An. vaneedeni*, *An. funestus*, *An. aruni* and *An. parensis*. *Anopheles confusus*, *An. rivulorum* and *An. brucei* can be identified on larval morphology. *Anopheles vaneedeni*, *An. funestus*, *An. aruni* and *An. parensis* can be identified using adult characteristics. Adult *An. parensis* can be identified by the presence of 66 - 75% of specimens having a small patch of pale scales at the apex of the 6th vein. However, 25-30% of specimens belonging to this species cannot be distinguished from *An. funestus*. Male *An. parensis* are characterized by the presence of a small patch of pale scales at the base of the club on the palps in 90% of specimens studied.

Little is known about *An. fuscivenosus* but it can be identified by the absence of the costal sector pale spot and the pre-accessory dark spot on the 1st vein being broader than the accessory sector spot. Female *An. aruni* differs from *An. funestus* by the presence of a broader banded palps and pale wings. The male palps of *An. aruni* are characterized by the presence of a well-developed pale patch at the base of the club (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).

Anopheles vaneedeni can only be separated from *An. funestus* by the presence of pale scales on the tarsal joints and the presence of a few pale scales at the base of the club on the male palps (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). Morphological analysis completed for this study revealed that in certain *An. funestus* specimens pale scales were also found, but they were never as obvious as those *An. vaneedeni* also carrying this character. Male palps of *An. funestus* were always dark at the base of the club.

Apart from morphological identification it is also possible to identify members of the *An. funestus* group using banding patterns on the polytene chromosomes (Green and Hunt, 1980; Green, 1982). This method requires half gravid female mosquitoes where ovarian chromosomes are polytenized. Polytene chromosomes can be used to distinguish most species, but *An. vaneedeni* and *An. funestus* share homosequential banding patterns and are only distinguished by a floating inversion on arm two. This method of identification is far quicker than using morphological studies but only half gravid females can be used. Analysing these banding patterns is difficult and expertise is essential.

8.2 MOLECULAR IDENTIFICATIONS

The Polymerase Chain Reaction (PCR) method is a rapid and species-specific method used successfully for the identification of members of the *An. gambiae* complex (Scott *et al.*, 1993). This method uses species-specific primers to amplify a variable region of rDNA, resulting in different size products after amplification. Differences in fragment sizes are used to identify members of the group.

Variable regions were sequenced in the *An. funestus* group in order to investigate the possibility of designing species-specific primers. Initial sequencing results were compared against *An. gambiae s.s.* sequences available on the GenBank database to determine variation between two unrelated species. The ITS1 region was never sequenced owing to the presence of a secondary band after amplification.

Alignment with *An. funestus* COII region against that of *An. gambiae* revealed areas with low homology. Sequencing alignment between the COII regions of *An. vaneedeni* and *An. funestus*, revealed a 2.7% sequence difference. A high degree of homology decreases the possibility of designing species-specific primers, especially when other members of the group are incorporated.

The D3 variable domain in the 28S gene on the rDNA was also analysed. Alignment of the D3 region of *An. gambiae* against that of *An. funestus* (Figure3.6) revealed a high degree of homology, but the 5' end of the region showed less homology.

The D3 region of *An. vaneedeni* was sequenced and this information made it possible to

develop a RFLP assay able to distinguish *An. funestus* from *An. vaneedeni* (Koekemoer *et al.*, 1998). Specimens belonging to these two species were the only specimens available at that time. *Anopheles rivulorum* and *An. leesoni* were only collected the following summer. The RFLP assay failed to distinguish between *An. rivulorum* and *An. leesoni*.

The high degree of homology found by sequencing the D3 region of members of the *An. funestus* group can be explained by the hypothesis that they are recently evolved species. It is possible that because the D3 region is located within a gene it is under selective pressure and mutations are only viable if they do not interfere with gene function. In the different species the areas showing mutation differences are located in approximately the same region of the amplified fragment. This indicates that the region is less important for gene function and can therefore accumulate more mutations over time without affecting gene function.

The fact that these sequence differences between species occur at approximately the same sites of the amplified fragment eliminates the possibility of designing a multiplex PCR method (primers are added in the same reaction mixture to amplify different size fragments visualized after electrophoresis). Designing a PCR method for identification was possible, but would have needed at least two individual PCR reactions to distinguish the four species. This would require the setting up two PCR reactions to identify one unknown specimen. The incorporation of more species in the assay would probably require additional PCR reactions. This would increase the cost of routine identification of specimens making it unfavourable as a routine assay. This led to the search for a more economical method to identify members of the *An. funestus* group.

Single Strand Conformation Polymorphism (SSCP) is a technique developed for the detection of DNA sequence differences between DNA molecules (Orita *et al.*, 1989a). SSCP analysis was used to detect DNA differences in the amplified D3 region of individual species. Using this method, it was possible to identify four members of the *An. funestus* group (Koekemoer *et al.*, 1999). Samples from throughout Africa were analysed and species-specific banding patterns were observed after SSCP electrophoresis.

Polymorphic banding patterns observed between *An. funestus* populations from East and West Africa may be useful if the polymorphism can be used as a marker to distinguish the population. Future genetic studies would be needed to explain the presence of the polymorphism.

8.3 ELISA STUDIES BEFORE AND AFTER MOLECULAR IDENTIFICATION

Using the SSCP method, it was possible to identify the positive ELISA specimens that had been sent to the SAIMR by Mpumalanga Malaria Control Programme and identified morphologically by them as members of the *An. funestus* group. DNA extractions were performed on the remains of the legs or abdomens of the specimens. SSCP analysis revealed two different banding patterns compared to previous work. This suggested that they were different species. To establish species identification, morphological analysis was conducted on the remaining wings (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).

From wing analysis, together with the different SSCP banding patterns, some specimens were identified as belonging to the *An. marshallii* group. The other specimens were identified as *An.*

demeilloni. To confirm that the different SSCP banding patterns gave identification of the above-mentioned species, pre-identified specimens stored in nitrogen were also analysed on SSCP electrophoresis.

Neither *An. demeilloni* nor the *An. marshallii* group have ever been implicated as vectors in South Africa (Gillies and De Meillon, 1968). To confirm the presence of *P. falciparum* in these non-vector species, a *P. falciparum*-specific PCR assay was performed on the homogenized head and thorax used in the ELISA procedure (Snounou *et al.*, 1993). Most of the specimens analysed did not give any PCR results suggesting that the positive-ELISA results were false and not due to infection with *P. falciparum*. Future studies need to be conducted to determine the cause of false positive-ELISA results and to eliminate these problems in future transmission studies.

8.4 ISOENZYME STUDIES

Isoenzyme electrophoresis revealed nine potential enzyme systems that can be used in the identification of either *An. vaneedeni*, *An. funestus* or *An. rivulorum*. It suggested potential systems that can be used in population genetic studies in the future to determine the breeding structure of species of the *An. funestus* group. This will prove important considering that future malaria control may involve genetic manipulation of vector species. Predicting how refractory genes will move between different population within a species as well as between species is important.

8.5 CONCLUSIONS

The fact that it is now possible to identify at least four members of the *An. funestus* group will assist in transmission and behavioural studies of these species. Future studies need to be conducted to determine the vector status of other members of the *An. funestus* group. The biology of most members of the *An. funestus* group, excluding *An. funestus*, is understudied and behavioural studies of the other members of the group is essential to give insight into this species group. The method developed for species identification needs to be evaluated in various regions outside of the tested areas to confirm the reliability of the assay. To predict what banding patterns will appear using other members of the *An. funestus* group is impossible. Developing a PCR method such as that currently being used to identify members of the *An. gambiae* complex would be more successful. The SSCP method is more time consuming than a multiplex PCR since it involves PCR plus an additional SSCP electrophoresis step. The PCR-SSCP identification method is more expensive since it requires this additional step, the use of polyacrylamide solution and additional equipment. Future studies would need to sequence part of the IGS region where more species-specific variation may be present.

APPENDIX I

MORPHOLOGICAL DIFFERENCES IN THE *AN. FUNESTUS* GROUP

Schematic representation of morphological differences in the *An. funestus* group (Gillies and De Meillon, 1968 and Gillies and Coetzee, 1987)

I-A:

An. aruni adult morphological characteristics on female adult wing (a) and palp (b). c- represents the darker form of the female palp. d- club of male palp.

An. parensis adult wing (e) and female palp (f) with the club of male palp (g).

I-B:

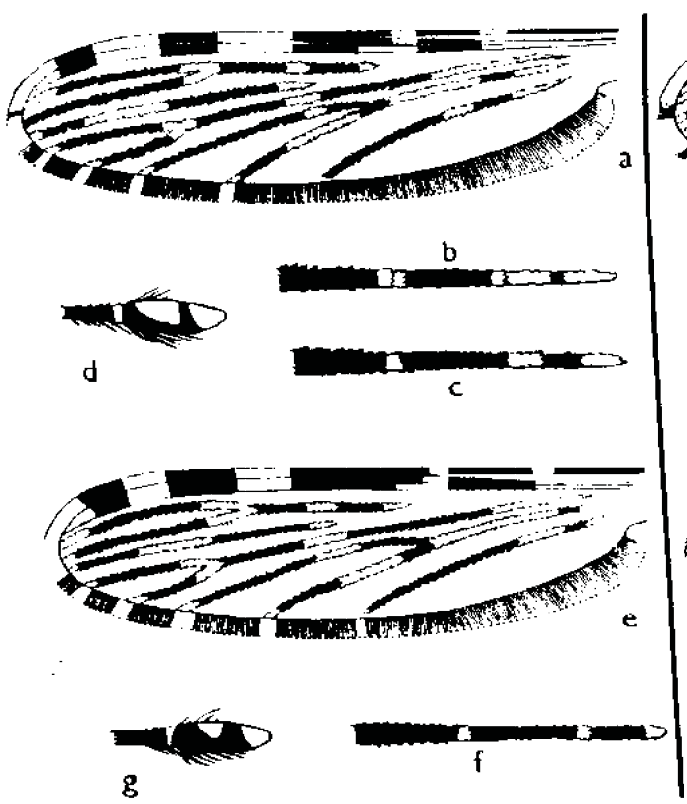
An. brucei depicting the female wing (a), palp (b) and pharynx, post-pharyngeal ridges (c).

An. fuscivenosus showing the female wing (d) and pharynx, post-pharyngeal ridges (e).

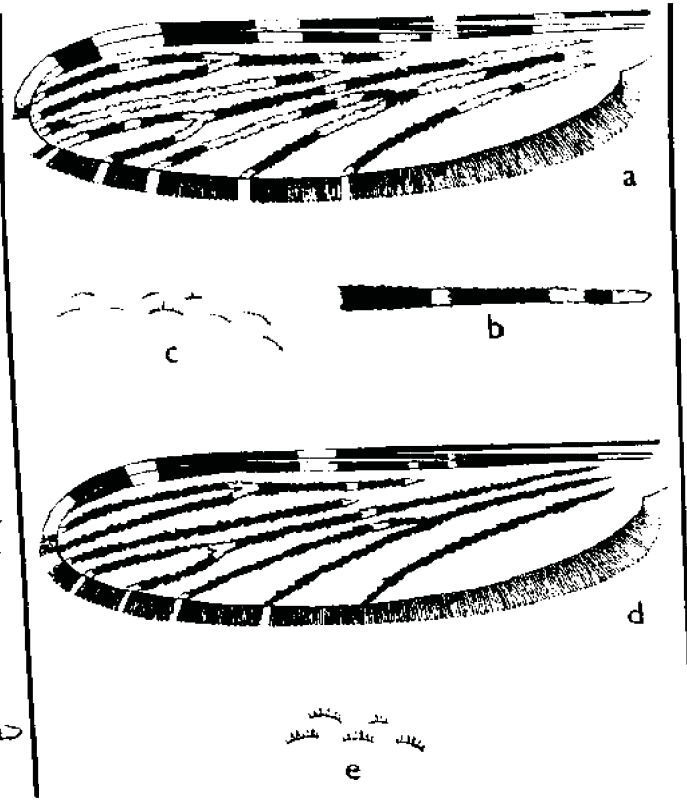
I-C:

Diagrammatic representation of the method use to estimate wing-tip ratio (a) and palpal band ratio (b) to distinguish *An. vaneedeni* from other members of the *An. funestus* group.

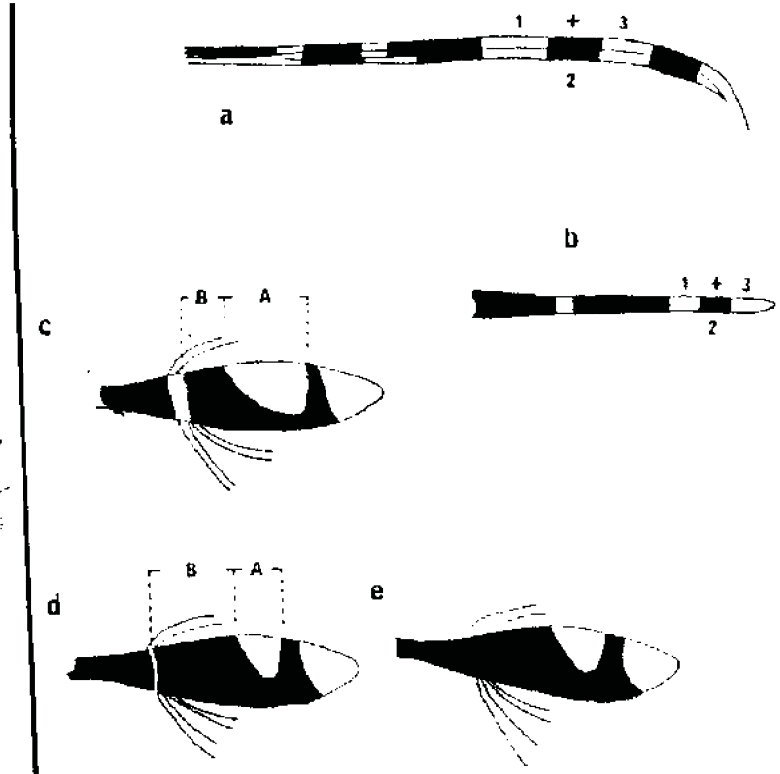
c- male palpal club in *An. aruni*, d- *An. vaneedeni* (5-10%) and e- *An. vaneedeni* (90-95%).



1A



1B



1C

II A :

Larval morphological differences of members of the *An. funestus* group. Clypeal patterns and abdominal plates of *An. funestus* subgroup (*An. vaneedeni, An. funestus, An. aruni and An. parensis*) (a,b), *An. confusus* (c,d), *An. leesoni* (e,f) and *An. brucei* and *An. rivulorum* (g,h).

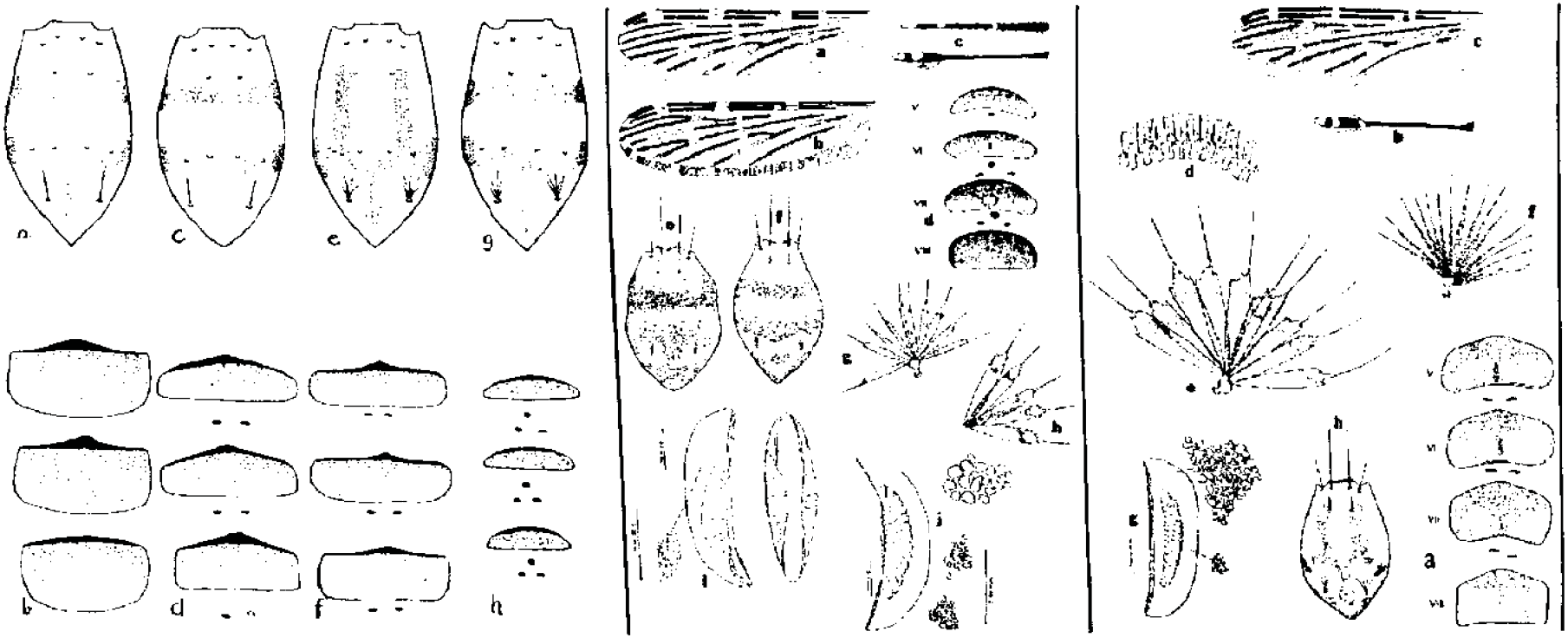
IIB:

Female *An. rivulorum* wings with two spot (a) and one spot (b) on vein 5a, female and male palps (c). d- Larval abdominal segments 5-8 of *An. rivulorum.* e, f - show the variation in pigmentation on the frontoclypeus, g, h- depicting the palmate hairs from the 1st and 5th abdominal segments respectively. I- morphology of *An. rivulorum* egg.

An. confusus egg are shown in j.

IIC:

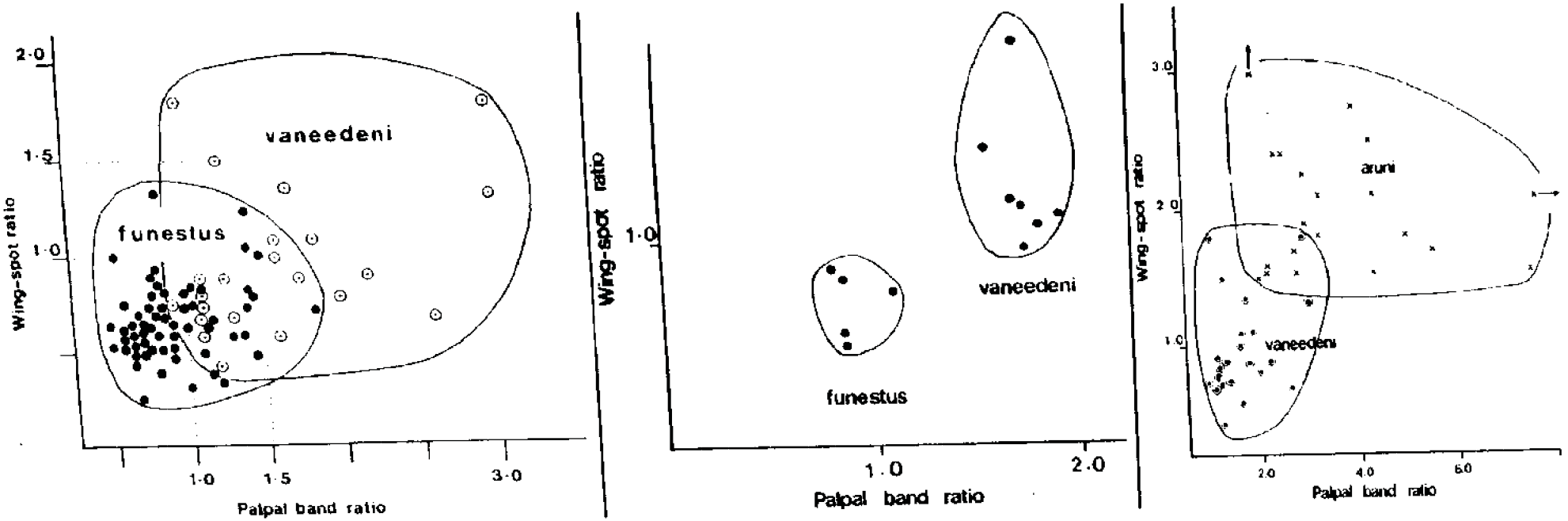
Morphological characteristics of *An. leesoni*. a- larva abdominal segments 5-8, b- male palp, c- female wing, d- female pharynx, e- larva, leaflets from the 5th abdominal palmate hair and f- larva, thoracic palmate hair. g- egg morphology and h- larva, fontoclypeus showing pattern.



IIA IIB IIC

III

Segregation of populations of *An. vaneedeni* and *An. funestus* (A) and segregation of *An. vaneedeni* and *An. aruni* by plotting wing-spot ratio against female palpal band ration. B depicts the segregation of individual females of *An. vaneedeni* and *An. funestus* by wing-spot ratio and female palpal band ratio.



IIIA IIIB IIIC

APPENDIX II

ALIGNMENTS OF SEQUENCED PCR PRODUCTS

PCR products were sequenced and aligned to confirm the correct region were amplified before continuing sequencing a larger sample. Sequence alignment after initial sequencing of PCR products, A-COII sequence from *An. funestus*, B- *An. vaneedeni* sequence from D3.

A

An funestus COII alignment results

High Probability

Sequences producing High-scoring Segment Pairs:		Score
gb|L20934|MSQMTCG	Anopheles gambiae mitochondrial geno...	1122
gb|L04272|MSQNCATR	Anopheles quadrimaculatus NADH dehyd...	1177
gb|L34351|CULCYTOXII	Culex quinquefasciatus mitochondrial...	985
gb|L14946|PKQMTPIEA	Phormia regina mitochondrial cytochr...	968
gb|U53265|ZEU53265	Zonosemata electa cytochrome oxidase...	823
gb|M95142|DROMTCOIIC	Drosophila lowei cytochrome oxidase ...	879

B

An. vaneedeni alignment D3 region:

Sequences producing significant alignments:		Score
b|L78065|MSQINSP	Anopheles albimanus ribosomal RNA gene.	256
gb|L22060|MQSRNAGN	Aedes albopictus 18S, 5.8S, and 28S ribos	254
emb|X93393|CPD3X528S	C.pipiens 28S rRNA gene, expansion	254
emb|X99212|CTRRNAS	C.tentans 18S, 5.8S & 28S rRNA genes	240
emb|X93392|CTD3X528S	C.tentans 28S rRNA gene, expansion segmen	240
emb|X80912|CT28SRR	C.thummi 28S rDNA gene	224

APPENDIX III

ISOENZYME PROTOCOL

Electrode buffers

A. Buffers.

1.Tris-citrate (TC) (pH 7.5) : Use 1M buffer and dilute 1:20 for preparation of gel solution. A dilution of 1: 40 is used as electrode buffer.

2.Tris-borate EDTA (TBE) (pH 8.9): Use 1:10 dilution for electrode buffer as well as in gel solution.

B. Gel preparation.

Wash glass plates with soap and rinse thoroughly with water, wipe plates dry and with 70% ethanol. Assemble glass plates according to manufactures instructions. Prepare the gel solution using 40% polyacrylamide solution (33:1). 10% of polyacrylamide gel solution are removed and add 200 µl 10% APS and 20 µl TEMED. As soon as base gel is set add 0.6ml (600 µl 10% APS and 60 µl TEMED, mix and pour. Insert the combs and remove air bubles. Leave 1hr or overnight to polymerize.

Pre-cool electrode buffer through use of the central cooling core.

Specimen preparation

Mosquito were homogenized in 30µl grinding buffer and kept on ice. Specimens not loaded directly on gel were kept at -70°C. 1-2µl of homogenate were loaded per well prior to electrophoresis. Gel was electrophoresed for 10-15 min at 100V, the remainder of electrophoresis was conducted at constant power of 50mA. Electrophoresis were generally halted once hb has migrated 2cm if TBE was used as electrode buffer or after 3 hours for TC electrode buffer.

Staining procedure

Staining chemicals were mixed with buffer and pour over gel after electrophoresis.

Following are the recipies used for electrophoresis as well as staining.

Grinding solution:

10% sucrose
1% Triton X- 100 detergent
1:3 diluton of tris-citrate tant buffer
Bromophenol blue (add enough for good colour)

TBE Buffer - 10X (Tris-Borate-EDTA, pH 8.9)

-- for 16 liters--

1572 g Tris; m.w. =121.1
280 g Boric acid; m.w.=62
89 g EDTA, tetrasodium; m.w.=380.2

TC Buffer 40X (Tris-Citrate, pH 7.1).

-- for 16 liters--

1467 g Tris; m.w.=121.1
751 g Citric acid; m.w.=210

STAINS

AAT (Aspartate Aminotransferase)
(Munstermunn, pers. comm., unpublished laboratory notes)

Stain 50ml:

Cysteine Sulphonic acid	50mg
∝-ketoglutaric acid	50mg
Glutamate dehydrogenase	32 units
NAD	20mg
NBT	20mg
2M Tris-cl	5ml

Incubate at 37°C, add 0.6ml PMS after 15 minutes.

ACP (Acid Phosphatase) E.C.3.1.3.2
(Steiner and Joslyn, 1979)

Stain 50ml:

Na+ a-Naptyl acid phosphate	25mg
0.1M MgCl	0.250ml
10%Manganese chloride	0.250ml
20% Sodium chloride	5ml
op: polyvinylpyrrolidone	50mg
Fast blue BB	50mg
1M acetate, pH5.0	2.5ml

Incubate at 4C for 20 min

AK (Adenylate Kinase) E.C.2.7.4.3
(Munstermunn, pers. comm., unpublished laboratory notes)

Stain 50ml

Glucose	100mg
0.1M MgCl	2.5ml
ADP	25mg
NADP+	12.5mg
Hexokinase	80ut
G-6-PDH	40ut
NBT	15mg
2M Tris-cl, pH7.1	1.25ml

Incubate 1\2 hr and add 2mg PMS

ALP (Alkaline Phoshatase) E.C.3.1.3.1
(Steiner and Joslyn, 1979)

Stain 50ml:

Na- a- Naphthyl acid phosphate	25mg
0.1M MgCl	1ml
0.1M Manganese chloride	2.5ml
polyvinylpyrrolidone	50mg
Fast Blue RR	50mg
2M Tris-Cl, pH 10.4	50ml

Soak gel 1hr in buffer, pour off. Add substrate, cofactors and dye with fresh buffer inc 3 hrs.

AO (Aldehyde Oxidase) E.C.1.2.3.1
(Steiner and Joslyn, 1979)

Stain 50ml:

Benzaldehyde	0.5ml
NBT	5mg
2M Tris-Cl pH 7.4	1.25ml

Incubate 1\2hr, add 2mg PMS

DIA (Diaphorase) E.C.1.6.2.2
(Harris and Hopkinson, 1976)

Stain 50ml:

NADH	12.5 mg
2,6-Dichlorophenol indophenol (DCIP)	5mg
NBT	10mg
2M Tris-Cl pH 8.5	0.7ml

EST (Esteras)
(Steiner and Joslyn, 1979)

Stain: 50ml

NaPO4 (monobasic)	0.66g
NaPO4 (Dibasic)	0.27g
dist. water	50ml
∝-Naphtyl acetate in acetone	4ml
Fast Blue RR (FBRR)	0.05g

Incubate and add FBRR after 15min

G6PDH (Glucose-6-phosphate Dehydrogenase)
EC.1.1.1.49 (Steiner and Joslyn, 1979)
Stain 50ml:

Glucose-6-phosphate	25mg
NADP	5mg
NBT	10mg
2M Tris-Cl pH 8	1.25

Incubate 1\2hr add 2mg PMS

GAPD (Glyceraldehyde-3-phosphate Dehyd'se)
E.C. 1.2.1.12 (Steiner and Joslyn, 1979)
Stain 50ml:

Na fructose-1,6-diphosphate	200mg
Sodium arsenate	50mg
NAD	10mg
Sodium pyruvate	50mg
Aldolase	50ut
NBT	7.5mg
2M Tris-Cl pH 7.5	1.25ml

Mix aldolase and substrate (Na-Fructose-1,6-diphosphate) in 20ml of buffer,
incubate 1hr.
Mix with co-factors and dye apply to gel.
Inc. 1\2 hr and add 2mg PMS

GCD (Glycerol Dehydrogenase) E.C.1.1.1.72 (Modified Harris and Hopkinson, 1976)
Stain: 50ml

Glycerol	5ml
NADP	7.5mg
0.1M MgCL	2.5ml
NBT	7.5mg
2M Tris-Cl pH 7.5	2.5ml

Add 2mg PMS.

GPI (Glucosephosphate Isomerase)
E.C .5.3.1.9 (Shaw and Prasad, 1970)
Sain: 5ml

Fructose-6-phosphate	5mg
0.1M MgCl	2.5ml
NADP	5mg
G-6-PDH	10ut
NBT	7.5mg
2M Tris-Cl pH 8.0	1.25ml

1\2 hr incubation add 2mg PMS

GPD (glycerol-3-phosphate dehydrogenase)
E.C.1.1.1.8 (Ayala *et al.*, 1972)
Stain 50ml:

1M alpha,beta - Glycerophosphate	2.5ml
NAD	7.5mg
NBT	7.5mg
2M Tris-Cl pH8.5	2.5ml

Inc. 1\2 hr add 2mg PMS.

GDH (glucose dehydrogenase)
E.C.1.1.1.47 (Harris and Hopkinson, 1976
Stain 50ml:

D- Glucose	9g
NAD	20mg
NBT	5mg
PMS	5mg
2M Phosphate buffer	1.25ml

GOT (Glutamate-oxaloacetate transaminase) E.C. 2.6.1.1 (Harris and Hopkinson, 1976

L- Asparic acid (100mg/ml)	1ml
α-ketoglutaric acid (100mg/ml)	1ml
Pyricoxal-5'-Phosphate	10mg
Fast Blue RR	50mg
2M Tris-Cl pH8	5ml

Add Fast Blue RR after 30 min incubation

GSR (Glutathione reductase)
E.C. 1.6.4.2.
(Harris and Hopkinson, 1976

Oxidase glutathione	20mg
NADPH	5mg
2.6-dichlorophenol-indophenol	1mg
NBT	5mg
2M Tris-Cl pH 8.4	1.125ml

HAD(Hydroxyacid dehydrogenase)
E.C. 1.1.1.30 (Shaw and Prasad, 1970)
Stain 50ml:

Gluconic acid	50mg
0.1M MgCl	0.250ml
NAD	15mg
NBT	10mg
2M Tris-Cl pH 8.0	1.25ml

Inc. 1\2 hr. add2mg PMS.

HK (Hexokinase) E.C. 2.7.1.1
(Tabachnick, 1978)
Stain: 50ml

Fructose	450mg
Glucose	450mg
0.1M MgCl	1.25ml
NADP	12.5mg
ATP	25mg
G-6-PDH	20ut
NBT	10mg
2M Tris-Cl pH 7.1	1.25ml

Inc. 1\2 hr add 2mg PMS

IDH (Isocitrate Dehydrogenase)
E.C.1.1.1.42 (Harris and Hopkinson, 1976)
Stain 50ml:

0.06M Na Isocitrate	0.5ml
0.1 M MgCl	2.5ml
NADP	7.5mg
NBT	7.5mg
2M Tris-Cl pH8.5	1.25ml

Inc. 1\2 hr add 2mg PMS

LAP (Leucine Aminopeptidase) E.C.3.4.11
(Steiner and Joslyn, 1979)
Stain 50ml:

L-Leucyl-b- napthylamide HCL	10mg
Black K salt	25mg
2 M Tris-Cl pH5.2	5ml

Inc. 1 hr in 0.5M boric acid. Rinse, inc. with substr 1hr add fresh buffer with dye

LDH (Lactate Dehydrogenase) E.C.1.1.1.27
(Shaw and Prasad, 1970)
Stain 50ml:

1M Na Lactate, pH 7.0	5ml
NAD	7.5mg
NBT	12.5mg
2M Tris-Cl pH 8.5	2.5 ml

Inc. 1hr add 2mg PMS.

MPI (Monophosphate Isomerase)
E.C. 5.3.1.8 (modified from Harris and Hopkinson, 1976)
Stain: 30ml

NADP	14mg
NBT	7mg
Mannose-6-phosphate	14mg
Magnesium chloride	28mg
Glucose phosphate isomerase	8mg
Glucose-6-Phosphate dehydrogenase	18U
2M Tris-cl pH 7.5	3ml
Dist. water	27ml

Add 0.6ml PMS and incubate at 37°C

MDH (Malate Dehydrogenase)
E.C. 1.1.1.37 (Shaw and Prasad, 1970)
Stain 50ml:

1M Na- Malate pH7	0.5ml
NAD	7.5mg
NBT	12.5mg
2M Tris-Cl pH 8.5	2.5ml

Inc 1\2 hr add 2mg PMS .

ME(Malate Dehydrogenase)
E.C.1.1.1.40 (Ayala et *al.*, 1972)
Stain: 50ml

1M Na malate pH 7.0	0.250ml
0.1M MgCl	1.25ml
NBT	10mg
2M Tris-Cl pH 8.5	1.25ml
NADP	10mg

Inc. 1\2hr add 2mg PMS

ODH (Octanol Dehydrogenase)
E.C. 1.1.1.73 (Mahon *et al.*, 1976)
Stain: 50ml

NAD	12.5 mg
NBT	10mg
PMS	2mg
2M Tris-Cl pH 8.5	1.25ml
Ethanol	0.5ml
Octan-2-ol	0.1ml

Inc. for 2hrs add more PMS if needed

PGD (6-phosphogluconate Dehydrogenase)
E.C.1.1.1.43 (Shaw and Prasad, 1970)
Stain: 50ml

Na 6-phosphogluconate	5mg
0.1M MgCl	2.5ml
NADP	2.5mg
NBT	5mg
2M Tris-Cl pH 8.0	1.25ml

Inc 1\2hr add 2mg PMS,

PGM (Phosphoglucomutase)
E.C. 2.7.5.1 (Shaw and Prasad, 1970)
Stain: 50ml

Na-glucose-1-phosphate	40mg
0.1M MgCl	2.5ml
NADP	5mg
G-6-PDH	20ut
NBT	10mg
2M Tris-Cl pH 7.1	1.25ml

After 1\2 hr add 2mg PMS

TREHALASE
E.C.3.2.1.2.8 (Meredith, 1980)
Stain 50ml:

Trehalose dihydrate	200mg
0.1 M MgCl	2.5ml
ATP	10mg
NADP	10mg
G6-PDH	20ut
NBT	10mg
2M Tris-Cl pH 7.1	1.25ml
Hexokinase	80ut

Inc. 1\2 hr add 2mg PMS

XDH (Xanthine Dehydrogenase)
E.C. 1.2.1.37 (Steiner and joslyn, 1979)
Stain: 50ml

NAD	20mg
NBT	12mg
PMS	1mg
Hypoxanthine	20mg
10% Potassium hydroxide	10 drops

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