Table 4. Frequency data for four enzyme systems studied.

Species.	Non-specific Esterase: Est. 1					
	1	2	3	4	5	6
	110	105	100	95	90	85
coustani A	0.04	0.12	0.45	0.27	0.11	0.01
coustani B	0.04	0.17	0.33	0.38	0.08	
tenebrosus		0.09	0.70	0.21		
ziemanni A	0.09	0.33	0.24	0.26	0.07	0.01
ziemanni B		0.09	0.29	0.24	0.27	0.11

Species.	Non-specific Esterase: Est. 2						
	7	8	9	10	11	12	13
	115	110	105	100	95	90	85
coustani A			0.07	0.42	0.40	0.11	
coustani B	0.30	0.70					
tenebrosus	-	-	-	-	-	-	-
ziemanni A				0.19	0.54	0.16	0.01
ziemanni B			0.05	0.13	0.55	0.23	0.04

Species.	Non-specific Esterase: Est. 3				
	14	15	16	17	18
	105	100	95	90	85
coustani A	0.18	0.52	0.28	0.02	
coustani B		0.92		0.08	
tenebrosus		0.05	0.77	0.14	0.04
ziemanni A		0.12	0.46	0.32	0.10
ziemanni B	0.06	0.47	0.44	0.02	0.01

Species.	Octanol dehydrogenase: Odh.		
	19	20	21
	100	95	90
coustani A		0.99	0.01
coustani B		1.00	
tenebrosus	0.04	0.86	0.10
ziemanni A		0.74	0.26
ziemanni B		0.84	0.16

Species.	Superoxide dismutase: Sod.				
	22	23	24	25	26
	105	100	95	90	85
coustani A			0.90	0.09	0.01
coustani B		0.70	0.30		
tenebrosus	1.00				
ziemanni A			0.95	0.05	
ziemanni B			1.00		

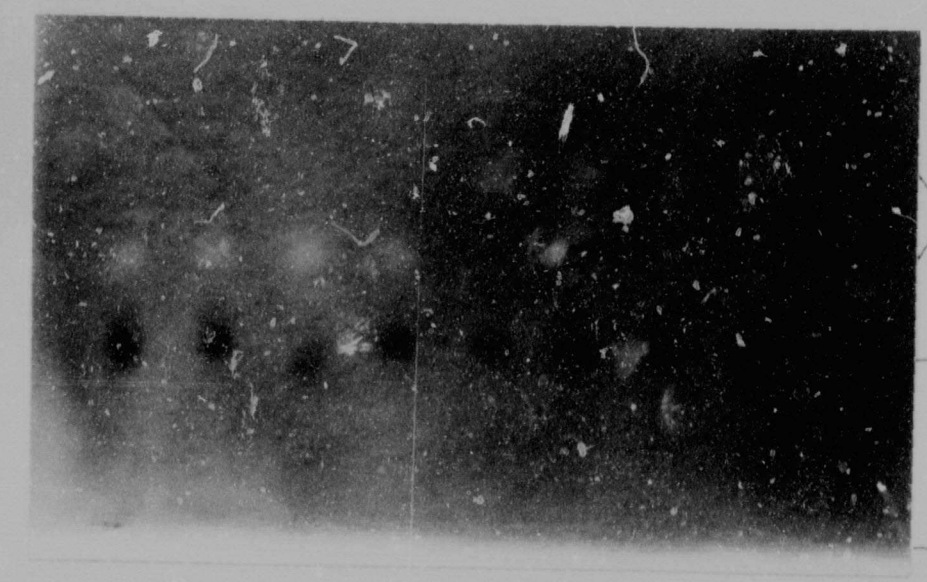
Species.	Glutamic-oxaloacetic transaminase: Got.				
	27	28	29	30	31
	110	105	100	95	90
coustani A	0.02	0.12	0.62	0.22	0.02
coustani B			1.00		
tenebrosus		0.14	0.43	0.43	
ziemanni A		0.21	0.61	0.18	
ziemanni B		0.15	0.31	0.44	0.10

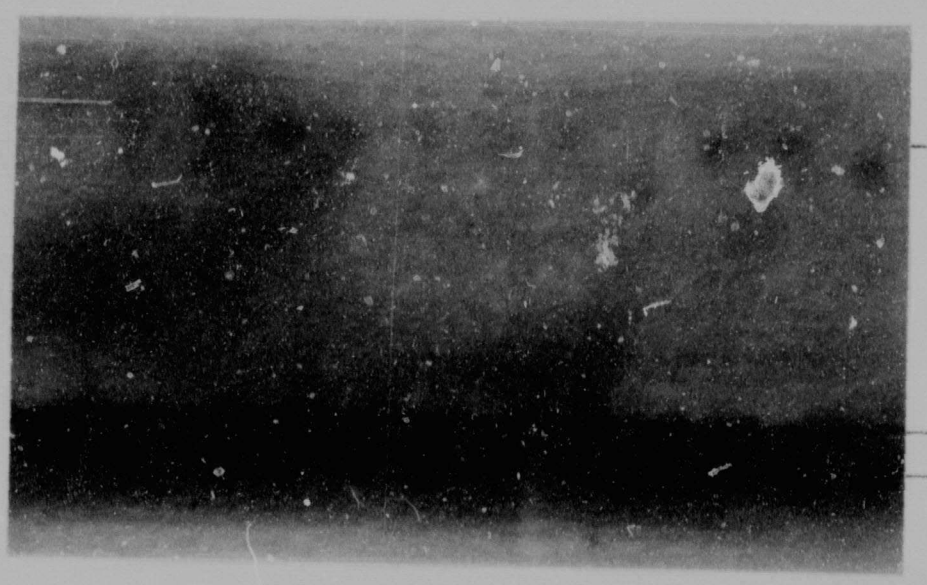
Sod are unique to tenebrosus. It also seems that the length of time the gel is allowed to run is crucial for the detection of the fast Est-2 bands in coustani B. In one gel run of 7.5cm, these bands were obscured by the darker Est-1 bands.

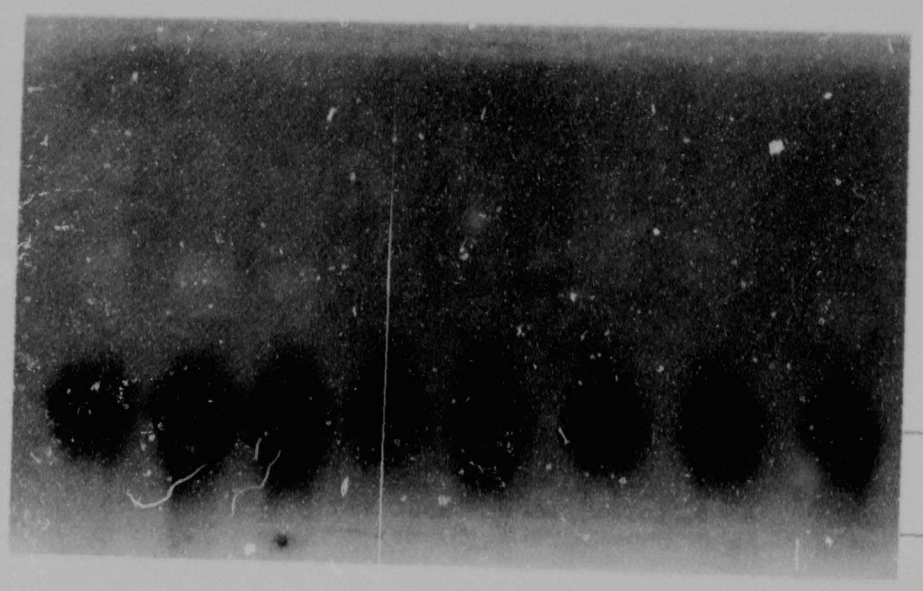
The separation of the ziemanni sibling species would depend on the relative frequencies of certain alleles in the populations. For this, Est-3 and Got show promise. A study of enzyme systems not tested here might reveal species-specific allozymes.

This study employed a standard matrix with a constant pH and, due to the lack of time and material, no attempts were made to vary the techniques. However, it has been shown that identical mobility in a given gel does not necessarily mean that single enzymes are involved. For example, Johnson (1976) showed that by varying the pore size of polyacrylamide gels, the α-glycerophosphate dehydrogenase locus of Colias butterflies contained at least five alleles rather than the two previously reported. Heat denaturation studies (eg. Singh et al, 1975, Cochrane, 1976) and iso-electric focusing (Milkman & Koehler, 1976) have also revealed variation within different loci.

Fig. 11. Examples of some other enzyme systems tested.


Sod
Odh
origin


Got 1
origin
Got 2


∝ Gpdh
origin

CHAPTER FOUR

THE MORPHOLOGICAL ANALYSIS

4.1 Introduction

The classification and naming of things is an absolute necessity for sensible discussion. While the classification of the higher orders is to some extent arbitrary, it is widely accepted that genetical species are real biological entities. Until recently, these classifications were based on morphological characters only, though now it has been shown that mating and behavioural differences are important species-specific characters (eg. Paterson, 1962).

With the application of genetical analysis to anophelines, species complexes have been revealed in many of the taxa (Paterson, 1964, Green, 1972, Lambert, 1979). This calls for a complete revision of the morphological data on these groups. With regard to the A.gambiae complex, which includes important vectors of plasmodia in Africa, many morphological studies have been carried out (eg. Coluzzi, 1964, Reid, 1973, 1975a, 1975b, Bryan, 1980). Unfortunately, the authors used either laboratory

colony material or restricted themselves to one locality and so none have proved generally useful. Not all members of a sibling species complex may be vectors and the need for easy identification of species of medical importance is obvious. This identification can only be achieved by the correlation of detailed morphological studies with those of genetical analysis.

Appendix III gives a short summary of my study done on the immature stages of the original three species of the coustani group (Coetzee & Newberry, 1980). These data were used as a basis for both the pupal and larval studies and adapted to accommodate the sibling species.

Appendix I gives the detailed collection records of the families used in the present study.

4.2 Adult identification

4.2.1 Methods

The adults in excellent condition were glued to small triangles of stiff paper held on insect pins. The pharynx and genitalia of certain specimens were cleared in 10% potassium hydroxide and mounted in Canada Balsam. The details of collecting and rearing methods are given in chapter two (2.2.1).

Measurements of certain wing, palp and leg spots (fig. 12)

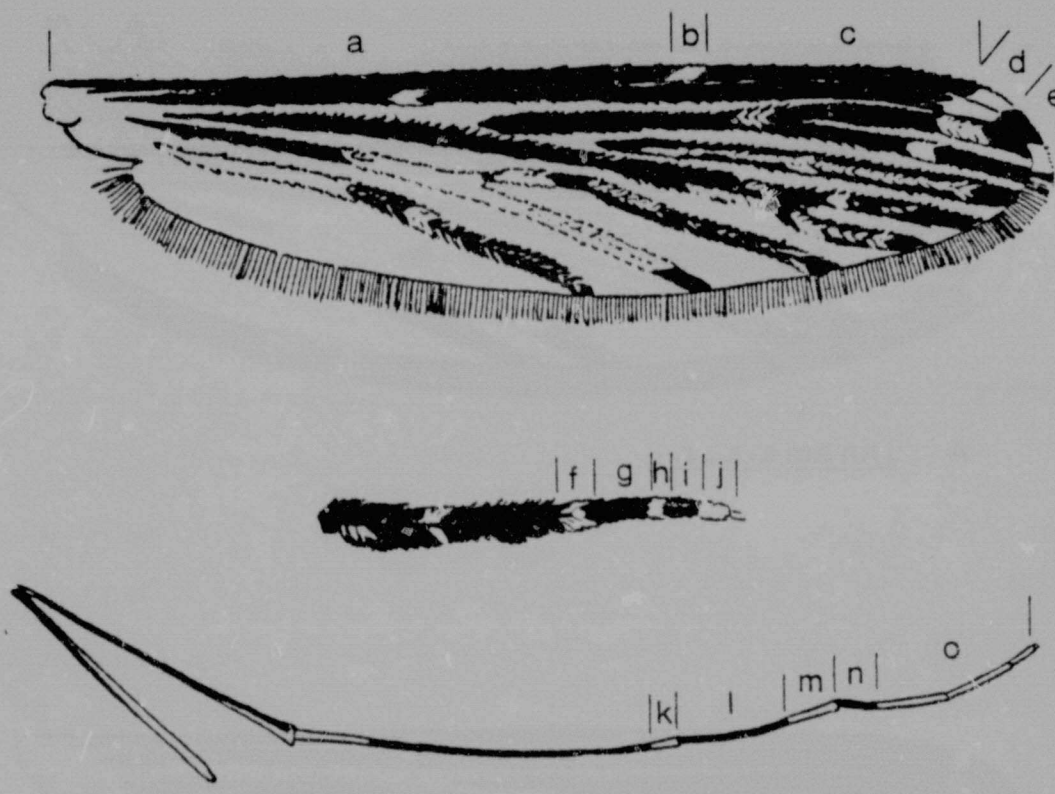
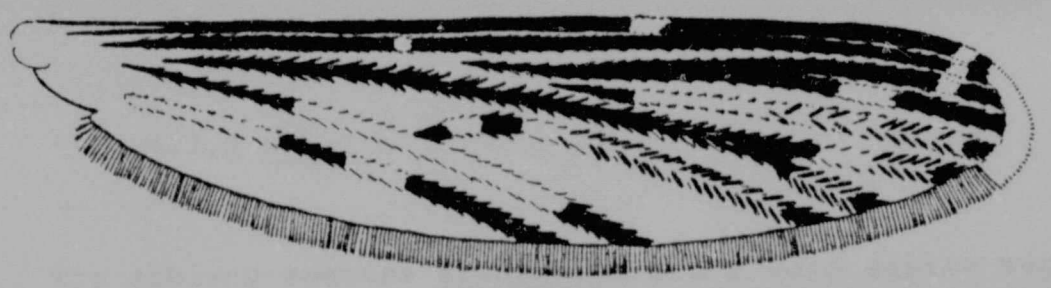
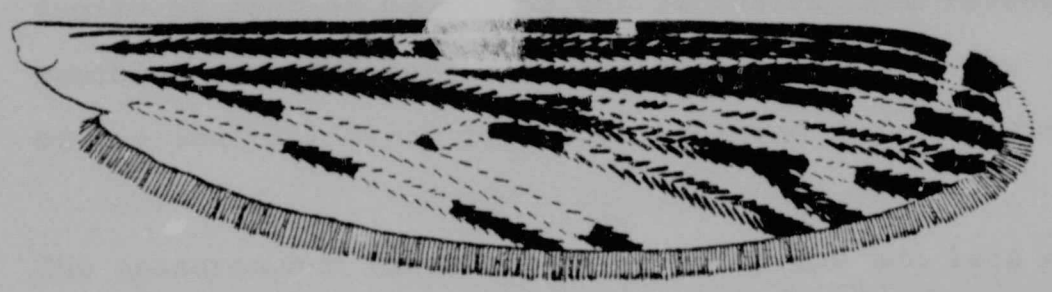


Fig. 12. The 15 spots measured on the wings, palps and legs of adults of A.coustani A and B.



A. ziemanni A



A. ziemanni B

Fig. 13. The wings of A. ziemanni A and B showing the difference in size of the apical pale spot.

were recorded from 50 individuals, mostly from different families, in an attempt to differentiate between coustani A and B.

4.2.2 Results

The sibling species ziemanni A and B were easily separated on the size of the apical pale wing spot (fig. 13), ziemanni A having it extending from the tip of vein 1 to beyond the third vein. A.ziemanni B has this spot greatly reduced and is about half the size of species A. One family of species B, out of the 34 reared from Kavango, Namibia, showed intergrades for this character with three of the adults looking like species A and two like species B.

The measurements taken of the wings, palps and legs of the coustani sibling species showed no variation between the species. The female pharynx and the male genitalia also showed no differences and correspond with the description given by Gillies & De Meillon (1968).

4.3 Pupal identification

4.3.1 Methods

Pupal skins were collected and preserved in 80% alcohol. They were then transferred to absolute ethanol overnight. The ethanol was siphoned off and the skins were allowed

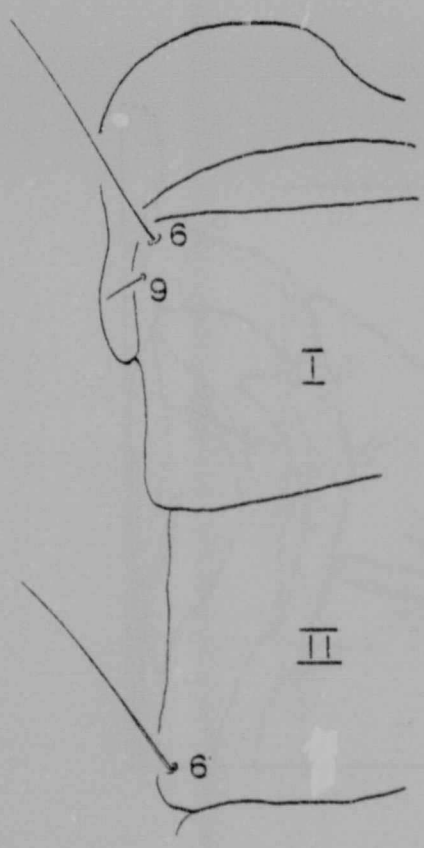
to soak in saturated phenol/ethanol for a few hours at least. A drop of phenol/ethanol/balsam was placed on a slide and the skins arranged therein for microscopy. This slide was allowed to dry for ±2 days and then protected with a coverslip on pure Canada balsam. All skins were, of course, correlated with individual adults.

Initially, full setal counts were done on a few individuals, following the system of Belkin (1962) where each hair is numbered and pupal setae are correlated to those on the larva. Certain promising characters were then chosen and all families were represented, with a maximum of ten individuals per family being used (see Appendix I for full details). Setal counts were done on a Leitz phase-contrast microscope at X400 magnification.

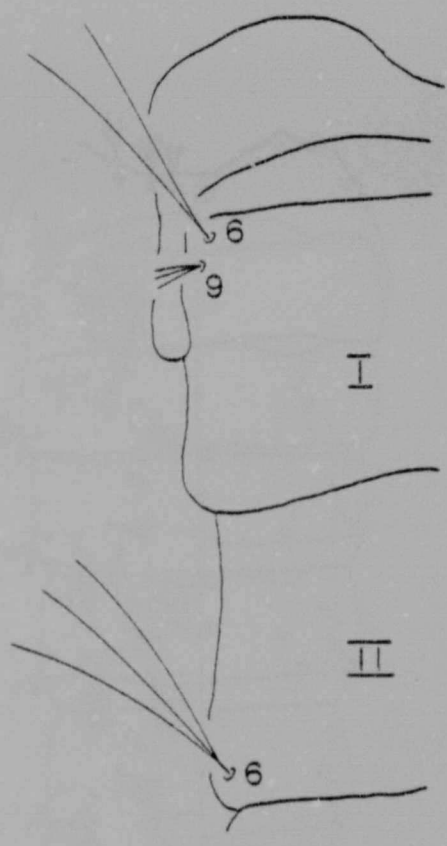
Full illustrations were done on good quality drawing paper three times the size of standard A4 paper and then reduced and photographed. They constitute part of Appendix III but it was thought more appropriate to include them in the text. The main differences between the sibling species are depicted in brief sketches (fig. 14).

4.3.2 Results

The data recorded for the original three species (Appendix III) were checked against the genetical species and corresponded with those of *tenebrosus*, *coustani* A and

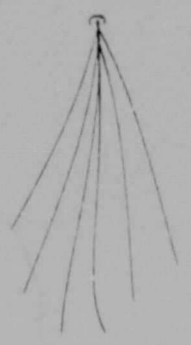


sp A

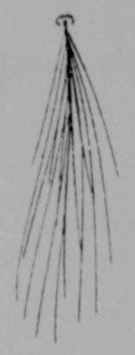


sp B

A. coustani



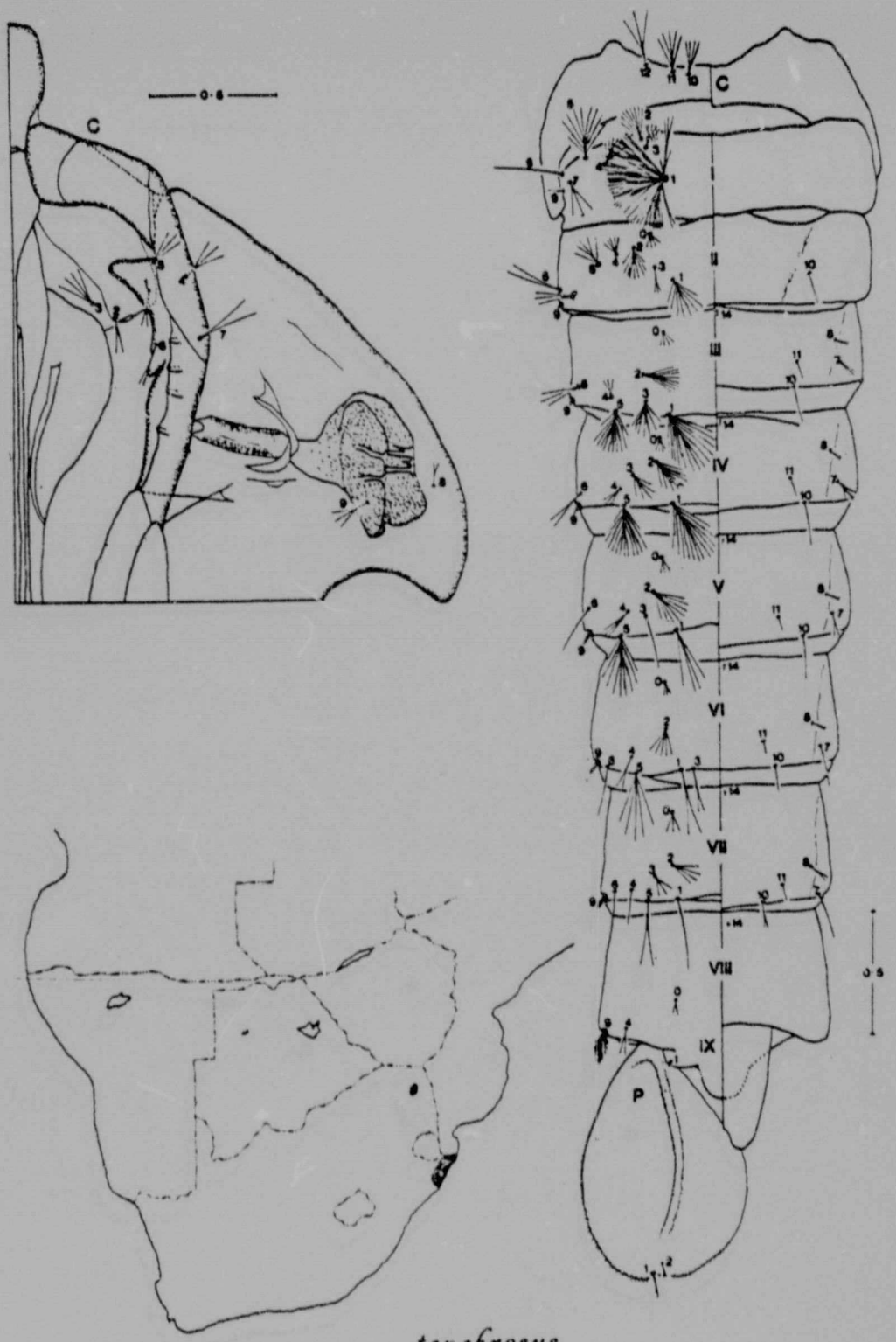
sp A



sp B

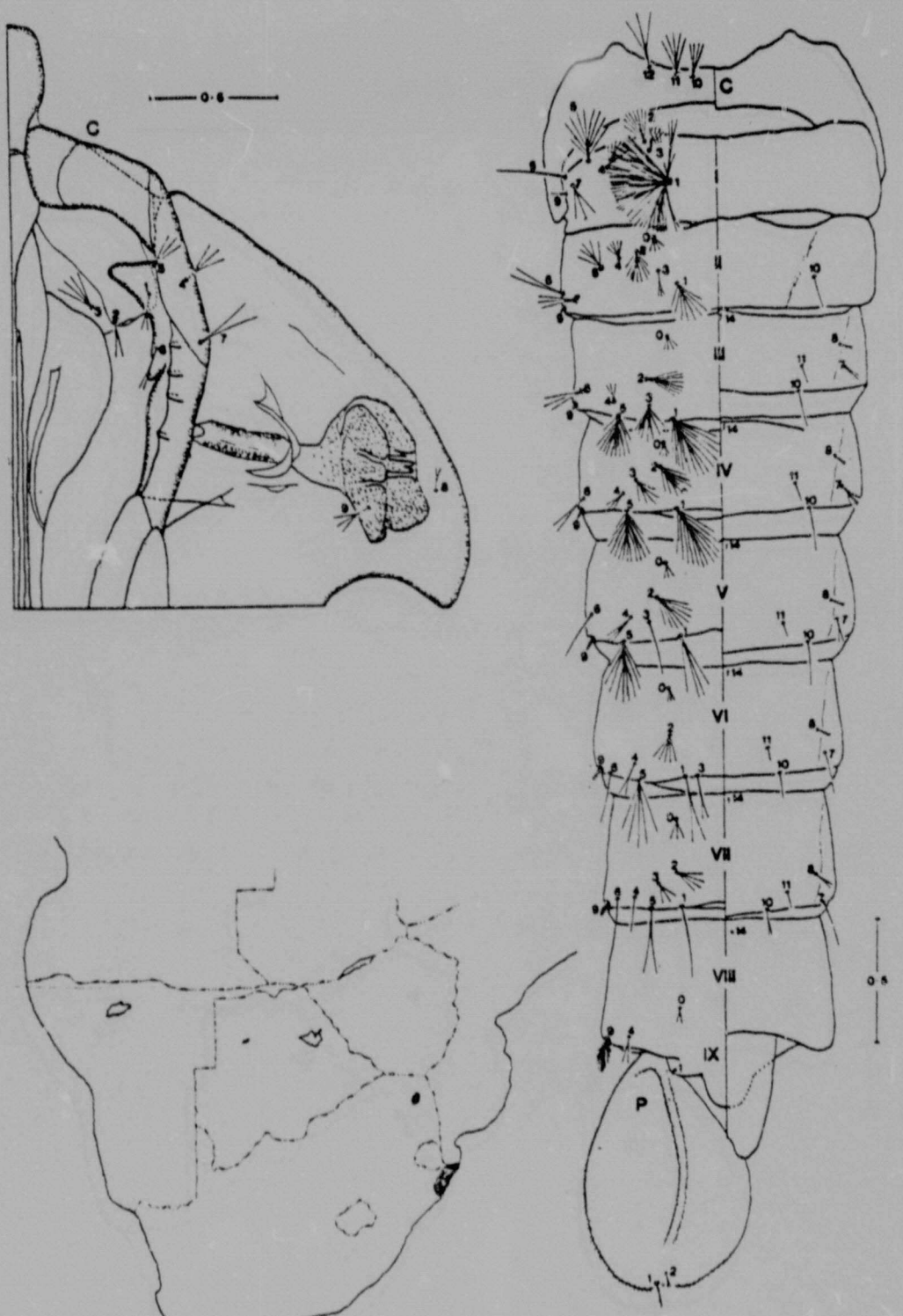
A. ziemanni seta 5 · VI

Fig. 14. Pupal characters of the sibling species.



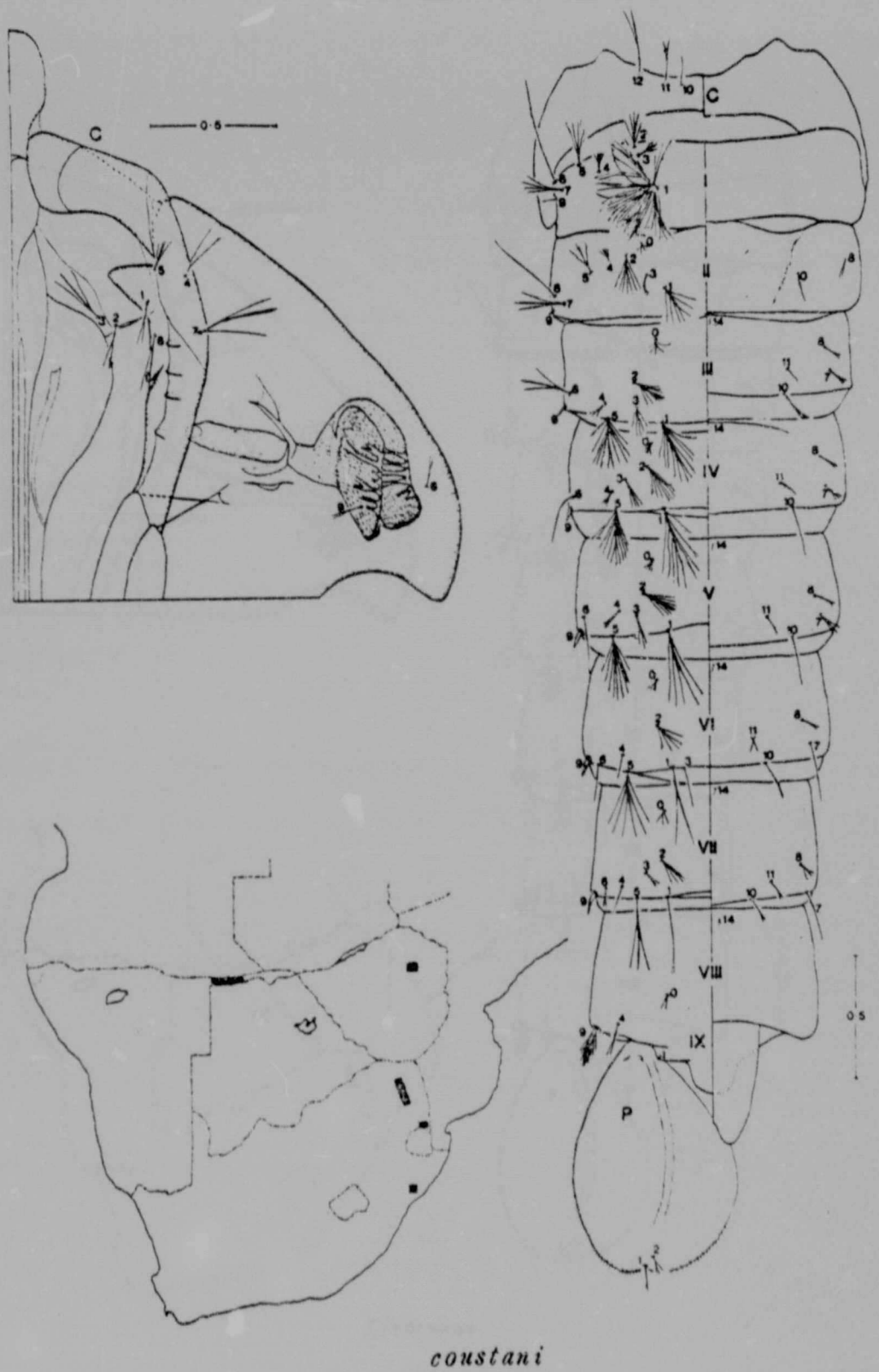
tenebrosus

Fig. 15. The pupa of A.tenebrosus.



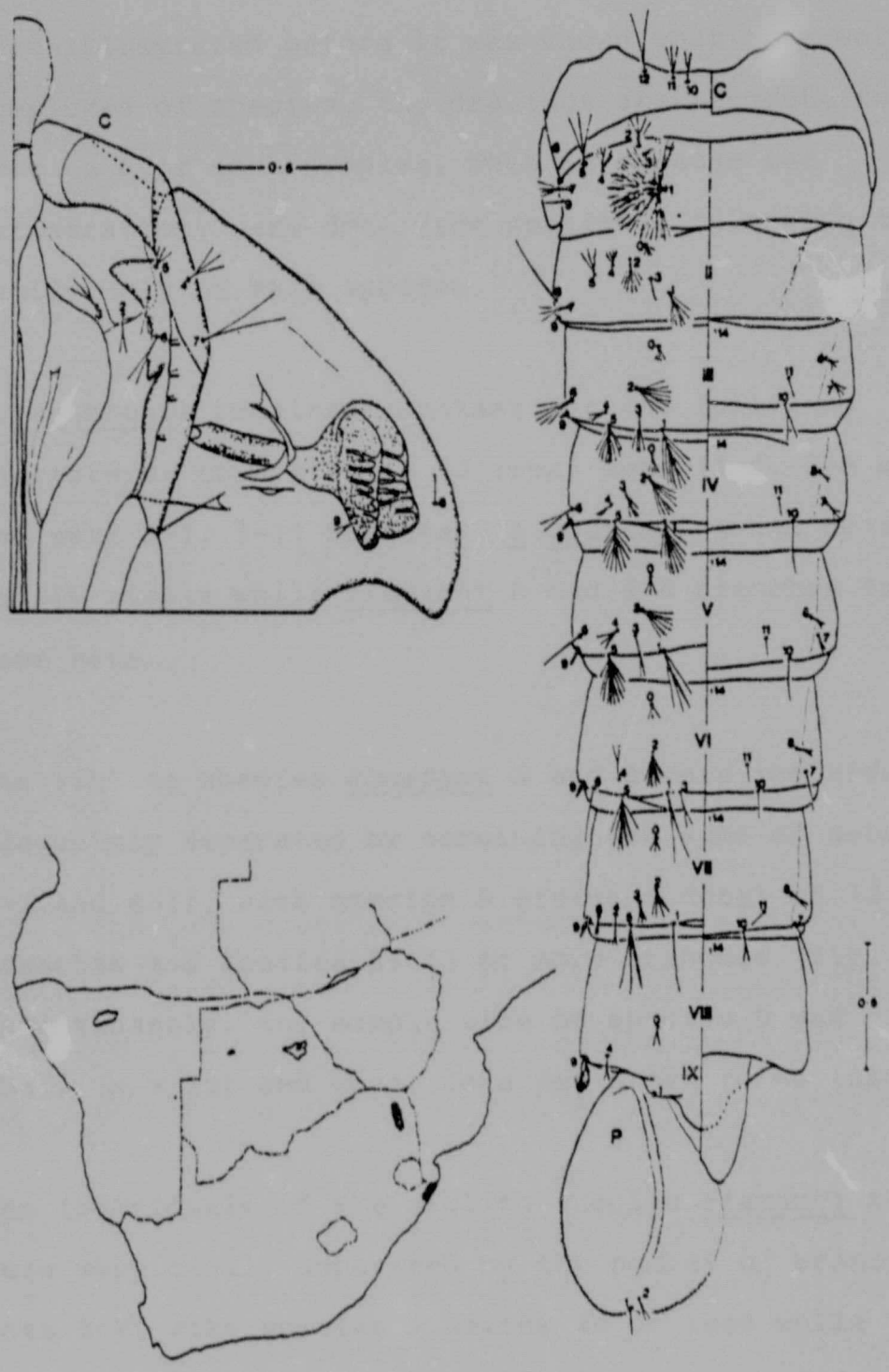
tenebrosus

Fig. 15. The pupa of A.tenebrosus.



coustani

Fig. 16. The pupa of A.coustani A.



Ziemanni

Fig. 17. The pupa of A.ziemanni A.

ziemanni A. Figs. 15, 16 and 17 show full illustrations of these three species. Although coustani and ziemanni were illustrated before it was known that they were complexes of species, the drawings are accurate for species A of each complex. This is because the illustrations were done from specimens belonging to one family only of each species.

A.tenebrosus remained constant for the following characters: trumpets 4 - 12 ribs; seta 11-C, 3-6 branches; and seta 5-I, 3-11 branches. A.coustani A had seta 6-IV mainly simple while ziemanni A had 2-4 branches for the same seta.

The sibling species coustani A and B were individually adequately separated by combining the sums of setae 6-I, 9-I and 6-II, with species A giving a total of 12 or less branches and species B, 13 or more branches (fig. 14). Unfortunately, the sample size of species B was very small (n = 25) and these data may prove to be insufficient.

The individuals of the sibling species ziemanni A and B were very easily separated by the number of branches on seta 5-VI with species A having 10 or less while species B usually had very much more than 10, averaging 15.5 (n = 70) (fig. 14). This character is so marked in the specimens of "ziemanni" previously collected from Namibia that they were not included in the immature study

summarized in Appendix III as it was suspected then that they were not the same species.

4.4 Larval identification

4.4.1 Methods

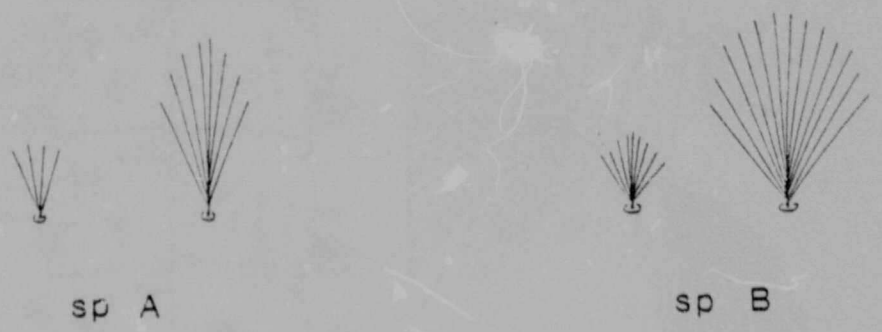
The same methods that were applied to the pupae were used for the larvae. This includes mounting of pelts, setal counts and illustrations. Fig. 18 shows the extreme differences between coustani A and B and ziemanni A and B. Figs. 19, 20 and 21 show full illustrations of the original three species.

4.4.2 Results

Again the data recorded in Appendix III were checked against the five genetical species and tenebrosus remained outstanding with the antennal shaft hair (1-A) having 12 or more branches, ranging from 12-19 branches.

Both the coustani and ziemanni complexes could only be distinguished by using family material as a whole and not on separate individuals as was possible in the pupal stage. For the coustani complex it was possible to separate the families by using the mean number of branches for seta 9-V with species A having 12.9 or less and species B having 13.0 or more. Again, due to the small sample size

A. coustani seta 9 · V



A. ziemanni setae 2 · I and 5 · II

Fig. 18., Larval characters of the sibling species.

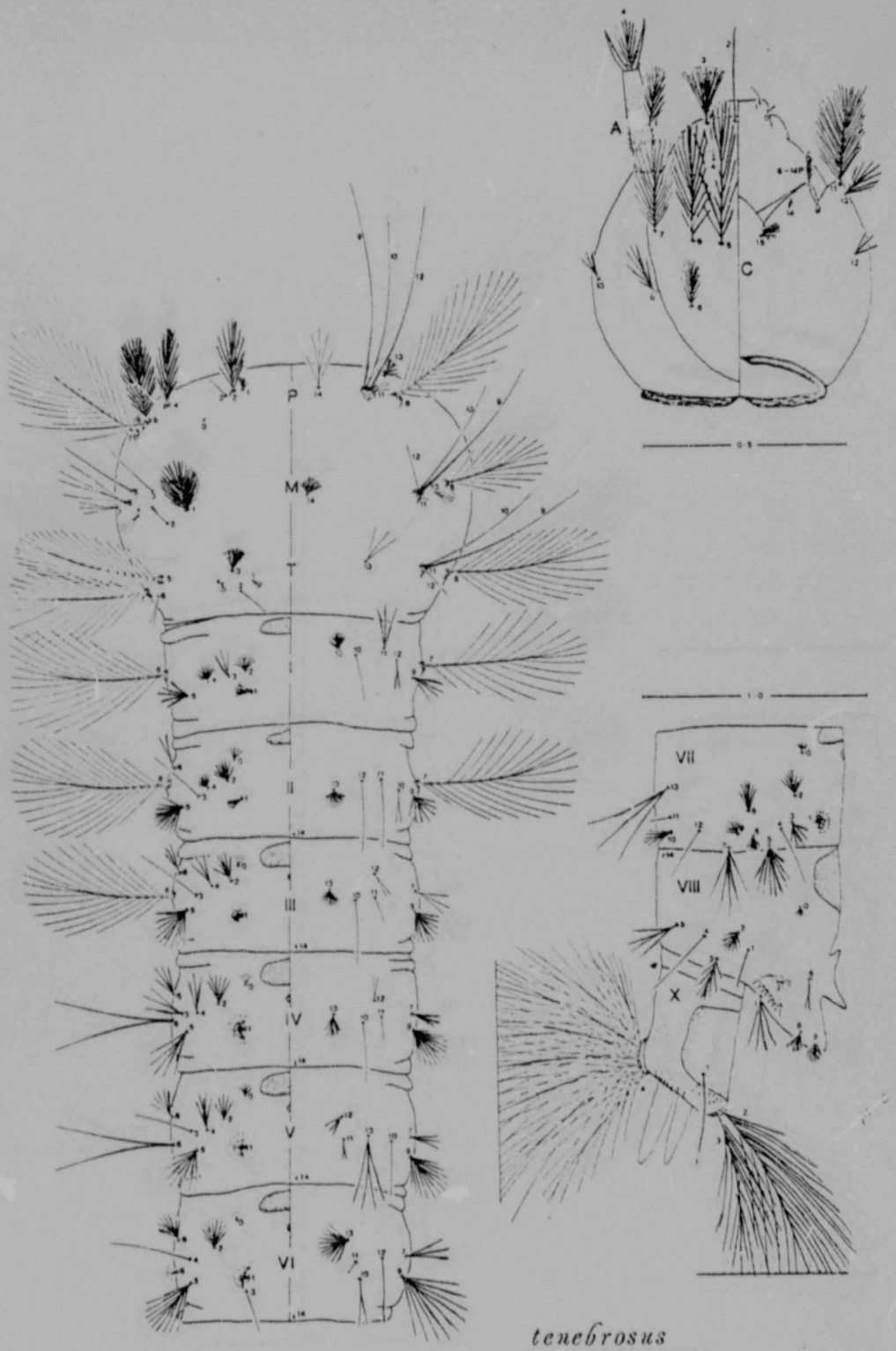


Fig. 19. The larva of A.tenebrosus.

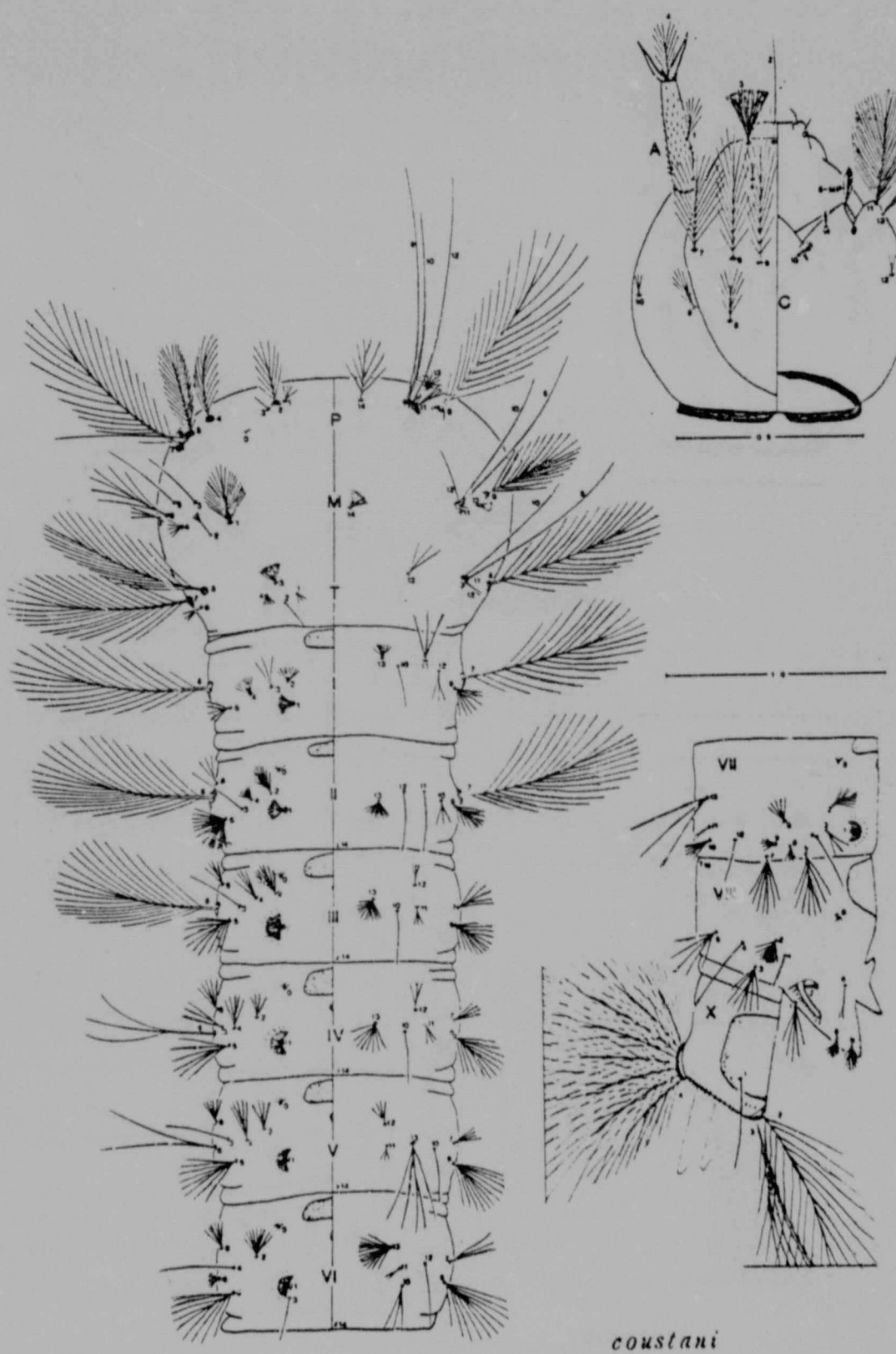


Fig. 20. The larva of A.coustani A.

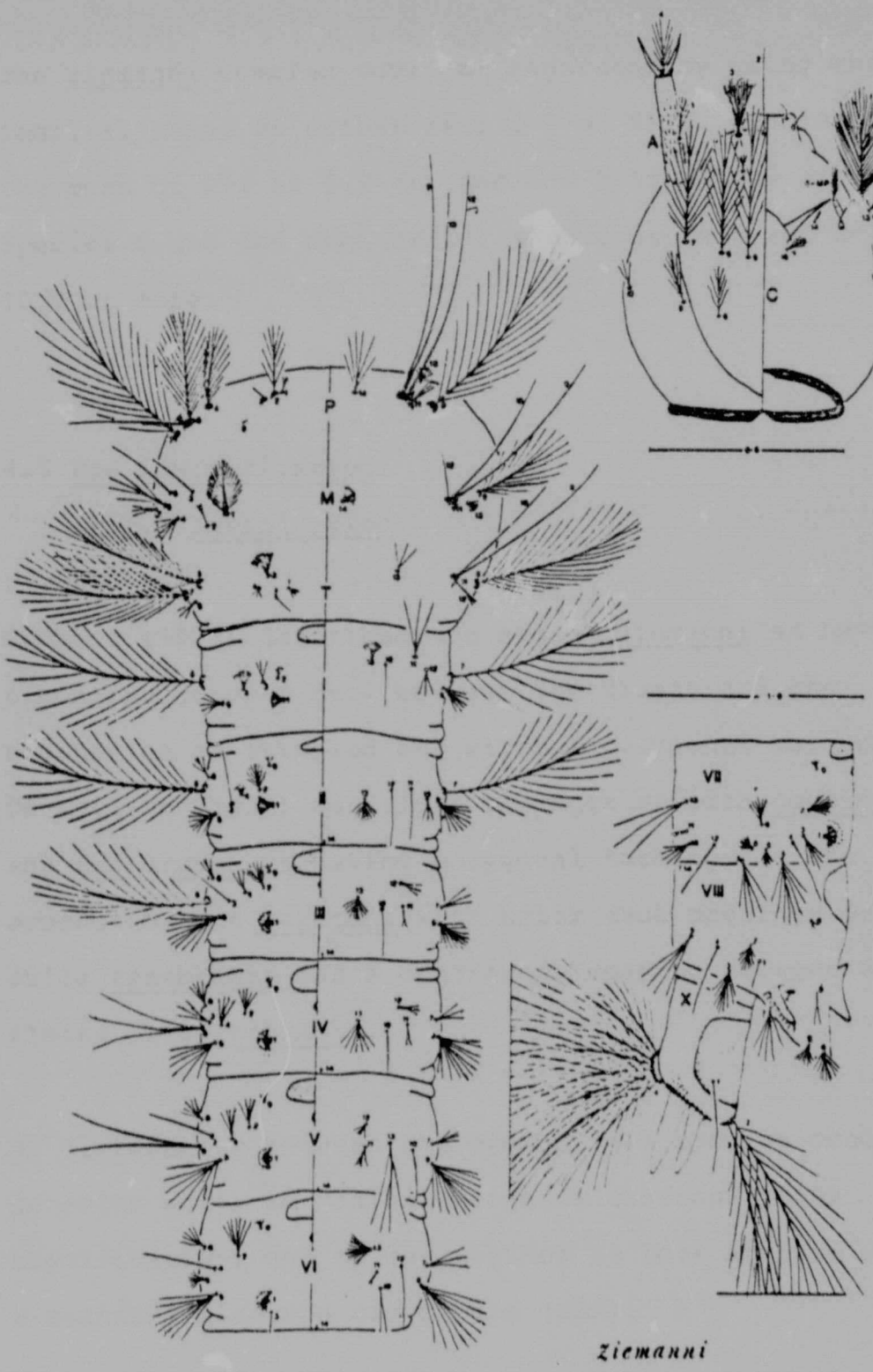


Fig. 21. The larva of A.ziemanni A.

of species B, this is open to question.

The ziemanni complex could be separated by using the familial means of either seta 2-I or 5-II. Species A had the mean of 2-I as 5.9 or less and 5-II as 9.9 or less. Species B had the mean of 2-I as 6.0 or more and 5-II as 10.0 or more.

4.5 Egg identification

4.5.1 Introduction

Gibbins (1933) described the egg of ziemanni as having a continuous open deck between the floats and the exochorion as stippled but without polygonal markings. De Meillon (1937) described the eggs of both coustani and tenebrosus as having polygonal markings on the exochorion but coustani with polar deck openings only, while tenebrosus has a continuous opening between the floats as in ziemanni.

No differences between the eggs of all species could be detected under an ordinary light microscope (X100 magnification) and it was decided to look at them under a scanning electron microscope (SEM).