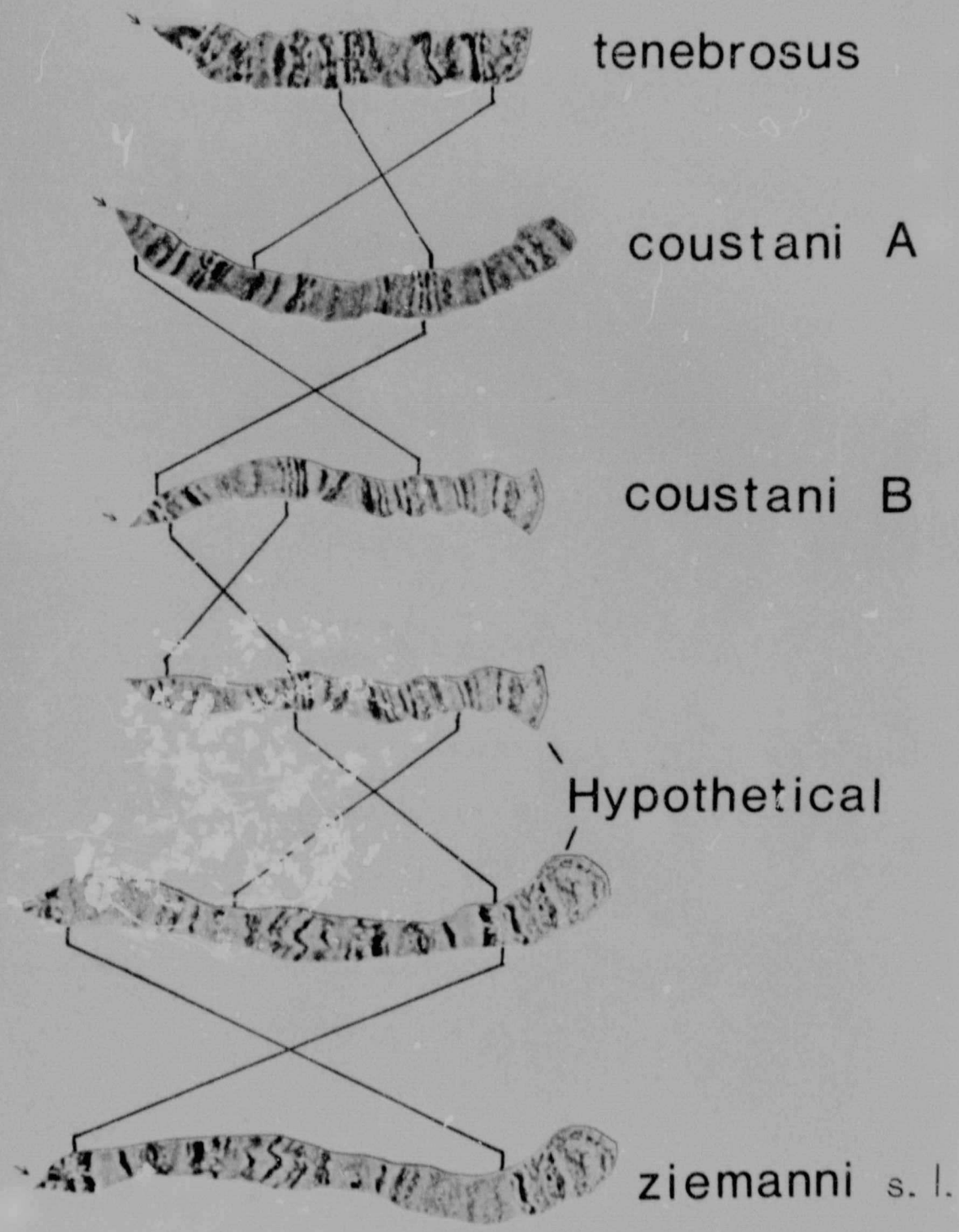

tenebrosus
coustani A
coustani B
Hypothetical
ziemanni s. l.

tenebrosus and ziemanni A. Two inversions were scored on arms 2L and 3R respectively of ziemanni B. Table 1 shows the frequencies found in the population. Although the sample size is rather small, it was decided to test these figures against the Hardy-Weinberg expectations.

Table 1. The numbers of 2La and 3Ra chromosome sequences found in the A.ziemanni B population.

| | | | |
|---|---|---|---|
| 2La | 9 | 3Ra | 5 |
| $2La/a^+$ | 20 | $3Ra/a^+$ | 22 |
| $2La^+$ | 2 | $3Ra^+$ | 3 |
| Total | 31 | | 30 |

The Hardy-Weinberg law states that frequencies of two alleles remain constant from generation to generation in a Mendelian population under certain conditions i.e. random mating, no selection and no immigration/emmigration (Wilson et al, 1973, p776). The predictions of this law allows one to derive gene frequencies from genotype frequencies and vice versa. The formula is:

$(p + q)^2$ i.e. $p^2 + 2pq + q^2 = 1$

where p and q are the relative frequencies of the two alleles being tested and hence $p^2$ is the expected relative frequency of the one homozygote, $q^2$ the relative frequency of the other and 2pq the relative frequency of the heterozygotes all at equilibrium.

Using the information in table 1, let the sample frequency of 2La = p and the sample frequency of $2La^+$ = q. The relative frequency of p is 0.613 (9 + 10/31) and q is 0.387 (10 + 2/31). Using the above formula with these gene frequencies, the genotype frequencies at equilibrium should be as follows:

$$0.3758 + 0.4744 + 0.1498 = 1$$

or at a population of 31

11.6 14.7 4.6

It can be seen that there is some discrepancy between the observed number and the expected. The Chi-square test of significance of differences ($X^2 = \frac{\Sigma(O-E)^2}{E}$) was carried out and $X^2 = 3.96$. At one degree of freedom, this figure just falls into the significant range ($p<0.05$).

The same procedure was gone through for the inversion 3Ra and a significant difference was noted ($X^2 = 6.70$, $p<0.01$). Either the sample is biased, or natural selection is favouring the heterozygotes.

A.coustani A also has two floating inversions, one on arm 2R and the other on arm 3R. Table 2 shows the frequencies found in the populations.

Inversion 2Ra shows significant deviation from the Hardy-Weinberg equilibrium ($X^2 = 5.92$, $p<0.05$) and again there is a relative excess of heterozygotes. Inversion 3Rc does not deviate significantly from the expected frequencies

Table 2. The numbers of 2Ra and 3Rc chromosome sequences found in the A.coustani A populations.

| | | | |
|---|---|---|---|
| 2Ra | 1 | 3Rc | 1 |
| $2Ra/a^+$ | 27 | $3Rc/c^+$ | 5 |
| $2Ra^+$ | 18 | $3Rc^+$ | 24 |
| Totals | 46 | | 30 |

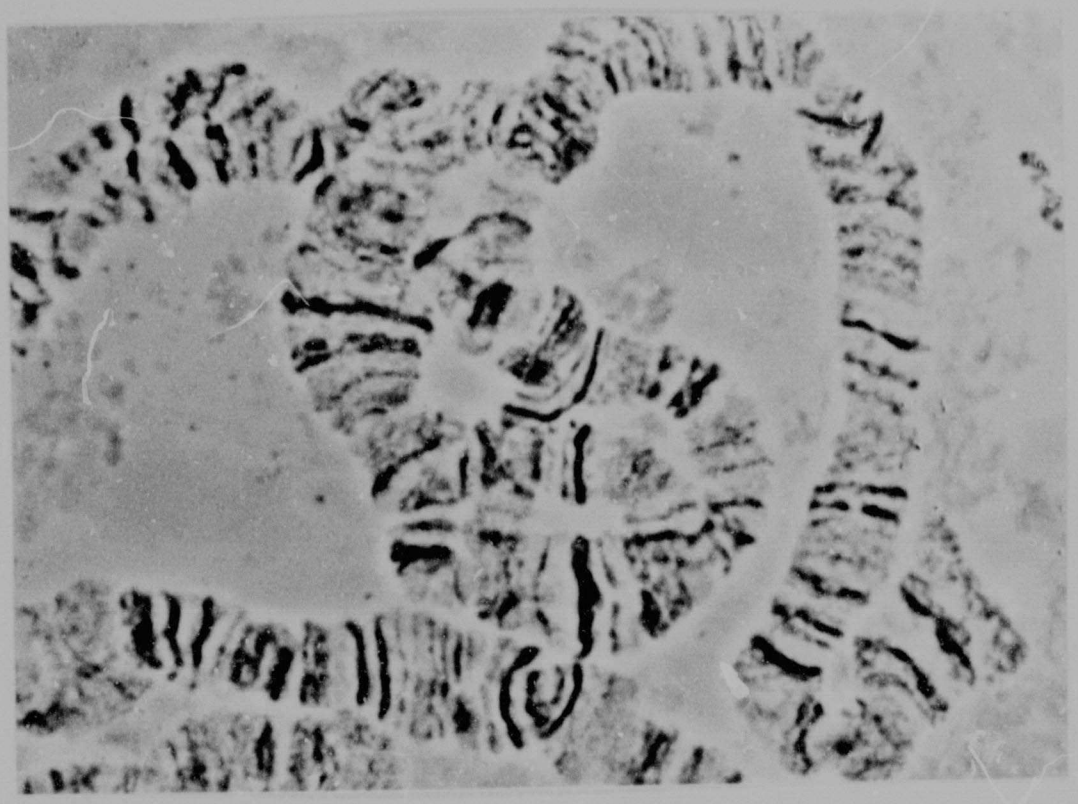
derived from Hardy-Weinberg equilibrium ($X^2 = 2.03$, $p<0.05$).

The only inversions seen in coustani B were the two heterozygotes $3Ra/a^+b/b^+$ which were always present in the few specimens examined. Fig. 6 illustrates the double heterozygote.

## 2.4 Cross-mating experiments between the coustani sibling species

Wild females were caught and the progeny reared as described in 2.2.1. Some males and females were separated and fed on sugar-water for two days and then the females were offered a blood meal. When the males were at least five days old, artificial mating was attempted as follows. The males were decapitated and the wings and legs removed. The thorax was glued to the edge of a petri dish with ventral surface uppermost with the abdomen hanging free. The female was anaesthetized and held on a needle attached to a suction pump. The abdomen of the female

Fig. 6. The double heterozygote loops on arm 3R of A.coustani B.



was brought into contact with the genitalia of the male and copulation was spontaneous when it occurred. The surviving females were again offered a blood meal and then put aside for egg-laying.

From five matings of coustani A female x coustani B male, two batches of eggs were obtained but none hatched. From the five reciprocal crosses, B female x A male, also two batches of eggs were obtained, of which one hatched and the larvae reared to adulthood.

Chromosomes were obtained from the fourth instar larvae and fig. 7 shows the extensive asynapsis and coiling of the homologous species arms. This is known to be a clear indication of genetic incompatibility in most anopheline species. In addition, the testes of the hybrid males were devoid of sperm. Fig. 8 shows a comparison between the testes of fertile and sterile males.

## 2.5 Discussion

The discovery of sibling species within two of the taxa is in keeping with other similar studies done on anophelines. Lambert (1979) discovered four chromosomal species within A.marshallii. Anopheles annulipes (Green, 1972), A.culicifacies (Green & Miles, 1980), A.squamosus and A.pharoensis (Green, pers. comm.) and of course, the classical A.gambiae are all species complexes.

Fig. 7. Hybrid chromosomes of A.coustani B x A.

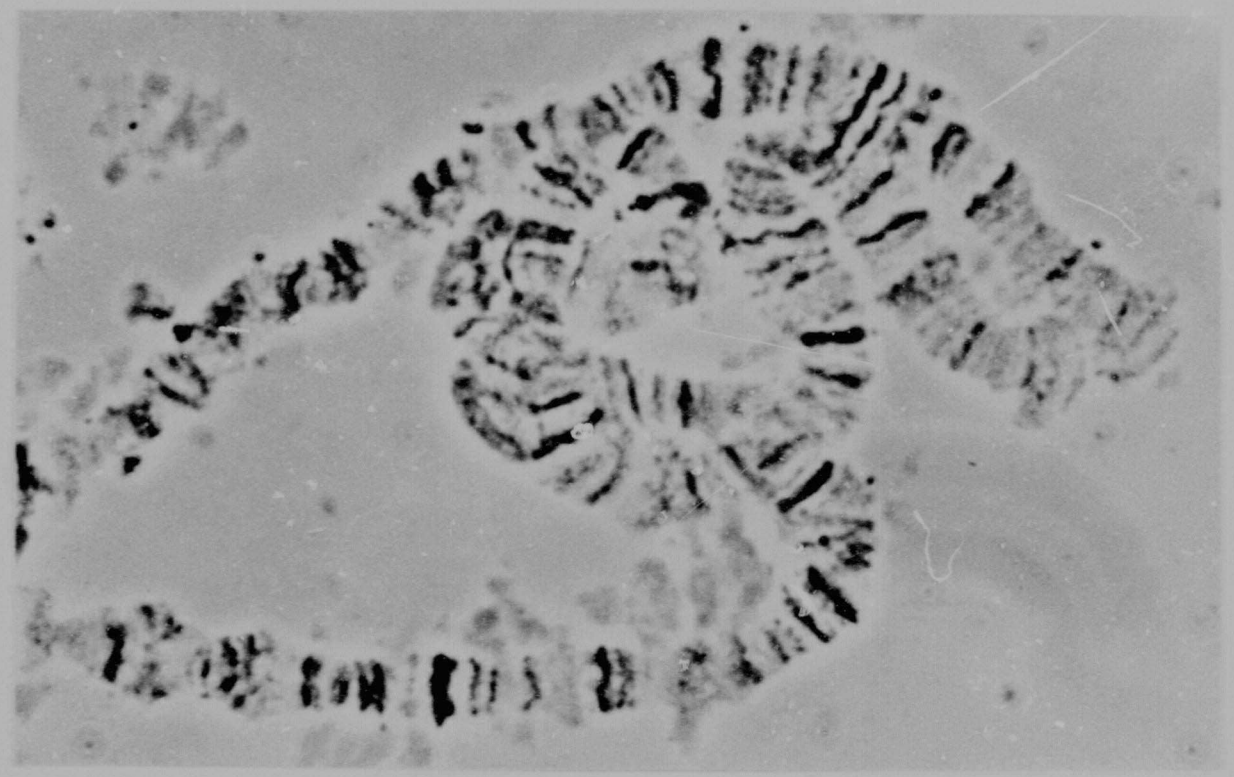
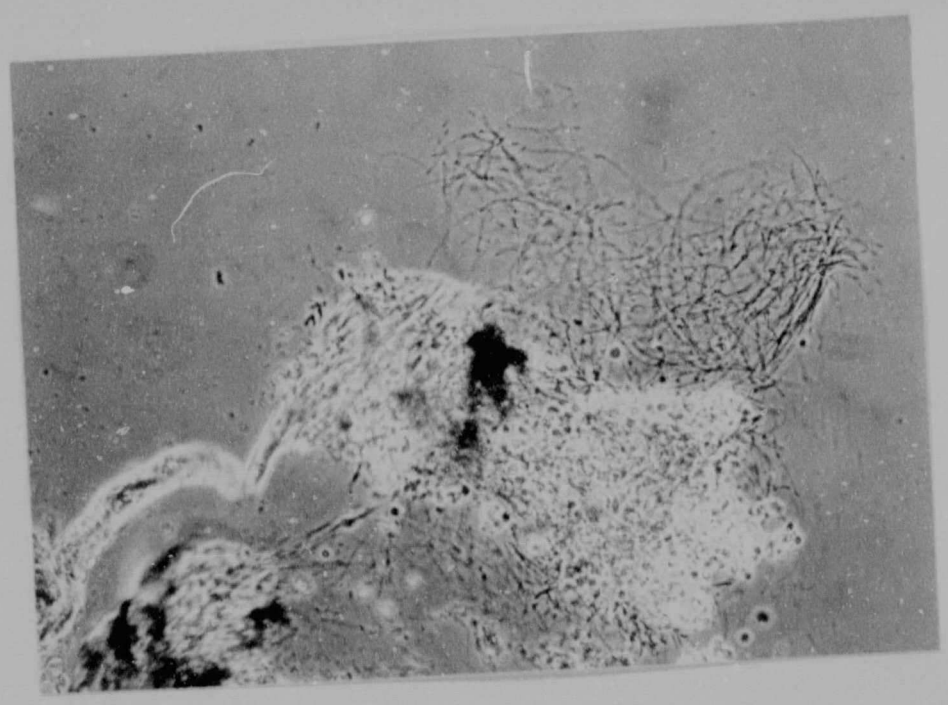
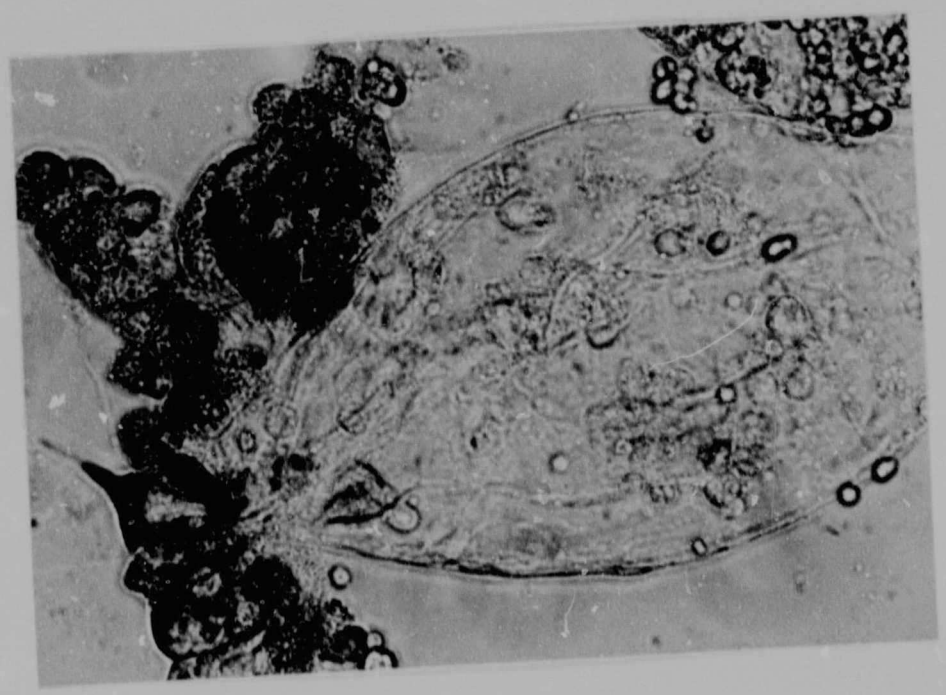


Fig. 8. a) Spermatozoa emerging from the squashed testes of a normal fertile A.coustani A male. b) The testes of a F1 hybrid male A.coustani B x A with no evidence of spermatozoa being present, only early spermatids.



a



b

The fixed inversion on the X chromosome is an easy character for identifying the coustani sibling species. The fact that there are apparently no floating inversions in ziemanni A , makes it easy to distinguish it from ziemanni B. No specimens of the latter species were observed where both inversions 2La and 3Ra were homozygous at the same time. There was always one heterozygote and often both inversions were heterozygous. The typical loops formed by the heterozygote make an easy means of identifying species B.

While fixed inversion differences between sympatric populations is a definite indication of separate gene pools and therefore separate species, when occurring between allopatric samples, they could possibly be geographic variations within a single species. It was therefore decided to do cross-mating experiments with the coustani and ziemanni sibling species because of the lack of sympatry in their distribution. It may seem that one family from ten coustani A x B matings is a high success rate, but these figures do not include all the females who died prior to mating because they would not take a blood meal, and all the males which had to be discarded when they would not copulate. Due to these laboratory difficulties, only two successful matings of the ziemanni A x B cross were obtained and both failed to lay eggs.

Due to the very poor quality of the polytene chromosomes, it was not possible to work out homologies between the autosomes of the five species. At times it was impossible to score the homozygous inversions and this no doubt has put a bias on the sample. It seems that other authors have experienced the same difficulty judging from the quality of their published plates (Baker & Kitzmiller, 1964, 1965, Seetharam & Chowdaiah, 1971, Oguma, 1976, Kanda & Oguma, 1977a, 1977b). The only published set of chromosomes I could use for the correlation of arm notations, was those of Anopheles barbirostris (Chowdaiah et al, 1970). The method of producing chromosome maps from montages of photographs, is considered superior to that of line drawings, as no detail is lost.

# CHAPTER THREE

## THE ELECTROPHORETIC ANALYSIS

### 3.1 Introduction

Starch-gel electrophoresis of the intermediate metabolic enzymes is a useful tool for identifying morphologically similar species. Ayala & Powell (1972) suggested that allozymes might prove useful for identification of sibling species and demonstrated diagnostic allozymes for identification of members of the Drosophila willistoni species group. Mahon et al (1976) applied this technique to chromosomally identified members of the Anopheles gambiae complex in southern Africa and several diagnostic allozymes were found. Miles (1979) provides a biochemical key for the identification of the gambiae complex.

At some loci electromorphs are polymorphic, while at others they are species-specific (fixed), and so are of value in taxonomy. Species-specific electromorphs also provide useful markers for the study of gene flow between natural populations. The overall objective of this section of the study is to make a preliminary estimate of possible diagnostic electromorphs, that is, electromorphs determined by fixed alleles.

# CHAPTER THREE

## THE ELECTROPHORETIC ANALYSIS

### 3.1 Introduction

Starch-gel electrophoresis of the intermediate metabolic enzymes is a useful tool for identifying morphologically similar species. Ayala & Powell (1972) suggested that allozymes might prove useful for identification of sibling species and demonstrated diagnostic allozymes for identification of members of the *Drosophila willistoni* species group. Mahon *et al* (1976) applied this technique to chromosomally identified members of the *Anopheles gambiae* complex in southern Africa and several diagnostic allozymes were found. Miles (1979) provides a biochemical key for the identification of the *gambiae* complex.

At some loci electromorphs are polymorphic, while at others they are species-specific (fixed), and so are of value in taxonomy. Species-specific electromorphs also provide useful markers for the study of gene flow between natural populations. The overall objective of this section of the study is to make a preliminary estimate of possible diagnostic electromorphs, that is, electromorphs determined by fixed alleles.

## 3.2 Methods

Starch-gel electrophoresis was used routinely because the banding patterns were so distinct and the technique allows staining for three different enzyme systems per gel, as the gel can be cut into three horizontal slices for scoring different systems.

Horizontal electrophoresis (Smithies, 1955) was used employing the buffer system of Barlow & Ridgway (1971). The gel contained 10g of hydrolysed starch per 100ml of dilute electrode buffer pH8.2. Mosquitoes were homogenised in 5µl distilled water, absorbed onto 6 x 5 mm of chromatography paper and inserted vertically along one end of the gel. The gel was then placed between two copper plates pre-cooled to 2°C, and a current of 500 volts passed through the gel for approximately 3 hours or until the bromophenol blue marker had moved 10cm from the origin. The gel was then cut into three equal slices with one slice always being used to detect esterase (Est) enzyme activity. The other two slices were used initially to test a variety of enzyme activities but eventually used only to detect octanol dehydrogenase (Odh), superoxide dismutase (Sod) and glutamic-oxaloacetic transaminase (Got) activities. The other enzymes examined were lactic dehydrogenase (Ldh), ∝-glycerophosphate dehydrogenase (∝Gpdh), xanthine dehydrogenase (Xdh), isocitric dehydrogenase (Idh), malic dehydrogenase (Mdh),

Table 3. The number of specimens per species tested for each enzyme system.

| Enzyme | tenebrosus | coustani A | coustani B | ziemanni A | ziemanni B |
|---|---|---|---|---|---|
| Est | 28 | 96 | 12 | 42 | 59 |
| Odh | 24 | 71 | 3 | 36 | 45 |
| Sod | 27 | 90 | 10 | 41 | 56 |
| Got | 14 | 66 | 3 | 38 | 43 |
| Ldh | 3 | 2 | 1 | 2 | 2 |
| ∝Gpdh | - | 6 | 4 | 4 | 5 |
| Xdh | 4 | 3 | - | - | 4 |
| Idh | - | 3 | 4 | - | - |
| Mdh | 3 | 2 | 1 | 2 | 2 |
| Pgm | 4 | 4 | - | - | 4 |

and phosphoglucomutase (Pgm). The detailed recipes for the stains and buffers are given in Appendix II. The number of specimens per species tested for each enzyme are given in table 3.

## 3.3 Results

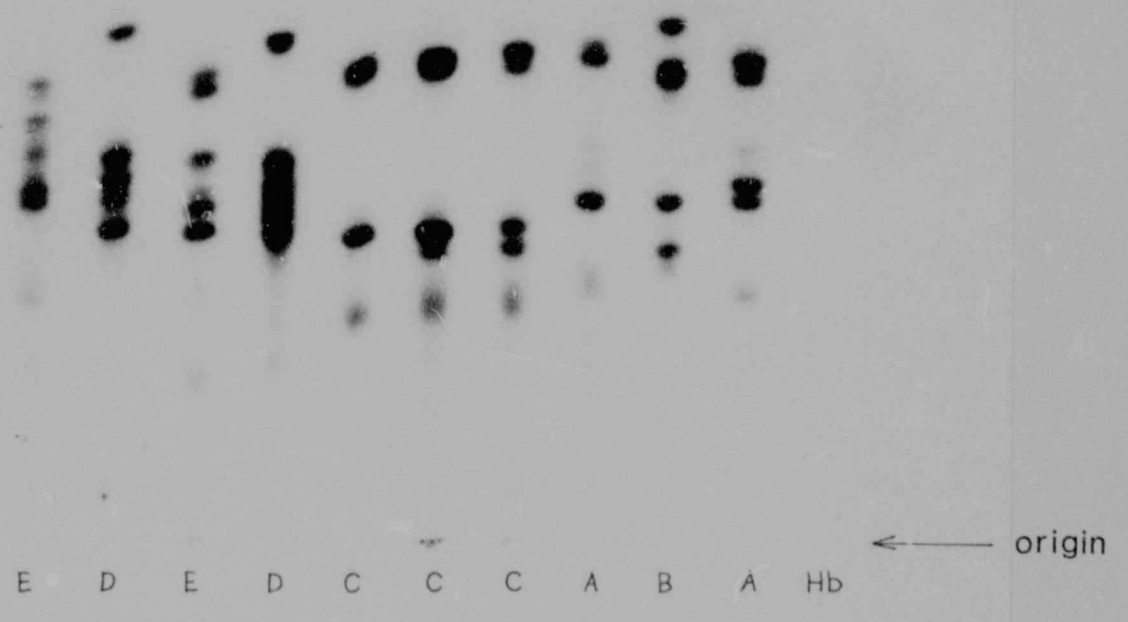
Four of the enzyme systems surveyed displayed inter- and/or intraspecific variation:

Est. This seems to consist of at least three loci (fig. 9). The gel run on the two coustani species and the F1 hybrids indicates that Est-2 can segregate for up to seven alleles (fig. 10). Est-$2^{115}$ and Est-$2^{110}$ have only been seen in

Fig. 9. Esterase allozymes of the five species studied.

ESTERASE ALLOZYMES.

A - coustani A
B - coustani B
C - tenebrosus
D - ziemanni A
E - ziemanni B



ESTERASE ALLOZYMES.

A - *coustani* A
B - *coustani* B
C - *tenebrosus*
D - *ziemanni* A
E - *ziemanni* B

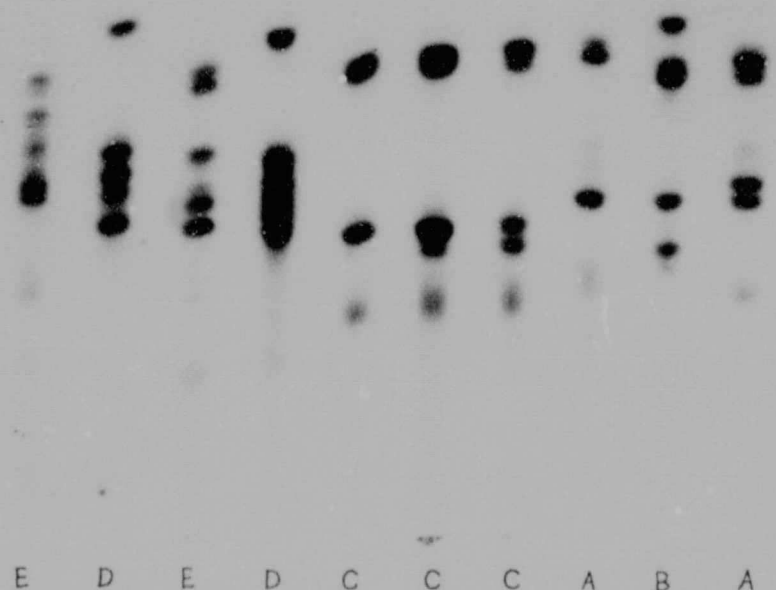
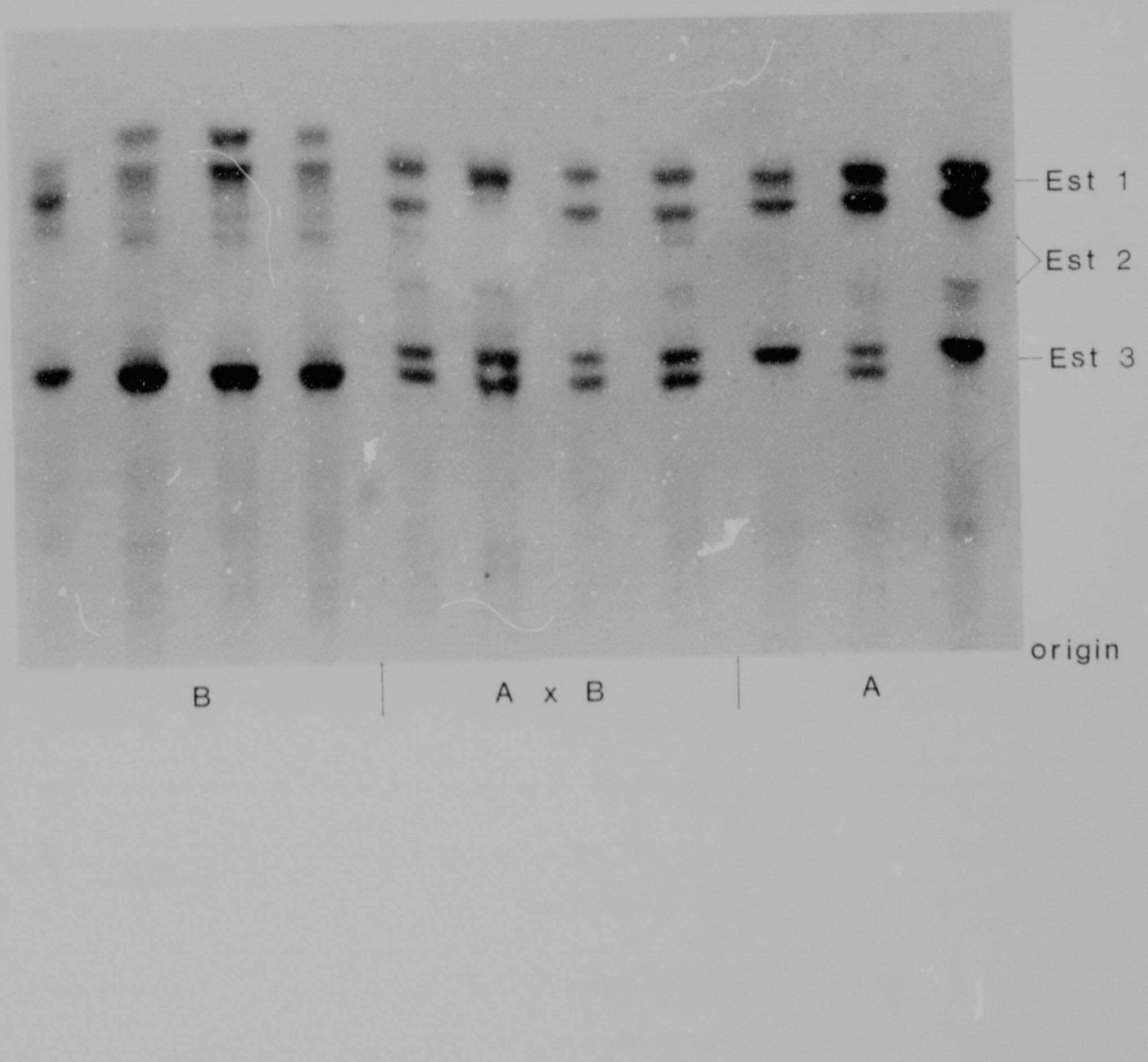


Fig. 10. Esterase allozymes of the two species of coustani with the F1 hybrids in the middle.


Est 1
Est 2
Est 3
origin
B
A x B
A

coustani B material while tenebrosus does not exhibit enzyme activity at this locus at all.

Odh. Three alleles at a single locus were found. Two-banded heterozygotes were seen in all species except coustani B.

Sod. Five alleles at a single locus were found. Sod 105 is fixed in tenebrosus and is species-specific (fig. 11). More detailed sampling is needed to show whether the relative frequencies of coustani A and B at the 100 and 95 alleles is a true reflection or not.

Got. Again five alleles at a single locus were found. All the species except coustani B were polymorphic. A common band of activity, migrating cathodally, was present in all species and was monomorphic (fig. 11).

The other enzyme systems tested showed no variation for the few specimens used. Relative frequencies for the above seven loci are presented in table 4.

## 3.4 Discussion

It is very difficult, with the limited sample size, to draw any but the most tentative conclusions from the data recorded. It seems that the absence of Est-2 and the fast