Multivariate Morphometrics of the Immature Stages of the *Anopheles balabacensis* complex (Diptera : Culicidae)

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ABSTRACT. Discriminant function analysis was made of morphometric data from laboratory stocks of *Anopheles dirus* species A (Thailand), species B (Perlis, Malaysia) and material from natural populations of a third species here designated *An. balabacensis sensu stricto* from Sabah which differ in their polytene chromosomes. The results showed that discrimination of all three types was successful for both larvae and pupae. The significance of these findings are discussed with consideration of their practical implications for those taxa which include individuals of public health importance.

INTRODUCTION

A genetical study based on chromosomal and cross-mating data established the specific distinctness of three populations of the *Anopheles balabacensis* complex from Thailand, Perlis (North Malaya) and Sabah (East Malaysia) (Hii, 1985). The first two taxa were designated as *An. dirus* species A and B respectively. Peyton & Harrison (1979) gave the name *An. dirus* to members of the *balabacensis* complex from Thailand. This species is morphologically distinct in the adult, pupal, and fourth larval stages from topotypic *An. balabacensis* from Balabac Island, Philippines and certain other areas of the Philippines and Malaysia. Previously the forms from Thailand and Perlis were treated as a single *'bal. balabacensis* Perlis/Thai form' by Reid (1968). The conclusion that this form is indeed two separate species has made it necessary to examine the extant type and living material of the three taxa previously all called *An. balabacensis balabacensis*. In an attempt to resolve this problem of identification and to give a genetic identity for type specimens, a morphological study of the three species was undertaken. This paper reports the use of multi-variate analysis to examine morphological data on the three sibling species of *An. balabacensis* complex and to develop objective criteria for identification.

Discriminant function analysis is a multivariate technique which is being increasingly used for identification of individuals in anthropology (Howells, 1969, Blackith & Reyment, 1971) and medical entomology (Coetzee & Newberry, 1980, Lambert & Coetzee, 1971). Because it generates a set of vectors that best separate *a priori* determined groups, canonical analysis is used to assess the

amount of differentiation that has occurred among groups. Therefore, this technique was used to provide optimal discrimination between the three species.

MATERIALS AND METHODS

Egg batches were obtained from three chromosomally-identified stock colonies which were maintained in the Ross Institute insectaries at the London School of Hygiene and Tropical Medicine. The designation of the colonies with their X chromosome types were: (1) *An. dirus* (a colony strain originating from Prachinburi Province, Thailand) - X_A, (2) *An. dirus* (Perlis strain originating from Padang Besar, Perlis state, Peninsular Malaysia) - X_B, (3) *An. balabacensis* (Sabah strain) - X_C. The origins and maintenance of the colonies were described in a previous paper (Hii, 1985).

Material was obtained as individual egg-batches from inseminated females and reared in an insectary at 25° C and 75% RH from egg to adulthood. Half of each egg batch was used for link-rearing and the other half for cytological preparations. Larval and pupal skins were preserved in 80% alcohol and later mounted in Euparal mounting medium. Readable polytene chromosomes were examined from ten fourth instar larvae of each family successfully reared using the chromosome preparations described for salivary glands (Kanda, 1979). Counts of setal branching of 70 larvae from 19 families and 99 pupae from 20 families were made using Belkin's (1962) system of nomenclature. Both the left and right lateral halves of the skins were examined. Table 1 shows the sample sizes of each chromosomally identified species that were measured for larval and pupal chaetotaxy (Fig. 1 and 2) and used for the analysis. Counts were made on 73 and 155 pairs of pupal and larval setae respectively under a Zeiss compound microscope with phase contrast illumination and green filter.

Data of setal counts were punched onto cards and the data were stored on a computer disc tape. Computations were carried out on the CDC 6600 at the University of London Computing Centre using three package programmes:- (1) BMD 07D - description of strata with histograms, (2) BMD PAM - description and estimation of missing values, (3) BMDP-7M - stepwise discriminant analysis program (Dixon, 1973, Dixon & Brown, 1977).

RESULTS

Pupa (Figure 3)

Having recorded the branching of 69 pairs of setae from each of 99 pupae, it became apparent from computer analysis that 8 of these setae could be of diagnostic value. They are 1-III, 7-II, 5-III, 1-IV, 3-IV, 9-VIII and 2-P (P = paddle). Using discriminant analysis, 97% discrimination was obtained with only 3 of the 99 specimens being misclassified. Figure 3 shows a scatterplot of the three groups; all specimens were almost perfectly discriminated for setal branching. A pupal key was constructed using only three of the eight characters, 1-II, 1-III and 5-III, whereby 82.6% of the 99 pupae could be correctly identified. The key is as follows: -

1. The sum of seta 1-11 on both sides lie in the range 19-66 branches ..... *An. dirus* species A

   The sum of seta 1-II on both sides lie in the range 50-99 branches 2

2. The sum of setae 1-III and 5-III on both sides lie in the range 16-27 and 15-24 branches respectively ..... *An. balabacensis*

   The sum of setae 1-III and 5-III on both sides lie in the range 19-34 and 20-39 branches respectively ..... *An. dirus* species B

Table 2 gives the branching of other significant setae of pupae in the three species.

Larvae (Figure 4)

Discriminant analysis of larval setae was also performed and 98.6% discrimination at 99.9% probability was obtained using the following ten characters: 7-M, 13-M, 9-I, 9-II, 2-III, 2-IV, 9-IV, 12-IV and 2-V. Three of the ten characters: 13-M, 9-I and 2-V could be used to construct a larval key whereby 98.6% correct identification was obtained for the larval material. The key is as follows:

1. The sum of seta 13-M on both sides lies in the range 9-17 branches ..... *An. dirus* species A

   The sum of seta 13-M on both sides lies in the range 5-12 branches 2

2. The sum of setae 9-I and 2-V on both sides lies in the range 5-8 and 6-8 branches respectively ..... *An. balabacensis*

   The sum of setae 9-I and 2-V on both sides lies in the range 7-12 and 6-10 branches respectively ..... *An. dirus* species B

Table 3 gives the branching of other significant setae in the three species.

DISCUSSION

Studies of the immature stages of *Anopheles balabacensis* and several forms of this taxon have been limited because Reid (1968) has combined the figures for *"b. balabacensis"* from North Malaya and Borneo with those of *bal. introlatus* and the "Fraser's Hill form." This suggests that these forms were virtually inseparable. However, the three newly distinguished species have been

satisfactorily separated using computer analysis in conjunction with Belkin's system. The clear separation of the three forms achieved by discriminant function analysis supports the previously determined genetic status of each of the three species. Thus multivariate analysis can be used as a tool to link population genetic studies of genetical species with formal taxonomy. When used in this way, it can give a genetic identity for type specimens and perhaps provide a practical means of identifying unknown specimens in malariometric entomology studies. A logical extension of this study is to use the canonical variate to identify type specimens and to study geographic variation of the three forms. Measurements of the types of *An. balabacensis* as well as those species currently recognized as synonyms will then be entered into the programme as unknown in order to determine whether any of these available names should be assigned to the species covered in this study. Preliminary tests using Smithsonian specimens collected from Chumphon and Phangnga provinces in southern Thailand indicates that the specimens of a chromosomal X_A type is *An. dirus* species A with a 99.9% probability. However the only limitation of this study is that setal counts from colony materials may differ from wild populations. For example, Peyton & Harrison (pers. comm.) has found the high branching counts of setae 1-II and 5-III of *An. dirus* species B did not match with wild specimens from Peninsular Malaysia. Unfortunately the precise genetic basis of morphological variation is rarely known and therefore one either knows or suspects that phenotypes are often a complex interplay between genes and environment. If variation appears homogeneously within and between broods of insects caught together there may be good grounds for suspecting two or more species present in samples.

It is felt that the work presented here adequately emphasize the advantages and usefulness of using modern multivariate analysis as a tool in taxonomy. Such an approach may increase our understanding of the epidemiology of vector-borne diseases and this may mean that control programmes against a group of species could be planned and monitored more carefully. This in turn requires an ability to identify single specimens especially from sympatric populations. For such a purpose, taxonomy must seek to define genetically-defined species within a formal framework and to provide practical means of identification appropriate to operational procedures in routine malariometric entomology.

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Table 1. Details of sample sizes used in multivariate analysis.

X - Karyotype	Number of: larvae	pupae	Total
X_A	24	32	56
X_B	24	31	55
X_C	31	36	67

Table 2. Mean number of branches for setae of significance other than those used in the pupal key.

Seta	*An. dirus* A	*An. dirus* B	*An. balabacensis*
7-II	3.1	5.2	4.7
1-IV	5.5	8.3	7.2
3-IV	6.9	8.3	7.0
3-V	2.9	3.8	2.7
9-VIII	11.3	17.2	1.2
2-P	1.9	2.0	2.6

Table 3. Mean number of branches for setae of significance other than those used in the larval key.

Seta	*An. dirus* A	*An. dirus* B	*An. balabacensis*
7-M	2.5	2.9	3.1
9-II	6.6	5.3	7.1
2-III	5.9	7.1	5.9
2-IV	3.7	4.1	3.0
12-IV	3.1	2.5	2.1

Fig. 1

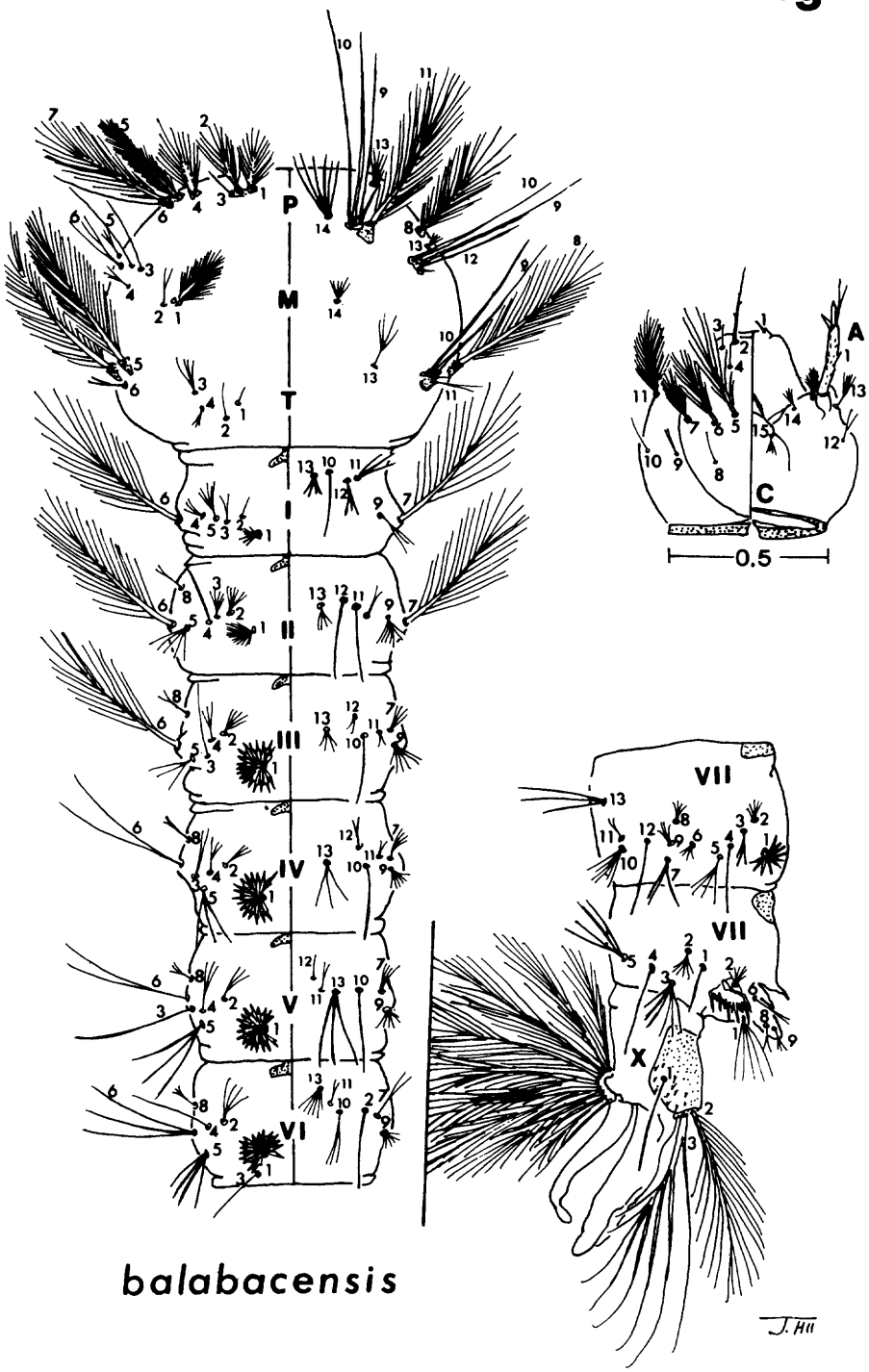

Figure 1. Larval chaetotaxy of *An. balabacensis*.

Fig. 2

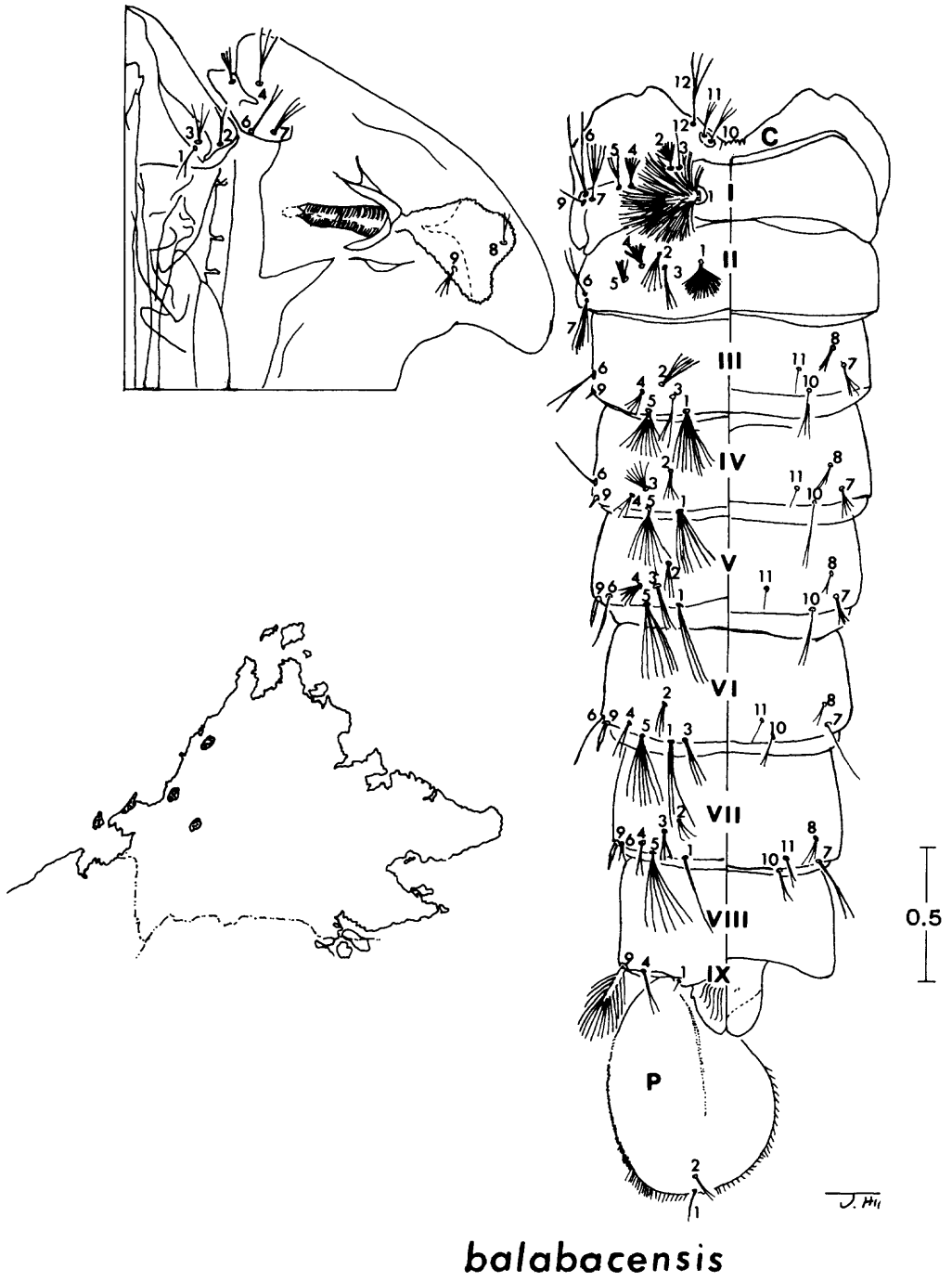

Figure 2. Pupal chaetotaxy of *An. balabacensis*.

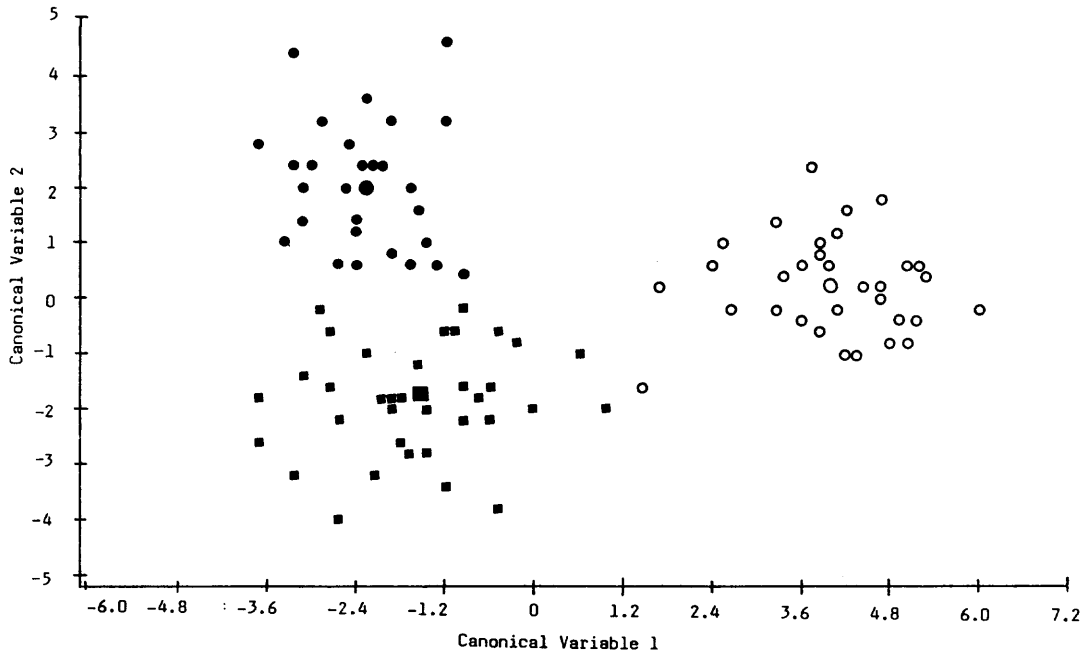

Figure 3. Discrimination of the pupae of *An. dirus* species A (o), *An. dirus* species B (●) and *An. balabacensis* (■) with respect to the first two canonical axes. Large symbols indicate a group centroid.

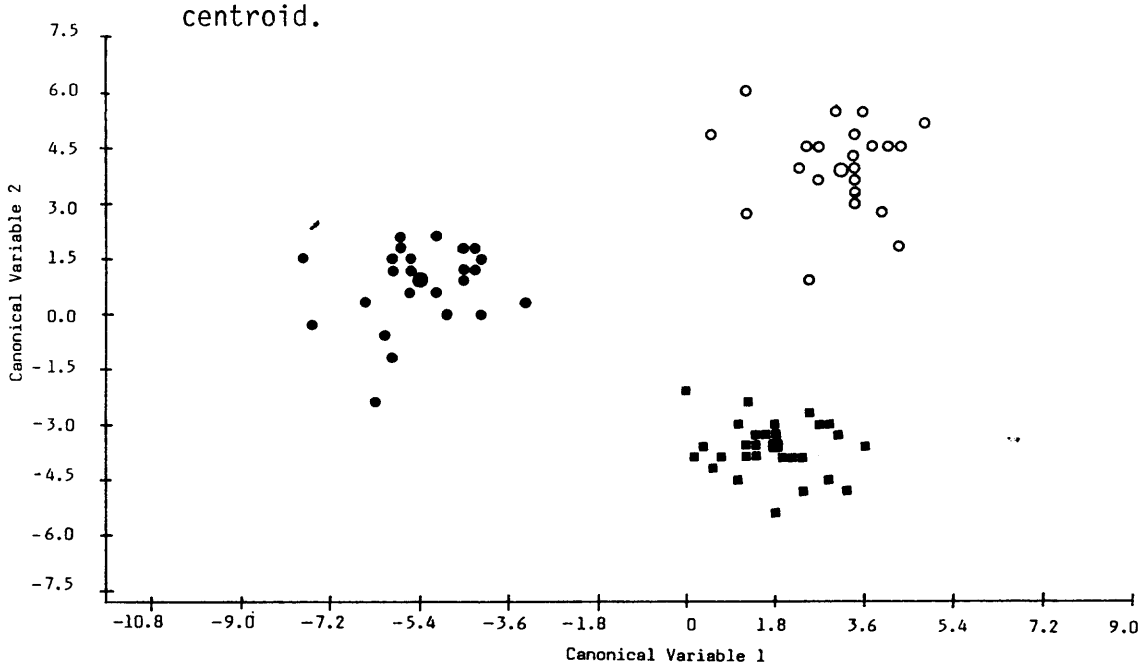

Figure 4. Discrimination of the larvae of *An.dirus* species A (o), *An. dirus* species B (●) and *An. balabacensis* (■) with respect to the first two canonical axes. Large symbols indicate a group centroid.