Morphometric Discrimination of Females of Five Species of *Anopheles* of the Subgenus *Nyssorhynchus* from Southern and Northwest Colombia

David A Calle L, Martha L Quiñones, Holmes F Erazo*, Nicolás Jaramillo O**/+

Programa de Estudio y Control de Enfermedades Tropicales **Grupo de Morfometría Biológica, Instituto de Biología, Universidad de Antioquia, A.A. 1226, Medellín, Colombia *Departamento Administrativo de Salud, Putumayo, Colombia

The most important vectors of human Plasmodium *in the neotropics belong to the subgenus* Nyssorhynchus. *These species are generally sympatric in terms of their geographical distributions. Some are difficult to identify based solely on examination of adult females using the available morphological keys, in these cases examination of immature stages and male genitalia is required to make correct determinations. However, in epidemiological studies it is necessary to identify the species of adult females which are found near humans, i.e. in studies of malaria transmission or evaluation of control measures. The purpose of the present study was to evaluate the discrimination of adult females of different species of* Nyssorhynchus *isolated mainly from Southern Colombia (department of Putumayo), using morphometric analysis. Adult females were obtained after rearing larvae collected in natural breeding places and from the progeny of females collected on humans. The morphological characteristics of the immature stages allowed the identification of four species of the subgroup Oswaldoi from Southern Colombia:* Anopheles rangeli Gabaldon, Cova Garcia & Lopez, *An. oswaldoi (Peryassu),* An. benarrochi Gabaldon, Cova Garcia & Lopez and *An. triannulatus (Neiva & Pinto). The species* An. nuneztovari (Gabaldon) from the Northwest of Colombia was included for comparison. Morphometric analysis allowed differentiation of the females of all species to a confidence level approaching 90% using principal components analysis of 10 wing and leg variables, followed by canonical variate analysis of the first four principal components. We conclude that morphometrics may represent a useful taxonomic tool for this group and that its use should be further studied.*

Key words: taxonomy - traditional morphometrics - malaria - canonical variate analysis - Southern Colombia

The subgenus *Nyssorhynchus* Blanchard (Diptera: Culicidae) contains the principal vector species of human *Plasmodium* in the neotropics including Colombia (Faran 1980). Some vectors are generally found to share the same geographical area and present high degree of morphological similarity at the adult stage, particularly among the females. This, together with the fact that the available keys do not take into account the great morphological variability of each species, produces confusion when attempting to identify an isolate based solely on specimens of adult females. For this reason, the characters recommended for accurate species identification are chaetotaxy of the fourth-instar larvae and morphological characters of the male genitalia (Faran 1980).

This problem is well illustrated by the frequent reports of isolation of *Anopheles evansae* from human bait collections in different areas of Colombia (Quiñones et al. 1987, 1992, Suárez et al. 1990, Perez et al. 1999, Villegas et al. 2000). In this context, Faran (1980) reported that *An. evansae* is restricted to the Brazilian Amazon, Bolivia and Argentina, suggesting that information on this species in

Colombia may reflect misidentification. In Southern Colombia, by obtaining isofamilies from adult females collected on human bait, it was verified that adult female specimens previously identified as *An. evansae* were in fact *An. benarrochi* (Quiñones et al. 2001). The high degree of morphological variability presented by adult females of *An. benarrochi* in this region also leads to misidentification with *An. oswaldoi*. In Southern Colombia a morphological variant of *An. benarrochi* was found in which adult females of this species have the dark portion of the hind tarsomere 2 ranging from 0.17 to 0.33 the length of the tarsomere (Quiñones et al. 2001), which is smaller than described for *An. benarrochi* in the different available keys (Faran 1980, Faran & Linthicum 1981, Rubio-Palis 2000), and overlaps the ranges reported for *An. oswaldoi*. The presence in Southern Colombia (Putumayo) of *An. benarrochi* instead of *An. evansae* was also confirmed by analysis of the nucleotide sequence of ITS-2 region and a fragment of cytochrome oxidase subunit I of mitochondrial DNA (Quiñones et al. 2000). These sequences were particularly useful for separating adult females of *An. benarrochi* from *An. oswaldoi*, these being the species whose Putumayo populations are indistinguishable using the available morphological keys.

Traditional multivariate morphometry could contribute towards resolving taxonomic problems such as those mentioned above. Furthermore, this method has been shown to be useful for the distinction of medically important insects that present problems in their identification (Añez et al. 1997, Gebre-Michael & Medhin 1997, Petrarca

et al. 1998). Morphometry of *Anopheles* of the subgenus *Nyssorhynchus* have been performed in Venezuela producing results that clearly separated different species i.e. *An. darlingi, An. marajoara, An. braziliensis* and *An. argyritarsis*, using cluster analysis and Euclidian distances (Rubio-Palis et al. 1997, Rubio-Palis 1998, 2000). In addition, morphometry has been useful in clarifying the taxonomic status of *An. darlingi*. This species shows considerable variation in biology and morphology along its distribution range suggesting that it could be a species complex. Euclidean distances were estimated by cluster analysis using wing, legs and head characteristics of 17 populations. This information, together with isozyme analysis, RAPD and sequencing of ITS-2, clarified its status as a single species (Manguin et al. 1999).

In the present study, canonical variate analysis, discriminant analysis and cluster analysis were used, based on the new variables generated by principal components analysis (PCA), to select an aggregate of morphological characters which collectively could differentiate females of five species [four species present in the South (Putumayo) and one species from Northwest Colombia] pertaining to the subgenus *An. (Nyssorhynchus)* with a high level of confidence.

MATERIALS AND METHODS

Collection of Anopheles - Adult mosquitoes were collected in three localities of the municipality of Puerto Asís (00°30'57"N; 76°29'42"W) and one locality in the municipality of Puerto Guzmán (00°58'13"N; 76°35'09"W) in the department of Putumayo, Southern Colombia. In addition, *An. nuneztovari* mosquitoes were collected in the municipality of Segovia (07°05'00"N; 74°42'13"W), department of Antioquia, Northwest Colombia.

Series were obtained by rearing larvae collected in the field. Larvae were reared individually and the exuviae preserved in 70% alcohol prior to mounting for taxonomy. Some larvae were also preserved for taxonomy. Once the adult emerged it was identified using traditional keys (Faran 1980) and one of its hind legs and wings were cut off for morphometric measurements.

Several isofamilies were also reared from females collected on human landing catches. The females were allowed to feed on mice in the laboratory and three days later were induced to oviposit by submitting them to stress by cutting off one wing and hind leg before placing them in pots containing water. Ten eggs were removed from each oviposition vial for species identification (Lounibos et al. 1997) and larvae were permitted to eclode from the others. These insects were raised to adult stage before continuing the same procedure describing above. Genitalia of the males of each isofamily were removed and retained for species identification.

Insects for morphometric study - The morphometric study was carried out with 115 females pertaining to five species of the subgenus *Nyssorhynchus. An. rangeli* (20 individuals), *An. oswaldoi* (24 individuals) and *An. triannulatus* (18 individuals) from Putumayo were obtained from series. The study also employed 33 individuals of *An. benarrochi* that had been identified based on morphology of eggs, larval and pupal exuviae of individuals

from each isofamily. Twenty individuals of *An. nuneztovari* were identified from larvae collected in the department of Antioquia and were also included in the analysis due to similarities observed between this species and *An. benarrochi* from Southern Colombia.

Measurements - To analyse the usefulness of the characteristics in Faran's key (1980) and those of Delgado and Rubio-Palis (1993) for taxonomic discrimination of the *Nyssorhynchus* species found in Southern Colombia, the following characters from legs and wings spots were measured using material fixed with transparent tape onto microscope slides: (1) the ratio of the humeral pale spot length of the costal vein to prehumeral dark spot length of the same vein, (2) subcostal pale spot length of the costal vein to subcostal dark spot of the same vein, length ratio, and (3) basal dark spot of hind tarsomere 2 to the total length of the same tarsomere. The morphological terms and abbreviations used follow Harbach and Knight (1980, 1982) and Wilkerson and Peyton (1990).

The following lengths of wing spots, whose measurements showed the greatest degree of precision and accuracy following the criteria of Sokal and Rohlf (1995), were selected for the multivariate analyses: (1) humeral dark, (2) presectoral pale, (3) sector pale, (4) preapical dark and (5) preapical pale. The following spots on the hind legs were selected in the same way: (1) dark of hind tarsomere 1 (Ta-III$_1$), (2) pale of hind tarsomere 2, (3) dark of hind tarsomere 2, (4) pale of hind tarsomere 3 and (5) pale of hind tarsomere 4.

A Nikon model SMZ-1 stereomicroscope with magnification of 20X and a graduated micrometer with a scale divided into 100 units, each equivalent to 38 µm, was used for the measurements. The outlying individuals in an analysis of the Mahalanobis distances were measured again to correct possible errors of measurement or data entry.

Multivariate analysis - A principal component analysis (PCA) was carried out, with the objective of examining morphometric relations between the diverse taxa. The PCA involves a priori assumption that all the individuals belong to a single group, within which it is hoped to detect the species (Pimentel 1992). Since the first four principal components gave rise to 94% of the original variance, they were used as new variables to carry out canonical variate analysis. The objective of this analysis was to maximise variation between the species and minimise intraspecific variation. The projection of the individuals onto the first two canonical factors permitted visual examination of the ordering of the diverse taxa in the multivariate space (Pimentel 1992).

To examine the relationship of morphological similarity between the species, cluster analysis was performed based on Mahalanobis distances matrix, obtained from the first four components of a PCA. These distances were used to construct a dendrogram using the unweighted pairs group method analysis (UPGMA). This dendrogram was compared visually with the hypothetical phylogenetic tree proposed by Faran (1980).

Mahalanobis distances were used for the discriminant analysis, these being a measure of the distance of each individual from the centroid of each species. Each dis-

tance is associated with a probability of pertaining to each species and this was used to classify the females into the most probable taxa. Such a reclassification was compared with that carried out a priori, verifying concordance between the two by means of the Kappa statistic. A concordance value of 0.81 to 0.99 is considered to be almost perfect (Landis & Koch 1977). Data were analysed using the computer programmes NTSYS (Applied Biostatistics Inc 1998), JMP (SAS Inst 1999), STATA (Stata Corp 2000) and Excel (Microsoft 2000).

RESULTS

Characteristics used in keys - Overlap was found between the ranges of the characteristics measured and those used in conventional keys (Faran 1980, Faran & Linthicum 1981, Delgado & Rubio-Palis 1993, Rubio-Palis 2000) for *An. oswaldoi*, *An. rangeli*, *An. benarrochi* and *An. nuneztovari* (Table). In contrast, *An. triannulatus* could be distinguished from the other species principally by the dark proportion of hind tarsomere 2 and the size of the subcostal pale spot, which also presented the greatest variability.

Canonical variate analysis - The centroids of each species could be differentiated statistically in the multivariate space formed by canonic factors 1 and 2, *An. triannulatus* being the species which was best discriminated. When each female was projected onto the canonical factors 1 and 2 and the most external individuals of each species connected by straight lines to form polygons, a good interspecific discrimination continued to be evident (Fig. 1). Nevertheless there was an overlap of less than 10% between the polygons corresponding to *An. benarrochi* and *An. oswaldoi* and also between *An. rangeli* and *An. nuneztovari*, indicating that the few individuals localized in these areas cannot be assigned with confidence to any of the species.

Cluster analysis - Based on the dendrogram, *An. triannulatus* showed the greatest morphological differentiation, while *An. nuneztovari* and *An. rangeli* were the most similar species (Fig. 2).

Reclassification of the females into their species - The Kappa statistic showed a degree of concordance of 89% between the classification predicted by the Mahalanobis distances and those obtained by the examination of series and isofamilies, representing an almost perfect reclassification according to the criteria of Landis and Koch (1977). *An. benarrochi* reclassified correctly for 91%, *An. oswaldoi* for 91.7 %, *An. rangeli* for 85 %, *An. nuneztovari* for 90 % and *An. triannulatus* for 100 % of specimens.

DISCUSSION

The morphometric multivariate techniques demonstrated that some combinations of morphological variables may serve to classify in their respective species approximately 90% of the females of the subgenus *An.* (*Nyssorhynchus*) included in the present study, quantitatively and without ambiguities. In contrast, conventional keys do no allow a correct classification to species in the majority of the same females. Thus, canonical variate analysis showed a clear separation of the different species in the space created by the first two canonical variables, which represent the greatest portion of the significant variance (93.087%).

The dendrogram obtained based on the Mahalanobis distances between the centroids of the species and projected onto the canonical factors (Fig. 2) presented a good correlation with the hypothetical phylogenetic tree constructed by Faran (1980) for the subgenus *Nyssorhynchus* (Fig. 2). *An. triannulatus* is the species furthest from the others in the two trees. It is noteworthy that Faran (1980)

TABLE

Mean values and ranges for the basal dark spot of hind tarsomere 2 (Ta-III$_2$) to the total length of the same tarsomere; the ratio of the humeral pale spot (HP) length of the costal vein to prehumeral dark spot (PHD) length of the same vein (HP/PHD); subcostal pale spot (SCP) length of the costal vein to subcostal dark spot of the same vein (SCD), length ratio (SCP/SCD), compared with ranges reported by Faran, 1980 (1) and Delgado and Rubio-Palis (1993)

| Species	Trait	Mean	SD	Range	Ranges reported in literature	
					1	2
Anopheles nuneztovari	TaIII$_2$	0.30	0.02	0.25 - 0.35	0.20 - 0.32	0.11 - 0.44
N = 20	HP/PHD	1.74	0.43	1.20 - 2.30	0.70 - 1.70	06.0 - 4.50
	SCP/SCD	0.50	0.09	0.36 - 0.71	0.20 - 0.55	0.03 - 0.73
Anopheles rangeli	TaIII$_2$	0.25	0.03	0.20 - 0.33	0.24 - 0.35	0.14 - 0.38
N = 20	HP/PHD	3.30	1.18	1.40 - 5.00	1.00 - 3.70	1.80 - 4.50
	SCP/SCD	0.77	0.18	0.54 - 1.20	0.45 - 1.00	0.53 - 0.80
Anopheles benarrochi	TaIII$_2$	0.26	0.03	0.17 - 0.33	0.36 - 0.55	ND
N = 33	HP/PHD	2.27	0.55	1.70 - 3.30	> 2.5	ND
	SCP/SCD	0.32	0.09	0.10 - 0.55	0.30 - 0.50	ND
Anopheles oswaldoi	TaIII$_2$	0.16	0.03	0.10 - 0.25	0.12 - 0.25	0.11 - 0.22
N = 24	HP/PHD	2.51	0.56	1.60 - 4.00	1.60 - 3.00	1.00 - 3.50
	SCP/SCD	0.20	0.14	0.04 - 0.66	0.10 - 0.50	0.11 - 0.44
Anopheles triannulatus	TaIII$_2$	0.37	0.07	0.26 - 0.54	0.40 - 0.70	0.20 - 0.43
N = 18	HP/PHD	1.35	0.46	0.60 - 2.30	0.50 - 1.30	0.40 - 1.10
	SCP/SCD	0.14	0.05	0.04 - 0.25	0.10 - 0.20	0.07 - 0.23
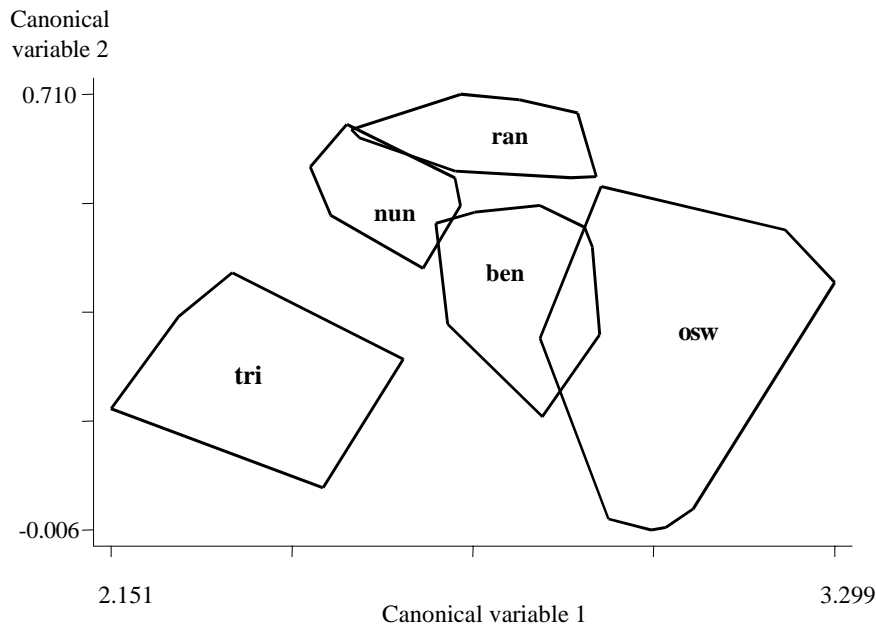
Fig. 1: polygons formed based on the projection of the individuals of each species onto the canonical variables 1 and 2, which gave rise to the greatest percentage of the total variation. ben: *An. benarrochi*, ran: *Anopheles rangeli*, nun: *An. nuneztovari*, tri: *An. triannulatus*, osw: *An. oswaldoi*.
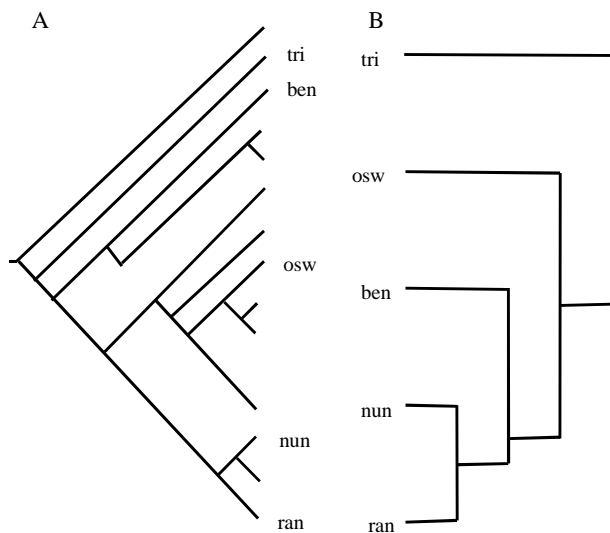

Fig. 2: comparison between the phenetic and phylogenetic relationship of five species of *Anopheles*. A: hypothetical phylogenetic tree proposed by Faran (1980) of the section Albimanus of the subgenus *Nyssorhynchus*; B: dendrogram constructed by UPGMA based on the Mahalanobis distances matrix. ben: *An. benarrochi*, ran: *An. rangeli*, nun: *An. nuneztovari*, osw: *An. oswaldoi*, tri: *An. triannulatus*.

groups, as in the hypothetical phylogenetic tree of Faran (1980).

The only unexpected position was that of *An. benarrochi*, which in our dendrogram occupies a position close to *An. nuneztovari* and *An. rangeli*. This is in disagreement with the hypothetical phylogenetic tree of Faran (1980), and may be due to the fact that populations from the south of Colombia represent a different morphologic variant to that studied by that author (Quiñones et al. 2001). According to Faran (1980) *An. benarrochi* is closely related to *An. strodei*, based on comparison of some structures of the male genitalia (basal lobe extending apically and rugose on the apical margin of the ventral clasper). Unfortunately we were not able to include the latter species in our study because no specimens were available. In the strict sense, comparing the hypothetical phylogenetic tree of Faran (1980) with our dendrogram only makes sense if the species evolved at a constant velocity such that the lengths of the branches on the tree represent equal evolutionary distances for each species; however our comparison only seeks to establish concordance between the phenetic similarities of the species and their phylogenetic relationships, considering that the morphological features grow in an integrated manner peculiar to each species.

The classification predicted by the discriminant functions derived from the Mahalanobis distances showed an almost perfect concordance (Kappa = 0.89) according to Landis and Koch (1977). This result shows the potentiality of morphometry to separate species of the series Oswaldoi, subgenus *Nyssorhynchus* of *Anopheles* in Southern Colombia, whose adult females present morphological characteristics that do not permit accurate determination using existing keys.

removed this species from the subgroup Oswaldoi, which contains all those in the present study, placing it in the subgroup Triannulatus. *An. rangeli* and *An. nuneztovari* are located very close to each other in the dendrogram, supporting the hypothesis that they are sister groups (Faran 1980). On the other hand, *An. oswaldoi* and *An. benarrochi* present themselves as belonging to separate

In conclusion, the traditional multivariate morphometry employed during the present study demonstrated its usefulness in separating females of five species of the subgenus *Nyssorhynchus* from Southern and Northwest Colombia, that are difficult to identify using the dichotomous morphological keys currently available. It is important to point out that this is the first study to use multivariate techniques to resolve taxonomic problems in the subgenus *Nyssorhynchus* in Colombia. The evidence presented here justifies continuation of the search for groups of variables and techniques of traditional and geometric morphometry to achieve the maximum degree of discrimination among adult females of these and the other 25 species of *Nyssorhynchus*.

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