CHAPTER 20

MULTIVARIATE DISCRIMINATION BETWEEN TWO CRYPTIC *HAEMAGOGUS* SPECIES ASSOCIATED WITH THE TRANSMISSION OF YELLOW FEVER VIRUS IN THE AMERICAS

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ABSTRACT: Mosquitoes of the genus *Haemagogus* are important vectors of yellow fever virus and other arboviruses and are the principal reservoirs of these viruses in nature. *Haemagogus capricornii* and *Haemagogus janthinomys* are closely related species between which females are morphologically cryptic. A morphometric study of these species was performed on male and female specimens from 14 municipalities in Brazil. Morphometric analyses were able to distinguish females. Multivariate morphometrics may be a useful tool for taxonomic studies of cryptic species in this group.
KEYWORDS: *Haemagogus*, morphometry, Culicidae, vectors

INTRODUCTION

The principal genera of mosquitoes capable of becoming infected with and transmitting yellow fever virus (YFV) are *Aedes*, *Haemagogus*, and *Sabethes*. These act as biological vectors in forested areas of the Americas, and 98% of isolations of YFV by Arbovirus Laboratory of Evandro Chagas Institute, in Belém, ParáState, Brazil, were

obtained from mosquitoes of these genera (Vasconcelos et al. 1997). *Haemagogus* are essentially wild species, and they have diurnal and acrodendrophilous habits. Some of them show a tendency to invade domiciles, while others are usually found outside of the human environment (Forattini 2002).

The genus *Haemagogus* encompasses 28 species, some of which are epidemiologically very important due to their involvement in the transmission and maintenance of YFV and other arboviruses in nature. *Haemagogus capricornii* Lutz is an efficient vector for YFV (Waddell and Kumm 1948, Waddell, 1949). *Haemagogus janthinomys* Dyar, considered to be the principal vector for YFV, is widely distributed from northern Argentina and southern Brazil to Honduras and Nicaragua and has been reported in all Brazilian states from Paranánorthward, eastern areas of Peru and Colombia, all of Venezuela, the Guianas, and Trinidad and Tobago (Arnell 1973).

The systematics of *Haemagogus* species were revised by Arnell (1973). This author showed that although *Hg. capricornii* and *Hg. janthinomys* were distinct species on the basis of the morphology of the male genitalia, the females of the 2 species were indistinguishable. Arnell (1973) also showed similarities between the diagnostic characteristics of the adult female forms and 4th-stage larvae of *Hg. capricornii* and *Hg. janthinomys*. Variability in the apical process of the aedeagus was utilized by Martínez et al. (1960) as the basis for designating subspecies. Arnell (1973) also described variation in the apex of the aedeagus, which contained an apical process that varies between beak-like and elongated to a heavily sclerotized keel. Based on aedeagus variability, Martínez et al. (1960) considered *Hg. capricornii capricornii*, *Hg. capricornii falco*, *Hg. capricornii janthinomys*, and *Hg. capricornii petrocchiae* as valid taxa. Larval and pupal stages were not morphometrically analyzed due to the small number of specimens and the difficulty in finding the oviposition sites of these species, which are located in the tree canopies. However, scanning electron microscopy studies on eggs (Linley and Chadee 1991, Alencar et al. 2005a) showed differences in the ornamentation of the exocorium, confirming the existence of marked differences between these 2 species.

Secundino et al. (1994) observed in some *Haemagogus* species from the Culicidae Collection of the Ren é Rachou Research Center (Fiocruz, Belo Horizonte) that some of the morphological characteristics did not correspond to those described by Arnell (1973). There were divergences relating to: bristles of the lower sternopleura, scales of the abdominal terga, and size of the wing cells (R_{2+3} and R_2). These divergences are inconsistent with existing dichotomous keys. According to Forattini (2002), variation in metallic coloration is inaccurate in distinguishing these species and can only be used in geographic regions where the 2 species exhibit extreme color variation. Methods utilizing molecular biology polymerase chain reaction (PCR) or biochemistry (isozymes) could be utilized, but they are expensive and depend on the availability of specialized equipment.

Morphometric analyses are increasingly being used to resolve taxonomic problems in other vector groups, such as Triatominae (Matias et al. 2001, Gumiel et al. 2003), Culicidae (Calle et al. 2002), Phlebotominae (Dujardin et al. 1999, 2005), Glossinidae (Patterson and Schofield 2005), and Ixodidae (Hutcheson et al. 1995, Klimov and Connor 2004). Results are often congruent with other phylogenetic techniques, such as isoenzymes (Patterson et al. 2001) or deoxyribonucleic acid (DNA) sequence analysis (Patterson and Schofield 2005).

While both *Hg. capricornii* and *Hg. janthinomys* are YFV vectors, they differ in host preferences. For example, in Caxias, 25.6% of *Hg. janthinomys* reacted to bird antiserum (Alencar et al. 2005b), while 52.9% of *Hg. capricornii* reacted to the same antiserum (Alencar et al. 2008b). Additionally, *Hg. janthinomys* activity is more influenced by the humidity, and activity in *Hg. capricornii* is influenced by temperature (Alencar et al. 2008a).

Due the risk of the emergence of YFV in areas previously considered to be virus free (Costa et al. 2002) and the sympatry of these 2 species in certain regions of Brazil, it is necessary to distinguish these species by means of more sophisticated techniques such as morphometry. With the goal of discriminating between the species, multivariate analyses on certain characteristics of adult females and males of *Hg. capricornii* and *Hg. janthinomys* were conducted, and these characteristics were assessed as taxonomic tools.

MATERIALS AND METHODS

The areas studied were the tropical forest (Atlantic Forest) and savanna biomes. The Atlantic Forest biome typically consists of tropical rain forest and covers the slopes of the mountain ranges that run along the Atlantic coast of Brazil. The savanna biome includes different phytophysiognomic and floristic types known as true savanna, open savanna, closed savanna, and riverbank or gallery forest formations, which include vegetation of twisted trees and bushes. The occurrence of this type of vegetation is common in regions where dry periods of 4 to 5 months are common (Pádua and Filho 1979).

Mosquitoes

The adult mosquitoes analyzed in this study came from the Entomological Collection of the Department of Entomology of the Oswaldo Cruz Institute (Rio de Janeiro, RJ, Brazil). The study was conducted on 227 adult specimens: 147 *Hg. janthinomys* (94 females and 53 males) and 80 *Hg. capricornii* (63 females and 17 males), from 14 localities (Table 1).

The specimens were identified by direct observation of the morphological characteristics under stereoscopic and transmitted-light microscopes using the dichotomous keys of Arnell (1973) and Forattini (2002). For samples in which only 1 species was identified, females were identified by association with identified males.

Morphometrics

Measurements were made of certain mosquito characteristics (Figure 1) using a stereoscopic microscope (ZEISS Stemi SV6H) with a 103 eyepiece and 43 zoom. A micrometer (100 div 5 10 mm) was calibrated using a micrometric lamina (0.001 mm). Figure 1 shows the following characters that were measured: wing, total length of anterior femur (AnFe), total length of the posterior femur (PoFe), length of silver marking on posterior femurs (SilMa), length of the proboscis (Prob), and length of the palpus (Palp). For the 2 remaining variables, longitudinal vein (R_{2+3}) and secondary vein (R_2), 53 zoom was utilized (Fig. 1).

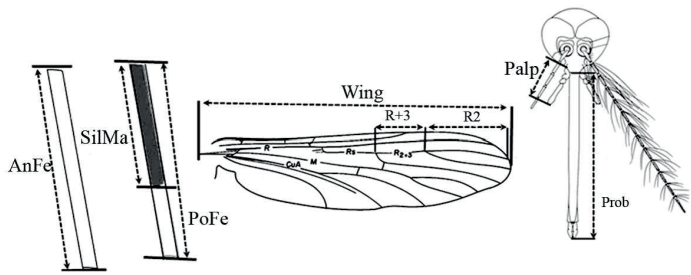

Fig. 1. Variables analyzed: total length of wing (Wing), total length of anterior femur (AnFe), total length of posterior femur (PoFe), length of silver marking on posterior femurs (SilMa), length of proboscis (Prob), length of palpus (Palp), longitudinal vein (R_{2+3}), and secondary vein (R_2).

Table 1. Geographic distribution of the *Haemagogus* species analyzed according to locality and sex in different municipalities with state abbreviations in Brazil.

Municipalities[1]	Latitude (S)	Longitude (W)	Altitude (m)	*Hg. janthinomys*		*Hg. capricornii*		Total
				Male	Female	Male	Female	
Caldas Novas GO	17º44′30″	48º37′30″	686	04	36			40
Canavieiras BA	15º40′30″	38º56′50″	04	17				17
Chapada dos Guimarães MT	15º27′38″	55º44′59″	811	06	22			28
Duque de Caxias RJ	22º47′08″	43º18′42″	19	12		02	18	32
Ibirama SC	27º03′25″	49º31′04″	150			02	02	04
Ituiutaba MG	18º58′08″	49º17′54″	544	14				14
Linhares ES	19º23′28″	40º04′20″	33	04				04
Pirapora MG	17º20′4″	44º56′31″	489			06	11	17
Maraú BA	14º06′11″	39º00′53″	36	03				03
Minaçu GO	13º31′59″	48º13′12″	351	02	16			18
Patrocínio MG	18º56′38″	46º59′33″	965	03				03
TinguáBiological Reserve RJ	22º45′33″	43º13′12″	25			03	27	30
São Luiz Gonzaga RS	28º24′30″	54º57′39″	231			09		09
Uruaçu GO	49º08′27″	14º31′29″	520			01	07	08
Total				65	74	23	65	227

[1] GO, Goiás; BA, Bahia; MT, Mato Grosso; RJ, Rio de Janeiro; SC, Santa Catarina; MG, Minas Gerais; ES, Espírito Santo; RS, Rio Grande do Sul.

Table 2. Univariate analysis of structures and dimensions of both sexes of *Haemagogus capricornii* and *Haemagogus janthinomys*.

Traits[1]	Samples	Mean[2]	Standard error	95% conf.	interval
Wing length	*Hg. capricornii* male	426.5	30.6	410.8	442.2
	Hg. capricornii female	459.6	35.6	450.6	468.5
	Hg. janthinomys male	404.9	31.0	396.3	413.4
	Hg. janthinomys female	447.7	42.1	439.1	456.2
R_{2+3}	*Hg. capricornii* male	76.4	6.8	72.9	79.9
	Hg. capricornii female	80.1	6.5	78.5	81.8
	Hg. janthinomys male	73.2	5.6	71.6	74.7
	Hg. janthinomys female	78.8	7.7	77.2	80.3
R_2	*Hg. capricornii* male	42.7	4.6	40.3	45.0
	Hg. capricornii female	56.2	6.3	54.6	57.8
	Hg. janthinomys male	42.7	5.3	41.2	44.2
	Hg. janthinomys female	57.2	9.8	55.2	59.2
Length AnFe	*Hg. capricornii* male	319.0	16.6	310.5	327.6
	Hg. capricornii female	327.4	27.5	320.4	334.3
	Hg. janthinomys male	301.8	30.4	293.4	310.1
	Hg. janthinomys female	322.4	33.0	315.6	329.1
Length PoFe	*Hg. capricornii* male	294.4	25.4	281.3	307.5
	Hg. capricornii female	316.2	26.5	309.6	322.9
	Hg. janthinomys male	277.9	25.5	270.9	284.9
	Hg. janthinomys female	308.4	30.8	302.1	314.7
SilMa	*Hg. capricornii* male	237.5	28.2	223.1	252.0
	Hg. capricornii female	260.8	28.7	253.6	268.0
	Hg. janthinomys male	241.9	38.2	231.4	252.4
	Hg. janthinomys female	282.5	33.8	275.6	289.4
Proboscis	*Hg. capricornii* male	377.6	29.9	362.2	392.9
	Hg. capricornii male	358.0	34.8	349.2	366.8
	Hg. janthinomys male	379.7	27.0	372.3	387.1
	Hg. janthinomys female	338.9	35.1	331.8	346.1
Length palpus	*Hg. capricornii* male	40.2	5.4	37.4	43.0
	Hg. capricornii female	46.9	8.1	44.9	49.0
	Hg. janthinomys male	46.2	7.6	44.1	48.3
	Hg. janthinomys female	41.8	7.6	40.2	43.3

[1] AnFe, anterior femur; PoFe, posterior femur; SilMa, length of silver marking on posterior femur.

[2] Means (values in microns), standard error (standard error), and 95% confidence interval (95% conf. interval) are given for 8 metric traits of male (C).

Table 3. Multiple analysis of variance (MANOVA) results for differences between sex and species.[1]

	Wilk's lambda	F	Df num	Df den	Prob.F
Sex	0.174507	34.887	8	59	<.0001
Species	0.519649	68.173	8	59	<.0001
Sex vs. species	0.544724	61.640	8	59	<.0001

[1] F, F values for the multivariate tests; Df num, the numerator degrees of freedom; Df den, the denominator degrees of freedom; Prob.F, the significance probability corresponding to the F ratio.

Numerical analyses

For all variables, log-transformed measurements (in mm) (Pimentel 1992) were used. Means, standard deviations, and 95% confidence inter- vals were calculated for the 8 variables in both sexes of the 2 species. When discriminant analysis is used, unequal group sizes may lead to a very high percent correct classification, but the improvement over random correct classification may be slight (Titus et al. 1984). For consistency regarding the small samples in some groups, a subset of each of these groups was obtained. The values for each subset were formed proportionally to the numbers of individuals per locality in the group. Means from every 2, 3, or 4 individuals were used. The intention was to conserve the information on the morphological variability between the individuals (c and j, *capricornii* and *janthinomys* male, respectively; f, female): cf 5 17, *c* 5 17, jf 5 18, and *j* 5 18. We then performed a 2-way multiple analysis of variance (MANOVA) to find significant differences between species, sexes, and the interaction between species and sexes. The degree of overlap among the females was assessed by discriminant analysis. The statistical significance from multivariate analyses was estimated (Wilks 1932). The results are displayed as points on the 1st 2 canonical axes. The discriminant correspondence classifications were summarized and verified statistically using Kappa statistics (Landis and Koch 1977, Viera and Garret 2005). Analyses were performed on the JMP package (SAS Institute 1995).

RESULTS

Means, standard errors, and confidence intervals are given in Table 2. These indicate that the females of these 2 species are generally larger than the males. MANOVA indicated significant differences between sexes and species and their interactions (Table 3). The 1st 3 canonical factors of the discriminant analysis summarized 100% of the total variation (Table 4). The discriminant analysis allowed perfect reclassification of female individuals (Table 5 and Figure 2). The functions of the first 2 canonical factors are:

Canonical factor 1: 0.92638507 (Wing) 2 2.5059695 (AnFe) 2 3.4588247 (PoFe) + 0.67753114 (SilMa) + 1.71218583 (Prob) 2 0.954102 (Palp) + 3.01858169 (R_{2+3}) + 0.65522522 (R_2).

Canonical factor 2: 0.48024958 (Wing) + 2.30227203 (AnFe) + 2.05631009 (PoFe) 2 4.7308625 (SilMa) + 4.59655113 (Prob) 2 6.7093122 (Palp) 2 0.214492 (R_{2+3}) + 2.55093866 (R_2).

In order to classify a new female specimen between the 2 cryptic females of *Hg. capricornii* and *Hg. janthinomys*, it is necessary to measure the 8 characters. The log-transformed measurements (in μm) of each variable of the new specimen must be multiplied by appropriate set of corresponding coefficients of the canonical factor 1 and 2, respectively. The sum of these products will result in a canonic value of each factor. These 2 values are plotted in Figure 2.

Table 4. Eigenvalues for the first 3 canonical factors of the discriminant analysis.[1]

	Cf 1	Cf 2	Cf 3
Eigen-values	4.9933134	0.9568273	0.5966037
%	76.27	14.62	9.11
Acc%	76.27	90.88	100

[1] Cf, canonical factor; %, percent of variance explained; Acc%, accumulated percent of variance explained.

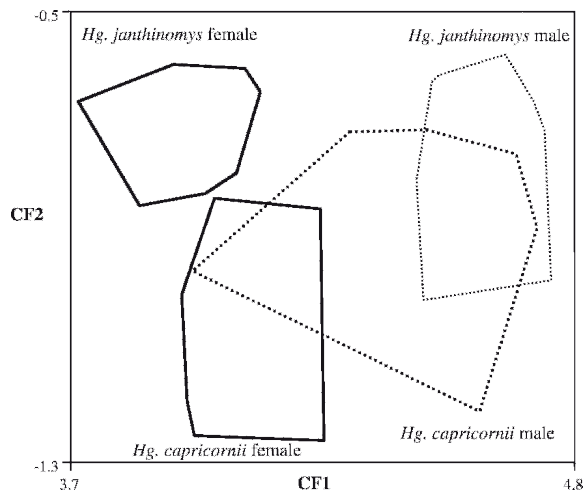

Fig. 2. Projections of *Haemagogus capricornii* female, *Haemagogus janthinomys* female, *Hg. capricornii* male, and *Hg. janthinomys* male onto the 1st canonical factor (horizontal axis) and 2nd canonical factor (vertical axis). The total variance explained by the 1st 2 canonical factors is 90.88% (76.27% and 14.62%, respectively). Polygons enclose the specimens in each group.

DISCUSSION

Haemagogus capricornii and *Hg. janthinomys* are sympatric in some localities in Brazil, but the distribution of each species is different, occupying ecosystems with important environmental differences (Alencar et al. 2008b). It is generally accepted that environmental changes will modify vectorborne disease transmission patterns (Patz et al. 2000), and empirical studies have shown that morphometric traits evolve via ecological selection (Rundle and Nosil 2005). Ecological speciation can proceed via divergence in just a few key genomic regions (Campbell and Bernatchez 2004, Emelianov et al. 2004) and can involve a small number of traits (Bradshaw and Schemske 2003, McKinnon et al. 2004).

The increase of average global temperatures and shifts in the climate on a global scale are becoming evident (Magnuson 2001, Moreno 2006). Vectorborne diseases may be relatively sensitive indicators of global changes, since transmission involves intermediate organisms, such as mosquitoes, that are strongly influenced by the environment (McMichael 2001). The effect of these changes on the geographic distributions of these 2 vectors remains an open question.

Due the risk of the emergence of YFV in areas previously considered to be free of the virus (Costa et al. 2002), the sympatry of these 2 species in certain regions of Brazil and the possibility of differential responses in transmission patterns and distribution make it critical to distinguish these species. The morphometric technique described here is an easy and accurate way to differentiate them. It is cheaper and faster than DNA (Gilchrist and Crisafulli 2006) and can be used with old museum material (Aytekin et al. 2007). The quantitative variables used in the present study permitted 100% discrimination of these species, greater than those of Calle et al. (2002) who, utilizing multivariate analyses, obtained 90% discrimination among females of 5 *Anopheles* species.

Table 5. Reclassification of females after discriminant function analysis.

Observed agreement for females (%)[1]			
Hg. capricornii	*Hg. janthinomys*	Expected agreement (%)[2]	Kappa[3]
100	100	50	0.913381

[1] Observed agreement indicates the proportion of individuals that have been correctly attributed to their respective group by the model.

[2] Expected agreement indicates the proportion of individuals correctly classified by chance alone.

[3] Kappa statistic measure of agreement is estimated between observed and expected classification; it is scaled from 0 to 1. A score between 0.81 and 1 is considered to be "almost perfect" or "perfect" (Landis and Koch 1977, Viera and Garrett 2005).

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