Comparison of Isoenzyme Electrophoresis and Morphometric Analysis for Phylogenetic Reconstruction of the Rhodniini (Hemiptera: Reduviidae: Triatominae)

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ABSTRACT A phylogenetic reconstruction of the medically important tribe Rhodniini (Hemiptera: Reduviidae) based on multilocus isoenzyme electrophoresis is compared with phylogenetic patterns derived from a traditional morphometric analysis. Even with non-normality in the morphometric data, and some inequalities in population variances, discriminant analysis of size-free variables provided broadly similar phylogenetic information to that derived from isoenzyme analysis, revealing 3 main species groups within the genus *Rhodnius*.

KEY WORDS Rhodniini, phylogeny, multilocus enzyme electrophoresis, morphometry.

THE TRIBE RHODNIINI Pinto comprises 2 genera, *Psammolestes* Bergroth and *Rhodnius* Stål, distinguished by the shape of the head and the width of femora. These genera differ from others of the Triatominae (Hemiptera, Reduviidae) by several morphological traits, particularly the apically inserted antennae and the presence of distinct callosities behind the eyes (Lent and Wygodzinsky 1979).

The genus *Psammolestes* contains 3 species distributed in South America east of the Andes from Venezuela to Argentina. They are invariably found in the woven stick nests of birds such as *Phacellodomus* sp., *Myiopsitta monacha*, *Pseudoseisura lophotes*, and others. The 13 recognized species of *Rhodnius* are also of primarily arboreal habitats, often occupying ecotopes in palm tree crowns or epiphytic bromeliads. However, the genus includes some highly domesticated species such as *R. prolixus* Stål, the major vector of Chagas disease in Venezuela, Colombia and parts of Central America (Schofield 1994), *R. pallescens* Barber, an important vector of Chagas disease in Panama (Pipkin 1968) and in some parts of northern Colombia (Guhl 1996, Moreno and Jaramillo 1996), and *R. ecuadoriensis* Lent & León in Ecuador (Aguilar and Yepez 1996). Several other species can enter dwellings and peridomestic habitats, and are of some local epidemiological importance, including *R. stali* Lent et al. (cited as *R. pictipes* Stål) in Bolivia (Tibayrenc and Le Pont 1984), *R. neglectus* Lent and *R. nasutus* Stål in Brazil (Carvalheiro and Barreto 1976; Silveira et al. 1983, 1984; Garcia-Zapata et al. 1985; Alencar 1987). *R. brethesi* Matta is primarily associated with piassaba palms (*Leopoldina piassaba*) but has been reported attacking humans in the Amazon region of Brazil (Coura et al. 1994). The remaining species (*R. dalessandroi* Carcavallo & Barreto, *R. domesticus* Neiva & Pinto, *R. neivai* Lent, *R. paraensis* Sherlock et al. *R. pictipes* Stål, and *R. robustus* Larousse) seem to be entirely silvatic, generally without epidemiological importance.

For some groups of *Rhodnius* species, such as *prolixus*, *robustus*, *neglectus* and *nasutus*, or *pictipes* and *stali*, there is no discrete morphological attribute that is reliably diagnostic, although molecular techniques can assist species determination (Dujardin et al. 1991, Solano et al. 1996). We wanted to see if a morphometric analysis could also be useful. Here we present a comparison of morphometrics and isoenzymes as methods for inferring phylogenetic relationships within the genus.

Materials and Methods

Insects. Adult specimens representing various populations of *Rhodnius* species ($n = 169$) were obtained from various sources (Table 1). In all cases, taxonomic determination was made by the originating laboratory using morphological characters following the keys of Lent and Wygodzinsky (1979) (Table 1). In our material, *R. prolixus*, from either natural populations or laboratory strains, was of domestic origin, collected inside dwellings (Table 1). One sample, supposed to be *R. prolixus*, was of silvatic origin, collected from palm trees in Colombia (Tolima) (Table 1). Thus, populations representing at least 8 species of *Rhodnius* were analyzed by both isoenzymes and morphometry.

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Table 1. Characteristics of the different samples analyzed

Code	1	2	3	*n*	4
RST	*R. stali*	Bermudez	Chapare, Bolivia	8	Natural 1993
RNA	*R. nasutus*	Jurberg	Ceara, Brazil	6	Insectary 1978
RNE	*R. neglectus*	Jurberg	Goias, Brazil	10	Insectary 1976
RDO	*R. domesticus*	Steindel	Santa Catarina, Brazil	8	Insectary 1984
			Santa Catarina, Brazil	4	Insectary 1996
			Santa Catarina, Brazil	14	Natural 1996
domRPR	*R. prolixus*	Jurberg	Cojedes, Venezuela	10	Insectary 1995
		Jurberg	Belem, Brazil	26	Insectary 1996
		Beard	Colombia	5	Insectary
		Ponce	Honduras	13	Natural 1996
		Shettino	Chiapas, Mexico	4	Insectary
		Moreno	Tolima, Colombia	7	Natural 1991
REC	*R. ecuadoriensis*	Jurberg	Peru	6	Insectary 1979
		Moreno	Ecuador	7	Insectary 1990
silvRPR	"*R. prolixus*"?	Moreno	Tolima, Colombia	8	Natural 1991
RPA	*R. pallescens*	Jurberg	Colombia	11	Insectary 1989
RBR	*R. brethesi*	Barrett	Brazil	4	Natural 1995
RPI	*R. pictipes*	Jurberg	Para, Brazil	10	Insectary 1989
RNV	*R. neivai*	Schofield	Venezuela	8	Insectary
PCO	*P. coreodes*	Noireau	Bolivia	18	Natural 1996
TIN	*T. infestans*	Dujardin	Bolivia	15	Natural 1996

1 Species identification based on external morphology; 2 Authority responsible for this morphological identification; 3 geographic origin of the strain; *n*, minimum number of specimens analyzed; 4 Natural or insectary population.

Two further species, *R. domesticus* ($n = 26$) and *R. neivai* ($n = 8$), could be analyzed by morphometrics alone. Three species were unavailable for this study: *R. dalessandroi*, which is considered of doubtful validity by Lent and Wygodzinsky (1979), possibly synonymous with *R. brethesi* (but see Martinez 1984), *R. paraensis*, which has never been encountered again since its original collection in 1976 despite several searches, and *R. robustus* Larrousse, 1927, which remains a controversial species very close to and frequently confounded with *R. prolixus*.

For this analysis, live insects were dissected for isoenzyme analysis of thoracic muscles, retaining the heads and wings for morphometric measurements. We made a parallel examination of a population of *Psammolestes coreodes* ($n = 18$) collected from furnariid bird nests in the Department of Santa Cruz, Bolivia, and a population of domestic *Triatoma infestans* ($n = 15$) also from Bolivia, which was used as the outgroup.

Isoenzyme Analysis. Twelve enzyme systems were assayed as follows: ACON (aconitate hydratase or aconitase, EC 4.2.1.3), FDP (fructose bisphosphatase,

Table 2. Genetic and metric distances

	TIN	PCO	RST	RNA	RNE	domRPR	REC	silvRPR	RPA	RBR	RPI
TIN		88.3	253.1	396.0	418.0	308.0	330.8	328.0	305.4	218.4	288.8
PCO	2.7 ±0.9		83.9	199.8	205.0	109.9	164.2	135.6	120.6	56.1	56.1
RST	3.4 ±1.3	0.9 ±0.3		81.7	92.2	41.4	54.1	35.9	24.9	43.6	15.8
RNA	2.7 ±0.9	1.1 ±0.4	1.1 ±0.4		9.9	46.3	43.9	37.7	27.7	169.3	54.1
RNE	2.7 ±0.9	1.1 ±0.3	1.0 ±0.3	0.1 ±nc		50.2	54.2	43.8	36.0	172.1	70.5
domRPR	2.2 ±0.7	1.3 ±0.4	1.3 ±0.4	0.8 ±0.3	0.7 ±0.2	41.4	24.8	31.0	61.3	28.9	
REC	2.7 ±0.9	1.0 ±0.3	1.3 ±0.4	0.9 ±0.3	1.1 ±0.3	1.1 ±0.3		26.6	27.5	107.1	28.9
silvRPR	2.7 ±0.9	2.0 ±0.6	2.0 ±0.6	2.0 ±0.6	2.3 ±0.7	1.5 ±0.5	0.5 ±0.2		14.6	84.9	24.6
RPA	1.9 ±0.6	2.0 ±0.6	1.8 ±0.5	1.4 ±0.4	1.4 ±0.4	1.7 ±0.5	0.7 ±0.2	0.6 ±0.2		82.7	14.6
RBR	1.8 ±0.5	1.4 ±0.4	1.8 ±0.5	1.4 ±0.4	1.8 ±0.5	1.1 ±0.3	1.0 ±0.3	1.3 ±0.4	1.8 ±0.5		60.5
RPI	2.1 ±0.6	1.5 ±0.5	1.1 ±0.4	1.4 ±0.4	1.5 ±0.5	1.9 ±0.6	1.4 ±0.4	1.6 ±0.5	1.3 ±0.4	0.5 ±0.2	

Below diagonal are the Nei's distances (Dj) ± SD derived from electrophoretic data. Standard deviations were computed after $[(1-I)/(Ir)]^{\wedge}(1/2)$ with I = the Nei's standardized identity and r the number of loci (17). Because the variance estimation is disallowed when I is higher than 0.65, the standard deviation was not computed (see "nc") for the *R. neglectus*–*R. nasutus* pair. Above diagonal are the Mahalanobis distances (Dm) computed from discriminant analysis on log-transformed measurements of head and wing characters. Calculations for metric analysis used the JMP software (SAS Institute 1995).

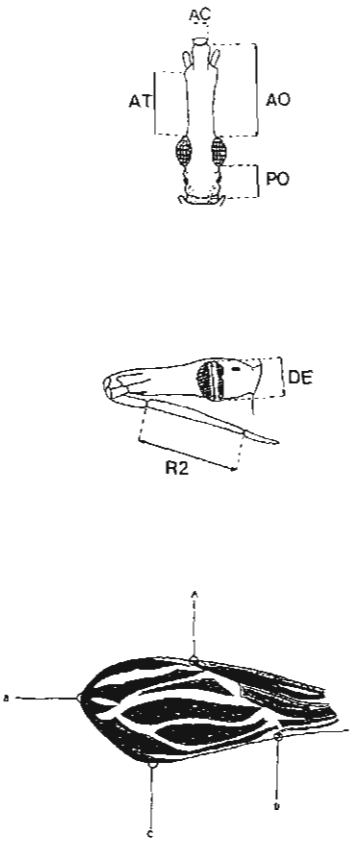

Fig. 1. Head and wing measurements. Measurements were made on head and wings of each specimen (Figs. 1, 2) as described for *T. infestans* (Dujardin et al. 1997a, b). Six head measurements: AO, anteocular distance; PO, postocular distance (excluding neck); DE, external diameter of eye; AT, length of antenniferous tubercle; AC, width of the antelypeus and R2, length of 2nd rostral segment (Fig 1). Three measurements were performed on each forewing after mounting the wings in Hoyer medium. The wing measurements were the linear distance between the points indicated in Fig. 2: WA, distance between 1 and 2; WB, between 1 and 3; and WC, between 1 and 4. Mean accuracy was 2.9%, and mean precision was 0.25 graduation, ranging from 0.00 (WA) to 0.80 (R2) after reexamining 15 individuals (15 head measurements and 30 wing measurements).

EC 3.1.3.11), αGPD (alpha glycerophosphate dehydrogenase, EC 1.1.1.8), GPI (glucose phosphate isomerase, EC 5.3.1.90), IDH (isocitrate dehydrogenase, EC 1.1.1.42), LAP (leucine aminopeptidase, EC 3.4.11), MDH (malate dehydrogenase, EC 1.1.1.37), ME (malic enzyme, EC 1.1.1.40), MPI (mannose phosphate isomerase, EC 5.3.1.8), 6PGD (phosphogluconate dehydrogenase, EC 1.1.1.44), PGM (phosphoglucomutase, EC 2.7.5.1), PEP-B (aminopeptidase B, EC 3.4.13, substrate l-leucyl-alanine). The electrophoretic conditions on cellulose acetate were according to Dujardin and Tibayrenc (1985) and Richardson et al. (1986).

For analysis of evolutionary relationships based on the isoenzyme patterns, allelic positions were polarized using *Triatoma infestans* (Klug) as an outgroup. *T. infestans* is a well-studied species of the related tribe Triatomini, representing a convenient sister taxon (Lent and Wygodzinsky 1979, Schofield 1988, Schofield and Dolling 1993). Only synapomorphies were used to construct the cladistic tree, following Hennig (1981). Nei's standard genetic distances (Nei 1987) were also computed (Table 2, below diagonal) for comparison with morphometric distances (Table 2, above diagonal). A Mantel test (Sokal and Rohlf 1995) was applied to assess the significance of correlation between these distances.

Morphometric Analysis. Using a dissection microscope with micrometer eyepiece, several measurements were taken of the head and wing of the same specimens analyzed by isoenzymes, following the system described for *T. infestans* (Dujardin et al. 1997a, b). Equality of variances among groups and normality of the distributions of log-transformed measurements were tested by Bartlett and Shapiro–Wilks tests, respectively.

Because our analysis used both adult sexes in the species comparisons, and because of other factors that could have influenced overall body size of the specimens, we wished to explore size-independent trends in morphological variation. The statistical procedure for removing size effects used the 1st principal component (PC1) as an indicator of global size (Bookstein 1989). Thus, a discriminant analysis was performed on the residuals of separate regressions of each variable with PC1. Only nonredundant variables showing positive and significant relationships with PC1 (9 measurements, Table 3) were included in the analysis (Fig. 1). The rationale for using discriminant analysis as a phylogenetic tool relied on its ability to focus upon "unshared" variation (Pimentel 1992), which may be considered an apomorphic variation (Sorensen 1992, Sorensen and Foottit 1992). The derived Mahalanobis distances (Table 2, above diagonal) were then submitted to parsimonious networking method using the program FITCH from the PHYLIP 3.4 package (Felsenstein et al 1995). Statistical significance of the multivariate analysis was estimated by the Wilks statistics.

Results

Isoenzyme Electrophoresis. Nine of the 12 enzyme systems showed a single locus, ACON and ME showed 2 loci, and PEP-B showed 4, giving a total of 17 loci for the analysis. These loci showed 83 allelic positions, which were polarized using *T. infestans* as an outgroup. Ten of the allelic positions were shared with those of *T. infestans* and were therefore classed as plesiomorphies. The other 73 were considered derived alleles (apomorphies); and of these, 46 were unique to a single species (autapomorphies). The 27 remaining alleles thus represented synapomorphies suitable for the construction of a cladistic tree. The tree allowing a minimum of homoplasy had 15 synapomorphies and

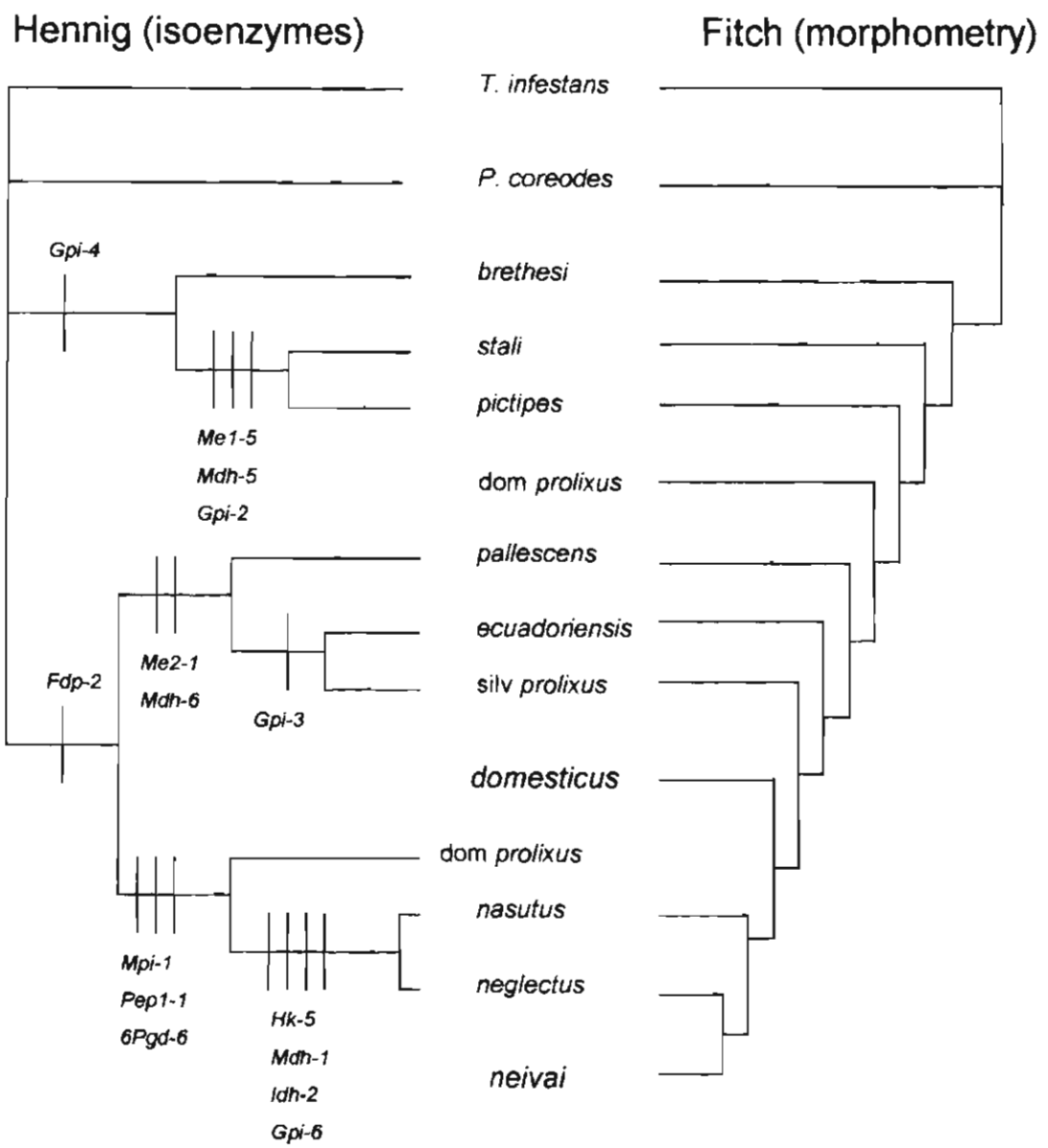

Fig. 2. Cladistic trees. Left tree: cladogram based on isoenzyme allelic synapomomorphies (indicated on each branch). Right tree: cladogram based on Mahalanobis distances derived from size-free canonical variate analysis, after the FITCH algorithm (PHYLIP package). Branch lengths were not reproduced. Both trees used *T. infestans* as outgroup. *R. domesticus* and *R. neivai* (right tree) were analyzed by morphometry only.

12 homoplasies (Fig. 2, left side). Excepting the outgroup, *T. infestans*, and *P. coreodes*, which was well separated from the *Rhodnius* species, this most parsimonious tree showed 3 clusters: the first one formed by *R. brethesi*, *R. stali*, and *R. pictipes*, the 2nd constituted by *R. pallescens*, *R. ecuadoriensis*, and the silvatic *R. prolixus*, and the last one grouping *R. nasutus*, *R. neglectus*, and the domestic *R. prolixus*.

Morphometric Analysis. Log-transformed data could not remove the significant departure from normality observed for most of the variables, and the variances between groups remained unequal for 3 measures after Bonferroni test at $P = 0.05$ (PO, WA, and WB, detailed results not shown). All variables were significantly correlated with the 1st principal component (PC1) ($P < 0.0001$ after Bonferroni test, Table 3) so that PC1 could be satisfactorily considered a general variable representing size (dos Reis et al. 1990). A space of shape measurements could therefore be constructed by explicit regression of size (PC1) out of the measured data, variable by variable. The regression residuals were then used as new variables on

Table 3. PC-1 as a general-size variable

Model Traits	*Triatoma, Psammolestes, Rhodnius* n = 202, PC1 (79%)		*Psammolestes, Rhodnius* n = 184, PC1 (86%)		*Rhodnius* n = 169, PC1 (85%)		
	LO	CO	LO	CO	LO	CO	AL
AO	0.49	0.98	0.48	0.99	0.44	0.96	1.72
PO	0.27	0.92	0.26	0.93	0.21	0.83	0.41
DE	0.15	0.57	0.11	0.51	0.26	0.82	0.59
AT	0.57	0.94	0.62	0.99	0.51	0.96	2.33
RR	0.44	0.97	0.47	0.99	0.4	0.98	1.45
AC	0.12	0.39	0.10	0.53	0.23	0.83	0.47
WA	0.22	0.75	0.18	0.76	0.29	0.89	0.75
WB	0.17	0.64	0.12	0.62	0.25	0.87	0.56
WC	0.22	0.79	0.18	0.79	0.28	0.91	0.73

Percent contribution of the 1st principal component (PC1) to the total variation (brackets), after a covariance-matrix based principal component analysis (PCA) performed on log-transformed data. The results of PCA are shown for the study group (i.e. *T. infestans, P. coreodes* and *Rhodnius* together), for *P. coreodes* and *Rhodnius*, and for *Rhodnius* exclusively (last 3 columns). Variables are listed with their coefficients for PC1 (columns LO), all positive and of the same sign, and their correlation coefficients with PC1 (columns CO), all positive and significant ($P < 0.0001$ after Bonferroni test). For *Rhodnius*, the corresponding static allometric coefficients are shown (columns AL): note the highly positive AL (2.33) for AT (antenniferous tubercle), which corresponds to the morphological criterion for the genus (apically inserted antennae). n, number of individuals.

which a discriminant or canonical variate analysis (CVA) was performed to explore size-free differences in form. This size-free CVA was highly significant ($P < 0.0001$). The correlation between Nei's standard genetic distances derived from the isoenzyme analysis, and the Mahalanobis distances derived from the size-free CVA was also significant ($P = 0.0248$ after 10,000 runs, Mantel test), and is illustrated by their 2nd-order polynomial relationship (Fig. 3). The tree derived from Mahalanobis distances was similar to the isoenzyme tree, except for the position of domestic *R. prolixus* (Fig. 2).

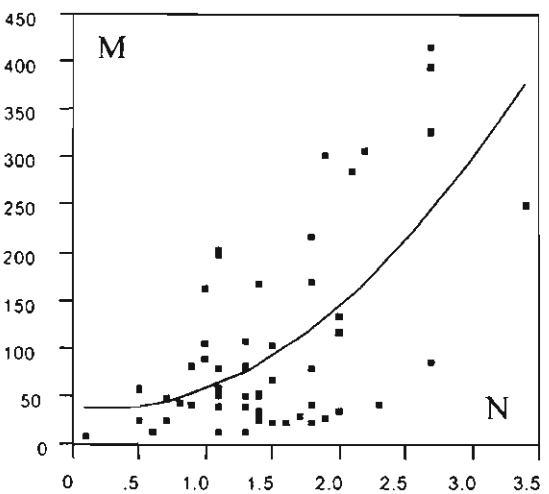

Fig. 3. Genetic basis of morphometric traits. Plotting of Mahalanobis (M, vertical axis) against Nei's (N, horizontal axis) distances, showing the positive and significant relationship between them. The Mantel test between distances matrices used 10,000 runs ($P = 0.0248$). The curved line represents the 2nd-order polynomial relationship (coefficient of determination is 0.41).

Discussion

Except for the relative position of the domestic *R. prolixus*, the isoenzyme-based tree and the morphometric tree were very similar to each other. A complementary illustration of the genetic basis of morphometric traits is provided by the significant correlation ($P < 0.001$) between the Mahalanobis and Nei's distances (Nei 1987). However, both trees left unresolved the position of *P. coreodes*, either included as an external species of the *Rhodnius* genus or as another paraphyletic lineage (Fig. 2).

Although originally identified as '*R. prolixus*' (Lopez and Moreno 1995), our silvatic sample of '*prolixus*' appeared to be genetically very different from the domestic samples of *R. prolixus* (Ds = 1.50 ± 0.50) but very near to *R. ecuadoriensis* (Ds = 0.50 ± 0.20), which is thought to be absent from Colombia (Schofield 1994). This was confirmed by morphometry (Fig. 2, right side), supporting the possibility that this sample may represent a distinct taxon. In previous studies, *ecuadoriensis* had been grouped with the more broadly distributed *R. pictipes* on the basis of morphological characteristics such as antennal sensilla patterns (Catalá and Schofield 1994) or the mottling on legs and body that is also shown by *R. pallescens* (Lent and Wygodzinsky 1979). In this study, however, the clustering of *R. ecuadoriensis* and *R. pallescens* with the silvatic *R. prolixus* from Colombia is in accord with their geographic distribution west of the Andes along the Pacific Coast in Peru, Ecuador, Colombia, and Panama. Pending further study, we consider the 'silvatic *prolixus*' as the 'Tolima form of *ecuadoriensis*' in view of its place of first capture and its relative closeness to *R. ecuadoriensis*.

Comparison of the isoenzyme and morphometric trees shows that only the domestic samples of *R. prolixus* were differently represented—grouped with *nasutus* and *neglectus* by the isoenzyme analysis, but closer to *pictipes* by morphometry. *R. prolixus* is distributed throughout Venezuela and Colombia, and

also in parts of Central America; our sample included specimens from all these areas. Recent genetic studies have suggested a South American origin for this important vector of Chagas disease, and support the idea that its introduction to Central America has been relatively recent—possibly resulting from human intervention (Dujardin et al. 1998). Its origin is most likely to be Venezuela, where both domestic and silvatic populations have been reported (e.g., Camboa 1962), whereas silvatic populations in Colombia—as shown by this study—have not been confirmed. By isoenzymes, the grouping of *R. prolixus* with 2 Brazilian species *(nasutus* and *neglectus)* is in agreement with an origin east of the Andes. The seemingly erratic position of *prolixus* in the morphometric tree may be the result of unequal sample sizes (there were 65 specimens of domestic *R. prolixus*, compared with 4–26 of each of the other species) having amplified the distorting effect of heteroscedasticity (Pimentel 1992).

The 2 species examined by morphometry alone, *R. domesticus* and *R. neivai*, were also grouped with the *nasutus–neglectus* pair. Again, this accords with geographical distribution because all of these species are from areas east of the Andes. *R. domesticus* is found only among epiphytic bromeliads in the Atlantic forest of southern Brazil, and is considered by some to be a derivative of *R. neglectus* from the central cerrado region of Brazil (C.J.S., unpublished data). However, little is known about *R. neivai*, which is distinguished from all other *Rhodnius* species by its almost uniform dark color, and apparent specialization to Cobija palms (*Copernicia tectorum*) in the llanos of northern Colombia and Venezuela.

Both isoenzyme and morphometric analyses suggest a possible basal group comprising *R. pictipes*, *R. stali*, and *R. brethesi*, which are all species of the Amazon–Orinoco forest region. *R. pictipes* and *R. stali* were expected to show a close relationship, because the latter had been included in *pictipes* until its recent description by Lent et al. (1993). Both share characteristics of the male genitalia, which are found in other Triatominae but not in other Rhodniini (Jurberg 1996). However, whereas both *pictipes* and *stali* are considered generalist species, occupying a wide variety of forest ecotopes and occasionally invading houses (Lent and Wygodzinsky 1979, Tibayrenc and Le Pont 1984, Schofield 1994), *brethesi* seems to be highly specialized and is recorded only from piassaba palms in the Amazon forest.

In summary, the monophyly of the tribe Rhodniini is neither confirmed nor rejected. Three groups of *Rhodnius* species can be recognized, a putative basal group including *brethesi*, *pictipes*, and *stali*, and 2 further groups separated geographically by the Andes: a western or Pacific group represented by *pallescens*, *ecuadoriensis*, and the Tolima form of *ecuadoriensis*, and an eastern or Atlantic group represented by *domesticus*, *nasutus*, *neglectus*, *neivai*, and domestic samples of *prolixus*. Both metric and proteic phylogenetic approaches were able to detect a species wrongly attributed to *prolixus*, and to relate it to 2 other species, *ecuadoriensis* and *pallescens*, from the same geographic region.

Thus, even with non-normal data distribution and some heteroscedasticity, a traditional morphometric study could accord with isoenzymes for the majority of the *Rhodnius* members. According to Pimentel (1992), non-normality has little, if any, influence on canonical variate analysis, where the only critical assumption is equality of population dispersion matrices (homoscedasticity). Violation of this assumption probably led to the different placing of *R. prolixus* in the morphometric tree, but the overall agreement observed here between isoenzymes and morphometrics illustrates the robustness of canonical variate analysis, and supports the idea that it may represent a valid phylogenetic approach (Sorensen and Foottit 1992).

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