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Isoenzyme electrophoresis of *Rhodnius* species: a phenetic approach to relationships within the genus

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Seventeen samples of *Rhodnius*, representing at least eight different species according to their morphological characteristics, were submitted to multilocus isoenzyme electrophoresis, revealing 17 different loci. A phenetic analysis of the enzyme data not only clustered the species in accordance with their geographical origin but also revealed interspecific relationships that differed from those expected from the morphology.

The genus *Rhodnius* Stal (Hemiptera: Reduviidae: Triatominae) currently comprises 13 species defined on the basis of morphological characters (Lent and Wygodzinsky, 1979; Schofield, 1994). All 13 are of Latin American distribution and several are of epidemiological significance as domestic vectors of *Trypanosoma cruzi* (Chagas), causative agent of Chagas disease (American trypanosomiasis). The species of greatest epidemiological significance are *R. prolixus* in Venezuela, Colombia and parts of Central America, and *R. pallescens* in Panama and southern Costa Rica. Several other species enter dwellings and peridomestic habitats and are of local importance in some areas, particularly *R. ecuadoriensis* in northern Peru and Ecuador, *R. stali* in Bolivia (previously cited as *R. pictipes*), and *R. neglectus* and *R. nasutus* in Brazil. *Rhodnius brethesi* is known to attack humans in parts of the Amazon region, although it is normally associated with piassaba palms. The other species seem to be entirely sylvatic, with little, if any, epidemiological importance.

The species of *Rhodnius* tend to be very similar in appearance, and their determination generally requires dissection of the male genitalia (Lent and Jurberg, 1969; Lent and Wygodzinsky, 1979). However, examination of large series often shows considerable overlap between key morphological characteristics, especially for the four species (*R. prolixus*, *R. robustus*, *R. neglectus*, and *R. nasutus*) sometimes known as the 'prolixus group' (Barrett, 1991; Harry, 1992, 1993), with some populations of the putative species being interfertile (Dujardin *et al.*, 1991; Barrett, 1996). Because of this overlap, the distribution of many of the species cannot be clearly determined, either at the geographical level (Dujardin *et al.*, 1991; WHO, 1991) or at the ecological level, in terms of the degree of domestic and sylvatic colonization. However, clarification of the distributions of each species is of considerable importance in assessing the species' relative

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epidemiological significance and in designing effective control strategies when appropriate (WHO, 1991). The present report is of a genetic study based on isoenzyme electrophoresis of most of the available species of *Rhodnius*. The study confirms the usefulness of this technique for species determination (Solano *et al.*, 1996) and allows a preliminary analysis of the geographical structuring of the genus.

MATERIALS AND METHODS

The Insects

Populations representing eight morphological species of *Rhodnius* were provided by different laboratories, or collected from natural habitats (Table 1). Together, these samples comprised 156 individuals, of which 23 were fifth-instar nymphs and 123 were adults. In all cases, the laboratory supplying the sample identified the bugs to species level on the basis of their morphological characters, following the keys of Lent and Wygodzinsky (1979).

Isoenzyme Electrophoresis

Cellulose-acetate electrophoresis was carried out according to Dujardin and Tibayrenc (1985) and Richardson *et al.* (1986). Twelve enzyme systems were assayed: ACON (aconitate hydratase or aconitase; EC 4.2.1.3); FDP (fructose bisphosphatase; EC 3.1.3.11); αGPD (α-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 (mannosephosphate isomerase; EC 5.3.1.8); 6PGD (phosphogluconate dehydrogenase; EC 1.1.1.44); PGM (phosphoglucomutase; EC 2.7.5.1); and PEP-B (aminopeptidase B, with L-leucyl-alanine as substrate; EC 3.4.13).

Data Analysis

Estimation of the relative genetic variability was limited to calculation of the proportion of polymorphic genes (*EP*). Jaccard's distances were calculated as a conservative measure of genetic divergence, since these do not depend on gene frequencies nor on number of loci. The phenetic (UPGMA) analysis of relationships between *Rhodnius* species was then derived from the matrix of Jaccard's distances.

RESULTS

Seventeen loci for the 12 enzyme systems investigated were consistently seen. Nine of the enzyme systems (GPD, GPI, LAP, PGM, 6PGD, MPI, FDP, MDH and IDH) showed a single zone of activity for all specimens. Two zones of activity were seen for ACON (and named *Acon1* and *Acon2*), two for ME (*Me1* and *Me2*), and four for PEP-B (*Pep1*, *Pep2*, *Pep3*, and *Pep4*) (see Table 2). Four of these enzyme loci displayed heterozygotic patterns in some individuals: *Idh* in *R. pictipes*, *R. neglectus* and *R. stali*; *Gpi* in *R. pictipes* and *R. stali*; *Fdp* in *R. neglectus* and domestic *R. prolixus*; and *Mpi* in *R. ecuadoriensis* and sylvatic '*R. prolixus*'. Different alleles, without the corresponding heterozygote patterns, were found at the: *Gpi* locus in *R. neglectus*; *Mdh* locus in *R. stali*, *R. ecuadoriensis*, *R. neglectus* and *R. nasutus*; *Pep1* locus in *R. pallescens* and *R. nasutus*; *Pep3* and *Pep4* loci in *R. ecuadoriensis*; *Fdp* locus in *R. pallescens*; *Pep2* and *Acon1* loci in *R. nasutus*; and *6pgd*, *Acon2*, *Gpd* and *Pgm* loci in *R. pictipes*. None of the 17 loci varied in *R. brethesi*. Thus the average enzyme polymorphism for all species (*EP*) was 0.15. Amongst them, *R. brethesi* showed the lowest variability ($EP = 0$), followed by domestic and sylvatic samples of *R. prolixus* ($EP = 0.06$), *R. pallescens* and *R. stali* ($EP = 0.12$ for each), *R. neglectus* ($EP = 0.18$), and *R. nasutus* and *R. ecuadoriensis* ($EP = 0.24$ for each). The highest variability was shown by *R. pictipes* ($EP = 0.35$).

From the 68 alleles revealed by this analysis, 29 (unique alleles) were limited to one species only (see Table 3). *Rhodnius pictipes* showed seven unique alleles, followed by *R. stali* (six unique alleles), *R. pallescens* (five), *R. ecuadoriensis* (three), domestic and sylvatic

TABLE 1
Origins of the Rhodnius *investigated*

Species	*Code*	*No. of bugs*	*Notes*
R. brethesi	BRE	4	Collected by Dr T. V. Barrett from piassaba palms (*Leopoldinia piassaba*) in Amazonas, Brazil, in 1995
R. ecuadoriensis	ECU1	10	FIOCRUZ reference strain (Department of Entomology, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil) believed to have originated from specimens obtained from Peru in 1979 by Dr J. M. S. Barata
	ECU2	4	Insectary strain from the University of Antioquia, Medellin, Colombia, originating from specimens collected in southern Ecuador by Dr. C. Romaña in 1990
	ECU3	2	Collected by Dr C. J. Schofield from Tagua palms (*Phytolephas aequatorialis*) in Manabi, Ecuador in 1995
R. nasutus	NAS	6	FIOCRUZ reference strain, developed from bugs collected in Ceará, Brazil, in 1978
R. neglectus	NEG	10	FIOCRUZ reference strain, founded in 1976 from specimens collected in Goiás, Brazil
R. pallescens	PAL	13	FIOCRUZ reference strain, acquired from the insectary of the University of Antioquia, Medellin, Colombia in 1989
R. pictipes	PIC	10	FIOCRUZ reference strain, originating from Pará state, Brazil, in 1989
R. prolixus (domestic origin)	DomPR1	10	FIOCRUZ reference strain, shown to be completely inter-sterile with the FIOCRUZ reference strain of *R. neglectus* (NEG; Dujardin *et al.*, 1991); from an old colony derived from Venezuelan specimens (believed to be from the state of Cojedes)
	DomPR2	10	From the insectary of the Instituto Evandro Chagas, Belem, Brazil (origin unknown)
	DomPR3	4	From the insectary of the Central Reference Laboratory, Tegucigalpa, Honduras; derived from bugs from Zapaca, Guatemala
	DomPR4	10	From the insectary of the Central Reference Laboratory, Tegucigalpa, Honduras; derived from bugs from Chiapas, Mexico
	DomPR5	13	Collected by Dr C. Ponce from houses in central Honduras, in 1996
	DomPR6	5	From the insectary of the Centers for Disease Control (Atlanta, GA), and derived from Colombian bugs
	DomPR7	6	Collected by Dr J. Moreno from houses in Tolima, Colombia, in 1991 (see Lopez and Moreno, 1995)
'*R. prolixus*' (sylvatic origin)	SylvPR	8	Collected by Dr J. Moreno from palms (*Attalea butyracea*) in Tolima, Colombia, in 1991 (see Lopez and Moreno, 1995)
R. stali	STA	4	Collected by Dr H. Bermudez from houses in Chaparé, Bolivia, in 1993

TABLE 2

Allelic scores (1 = allele present and 0 = allele absent) and comparative levels of electrophoretic migration for 17 enzyme loci of the Rhodnius *species studied**

	Score for:								
Allele	*STA*	*NAS*	*NEG*	*DomPr*	*ECU*	*SylvPr*	*PAL*	*BRE*	*PIC*
6pgdh-1	0	0	0	0	0	0	0	0	1
6pgdh-2	0	0	0	0	0	0	0	1	1
6pgdh-3	1	0	0	0	1	1	1	0	0
6pgdh-4	0	1	1	1	0	0	0	0	0
Acon1-1	0	0	0	0	0	1	0	0	0
Acon1-2	0	1	0	1	0	0	0	1	0
Acon1-3	1	1	1	0	1	0	1	0	1
Acon2-1	0	1	1	0	0	0	1	1	1
Acon2-2	0	0	0	1	1	1	0	0	1
Acon2-3	1	0	0	0	0	0	0	0	0
Fdp-1	0	0	1	1	0	0	0	0	0
Fdp-2	0	1	1	1	1	1	1	0	0
Fdp-3	0	0	0	0	0	0	1	1	1
Fdp-4	1	0	0	0	0	0	0	0	0
Gpd-1	0	0	0	0	0	0	0	1	0
Gpd-2	0	0	0	0	0	0	0	0	1
Gpd-3	0	0	0	1	1	1	1	0	0
Gpd-4	1	1	1	0	0	0	0	0	1
Gpd-5	0	0	0	0	0	0	0	0	1
Gpi-1	0	0	0	0	0	0	1	0	0
Gpi-2	1	0	0	0	0	0	0	0	1
Gpi-3	0	0	0	0	1	1	0	0	0
Gpi-4	1	0	0	1	0	0	0	1	1
Gpi-5	0	1	1	0	0	0	0	0	0
Gpi-6	0	0	1	0	0	0	0	0	0
Idh-1	0	0	0	1	0	0	0	0	0
Idh-2	0	1	1	0	0	0	0	0	0
Idh-3	0	0	0	0	0	0	1	0	0
Idh-4	0	0	0	0	1	1	0	1	1
Idh-5	0	0	0	0	0	0	0	0	1
Idh-6	1	0	0	0	0	0	0	0	0
Lap-1	0	0	0	0	0	0	0	1	1
Lap-2	1	1	1	1	1	1	1	0	0
Mdh1-1	0	1	1	0	0	0	0	0	0
Mdh1-2	1	1	1	1	0	0	0	1	0
Mdh1-3	0	0	0	0	1	0	0	0	0
Mdh1-4	1	0	0	0	0	0	0	0	1
Mdh1-5	0	0	0	0	1	1	1	0	0
Me1-1	0	1	1	1	0	0	0	0	0
Me1-2	0	0	0	0	1	1	1	1	0
Me1-3	1	0	0	0	0	0	0	0	1
Me2-1	0	0	0	0	1	1	1	0	0
Me2-2	1	1	1	1	0	0	0	1	1
Mpi-1	0	1	1	1	0	0	0	0	0
Mpi-2	0	0	0	0	1	1	1	1	1
Mpi-3	0	0	0	0	1	1	0	0	0
Mpi-4	1	0	0	0	0	0	0	0	0

TABLE 2 (*Continued*)

Allele	*Score for:* *STA*	*NAS*	*NEG*	*DomPr*	*ECU*	*SylvPr*	*PAL*	*BRE*	*PIC*
Pep1-1	0	1	1	1	0	0	0	0	0
Pep1-2	1	1	0	0	1	1	1	1	1
Pep1-3	0	0	0	0	0	0	1	0	0
Pep2-1	0	1	1	0	0	0	0	1	1
Pep2-2	0	0	0	1	1	1	1	0	0
Pep2-3	0	1	0	0	0	0	0	0	0
Pep2-4	1	0	0	0	0	0	0	0	0
Pep3-1	0	0	0	0	0	0	0	1	0
Pep3-2	0	0	0	0	0	0	0	0	1
Pep3-3	0	0	0	0	0	0	1	0	0
Pep3-4	1	1	1	1	1	1	0	0	0
Pep3-5	0	0	0	0	1	0	0	0	0
Pep4-1	1	1	1	1	1	1	1	1	1
Pep4-2	0	0	0	0	1	0	0	0	0
Pgm-1	0	0	0	1	0	0	0	0	0
Pgm-2	0	0	0	0	0	0	0	0	1
Pgm-3	0	0	0	0	0	0	0	0	1
Pgm-4	0	1	1	0	1	0	0	1	0
Pgm-5	0	0	0	0	0	1	0	0	0
Pgm-6	1	0	0	0	0	0	0	0	0
Pgm-7	0	0	0	0	0	0	1	0	0

* Alleles are numbered from fastest to slowest migrating bands on the gels.

STA, *Rhodnius stali*; NAS, *R. nasutus*; NEG, *R. neglectus*; DomPr, domestic *R. prolixus*; ECU, *R. ecuadoriensis*; SylvPr, sylvatic '*R. prolixus*'; PAL, *R. pallescens*; BRE, *R. brethesi*; PIC, *R. pictipes*.

R. prolixus and *R. brethesi* (two each), and *R. neglectus* and *R. nasutus* (one). Most of the remaining 39 unique alleles were shared by three species groups (see Table 2): Group 1—*R. pictipes*, *R. stali* and *R. brethesi* (four commonly shared alleles, nine shared between *R. pictipes* and *R. stali*, and 11 between *pictipes* and *brethesi*); Group 2—domestic *R. prolixus*, *R. neglectus* and *R. nasutus* (10 commonly shared alleles, 18 shared between *R. neglectus* and *R. nasutus*); and Group 3—*R. pallescens*, sylvatic *R. prolixus* and *R. ecuadoriensis* (11 commonly shared alleles, with 16 shared between *R. ecuadoriensis* and sylvatic '*prolixus*'). The UPGMA tree derived from Jaccard's distances (Table 3) reproduced this pattern of allelic distribution, with the three species groupings clearly clustered (see Fig.).

DISCUSSION

The absence of heterozygotic forms at several loci in *R. ecuadoriensis*, *R. nasutus*, *R. neglectus*, *R. pallescens*, *R. pictipes* and *R. stali* may be attributable to the small number of individuals of these species that could be examined in the present study. In the case of *R. ecuadoriensis*, this absence could be due to geographical differentiation, since the different alleles of *Mdh*, *Pep3* and *Pep4* were distributed according to the geographical origin of the specimens from Ecuador or Peru.

The small samples used for most of the species also mean that the present estimates of their genetic variability should be regarded with caution. It is notable, however, that the species of widest geographical distribution,

TABLE 3

Jaccard's distances computed from the allelic frequencies

	STA	*NAS*	*NEG*	*DomPr*	*ECU*	*SylvPr*	*PAL*	*BRE*	*PIC*
STA									
NAS	0.75	0							
NEG	0.78	0.217	0						
DomPr	0.81	0.607	0.59	0					
ECU	0.82	0.8	0.83	0.781	0				
SylvPr	0.84	0.853	0.88	0.759	0.304	0			
PAL	0.85	0.824	0.85	0.844	0.571	0.577	0		
BRE	0.84	0.733	0.81	0.833	0.813	0.833	0.8	0	
PIC	0.74	0.816	0.84	0.895	0.846	0.865	0.838	0.633	0

STA, *Rhodnius stali*; NAS, *R. nasutus*; NEG, *R. neglectus*; DomPr, domestic *R. prolixus*; ECU, *R. ecuadoriensis*; SylvPr, sylvatic '*R. prolixus*'; PAL, *R. pallescens*; BRE, *R. brethesi*; PIC, *R. pictipes*.

R. pictipes, was also the one showing the highest levels of enzyme polymorphism as well as the highest number of unique alleles. In contrast, the species with the lowest enzyme polymorphism, *R. brethesi*, is also one of the most specialised, generally only being found on piassaba palms and locally known as '*piolho da piaçaba*' (literally 'piassaba louse'; Coura *et al.*, 1994).

In previous studies, *R. ecuadoriensis* and *R. pallescens* have been grouped with *R. pictipes* on the basis of shared morphological characters such as diffuse mottling over the legs and body (Lent and Wygodzinsky, 1979) or similar patterns of antennal sensilla (Catalá and Schofield, 1994). However, the present results indicate considerable genetic distance between *R. pictipes* and the *R. ecuadoriensis/R. pallescens* pair, as well as a different pattern of relationship with the other *Rhodnius* species. The results place the sylvatic '*R. prolixus*' with the *R. ecuadoriensis/R. pallescens* pair. The present study included the second isoenzymatic comparison between domestic and sylvatic samples of *R. prolixus* from the Tolima region of central Colombia. In the first (Lopez and Moreno, 1995), the sylvatic sample from palm trees was assumed to be *R. prolixus*, but a complete lack of gene flow was revealed by isoenzyme comparison with domestic *R. prolixus* from houses in the same region. In the present analysis, the sylvatic '*R. prolixus*' also appears very different from domestic *R. prolixus*, and may merit new status as a distinct taxonomic entity. Until further studies are complete, however, this population should be considered as the Tolima or Colombian form of *R. ecuadoriensis*, because of its relative proximity to other *R. ecuadoriensis* populations.

The present results also reveal a clear distinction between *R. stali* and *R. pictipes*. *Rhodnius stali* is a recently described but poorly known species from the states of Acre and Mato Grosso (Brazil), and from the department of Chaparé (Bolivia), where it has been found invading human dwellings (H. Bermudez, unpubl. obs.). It has long been confused with *R. pictipes*, from which it was recently separated on the basis of the characters of the male genitalia (Lent *et al.*, 1993); the present results support the validity of this separation, revealing considerable enzymatic divergence between the two species.

The results of the present study clearly distinguish domestic *R. prolixus* from *R. neglectus* and *R. nasutus* but, in agreement with their morphological similarity, these three species were closely clustered in the phenetic analysis (Fig.). The small distance between *R. neglectus* and *R. nasutus* may indicate that their speciation was very recent and/or the possibility of gene exchange in parapatric zones. In fact, these two species of *Rhodnius* showed fewer isoenzymatic differences than seen between populations of *T. infestans* and

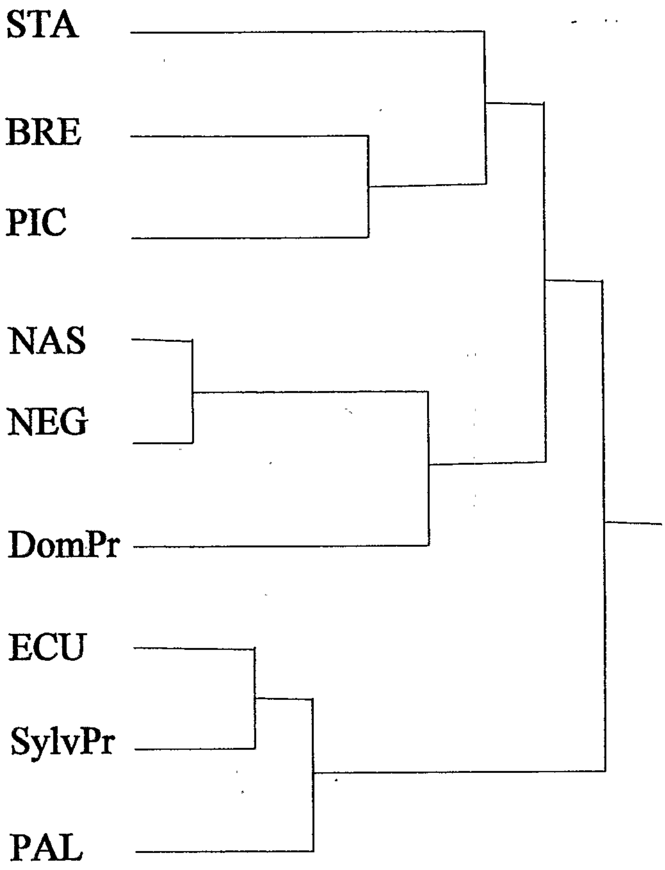

Fig. An UPGMA tree derived from Jaccard's distances between *Rhodnius* species, with the positions of *Rhodnius stali* (STA), *R. brethesi* (BRE), *R. pictipes* (PIC), *R. nasutus* (NAS), *R. neglectus* (NEG), domestic *R. prolixus* (DomPr), *R. ecuadoriensis* (ECU), sylvatic '*R. prolixus*' (SylvPr) and *R. pallescens* (PAL) indicated.

T. platensis examined using identical techniques (Pereira *et al.*, 1996), and these two species of *Triatoma* are known to be interfertile (Usinger *et al.*, 1966). The clustering of domestic *R. prolixus* with *R. nasutus* and *R. neglectus* into one group may indicate that they had a common ancestral form—a hypothesis which should be verified by other, phylogenetic approaches.

With the exception of domestic *R. prolixus*, the present isoenzyme analysis grouped the *Rhodnius* species in accordance with their broad geographical origin; the members of Group 1 (*R. pictipes*, *R. brethesi* and *R. stali*) are all species of the Amazon–Orinoco forest, whereas the members of Group 2 (*R. neglectus* and *R. nasutus*) are from the drier, *cerrado* and *caatinga* regions of central and north–eastern Brasil. In contrast, the Group-3 species (*R. pallescens*, *R. ecuadoriensis* and the sylvatic '*R. prolixus*' or Tolima form) are all from valleys to the west of the Andean mountains. Only domestic *R. prolixus* would appear to distort this pattern, since they can be found in regions both east and west of the Andes, and in parts of Central America. Nevertheless, the present isoenzyme data indicate that domestic *R. prolixus* originate in the eastern part of South America, together with the other species of Groups 1 and 2; its occurrence in Central America is probably the result of human intervention (Dujardin *et al.*, 1998) and its occurrence in Colombia west of the Andes may also the result of its passive transport in association with humans.

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