Random Amplification of Polymorphic DNA as a Tool for Taxonomic Studies of Triatomine Bugs (Hemiptera: Reduviidae)

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ABSTRACT Eleven of 27 decameric primers were found to be suitable for random amplification of polymorphic DNA (RAPD) from triatomine bugs on the basis that they produced discrete profiles and distinguished among Panstrongylus megistus (Burmeister), Rhodnius prolixus Stål, and Triatoma infestans (Klug). The legs, or single leg segments, of individual bugs were used as the source of DNA so that the taxonomic value of the bug was conserved. Within the scope of the specimens studied, RAPD profiles allowed assignment to species even when bugs were kept dry for up to 12 mo. Profiles for individuals within a species were not identical. RAPD profiles, with the specimens tested, distinguished among species of 3 pairs considered to be morphologically similar and closely related, namely, Rhodnius ecuadorensis Lent & León and Rhodnius pictipes Stål; Rhodnius nasutus Stål, and Rhodnius neglectus Lent; Rhodnius prolixus Stål and Rhodnius robustus Larrousse. RAPD data conformed with the perceived affinities among these species. RAPD polymorphisms were seen with T. infestans from 3 different localities, but none of the polymorphisms was confined to 1 source. RAPD provided a molecular basis to reassess taxonomic relationships within the Triatomine subfamily. The accurate distinction of triatomine species and of intraspecific bug populations may contribute to elimination of vector-borne Chagas disease from the Americas.

KEY WORDS Panstrongylus, Rhodnius, Triatoma, triatomines, random amplification of polymorphic DNA, molecular taxonomy

Chagas disease (American trypanosomiasis) is one of the most important public health problems in Latin America. The kinetoplastid protozoan parasite responsible, Trypanosoma cruzi, is thought to infect up to 18 million people, and another 100 million live in endemic areas where they are at risk of acquiring infection (WHO 1991). Arthropod vectors of T. cruzi are blood-sucking triatomine bugs (Hemiptera: Reduviidae). Transmission of the parasite occurs while bugs are feeding if T. cruzi infected bug feces contaminates the mucous membranes or abraded skin of the mammalian host. Transmission can also occur through contaminated blood transfusion, organ transplant, congenitally, and by oral contamination. The most frequent cause of infection is triatomine attack in infested houses. The initial acute phase of T. cruzi infection is seldom recognized and may be asymptomatic. There is no satisfactory treatment for chronic infections, and no vaccine, in part because pathogenesis appears to involve autoimmunity. Therefore, control of Chagas disease relies principally on elimination of domestic triatomine populations by spraying with residual insecticides, supported by health education and housing improvement, in conjunction with screening and treatment of suspect transfusion blood. If sustained by adequate resources, these public health measures are effective. They have been used to eliminate domestic Triatoma infestans (Klug) from Sao Paulo state, Brazil (Wanderley 1993), and, with residual pyrethroid insecticides, are the basis for the international Southern Cone Programme to eliminate T. infestans in Argentina, Bolivia, Brazil, Chile, Paraguay, and Uruguay (Dias 1993).

Residual infestation and reinfestation of sprayed houses obstruct control campaigns, especially if spraying does not cover the entire endemic focus. Recolonization can arise from untreated domestic habitats or by immigration of bugs from silvatic foci (Schofield 1995, Harry et al. 1992), and peridomestic or silvatic species may replace a domestic vector that has been removed by insecticides. Thus, the accurate identification of triatomine species, some of which are morphologically similar but differ in habitat, behavior, and potential for domiciliation, and the distinction of intraspecific bug populations, has a significant role to play in the elimination of Chagas disease from the Americas.

Triatomine species are traditionally identified using morphological criteria, to which the most comprehensive guide is the monograph by Lent and
Wygodzinsky (1979). On the basis of morphology, 123 species of the subfamily Triatominae have been described, grouped into 5 tribes and 14 genera (Lent and Wygodzinsky 1979, Schofield 1994). There are 110 species restricted to the Americas, and of the 13 Old World species, 8 are part of the T. rubrofasciata group and 5 species form the Indian genus Linscostus (Gorla et al. 1997). Most triatomine species live in silvatic habitats, such as palm trees, hollow trees, burrows, among rocks, or in caves, feeding from mammals, birds, or reptiles (only mammals are susceptible to T. cruzi infection). The majority of New World triatomine species have been recorded as infected with T. cruzi but the 5 most important domestic vectors fall into 3 genera and the 2 tribes Triatomini [Panstrongylus megistus (Burmeister), T. infestans, T. brasiliensis Neiva, T. dimidiatia (Latreille)] and Rhodniini (Rhodnius prolizus Stål).

Some bug species, such as R. prolizus and R. robustus Larrousse, are difficult to separate taxonomically with confidence (Schofield 1994) and others have controversial taxonomic status. Few studies have attempted any cladistic analysis within a major genus, or between the 5 tribes of triatominae, or the abundant New World species and those few species restricted to the Old World (Gorla et al. 1997). The evolutionary histories of triatomine taxa are therefore uncertain. Isoenzyme electrophoresis (Dorea et al. 1982, Dujardin et al. 1987, Harry et al. 1992, Garcia et al. 1995a, Pereira et al. 1996) and cytogenetic studies (Perez et al. 1992) have been introduced to assist morphological characterization. Isoenzyme profiles have been applied to examine the population genetics of silvatic and domestic populations of T. infestans (Dujardin et al. 1987, Garcia et al. 1995b) and R. prolizus (Harry et al. 1992, Lopez and Moreno 1995) and to detect gene flow between populations, but a small number of polymorphic loci are amenable to isoenzyme analysis and living or freshly frozen samples are necessary. Cytogenetic methods are time consuming and affected by both the life cycle stage and the sex of the triatomine specimen.

Molecular biological methods can provide highly discriminatory markers to clarify triatomine taxonomy and population genetics. One relatively straightforward approach is genetic fingerprinting by random amplification of polymorphic DNA (RAPD) in polymerase chain reactions (PCR) with single short oligonucleotide primers of arbitrary sequence. RAPD has become widely adopted for genome analysis and population genetics of various insect species (Black 1993, Haymer 1994, Wilkerson et al. 1995) especially those species that are problematic with classical taxonomic approaches (Adamson et al. 1991, Ballinger-Crabtree et al. 1992, Kambhapati et al. 1992, Favia et al. 1994). Here we have examined the potential of RAPD protocols for taxonomic and epidemiologic studies of triatomine bug populations.

<table>
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<tr>
<th>Table 1. Species and origins of triatomine bugs (colonies or collections) used as sources of DNA for RAPD analyses</th>
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<tbody>
<tr>
<td>P. megistus</td>
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<tr>
<td>R. ecuadoriensis</td>
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<td>R. nasutus</td>
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<tr>
<td>R. neglectus</td>
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<td>R. pallescens</td>
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<td>R. pictipes</td>
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<td>R. prolizus</td>
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<tr>
<td>R. prolizus</td>
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<td>R. robustus</td>
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<td>T. infestans</td>
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<tr>
<td>T. infestans</td>
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<td>T. protracta</td>
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* Red-eyed mutant; courtesy of Chris Curtis and the Instituto Nacional de Salud (Bogota, Colombia).

Materials and Methods

Triatomine Bugs. The domestic triatomines P. megistus, R. prolizus, and T. infestans were selected to represent distinct and divergent species that are epidemiologically important as vectors of T. cruzi. Within the genus Rhodnius we examined 6 species (Table 1) and included 3 pairs of species that are said to be morphologically similar and closely related, namely: R. ecuadoriensis Lent & León and R. pictipes Stål; R. nasutus Stål and R. neglectus Lent; R. prolizus and R. robustus Larrousse. T. infestans came from 3 geographically separate collection sites at Tarija, Cochabamba, and Sucre in Bolivia. The majority of bugs were from established laboratory colonies (Table 1), except that R. pictipes and R. robustus were freshly collected in 1995 from their silvatic habitats in Pará state, Brazil, using methods described by Miles et al. (1981); T. infestans were progeny of recent field collections in Bolivia. In the case of R. pictipes and R. robustus, new field collections were made because pure bred laboratory colonies were not available. Species identification was determined by morphology as described by Lent and Wygodzinsky (1979). Some bug specimens were kept dry for up to 12 mo so that RAPD profiles could be compared with those of living insects.

DNA Samples. Each bug was examined individually. Legs were selected as the tissue of choice for the source of DNA because they were free of host (blood meal) contaminants and gut symbionts, and they could be removed without destroying the taxonomic value of the specimen. For some individuals the 6 legs were pooled before DNA extraction; with others, samples were obtained from a single leg or 1 leg segment. Legs were ground to a fine powder with tissue grinders (Kontes, Vineland, NJ) in a 1.5-ml microcentrifuge tube that contained liquid nitrogen. After grinding, the liquid nitrogen was allowed to evaporate and the powder resuspended in 1 ml of lysis buffer (50 mM NaCl, 50 mM EDTA,
Table 2. Eleven (of 27) decameric oligonucleotide primers that gave RAPD profiles with template DNA from triatomine bugs and distinguished *P. megistus*, *P. prolixus*, and *T. infestans*

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>5'–3' Sequence</th>
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<tbody>
<tr>
<td>L2</td>
<td>CGGACCTGCCC</td>
</tr>
<tr>
<td>L4</td>
<td>GTGGATGCGGA</td>
</tr>
<tr>
<td>L5</td>
<td>AAGACCTCGGT</td>
</tr>
<tr>
<td>A1</td>
<td>TCAGATAGCA</td>
</tr>
<tr>
<td>A2</td>
<td>GAAGACCGGTT</td>
</tr>
<tr>
<td>A5</td>
<td>GTGACGTAGG</td>
</tr>
<tr>
<td>H2</td>
<td>TTTCCCCGCTG</td>
</tr>
<tr>
<td>H3</td>
<td>CATCCCCCTG</td>
</tr>
<tr>
<td>H5</td>
<td>TAGGATCAGA</td>
</tr>
<tr>
<td>L1</td>
<td>CGGCCTCTGG</td>
</tr>
<tr>
<td>L6</td>
<td>AAGGATACGA</td>
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Random amplification of polymorphic DNA. The RAPD protocol (Carrasco et al. 1996) was modified from Williams et al. (1990). Reaction mixtures of 20 μl contained 0.2 mM dATP, dCTP, dGTP, and dTTP (Pharmacia LKB Biotechnology, Uppsala, Sweden), 20 pmol oligonucleotide primer, 1.0 U Taq DNA polymerase (Stratagene, Cambridge, U.K.), ~5 ng template DNA, 0.001 gelatin (in 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris HCl, pH 8.8) overlaid with 40 μl of mineral oil (3416, Sigma). PCR primers (British Biotechnology Products, Oxon, U.K.) giving RAPD profiles that distinguish between triatomine genera are listed in Table 2. Amplifications were performed on a programmable thermal cycler (Hybaid, Middlesex, U.K.) with the following cycle conditions: 94°C for 5 min, 30°C for 2 min, 72°C for 1 min (2 cycles); 94°C for 30 s, 40°C for 2 min, 72°C for 1 min (32 cycles); 95°C for 30 s, 40°C for 2 min, 72°C for 5 min (1 cycle). Negative control PCR reactions included all reaction components except template DNA, made up to an equal volume with sterile water.

Random amplification of polymorphic DNA products were electrophoresed in agarose (2%, in TAE) stained with ethidium bromide and recorded on a gel documentation system with a UV light transilluminator. In addition, the RAPD products from *R. prolixus* and *R. robustus* were compared by electrophoresis in polyacrylamide (6%, in 1 × TBE buffer) and silver staining according to the conditions described by Vidigal et al. 1994.

Results

Discrete RAPD profiles were obtained with tissues from live triatomine bugs and from dry bugs stored for up to 12 mo at room temperature (Fig. 1). Profiles for all individuals within a species were not precisely identical, but profiles for dried specimens were compatible with those for live insects. Specimens were, with the limited scope of the samples studied, readily assignable to species on the basis of RAPD profiles (Fig. 1, tracks 5–8). Conditions were controlled such that the concentrations of reactants and cycling conditions were unaltered, and a single commercial source of Taq polymerase was used. Suitable templates could be obtained without Geneclean (not shown). Clearly, identifiable profiles were given from all life cycle stages from eggs (whole organism as samples), nymphs (legs or single leg segment), and adults (legs or single leg segment) although some profile diversity was again seen between individuals (Fig. 2, primer A2).
Of 27 decameric primers tested, 11 gave both discrete RAPD profiles and distinguished among P. megistus, R. prolixus, and T. infestans. Examples of results with 4 of the 11 primers are shown in Figs. 1–6. Examples of RAPD profiles for the 3 genera with the 2 primers A2 and L5 are shown in Fig. 3; few profile components were shared across the genera.

We determined whether RAPD profiles could distinguish between species within the same genus, including species that were thought to be closely related, such as R. ecuadorensis and R. pictipes, R. nasutus, and R. neglectus, or for which the taxonomic status is controversial (R. prolixus and R. robustus) and also to see whether RAPD data gave any indication of affinities among species. The sharing of profile components across such species, with primers that were highly discriminatory for the 3 genera, did suggest a relationship between the species pairs (Fig. 4). Yet in each case, profile components separated species pairs and might prove to be species-specific characters. Thus, 3 bands distinguished between R. pictipes and R. ecuadorensis, 2 between R. nasutus and R. neglectus, and 2 between R. prolixus and R. robustus, with primer A2.

Random amplification of polymorphic DNA profiles of individual adult T. infestans from 3 different localities in Bolivia were compared using 4 primers. Polymorphisms were seen with primers H3 (Fig. 5) and L5 (not shown), but none of these polymorphisms was confined to a single locality. T. infestans from 2 further Bolivian localities showed the same range of polymorphisms (not shown). Colonies of R. prolixus from Brazil, Colombia, and the United Kingdom, but of unknown early history, were also compared and no colony-specific characteristics were seen with 6 primers tested.

The resolution of RAPD products by nondenaturing polyacrylamide gel electrophoresis and silver
Fig. 5. RAPD profiles of T. infestans from 3 different localities in Bolivia with primer H3. Tracks: (1-3) Tarija, (4-6) Cochabamba, (7 and 8) Sucre. Track M contains 1-kb ladder marker.

staining was checked with R. prolixus and R. robustus, allowing, for the samples available to us, correct assignment to species; variation was seen between individuals within the same species.

Discussion

We have obtained consistent RAPD profiles from living specimens and dried specimens stored for up to 12 mo, and from all life cycle stages from egg to adult by using rigorously standardized PCR mixtures.

The use of legs or leg segments as source of DNA template avoids the complication of contamination with host blood meal templates or with DNA from parasites, such as T. cruzi, or from symbionts in the triatomine gut. As expected, occasional differences were seen in the RAPD profiles for individuals within the same species. Usually these differences were in intensity of common components but in some cases in the presence or absence of discrete intense staining bands (Figs. 2 and 4, R. prolixus; Fig. 5, T. infestans). Obviously, much greater diversity might be found if specimens representing the entire geographical range of a given species are examined.

The profiles for representatives of the 3 genera Panstrongylus, Rhodnius, and Triatoma were very different with few components shared (Fig. 3).

A few species of triatomine bugs are so alike morphologically that their independent taxonomic status has been questioned. We selected 3 species pairs, R. pictipes, R. ecuadorensis; R. neglectus, R. nasutus; R. robustus, R. prolixus, to give us an indication of the capacity of RAPD profiles to assess and resolve relationships between closely related species of the same genus. R. neglectus is distinguished from R. nasutus by various aspects of the color, the wider basis of the median process of the pygophore...
of the male genitalia in the latter species, and by isoenzyme profiles (J. P. Dujardin, ORSTOM/IBBA, La Paz, Bolivia, personal communication). *R. robustus* shares most morphological and color characters with *R. prolixus* and its taxonomic status as a distinct species has been doubted (Harry et al. 1992). In their monograph, Lent and Wygodzinsky (1979) regard *R. robustus* and *R. prolixus* as valid taxa based on differences in color of the hind tibia of 4th and 5th stage nymphs (light in *R. robustus*) and small differences in the shape of the modified basal plate struts of the aedeagus of the male genitalia. Harry et al. (1992) found no isoenzyme differences between what they considered to be silvatic *R. robustus* and domestic *R. prolixus* from Trujillo, Venezuela. The taxonomic status of these 2 species is of considerable epidemiological importance because *R. robustus* is widely distributed in silvatic habitats where no domestic *R. prolixus* have been found. In the Amazon basin, *R. robustus* is abundant in palm trees (Miles et al. 1981, 1983) and yet truly domiciliated triatomine bug species have not been found anywhere in the Amazon region.

Although we have not yet attempted any analysis based on pairwise presence/absence comparisons, the RAPD profiles for species within the genus *Rhodnius* broadly accorded with perceived relationships from traditional morphological comparisons. Thus, in Fig. 4, *R. prolixus* and *R. robustus*, *R. neglectus* and *R. nasutus* share a number of features in common, *R. pictipes* and *R. ecuadorensis*, whilst similar to each other, appeared to diverge from the other 4 species of *Rhodnius* (Figs. 4 and 6). Nevertheless, in each case, with the samples examined here, RAPD criteria could assign individual specimens to species. Clearly the ability to define genus and species-specific criteria must depend on broader comparisons within and across species using samples representing wider areas of distribution, for example, to exclude differences caused by genetic drift between populations of the same species. These results imply that RAPD profiles provide a molecular basis to reassess taxonomic relationships in the triatomine subfamily.

A further important aspect of vector biology with important implications for control of Chagas disease is the ability of populations of the same bug species to move between silvatic and domestic habitats. Other authors have used RAPD for the identification of cryptic species (Hadrys and Siva-Jothy 1994, Wilkerson et al 1995), for genome analysis of the malaria vector *Anopheles gambiae* (Favia et al. 1994), to identify related species of the mosquito genus *Aedes* and populations of *Aedes albopictus* (Kambhapati et al. 1992) and to identify the sandfly species *Lutzomyia spinicrassa* Morales, Osorno-Mesa, Osorno & Hoyos, and *Lutzomyia youngi* Feliciangeli & Murillo (Adamson et al. 1993). Not surprisingly, RAPD analysis has shown high levels of polymorphism where little allozyme diversity has been detected (Black et al. 1992). *T. infestans* is considered to be entirely domestic throughout most of its distribution and this has simplified the Southern Cone Programme for elimination of *T. infestans*. In Cochabamba, Bolivia, however, *T. infestans* is found in both silvatic and domestic habitats. Isoenzyme characters (Dujardin et al. 1987) did not detect any signs of speciation between the silvatic and domestic *T. infestans* populations, although morphometric analysis suggested separation between them (Dujardin et al. 1996a). Nevertheless, isoenzyme analysis has helped to distinguish between residual infestation and reinfestation of sprayed houses (Dujardin et al. 1996b). We used 3 geographically separate Bolivian populations to show that some RAPD primers can detect polymorphisms among populations of *T. infestans* (Fig. 5) and thus, in principle, might provide a means of following bug migration and establishing source of reinfestation. Carlier et al. (1996) report that RAPD with *T. infestans* reveals higher intraspecific variability than isoenzymes.

Combinations of biochemical, cytogenetic, and morphological criteria have been used to address the limitations of classical taxonomic methods. It seems likely that molecular methods of genome analysis, of which RAPD is but one approach, will provide a valuable means of reassessing triatomine phylogeny, including relationships between the Old and New World species and with other Reduviidae without destroying important morphological characteristics. It is likely that automated fragment analysis of fluorescently labeled PCR products of known identity will provide the basis for informative qualitative comparisons. The possibility of using dried specimens, for example from museum collections, suggests that such a study could, in part, be retrospective. As well as the intrinsic academic interest, a clear view of triatomine taxonomy and vector potential will assist the worthy and achievable goal of eliminating vector-borne *T. cruzi* transmission in the Americas.
Acknowledgments

We thank H. Bermudez (Bolivia), C. Curtis (U.K.), and the Instituto Nacional de Salud (Bogota, Columbia) for their encouragement and cooperation and the World Health Organization, European Commission, Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICIT, Venezuela), Fundação Nacional de Saúde (Brazil), and the Wellcome Trust (U.K.) for financial support.

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Received for publication 24 October 1996; accepted 19 June 1997.