Annals of Tropical Medicine & Parasitology, Vol. 101, No. 3, 247–253 (2007)

The identification of sandfly species, from an area of Argentina with endemic leishmaniasis, by the PCR-based analysis of the gene coding for 18S ribosomal RNA

P. A. BARROSO[*], J. D. MARCO[*,†], H. KATO[‡], R. TARAMA[‡], P. RUEDA[§], S. P. CAJAL[§], M. A. BASOMBRÍO[†], M. KORENAGA[*], N. J. TARANTO[§] and Y. HASHIGUCHI[*]

[*]*Department of Parasitology, Kochi Medical School, Kochi University, Kohasu, Okoh, Nankoku, Kochi 783-8505, Japan*
[†]*Instituto de Patología Experimental, Facultad de Ciencias de la Salud, Universidad Nacional de Salta – Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires 177, Salta 4400, Argentina*
[‡]*Department of Veterinary Hygiene, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan*
[§]*Instituto de Enfermedades Tropicales, Sede Regional Orán, Universidad Nacional de Salta, Alvarado 751, San Ramón de la Nueva Orán, Salta 4530, Argentina*

Received 10 February 2006, Revised 18 July 2006, Accepted 21 July 2006

Published by Maney Publishing (c) Liverpool School of Tropical Medicine

The area around Río Blanco, in the Orán department in the north of the Argentinian province of Salta, is endemic for American tegumentary leishmaniasis. In an attempt to facilitate the identification of the *Lutzomyia* species in this area, sequences of the gene coding for the 18S ribosomal RNA (rRNA) of sandflies caught in a Shannon trap were explored, by a combination of PCR and analysis of restriction-fragment-length polymorphism (RFLP). The products from the PCR, which employed two primers developed specifically for this study (Lu.18S 1S and Lu.18S AR), were cloned into a commercial vector (pGEM-T Easy) so that their nucleotide sequences could be investigated. In the RFLP analysis, the products of single and double digestion with the *Afa*I and *Hap*II restriction enzymes were separated by electrophoresis in 3% or 4% agarose. Taken together with the results of a morphological investigation of the flies, the resultant DNA fragment patterns were sufficient to identify most of the sandflies caught as *Lu. neivai*. Although two other species, *Lu. cortelezzii* and *Lu. sallesi*, were collected, they were relatively rare and only identified morphologically. A single digestion of the 18S-rRNA gene sequences with *Afa*I or *Hap*II appeared sufficient and useful for the identification of *Lu. neivai* from the north of Salta province, and for several other *Lutzomyia* species.

During the 1980s and 1990s, three epidemic outbreaks of American tegumentary leishmaniasis (ATL) occurred in the north of the Argentinian province of Salta, where *Leishmania* (*Viannia*) *braziliensis* is the predominant causative agent of the disease, followed by *L.* (*Leishmania*) *amazonensis* and *L.* (*V.*) *guyanensis* (Cupolillo *et al.*, 1994; Segura *et al.*, 2000; Frank *et al.*, 2003; Marco *et al.*, 2005). Although five species of sandfly have been reported in the Orán and San Martín departments of Salta province — *Lutzomyia neivai*, *Lu. migonei*, *Lu. cortelezzii*, *Lu. punctigeniculata* and *Lu. shannoni* (Salomón *et al.*, 2004) — little is known about their vectorial capacity. In most studies on the transmission of *Leishmania* spp., sandflies are identified by the morphological examination of internal

Reprint requests to: P. A. Barroso.; E-mail: paola@med.kochi-u.ac.jp; fax: +81 88 880 2415.

© 2007 The Liverpool School of Tropical Medicine
DOI: 10.1179/136485907X156988

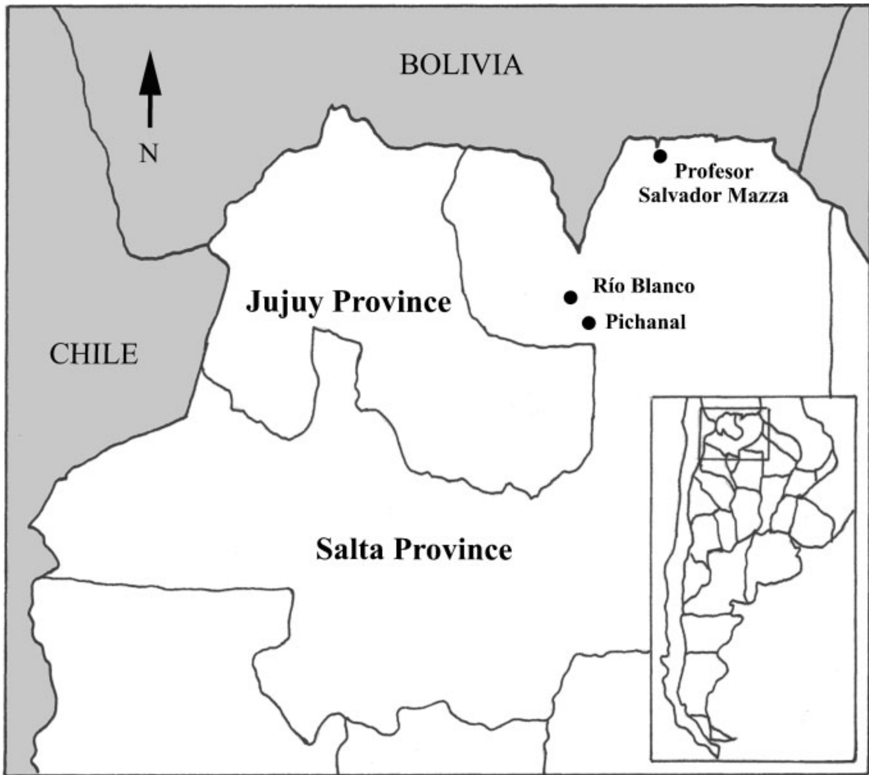

FIG. 1. Map of the north of Argentina, showing the locations of the sites, in Salta province, where the sandfly specimens were caught.

structures — often the spermathecae, cibaria and pharynges of the female flies and the terminal genitalia of the males (WHO, 1990). This technique, however, requires special taxonomic skills, is time-consuming, cannot be applied simultaneously to two or more flies, and is often not useful in distinguishing between species that are morphologically very similar. Although iso-enzyme electrophoresis or the analysis of cuticular hydrocarbons by gas chromatography can be useful in identifying sandfly species, including those that are difficult to distinguish by morphology (WHO, 1990), they require refined storage conditions for the samples. Within the last decade, sandflies from the Old World have been successfully identified, to species level, by restriction-fragment-length-polymorphism (RFLP) analysis, using the products of the PCR-based amplification of the flies' 18S ribosomal RNA (rRNA) genes (Aransay *et al.*, 1999). In the present study, this molecular technique, which appears to be reliable and does not need either refined storage conditions for the samples or taxonomic expertise, was applied, in a modified form, to the sandflies of northern Argentina.

MATERIALS AND METHODS

Study Areas

Sandflies were collected at two sites (A and B) in Río Blanco and at one site (C) in Pichanal, all in the Orán department, and at two sites (D and E) in Profesor Salvador Mazza, all in the northern part of Salta province, Argentina (Fig. 1). Cases of ATL have been reported at Río Blanco and Pichanal. Profesor Salvador Mazza has no recorded history of the disease, although it

Published by Maney Publishing (c) Liverpool School of Tropical Medicine

does possess phyto–geographical characteristics that appear favorable for sandflies (unpubl. obs.).

Sandfly Collections

The sandflies were collected, in an illuminated Shannon trap, during December 2002 and January 2003 (a period that coincides with the seasonal peak in the abundance of adult sandflies). Each collection was made between 20.30 to 22.30 hours, with the sandflies caught kept cool (4°C) until the next morning, when most were dissected and identified by morphology. A subsample of the large collection made at site B in Río Blanco was fixed in 70% ethanol and stored until it could be investigated by PCR–RFLP (see below).

Sandfly Species Identification

MOLECULAR CLONING AND NUCLEOTIDE SEQUENCING OF SANDFLY 18S-RRNA GENE

Since the primers used by Aransay *et al.* (1999), in their studies of Old-World *Phlebotomus*, did not work on the New-World *Lutzomyia* collected in the present study, new primers, based on conserved sequences of the 18S-rRNA genes of *Lutzomyia* species, had to be developed. The new primers were named Lu.18S 1S (5′-TGC CAG TAG TTA TAT GCT TG-3′) and Lu.18S AR (5′-CAC CTA CGG AAA CCT TGT TAC-3′). They were used in PCR similar to those described by Aransay *et al.* (1999), and each reaction used 25 µl of a commercial PCR mixture (Premix *Taq*™; Takara Bio, Shiga, Japan) and DNA extracted (Aransay *et al.*, 1999) from flies, from the site-B collection, that had either been identified, by morphology, as *Lu. neivai* or were 'unidentified'. The thermocycler used was set to give an initial denaturation at 95°C for 2 min, followed by 40 cycles, each of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and polymerization at 72°C for 2 min. The product of each PCR was cloned into a commercial vector (pGEM-T Easy Vector; Promega, Madison, WI), and then sequenced (Kato *et al.*, 2005). The levels of homology between the sequence of the PCR-amplified fragment from the flies morphologically identified as *Lu. neivai* (GenBank accession number AB214970) and the 18S-rRNA genes from *Lu. shannoni*, *Lu. geniculata*, *Lu. vattieri*, *Lu. toroensis*, *Lu. verrucarum* and *Lu. longipalpis* were evaluated using the BLAST program (Altschul *et al.*, 1990).

RESTRICTION-FRAGMENT ANALYSIS

For the RFLP analysis, 5 µl of each PCR product were digested with the restriction enzymes *Afa*I (Takara Bio) and *Hap*II (Takara Bio), which were used either separately or in combination. Each enzyme-digested sample was separated by electrophoresis in 3% or 4% agarose gel, to produce a DNA fragment pattern.

MORPHOLOGICAL CHARACTERISTICS

The morphological identification of the *Lutzomyia* caught was based mainly on the spermathecae and cibarial armatures, although, in some cases, it was also necessary to examine the male terminalia (Young and Duncan, 1994; Marcondes, 1996).

RESULTS

In the present study, in order to establish a molecular-genetics basis for the classification of *Lutzomyia*, analysis of the 18S-rRNA gene by PCR–RFLP was extended to samples from this New-World genus. The new primer pair, Lu.18S 1S and Lu.18S AR, amplified a single, identical DNA fragment, of about 2 kbp, from the total DNA of each of the site-B flies identified morphologically as *Lu. neivai* (Fig. 2) and that of each of the 19 unidentified sandflies from site B that were also tested. The sequence of this PCR-amplified fragment showed 98% homology with the corresponding (18S-rRNA) genes from *Lu.*

Published by Maney Publishing (c) Liverpool School of Tropical Medicine

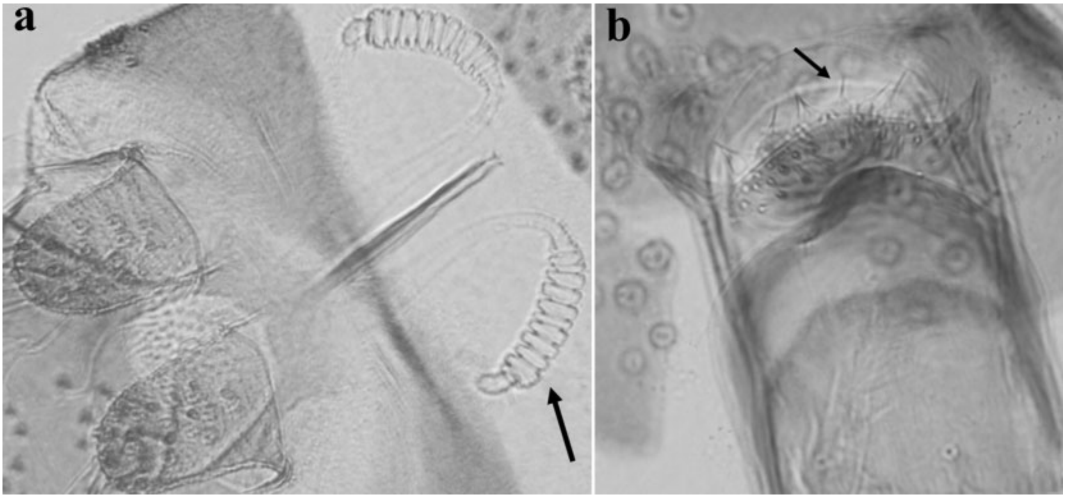

FIG. 2. Photomicrographs of a female *Lutzomyia neivai*, showing the spermathecae (a) and a horizontal tooth in the cibarium (b).

Published by Maney Publishing (c) Liverpool School of Tropical Medicine

shannoni, *Lu. geniculata*, *Lu. vattieri* and *Lu. toroensis*, and 97% homology with those from *Lu. verrucarum* and *Lu. longipalpis*. The *Lu. neivai* 18S-rRNA gene sequence was split at seven points (nucleotide positions 50–51, 87–88, 529–530, 671–672, 863–864, 1579–1580 and 1864–1865) by *Afa*I, and at six points (390–391, 755–756, 859–860, 1225–1226, 1249–1250 and 1806–1807) by *Hap*II. As shown in Figure 3(a), digestion of the PCR product with *Afa*I alone therefore gave eight fragments (of 37, 50, 142, 155, 192, 285, 442 and 716 bp), whereas digestion with *Hap*II alone gave seven fragments (of 24, 104, 213, 365, 366, 390 and 557 bp). Double digestion of the *Lu. neivai* 18S-rRNA gene sequence, with both *Afa*I and *Hap*II, resulted in 14 fragments [of 4, 24, 37, 50, 58, 84, 104, 139, 142, 155, 227, 303, 330 and 362 bp; Fig. 3(b)]. As the 19 'unidentified' sandflies from Río Blanco gave fragment patterns similar to that produced using the morphologically identified *Lu. neivai*, they were all identified as this species [Fig. 3(b)].

Usefully, in terms of taxonomy, the same PCR–RFLP analysis, using single digestion with either *Afa*I or *Hap*II and DNA from *Lu. shannoni*, *Lu. geniculata*, *Lu. vattieri*, *Lu. toroensis*, *Lu. verrucarum*, *Lu. longipalpis* or any of several other *Lutzomyia* species, always gave a species-specific pattern that was distinct from that of *Lu. neivai* and that of any other species tested (data not shown).

Almost all the sandflies collected in the present study were identified as *Lu. neivai* (see Table). The samples of the other species collected, which were identified by morphology as *Lu. cortelezzii* and *Lu. sallesi*, were judged too small to make their investigation by PCR–RFLP worthwhile (Table).

DISCUSSION

In the present study, attempts were made to modify and standardize the '18S-rRNA-gene' technique developed for *Phlebotomus* in the Old World (Aransay *et al.*, 1999), for use in the identification of Argentinian *Lutzomyia* (initially those caught at Río Blanco, in the department of Orán, where ATL is endemic). Since PCR based on the F1 and R1 primers developed by Aransay *et al.* (1999) did not give any useful product when run with *Lutzomyia* DNA (data not shown), a new pair of primers, Lu.18S 1S

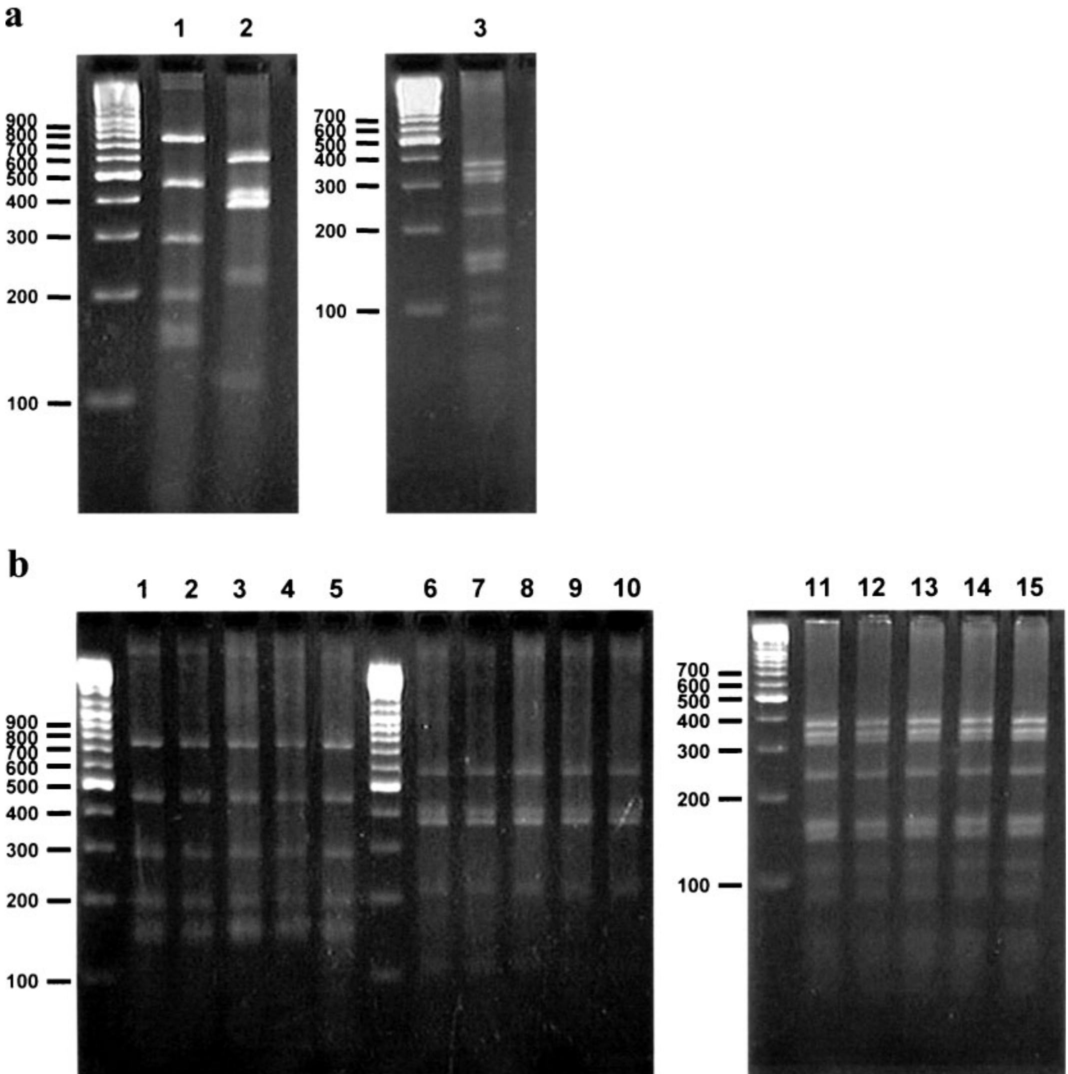

FIG. 3. Classification of Argentinian *Lutzomyia* by the PCR–RFLP analysis of their 18S-rRNA gene fragments. Panel (a) shows the fragments, from a fly morphologically identified as *Lu. neivai*, produced by digestion with *Afa*I (lane 1), *Hap*II (lane 2) or both *Afa*I and *Hap*II (lane 3). Panel (b) illustrates how five of the 19 unidentified flies showed identical fragment patterns after the PCR-amplified fragment of their 18S-rRNA genes was digested with *Afa*I alone (lanes 1–5), *Hap*II alone (lanes 6–10), or the combination of both of these enzymes (lanes 11–15); the other 14 unidentified flies showed the same patterns.

Published by Maney Publishing (c) Liverpool School of Tropical Medicine

and Lu.18S AR, had to be produced. By using this pair of primers, a fragment of the *Lu. neivai* 18S-rRNA gene could be amplified and compared with the corresponding sequences of *Lu. shannoni*, *Lu. geniculata*, *Lu. vattieri*, *Lu. toroensis*, *Lu. verrucarum* and *Lu. longipalpis*, which showed 97%–98% homology. Usefully, at least for *Lu. neivai* from Salta province and several other species of *Lutzomyia*, the interspecific differences in the fragments amplified in PCR based on the Lu.18S 1S and Lu.18S AR primers were sufficient to give species-specific patterns when those products were digested with *Afa*I or *Hap*II and then subjected to electrophoresis.

TABLE 1. *The results of the morphological or molecular identification, to species level, of the sandflies collected in Salta province, Argentina, in December 2002–January 2003*

Species	No. of sandflies caught in:					
	Río Blanco			Profesor Salvador Mazza		
	Site A	Site B	Pichanal (site C)	Site D	Site E	All sites
Lu. neivai	13	268*	1	0	1	283
Lu. cortelezzii	0	0	17†	0	0	17
Lu. cortelezzii/Lu. sallesi	0	0	0	6‡	1	7

*Nineteen identified by the PCR–RFLP analysis of their 18S-rRNA gene.
†Sixteen females assumed to be *Lu. cortelezzii* as the only male caught at this site was identified as this species
‡The four females were identified as *Lu. cortelezzii/Lu. sallesi* since one male identified as *Lu. cortelezzii* and one male identified as *Lu. sallesi* were caught at this site.

Published by Maney Publishing (c) Liverpool School of Tropical Medicine

Although, in the PCR–RFLP study of Old-World sandflies by Aransay *et al.* (1999), double digestion, with *Rsa*I (which cleaves the same sites as *Afa*I) and *Hpa*II (which cleaves the same sites as *Hap*II), sometimes gave more useful results than digestion with a single restriction enzyme, this was not the case with the Argentinian *Lutzomyia* (present study). Aransay *et al.* (1999) found that double digestion gave species-specific patterns for *P. papatasi*, *P. alexandri*, *P. sergenti*, *P. simici*, *Sergentomyia minuta*, *S. fallax cypriotica* and *S. dentata*, although three other *Phlebotomus* species, all belonging to the subgenus *Larroussius*, required a second double digestion, with *Acc*I and *Ban*I, to distinguish them. In the present study, double digestion, with *Afa*I and *Hap*II, gave many, relatively small fragments and a fragment pattern that was, for the identification of the species investigated, no more useful and slightly harder to 'read' than the pattern produced when either of these enzymes was used alone.

In conclusion, the present results indicate that PCR–RFLP analysis of the 18S-rRNA gene could be a suitable and useful method for the classification of the *Lutzomyia* species found in the north of Salta province. The usefulness of this technique in the identification of other *Lutzomyia* species, from Salta province and elsewhere, will be explored in future studies.

ACKNOWLEDGEMENTS. The authors thank D. B. Ribble, for his constructive comments on the manuscript. This study was supported financially by the Ministry of Education, Science, Culture and Sports of Japan (via grants 14256002 and 18256004).

REFERENCES

Altschul, S. F. , Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403–410.

Aransay, A. M., Scoulica, E., Chaniotis, B. & Tselentis, Y. (1999). Typing of sandflies from Greece and Cyprus by DNA polymorphism of 18S rRNA gene. *Insect Molecular Biology*, **8**, 179–184.

Cupolillo, E., Grimaldi Jr, G. & Momen, H. (1994). A general classification of New World *Leishmania* using numerical zymotaxonomy. *American Journal of Tropical Medicine and Hygiene*, **50**, 296–311.

Frank, F. M., Fernández, M. M., Taranto, N.J., Cajal, S. P., Margni, R. A., Castro, E., Thomaz-Soccol, V. & Malchiodi, E. L. (2003). Characterization of human infection by *Leishmania* spp. in the northwest of Argentina: immune response, double infection with *Trypanosoma cruzi* and species of *Leishmania* involved. *Parasitology*, **126**, 31–39.

Kato, H., Uezato, H., Katakura, K., Calvopiña, M., Marco, J. D., Barroso, P. A., Gomez, E. A. L., Mimori, T., Korenaga, M., Iwata, H., Nonaka, S. & Hashiguchi, Y. (2005). Detection and identification

of *Leishmania* species within naturally infected sand flies in the Andean areas of Ecuador by a polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene*, **72**, 87–93.

Marco, J. D., Barroso, P. A., Calvopiña, M., Kumazawa, H., Furuya, M., Korenaga, M., Cajal, S. P., Mora, M. C., Rea, M. M. J., Borda, C. E., Basombrío, M. A., Taranto, N. J. & Hashiguchi, Y. (2005). Species assignation of *Leishmania* from humans and canine American tegumentary leishmaniasis cases by multilocus enzyme electrophoresis in north Argentina. *American Journal of Tropical Medicine and Hygiene*, **72**, 606–611.

Marcondes, C. B. (1996). A redescription of *Lutzomyia* (*Nyssomyia*) *intermedia* (Lutz & Neiva, 1912), and resurrection of *L. neivai* (Pinto, 1926) (Diptera, Psychodidae, Phlebotominae). *Memórias do Instituto Oswaldo Cruz*, **91**, 457–462.

Salomón, O. D., Wilson, M. L., Munstermann, L. E. & Travi, B. L. (2004). Spatial and temporal patterns of phlebotomine sand flies (Diptera: Psychodidae) in a cutaneous leishmaniasis focus in northern Argentina. *Journal of Medical Entomology*, **41**, 33–39.

Segura, E. L., Juan, N., Piquin, A. L. M., Cuba-Cuba, C. A., Abramo-Orrego, L., McMahon-Pratt, D., Montamat, E. E., Momen, H. & Grimaldi Jr, G. (2000). Molecular and biologic characterization of *Leishmania* parasites implicated in an epidemic outbreak in northwestern Argentina. *Parasitology Research*, **86**, 504–508.

World Health Organization. (1990). *Control of the Leishmaniases*. Technical Report Series No. 793. Geneva: WHO.

Young, D. G. & Duncan, M. A. (1994). Guide to the identification and geographic distribution of *Lutzomyia* sand flies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae). *Memoirs of the American Entomological Institute*, **54**, 1–881.

Published by Maney Publishing (c) Liverpool School of Tropical Medicine