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Sand fly typing: a simple and morphologically-supported method based on polymorphism of 18S rRNA gene in a Leishmaniasis endemic area of Argentina

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ABSTRACT

Leishmaniases are vector-borne diseases that in the Americas are distributed from southern United States to northern Argentina. The vectors for this disease are small dipterans known as sand flies that are usually identified morphologically by observing structures with taxonomic value; but it is time-consuming, laborious, and requires entomological expertise. Then, this work was aimed at identifying sand flies with molecular techniques, using the morphological identification as a reference technique, in an endemic area of American Tegumentary Leishmaniasis (ATL) located in northern Argentina. For this, sand flies were caught at two patches of vegetation adjacent to rural areas in Orán department, Salta Province. Females were dissected with sterile needles; the head and last abdominal segments were analyzed for morphological identification. The remaining thorax and abdominal segments were used to extract DNA, which was amplified by PCR of the small subunit (SSU), 18S rRNA gene. PCR products were digested with CviQI and DdeI enzymes to identify sand fly species by Restriction Fragment Length Polymorphism (RFLP) analysis. Thus, the restriction pattern of each caught species was defined according to morphological identification. A total of 1501 females, belonging to four sand fly species, were captured. *Nyssomyia neivai* (1347/1501) was the most abundant species, followed by *Migonemyia migonei* (90/1501). From the total, 801 females were morphologically and molecularly identified, while 700 females were characterized only molecularly. For those females analyzed by both methods, there was total coincidence in the achieved result. Besides, the 5% (38/801) of females that could not be determined morphologically due to inadequate mounting were molecularly identified. All the females characterized just by PCR-RFLP, were successfully identified. Our results indicate that the explored method is capable of identifying the sand fly species that circulate in an ATL endemic area. Since this method is based on the analysis of markedly different patterns, the identification process might be more easily reproduced, as the bias introduced by the technician's lack of experience is removed.

1. Introduction

Vector-borne diseases stand for more than 17% of all infectious diseases, causing more than 700 000 deaths annually. The economic burden of these illnesses is significant and wide; the expenses range from vector control activities and clinical case management to personal protective measures as insect repellent (WHO, 2017). Particularly, the leishmaniases are a group of parasitic diseases caused by kinetoplastid flagellates from *Leishmania* genus that are transmitted by female sand fly bite (Diptera: Psychodidae: Phlebotominae) (Killick-Kendrick,

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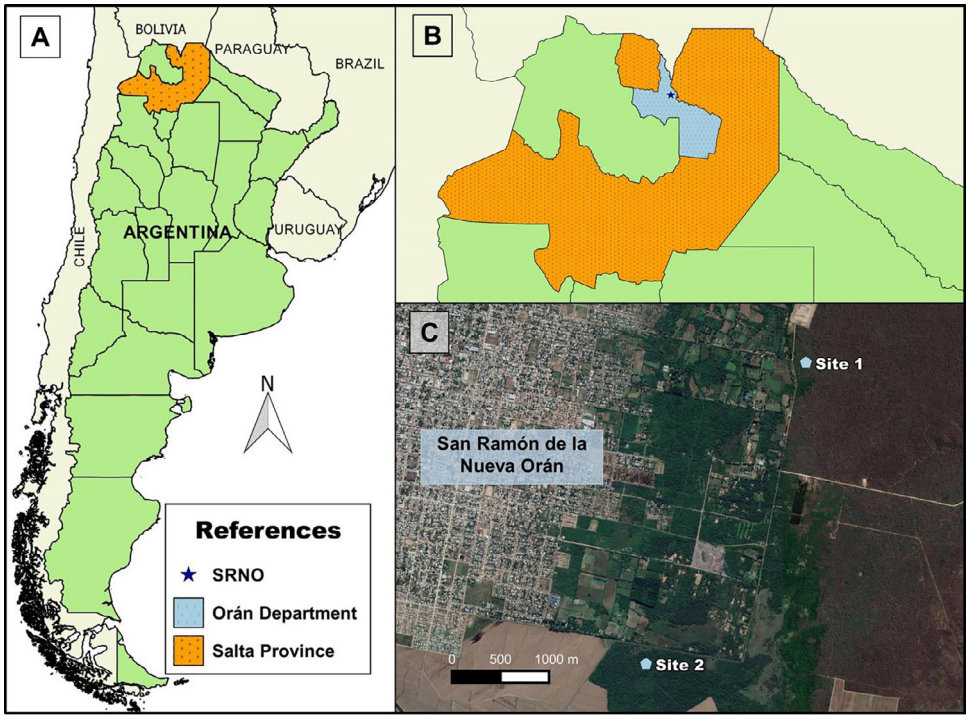

Fig. 1. A: Location of Salta Province in Argentina. **B:** Location of Orán department and San Ramón de la Nueva Orán (SRNO) city. **C:** Sampling sites.

1990). Also, the leishmaniases comprise a wide spectrum of clinical manifestations with an estimated annual incidence of 1-1.5 million cases for cutaneous forms and half a million cases for visceral forms (Bruschi and Gradoni, 2018; Desjeux, 2004).

In the Americas, the leishmaniases occur from southern United States to northern Argentina, except for Chile (OPS, 2019). Singularly, in Argentina the American Tegumentary Leishmaniasis (ATL) is endemic to ten provinces (Salomón et al., 2012) and in the north of Salta province it has mainly been described in Orán and San Martín departments. This zone reported 53% of human ATL cases for the country, although only 0.7% of the Argentinian population lives there (Salomón et al., 2008). Furthermore, Orán department has gained more epidemiological relevance due to two main outbreaks: the first ATL epidemic outbreak occurred during 1984-1985 (Sosa-Estani et al., 2000), and the largest outbreak recorded in the country with more than 900 human cases in 1998 (Salomón et al., 2001).

Regarding Argentinian phlebotomine fauna, entomological studies showed the presence of 32 sand fly species distributed in 14 provinces (Szelag et al., 2016). The following species have been found in Salta province: *Nyssomyia neivai, Migonemyia migonei, Psathyromyia bigeniculata, Psathyromyia punctigeniculata, Evandromyia cortelezzii, Evandromyia sallesi, Micropygomyia quinquefer*, and *Lutzomyia longipalpis* (Barroso et al., 2007; Bravo et al., 2013; Chanampa et al., 2018; Copa et al., 2018; Quintana et al., 2010; Salomón et al., 2004). Most of these species are also present in the other provinces where *Nyssomyia whitmani, Pintomyia pessoai, Pintomyia fischeri*, and *Brumptomyia* spp. can be found too (Salomón et al., 2009).

Usually, the observation of internal structures like the cibarium and the spermathecae for females and the terminal genitalia for males allow the identification of sand fly species (Galati, 2003; Young and Duncan, 1994). However, this procedure is laborious, time-consuming, and dependent on the experience and entomological skills of technicians (Giantsis et al., 2017). Moreover, insects are frequently damaged during capture, transport or mounting process, hindering the traditional taxonomy (Lane and Crosskey, 1994).

Due to the restrictions or limitations above mentioned, several molecular markers have been studied to develop a simpler method to identify sand fly species. Most of the molecular techniques are DNA-based and consist mainly of the sequencing of PCR products (Depaquit, 2014). Some of the latest and most cutting-edge methods are DNA-barcoding studies (Nzelu et al., 2015; Romero-Ricardo et al., 2016), and Next Generation Sequencing assays that identify *Leishmania* parasites, blood sources, and plant meals simultaneously (Abbasi et al., 2019). Nevertheless, when numerous sand flies need to be analyzed for epidemiological or surveillance tasks, those molecular techniques are not suitable for low-resource endemic areas. In contrast, the analysis of PCR products by Restriction Fragments Length Polymorphism (RFLP) might be a more convenient alternative, since no sophisticated equipment nor a high budget are required.

The analysis of the small subunit (SSU), 18S ribosomal RNA gene (18S rRNA) by PCR-RFLP allowed the successful species determination of sand flies from the Old and New World (Aransay et al., 1999; Terayama et al., 2008). The 18S rRNA target has a slow mutation rate, which allows the design of universal primers, and its high number of copies simplifies PCR assay (Aransay et al., 2000; Turbeville et al., 1991). Previously, our work group analyzed with these techniques, sand flies from northern Salta province, Argentina, which is a hyper endemic region for ATL (Barroso et al., 2007). The authors focused on determining the restriction pattern of *Ny. neivai*, which is the main ATL vector in the country. Nevertheless, at least five other sand fly species were recorded for the area (Salomón et al., 2008, 2004), which allowed us to deepen the molecular analysis.

In this context, the aim of this work was to identify sand fly species that circulate in Orán department by PCR-RFLP of the 18S rRNA gene, considering the morphological determination as the Gold standard.

2. Materials and methods

2.1. Study area and sand fly collection

The study took place in San Ramón de la Nueva Orán (23°01′25″S and 64°23′29″W), which is the main city of Orán department located in the north of Salta Province, Argentina (Fig. 1A and 1B). Sand flies were caught with Centers for Disease Control (CDC) light traps placed at 1.5

m above ground during 12 h (from 7 pm to 7 am).

Sand flies were captured at two patches of secondary vegetation adjacent to rural areas; the CDC traps were placed in extra-domiciliary environments. Site 1 was located to the East of San Ramón de la Nueva Orán city (23°8′0.43"S and 64°17′4.13"W) (Fig. 1C). There, captures were done monthly from March to December, 2016, and sample collection consisted of a night of sampling using nine CDC. Site 2 was located to the SE of San Ramón de la Nueva Orán city (23°9′39.21"S and 64°18′5.79"W) (Fig. 1C). In that site, captures were done bimonthly, from December, 2016 to November 2017, and sample collection consisted of two or three nights per sampling using ten CDC. Captured sand flies were fixed in 70% ethanol and stored until processed. For this study, only females were analyzed.

2.2. Morphological sand fly identification

All 102 females (100%) caught at site 1 were analyzed morphologically and molecularly. On the other hand, only 699 females (50%) of females of site 2 were analyzed by both methods, while the remaining 700 were analyzed by the molecular method exclusively. Female sand flies were dissected with sterile needles to separate the head and the last three abdominal segments that were placed in individual wells of 96-well plates. To clarify the anatomical structures, lacto-phenol (lactic acid: phenol) was added to each well. After 48-72 h, the clarified fragments were mounted over glass slides in Canada balsam for microscopic examination. Sand fly identification was made by observing the cibarium, the spermathecae, and other structures of taxonomic relevance following morphological keys (Galati, 2003; Sábio et al., 2016). Females belonging to *cortelezzii* complex (*Evandromyia cortelezzii* and *Evandromyia sallesi*) were not classified up to the species level due to the slight morphological differences between species. Although several male specimens of *Ev. cortelezzii* were trapped at site 2 (data not included nor discussed in this work), we did not consider this data sufficient to extend the taxonomic classification of females beyond complex level.

2.3. Molecular sand fly identification

2.3.1. Sand fly DNA extraction

The remaining ethanol-fixed thorax of each dissected female was dried out to remove ethanol, and then was put in individual Eppendorf tubes. Subsequently, DNA was extracted by adding 100 µl of lysis buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 0.1% SDS, and proteinase K [200 µg/mL]), without homogenization (Kato et al., 2007). Tubes were incubated at 56°C for 3 h and later were kept at -20°C until processed. The extracted DNA was diluted to 6 ng/µL and then used directly as template for PCR amplification.

2.3.2. PCR amplification and sequencing

A fragment of 1900 bp of the SSU, 18S rRNA gene was amplified by PCR using 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') primers (Barroso et al., 2007). Amplification reactions were performed on a Bio-Rad T100 thermal cycler (Bio-Rad, CA, USA) in a final volume of 25 µl. Briefly and according to manufacturer's instructions, two µl of sand fly DNA were added to a PCR mixture, containing buffer solution 1X, 0.2 µM of each dNTP's, 0.8 µM of each primer and 5 U/µL TaKaRa Ex Taq DNA polymerase hot-start version (Takara-Bio, Shiga, Japan). Conditions for cycling were 3 min at 94°C for initial denaturation, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 57.5°C for 1 min, and a final extension at 72°C for 2 min, concluding with a polymerization step at 72°C for 7 min. *Nyssomyia neivai* DNA was used as a positive control in each reaction, and sterile MQ water as blank control. After the amplification, PCR products were analyzed on 1% agarose gel by electrophoresis, stained with ethidium bromide and visualized under ultraviolet light, then precipitated with 70% ethanol and quantified using a Spectrophotometer NanoVue Plus (Biochrom, a division of Harvard Bioscience, Inc., USA). The amplicons were sequenced using a BigDye terminator cycle sequencing kit, version 1.1 (Applied Biosystems, Foster City, CA), purified by FastGene Dye Terminator Removal kit (Nippon Genetics Co.,Ltd, Tokyo, Japan) and analyzed on a Applied Biosystems Hitachi 3130 Genetic Analyzer automated sequencer, employing the primers Lu.18S 1S and Lu.18S AR. The sequences obtained were assembled and edited on Chromas Lite V 2.6.5 (https://technelysium.com.au/wp/chromas/). In order to find a restriction enzyme suitable to distinguish between *Ny. neivai* and *Psathyromyia bigeniculata* species, DNA sequences were analyzed by *in silico* approaches. Sequences were deposited on GenBank (accession numbers MH367275-77).

2.3.3. RFLP analysis

Digestions of PCR products were carried out with the CviQI restriction enzyme following the manufacturer's instructions (Thermo Scientific, USA) (Barroso et al., 2007). DdeI enzyme (Thermo Scientific, USA) was also used to differentiate the sand fly species that CviQI could not; it was chosen by *in silico* probes performed with NEBCutter 2.0 online version (nc2.neb.com/NEBcutter2/). The digested fragments were separated by electrophoresis in 2% agarose, stained with ethidium bromide and visualized under ultraviolet light. Species identification was reached by contrasting the restriction patterns generated for each specimen analyzed with those previously defined according to morphological determination.

2.4. Statistical analysis

All specimens molecularly processed were considered for statistical analyzes. Chi-squared test was used to compare the abundance of the most prevalent species between sites. For analyses, abundance results were expressed as capture effort (abundance of females per night-trap). Analyses with p-values <0.05 for a level of significance of 95% were considered statistically significant. The Infostat software version 2018 (Infostat v 2018p. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. http://www.infostat.com.ar) was used for statistical analysis, while QGIS 3.4 (http://www.qgis.org/es/site/) was used for map drawing.

3. Results

3.1. Morphological sand fly identification

A total of 1501 sand fly females were caught; 102 of them at site 1 and the remaining 1399 at site 2. Out of the total, 801 females (102 from site 1 and 699 from site 2) were morphologically identified and belonged to four sand fly species (Table 1). The 5% (38/801) of analyzed females could not be determined by morphology due to the loss of anatomical structures or inadequate mounting.

3.2. Molecular sand fly identification

All female sand flies (1501) were analyzed by the PCR-RFLP method. All PCR products exhibited the same size (~1900 bp) and no DNA amplification was detected for blank controls (Fig. 2). Three of the four species caught were present in both sites. Total abundance by species was as follows: *Ny. neivai* (89.7%), *Mg. migonei* (6.0%), *cortelezzii* complex (1.5%), and *Pa. bigeniculata* (0.3%) (Table 1). *Ny. neivai* was the most abundant taxon in both sites, with statistically significant differences (abundance expressed as capture effort: 7.26 females/night/trap for site 1 and 63.25 females/night/trap for site 2; $p<0.05$).

The identification by RFLP of the four caught species was reached employing the restriction enzymes CviQI and DdeI, which generated three and two restriction patterns, respectively (Table 2). Furthermore, the CviQI enzyme was able to distinguish *Mg. migonei* from *cortelezzii*

TABLE 1
Total abundance of species by taxonomic identification method.

	Abundance of species caught (%)			
	Morphological Identification		Molecular Identification* (PCR-RFLP)	
Sand fly species	**Site 1**	**Site 2**	**Site 2**	**Total per species**
Ny. neivai	68 (66.7)	617 (88.3)	662 (94.6)	1347 (89.7)
Mg. migonei	17 (16.7)	44 (6.3)	29 (4.1)	90 (6.0)
***cortelezzii* complex**	5 (4.9)	8 (1.1)	9 (1.3)	22 (1.5)
Pa. bigeniculata	4 (3.9)	0 (0)	0 (0)	4 (0.3)
Not identified	8 (7.8)	30 (4.3)	0 (0)	38 (2.5)
Total per site	102 (100)	699 (100)	700 (100)	1501 (100)

*Molecular results of females that were identified by both methods are not detailed since their abundance was already reported under Morphological Identification.

complex; but not *Ny. neivai* from *Pa. bigeniculata*, as they generated the same unique restriction pattern (Fig. 3). Fortunately, DdeI enzyme was able to identify *Ny. neivai*; the RFLP patterns corresponded to those predicted by *in silico* analyses (data not shown). With this enzyme, *Pa. bigeniculata, Mg. migonei* and *cortelezzii* complex produced the same indistinguishable pattern (Fig. 3).

PCR-RFLP assays using the two restriction enzymes allowed the molecular identification of 100% (1501/1501) of analyzed females. All individuals identified by both methods were classified identically by them. On the other hand, as seen in Table 3, PCR-RFLP combination was able to identify 5% (38/801) more females than the morphological method. *Mg. migonei* was the most difficult species to be identified by morphology, probably due to its thin and faint spermathecae (Table 3).

Discussion

Phlebotomine sand flies have medical relevance because they spread pathogens to human and non-human animals worldwide. Of over 1000 sand fly species recorded in the world, 98 are proven or suspected vectors of leishmaniases (Maroli et al., 2013; Shimabukuro et al., 2017). Similarly, sand fly insects have also been associated to the transmission of other pathogens as arboviruses (Depaquit et al., 2010) and the bacterium *Bartonella bacilliformis* (Chamberlin et al., 2002; Herrer and Christensen, 1975). Since sand flies are generally identified through morphology, which has some limitations, this work aimed successfully to test a simple and economic method to identify sand fly species molecularly, in an ATL hyper endemic area.

The molecular sand fly identification by PCR-RFLP reached total concordance with the morphological identification (801/801). For all sand flies, the generated restriction patterns were precise as measured by the concordance with traditional species determination. Moreover, those specimens unidentified by morphology (5%) were successfully classified molecularly. Being able to determine sand fly species with no more than two enzymes and through single digestions is a great advantage of the technique. Methods based on double digestions generate many small fragments that produce restriction patterns hard to read and comprise (Aransay et al., 1999). Furthermore, DNA extraction was not based on commercial kits and could be applied to ethanol-fixed sand flies, implying that no special conditions are required for sample storage during field work for similar studies.

The molecular procedure we used is based on a previous work of our group that focused on *Ny. neivai* identification (Barroso et al., 2007). In the current study, we tested in greater depth its potential, extending the analysis to the species that were circulating in areas with ATL transmission. The species composition of sand flies reported in this work was concordant with past entomological captures performed in Orán department (Chanampa et al., 2018; Copa et al., 2018; Salomón et al., 2008, 2004), in which *Ny. neivai* was also found as the most abundant species (Krolewiecki et al., 2013; Quintana et al., 2010). In Argentina, *Ny. neivai* and *cortelezzii* complex have been found naturally infected with *L. (V.) braziliensis* (Córdoba-Lanús et al., 2006; Rosa et al., 2012) that is the causal agent of cutaneous and mucocutaneous clinical forms of the disease typically diagnosed in our study area (Krolewiecki et al., 2017). *Mg. migonei* was also found naturally infected with *L. (L.) infantum* (Moya et al., 2015), which highlights the importance of systematic entomological surveillance in this region with a high risk of infection.

Although both sampling sites were located in the border of vegetation patches (Fig. 1C), site 2 showed greater abundance of sand flies (Table 1). Sand fly abundance is positively correlated to vegetation cover (Chanampa et al., 2018) since these insects find suitable resting, breeding and mating sites, as well as sugar sources, in vegetated areas (Alencar et al., 2011; Killick-Kendrick, 1999; Poché et al., 2017). During the study period, site 1 was altered by deforestation for wood extraction (personal observation), which might have lead the

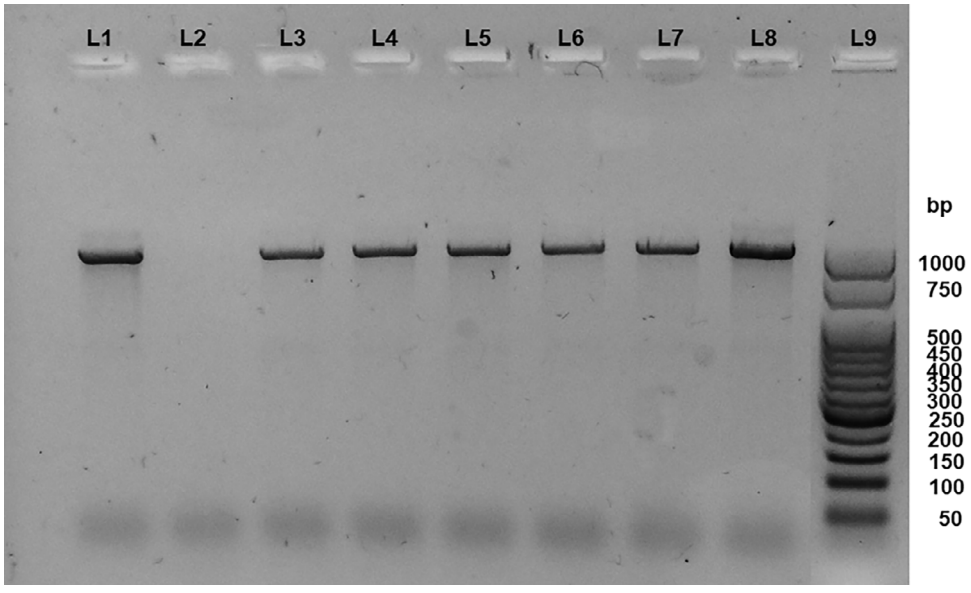

Fig. 2. PCR of 18S rRNA gene. L1: Positive control of *Ny. neivai*. L2: Blank control. L3-8: Wild females. L9: 50 bp molecular marker.

TABLE 2
Number of cuts and restriction fragment length for sand fly species.

	Restriction enzymes					
	CviQI			DdeI		
Sand fly species	Number of cuts	Restriction Pattern (RP)	Restriction Fragments (bp)*	Number of cuts	Restriction Pattern (RP)	Restriction Fragments (bp)*
Ny. neivai	5	a	135, 142, 192, 285, 442, 716	2	d	338, 352, 1253
Pa. bigeniculata	5	a	110, 142, 192, 285, 442, 717	3	e	249, 340, 350, 1005
Mg. migonei	5	b	109, 113, 237, 240, 334, 1006	3	e	253, 341, 351, 1005
***cortelezzii* complex**	3	c	109, 328, 477, 1005	3	e	252, 341, 350, 999

*: the sum of the DNA fragments is equal to the size of the undigested PCR products

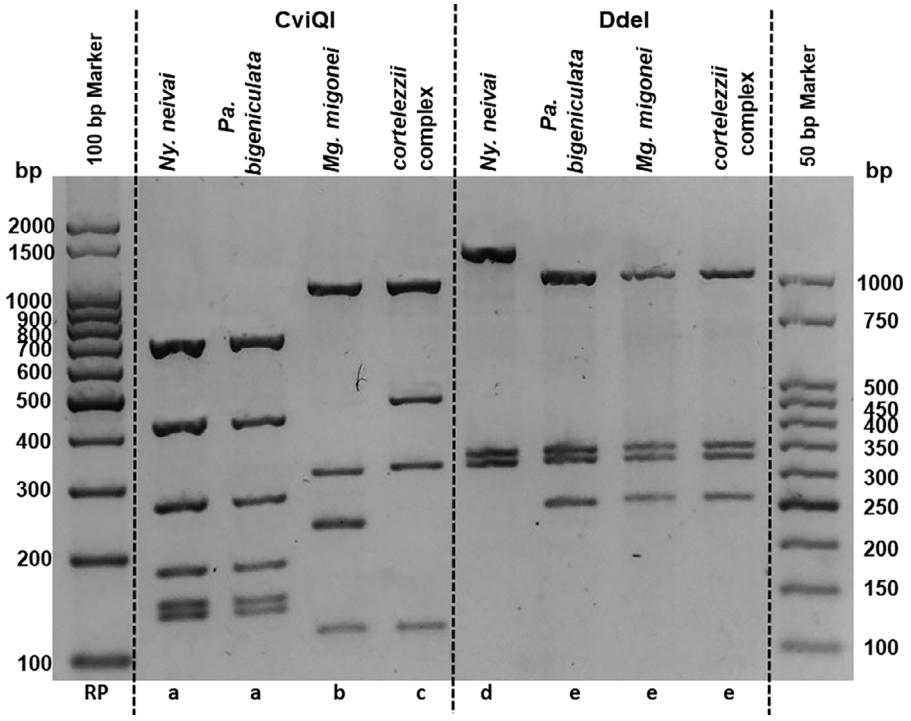

Fig. 3. Restriction patterns of sand fly species by CviQI and DdeI enzymes. RP: Restriction pattern.

TABLE 3
Comparison of identification method performance by caught species.

	Method of sand fly Identification		
Sand fly species	Morphological (M)	Molecular (RFLP)	Proportion of unidentified females by Morphology: 1-(M/RFLP)
Ny. neivai	685	702	0.02
Mg. migonei	61	79	0.23
***cortelezzii* complex**	13	16	0.19
Pa. bigeniculata	4	4	0.00
Not identified	38	0	*NA*
Total	801	801	0.44

NA: not applicable.

abundance of sand flies to decrease. On the other hand, site 2 is close to rural dwellings (70 m) with pens of chickens, goats and pigs, and is also placed near sugar cane crops that favor the proliferation of rodent plagues (Zamorano et al., 1988). Therefore, the availability of blood sources could be related to the greater sand fly abundance recorded there (Quintana et al., 2012, 2010). Regardless, our hypotheses should be tested through specific analysis, e.g.: correlations between sand fly abundance and cover vegetation estimated by Normalized Difference Vegetation Index (NDVI) to reach formal and valid conclusions. Finally, samplings were not executed during the same period of time and hence, the effect of differences in climate conditions should also be considered (Gálvez et al., 2010). In fact, variables as temperature, humidity and rainfall have shown to modulate the abundance of *Ny. neivai* in our study area (Salomón et al., 2004).

In previous research where the 18S rRNA gene was also analyzed by PCR-RFLP, the authors found polymorphic patterns for some species of sand flies (Aransay et al., 1999; Terayama et al., 2008). In each of these studies, the samples came from different geographic regions or countries. In this study, sampling sites were 4 km away from each other and belonged to the Yungas ecoregion. Our results showed that the same restriction pattern was obtained for all the specimens of each analyzed species. Therefore, we believe that the absence of polymorphic patterns for the analyzed species might be related to the small scale of sampling or to the fact that both sampling sites are part of a continuous strip of vegetation (Fig. 1C). Nevertheless, it is important to highlight that the molecular target, the RFLP technique and the amount of restriction enzymes used in this work do not allow making inferences on the genetic diversity of the analyzed populations. For this purpose, a larger sample size and more appropriate molecular markers, such as mitochondrial, should be considered.

We think that both molecular and morphological identification are necessary to reach a better sand fly study and characterization.

Traditional morphological identification should not be replaced for the molecular one because the former is indeed the source of basic systematic required for any molecular approach. However, molecular methods have undisputed advantages related to common taxonomic problems for insect vectors (Depaquit, 2014). Firstly, sand flies are minute arthropods that require meticulous and time-consuming preparation before morphological identification. This, in most of cases results in the impossibility of sand fly identification because of the damage of taxonomic value structures or inadequate material preparation, which does not affect molecular analysis at all. Secondly, most taxonomic keys allow the identification of sand fly species only during adult stage (Young and Duncan, 1994), whereas molecular studies can be done also from larval stages (Vivero et al., 2017). Thirdly, there are morphologically identical species that are reproductively isolated; hence, the traditional classification is unable to distinguish among them. In contrast, cryptic species can be approached and identified successfully by molecular methods (Scarpassa and Alencar, 2012), which might improve the phylogeny of sand flies (Beati et al., 2004). Therefore, the combination of both approaches might solve sand fly study issues.

To finish, we would like to state that we consider this technique useful to complement, not replace, the traditional identification method. The failure of the CviQI enzyme for differencing between *Ny. neivai* and *Pa. bigeniculata* (species with apparent morphological differences) represents a limitation of the technique. In any case, according to the morphological identification, the explored method was able to identify the species that circulate in our study area, which are also present in the other Argentinian provinces endemic for leishmaniases (Rosa et al., 2012; Salomón et al., 2010, 2009, 2006, 2002) and Paraguay (Salomón et al., 2003). Finally, the PCR-RFLP combination compared to the morphological method allows processing more sand flies in less time, but it is more expensive. Nonetheless, PCR-RFLP is cheaper, and even less time-consuming than commonly used PCR-sequencing methods.

Conclusions

In conclusion, the analysis of PCR-RFLP of SSU, 18S rRNA gene represents a simple and effective method for a molecular large screening of wild sand flies from an ATL endemic area. Since this method is based on the analysis of markedly different patterns, the identification process might be more easily reproduced, as the bias generated by the technician's lack of experience is completely removed. Also, it is important to take into account that morphological identification is suitable when dealing with small-size sand fly samples and well-maintained specimens. The extraction method of DNA we used is compatible with the search for natural infection (Kato et al., 2007). Hence, combining the vector identification and search for *Leishmania* spp. would serve to delimit areas of high transmission risk. Further studies, including larger sample of sand flies and a more diverse geographical area, are necessary to confirm the species-specific RFLP patterns. Of particular interest to us is to sample and molecularly analyze males of the *cortelezzii* complex (*Ev. cortelezzii* and *Ev. sallesi,* reported in our study area), to determine if the technique is capable of differentiating them.

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Author statement

MCA and **PAB** designed the study, undertook laboratory and field work, and wrote the manuscript.

GNC and **JDM** undertook laboratory and field work, and revised the manuscript.

JJL, JFG, JRN and **MK** reviewed the manuscript for important intellectual content.

ILQ, CLH and **MEDF** undertook laboratory and field work.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2020.105609.

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