Infection, Genetics and Evolution 30 (2015) 308–317

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Multilocus sequence typing approach for a broader range of species of *Leishmania* genus: Describing parasite diversity in Argentina

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ARTICLE INFO

Article history:
Received 4 October 2014
Received in revised form 19 December 2014
Accepted 24 December 2014
Available online 31 December 2014

Keywords:
Leishmania
Argentina
Multilocus sequence typing

ABSTRACT

Leishmaniasis is a vector-borne protozoan infection affecting over 350 million people around the world. In Argentina cutaneous leishmaniasis is endemic in nine provinces and visceral leishmaniasis is spreading from autochthonous transmission foci in seven provinces. However, there is limited information about the diversity of the parasite in this country. Implementation of molecular strategies for parasite typing, particularly multilocus sequence typing (MLST), represents an improved approach for genetic variability and population dynamics analyses. We selected six loci as candidates implemented in reference strains and Argentinean isolates. Phylogenetic analysis showed high correlation with taxonomic classification of the parasite. Autochthonous *Leishmania* (*Viannia*) *braziliensis* showed higher genetic diversity than *L.* (*Leishmania*) *infantum* but low support was obtained for intra-*L. braziliensis* complex variants suggesting the need of new loci that contribute to phylogenetic resolution for an improved MLST or nested-MLST scheme. This study represents the first characterization of genetic variability of *Leishmania* spp. in Argentina.

1. Introduction

According to the World Health Organization (WHO) leishmaniasis is considered one of the world's most neglected diseases, affecting largely the poorest populations in most tropical and subtropical regions. About 350 million people are considered at risk of acquiring leishmaniasis (WHO, 2010). The causative agent corresponds to flagellated protozoan parasites of the genus *Leishmania*. About 21 *Leishmania* species are known to infect humans and at least 30 species of *Phlebotomine* sand flies are implicated in the transmission of different species (Pavli and Maltezou, 2010). In addition, phenotypic diversity among species determines different parasite-vector-host relationships and clinical presentations that differ from self-healing cutaneous leishmaniasis (CL) to recurrent mucocutaneous presentation (MCL) or deadly visceral leishmaniasis (VL).

In Argentina, American tegumentary leishmaniasis (ATL), which includes CL and MCL, has significantly increased in incidence since the first reported cases at the beginning of the 20th century while the first human autochthonous case of VL was diagnosed in 2006 (Salomón et al., 2008). Endemic areas span ten provinces in the north of the country involving four *Leishmania* and 28 *Phlebotominae* species. *Leishmania* (*Viannia*) *braziliensis, L.* (*Leishmania*) *ama-*

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http://dx.doi.org/10.1016/j.meegid.2014.12.031
1567-1348/

zonensis, and *L.* (*V.*) *guyanensis* have been incriminated as the causative agent of ATL in Argentina (Marco et al., 2005; Frank et al., 2003). In addition, we recently identified *Leishmania* (*V.*) *panamensis* and *Leishmania* (*L.*) *infantum* in autochthonous cases of single CL and VL, respectively (Marco et al., 2012; Barrio et al., 2012).

Multilocus enzyme electrophoresis (MLEE) is the current gold standard for identification and typing of the genus *Leishmania*, particularly the Montpellier system which is based on the mobility patterns of 15 enzymes (Rioux et al., 1990). Several DNA based methods have proved their usefulness for diversity description both at species and strain levels (Schönian et al., 2011). In particular, multilocus sequence typing (MLST) has been proposed as an efficient and portable method for molecular epidemiology studies of bacterial pathogens (Maiden et al., 1998). This strategy has also been applied for *Leishmania* including ten loci for the *Leishmania donovani* complex (Mauricio et al., 2006; Zemanova et al., 2007) and five or four markers for *L.* (*Viannia*) spp. (Tsukayama et al., 2009; Boite et al., 2012, respectively). These studies pointed out established that MLST provides higher resolution than MLEE but also discussed the need for analysis of more candidate genes in a wider range of *Leishmania* species.

Consequently, we report here the evaluation of seven housekeeping gene fragments as candidate loci for a MLST scheme. Our gene set analysis spanned the *Leishmania* genus including the two subgenera *Leishmania* and *Viannia*, seven species complexes and 16 species (Table 1). Primers were designed according to species with available genome sequences having major impact in Argentina, *L.* (*L.*) *infantum and L.* (*V.*) *braziliensis*, and specifically amplified genus *Leishmania* excluding the causal agent of Chagas disease, *Trypanosoma cruzi*, since endemic regions of both diseases overlap in the north of the country (data not shown).

The analysis of a wider spectrum of species applying the MLST strategy enables the analysis of genetic diversity of the whole genus on a global scale.

2. Materials and methods

2.1. Parasite stocks and genome available sequences

Argentinean autochthonous isolates of *Leishmania* were obtained at the Instituto de Investigaciones en Enfermedades Tropicales, Orán, Universidad Nacional de Salta (Salta, Argentina) and the Veterinaria del Oeste, Posadas, Misiones, Argentina. This collection of strains has been recently isolated from lesions of rural workers, who have indicated as infection sites the ones shown in the Fig. 1. They were isolated by using USMARU-PBSS medium, as previously described (Marco et al., 2005). Parasite isolates and reference strains used in this study were cryopreserved and stored at the Instituto de Patologia Experimental, Facultad de Ciencias de la Salud, Universidad Nacional de Salta (Salta, Argentina) and the Department of Parasitology, Kochi Medical School, Kochi University (Nankoku, Kochi, Japan) (Table 1). All *Leishmania* stocks were cultured using RPMI 1640 medium supplemented with 100 U/mL penicillin, 50 μg/mL streptomycin and 10% heat inactivated fetal bovine serum. Promastigote cultures were spotted onto FTA cards and stored at room temperature until PCR amplification. *Leishmania* species from Argentinean isolates had been previously

Table 1
Argentinean isolates and reference strains included in the study.

Strain ID	WHO code	Species	Argentinean location
Argentinean isolates			
AFO15*	MHOM/AR/12/AFO15	*L.* (*V.*) *braziliensis*	Hipólito Yrigoyen, Salta, NWA
CRO9*	MHOM/AR/11/CRO9	*L.* (*V.*) *braziliensis*	Rivadavia Banda Sur, Salta, NWA
DSO7*	MHOM/AR/11/DSO7	*L.* (*V.*) *braziliensis*	SRN Orán, Salta, NWA
MSO6*	MHOM/AR/10/MSO6	*L.* (*V.*) *braziliensis*	Hipólito Yrigoyen, Salta, NWA
NNO10*	MHOM/AR/12/NNO10	*L.* (*V.*) *braziliensis*	Tabacal, Salta, NWA
NNO11*	MHOM/AR/12/NNO11	*L.* (*V.*) *braziliensis*	SRN Orán, Salta, NWA
OOO8*	MHOM/AR/11/OOO8	*L.* (*V.*) *braziliensis*	SRN Orán, Salta, NWA
RDO12A*	MHOM/AR/12/RDO12a	*L.* (*V.*) *braziliensis*	Pichanal, Salta, NWA
RDO12B*	MHOM/AR/12/RDO12b	*L.* (*V.*) *braziliensis*	Pichanal, Salta, NWA
RTO14*	MHOM/AR/12/RTO14	*L.* (*V.*) *braziliensis*	SRN Orán, Salta, NWA
MDP1*	MCAN/AR/10/MDP1	*L.* (*L.*) *infantum*	Posadas, Misiones, NEA
NNP2*	MCAN/AR/10/NNP2	*L.* (*L.*) *infantum*	Posadas, Misiones, NEA
Reference strains			
L 147	MHOM/ET/72/L100	*L.* (*L.*) *aethiopica*	–
M2269	MHOM/BR/73/M2269	*L.* (*L.*) *amazonensis*	–
JISH220	MPSA/SA/83/JISH220	*L.* (*L.*) *arabica*	–
BHU 1220	ND	*L.* (*L.*) *donovani*	–
BPK282A1	MHOM/NE/03/BPK282A1	*L.* (*L.*) *donovani*	–
DD8*	MHOM/IN/80/DD8	*L.* (*L.*) *donovani*	–
CUR 3	MCAV/BR/95/CUR 3	*L.* (*L.*) *enrietti*	–
GERBILLI	MRHO/CN/60/GERBILLI	*L.* (*L.*) *gerbilli*	–
LLM-877	MCAN/ES/98/LLM-877	*L.* (*L.*) *infantum*	–
5ASKH*	MHOM/SU/73/5ASKH	*L.* (*L.*) *major*	–
LV39c5	RHO/SU/59/P (clone 5)	*L.* (*L.*) *major*	–
Friedlin	MHOM/IL/81/Friedlin	*L.* (*L.*) *major*	–
SD 75.1	MHOM/SN/74/SD	*L.* (*L.*) *major*	–
U1103	MHOM/GT/01/U1103	*L.* (*L.*) *mexicana*	–
L 590	MHOM/IL/90/P283	*L.* (*L.*) *tropica*	–
MEL	MMEL/SU/79/MEL	*L.* (*L.*) *turanica*	–
M2903	MHOM/BR/75/M2903	*L.* (*V.*) *braziliensis*	–
M2904	MHOM/BR/79/M2904	*L.* (*V.*) *braziliensis*	–
M4147*	MHOM/BR/75/M4147	*L.* (*V.*) *guyanensis*	–
LC39*	MHOM/PE/84/LC39	*L.* (*V.*) *peruviana*	–
L13	MHOM/COL/81/L13	*L.*(*V.*) *panamensis*	–
Parrot-TarII	ND	*S. tarentolae*	–

Twelve Argentinean isolates and four reference strains were sequenced "de novo" (*) and compared to 18 genome wide sequences. NWA: North West Argentina; NEA: North East Argentina.

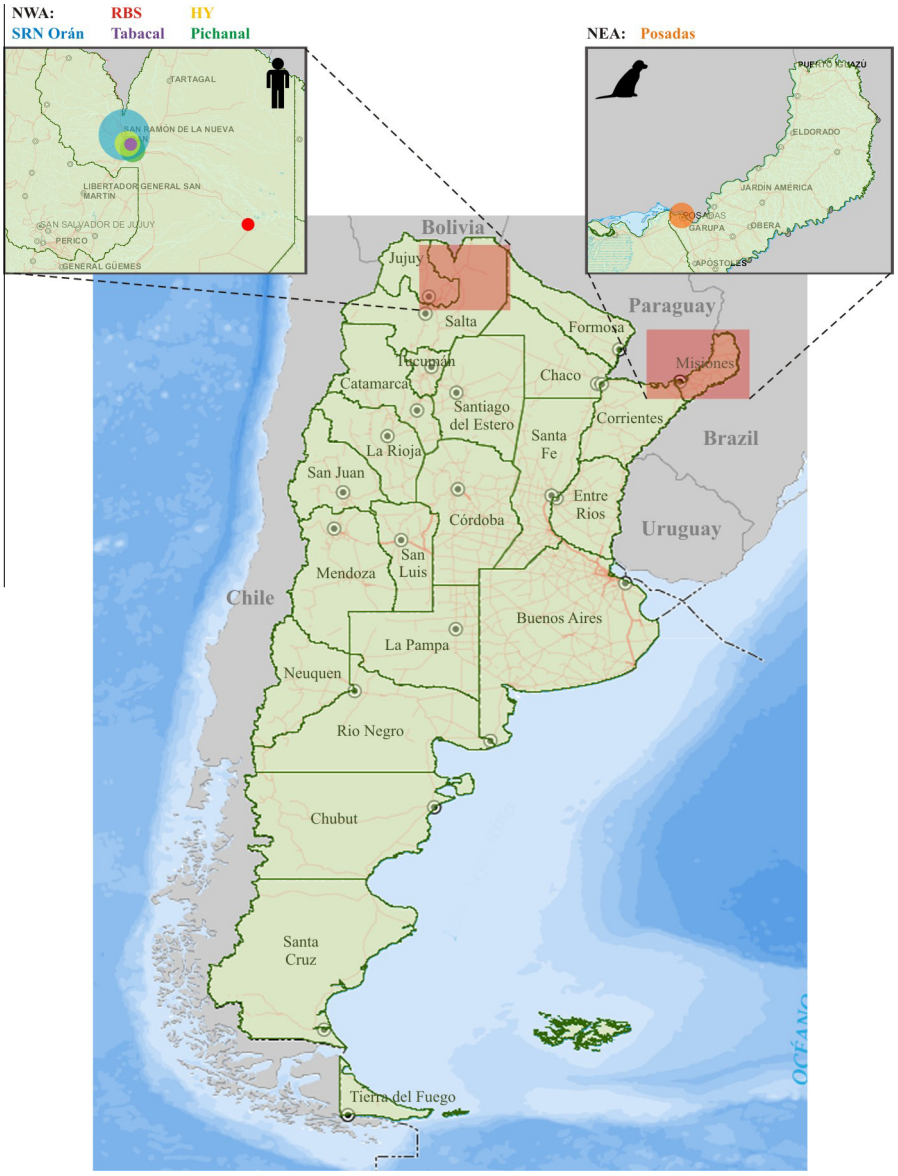

Fig. 1. Geographic distribution of *L.* (*V.*) *braziliensis* Argentinean isolates. Geographic origins were mapped based on localities reference coordinates on a .kml format file and mapped by gvSIG software (http://www.gvsig.com/). The two regions (NEA and NWA) from where the isolates were obtained are amplified from the general map. Isolates were obtained from human cases (NWA) or canine cases (NEA). Dots are shown in the map according the number of studied isolates (size) and to the isolate origin (color). Blue: SRN Orán, San Ramón de la Nueva Orán (four isolates); Yellow: HY, Hipólito Yrigoyen (two isolates); Violet: Tabacal (one isolate); Green: Pichanal (two isolates); Red: RBS, Rivadavia Banda Sur (one isolate) and Orange: Posadas (two isolates). NWA: Northwestern Argentina; NEA: Northeastern Argentina. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

identified by *cytochrome b* (*cyt* b) gene sequence analysis (Marco et al., 2005; Luyo-Acero et al., 2004). Eighteen available genome sequences on TriTrypDB Kinetoplastid Genomics Resourse were included in the analysis (http://tritrypdb.org/tritrypdb/, Table 1).

2.2. Selection of loci and PCR amplification

The search for candidate genes was performed based on the previous MLEE typing scheme and metabolic pathways enzymes established by the Kyoto Encyclopedia of Genes and Genomes (KEGG database, http://www.genome.jp/kegg/kegg1.html). Furthermore, we selected genes distributed among the parasite genome with orthologs present throughout the genus *Leishmania*. Primers were designed according to genome sequences available at GeneDB (http://www.genedb.org/): *L.* (*V.*) *braziliensis*, MHOM/BR/79/M2904 and *L.* (*L.*) *infantum*, MCAN/ES/98/LLM-877 since they represent the main species that cause ATL and VL in Argentina. We explored seven single copy genes regions as candidate loci distributed in six different chromosomes that spanned from 329 to 544 bp. Target genes selected were: aconitase (*aco*; EC: 2.5.1.16), alanine aminotransferase (*alat*; EC: 2.6.1.21), enolase (*enol*; EC: 4.2.1.11), hypoxanthineguanine phosphoribosyltransferase (*hgprt*; EC: 2.4.2.8), phosphoglucomutase (*pgm*; EC: 5.4.2.2), Phosphomannomutase (*pmm*; EC: 5.4.2.8) and spermidine synthase (*spdsyn*; EC: 2.5.1.16). Candidate genes and primers are described in Table 2. One 1,2-mm diameter of FTA™ Classic Cards disks containing the culture sample were punched using a Harris 1.2 mm Micro Punch (Whatman International Ltd, Maidstone, Kent, UK) washed three times with FTA purification reagent (Whatman International Ltd) and once with TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). The disks were air dried and subjected directly to PCR amplification. PCR amplification reactions were performed in a 50 μL reaction mixture containing 0.4 μM of each primer, 0.2 mM of each deoxyribonucleotide triphosphate (Promega, Madison, Wi, US), 1.25 U of

Table 2
Candidate loci information.

Target Gene	Enzyme entry	Locus TAG	Gene length (bp)	Amplicon size (bp)	Primers name and sequence (5′-3′)
Aconitase (*aco*)	EC 2.5.1.16	LbrM.18.0570 LinJ.18.0510	2691	579	ACO.F: CAAGTTCCTGRCGTCTCTGC ACO.R: GAGTCCGGGTATAGCAKCCC
Alanine aminotransferase (*alat*)	EC 2.6.1.21	LbrM.12.0630 LinJ.12.0580	1494	589	ALAT.F: GTGTGCATCAACCCMGGGAA ALAT.R: CGTTCAGCTCCTCGTTCCGC
Enolase (*enol*)	EC 4.2.1.11	LbrM.14.1330 LinJ.14.1240	1290	431	ENOL.F: GCTGCCGATCCTGATGGAGG ENOL.R: ACCCGTTCTCCATGCACAGC
Hypoxanthine-guanine phosphoribosyltransferase (*hgprt*)	EC 2.4.2.8	LbrM.21.0990 LinJ.21.0980	636	412	HGPRT.F: GCTCTACCTGCTGTGCGTGC HGPRT.R: ATCGCGCAGCTCGCGRTACG
Phosphoglucomutase (*pgm*)	EC 5.4.2.2	LbrM.21.0700LinJ.21.0700	1770	529	PGM.F: CAGAGAAGCTGACGTCCCAG PGM.R: GACGGGTTCACGAAGAAGCG
Phosphomannomutase (*pmm*)	EC 5.4.2.8	LbrM.35.2180 LinJ.36.2070	744	536	PMM.F: TTCAAGCTTGGCGTCGTCGG PMM.R: TAATCGTTRCCGCCCTCTGA
Spermidine synthase (*spdsyn*)	EC 2.5.1.16	LbrM.04.0630 LinJ.04.0570	903	394	SPDSYN.F: CGAACCTGTCGCTGACGTG SPDSYN.R: GAYTCGCCCTGGTTGCACAC

Seven candidate loci were mapped on *L.* (*V.*) *braziliensis* M2904 and *L.* (*L.*) *infantum* JCMP5 genomes (locus TAG).

GoTaq DNA polymerase (Promega), 10 μL of 5× PCR buffer and 1.2-mm-diameter disks from punched FTA cards. Amplification was carried out in a Bio-Rad MyCycler Thermal Cycler (Bio-Rad, Hercules, California, US) with an initial 3 min denaturation at 94 °C, followed by 35 amplification cycles (denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s) followed by a final extension step at 72 °C for 10 min. Five microliter of each amplified product were electrophoresed in a 1% agarose gel stained with ethidium bromide and molecular size marker (100 bp Ladder, Invitrogen) to confirm PCR product size. The remaining 45 μL of the amplified product was purified by precipitation with 11.25 μL of 125 mM EDTA and 135 μL of absolute ethanol, centrifugation at 10,000*g*, precipitation with 70% ethanol and resuspension in pure water. Both strands were sequenced on a Big Dye Terminator v3.1 kit and analyzed on an ABI 3130XL genetic analyzer (Applied Biosystems, California, US).

2.3. Sequences analysis and allelic profiles coding

Consensus sequences for each gene were obtained through Forward and Reverse strands assembly using STADEN Package software (MRC-LMB, UK). We also visually checked both strands for the detection of ambiguous (heterozygous) sites when two peaks overlapped in both chromatograms. Consensus sequences were aligned and trimmed in frame using MEGA6 software (Tamura et al., 2013). Sequences were deposited in GenBank under accession numbers KJ643143–KJ643238. MLSTest software (http://www.ipe.unsa.edu.ar/software) was used to calculate the number of alleles, the number of polymorphic nucleotides and the discriminatory power for each locus (Tomasini et al., 2013). Diploid Sequence Types (DSTs) and a minimal number of loci based on Genotypic Diversity (GD) were assigned according to allelic profiles using MLSTest software.

2.4. Phylogenetic and haplotype analysis

Phylogenetic relationships among single and concatenated sequences of selected genes were inferred by Neighbor Joining (NJ) method tested with 500 bootstrap replications using MLSTest software. Ambiguous data was established as average states as heterozygosis resolution strategy in order to avoid possible phylogenetic signal misinterpretation during the analysis. The BIONJ-based incongruence length difference test (BIONJ-ILD) was carried out in MLSTest software using 1000 random permutations to evaluate statistical significance (ILDp). Congruence among distance matrices using Kendall's W correspondence index was evaluated in MLSTest using 1000 random permutations. *Sauroleishmania tarentolae* Parrot-TarII was considered as the outgroup.

Haplotype reconstruction was performed by DNAsp 5 (Librado and Rozas, 2009) using the PHASE algorithm, which automatically assigns the haplotype number (*h*) for each unique haploid sequence. Then, haplotype data were used in DNAsp to calculate the synonymous and non-synonymous substitution rates (d_N/d_S) and haplotype diversity (Hd). Haplotypes networks were built by TCS software based on a statistical parsimony estimation approach (Clement et al., 2000).

3. Results

3.1. Seven candidate loci in a wider spectrum of Leishmania species

Seven target genes were selected as candidates according to their involvement in metabolic pathways in the parasite (presumably under negative selection) following the basic concepts of the MLST typing strategy. We also selected these single copy gene regions due to the presence of a high number of SNPs among *Leishmania* genus genomic data and their distribution on different chromosomes.

Aconitase (*aco*), alanine aminotransferase (*alat*), enolase (*enol*) and hypoxantine-guanine phosphoribosyl transferase (*hgprt*) genes are presented for the first time as candidates for DNA based molecular typing strategies for *Leishmania*. Among them, only *hgprt* was previously included for hybrid determination between *L.* (*L.*) *infantum* and *L.* (*L.*) *major* (Ravel et al., 2006). Enolase corresponds to a single copy gene located in chromosome 14. However, *L.* (*V.*) *braziliensis* strain M2904 genome analysis revealed the presence of two tandem copies of this gene (LbrM.14.1320 and LbrM.14.1330 with 1499 and 1741 bp, respectively) with a high homology region of 1045 bp while LbrM.14.1320 N-ter and LbrM.14.1330 C-ter regions are unique for each locus (454 bp and 242 bp, respectively). We therefore designed primers in order to specifically amplify a 392 region of LbrM.14.1330 which belongs to the orthologous group of genes with other species included in this analysis. New regions from previously studied genes (*pgm*, *pmm* and *spdsyn*) were studied in order to facilitate the analysis of a wider spectrum of species, mainly *L.* (*V.*) *braziliensis* and *L.* (*L.*) *infantum*, major species that cause ATL and VL in Argentina.

3.2. Genetic composition of Leishmania strains

Six genes were successfully amplified *do novo* in 12 Argentinean isolates and four reference strains (Table 1) whereas *pmm* loci could not be amplified in two Argentinean *L.* (*L.*) *infantum* isolates

(MDP1 and NNP2) and two reference strains from *L.* (*L.*) *major* and *L.* (*L.*) *donovani* species (5ASKH and DD8, respectively). Both *pmm* primers showed a 100% identity with genomic sequences from these species suggesting that the lack of amplification could be due to the presence of a nucleotide polymorphism within the primer region in these specific *Leishmania* strains.

As shown in Table 3 gene fragments had 82–167 polymorphic (segregating) sites with *hgprt* having the least and *alat* the most variable loci according to the Discriminatory Power parameter (DP). Several species-specific polymorphic sites were distributed along one to six loci (excluding *pmm*). Non-pathogenic to humans, *L.* (*L.*) *enrietti* and *S. tarentolae*, displayed the highest number of specific SNPs (183 and 145, respectively) whereas *L.* (*L.*) *major* and *L.* (*V.*) *braziliensis* had intra-species polymorphisms (3 and 4, respectively).

Eight out of nine "de novo" sequences from Argentinean *L.* (*V.*) *braziliensis* isolates showed ambiguous sites at *aco* (two sites), *alat* (one site) and *pgm* (two sites) loci (Table 3). Diploid Sequence Types (DSTs) were defined including these heterozygous sites in which the distance matrix for the complete set of six loci was successfully amplified in the collection (Table 4). The number of MLST alleles varied from 14 to 20 (*spdsyn-hgprt* and *pgm*, respectively) describing 25 DSTs with a DP value of 0.975.

Ten Argentinean *L.* (*V.*) *braziliensis* isolates described eight DSTs. These DSTs were different from Brazilian DSTs. The *L.* (*V.*) *braziliensis* isolate CRO9 from Rivadavia Banda Sur, was the most geographically distant isolate in the collection with two unique alleles for *aco* and *pgm* genes (Fig. 1). As shown in Table 4 the *L.* (*V.*) *peruviana* LC39 reference strain had a unique DST (DST 13) described by unique alleles for *alat*, *hpgrt* and *pgm* loci (11, 11 and 13, respectively) that derived from one exclusive SNP in each locus. On the other hand, both Argentinean *L.* (*L.*) *infantum* isolates shared the same DTS with LLM-877, a strain from Spain.

3.3. Phylogenetic analysis of concatenated sequences

Neighbor joining dendrograms were built for each candidate loci separately (data not shown) or the concatenated sequences (2626 bp excluding *pmm*). The dendrogram based on concatenated sequences showed high bootstrap support both at specie-complexes and species level organization (Fig. 2A). No statistically-significant incongruence was detected among loci (ILDp = 0.719) and distance matrices had high and statistically significant correspondence ($W = 0.8575$, $p = 0.001$) suggesting no major concerns in concatenating loci.

Dendrogram branches within the *L. braziliensis* complex cluster showed low bootstrap values among species and isolates due to the low number of specific SNPs (Fig. 2B). However, these few SNPs described nine DTs within this group defined by allele variation in genes *aco*, *alat*, *enol*, *hgprt* and *pgm*.

Table 3
Gene diversity parameters.

Gene	*S*	NHI	NHS	No. alleles	DP	*h*	Hd	d_N/d_S
aco	157	5	2	17	0.923	17	0.875	0.15109
alat	167	5	1	17	0.929	16	0.89	0.18303
enol	92	0	0	16	0.874	16	0.853	0.10386
hgprt	82	0	0	14	0.857	14	0.836	0.12335
pgm	145	2	2	20	0.946	19	0.921	0.26331
spdsyn	101	0	0	14	0.81	14	0.787	0.21049

Studied diversity parameters included: *S*: Number of polymorphic (segregating) sites; NHI: Number of heterozygous isolates; NHS: Heterozygous sites; DP: discriminatory power; *h*: Number of Haplotypes; Hd: Haplotype diversity; d_N/d_S: ratio of non-synonymous (d_N) to synonymous (d_S) substitutions per nucleotide site.

Table 4
Diploid sequence type definitions.

Strain/isolate	*aco*	*alat*	*enol*	*hgprt*	*pgm*	*spdsyn*	DST
L 147	9	9	9	9	11	8	11
M2269	12	13	12	12	15	11	15
JISH220	7	7	7	7	9	7	9
BHU 1220	3	3	3	3	3	3	3
BPK282A1	3	3	3	3	3	3	3
DD8	3	3	3	3	3	3	3
CUR 3	5	4	4	4	5	4	5
GERBILLI	6	6	6	6	8	6	8
LLM-877	11	12	11	3	14	10	14
MDP1	11	12	11	3	14	10	14
NNP2	11	12	11	3	14	10	14
5ASKH	1	1	1	1	1	1	1
Friedlin	1	1	5	5	7	5	7
LV39c5	1	1	1	1	1	1	1
SD 75.1	1	1	5	5	1	5	24
U1103	17	17	16	14	20	14	25
L 590	10	10	10	10	12	9	12
MEL	14	15	14	6	18	12	19
AFO15	2	2	2	2	2	2	2
CRO9	4	2	2	2	4	2	4
DSO7	2	5	2	2	6	2	6
M2903	2	2	2	2	16	2	16
M2904	2	2	13	2	16	2	17
MSO6	2	5	2	2	2	2	20
NNO10	2	2	2	2	6	2	21
NNO11	15	5	2	2	2	2	22
OOO8	2	2	2	2	2	2	2
RDO12A	15	5	2	2	2	2	22
RDO12B	15	5	2	2	2	2	22
RTO14	15	5	2	2	2	2	22
M4147	13	14	8	8	17	2	18
LC39	2	11	2	11	13	2	13
L13	8	8	8	8	10	2	10
Parrot-TarII	16	16	15	13	19	13	23

Diploid sequence types were obtained by MLSTest software.

3.4. Viannia subgenus haplotype analysis

A low number of SNPs prevented phylogenetic analysis from resolving a potential population structure of *L. braziliensis* complex in the studied region. We therefore analyzed gene genealogies through haplotype resolution and networks. For this purpose we resolved haplotypes using two different approaches, manual resolution or PHASE algorithm (bayesian criterion). As most loci included only one heterozygous site both strategies displayed the same haplotypes. However, *pgm* locus for CRO9 isolate had two ambiguous sites that resulted in two haplotypes by bayesian approach (Table 5). All loci were shown to be under stabilizing selection. Haplotype number and haplotype diversity showed *spdsyn* and *pgm* as the least and most variable loci, respectively, consistently with diploid allele diversity (Table 3).

As shown in Table 4, isolates NNO11, RDO12A, RDO12B and RTO14 shared DST 22 defined by ambiguous sites at three loci (*aco*, *alat* and *pgm*) and corresponded to geographically nearby locations SRN Orán and Pichanal (24.7 km). They presented H1, ancestor and most frequent haplotype in the complete set of loci, while they showed unique haplotype H2 in *aco* locus and shared H2 from *alat* and *pgm* loci with other *L.* (*V.*) *braziliensis* isolates from nearby areas (SRN Orán and Hipólito Yrigoyen located 14.4 km from SRN Orán). On the other hand, most distant isolate CRO9 shared H1 ancestor haplotype and described unique H3 in *aco* and *pgm* loci. Single haplotypes were obtained for LC39 in *hgprt* (H2) and *pgm* (H5) loci in agreement with DSTs alleles as this strain did not present ambiguous sites. Haplotype genealogies by maximum parsimony-based haplotype networks analysis displayed H1 as the ancestor haplotype (Fig. 3) with single mutation connections to specific *L. braziliensis* complex haplotypes from diverse localities according to the low number of SNPs among all loci.

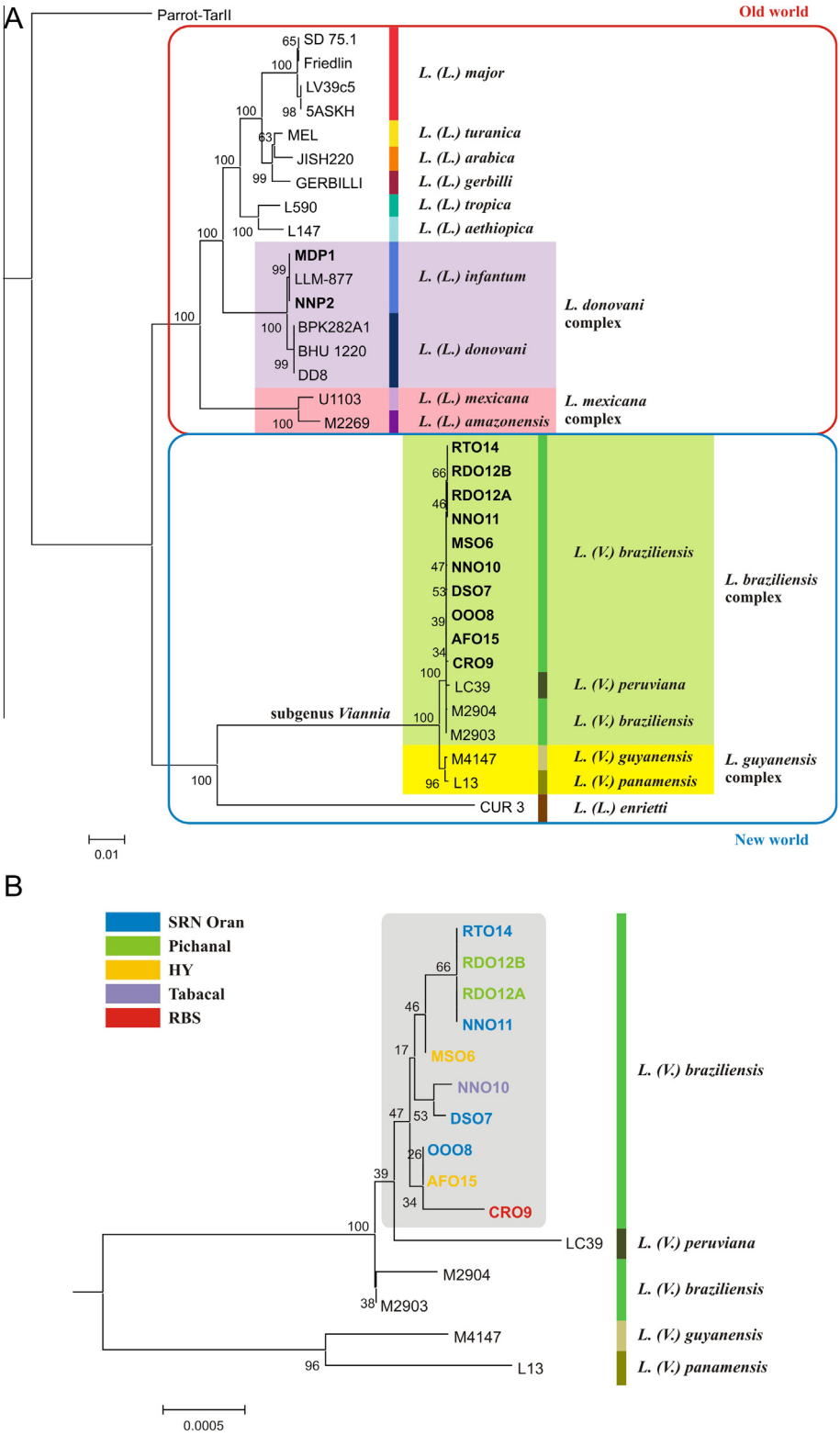

Fig. 2. Concatenated NJ dendrogram for *Leishmania* spp. (A) *Leishmania* spp. dendrogram: six loci sequences were concatenated (2626 bp: *aco*, *alat*, *enol*, *hgprt*, *pgm* and *spdsyn*) and phylogenetic relations among genus *Leishmania* were established by NJ, 1000 bootstrap support. Argentinean isolates are indicated in bold fonts and different taxonomic levels are indicated: Old/New World, subgenus, species complexes and species. *Sauroleishmania taretolae* was established as outgroup. (B) Subgenus *Viannia* subtree: Argentinean isolates were colored according to their geographic origin. SRN Orán: San Ramón de la Nueva Orán; Pichanal; HY: Hipólito Yrigoyen; Tabacal and RBS: Rivadavia Banda Sur. Neighbor Joining (NJ) tree tested with 1000 bootstrap replications was built using MLSTest software.

4. Discussion

Multilocus sequence typing has been applied to *Leishmania* parasites from Old and New World isolates and clinical samples. These studies established that MLST provides higher resolution than MLEE but also discussed the need for more candidate gene analysis in a wider range of *Leishmania* species (Mauricio et al., 2006; Zemanova et al., 2007; Tsukayama et al., 2009; Boite et al., 2012).

Table 5
Argentinean isolates and reference strains of *L.* (*V.*) *braziliensis* haplotype resolution.

Strain/isolate	*aco*	*alat*	*enol*	*hgprt*	*pgm*	*spdsyn*
AFO15	1/1	1/1	1/1	1/1	1/2	1/1
CRO9	1/3	1/1	1/1	1/1	1/3	1/1
DSO7	1/1	1/2	1/1	1/1	2/2	1/1
M2903	1/1	1/1	1/1	1/1	1/1	1/1
M2904	1/1	1/1	2/2	1/1	1/1	1/1
MSO6	1/1	1/2	1/1	1/1	1/2	1/1
NNO10	1/1	1/1	1/1	1/1	2/2	1/1
NNO11	1/2	1/2	1/1	1/1	1/2	1/1
OOO8	1/1	1/1	1/1	1/1	1/2	1/1
RDO12A	1/2	1/2	1/1	1/1	1/2	1/1
RDO12B	1/2	1/2	1/1	1/1	1/2	1/1
RTO14	1/2	1/2	1/1	1/1	1/2	1/1

Ambiguous sites from Argentinean *L.* (*V.*) *braziliensis* isolates were resolved by PHASE algorithm. Both haplotypes are shown for each homozygous and heterozygous isolate.

Thus, the present study proposes alternative target genes to be considered for a MLST strategy for the description of the genetic variability of a wider spectrum of species among the genus *Leishmania* with a spotlight on relevant species involved in ATL and VL cases in Argentina. Genetic variability of Argentinean isolates was studied in the context of reference strains and genomic sequences including the three subgenera, seven species complexes and 16 species (Table 1).

The seven single copy gene candidates were shown to be under stabilizing selection. In addition, dendrogram topologies and haplotype networks showed high correlation with *Leishmania* taxonomic organization. Recently, the gene *spdsyn* was included in a seven loci scheme for the analysis of the genetic structure of the genus *Leishmania* in Africa and Eurasia showing high correlation with taxonomic classification of species such as *L.* (*L*) *aethiopica*, *L.* (*L.*) *arabica*, *L.* (*L.*) *turanica*, *L.* (*L.*) *gerbilli* and *L.* (*L*) *major* (El Baidouri et al., 2013). On the other hand, *pgm* and hexokinase genes enabled the distinction of different zymodemes within *L.* (*L.*) *tropica* (Azmi et al., 2013). Phosphomannomutase locus could not be amplified in two local isolates and two reference strains. In contrast to the other six loci it showed no intra-specific variability. Although the primers used for amplification of *pmm* showed 100% identity to the genomic sequences of the species that could not be amplified, primers used for the different candidates genes showed polymorphisms within the genomic sequences of the full spectrum of species analyzed. This reveals the need to focus on primer sequences that must co-evolve as more genome sequences become available. In addition, relevant species in a particular study should be considered in order to select the primers.

Both Argentinean *L.* (*L.*) *infantum* isolates shared the same DTSs as LLM-877 strain from Spain. This is in agreement with previous studies by Ferreira and colleagues that showed low genetic variability among *L.* (*L.*) *infantum* isolates from Brazil using microsatellite markers which vary more rapidly than housekeeping genes (Ferreira et al., 2012). Moreover, comparative microsatellite typing studies found a very homogenous population structure of this species in Brazil and Paraguay consisting exclusively of MON-1 strains from the Old World (Spain, Portugal, France and Italy) (Kulhs et al., 2011). New human VL foci were recently described in the country and, according with to the Argentinean Department of Health, 103 human cases where reported up until July 2012 (Barrio et al., 2012; Gould et al., 2013). More canine and human isolates should be obtained and analyzed in order to describe the genetic background of this population in our country.

On the other hand, with respect to ATL cases in Argentina, the situation is different. The first reports of autochthonous MCL cases date back to 1918 with several outbreaks since the 80 s (Salomón et al., 2001). However, there is scarce information about the taxonomic classification of the parasites involved in those cases, in which the *Leishmania* spp. was inferred according to clinical presentation and the known species distribution within the region. In addition, worldwide genetic diversity studies about *Leishmania* spp. parasites do not include Argentinean cases. *Leishmania* (*L.*) *braziliensis* is the causal agent of the 90.3% of ATL cases in this region. In addition, its local zymodemes were found to have an ability to metastasize to mucocutaneous tissues and lymph nodes. Other species such as *L.* (*V.*) *guyanensis*, *L.* (*V.*) *amazonensis*, and recently *L.* (*V.*) *panamensis* were also found within the northwestern Argentinean region (Marco et al., 2005; Frank et al., 2003; Marco et al., 2006, 2012). Recently, Locatelli and colleagues studied 66 patients diagnosed as positive ATL cases from northwest Argentina by *cytochrome* b (*cyt* b) sequence analysis (Locatelli et al., 2014). Even though this analysis allowed a precise identification at species level, it failed to provide further information on the genetic diversity of prevalent *Leishmania* spp. in the area and highlighted the need for the implementation of new molecular markers (MLMT or MLST) to study this issue. Thus we proposed to deepen the study of genetic variants by searching new candidate genes. The sequence analysis of the six loci raised the genetic variability description from two variants described by *cyt* b to eight DSTs in the studied population raising the discriminatory power from 0.725 to 0.91 among the subgenus *L. braziliensis* complex (including *L.* (*V.*) *peruviana* LC39 ref. strain). As all candidates were single copy genes the presence of ambiguous sites in three loci (*aco*, *alat* and *pgm*) showed the presence of heterozygosity or host coinfection with isolation of different populations in the same culture. Cloning of each sample for MLST analysis is detrimental to the high-throughput perspective of multilocus approaches applied to genetic population studies. This underlines the need to describe genetic variability through strategies that incorporate or resolve ambiguous data by specific algorithm implementations. Thus, the analysis of the population ambiguous sites were included in the analysis defining genetic variants such as Diploid Sequence Types (DSTs) that included diploid allele's combinations when appropriated. This was the case of nine of the ten Argentinean *L.* (*V.*) *braziliensis* isolates. Polymorphic sites were mostly bi-allelic but we also found possible tri- and tetra-allelic variants (CRO9). Previously, Boite and colleagues described triallelic variants in four housekeeping genes in *Viannia* subgenus and proposed that multiallelic frequencies might be quite frequent in the subgenus *Viannia* species group (Boite et al., 2012). Here we showed that this also occurred in species from the *Leishmania* subgenus which is consistent with the presence of trisomic and tetrasomic chromosomes in other species as reported by Rogers and colleagues (Rogers et al., 2011).

Even though a small number of SNPs where described within the *Viannia* subgenus, one to three mutational steps between *L. braziliensis* and *L. guyanensis* complexes, these variations contributed with informative phylogenetic signals in order to obtain high bootstrap support for the clustering of each species complex. Within the *L. braziliensis* complex cluster in NJ dendrogram we obtained low branch bootstrap values avoiding subclusters resolution and thus possible correlation analysis with parameters such as clinical presentation, transmission cycle components, or phytogeographic distribution. A key factor to be considered in this lack of correlation is that cases from which parasites were isolated and analyzed corresponded to patients that might migrate for rural jobs. However, haplotype resolution and network analysis suggested some phytogeographic structuring. Isolates mostly originated from human cases from Orán, Hipólito Yrigoyen and Pichanal that cover a distance of 35.2 km, while CRO9 isolate corresponded to a multiple CL case from Rivadavia Banda Sur, a distant area (187 km) with respect to previous locations. The Orán, Hipólito Yrigoyen and Pichanal cities are located in the "Selva de Transición" a subtropical humid forest, while Rivadavia Banda

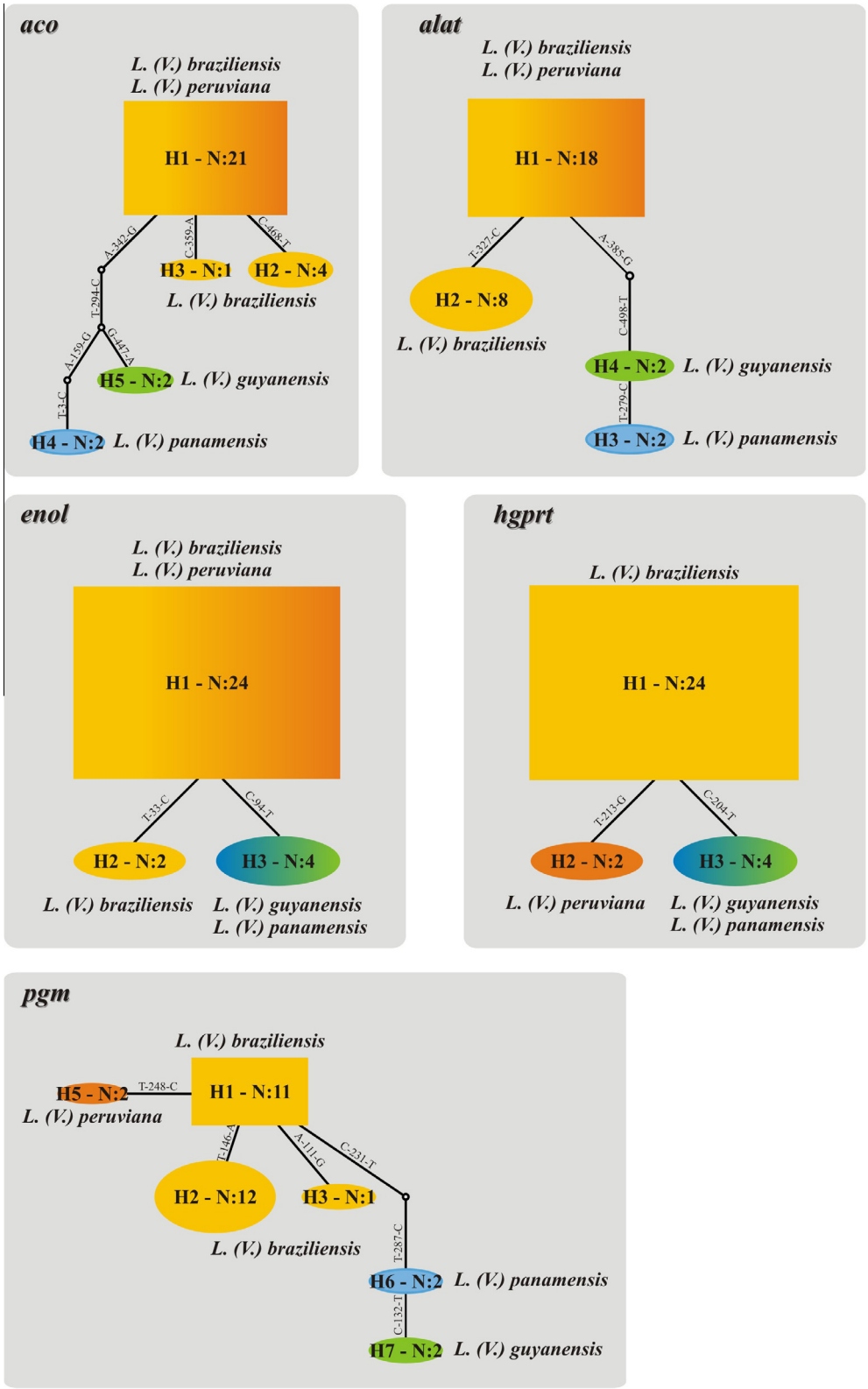

Fig. 3. Subgenus *Viannia* haplotype networks. Haplotype networks were built by a statistical parsimony estimation approach based on haplotype reconstruction by PHASE algorithm using TCS software. Mutational steps were specified for each network connection and haplotype codes for *L. braziliensis* complex are as specified in Table 5. No network was obtained for *spdsyn* locus since the whole subgenus *Viannia* presented a unique haplotype. N: number of isolates included in each haplotype.

Sur corresponds to the region called Chaco Occidental, a xerophytic lowland plain with a semiarid climate (Marco et al., 2005). The CRO9 isolate described unique alleles for *aco* and *pgm* loci that resulted in unique haplotypes related to the ancestor haplotypes by one mutation step. Therefore, CRO9 describes the first indication of a possible phytogeographic correlation with haplotype variants, while more isolates should be analyzed in order to deepen a possible phytogeographic structuring of this species in the region, suggesting the involvement of different reservoirs/vectors associated with their transmission cycles.

There is some controversy regarding whether *L.* (*V.*) *peruviana* should be considered a sole species or a *L.* (*V.*) *braziliensis* subspecies. In a recent work Fraga and colleagues suggested that, based on *hsp*20 and *hsp*70 phylogenetic analysis, *L.* (*V.*) *peruviana* could be considered as subspecies of *L.* (*V.*) *braziliensis* (Fraga et al., 2013). In our study *L.* (*V.*) *peruviana* LC39 WHO reference strain was not the exception and clustered together with *L.* (*V.*) *braziliensis* with low bootstrap support (Fig. 2B).

Based on the data obtained in this study we propose six candidate genes that enable a precise discrimination of *Leishmania* spp. according to species complex and species levels excluding *L.* (*V.*) *braziliensis* complex species. The maximum number of DSTs (Gould et al., 2013) was also obtained after the analysis of four of the six loci after an optimum number of loci analysis which estimates genotypic diversity for every possible combination of two to n-1 loci. These four loci corresponded to *aco*, *alat*, *enol* and *pgm* with the highest discriminatory power among the analyzed set. We therefore propose to continue with these genes adding other candidates with the aim of improving the resolution of clusters among *L.* (*V.*) *braziliensis* complex genetic variants, such as the malate dehydrogenase (E.C.1.1.1.37, MDH), the malic enzyme (E.C.1.1.1.40, ME) genes, and mannose-phosphate isomerase (E.C.5.3.1.8, MPI) genes (Marco et al., 2005). Finally, we also consider that maybe a unique set of loci for the entire genus may not be a final solution in order to get a proper resolution level among intra-specific variants but instead we must study different sets of genes according to different taxonomic levels proposing a typing algorithm or nested MLST scheme.

5. Conclusions

Even though leishmaniasis represents a health problem in northern Argentina there are no genetic diversity studies of the parasite in the country so far. This work describes a higher genetic diversity among isolates of *L.* (*V.*) *braziliensis* in accordance to the history of these diseases in the country. At the same time, we proposed six genes as reliable candidates to be considered in a MLST typing approach for population diversity studies of the genus *Leishmania*. Finally, MLST was firstly proposed for bacterial molecular epidemiology studies. However, protozoan parasites genomic composition is more complex. In particular, the genus *Leishmania* displays high nucleotide diversity among housekeeping genes coding regions throughout genomic sequences. Thus, future studies should be focus on developing a molecular typing algorithm with nested MLST schemes according relevant *Leishmania* taxonomic levels in each region.

Acknowledgements

Financial support was obtained from the National Agency for Science and Technology, Argentina (PICT 2009-0135, PICTO-GLAXO 2011-0017); The Research Council of the National University of Salta (Grant No. 2041).

We are grateful to Patricio Diosque, Miguel A. Basombrío, Alejandro Uncos, Maria Celia Mora, Karina Caimi, Daniel Ribble, Alejandro Krolewiecki, Keiko Morisawa, Kyoko Imamura and Federico Ramos for their contributions and valuable comments.

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