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Genome Analysis of Triploid Hybrid *Leishmania* Parasite from Neotropics

Appendix 2

Genome sequence analyses of MHOM/CR/2020/StPierre

PART A: Decomposing of haplotype sequences for the HSP70 gene

The ambiguity of bases at 11 positions in the HSP70 fragment (Table 1) prompted us to decompose haplotype sequences to infer the parental species of MHOM/CR/2020/StPierre. To this end, 150bp read sequences were mapped with SMALT against one of the HSP70 genes within the *L. braziliensis* M2904 reference genome. Sequence data spanning all six neighboring ambiguous positions (1576–1679) in one read were extracted. This revealed five sets of identical sequences (i.e., haplotypes) found within a total of 83 high-quality read sequences. Haplotype 1 (H1) was found in 26 read sequences (32%) and has alleles equal to the *L. braziliensis* M2904 reference (Table 1). Haplotype 2 (H2) was found in 31 read sequences (38%) and contains alleles specific to *L. guyanensis* (Table 1). The remaining 3 haplotypes (26 sequences) showed signatures of mosaic ancestry, with allelic combinations similar to haplotypes 1 and 2.

PART B: Whole genome sequence analyses

Publicly available sequence data

To capture as much as possible the genome diversity of *Leishmania (Viannia)* in South-America, we included publicly available sequence data from 35 strains and generated sequence data for 5 strains. Specifically, we included sequence data of the following seven *Leishmania (Viannia)* species: *L. lainsoni* (N = 1) (*1*), *L. naiffi* (N = 3) (*2–4*), *L. shawi* (N = 1) (*4*), *L.*

guyanensis (N = 3) (*2–5*), *L. panamensis* (N = 15) (*4*,*6–8*), *L. peruviana* (N = 2) (*7*) and *L. braziliensis* (N = 15) (*7*,*9*) (Table 2).

The three *L. guyanensis* strains originated from French Guiana, Brazil and Venezuela. The 15 *L. panamensis* strains originated from Colombia (N = 13) and Panama (N = 2) (Table 2). Note that one strain (LgCL085) was previously considered as *L. guyanensis* (*3*), but was here classified as *L. panamensis* following the phylogenomic analyses presented below.

We considered a total of four genetically divergent subgroups within the *L. braziliensis* species complex. The first subgroup, here-after referred to as *L. braziliensis* 1, is regarded as the main *L. braziliensis* species and is responsible for human (muco-)cutaneous leishmaniasis. The second subgroup, here-after referred to as *L. braziliensis* 2, has been occasionally diagnosed in Peru and Bolivia (*10–12*). The third subgroup, here-after referred to as *L. braziliensis* 3, was described within a geographically restricted ecotype in the Pernambuco state in Brazil (*13*). The fourth subgroup, here-after referred to as *L. braziliensis* 4, includes two strains from Colombia (*9*), and was classified as such by us following the phylogenomic analyses presented below.

Genome sequencing of five *L. braziliensis* strains

Sequence data was generated for three *L. braziliensis* 2 strains (CUM555, CUM663 and PER163) and two *L. braziliensis* 3 strains (HBO and LIS) (Table 2). To this end, parasites were grown in culture medium for 3 to 4 days at the Antwerp Institute of Tropical Medicine or FIOCRUZ in Brazil, and their DNA was extracted using a commercial column DNA extraction protocol. At the Wellcome Sanger Institute, genomic DNA was sheared into 400 to 600 bp fragments by focused ultrasonication (Covaris Inc.), and amplification-free Illumina libraries were prepared (*14*). One hundred base pair paired end reads were generated on the HiSeq 2000, and 150 bp paired end reads were generated on the HiSeq ×10 according to the manufacturer's standard sequencing protocol.

Bioinformatic analyses

Genomic sequence reads of all strains were mapped against the *L. braziliensis* M2904 reference genome (available on https://tritrypdb.org as LbraziliensisMHOMBR75M2904_2019) using SMALT v0.7.6 (https://www.sanger.ac.uk/tool/smalt-0/). This reference includes 35 major chromosomes (32.73Mb) and a complete mitochondrial maxicircle (27.69kb) (*7*).

Given the diversity of species included in this study, we tested the quality of sequence alignments against the M2904 reference by characterizing the accessible genome (*15*), i.e., genomic regions with a minimum read depth of 5, mapping quality of 25 and base quality of 25. The coverage of the accessible genome ranged between 28.6Mb (87.2% of the chromosomal region) for *L. shawi* strain M8408 and 30.4Mb (92.9%) for *L. braziliensis* strain Lb8025 (Table 3). The coverage for strain MHOM/CR/2020/StPierre was equal to 30.0 Mb (91.8%) (Table 3). These results showed that a substantial fraction of the reference genome can be used for genotyping and ancestry analyses.

Large number of heterozygous sites (where one haplotype is similar to M2904) points to a member of the *L. braziliensis* species complex as one of the parents

A total of 125,632 SNPs were identified within the strain from Costa Rica, including 21,168 homozygous SNPs (where both haplotypes were different to the consensus sequence of M2904) and 104,464 heterozygous SNPs (where one haplotype was similar to M2904 and the other different). This latter observation indicates *L. braziliensis* as one of the species that contributed sequences. This number of heterozygous SNPs was at least 4.1 and 16.4 times higher compared to the numbers observed for *L. braziliensis* and *L. guyanensis* respectively (Figure 1), confirming that the Costa Rica strain is either the result of hybridization or a mixed infection.

Phylogenetic analyses based on the mitochondrial maxicircle points to a member of the *L. guyanensis* species complex as one of the parents

Single Nucleotide Polymorphisms (SNPs) were called jointly across all genomes using bcftools mpileup/call (*16*), retaining only SNPs with a minimum SNP quality (QUAL) of 100, mapping quality of 50 and genotype quality of 60. Genotyping uncovered - across all genomes - a total 1,103,461 SNPs within the 35 major chromosomes and 467 SNPs within the coding region of the mitochondrial maxicircle. A phylogenetic network reconstructed with SplitsTree (*17*) using nuclear SNPs showed that the Costa Rica strain occupied a central position between the *L. guyanensis* and *L. braziliensis* species complexes, highlighting its uncertain ancestry (Figure 2). A phylogeny based on the uniparentally inherited mitochondrial maxicircles (*7*,*18*,*19*) showed that the Costa Rica strain clustered with *L. guyanensis* (Figure 3), pointing to a member of the *L. guyanensis* species complex as one of the parental species.

Genomic distribution of heterozygous sites suggests that MHOM/CR/2020/StPierre is a hybrid parasite, rather than the result of a mixed infection

When analyzing the genome-wide SNP distribution in non-overlapping 10kb windows (3,091 windows in total), 2,890 windows (93.5%) were found where at least half the SNPs were heterozygous (Figure 4), and only 108 windows (3.5%) were entirely homozygous. The majority of homozygous 10kb windows (95/108, 88%) covered almost entirely chromosomes 1 and 11, the first 140 kb of chromosome 20 and the last 60kb of chromosome 27 (Figure 5). The observation of a largely heterozygous genome that is interrupted by homozygous stretches strongly suggests that the isolate is a hybrid parasite, rather than the result of a mixed infection.

Distribution of allelic read depths at heterozygous sites shows that the hybrid is a triploid parasite, and reveals major recombination breakpoints

The genetic complexity of *Leishmania* infections is of particular interest because of the existence of aneuploidy, which was genomically inferred by investigating allelic read depth frequencies (ARDF) at heterozygous sites and standardized chromosomal read depths (*20*). The genome-wide ARDF distribution was bimodal for the hybrid parasite, with modes 0.33 and 0.67 (Figure 6) suggesting that the hybrid is triploid (*21*), with the exceptions of chromosomes 1 and 11 (no distribution because of absence of heterozygous sites), 3 and 12 (trimodal distributions). Assuming triploidy, standardized chromosomal read depths showed that chromosome 10 was trisomic, chromosomes 1, 3 and 12 were tetrasomic and chromosome 31 was hexasomic (Table 4). Shifts in the ARDF distribution of species-specific alleles between the two modes along chromosomes (Figure 7) represent recombination events that occurred since the hybridization event (*18*) and further exclude the possibility of a mixed infection.

Neighbor-Joining phylogenetic trees

The low bootstrap estimates in the ML phylogenetic trees based on SNPs detected in the telomeric region of chromosome 20 (Figure in main article) and the maxicircle coding region (Figure 3) prompted us to reconstruct a different type of phylogeny for complementary insights. To this end, we reconstructed Neighbor-Joining phylogenetic trees based on the number of nucleotide differences between *Leishmania* strains using the R package ape (*23*), which are shown in Figures 8 and 9.

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Table 1. Sequence variation across 11 positions in the multicopy heat-shock protein 70 gene (*hsp70*)*

POSITION on full CDS	SANGER	WGS	H1 (M2904)	H2 (*L. guyanensis*)
504	R	G, A	NA	NA
731	S	G, C	NA	NA
762	R	G, A	NA	NA
1173	R	G, A	NA	NA
1576	K	T, G	T	G
1600	R	A, G	A	G
1632	Y	C, T	C	T
1666	R	G, A	G	A
1669	K	T, G	T	G
1679	R	A, G	A	G
1734	S	C, G	NA	NA

*A 1,245 bp fragment of the *hsp70* locus was sequenced for species typing, revealing ambiguous bases at 11 positions (column 'SANGER'). Underlying alleles were identified through genotyping of Single Nucleotide Polymorphisms (SNPs) after mapping sequencing reads against a single *hsp70* gene sequence of the M2904 reference genome. This revealed heterozygous SNPs at all 11 ambiguous positions: the two alleles in column 'WGS' represent the reference allele (similar to the M2904 consensus sequence) and alternate allele (different to the M2904 consensus sequence), respectively. Haplotypes (columns 'H1' and 'H2') were decomposed for six neighboring ambiguous positions (1576–1679) by extracting 150bp sequence reads covering all six positions.

Table 2. List of 40 publicly and in-house available sequence data from seven *Leishmania (Viannia)* species that were used for whole genome sequence analyses*

SRA Run Accession Number	Name	Species	Country	References
ERR471302	CUM29	*L. braziliensis* 1	Bolivia	(*7*)
ERR3988461	Lb7529	*L. braziliensis 1*	Colombia	(*9*)
ERR3988463	Lb7740	*L. braziliensis 1*	Colombia	(*9*)
ERR3988465	Lb7933	*L. braziliensis 1*	Bolivia	(*9*)
ERR3988466	Lb8025	*L. braziliensis 1*	Colombia	(*9*)
ERR3988467	Lb8102	*L. braziliensis 1*	Colombia	(*9*)
ERR377654	LC1565	*L. braziliensis 1*	Peru	(*7*)
ERR3150801	RO393	*L. braziliensis 1*	Brazil	(*7*)
ERR877281	CUM555	*L. braziliensis 2*	Bolivia	this study
ERR877282	CUM663	*L. braziliensis 2*	Bolivia	this study
ERR467298	PER163	*L. braziliensis 2*	Peru	this study
ERR3150831, ERR3150735	HBO	*L. braziliensis 3*	Brazil	this study
ERR3150728, ERR3150824	LIS	*L. braziliensis 3*	Brazil	this study
ERR3988462	Lb7616	*L. braziliensis 4*	Colombia	(*9*)
ERR3988464	Lb7864	*L. braziliensis 4*	Colombia	(*9*)
SRR8179913	204–365	*L. guyanensis*	French Guyana	(*5*)
SRR1662195	M4147	*L. guyanensis*	Brazil	(*4*)
ERR6188759	S8104	*L. guyanensis*	Venezuela	(*2*)
SRR8179821	216_34	*L. lainsoni*	Peru	(*1*)
ERR6188758	HOMI-81	*L. naiffi*	Colombia	(*2*)
ERR205764	LnCL223	*L. naiffi*	Colombia	(*3*)
SRR1657911	M5533	*L. naiffi*	Brazil	(*4*)
ERR205773	LgCL085	*L. panamensis*	Colombia	(*3*)
ERR3648455	LpS7762	*L. panamensis*	Colombia	(*8*)
ERR3648456	LpS7842	*L. panamensis*	Colombia	(*8*)
ERR3648457	LpS8036	*L. panamensis*	Colombia	(*8*)
ERR3648458	LpS8046	*L. panamensis*	Colombia	(*8*)
ERR3648459	LpS8049	*L. panamensis*	Colombia	(*8*)
ERR3648460	LpS8056	*L. panamensis*	Colombia	(*8*)
ERR3648461	LpS8061	*L. panamensis*	Colombia	(*8*)
ERR3648463	LpS8087	*L. panamensis*	Colombia	(*8*)
ERR3648466	LpS8117	*L. panamensis*	Colombia	(*8*)
ERR3648467	LpS8124	*L. panamensis*	Colombia	(*8*)
ERR3648469	LpS8136	*L. panamensis*	Colombia	(*8*)
SRR1552486	PSC1	*L. panamensis*	Panama	(*6*)
ERR3656054	REST417	*L. panamensis*	Colombia	(*7*)
SRR1662198	WR120	*L. panamensis*	Panama	(*4*)
ERR662608	HB83	*L. peruviana*	Peru	(*7*)
ERR662626	LCA04	*L. peruviana*	Peru	(*7*)
SRR1657909	M8408	*L. shawi*	Brazil	(*4*)

*RO393, LIS and HBO are *Leishmania* strains available at the *Leishmania* collection from the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil (http://clioc.fiocruz.br/).

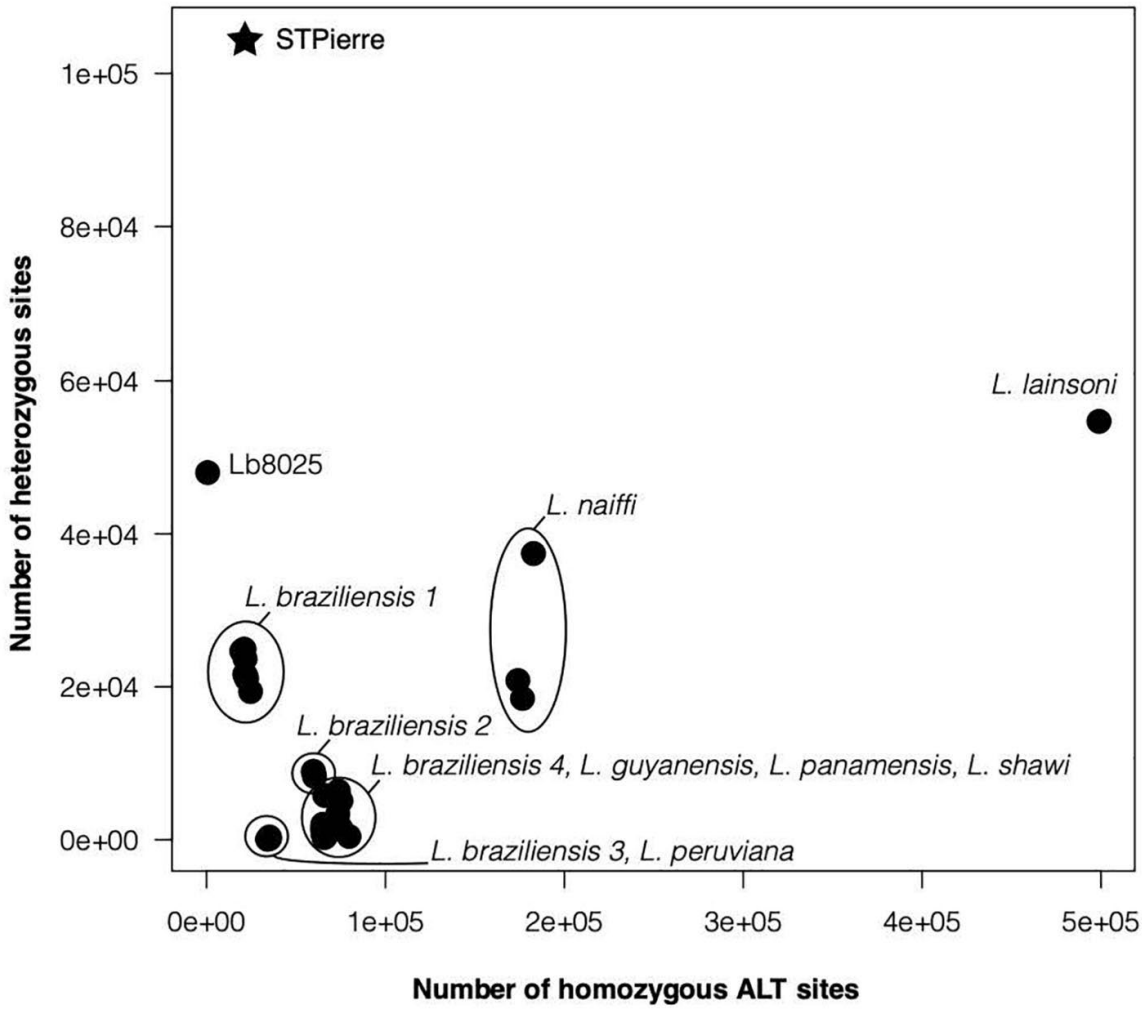
Table 3. Coverage and number of accessible genomic regions in each of the 41 *Leishmania* strains

Species	Strain	Coverage	Fraction	Number of regions
Leishmania shawi	M8408	28552102	87,23%	65585
Leishmania panamensis	WR120	28586898	87,34%	64401
Leishmania naiffi	M5533	28604295	87,39%	61373
Leishmania panamensis	REST417	28765477	87,88%	77484
Leishmania lainsoni	216_34	28788593	87,96%	94726
Leishmania naiffi	LnCL223	28832216	88,09%	94858
Leishmania guyanensis	M4147	28867457	88,20%	50537
Leishmania naiffi	HOM81	29366435	89,72%	38873
Leishmania panamensis	LgCL085	29410263	89,85%	41655
Leishmania panamensis	PSC1	29501473	90,13%	30825
Leishmania guyanensis	204_365	29585997	90,39%	62565
Leishmania panamensis	LpS8124	29638319	90,55%	24813
Leishmania panamensis	LpS8136	29642314	90,56%	25099
Leishmania panamensis	LpS8117	29646217	90,58%	23988
Leishmania panamensis	LpS8049	29661360	90,62%	23912
Leishmania panamensis	LpS8061	29678344	90,67%	24194
Leishmania panamensis	LpS8046	29682946	90,69%	24614
Leishmania guyanensis	S8104	29684216	90,69%	23465

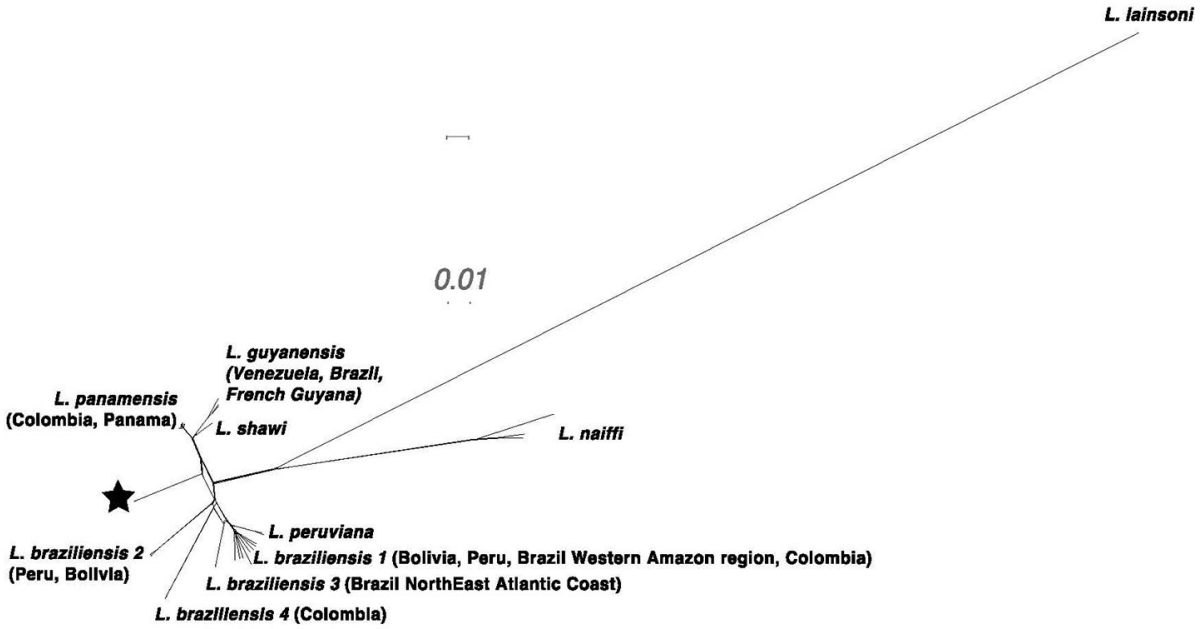
Species	Strain	Coverage	Fraction	Number of regions
Leishmania panamensis	LpS8056	29686912	90,70%	24218
Leishmania panamensis	LpS7762	29692655	90,72%	21681
Leishmania panamensis	LpS7842	29715153	90,79%	22423
Leishmania panamensis	LpS8087	29716249	90,79%	21868
Leishmania panamensis	LpS8036	29728357	90,83%	21984
Leishmania braziliensis 2	PER163	29791821	91,02%	32749
Leishmania braziliensis 4	Lb7864	29815765	91,09%	17887
Leishmania braziliensis 1	LC1565	29861013	91,23%	27927
Leishmania braziliensis 2	CUM663	29872352	91,27%	32131
Leishmania braziliensis 2	CUM555	29887936	91,31%	31159
Leishmania braziliensis 4	Lb7616	29944008	91,49%	15486
Leishmania peruviana	HB83	29984415	91,61%	29030
Leishmania peruviana	LCA04	30014816	91,70%	25532
Leishmania braziliensis 1	CUM29	30025178	91,73%	21921
Hybrid strain	MHOM/CR/2020/StPierre	30032613	91,76%	16291
Leishmania braziliensis 3	HBOA1	30039532	91,78%	28782
Leishmania braziliensis 3	LISA1	30046468	91,80%	27611
Leishmania braziliensis 1	Lb8102	30054846	91,82%	11135
Leishmania braziliensis 1	Lb7740	30062478	91,85%	11505
Leishmania braziliensis 1	Lb7529	30082397	91,91%	10581
Leishmania braziliensis 1	Lb7933	30129942	92,05%	10957
Leishmania braziliensis 1	RO393	30160429	92,15%	27477
Leishmania braziliensis 1	Lb8025	30412185	92,92%	8012

Table 4. Somy variation was genomically inferred by investigating allelic read depth frequencies (ARDF) at heterozygous sites and standardized chromosomal read depths assuming triploidy (*20*)

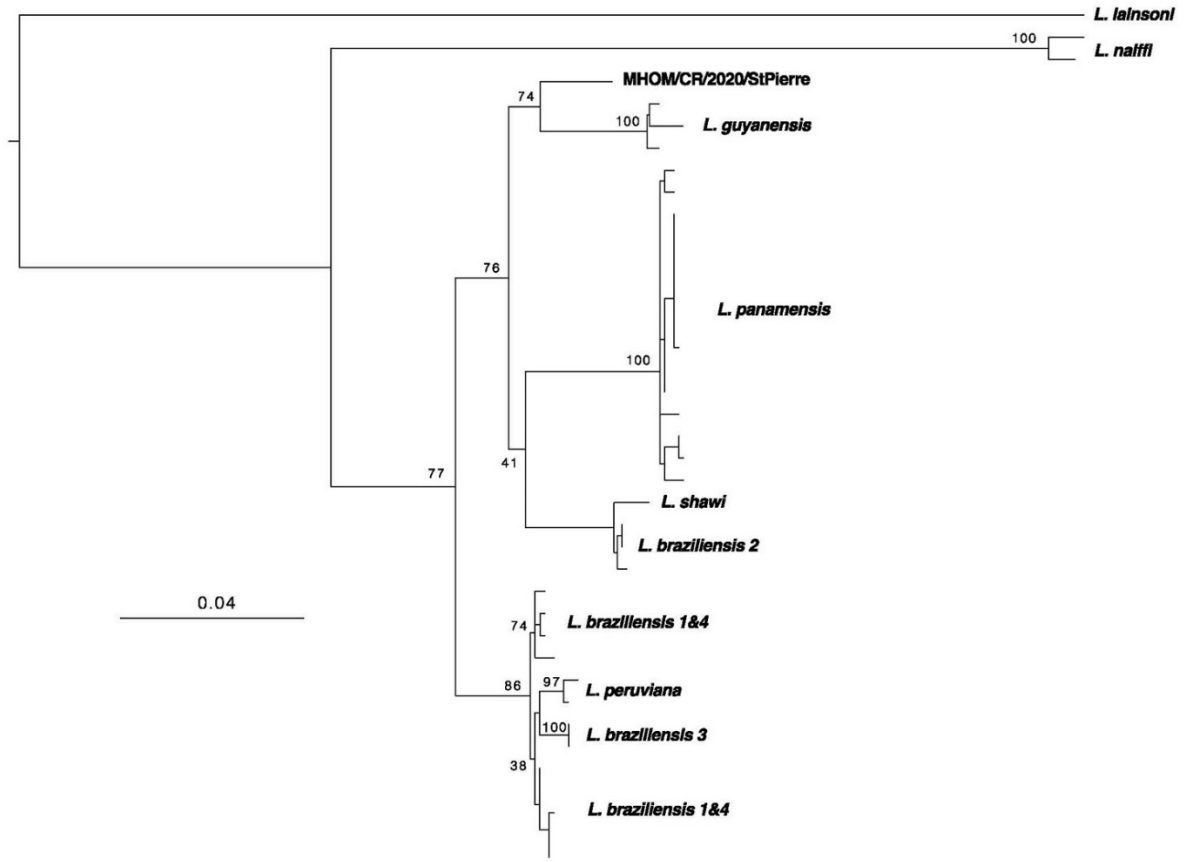
Chromosome	Haploid Chromosomal Read Depths	Somy based on read depths, assuming triploidy	Somy based on ARDF
1	1,4	4,3	NA
2	0,9	2,6	3
3	1,3	3,9	4
4	1,0	2,9	3
5	1,0	3,1	3
6	1,0	3,0	3
7	1,0	3,0	3
8	1,0	2,9	3
9	1,0	3,0	3
10	0,9	2,8	3
11	1,1	3,2	NA
12	1,4	4,1	4
13	1,0	2,9	3
14	1,0	3,0	3
15	1,0	3,1	3
16	1,0	3,0	3
17	1,0	3,1	3
18	1,0	3,0	3
19	1,0	2,9	3
20	1,0	2,9	3
21	1,0	3,0	3
22	1,0	3,0	3
23	1,0	3,1	3
24	1,0	3,0	3
25	1,0	3,1	3
26	1,0	3,0	3
27	1,0	3,0	3
28	1,0	3,0	3
29	1,0	3,0	3
30	1,0	3,0	3
31	2,0	6,0	6
32	1,0	3,0	3
33	1,0	3,0	3
34	1,0	3,0	3
35	1,0	3,0	3



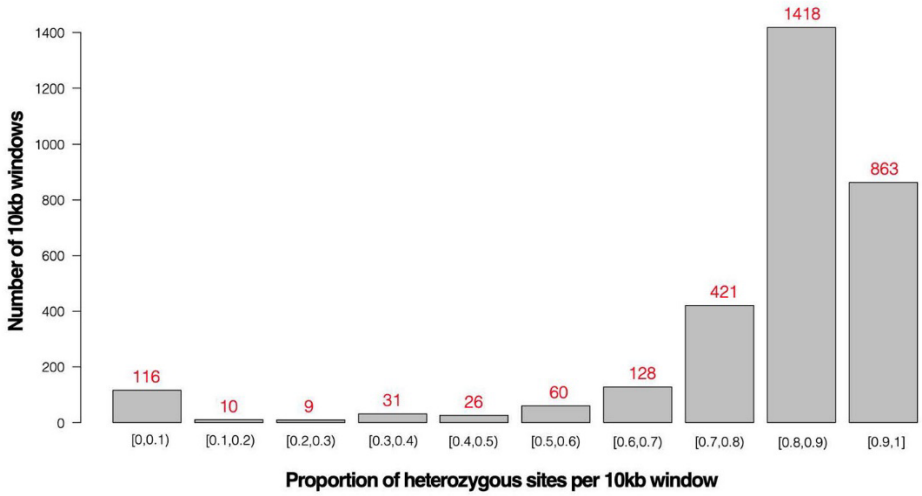
Appendix 2 Figure 1. Number of heterozygous versus homozygous sites for each of the 41 *Leishmania* genomes included in this study. ALT = alternate alleles, i.e., alleles different to the reference genome.



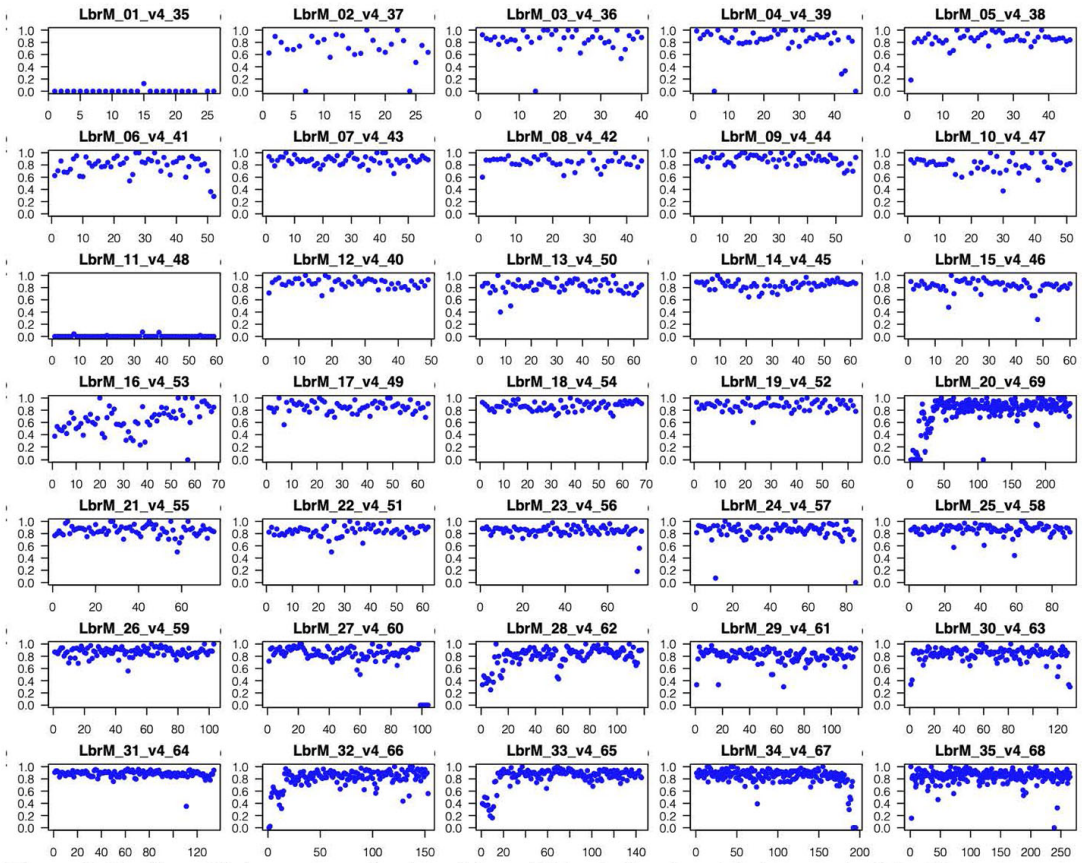
Appendix 2 Figure 2. Phylogenetic network as obtained with SplitsTree (*17*) using 1,103,461 genome-wide SNPs called across 41 *Leishmania* genomes. The star indicates the position of MHOM/CR/2020/StPierre. For each strain, sequences were composed based on concatenated SNPs that were each coded by two base pairs, resulting in sequences of 2,206,922 nt. The distance scale on top shows substitutions/sites.



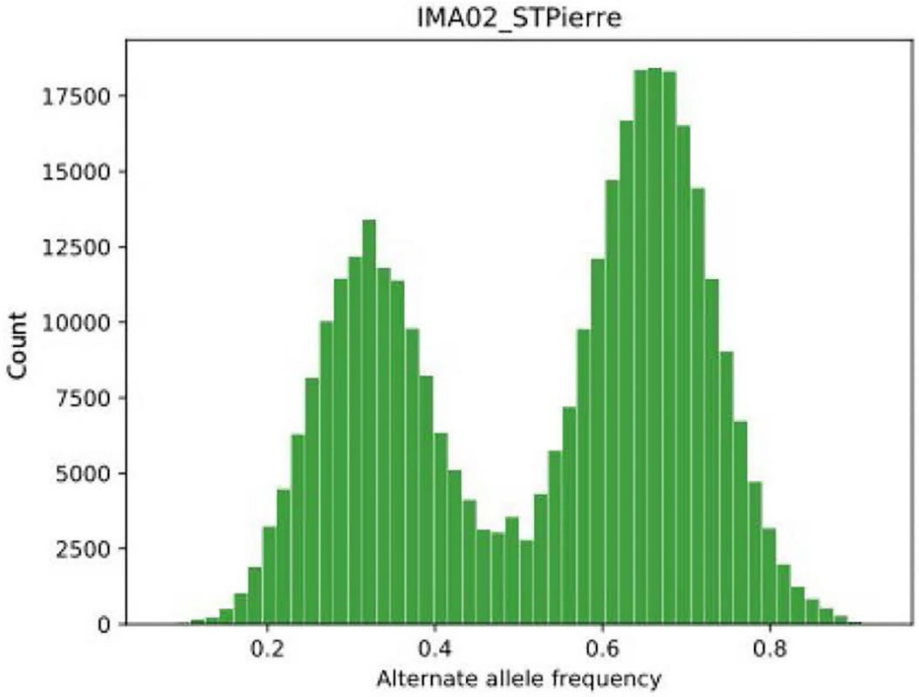
Appendix 2 Figure 3. Midpoint rooted Maximum Likelihood phylogenetic trees based on 467 SNPs called within the mitochondrial maxicircle coding region. Consensus phylogenetic trees were generated from 1,000 bootstrap trees using IQTREE (*22*) under the TN+F+ASC substitution model (TN = unequal transition/transversion rates and unequal purine/pyrimidine rates, *F* = empirical base frequencies, ASC = ascertainment bias correction), which was the best-fit model revealed by ModelFinder as implemented in IQTREE. Assuming that the maxicircle is haploid and because we observed no signatures of heteroplasmy (all SNPs were homozygous), SNPs were coded by one base pair, resulting in sequences of 467 nt. The distance scale (bottom) shows substitutions/sites. Branch support values are presented near each node following 1000 bootstrap replicates. Note that the *L. braziliensis* lineage 2 is closely related to *L. shawi* and positioned with low bootstrap support (41%) within the *L. guyanensis* species complex. This discrepancy between the nuclear (Figure 2) and maxicircle (Figure 3) phylogenies suggest a complex ancestry for the *L. braziliensis* lineage 2, an observation that warrants more detailed analyses in future research.



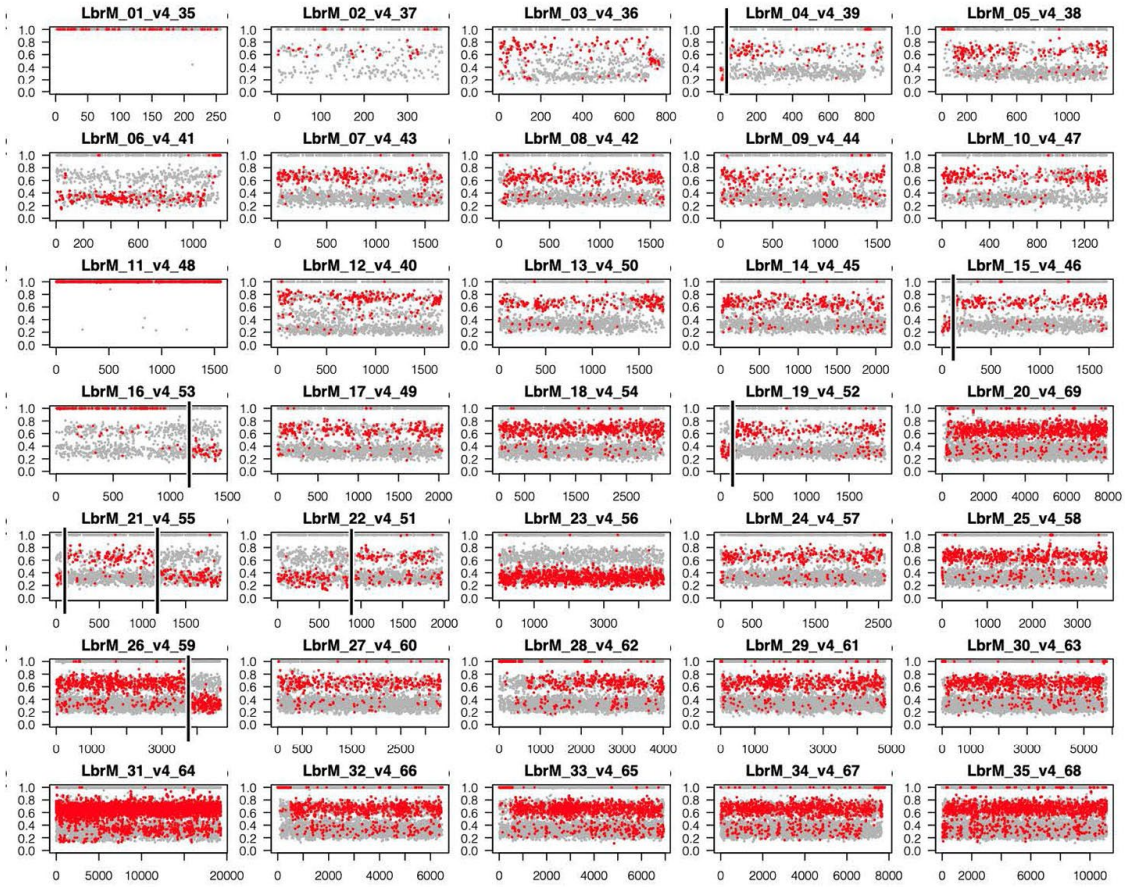
Appendix 2 Figure 4. Number of 10kb windows (y-axis) with a given fraction of heterozygous SNP sites (x-axis).



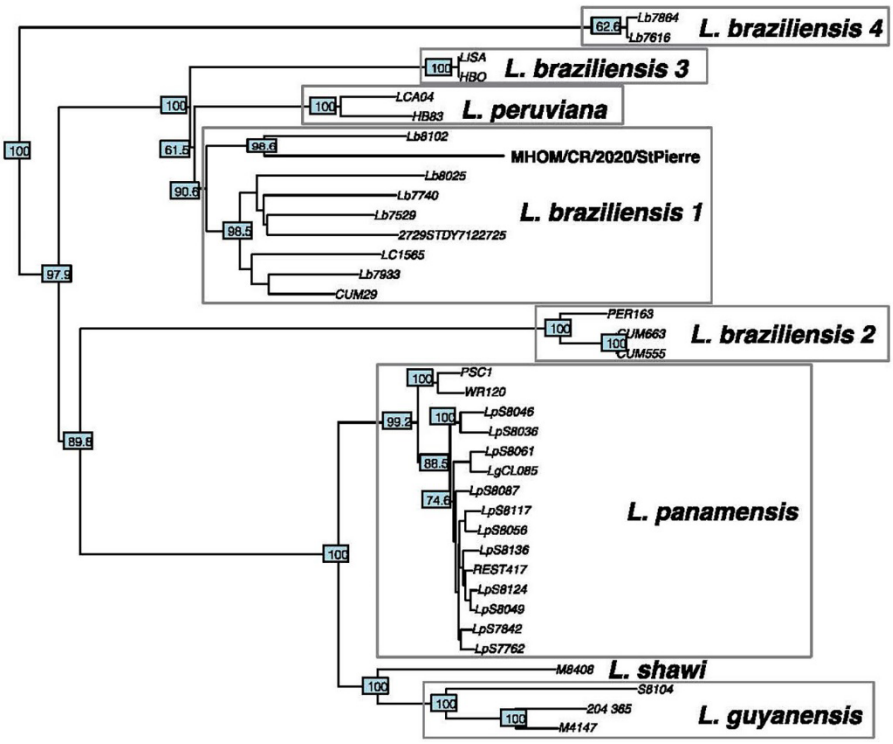
Appendix 2 Figure 5. Fraction of heterozygous sites (y-axis) per 10kb window (x-axis) along each of the 35 chromosomes. The majority of homozygous 10kb windows (95/108, 88%) covered almost entirely chromosomes 1 and 11, the first 140 kb of chromosome 20 and the last 60kb of chromosome 27.



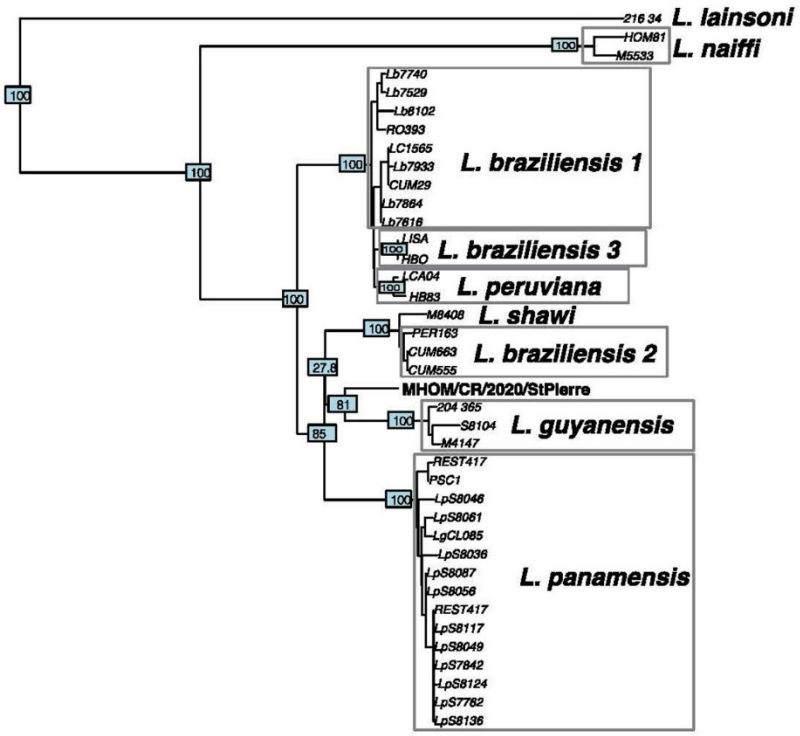
Appendix 2 Figure 6. Genome-wide distribution of read depth frequencies of alternate alleles at heterozygous sites for MHOM/CR/2020/StPierre.



Appendix 2 Figure 7. Distribution of read depth frequencies of all alternate alleles (gray and red dots) at heterozygous sites for each of the 35 chromosomes in the hybrid genome. Red dots reflect alternate alleles that were specific to the *L. guyanensis* species complex, i.e., alleles found in *L. guyanensis* and/or *L. panamensis* and/or *L. shawi* strains and not in any other *Viannia* species. Black bars denote position of major shifts in read depth frequencies, which represent recombination events since the initial hybridization event.



Appendix 2 Figure 8. Neighbor-Joining tree based on the number of nucleotide differences between *Leishmania* strains. Sequences contained 3,015 nt from the telomeric region of chromosome 20 and are the same as used in Figure 1 of the main text. Node support is based on 1,000 bootstrapped phylogenetic trees.



Appendix 2 Figure 9. Neighbor-Joining tree based on the number of nucleotide differences between *Leishmania* strains. Sequences contained 467 nt from the maxicircle coding region and are the same as used in Figure 3 of Appendix 2. Node support is based on 1000 bootstrapped phylogenetic trees.