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Leishmania naiffi and Leishmania guyanensis reference genomes highlight genome structure and gene evolution in the Viannia subgenus

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The unicellular protozoan parasite *Leishmania* causes the neglected tropical disease leishmaniasis, affecting 12 million people in 98 countries. In South America, where the *Viannia* subgenus predominates, so far only *L. (Viannia) braziliensis* and *L. (V.) panamensis* have been sequenced, assembled and annotated as reference genomes. Addressing this deficit in molecular information can inform species typing, epidemiological monitoring and clinical treatment. Here, *L. (V.) naiffi* and *L. (V.) guyanensis* genomic DNA was sequenced to assemble these two genomes as draft references from short sequence reads. The methods used were tested using short sequence reads for *L. braziliensis* M2904 against its published reference as a comparison. This assembly and annotation pipeline identified 70 additional genes not annotated on the original M2904 reference. Phylogenetic and evolutionary comparisons of *L. guyanensis* and *L. naiffi* with 10 other *Viannia* genomes revealed four traits common to all *Viannia*: aneuploidy, 22 orthologous groups of genes absent in other *Leishmania* subgenera, elevated TATE transposon copies and a high NADH-dependent fumarate reductase gene copy number. Within the *Viannia*, there were limited structural changes in genome architecture specific to individual species: a 45 Kb

amplification on chromosome 34 was present in all bar *L. lainsoni*, *L. naiffi* had a higher copy number of the virulence factor leishmanolysin, and laboratory isolate *L. shawi* M8408 had a possible minichromosome derived from the 3' end of chromosome 34. This combination of genome assembly, phylogenetics and comparative analysis across an extended panel of diverse *Viannia* has uncovered new insights into the origin and evolution of this subgenus and can help improve diagnostics for leishmaniasis surveillance.

1. Introduction

Most cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) cases in the Americas are the result of infection by *Leishmania* parasites belonging to the *Viannia* subgenus. The complexity of the molecular, epidemiological and ecological challenges associated with *Leishmania* in South America remains opaque due to our limited understanding of the biology of *Viannia* parasites. Nine *Viannia* (sub)species have been described so far: *L. (V.) braziliensis*, *L. (V.) peruviana*, *L. (V.) guyanensis*, *L. (V.) panamensis*, *L. (V.) shawi*, *L. (V.) lainsoni*, *L. (V.) naiffi*, *L. (V.) lindenbergi* and *L. (V.) utingensis*. CL and MCL are endemic in 18 out 20 countries in the Americas [1] and are mainly associated with *L. braziliensis*, *L. guyanensis* and *L. panamensis*, whose frequency varies geographically. Other species are less frequently associated with human disease, and some are restricted to certain areas [2].

Human CL is partially driven by transmission from sylvatic and peridomestic mammalian reservoirs [3], via sand flies of the genus *Lutzomyia* (*sensu* Young and Duncan, 1994) in the Americas, distinct from *Phlebotomus* sand flies in the Old World [4]. Although CL has spread to domestic and peridomestic niches due to migration, new settlements and deforestation [5–7], there is still a high incidence of some *Leishmania* in sylvatic environments, such that human infection is accidentally acquired due to sand fly bites when handling livestock [8]. *Leishmania naiffi* and *L. guyanensis* are among the *Viannia* species that show variable responses to treatment, and diversity in the types of clinical manifestations presented, and are adapting to environmental niche and transmission changes driven by humans.

Leishmania naiffi was formally described from a parasite isolated in 1989 from its primary reservoir, the nine-banded armadillo (*Dasypus novemcinctus*), in Pará state of northern Brazil [9–11]. *Leishmania naiffi* was initially placed in the *Viannia* subgenus based on its molecular and immunological characteristics [9]. Many phlebotomine species are likely to participate in the transmission of *L. naiffi* in Amazonia [12], including *Lu.* (*Psathyromyia*) *ayrozai* and *Lu.* (*Psychodopygus*) *paraensis* in Brazil [13], *Lu.* (*Psathyromyia*) *squamiventris* and *Lu. tortura* in Ecuador [14], and *Lu. trapidoi* and *Lu. gomezi* in Panama [15]. *Leishmania naiffi* has been isolated from humans and armadillos [9,10] and detected in *Thrichomys pachyurus* rodents found in the same habitat as *D. novemcinctus* in Brazil [16]. The nine-banded armadillo is hunted, handled and consumed in the Americas and is regarded as a pest [11,17,18]. People in the same vector range as these armadillos could be exposed to infective sand flies: three *L. naiffi* CL cases followed contact with armadillos in Suriname [19]. *Leishmania naiffi* causes localized CL in humans with small discrete lesions on the hands, arms or legs [10,20,21], which has been observed in Brazil, French Guiana, Ecuador, Peru and Suriname [19,22]. CL due to *L. naiffi* usually responds to treatment [10,22] and can be self-limiting [23], though poor response to antimonial or pentamidine therapy was reported in two patients in Manaus, Brazil [20].

L. guyanensis was first described in 1954 [24] and its primary hosts are the forest dwelling two-toed sloth (*Choloepus didactylus*) and the lesser anteater *Tamandua tetradactyl* [25]. Potential secondary reservoirs of *L. guyanensis* are *Didelphis marsupialis* (the common opossum) [26,27], rodents from the genus *Proechimys* [25], *Marmosops incanus* (the grey slender opposum) [28] in Brazil and *D. novemcinctus* [29]. *Lu. umbratilis*, *Lu. anduzei* and *Lu. whitmani* are prevalent in forests [30] and act as vectors of *L. guyanensis* [31–33]. *Leishmania guyanensis* has been found in French Guiana, Bolivia, Brazil, Colombia, Guyana, Venezuela, Ecuador, Peru, Argentina and Suriname [34–39].

More precise genetic screening of *Viannia* isolates is necessary to trace hybridization between species. Infection of humans, dogs and *Lu. ovallesi* with *L. guyanensis*/*L. braziliensis* hybrids was reported in Venezuela [40,41]. A *L. shawi*/*L. guyanensis* hybrid causing CL was detected in Amazonian Brazil [42], and *L. naiffi* has produced viable progeny with *L. lainsoni* [43] and *L. braziliensis* (Elisa Cupolillo 2018, unpublished data). There is extensive evidence of interbreeding among *L. braziliensis* complex isolates, including more virulent *L. braziliensis*/*L. peruviana* hybrids with higher survival rates within hosts *in vitro* [44].

Leishmania genomes are characterized by several key features. Genes are organized as polycistronic transcription units that have a high degree of synteny across *Leishmania* species [45]. These polycistronic

transcription units are co-transcribed by RNA polymerase II as polycistronic pre-mRNAs that are 5′-transpliced and 3′-polyadenylated [46,47]. This means translation and stability of these mature mRNAs determine gene expression rather than transcription rates. In addition, *Leishmania* display extensive aneuploidy, frequently possess extrachromosomal amplifications driven by homologous recombination at repetitive sequences, and have variable gene copy numbers [48]. The *Leishmania* subgenus genomes of *L. infantum*, *L. donovani* and *L. major* have 36 chromosomes [49], whereas *Viannia* genomes have 35 chromosomes due to a fusion of chromosomes 20 and 34 [45,50]. In contrast to the species of the *Leishmania* subgenus, *Viannia* parasites possess genes encoding functioning RNA interference (RNAi) machinery that may mediate infective viruses and transposable elements [51].

Fully annotated genomes have been described in detail for only two *Viannia* species: *L. panamensis* [51] and *L. braziliensis* [45,48], limiting our comprehension of their evolutionary origin, genetic diversity and functional adaptations. Consequently, we present reference genomes for *L. guyanensis* LgCL085 and *L. naiffi* LnCL223 to address these critical gaps. These new annotated reference genomes were compared with other *Viannia* species genomes to examine structural variation, sequence divergence, gene synteny and chromosome copy number changes. We contrasted the genomic configuration of *L. guyanensis* LgCL085 and *L. naiffi* LnCL223 with the *L. braziliensis* MHOM/BR/1975/M2903 assembly, two unannotated *L. peruviana* chromosome-level scaffold assemblies [52], the *L. panamensis* MHOM/PA/1994/PSC-1 reference and the *L. braziliensis* MHOM/BR/1975/M2904 reference. Furthermore, we assessed aneuploidy in five unassembled *Viannia* datasets originally isolated from humans, armadillos and primates, which are commonly used in studies on *Viannia* parasites [53–56]: *L. shawi* reference isolate MCEB/BR/1984/M8408 also known as IOC_L1545, *L. guyanensis* MHOM/BR/1975/M4147 (iz34), *L. naiffi* MDAS/BR/1979/M5533 (IOC_L1365), *L. lainsoni* MHOM/BR/1981/M6426 (IOC_L1023), *L. panamensis* MHOM/PA/1974/WR120 [53] (IOC stands for Instituto Oswaldo Cruz).

2. Results

2.1. Genome assembly from short reads

The genomes of *L.* (*Viannia*) *guyanensis* LgCL085 and *L.* (*V.*) *naiffi* LnCL223 were assembled from short reads, along with an assembly of *L. braziliensis* M2904 generated in the same way as a positive control [48] (table 1). This facilitated comparison with the published M2904 genome, which was assembled by capillary sequencing of a plasmid clone library together with extensive finishing work and with fosmid end sequencing [45], so that the ability of short reads to correctly and comprehensively resolve *Leishmania* genome architecture could be quantified.

Firstly, the *L. guyanensis* LgCL085, *L. naiffi* LnCL223 and the *L. braziliensis* M2904 control reads were filtered to remove putative contaminant sequences identified by aberrant GC content, trimmed at the 3′ ends to remove low-quality bases, and polymerase chain reaction (PCR) primer sequences were removed (see Methods for details) resulting in 26 067 692 properly paired reads for *L. guyanensis*, 13 979 628 for *L. naiffi*, 34 592 618 for the *L.* (*V.*) *braziliensis* control (electronic supplementary material, table S1). These filtered reads for *L. guyanensis*, *L. naiffi* and *L. braziliensis* were de novo assembled into contigs using Velvet [57] with k-mers of 61 for *L. guyanensis*, 43 for *L. naiffi* and 43 for the *L. braziliensis* control optimized for each library.

The initial contigs were scaffolded using read pair information with SSPACE [58] to yield 2800 *L. guyanensis* scaffolds with an N50 of 95.4 Kb, 6530 *L. naiffi* scaffolds with an N50 of 24.3 Kb, and 3782 *L. braziliensis* scaffolds with an N50 of 20.6 Kb (table 2). The corrected scaffolds for *L. guyanensis*, *L. naiffi* and the *L. braziliensis* control were contiguated (aligned, ordered and oriented) using the extensively finished *L. braziliensis* M2904 reference with ABACAS [59]. The output was split into 35 pseudo-chromosomes and REAPR [60] broke scaffolds at possible misassemblies to assess contiguation accuracy. The pseudo-chromosome lengths of each sample approximated the length of each corresponding *L. braziliensis* M2904 reference chromosome with the exceptions of shorter *L. guyanensis* chromosomes 2, 4, 12 and 21, and a longer *L. naiffi* chromosome 1 (electronic supplementary material, figure S1). Post-assembly alignment of all bin contigs using BLASTn identified 44 *L. guyanensis* sequences spanning 4 566 791 bp as putative contaminants that were removed: half had high similarity to bacterium *Niastella koreensis* (electronic supplementary material, table S2).

When the reads for each were mapped to its own assembled genome, the median read coverage was 56 for *L. guyanensis*, 36 for *L. naiffi* and 75 for the *L. braziliensis* control. The latter was on par with the

Table 1. Data used in this study. The World Health Organization (WHO) numbers are structured such that M is mammal, R is reptile, HOM is *Homo*, CAN is canine, DAS is *Dasypus* (an armadillo), CEB is *Cebus* (a primate), ARV is *Arvicanthis* (a rodent), TAR is *Tarentolae* and LAT is *Latastia* (a long-tailed lizard). The top two rows indicate the isolates for *L. guyanensis* and *L. naiffi* genomes published here.

species	source	data type	name or WHO number	SRA[a]	number and length of reads	reference
L. guyanensis	SRA	reads	LgCL085	ERX180458	15 272 969 (100 bp paired-end)	this study
L. naiffi	SRA	reads	LnCL223	ERX180449	8 131 246 (100 bp paired-end)	this study
L. braziliensis	Sanger FTP site	genome and reads	MHOM/BR/1975/M2904	ERX005631 (LbrM2904 v3)	26 007 384 (76 bp paired-end)	Rogers et al. [48]
L. guyanensis	SRA	reads	MHOM/BR/1975/M4147	SRX767379	6 225 035 (100 bp paired-end)	Harkins et al. [53]
L. lainsoni	SRA	reads	MHOM/BR/1981/M6426	SRX764333	4 630 952 (100 bp paired-end)	Harkins et al. [53]
L. naiffi	SRA	reads	MDAS/BR/1979/M5533	SRX764332	9 646 461 (100 bp paired-end)	Harkins et al. [53]
L. panamensis	Genbank and SRA	genome and reads	MHOM/PA/1994/PSC-1	SRX681913; (CP009370: CP009404)	5 875 837 (100 bp paired-end)	Llanes et al. [51]
L. panamensis	SRA	reads	MHOM/PA/1974/WR120	SRX767384	4 536 341 (100 bp paired-end)	Harkins et al. [53]
L. shawi	SRA	reads	MCEB/BR/1984/M8408	SRX764331	5 110 479 (100 bp paired-end)	Harkins et al. [53]
L. peruviana	Genbank and SRA	genome and reads	PAB-4377	ERX556165 (Bioproject ID: PRJEB7263)	16 117 316 (100 bp paired end)	Valdivia et al. [52]
L. peruviana	Genbank and SRA	genome and reads	LEM1537 (MHOM/PE/1984/LC39)	ERX556164 (Bioproject ID: PRJEB7263)	9 378 317 (100 bp paired end)	Valdivia et al. [52]

[a] SRA stands for SRA or TriTrypDB accession ID.

Table 2. Summary of *L. braziliensis* reference M2904, *L. braziliensis* control, *L. guyanensis* LgCL085 and *L. naiffi* LnCL223 genome assembly contigs, scaffolds, gaps, read coverage, assembled chromosomal and contig sequence and levels of gene annotation.

| | *L. braziliensis* | | *L. guyanensis* LgCL085 | *L. naiffi* LnCL223 |
	M2904	control		
initial number of contigs		13 601	10 308	14 682
initial contig N50 (Kb)		5.1	9.6	5.7
number of scaffolds		3782	2800	6530
scaffold N50 (Kb)		20.6	95.4	24.3
number of gaps	919	3352	1557	3853
median read coverage	75	74	56	36
N content (%)	0.29	0.99	0.45	1.07
chromosomes total length (bp)	31 238 104	28 985 156	28 274 008	29 179 723
bin sequence total length (bp)	850 747	1 024 497	2 740 314	1 161 372
total genome length (bp)	32 088 851	30 009 653	31 014 322	30 341 095
protein-coding genes	8357	8001	8230	8104
genes on chromosomes	8432	7873	7757	7952
genes on bin contigs	188	288	619	310
total number of genes	8620	8161	8376	8262

74-fold median coverage observed when M2904 short reads were mapped to the *L. braziliensis* reference [45,48] (electronic supplementary material, table S3). The differing coverage levels correlated with the numbers of gaps in the final genome assembly of *L. guyanensis* (1557, table 2) and *L. naiffi* (3853).

2.2. Multi-locus sequencing analysis of *L. guyanensis* LgCL085 and *L. naiffi* LnCL223 with the *Viannia* subgenus

As a first step in investigating the genetic origins of these isolates, we examined their species identity using MLSA (multi-locus sequencing analysis). Four housekeeping gene sequences published for 95 *Viannia* isolates including *L. braziliensis, L. lainsoni, L. lindenbergi, L. utingensis, L. guyanensis, L. shawi* and *L. naiffi* [56] were compared with orthologues of each gene extracted from assemblies of *L. naiffi* LnCL223, *L. guyanensis* LgCL085, the *L. braziliensis* reference, *L. panamensis* PSC-1 and *L. peruviana* PAB-4377. Among the 95 were four samples with reads available [53]: *L. shawi* MCEB/BR/1984/M8408 (IOC_L1545), *L. guyanensis* MHOM/BR/1975/M4147 (iz34), *L. naiffi* MDAS/BR/1979/M5533 (IOC_L1365) and *L. lainsoni* MHOM/BR/1981/M6426 (IOC_L1023). The genes were aligned using Clustal Omega v1.1 [61] to create a network for the 102 isolates with SplitsTree v4.13.1 [62]. This replicated the expected highly reticulated structure [56], where *L. braziliensis* M2904 and *L. peruviana* PAB-4377 were in the *L. braziliensis* cluster (figure 1).

Previous work suggests that the *L. guyanensis* species complex includes *L. panamensis* and *L. shawi* because they show little genetic differentiation from one another [56,63–65]. The MLSA here showed that the new *L. guyanensis* LgCL085 reference clustered phylogenetically in the *L. guyanensis* species complex, had no sequence differences compared with *L. panamensis* PSC-1, and seven differences versus *L. shawi* M8408 across the 2902 sites aligned (figure 1). *Leishmania guyanensis* LgCL085 grouped with isolates classified as zymodeme Z26 by multi-locus enzyme electrophoresis (MLEE) associated with *L. shawi* [54]. This was supported by the number and the alleles of genome-wide single-nucleotide polymorphisms (SNPs) called using reads mapped to the *L. braziliensis* M2904 reference for *L. guyanensis* (355 267 SNPs), *L. guyanensis* M4147 (326 491), *L. panamensis* WR120 (294 459) and *L. shawi* M8408 (296 095) (electronic supplementary material, table S4).

The *L. naiffi* LnCL223 was closest to *L. naiffi* ISQU/BR/1994/IM3936, with two differences. It clustered with MLEE zymodeme Z49 based on the correspondence between the MLSA network and previously typed zymodemes, though *L. naiffi* is associated with more zymodemes than other *Viannia*. The number and the alleles of genome-wide SNPs called using reads mapped to the *L. braziliensis* reference were
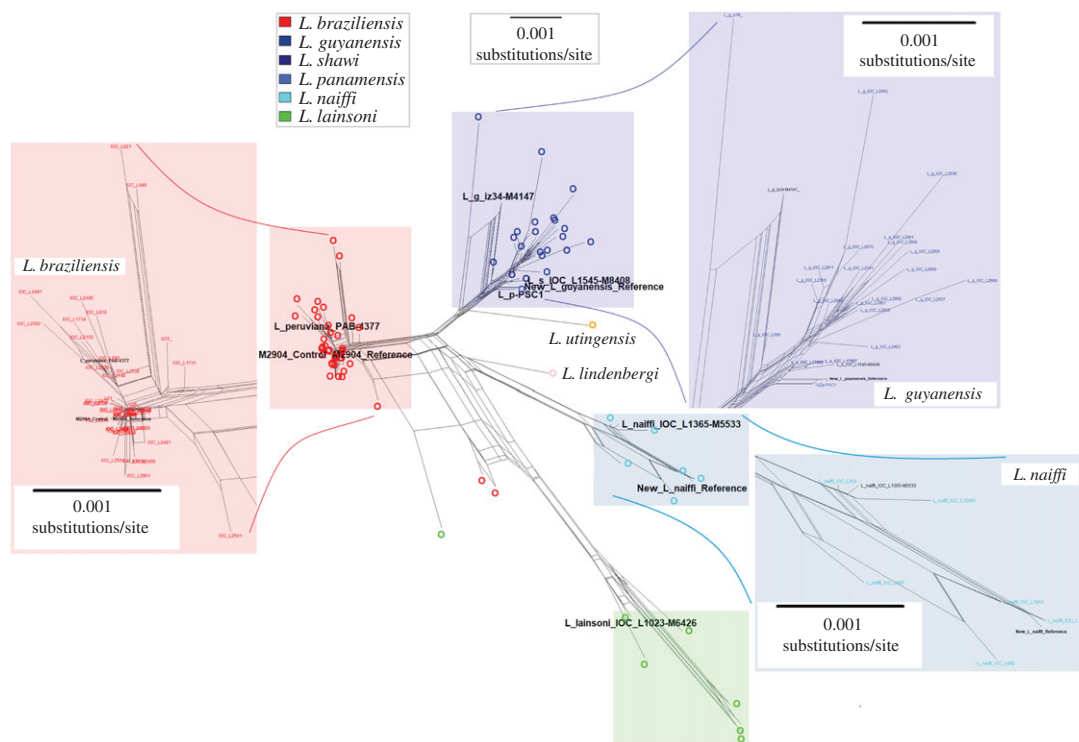
Figure 1. Middle: a neighbour-Net network of the uncorrected p-distances from concatenated 2902-base sequences from four housekeeping genes for 102 *Viannia* samples. The genes were glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), mannose phosphate isomerase (MPI) and isocitrate dehydrogenase (ICD). *Leishmania naiffi* LnCL223 (cyan) is 'New_L_naiffi_Reference' and is related to M5533 (IOC_L1365). *Leishmania guyanensis* LgCL085 (blue) is 'New_L_guyanensis_Reference' and is related to the *L. shawi* M8408 (IOC_L1545) assembly and the *L. panamensis* PSC-1 genome, but less so to *L. guyanensis* M4147 (iz34). The *L. braziliensis* M2904 reference and control are 'M2904_Reference' and 'M2904_Control', proximal to *L. peruviana* PAB-4377. *L. lainsoni* M6426 (IOC_L1023) (green), *L. utingensis* (orange) and *L. lindenbergi* (pink) are shown. The isolate names and detail for each species complex are shown by insets in red (*L. braziliensis*), dark blue (*L. guyanensis*) and light blue (*L. naiffi*). For detailed viewing, the nexus file can be downloaded at https://figshare.com/s/eecf1c6b42ac4deb6acc and high-resolution PDF at https://doi.org/10.6084/m9.figshare.5687329.

similar for *L. naiffi* (548 256) and M5533 (633 560) (electronic supplementary material, table S4) and consistent with the MLSA genetic distances.

There was no evidence of recent gene flow between these three species at any genome-wide 10 Kb segment and *L. naiffi* LnCL223 had fewer SNPs compared with *L. braziliensis* M2904 than *L. guyanensis* LgCL085 (electronic supplementary material, figure S2). Linking the MLSA network topology with previous work [56,63–65], four genetically distinct species complexes are represented by the genome-sequenced *Viannia* at present: (i) *braziliensis* including *L. peruviana*, (ii) *guyanensis* including *L. panamensis* and *L. shawi*, (iii) *naiffi* and (iv) *lainsoni* (electronic supplementary material, table S4), and the less explored (v) *lindenbergi* and (vi) *utingensis* complexes (figure 1).

2.3. Ancestral diploidy and constitutive aneuploidy in *Viannia*

The normalized chromosomal coverage of the *L. guyanensis* LgCL085 and *L. naiffi* LnCL223 reads mapped to *L. braziliensis* M2904 showed aneuploidy on a background of a diploid nuclear genome (figure 2). The coverage levels of reads for *L. peruviana* LEM1537, *L. peruviana* PAB-4377, *L. panamensis* PSC-1 and the triploid *L. braziliensis* control mapped to the M2904 reference, confirmed previous work (electronic supplementary material, figure S3), including the *L. braziliensis* control (electronic supplementary material, figure S4), and demonstrated that assemblies from short read data were sufficient to estimate chromosome copy number differences. Repeating this for *L. shawi* M8408, *L. naiffi* M5533, *L. guyanensis* M4147, *L. panamensis* WR120 and *L. lainsoni* M6426 showed that all these *Viannia* were predominantly disomic and thus diploidy was the likely ancestral state of this subgenus (figure 2).
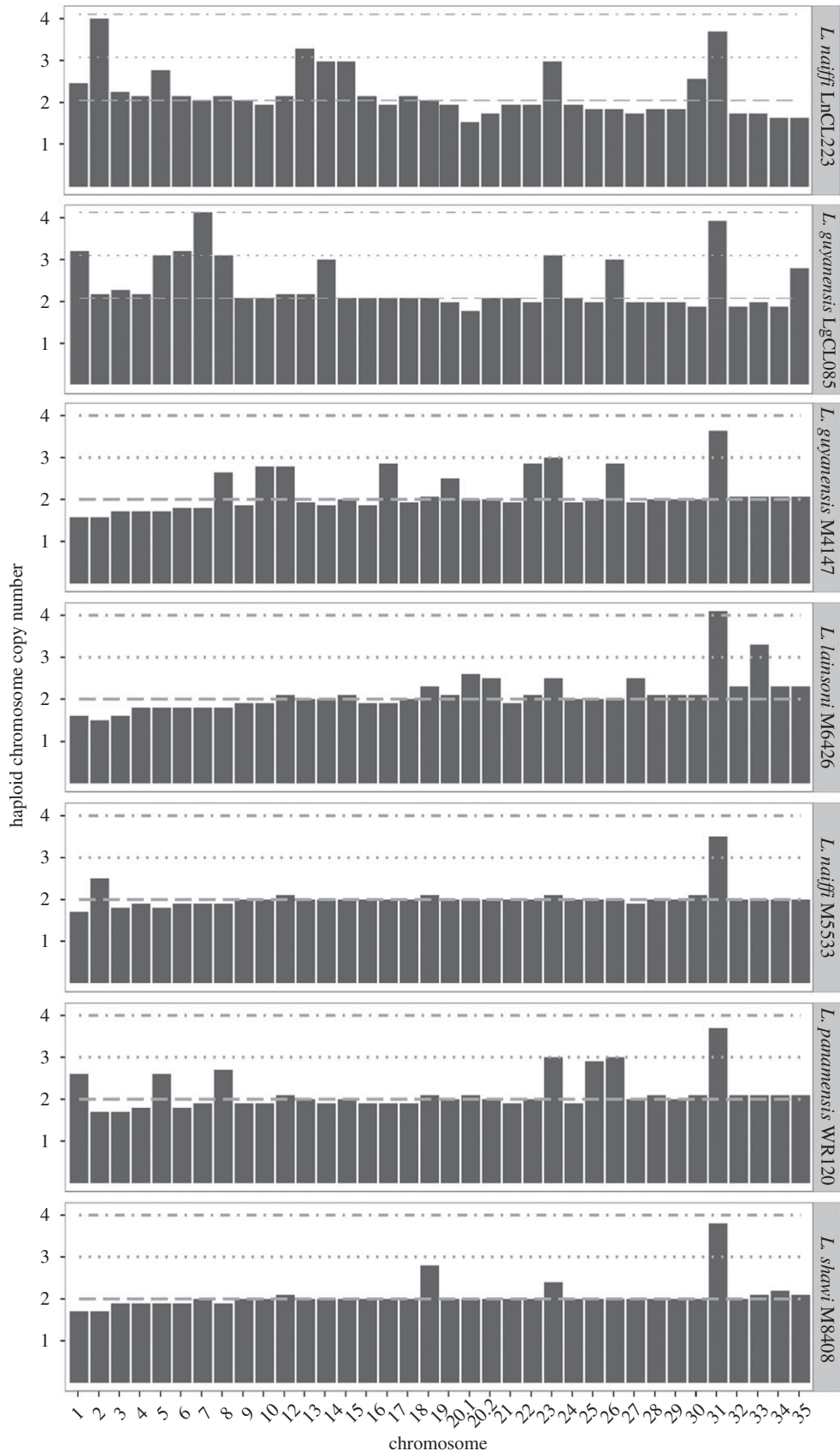
Figure 2. Normalized chromosome copy numbers of *L. naiffi* LnCL223 reads mapped to its assembly, *L. guyanensis* LgCL085 reads mapped to its assembly, and *L. guyanensis* M4147, *L. lainsoni* M6426, *L. naiffi* M5533, *L. panamensis* WR120 and *L. shawi* M8408 reads mapped to *L. braziliensis* M2904. Dashed lines indicate disomic, trisomic and tetrasomic states. Results for *L. panamensis* PSC-1 and *L. peruviana* PAB-4377 were previously published and are in electronic supplementary material, figure S3.

The somy patterns were supported by the results of mapping the reads of each sample to their own assembled genome or to the M2904 reference to produce the read depth allele frequency (RDAF) distributions from heterozygous SNPs. The majority of *L. braziliensis* M2904 control chromosomes had peaks with modes at approximately 33% and approximately 67% indicating trisomy, rather than a single peak at approximately 50% consistent with disomy (electronic supplementary material, figure S5). The RDAF distributions from reads mapped to its own assembly for *L. guyanensis* LgCL085 and *L. naiffi* LnCL223 had a mode of approximately 50% (electronic supplementary material, figure S6), including peaks indicating trisomy for LgCL085 chromosomes 13, 26 and 35 (electronic supplementary material, figure S7).

2.4. 8262 *L. naiffi* and 8376 *L. guyanensis* genes annotated

A total of 8262 genes were annotated on *L. naiffi* LnCL223: of these 8104 were protein-coding genes, 78 were tRNAs, 15 rRNA genes, four snoRNA genes, two snRNA genes and 59 pseudogenes. In total, 310 genes were on unassigned contigs (electronic supplementary material, table S3) and 8376 genes were annotated on *L. guyanensis* LgCL085: of these, 8230 were protein-coding genes, 75 tRNAs, 14 rRNA genes, four snoRNA genes, two snRNA genes and 51 pseudogenes. Six hundred and nineteen genes were on unassigned contigs.

There were 8161 genes (8001 protein coding) transferred to the control *L. braziliensis* genome, along with 76 tRNAs, two snRNA genes, four snoRNA genes, 13 rRNA genes and 65 pseudogenes (table 2). There were 7719 of the protein-coding genes (96.5%) clustered into 7244 orthologous groups (OGs), whereas 8137 of the 8375 (97.2%) protein-coding genes on the *L. braziliensis* reference grouped into 7383 OGs. This indicated that 97% of protein-coding genes in OGs were recovered, and only 2.8% (235) across 201 OGs were absent in the M2904 control, mainly hypothetical or encoded ribosomal proteins (electronic supplementary material, table S5). In the same way, we found 70 protein-coding genes (electronic supplementary material, table S6) in 62 OGs on the M2904 control absent in the published *L. braziliensis* annotation.

Few genes were present in *L. braziliensis* but absent in *L. guyanensis* LgCL085 and *L. naiffi* LnCL223. Coverage depth was used to predict each gene's haploid copy number, such that genes with haploid copy numbers at least twice the assembled copy number indicated partially assembled genes in the reference assembly. Thus, we investigated all OGs with haploid copy numbers at least twice the assembled copy number to quantity completeness of the assembly. Only 145 genes in 92 OGs on *L. guyanensis* LgCL085 (electronic supplementary material, table S7), 142 genes in 90 OGs on *L. naiffi* LnCL223 (electronic supplementary material, table S8) and 102 genes in 71 OGs (electronic supplementary material, table S9) on the *L. braziliensis* control met this criterion, indicating few unassembled genes in each assembly. One hypothetical gene (LnCL223_272760) in *L. naiffi* LnCL223 with no retrievable information had a haploid copy number of 15 (OG5_173495), whereas all other genomes examined here had zero to two copies.

2.5. A 245 Kb rearrangement akin to a minichromosome in *L. shawi* M8408

We discovered a putative minichromosome or amplification at the 3′ end of *L. shawi* M8408 chromosome 34 based on elevated coverage across a pair of inverted repeats spanning 245 Kb (figure 3). This locus spanned at least bases 1 840 001 to 1 936 232 (the end) of *L. braziliensis* M2904 chromosome 34 (electronic supplementary material, figure S8 and table S10). It was orthologous to a known 100 Kb amplification on *L. panamensis* PSC-1 chromosome 34 that was predicted to produce a minichromosome when amplified, and contained the frequently amplified LD1 (*Leishmania* DNA 1) region [66]. In contrast to the *L. panamensis* PSC-1 minichromosome, the *L. shawi* M8408 amplification was approximately 30 Kb longer and closer in length to the *L. braziliensis* M2903 245 Kb minichromosome [67].

2.6. A 45 Kb locus was amplified in most *Viannia* genomes

A 45 Kb amplification on chromosome 34 spanning a gene encoding a structural maintenance of chromosome family protein and ten hypothetical genes had between two and four copies in all samples except *L. lainsoni* M6426 (figure 3; electronic supplementary material, table S10). Using the *L. guyanensis* gene annotation, putative functions were assigned to five of the ten hypothetical genes. This duplication spanned chromosomal location 1.32–1.35 Mb in the *L. braziliensis* M2904 reference and had two additional hypothetical genes in *L. naiffi* LnCL223 (LnCL223_343280 and LnCL223_343290; figure 4).
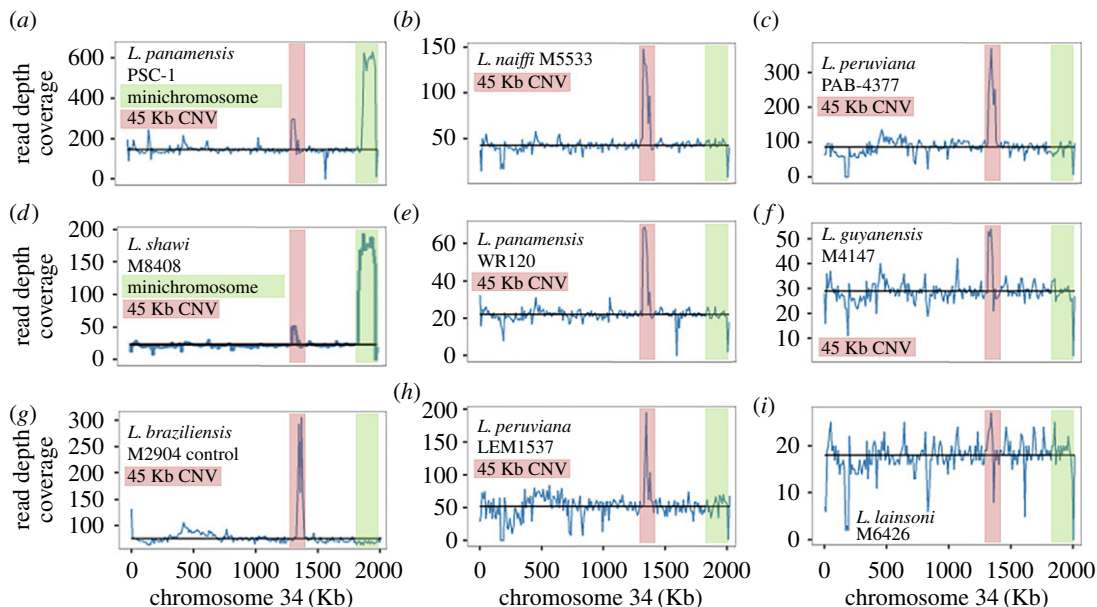
Figure 3. Read depth coverage (blue, *y*-axis) in 10 Kb blocks for reads mapped to *L. braziliensis* M2904 chromosome 34 (*x*-axis) for nine *Viannia* isolates. The black horizontal line is the median chromosome 34 coverage. *L. panamensis* PSC-1 (*a*) and *L. shawi* M8408 (*d*) showed a 3′ jump in coverage (green) consistent with an amplification of inverted repeats that could form a linear minichromosome. In addition, this pair shared a 45 Kb amplification (pink) also found in the *L. braziliensis* M2904 control (*g*), *L. naiffi* M5533 (*b*), *L. panamensis* WR120 (*e*), *L. peruviana* LEM1537 (*h*), *L. peruviana* PAB-4377 (*c*) and *L. guyanensis* M4147 (*f*). This was absent in *L. lainsoni* M6426 (*i*).
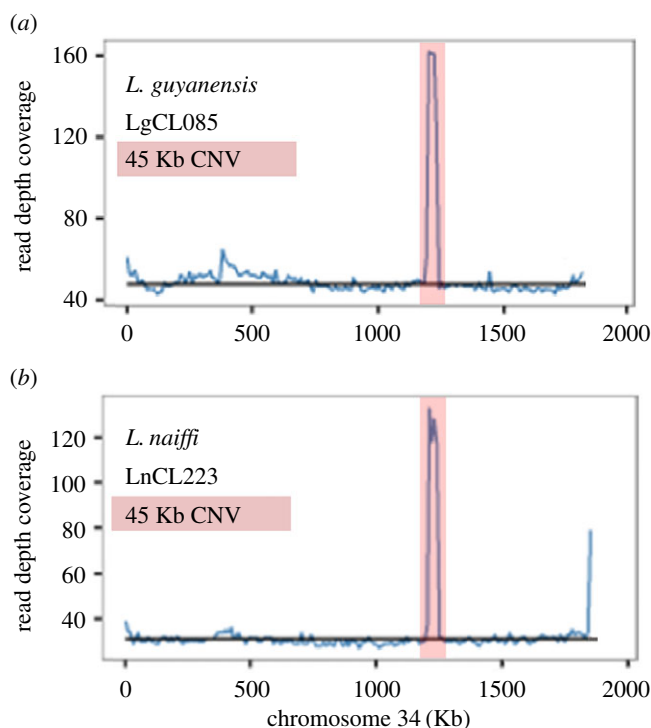

Figure 4. Median coverage (blue) in 10 Kb blocks for *L. guyanensis* LgCL085 reads mapped to its own assembled chromosome 34 (*a*) and *L. naiffi* LnCL223 reads mapped to its own assembled chromosome 34 (*b*). The black horizontal line is the median chromosome 34 read coverage. There was a 45 Kb amplification to three copies (pink) in *L. guyanensis* LgCL085 (at chromosome 34 bases 1 195 232–1 239 355, 44 123 bases in length). Similarly, there was a 45 Kb fourfold amplification (pink) in *L. naiffi* LnCL223 (at chromosome 34 bases 1 206 328–1 251 119, 44 791 bases in length). The latter encompassed two additional hypothetical genes relative to *L. guyanensis* LgCL085. Neither had evidence of a 3′ minichromosome.

2.7. Genes exclusive to *Viannia* genomes

A total of 7961 (96.7%) of the 8230 genes annotated for *L. guyanensis* LgCL085 were assigned to 7381 OGs, 7893 (97.4%) of the 8104 *L. naiffi* LnCL223 genes to 7324 OGs, and 7692 (99.3%) of the *L. panamensis* PSC-1 7748 to 7245 OGs. A total of 6835 of these OGs were shared with nine species from the *Leishmania*, *Sauroleishmania* and *Viannia* subgenera: *L. (L.) major*, *L. (L.) mexicana*, *L. (L.) donovani* (*infantum*), *L. (V.) guyanensis*, *L. (V.) naiffi*, *L. (V.) braziliensis*, *L. (V.) panamensis*, *L. (S.) adleri*, *L. (S.) tarentolae* (electronic supplementary material, table S11).

We identified 22 OGs exclusive to *Viannia* (electronic supplementary material, table S12): three OGs contained the RNAi pathway genes (DCL1, DCL2, RIF4). Another OG was the telomere-associated mobile elements (TATE) DNA transposons (OG5_132061), a dynamic feature of *Viannia* genomes [51] (electronic supplementary material, Results). Four OGs encoded a diacylglycerol kinase-like protein (OG5_133291), a nucleoside transporter (OG5_134097), a beta-tubulin/amastin (OG5_183241) and a zinc transporter (OG5_214682). The remaining 14 OGs contained hypothetical genes.

An NADH-dependent fumarate reductase gene (OG5_128620) was amplified in the *Viannia* examined here: *L. guyanensis* LgCL085 had 14 copies, *L. naiffi* LnCL223 had 16, *L. panamensis* PSC-1 had 16, *L. peruviana* PAB4377 had 23, *L. peruviana* LEM1537 had 14 and *braziliensis* M2904 had 12. This contrasted with the *Leishmania* and *Sauroleishmania* subgenera for which three to four copies had been reported for *L. infantum*, *L. mexicana*, *L. major*, *L. adleri* and *L. tarentolae* [68,69]. This gene has been implicated in enabling parasites to resist oxidative stress and potentially aiding persistence, drug resistance and metastasis [70,71].

2.8. Few species-specific genes in *L. guyanensis* LgCL085 and *L. naiffi* LnCL223

Four genes from four OGs unique to *L. naiffi* LnCL223 were identified compared with other *Leishmania* (electronic supplementary material, table S13). Of these four, hypothetical genes LnCL223_312570 and LnCL223_292920 had orthologues in *T. brucei* and *T. vivax,* respectively. The LnCL223_341350 protein product had 44–45% sequence identity with a *Leptomonas* transferase family protein, and LnCL223_352070 was a methylenetetrahydrofolate reductase (OG5_128744), but had no orthologues in the other eight *Leishmania* or five *Trypanosoma* species investigated here. *Leishmania guyanensis* LgCL085 had 31 unique genes in 30 OGs, 25 of which were on unplaced contigs. Four of the six chromosomal genes were also in *Trypanosoma* genomes, encoding two hypothetical proteins (a tuzin and a poly ADP-ribose glycohydrolase). Twenty eight of the 31 had orthologues in eukaryotes, of which three had orthologues in the free-living freshwater ciliate protozoan *Tetrahymena thermophile* (electronic supplementary material, table S14) [72].

2.9. *Leishmania guyanensis* LgCL085 and *L. naiffi* LnCL223 had over 300 gene arrays

Gene arrays are genes in the same OG with more than two haploid gene copies: they can be *cis* or *trans*. There were 327 gene arrays on *L. naiffi* LnCL223 (electronic supplementary material, table S15), 334 on *L. guyanensis* LgCL085 (electronic supplementary material, table S16) and 255 on the control *L. braziliensis* M2904 (electronic supplementary material, table S17)—half the arrays on each genome had two copies of each gene. Twenty-two of the *L. guyanensis*, 18 of the *L. naiffi* LnCL223 and 15 of the control *L. braziliensis* gene arrays contained 10+ haploid gene copies (table 3). The *L. panamensis* PSC-1 genome had approximately 400 tandem arrays, of which 71% had more than two copies. The *L. braziliensis* M2904 genome had 615 arrays corresponding to 763 OGs in OrthoMCL v5. Thus, the control genome underestimated the number of gene arrays due to either gene absence or incomplete assembly, indicating that the number of arrays on *L. naiffi* LnCL223 and *L. guyanensis* LgCL085 was underestimated.

The most expanded array on *L. guyanensis* LgCL085 contained TATE DNA transposons (OG5_132061) with 50 haploid gene copies (table 3) compared with 11 on *L. naiffi* LnCL223, 21 on the *L. braziliensis* control and 16 on *L. panamensis* PSC-1. The *L. braziliensis* M2904 assembly had 40 TATE DNA transposons, but only two were annotated on the control here, illustrating that more accurate estimates of copy number may be possible.

Leishmania naiffi LnCL223 had the highest haploid gene copy number of the M8 family metalloprotease leishmanolysin (GP63) array (OG5_126749) with 56 haploid gene copies, compared with 33 in *L. guyanensis* LgCL085, 28 in *L. panamensis* PSC-1 and 31 in *L. braziliensis* M2904. This was the sole protease-related OG amplified in all three species (electronic supplementary material, table S23). This family was not expanded in *L. peruviana* LEM1537 or PAB4377. This was consistent with previous work

Table 3. Arrays with 10 or more gene copies predicted by read depth for each species. OG stands for orthologous group. Genes in OG show the number of genes associated with that OG functional category. OG haploid copy number indicates the numbers of haploid gene copies found in each genome: B stands for the *L. braziliensis* M2904 control, G for *L. guyanensis* LgCL085 and N for *L. naiffi* LnCL223. The grey shading highlights the genes with elevated OG haploid copy numbers.

OG		genes in OG			OG haploid copy number		
ID	description	B	G	N	B	G	N
L. guyanensis LgCL085, L. naiffi LnCL223 and L. braziliensis M2904 control							
OG5_132 061	TATE DNA transposon	2	14	3	21	50	11
OG5_126 605	alpha tubulin	1	2	1	17	36	13
OG5_130 729	amastin-like surface protein	8	24	20	24	26	33
OG5_126631	elongation factor 1-alpha	1	1	1	12	18	20
OG5_126703	polyubiquitin	2	2	1	21	16	43
OG5_126558	dynein heavy chain, cytosolic	14	13	14	13	13	14
OG5_129265	pteridin transporter; folate/biopterin transporter	8	10	9	14	13	13
OG5_143904	amastin-like surface protein	5	4	6	48	12	14
OG5_126623[a]	lipophosphoglycan biosynthetic protein / glucose regulated protein 94; heat shock protein 90 / 83–1	2	2	2	11	10	12
L. guyanensis LgCL085 and L. naiffi LnCL223							
OG5_126749	GP63 leishmanolysin	4	8	9	5	33	56
OG5_126617	receptor-type adenylate cyclase	3	5	5	5	14	13
OG5_126611	beta tubulin	1	1	2	0	14	27
OG5_128620	NADH-dependent fumarate reductase	4	3	4	2	14	16
OG5_126585	kinesin K39; hypothetical protein	7	10	10	7	12	11
OG5_126573	histone H4	1	7	4	3	11	10
L. naiffi LnCL223							
OG5_173495	hypothetical protein	0	1	1	0	2	15
OG5_126568	ABC1 transporter	9	10	12	10	9	14
OG5_127342	peptidase m20/m25/m40 family protein	2	2	2	6	3	10
L. guyanensis LgCL085							
OG5_173452	tuzin	2	3	1	1	19	1
OG5_145872	ATG8/AUT7/APG8/PAZ2	1	2	1	8	19	1
OG5_143922	ATP-dependent DEAD-box helicase	1	2	0	6	17	0
OG5_148241	conserved hypothetical protein	1	1	1	0	14	1
OG5_137181	ATG8/AUT7/APG8/PAZ2	1	1	0	0	13	0
L. braziliensis M2904 control							
OG5_126588[a]	heat-shock protein hsp70; glucose-regulated protein 78	4	3	3	14	7	8
OG5_138994	tuzin	5	3	2	13	4	2
OG5_129839	phosphoglycan beta 1,3 galactosyltransferase	2	4	1	12	5	2
OG5_127067	thimet oligopeptidase; metallo-peptidase, Clan MA(E), Family M3	3	3	3	12	9	7
L. guyanensis LgCL085 and L. braziliensis control							
OG5_169610	surface antigen-like protein	1	1	0	16	11	0
OG5_127518	SLACS like gene retrotransposon element	2	3	1	18	10	1

[a]For OG5_126623 and OG5_126588, the elevated copy numbers were due to amplified heat shock protein (*hsp*) genes rather than the glucose-regulated protein (*grp*) loci, a potential limitation of OG analyses.

on *L. guyanensis* leishmanolysin [73] indicating it is a highly expressed virulence factor in promastigotes [74] affecting the survival during the initial stages of infection [74–77]. *Sauroleishmania* genomes also had high array copy numbers: 37 for *L. adleri* [69] and 84 for *L. tarentolae* (electronic supplementary material, table S12). *Leishmania* subgenus genomes had lower copy numbers, with 13 for *L. mexicana*, 15 for *L. infantum* and five for *L. major* (OG4_10176 for *L. braziliensis* M2904, *L. mexicana*, *L. infantum* and *L. major*).

A tuzin gene array (OG5_173452) had higher haploid copy numbers on *L. guyanensis* LgCL085 (19) and *L. panamensis* PSC-1 (22) compared with the two copies in *L. naiffi*, *L. mexicana*, *L. infantum*, *L. major*, *L. braziliensis*, *L. adleri* and *L. tarentolae*. Tuzins are conserved transmembrane proteins in *Trypanosoma* and *Leishmania* associated with surface glycoprotein expression [78]. They are often contiguous with δ-amastin genes, whose products are abundant cell surface transmembrane glycoproteins potentially involved in the infection or survival within macrophages. They are absent in *Crithidia* and *Leptomonas* species, who lack a vertebrate host stage [78]. Tuzins may play a role in pathogenesis [79], which may be related to leishmaniasis caused by *L. guyanensis*.

3. Discussion

3.1. *Leishmania* (*Viannia*) *guyanensis* and *L.* (*V.*) *naiffi* draft reference genomes

We assembled high-quality reference genomes for two isolates, *L.* (*Viannia*) *guyanensis* LgCL085 and *L.* (*V.*) *naiffi* LnCL223, from short read sequence libraries to illuminate genomic diversity in the *Viannia* subgenus and extend previous work [52]. This process combined the de novo assembly with a reference-guided approach using the published genome of *L. braziliensis* M2904 to assemble the *L. guyanensis* LgCL085 and *L. naiffi* LnCL223 into 35 chromosomes each (table 2). An essential feature of this process was to identify and remove contamination in the *L. guyanensis* and *L. braziliensis* M2904 libraries and to trim low-quality bases in *L. naiffi* LnCL223 to ensure that the reads used were informative and free of exogenous impurities. A second screen for contamination in unassigned contigs also removed several *L. guyanensis* LgCL085 contigs, which improved subsequent annotation and gene copy number estimates.

3.2. Genomes assembled from short reads capture aneuploidy and nearly all genes

Our strategy was tested by applying the same protocol to the *L. braziliensis* M2904 short read library, which acted as a positive control and quantified the precision of the final output. This facilitated the detection of structural variation or annotation problems, chiefly underestimated copy numbers at certain genes and the incorrect assembly of some loci that were fixed manually. The resulting genomes were largely complete: for comparison, the control *L. braziliensis* M2904 genome had only four homozygous SNPs, 97.2% of the protein-coding genes of the reference (231 were missing) and 70 additional genes missed in the reference sequence. These findings highlight scope to resolve *Leishmania* chromosomal architecture more accurately, particularly at repetitive regions and gene arrays, using longer sequencing reads and hybrid assembly approaches.

We showed that the majority of *Viannia* were diploid and had 35 chromosomes. Aneuploidy was evident for *L. guyanensis* LgCL085, *L. guyanensis* M4147, *L. naiffi* LnCL223, *L. naiffi* M5533, *L. lainsoni* M6426, *L. panamensis* WR120 and *L. shawi* M8408 as anticipated [80]. This was verified using read depth allele frequency distributions of reads mapped to *L. braziliensis* M2904 and to their own assemblies.

The *L. guyanensis* LgCL085 genome had more protein-coding genes (8230) than *L. naiffi* LnCL223 (8104). These numbers were similar to those for *L. panamensis* PSC-1 (7748) [51] and *L. braziliensis* M2904 (8357) [48]. The vast majority of protein coding gene models were computationally transferred [81] from the *L. braziliensis* M2904 reference with perfect matching, and were verified and improved manually. Both the *L. guyanensis* and *L. naiffi* reference genomes contained unassigned bin contigs, and chromosomal regions homologous to multiple chromosomal loci or containing partially collapsed gene arrays. Ninety (*L. naiffi*) and 92 (*L. guyanensis*) collapsed gene arrays were identified where haploid gene copy numbers were at least twice the assembled copy number when the reads were mapped to the assembled genomes.

3.3. A better resolution of the *Viannia* species complexes

This study illustrated that high-throughput sequencing approaches, alignment methods and annotation tools can improve the accuracy of *Leishmania* gene copy number estimates, gene organization and genome structure resolution. This yielded insights into features differentiating the isolates examined

here, including a 45 Kb duplication on chromosome 34 of most *Viannia*, variable gene repertoires across *Viannia* species, and a potential minichromosome derived from the 3′ end of *L. shawi* M8408 chromosome 34. Further work is required to investigate *L. utingensis* and *L. lindenbergi* and other potential distinct lineages [82].

Both single-gene and large-scale copy number variations (CNVs) were tolerated by all *Leishmania* genomes. *Leishmania* genomes have extensive conservation of gene content with few species-specific genes [45,48]: here, only 31 *L. guyanensis* LgCL085 and four *L. naiffi* LnCL223 species-specific genes were found. These four genes unique to *L. naiffi* LnCL223, its leishmanolysin hyper-amplification, the 31 genes only in *L. guyanensis* LgCL085 and its tuzin arrays all represent potential targets for improving species-specific typing and better disease surveillance. This is important because infections by the *Viannia* are spread by many hosts and all sources of infections need to be addressed. Immunological screening of anti-*Leishmania* antibodies could be enhanced by genetic testing to identify infections from non-endemic or rarer sources like *L. naiffi*, which has longer parasite survival rates in macrophages *in vitro* [83].

MLSA of 100 *Viannia* isolates across four genes and genome-wide diversity inferred from mapped reads indicated that *L. guyanensis* LgCL085 was closest to *L. panamensis* PSC-1 within the *L. guyanensis* species complex, but was assigned the *L. guyanensis* classification because *L. guyanensis*, *L. panamensis* and *L. shawi* were a monophyletic species complex as shown by MLSA [56], multi-locus microsatellite typing [64], *hsp70* [65], internal transcribed spacer [84,85], MLEE [86] and random amplified polymorphic DNA data [87]. Further typing of a more extensive *L. guyanensis, L. panamensis* and *L. shawi* isolate set might clarify if these are distinct species or a single genetic group.

More precise genetic screening of *Viannia* isolates is necessary to trace hybridization between species. Infection of humans, dogs and *Lu. ovallesi* with *L. guyanensis/L. braziliensis* hybrids was reported in Venezuela [40,41]. A *L. shawi/L. guyanensis* hybrid causing CL was detected in Amazonian Brazil [42], and *L. naiffi* has produced viable progeny with *L. lainsoni* [43] and *L. braziliensis* (Elisa Cupolillo 2018, unpublished data). There is extensive evidence of interbreeding among *L. braziliensis* complex isolates, including more virulent *L. braziliensis/L. peruviana* hybrids with higher survival rates within hosts *in vitro* [44].

4. Conclusion

This study highlighted the utility of genome sequencing for the identification, characterization and comparison of *Leishmania* species. We demonstrated that short reads were sufficient for assembly of most *Leishmania* genomes so that SNP, chromosome copy number, structural and somy changes can be investigated comprehensively. The *L. (V.) guyanensis* and *L. (V.) naiffi* genomes represent a further advance in refining the taxonomical complexity of the *Viannia* by illustrating their genomic characteristics and the extent to which these are shared across *Viannia* species, which will assist examining the extent to which they can hybridize. This improved understanding of *Leishmania* genomes should be used to explore the complex epidemiology of CL and MCL pathologies in the Americas and the roles of non-human reservoirs and sand flies in these processes. Future work could tackle transmission, drug resistance and pathogenesis in the *Viannia* by applying long-read high-throughput sequencing to examine broader sets of isolates, their genetic diversity, contributions to microbiome variation, and control of transcriptional dosage at gene amplifications.

5. Methods

5.1. *Leishmania guyanensis* and *L. naiffi* whole genome sequencing

Extracted DNA for *L. guyanensis* LgCL085 and *L. naiffi* LnCL223 was received from Charité University Medicine (Berlin) at the Wellcome Trust Sanger Institute on 6 February 2012. Paired-end 100 bp read Illumina HiSeq 2000 libraries were prepared for both during which *L. guyanensis* required 12 cycles of PCR. The DNA was sequenced (run 7841_5#12) on 15 (*L. guyanensis*, run 7841_5#12) and 23 (*L. naiffi*, run 7909_7#9) March 2012. The library preparation, sequencing and read quality verification were conducted as outlined previously [69]. The resulting *L. guyanensis* library contained 15 272 969 reads with a median insert size of 327.0 (NCBI accession ERX180458) and the *L. naiffi* one had 8 131 246 reads with a median insert size of 335.4 (ERX180449).

5.2. *Viannia* comparative genome, annotation and proteome files

The *L. braziliensis* reference genome (MHOM/BR/1975/M2904) was a positive control whose short reads were examined using the same methods. It was originally sequenced using an Illumina Genome Analyzer II [48] yielding 26 007 384 76 bp paired-end reads with a median insert size of 244.1 bp (ERX005631). Protein sequences were retrieved from the EMBL files using Artemis [88]. Two *L. panamensis* genomes, two *L. peruviana* genome assemblies and five 100 bp paired-end Illumina HiSeq 2000 read libraries of other *Viannia* isolates [53] were used for comparison (table 1). We included the genomes of *L. panamensis* MHOM/PA/1994/PSC-1, *L. peruviana* PAB-4377 and LEM1537 (MHOM/PE/1984/LC39), and the 100 bp Illumina HiSeq 2000 paired-end reads for each *L. peruviana* PAB-4377 (16 117 316 reads) and *L. peruviana* LEM1537 (9 378 317 reads).

5.3. Library quality control, contaminant removal and screening

Electronic supplementary material, figure S9, presents an overview of the bioinformatic steps used in this paper. Quality control of the *L. guyanensis* LgCL085, *L. naiffi* LnCL223, *L. braziliensis* M2904, the five *Viannia* libraries from [53], two *L. peruviana* libraries and *L. panamensis* PSC-1 read library was carried out using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). No corrections were required for the other libraries. An abnormal distribution of GC content per read observed as an extra GC content peak outside the normal peak for the *L. braziliensis* M2904 and *L. guyanensis* reads indicated sequence contamination that was removed (electronic supplementary material, figure S10). Two Illumina PCR primers in the *L. braziliensis* M2904 reads were removed (electronic supplementary material, table S1). Further evaluation using GC content filtering and the non-redundant nucleotide database with BLASTn [89] to remove contaminant sequences (electronic supplementary material, figure S10) with subsequent correction of read pairing arrangements reduced the initial 52 014 768 reads to 34 592 618 properly paired reads for assembly.

The M2904 reads used to assemble a control genome were used for read mapping, error correction and SNP calling, so the contamination did not affect the published reference. However, it did reduce the number of reads mapped as shown in [48] where only 84% of the *L. braziliensis* M2904 short reads mapped to the *L. braziliensis* assembly, compared with 92% of reads for *L. infantum* reads mapped to its own assembly, 93% of *L. major* reads mapped to its own assembly and 97% of *L. mexicana* reads mapped to its own assembly.

The 8 131 246 100 bp paired-end *L. naiffi* LnCL223 reads and 15 272 969 100 bp paired-end *L. guyanensis* LgCL085 reads were filtered (electronic supplementary material, table S1) in the same manner using BLASTn and the smoothness of the GC content distribution to remove putative contaminants. Low-quality bases were trimmed at the 3' end of *L. naiffi* LnCL223 reads to remove bases with a phred base quality less than 30 using Trimmomatic [90] (electronic supplementary material, table S1 and figure S11). This resulted in 13 033 846 paired-end *L. guyanensis* LgCL085 sequences and 6 989 814 paired-end *L. naiffi* LnCL223 sequences—85% and 86% of the initial reads, respectively (electronic supplementary material, table S1).

5.4. Genome evaluation, assembly and optimization

Processed reads were assembled into contigs using Velvet v1.2.09 and assemblies for all odd-numbered k-mer lengths from 21 to 75 were evaluated. The expected k-mer coverage was determined for each assembly using the mode of a k-mer coverage histogram from the velvet-estimate-exp_cov.pl script in Velvet to maximize resolution of repetitive and unique regions [57]. This suggested optimal k-mers of 61 for *L. guyanensis* LgCL085 and 43 for both *L. naiffi* LnCL223 and *L. braziliensis*, which produced assemblies with the highest N50 lengths. Each assembly was assembled with this expected coverage, and contigs were removed if their average k-mer coverage was less than half the expected coverage levels. An expected coverage of 16 and a coverage cut-off of 8 was applied to *L. naiffi* reads, an expected coverage of 19 and coverage cut-off of 8.5 to *L. guyanensis* LgCL085, and an expected coverage of 28 and coverage cut-off of 14 to *L. braziliensis*.

The assembly with the highest N50 for each was scaffolded using SSPACE [58]. In the initial assemblies, 76% of gaps in scaffolds (3592/4754) were closed in for *L. guyanensis* LgCL085, 63% (4096/6530) for *L. naiffi* LnCL223 and 67% (4834/8786) for *L. braziliensis* using Gapfiller [58]. Erroneous bases were corrected by mapping reads to the references with iCORN [91] (electronic supplementary material, figure S12). Misassemblies detected and broken using REAPR [60] were aligned to the

L. braziliensis M2904 reference (excluding the bin chromosome 00). Scaffolds were evaluated and broken at putative misassemblies detected from the fragment coverage distribution (FCD) error and regions with low coverage when the reads were mapped to both broken and unbroken options. Additionally, the *L. braziliensis* broken and unbroken scaffolds were used to verify that removing misassemblies prior to (but not after) the contiguation of scaffolds resulted in more accurate assembled chromosomes. Mis-assembled regions without a gap were replaced with N bases. REAPR corrected 444 errors in *L. naiffi* LnCL223, of which 59 were caused by low fragment coverage, 206 in *L. guyanensis* LgCL085 (eight due to low fragment coverage) and 232 in the *L. braziliensis* control (57 caused by low fragment coverage). Each assembly step improved the corrected N50 and percentage of error-free bases (EFB%) assessed using REAPR (electronic supplementary material, table S18), with the sole exception of *L. braziliensis* control at the error-correction stage, likely due to its higher heterozygosity. The EFB% was the fraction of the total bases whose reads had no mismatches, matched the expected insert length, had a small FCD error and at least five read pairs oriented in the expected direction.

Gaps > 100 bp were reduced to 100 bp and 200 bp at the edge of each unplaced scaffold was aligned with the 200 bp flanking all pseudo-chromosome gaps using BLASTn to verify that no further gaps could be closed using unplaced scaffolds. Unplaced bin scaffolds less than 1 Kb were discarded, and the resulting assemblies were visualized and compared to *L. braziliensis* using the Artemis Comparison Tool [92]. *Leishmania guyanensis* LgCL085 bin sequences with BLASTn E-values less than 1×10^{-5} and percentage identities greater than 40% to non-*Leishmania* species in non-redundant nucleotide database were removed as possible contaminants. The final scaffolds were contiguated using the *L. braziliensis* reference with ABACAS [59], unincorporated segments were labelled as unassigned 'bin' contigs, and kDNA contigs were annotated (electronic supplementary material).

5.5. Phylogenomic multi-locus sequencing analysis characterization

An MLSA approach was adopted to verify the *Leishmania* species identity using four housekeeping genes: glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), mannose phosphate isomerase (MPI) and isocitrate dehydrogenase (ICD). Orthologues from other genomes and assemblies were obtained using BLASTn alignment with thresholds of E-value less than 0.05 and percentage identity greater than 70%. *Leishmania peruviana* LEM-1537 genome had gaps at the MPI and 6PGD genes and was excluded. The four housekeeping genes spanning 2902 sites were concatenated in the order G6PD, 6PGD, MPI and ICD, and aligned using Clustal Omega v1.1 to create a Neighbour-Net network of uncorrected *p*-distances using SplitsTree v4.13.1.

5.6. Genome annotation and manual curation

Annotation of the *L. guyanensis* LgCL085, *L. naiffi* LnCL223 and *L. braziliensis* control genomes was completed using Companion [80] using *L. braziliensis* M2904 as the reference as outlined previously [69], including manual checking and correction of gene models. A control run with the *L. braziliensis* M2904 reference genome using itself as a reference was performed. In *L. naiffi* LnCL223, 13 genes and one pseudogene were removed because they overlapped existing superior gene models that had improved sequence identity with *L. braziliensis* M2904 orthologues. Forty-six protein-coding genes were also manually added. Thirty-four protein-coding genes on *L. guyanensis* LgCL085 were manually added and one protein coding gene was removed. Two hundred and sixty-nine gene models on *L. naiffi* LnCL223 and 198 on *L. guyanensis* with multiple joins mainly caused by the presence of short gaps were corrected by extending the gene model across the gap where the gap length was known (less than 100 bp). If the gap length was unknown (greater than 100 bp), the gene was extended to the nearest start or stop codon.

5.7. Measuring ploidy, chromosome copy numbers and copy number variations

By mapping the reads with SMALT v5.7 (www.sanger.ac.uk/resources/software/smalt/) to *L. braziliensis* M2904, the coverage at each site was determined to quantify the chromosome copy numbers and RDAF distributions at heterozygous SNPs as per previous work [69]. The RDAF distribution was based on the coverage level of each allele at heterozygous SNPs and this feature differed across chromosomes for each isolate (electronic supplementary material, Results). The median coverage per chromosome was obtained, and the median of the 35 values combined with the RDAF distribution mode approximating 50% indicated that all isolates examined here were mostly diploid (except the triploid *L. braziliensis* M2904). These were visualized with R packages ggplot2 and gridExtra.

After PCR duplicate removal, the mapped reads were used to detect CNVs across genes or within non-overlapping 10 Kb blocks for all chromosomes and bin contigs using the median depth values normalized by the median of the chromosome (or bin contig). Loci with a copy number ≥ 2 were analysed for *L. naiffi* LnCL223, *L. guyanensis* LgCL085 and the *L. braziliensis* control using their reads mapped to their own assembly. This was also repeated for reads mapped to the *L. braziliensis* M2904 reference for *L. guyanensis* M4147, *L. naiffi* M5533, *L. shawi* M8408, *L. lainsoni* M6426, *L. panamensis* WR120, *L. panamensis* PSC-1, *L. peruviana* LEM1537 and *L. peruviana* PAB-4377. *Leishmania panamensis* PSC-1 reads were mapped to its own reference genome to verify that we could find previously identified amplified loci, and we mapped *L. panamensis* WR120 to it so that CNVs shared by both *L. panamensis* could be obtained. The BAM files of *L. naiffi* LnCL223, *L. guyanensis* LgCL085 and *L. braziliensis* M2904 reads mapped to its own assembly were visualized in Artemis to confirm and refine the boundaries of amplified loci.

5.8. Identification of orthologous groups and gene arrays

Protein-coding genes from *L. guyanensis* LgCL085, *L. naiffi* LnCL223 and the *L. braziliensis* M2904 control genome were produced from the EMBL files for each genome and these were submitted to the ORTHOMCLdb v5 webserver [93] to identify OGs. 11 825 OGs with associated gene IDs in at least one of four *Leishmania* species (*L. major* strain Friedlin, *L. infantum*, *L. braziliensis* and *L. mexicana*) or five *Trypanosoma* species (*T. vivax, T. brucei, T. brucei gambiense, T. cruzi* strain CL Brener and *T. congolense*) were retrieved from the OrthoMCL database and compared with OGs for each genome. The copy number of each OG was estimated by summing the haploid copy number of each gene in the OG. Gene arrays in each genome were identified by finding all OGs with haploid copy number ≥ 2. Large arrays (greater than or equal to 10 gene copies) were examined and arrays with unassembled gene copies were identified by finding those with haploid gene copy number at least twice the assembled gene number.

5.9. Single-nucleotide polymorphism screening and detection

The filtered reads with Smalt as mapped above were used for calling SNPs using Samtools Pileup v0.1.11 and Mpileup v0.1.18 and quality-filtered with Vcftools v0.1.12b and Bcftools v0.1.17-dev as previously [69] such that SNPs called by both Pileup and Mpileup post-screening were considered valid. These SNPs all had: base quality greater than 25; mapping quality greater than 30; SNP quality greater than 30; a non-reference RDAF greater than 0.1; forward–reverse read coverage ratios greater than 0.1 and less than 0.9; five or more reads; 2+ forward reads; and 2+ reverse reads. Low-quality and repetitive regions of the assemblies were identified and variants in these regions were masked as outlined elsewhere [69]. SNPs were classed as homozygous for an alternative allele to the reference if their RDAF ≥ 0.85 and heterozygous if it was greater than 0.1 and less than 0.85.

The high level of nucleotide accuracy of the assembled genomes was indicated by the low rate of homozygous SNPs when the reads mapped to its own assembly (50 for *L. naiffi* LnCL223, 12 for *L. guyanensis* LgCL085, 68 for the *L. braziliensis* reference and four for the *L. braziliensis* control). Likewise, the numbers and alleles of heterozygous SNPs for the *L. braziliensis* control (25 474) matched that for the reference (25 975), suggesting that the 705 (*L. naiffi* LnCL223) and 14 739 (*L. guyanensis* LgCL085) heterozygous SNPs were accurate. The difference in homozygous and heterozygous SNP rates for *L. braziliensis* here versus the original 2011 study [48] was likely due to differing methodology. The genetic divergence of *L. naiffi* LnCL223 and *L. guyanensis* LgCL085 compared with *L. braziliensis* was quantified using the density of heterozygous and homozygous SNPs per 10 Kb non-overlapping window on each chromosome, visualized using Bedtools.

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