Exploring Host-Specificity: Untangling the relationship between Leishmania (Viannia) species and its endosymbiont Leishmania RNA Virus 1

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Article

Exploring Host-Specificity: Untangling the Relationship between *Leishmania* (*Viannia*) Species and Its Endosymbiont *Leishmania* RNA Virus 1

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Abstract: A relevant aspect in the epidemiology of Tegumentary Leishmaniasis (TL) is the *Leishmania* parasites carrying a viral endosymbiont *Leishmania RNA Virus* 1 (LRV1), a dsRNA virus. *Leishmania* parasites carrying LRV1 are prone to causing more severe TL symptoms, increasing the likelihood of unfavorable clinical outcomes. LRV1 has been observed in cultured strains of five *L.* (*Viannia*) species, and host specificity was suggested when studying LRV1 from *L. braziliensis* and *L. guyanensis* strains. The coevolution hypothesis of LRV1 and *Leishmania* was based on phylogenetic analyses, implying an association between LRV1 genotypes, *Leishmania* species, and their geographic origins. This study aimed to investigate LRV1 specificity to *Leishmania* (*Viannia*) species hosts by analyzing LRV1 from *L.* (*Viannia*) species. To this end, LRV1 was screened in *L.* (*Viannia*) species other than *L. braziliensis* or *L. guyanensis*, and it was detected in 11 out of 15 *L. naiffi* and in 2 out of 4 *L. shawi*. Phylogenetic analyses based on partial LRV1 genomic sequencing supported the hypothesis of host specificity, as LRV1 clustered according to their respective *Leishmania* species hosts. These findings underscore the importance of investigating *Leishmania* and LRV1 coevolution and its impact on *Leishmania* (*Viannia*) species dispersion and pathogenesis in the American Continent.

Keywords: *Leishmania* (*Viannia*); *Leishmania* RNA Virus 1; phylogeny; host-specificity; coevolution

1. Introduction

Cutaneous leishmaniasis (CL) is a neglected disease that mainly affects the poorest populations in developing countries [1]. Brazil is the country with higher incidence of CL in the American Continent, where the disease is associated to at least seven species, one of the subgenus *Leishmania*: *L.* (*Leishmania*) *amazonensis* and the others of the subgenus *Viannia*: *L.* (*Viannia*) *guyanensis*, *L.* (*Viannia*) *braziliensis*, *L.* (*Viannia*) *shawi*, *L.* (*Viannia*) *lainsoni*, *L.* (*Viannia*) *naiffi* and *L.* (*Viannia*) *lindenbergi* [2].

A relevant aspect in the epidemiology of CL in Brazil, but not only observed in this region, is the circulation of parasites carrying a viral endosymbiont denominated *Leishmania* RNA Virus 1 (LRV1), found in *L.* (*Viannia*) sp. [3,4]. The outcome and severity of CL might be correlated with several factors, including the *Leishmania* species involved and the presence of LRV1 [4–10].

It was shown in experimental infections that *Leishmania* parasites carrying LRV1 are predisposed to aggravate the course of infection, leading to the mucosal form of leishmaniasis. The role of LRV1 in the exacerbation of the infection relies on Type I interferon (Type I IFNs) production by macrophages and in vivo signaling [11,12]. It is known that the virus can subvert innate immunity by acting as a negative regulator of the NLRP3 inflammasome, favoring parasites' survival and disease chronification [13]. Furthermore, it was described that the presence of LRV1 increases the risk of mucosal development in human patients by almost three times compared to individuals who present CL without virus detection [4]. However, the association of LRV1 and mucosal manifestation was not observed in studies evaluating other cohorts, but association with therapeutic failure was

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demonstrated [14–16]. Differences in the parasites circulating in the different regions evaluated and/or in the LRV1 infecting these parasites are possible explanations for the observed differences among some studies.

Previous studies with the complete genome sequences of LRV1 and sequences obtained from a phylogenetically informative region of the viral genome from *L.* (*V.*) *guyanensis* and *L.* (*V.*) *braziliensis* showed evidence of host-specificity in *L.* (*Viannia*) species-LRV1 interaction, clustering LRV1 sequences according to their *Leishmania* species host [17,18]. LRV1 from one *L.* (*V.*) *shawi* strain was also analyzed, clustering close to LRV1 from *L. guyanensis*, mirroring what is observed in the phylogenetic relationship of these two *Leishmania* species [18]. Although LRV1 was detected already in cultivated strains of *L. naiffi* and *L. panamensis* [15,19–21], there is no information on phylogenetic relationship of viruses from these species comparing to LRV1 from other species. LRV1 was already associated with human infections caused by *L. lainsoni* [4] and *L. peruviana* [22], but not in cultivated strains from these species.

Due to the significance of the *Leishmania*-LRV1 symbiosis in the epidemiology of cutaneous and mucosal leishmaniasis, it is crucial to gain a comprehensive understanding of the viruses's diversity and spread within parasite populations. Thus, our study aimed to examine the presence of LRV1 in various strains of Brazilian *L.* (*Viannia*) species, excluding *L. braziliensis* and *L. guyanensis*. LRV1 was not detected in different strains of three species with apparently restricted circulation in the Amazon region, and common in areas with reported circulation of LRV1: *L. lainsoni*, *L. lindenbergi* and *L. utingensis*. However, our findings revealed the presence of LRV1 in cultures identified as *L. naiffi* and *L. shawi*, confirming previous observations. Of note, phylogenetic analyses indicates *L. naiffi*, along with *L. lainsoni*, as the most divergent species of the *Viannia* subgenus [23]. Patients infected with *L. naiffi* experienced a poor response to antimonial or pentamidine therapy, indicating that the species could resist to first-line treatment. The presence of LRV1 in *L. naiffi* raised the possibility that this virus could increase *Leishmania* spp virulence and thereby influence therapeutic failure [15,24]. *L.* (*V.*) *shawi* is a species closely related to *L. guyanensis* [23] dispersed mainly in the Amazon region [25]. This species is responsible for CL, usually found as a single lesion, but cases of multiple lesions, clearly due to metastases, are occasionally seen [26]. A recent report showed the first *L. shawi* infection in mucosal secretion in Brazil, that represents a warning for the possible association between *L. (V.) shawi* and mucosal lesions [27]. In order to contribute with characterization of LRV1 from different *L.* (*Viannia*) species and strains and further investigate host-specificity in this combination, we conducted a comparative analyses of publicly available sequences of *L. braziliensis* and *L. guyanensis* to newly obtained LRV1 from both *L. naiffi* and *L. shawi*, reported herein.

2. Materials and Methods

2.1. Leishmania Culture

Strains from *L. lainsoni* (n=4), *L. lindenbergi* (n=3), *L. naiffi* (n= 18), *L. shawi* (n=4) and *L. utingensis* (n= 1), from different geographic regions, available at *Leishmania* Collection of Fiocruz (CLIOC), were screened (Table S1). Parasites were grown in NNN (Novy-MacNeal-Nicolle), and Schneider medium supplemented with 20% fetal bovine serum and incubated in a BOD (biochemical oxygen demand) incubator at 25º C until reaching the average amount of $5x10^5$ parasites.

Cultures were centrifuged at 1400 *g* for 10 minutes at 4°C, resuspended in DNA/RNA Shield™ (Zymo Research Corporation – Irvine, CA/USA), and stored at -20°C until RNA extraction.

2.2. RNA Extraction and cDNA Synthesis

RNA of the strains was extracted using the TRIzol® reagent (Invitrogen® – USA). RNA concentration and purity were determined using a NanoDrop® 2000 spectrophotometer (Thermo Scientific™ – Wilmington, MA/USA). Reverse transcription was performed using 2µg of RNA through the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™ – Foster City, CA/USA), following the manufacturer's recommendations.

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2.3. LRV1 Detection

To determine which strains deposited in the Fiocruz *Leishmania* Collection (CLIOC) were positive for LRV1, a screening protocol was performed using the primers LRV F - 5′-ATGCCTAAGAGTTTGGATTCG- 3′ and LRV R - 5′- ACAACCAGACGATTGCTGTG – 3′ [4] (Figure 1). For the HSP70 fragment amplification, which was used as an endogenous control of all RT-PCR reactions, primers Hsp70cF 5- GGACGAGATCGAGCGCATGGT-3′ and Hsp70cR 5′-TCCTTCGACGCCTCCTGGTTG-3′ were used [28]. For both reactions a final volume of 50 µl was used: 10X Buffer (1X), MgCl2 (1.5mM), dNTPs (0.2Mm), Primer F (0.2µM), Primer R (0.2mM), Taq Platinum (1.0U/µl) and 1 µl of cDNA. RT-PCR was performed at 94ºC for 2 minutes, 35 cycles at 94ºC for 30 seconds, 58ºC for 30 seconds and 72ºC for 45 seconds, with a final extension phase at 72ºC for 5 minutes.

Positive strains for LRV1 (n = 12; 11 *L. naiffi* and 1 *L. shawi*) were submitted to another PCR reaction that aimed to amplify a fragment of approximately 850 base pairs for LRV1, which is a phylogenetically informative fragment. For this, the primers LRV1 F orf1 5'-ATGCCTAAGAGTTTGGATTCG-3' and LRV1 R orf2 5'-AATCAATTTTCCCAGTCATGC-3' [18] were used, amplifying a fragment corresponding to part of the *orf1* region and the beginning of the *orf2* region, including the portion responsible for encoding the viral capsid protein (Figure 1). A final volume of 50 µl was used: 10X Buffer (1X), MgCl2 (1.5Mm), dNTPs (0.2Mm), Primer F (0.2µM), Primer Rg (0.2mM), Taq Platinum (1.0U /µl) and 3 µl of cDNA. RT-PCR was performed at 95ºC for 2 minutes, followed by 35 cycles at 95ºC for 30 seconds, 57ºC for 45 seconds and 72ºC for 45 seconds, with a final extension phase at 72ºC for 5 minutes. All RT-PCR products were stained with GelRed® (Biotium – Fremont, CA/USA) and visualized in a 2% agarose gel.

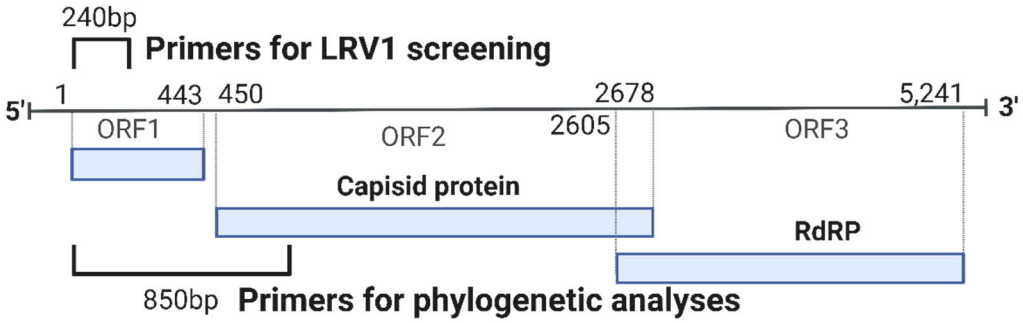

Figure 1. Schematic representation of the genome of LRV1 showing the annealing region for each primer used for screening (240bp) and phylogenetic analyses (850bp).

2.4. Sequencing

For sequencing, 45 µl of RT-PCR products were purified using the Wizard® SV Gel and RT-PCR Clean-Up System kit (Promega – Madison, WI/USA), following the manufacturer's recommendations, with a final elution volume of 45 µl. Sequencing was performed with the same primers for RT-PCR amplification on the Fiocruz DNA Sequencing Platform – Rio de Janeiro (RPT01A), where the technique employed was Sanger sequencing.

2.5. Analyses of LRV1 Sequences

The consensus sequences were created in the BioEdit program [29]. Then, the inference of the best model to build the phylogenetic tree was performed in MEGAX [30] software, where the Tamura 3-parameter model with gamma distribution parameter (G) and invariable sites (I) was the one presenting the lowest BIC (Bayesian Information Criterion) score. The tree was constructed using the maximum likelihood method employing 10.000 replicates (bootstrap).

In addition, to increase the chances of finding the most parsimonious connections, networks using NeighborNet were built by the SplitsTree program [31]. For that, we use the MEGAX function

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to exclude sites with missing/ambiguous data and gaps of the sequences, which resulted in sequences that were 509 nucleotides long and were common across groups of each species.

The analyzed sequences correspond to LRV1 detected in *L. naiffi* and *L. shawi* strains isolated from sandflies and humans from Amazonian regions, already deposited at GenBank (Table 1). The analyses was conducted comparing the new LRV1 sequences to those already available (Table S1).

Table 1. *Leishmania* (*Viannia*) strains from different species screened for the presence of the viral endosymbiont LRV1 and information on accession for LRV1 sequences obtained for the positive strains.

Leishmania Strain ID (IOCL)	Parasite Species	*Leishmania* International Code	Geographic Origin (City, State)	Sequence Length	GenBank Accession Number
854	*L. naiffi*	ISQU/BR/1985/IM2264	Cachoeira Porteira, Pará	759	OR147337
3007	*L. naiffi*	MHOM/BR/2003/IRCF	Manaus, Amazonas	701	OQ106956
3228	*L. naiffi*	MHOM/BR/2010/MS	Manaus, Amazonas	699	OQ106955
3316	*L. naiffi*	MHOM/BR/2011/58-AMS	Mojuí dos Campos, Pará	706	OQ106958
3515	*L. naiffi*	MHOM/BR/2013/49UAS	Manaus, Amazonas	709	OQ106952
3516	*L. naiffi*	MHOM/BR/2013/63DDL	Manaus, Amazonas	799	OQ106959
3517	*L. naiffi*	MHOM/BR/2013/65HCC	Manaus, Amazonas	710	OR147338
3518	*L. naiffi*	MHOM/BR/2013/66CPS	Manaus, Amazonas	679	OQ106951
3519	*L. naiffi*	MHOM/BR/2013/51FRS	Manaus, Amazonas	660	OQ106950
3520	*L. naiffi*	MHOM/BR/2013/62FJFM	Manaus, Amazonas	632	OQ106954
3531	*L. naiffi*	MHOM/BR/2013/56EGP	Manaus, Amazonas	708	OQ106953
991	*L. naiffi*	MDAS/BR/1987/IM3307	São Félix do Xingu, Pará	-	---
992	*L. naiffi*	MDAS/BR/1987/IM3280	São Félix do Xingu, Pará	-	---
993	*L. naiffi*	MDAS/BR/1987/IM3281	São Félix do Xingu, Pará	-	---
1123	*L. naiffi*	MHOM/BR/1986/IM2736	Manaus, Amazonas	-	---
1365	*L. naiffi*	MDAS/BR/1979/M5533	Almeirim, Pará	-	---
3310	*L. naiffi*	MHOM/BR/2011/S50	Santarém, Pará	-	---
3541	*L. naiffi*	MHOM/BR/2014/61AAM	Manaus, Amazonas	-	---
1594	*L. shawi*	MHOM/BR/1990/IM2842	Manaus, Amazonas	781	OQ106957
1067	*L. shawi*	IWHI/BR/1985/IM2324	Tucuruí, Pará	-	---
1068	*L. shawi*	IWHI/BR/1985/IM2326	Tucuruí, Pará	-	---
3481	*L. shawi*	MHOM/BR/2013/18	Manaus, Amazonas	-	---
1023	*L. lainsoni*	MHOM/BR/1981/M6426	Benevides, Pará	-	---
1266	*L. lainsoni*	MCUN/BR/1983/IM1721	Tucuruí, Pará	-	---
2497	*L. lainsoni*	MHOM/BR/2002/NMT-RBO 027P	Rio Branco, Acre	-	---
3398	*L. lainsoni*	MHOM/BR/2012/AP60A	Porto Velho, Rondônia	-	---
2690	*L. lindenbergi*	MHOM/BR/1966/M15733	Belém, Pará	-	---
3645	*L. lindenbergi*	MHOM/BR/2015/RO514	Porto Velho, Rondônia	-	---
3746	*L. lindenbergi*	MHOM/BR/2014/RO285	Porto Velho, Rondônia	-	---
2689	*L. utingensis*	ITUB/BR/1977/M4964	Belém, Pará	-	---

In gray are highlighted the negative strains for LRV1. MHOM = Mammalia, *Homo sapiens*; ISQU = Insecta, *Lutzomyia squamiventris*; MDAS = Mammalia, *Dasypus sp.*; IWHI = Insecta, *Lutzomyia whitmani*.

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3. Results

3.1. LRV1 Was Not Detected in all L. (Viannia) Species Analyzed, but Was Frequent in L. naiffi Strains

All RT-PCR reactions were performed with promastigotes of available strains identified as *L. lainsoni*, *L. lindenbergi*, *L. naiffi*, *L. shawi*, and *L. utingensis*, in addition to *L. guyanensis* (MHOM/BR/1975/M4147) and *L. braziliensis* (MHOM/BR/1975/M2903) strains, which were used as positive and negative controls for the experiments, respectively. In total, 4 *L. lainsoni*, 3 *L. lindenbergi*, 18 *L. naiffi*, 4 *L. shawi* and 1 *L. utingensis* strains were submitted to screening protocol for LRV1 detection. Of these, 7 *L. naiffi*, 3 *L. shawi*, and all *L. lainsoni*, *L. lindenbergi* and *L. utingensis* strains were negative for the virus. LRV1 has been detected in 11 *L. naiffi* strains (61%) and in one *L. shawi* analyzed, in addition to the strain of this species that had already been described presenting LRV1 [18]. After quality checking, sequences ranging from 632nt to 799nt (Table 1) were employed in the analyses described below.

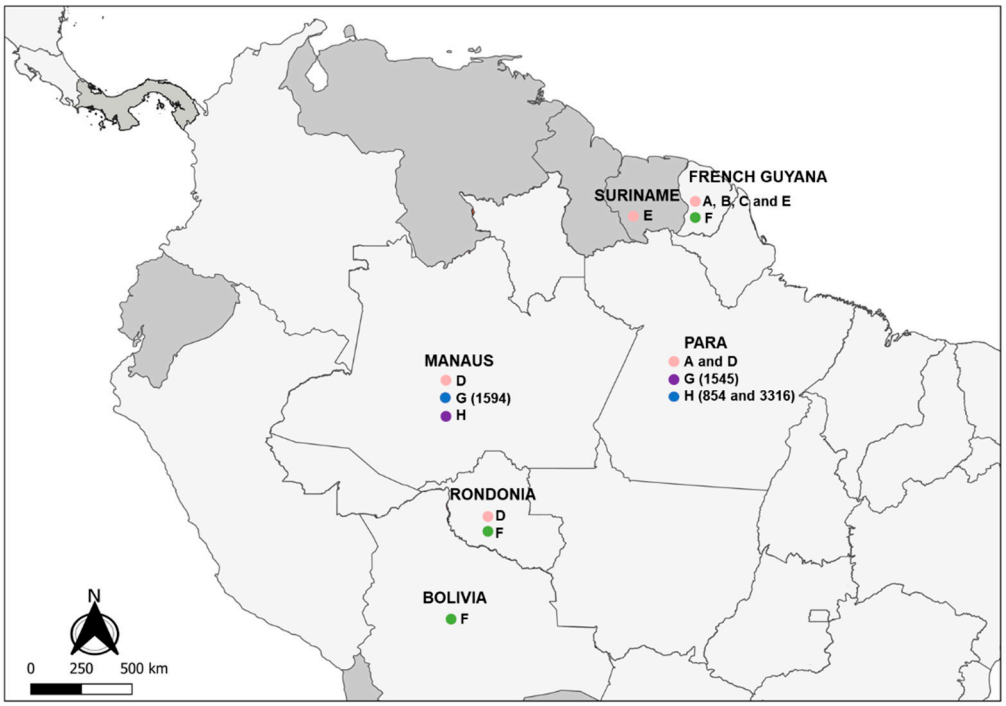

Figure 2. Partial map of South and Central America indicating the countries and Brazilian states where LRV1 has been detected in *Leishmania* (*Viannia*) spp. analyzed in this study. Colored circles refer to species presenting LRV1: pink= *L. guyanensis*; green = *L. braziliensis*; purple = *L. shawi* and blue = *L. naiffi*. Letters (A to H) indicate groups defined accordingly the phylogenetic analyses (Figure 3); numbers indicate specific strains (Table 1) in such group.

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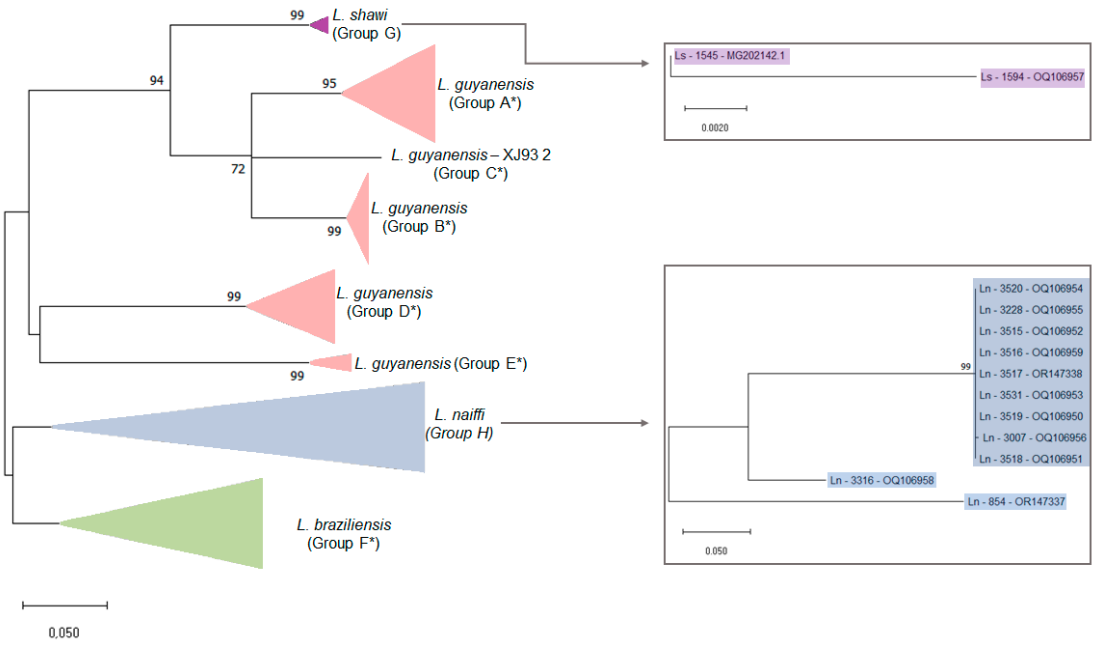

Figure 3. Maximum Likelihood phylogenetic tree of *Leishmania* RNA Virus 1 found in *Leishmania* (*Viannia*) species. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model [32]. The tree with the highest log likelihood (-4303.97) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.8242)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 28.96% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analyses involved 59 nucleotide sequences. There were a total of 442 positions in the final dataset. Ls = *L. shawi*; and Ln = *L. naiffi*. For details of each strains see Tables 1 and S1. *Groups defined following the proposal of Tirera et al. [17]; Groups G and H were defined in the present study.

3.2. *Variability of LRV1 Diversity across Leishmania Host Species*

In addition to the sequences obtained in the present study, another 47 LRV1 sequences from *L. braziliensis* (n=11), *L. guyanensis* (n=35) and *L. shawi* (n=1) strains available on GenBank were included in the analyses (Table S1), corresponding to sequences reported from studies in French Guiana [17], Bolivia [32,33] and Brazil [18,32,33]. Considering the LRV1 sequences analyzed, a higher diversity was observed within LRV1 from *L. guyanensis*, followed by LRV1 from *L. braziliensis*, *L. naiffi*, and *L. shawi* (Table 2). Of note, few differences were observed between the two LRV1 sequences from *L. shawi* analyzed despite being isolated from different hosts, in different regions. The high similarity among LRV1 sequences from *L. naiffi* is also interesting, since the two most divergent sequences correspond to strains from Pará. All the others (n=9), very close related, from Manaus (Amazonas, Brazil), despite of one strain from this group, IOCL 3007, was isolated ten years before the others from a patient who contracted the infection in Manaus, but it was attended in a referral center in Rio de Janeiro. The diversity of LRV1 from *L. guyanensis* is also depicted in the phylogenetic analyses, clustering these sequences in distinct groups (Figure 1) as previously demonstrated.

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Table 2. Estimates of average evolutionary divergence, based on number of differences and Tamura-3-parameter, over sequence pairs within mean groups.

	L. guyanensis	*L. braziliensis*	*L. naiffi*	*L. shawi*
Number of differences	448.96	295.15	31.75	7
Tamura-3-parameter model	0.18	0.16	0.06	0.01

3.3. Higher Similarity Is Observed for LRV1 Sequences among Closely Related Leishmania Species

Putting together the results obtained in this study and sequences of LRV1 publicly available, it was possible to compare LRV1 from four different species: *L. guyanensis*, *L. braziliensis*, *L. naiffi* and *L. shawi*. The analyses between group mean distance reveals that *L. shawi* and *L. guyanensis* exhibit lower distances, particularly within Group A, B, and C (Table S2). However, there is considerable diversity among LRV1 from *L. guyanensis*, with *L. braziliensis* and *L. naiffi* showing a significant distance between them (Table 3).

Table 3. Estimates of evolutionary divergence over sequence pairs between groups based on Tamura 3-parameter model (below diagonal) and on number of differences (above diagonal).

	L. naiffi	*L. shawi*	*L. guyanensis*	*L. braziliensis*
L. naiffi	-	109	109	100
L. shawi	0.208	-	88	114
L. guyanensis	0.214	0.147	-	550
L. braziliensis	0.190	0.204	0.260	-

3.4. Host-Specificity Is Clear Observed in the LRV1-L. (Viannia) Species Relationship

To investigate phylogenetic relationships between LRV1 from *L. naiffi* and *L. shawi*, along with other 47 LRV1 sequences available (from *L. guyanensis*, *L. braziliensis* and only one *L. shawi*), a Maximum Likelihood (ML) based tree (Figure 3) and phylogenetic network (Figure 4) were constructed. The Maximum Likelihood tree shows several groups with strong support (higher than 70%) and the results are in agreement with previously study [17,18], indicating LRV1 from *L. guyanensis* grouping in a cluster composed by different subclusters (here named accordingly to Tirera et al. [17]), and only one cluster for LRV1 from *L. braziliensis*, despite of the variability observed within this group (Figure 3 and Table 2). The new LRV1 sequence obtained for *L. shawi* clustered together with the other sequence available, keeping a close relationship to the *L. guyanensis* cluster (Figure 3). Although a clear group is observed for LRV1 sequences from *L. naiffi*, two strains presented very divergent LRV1 sequences and grouped with other LRV1 from *L. naiffi* with low bootstrap support, but the most parsimonious connections for these two sequences were observed with LRV1 from *L. naiffi* (Figure 4). As mentioned before, these two strains are from Pará and all the others are from another, but the same endemic region.

The phylogenetic network shows sequences of clustering similar to those observed with the ML tree. The network however suggests a structure of a common ancestor for all LRV1 (Figure 4). The structure could be the result of low amounts of recombination between sequences of the network, however, representing a bottleneck, or maybe simply due to divergent phylogenetic signals.

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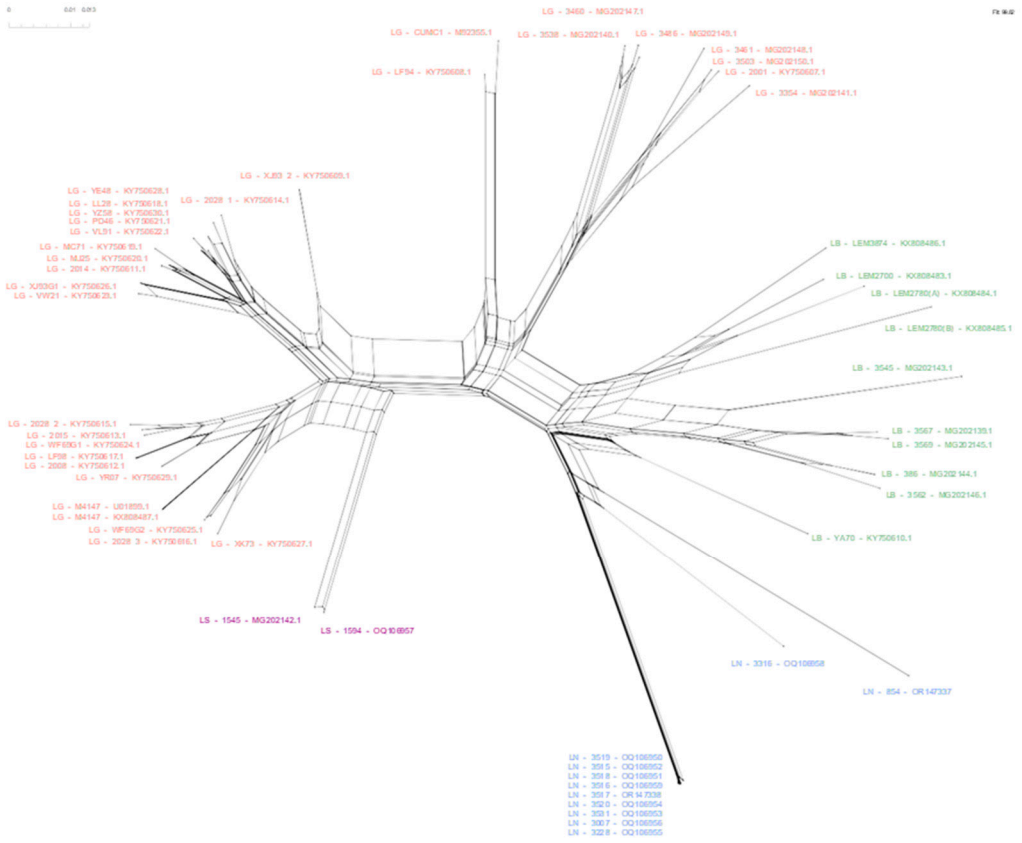

Figure 4. NeighborNet showing the relationship among LRV1 sequences from different *Leishmania* (*Viannia*) species. The network was computed using SplitsTree software. Text colors refer to each group analyzed. Pink = *L. guyanensis*; purple = *L. shawi*; blue = *L. naiffi* and green = *L. braziliensis*. Strains 854 and 3316, belonging to the species of *L. naiffi*, presented a divergent profile from the other strains of the same group.

4. Discussion

Virus-like particles were demonstrated in *Leishmania* parasites in the late 1970s [34] but the first molecular description of *Leishmania* RNA Virus was in 1988, for viruses found in the cytoplasm of a *L.* (*V.*) *guyanensis* strain [35]. Ever since LRV1 has been detected in clinical isolates from Peru [36], Brazil [4,15], Colombia [37], Bolivia [16], Costa Rica [38], French Guiana [39], and more recently in Panamá [19]. These viruses were observed not only in *L. guyanensis*, but also in strains identified as *L. braziliensis*, *L. shawi*, *L. naiffi* and *L. panamensis* [4,18–21,40,41], indicating an old relationship between LRV1 and *L.* (*Viannia*) subgenus. Furthermore, LRV1 was detected in clinical samples collected from patients infected by *L. lainsoni* [4] and *L. peruviana* [22], but no cultivated strains of these two strains positive for LRV1 are available yet, limiting our analyses. Considering the epidemiological and medical importance of the symbiosis between *Leishmania* and LRV, there is a distinct requirement to comprehend the variety and spread of the virus within parasite populations.

The coevolution hypothesis for LRV-*Leishmania* species emerged in 1995 when Widmer and Dooley performed a phylogenetic analyses and found that genetic distances between LRV types mirror the heterogeneity observed for *Leishmania* species based on random amplified polymorphic DNA (RAPD) fingerprints [42]. More than ten years later a study presenting a genetic characterization and phylogenetic analyses of LRV1 sequences from 27 *L. guyanensis* strains and two *L. braziliensis* was published and host-specificity for LRV1 began to be revealed [17]. A year later a robust phylogenetic analyses was presented including 35 LRV1 sequences from *L. guyanensis*, 11 from *L. braziliensis* and, for the first time, a sequence of LRV1 found in a *L. shawi* strain [18]. Both studies presented evidence corroborating the hypothesis of the coevolution of LRV1 and *L.* (*Viannia*) parasites, grouping LRV1 sequences according to the parasite species and geographical clustering.

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The mentioned above studies suggest a specific relationship of LRV1 to such *L.* (*Viannia*) species, combined with the observation of LRV1 in other species, motivate the present study to screen for LRV1 in strains representing *L.* (*Viannia*) species not analyzed so far and available at the *Leishmania* Collection from Fiocruz. To this end, we analyzed all available strains for *L. lainsoni*, *L. lindenbergi*, *L. naiffi*, *L. shawi* and *L. utingensis*. As previously demonstrated, LRV1 was detected in *L. naiffi* and *L. shawi* strains [18,24], but not in *L. lainsoni*, despite the fact that LRV1 was previously detected in clinical samples collected from a patient presenting cutaneous leishmaniasis caused by this species [4]. Of note, *Leishmania* parasites were isolated from this patient and the identified strain was included in our analyses (IOCL 3398), but it was negative for LRV1. There are some possibilities to explain these results, including the possibility of mixed infection by two or more *Leishmania* species, but with the isolation and growth in culture medium of *L. lainsoni* to the detriment of another *L.* (*Viannia*) species that does not grow very well in culture medium as *L. lainsoni* [43] Loss of LRV1 during the process of cultivation is also another possibility [44]. Thus, herein we were able to screen LRV1 in different strains from different *L.* (*Viannia*) species and to analyze nucleotide sequences of LRV1 from *L. naiffi* and *L. shawi*. Interestingly was the fact that more than 50% of *L. naiffi* strains analyzed were positive for LRV1, but we don't know yet if this symbiotic relationship confers any advance to *L. naiffi* parasites, such as the capacity of interacting with different sandfly species and/or dispersion in different geographic regions [45]. It has been demonstrated that *L. naiffi* infection could not have a self-healing nature as it was described years ago [46,47]. Patients could experience a poor response to antimonial or pentamidine therapy [24]. Only in 2019 was first reported a case of a patient infected by *L. naiffi* carrying LRV1, raising the possibility that the presence of this virus could increase *Leishmania* spp. virulence and thereby influence therapeutic failure [15], aspects already observed for *L. braziliensis* and *L. guyanensis*, but that must be better investigated.

Although it is still important to investigate the specificity of LRV1 to other *Leishmania* species such as *L. panamensis*, since LRV1 was already detected in cultivated strains of this species [19–21], our results strongly support this kind of relationship, keeping *L. shawi* in a separate cluster close related to *L. guyanensis*. Here we assumed the groups suggested by Tirera et al 2017 [17], where *L. guyanensis* was divided into five subclusters (A-E). The divergence within *L. guyanensis* is higher than the observed between *L. guyanensis* and *L. shawi*, corroborating the assumption of these as a complex of species [23]. Following this, LRV1 sequences from *L. shawi* formed another subcluster, named here as Group G, closely related to the *L. guyanensis* subclusters A and B and C. Although LRV1 sequences were obtained for only two *L. shawi* strains so far, the fact that they clustered together, despite the strains being from different geographic regions, is also an important aspect to support host-specificity for LRV1. The diversity of LRV1 from *L. guyanensis*, forming several subclusters, must be better explored, but the number of LRV1 sequences from *L. guyanensis* analyzed and the geographic dispersion of these parasites in the Amazon region might be contributing to this result.

Host specificity was also observed for LRV1 genotypes from *L. naiffi*. The phylogenetic tree and the NeighborNet (Figs 3 and 4) show that LRV1 sequences from *L. naiffi* clustered in a well-supported monophyletic clade. Of note, most of LRV1 sequences from *L. naiffi* analyzed were very similar. The diversity observed within LRV1 sequences from the same species must be further investigated, but it is important to consider that all but one *L. naiffi* strains presenting highly similar LRV1 were obtained from patients infected in the same endemic region and included in the same study, suggesting a possibility of problems during laboratory manipulation. However, this very similar group contained one strain isolated previously to the mentioned study and was not manipulated together with the other strains, suggesting a homogeneity for the *L. naiffi* population circulating in this area and causing human disease, which can represent an epidemic clone. LRV1 sequences obtained for two *L. naiffi* strains, IOCL 3316 and IOCL 854 strains, both isolated in Pará, showed a different phylogenetic pattern from the above-mentioned *L. naiffi* strains isolated from Manaus, Amazonas. The strain IOCL 854 was obtained from a sandfly species, *Lutzomyia squamiventris*, and LRV1 from this strain showed a basal position into the *L. naiffi* clade, despite the close relationship of this strain to other *L. naiffi*, including IOCL 3007, as previously demonstrated [23,48]. Considering *Leishmania* (*Viannia*) species depicted by microsatellite analyses, it is more expected that parasites from populations circulating in

the Amazon basin (POP2 and POP3 after Kuhls et al. [48]) carries LRV1 and each subpopulation has an association with specific LRV1 genotypes.

By analyzing LRV1 sequences from several strains representing different *L. (Viannia)* species we demonstrate that LRV1 genotypes form distinct clusters corresponding to their *Leishmania* species host, suggesting that the transfer of viral particles between strains from different species does not occur frequently. Altogether, our results reinforce the concordance between the phylogenetic patterns of LRV1 and *Leishmania (Viannia)* species, providing support for the prevailing hypothesis that LRV1 is an ancient virus that has undergone co-evolution with their hosts [42,49]. Recently it was shown that parasite hybridization might explain the high occurrence of the symbiotic interaction of LRV1 with *L. braziliensis* in Peru and Bolivia [50]. It is possible that this also explains the high frequency of LRV1 in parasites from the Brazilian Amazon Region, since many possible hybrids were described in the region [51], and analyses of microsatellite markers have shown extensive diversity in the subgenus *L. (Viannia)*, with an indication of both clonality and recombination as a strategy of reproduction [48].

5. Conclusions

Our study adds to the growing body of evidence supporting a specific relationship between *Leishmania* RNA Virus 1 (LRV1) and species within the *L.* (*Viannia*) subgenus. LRV1 has been detected in various clinical isolates from different species, including *L. guyanensis*, *L. braziliensis*, *L. shawi*, *L. naiffi*, and *L. panamensis*, but not in other species as well as not in *L. braziliensis* circulating outside the Amazon Basin, raising intriguing questions about host specificity and the potential impact on virulence and therapeutic response.

While the results provide strong support for the association of LRV1 with specific *Leishmania* species, more investigations are needed to understand its specificity to other species, such as *L. panamensis*. The divergence observed within LRV1 sequences from the same species warrants further scrutiny, especially considering potential issues during laboratory manipulation and the homogeneity of *L. naiffi* populations in certain endemic regions. Overall, the identification and characterization of LRV1 in different *Leishmania* species shed light on the complex interactions between these viruses and the parasites they infect. Future research in this area may uncover novel insights into the biology and pathogenesis of *Leishmania* infections, offering new perspectives on therapeutic strategies and disease management.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Information on LRV1 sequences from previous studies used for phylogenetic analyses; Table S2: Estimates of average evolutionary divergence over sequence pairs within groups and between groups using Tamura 3-parameter model and number of differences.

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