Fixed Differences in the \textit{paralytic} Gene Define Two Lineages within the \textit{Lutzomyia longipalpis} Complex Producing Different Types of Courtship Songs

Rachel M. M. A. Lins\textsuperscript{1}, Nataly A. Souza\textsuperscript{2}, Reginaldo P. Brazil\textsuperscript{3}, Rhayza D. C. Maingon\textsuperscript{4}, Alexandre A. Peixoto\textsuperscript{1,5}\textsuperscript{*}

\textsuperscript{1}Laboratório de Biologia Molecular de Insetos, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil, \textsuperscript{2}Laboratório de Transmissores de Leishmanioses, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil, \textsuperscript{3}Laboratório de Bioquímica e Fisiologia de Insetos, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil, \textsuperscript{4}Centre for Applied Entomology and Parasitology, Institute for Science and Technology in Medicine, School of Life Sciences, Keele University, Keele, United Kingdom, \textsuperscript{5}Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular (INCT-EM), Rio de Janeiro, Brazil

Abstract

The sand fly \textit{Lutzomyia longipalpis} (Diptera: Psychodidae: Phlebotominae), the most important vector of American visceral leishmaniasis, is widely distributed in Latin America. There is currently a consensus that it represents a species complex, however, the number and distribution of the different siblings is still uncertain. Previous analyses have indicated that Brazilian populations of this vector can be divided into two main groups according to the type of courtship song (Burst vs. Pulse) males produce during copulation. Nevertheless, no diagnostic differences have been observed between these two groups with most molecular markers used to date. We analyzed the molecular divergence in a fragment of the \textit{paralytic} gene, a locus involved in the control of courtship songs in \textit{Drosophila}, among a number of \textit{Lu. longipalpis} populations from Brazil producing Burst and Pulse-type songs. Our results revealed a very high level of divergence and fixed differences between populations producing the two types of songs. We also compared \textit{Lu. longipalpis} with a very closely related species, \textit{Lutzomyia cruzi}, which produces Burst-type songs. The results indicated a higher number of fixed differences between \textit{Lu. cruzi} and the Pulse-type populations of \textit{Lu. longipalpis} than with those producing Burst-type songs. The data confirmed our previous assumptions that the presence of different sibling species of the \textit{Lu. longipalpis} complex in Brazil can be divided into two main groups, one representing a single species and a second more heterogeneous group that probably represents a number of incipient species. We hypothesize that \textit{para} might be one of the genes directly involved in the control of the courtship song differences between these two groups or that it is linked to other loci associated with reproductive isolation of the Brazilian species.

Introduction

The study of species complexes provides an opportunity to investigate a number of unanswered questions about speciation [1]. Divergence between populations resulting in recent or incipient speciation can eventually lead to a number of molecular, behavioral and morphological changes, but very often these characters do not evolve at similar rates. This is particularly true in cases of cryptic speciation [2] where morphologically indistinguishable species can show striking behavioral differences, especially in aspects of courtship.

Acoustic communication is an important aspect of sexual behavior in a large number of insects [3], including disease vectors (e.g. [4–6]), and it has also a role in the reproductive isolation of many closely related species. In \textit{Drosophila}, for example, courtship song is usually species-specific, being one of the signals females use to recognize males of their own species (e.g. [7–10]). \textit{Drosophila} studies have also identified a number of genes controlling features of courtship songs (reviewed by [11–12]).

Acoustic signals can be also useful as one of the markers in an integrative analysis for species identification where classic morphologic differences fail to differentiate incipient sibling species [13]. One example in blood-sucking insects involves study of male copulation songs in the \textit{Lutzomyia longipalpis} species complex [14–16], the main neotropical vector of \textit{Leishmania infantum}, the etiological agent of American visceral leishmaniasis (AVL) [17]. As the main vector of an important parasitic disease, the existence of cryptic species in this insect may have important epidemiologic consequences [18–19] since divergence caused by genetic drift and/or natural selection may affect genes controlling aspects of the disease vector potential, resulting in sibling species that are more efficient as vectors than others as has been shown in the \textit{Anopheles gambiae} species complex [20–21].

Although \textit{Lu. longipalpis} is a species complex [22–25], the number and distribution of the different sibling species is still uncertain. Previous studies using a combination of crossing experiments [22,26], analyses of acoustic signals [14–16], male
sex pheromones [16,27–30] and molecular markers including orthologues of Drosophila courtship song genes period (per) and cacophony (cac) [16,31–35], and microsatellites [34–35], have indicated that the Brazilian populations of this vector can be divided into two main groups according to the type of copulation song (Burst vs. Pulse) and pheromones that males produce [16]. Males of the first group of populations produce Burst-type song and the dipteran Cembrene-1 pheromone and probably represent a single species while the second group consists of populations producing different subtypes of Pulse-type song in combination with different pheromones that probably represent a number of incipient species [16]. However, Lu. longipalpis genetic structure in Brazil is rather complex with evidence of incomplete reproductive isolation and introgression [16,22,33] and no observed diagnostic differences between these two groups in most molecular markers used so far that would allow for a rapid identification of the different species.

The only potential exception so far is the paralytic (para) gene, a locus also involved in the control of courtship songs in Drosophila [36], characterized by fixed differences between a pair of sympatric sibling species of the Lu. longipalpis complex from Sobral (Ceará State, Brazil), that produce different copulation songs and male sex pheromones [37]. In the present study, we have extended the analysis of the para gene to a number of other Lu. longipalpis populations from Brazil. In addition, we have also analyzed the differentiation between Lu. longipalpis and Lutzomyia cruzi, a closely related species [30] that also acts as a vector of Leishmania infantum in a region of Brazil [39]. Analyses of copulation songs, pheromones and molecular markers have indicated that Lu. cruzi is another species of the Lu. longipalpis complex [35,40–41].

Methods

We analyzed samples of Lu. longipalpis from eight different Brazilian localities: Lapinha, Minas Gerais State; Jaíba, Minas Gerais State; Jacobina, Bahia State; Pancas, Espírito Santo State; Estrela de Alagoas, Alagoas State; Natal, Rio Grande do Norte State; Marajó Island (Salvaterra), Pará State; and Teresina, Piauí State (Figure 1). A permit for sand fly collection in Brazil was obtained from the Brazilian Ministry of Environment (SISBIO #26066-1). Sand flies were captured using CDC light-traps near human habitation with permission from local homeowners. In addition, the collections were usually supported by the local vector surveillance authorities from local State Health Departments. Male Lu. longipalpis are characterized by polymorphism in the number of abdominal spots [22]: although this phenotype cannot be used to identify different allopatric species of the complex, it can be useful in some cases of sympathy, as previous work in Sobral [reviewed in [19,25]] and, more recently, in Estrela de Alagoas and Jaíba [16] has shown. In these three localities, the sympatric one spot (1S) and two spot (2S) males produce different copulation songs (Pulse-type and Burst-type, respectively) and represent different species [16]. Therefore, these samples were analyzed separately. Males from Natal that are highly polymorphic for the number of spots including very high numbers of intermediate forms which are rare in Sobral, Estrela de Alagoas and Jaíba, and Pancas (1S) produced Burst-type song, while males of Lapinha (1S), Jacobina (2S) and Teresina (1S) that represents a majority of this locality produce different subtypes of Pulse-type song [16]. We also analyzed a sample of Lu. cruzi from Corumbá, Mato Grosso do Sul State and two males of Lutzomyia pseudolongipalpis from Curarigua, Venezuela, used as an outgroup in the genealogical analysis.

Genomic DNA was isolated according to [42] and the PCR Master Mix (Promega) was used to perform PCR according to [37]. PCR products were purified using the Wizard SV Gel and PCR Clean-up System (Promega) or GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). Purified fragments were cloned using the pMOSBlue Blunt End Cloning Kit (GE Healthcare) or TOPO TA Cloning Kit (Invitrogen). Plasmid DNA was isolated using Flexigem Kit (GE Healthcare) or using 96 well microplates and the alkaline lysis method [43] followed by filtration in Millipore Multiscreen filter plates. DNA sequencing was carried out with an ABI 3730 sequencer using the Big Bye 3.1 Kit (Applied Biosystems).

Lu. longipalpis para gene fragments from all populations were initially processed using BioEdit Sequence Alignment Editor [44] before population genetics analyses, which also included previously published sequences from Sobral [37]. A minimum of eight sequences per individual were aligned to obtain two consensus sequences corresponding to the two alleles, A and B, or one consensus sequence where flies were treated as homozygotes and the sequences were duplicated. The estimated probability of misclassifying a heterozygous fly as a homozygous with this procedure was less than 1%.

Both polymorphism and population structure analyses were carried out using DnaSP v5 [45] and Proseq 2.91 [46]. A Minimum Evolution tree based on p distances was estimated using MEGA5 [47]. All sequences were submitted to GenBank (accession numbers JQ359112–JQ359437). Analysis of molecular variance (AMOVA) was carried out with Arlequin 3.11 [48]. A non-recombiant block of the initial fragment was obtained using the I Mage program [49] to construct the haplotype network with TCS v1.21 software [50].

Results

We analyzed a total 298 allele sequences from 149 males of a fragment of the para gene of Lu. longipalpis [37] of approximately 385 bp, including a variable sized intron of ~220 bp. Analyses included previously published and new sequences from the two Sobral sympatric sibling species [37] and new sequences from samples of the eight Brazilian localities analyzed here (Figure 1). Sympatric one spot (1S) and two spot (2S) males found in Estrela de Alagoas and Jaíba were analyzed separately since these males
produce different copulation songs (Pulse and Burst, respectively) and represent different species, as previously observed in Sobral [16]. We also analyzed 24 allele sequences of Lu. cruzi males from Corumbá, State of Mato Grosso do Sul, a closely related sibling of the Lu. longipalpis complex producing Burst-type song [41], and used 4 sequences obtained from two males of Lu. pseudolongipalpis, a more distantly related sibling species [35,31], as an outgroup in the genealogical analysis (see below). Figure S1 shows the alignment of the whole fragment. Most of the variation was found within the intron, that included a number of indels. However, some rare non-synonymous substitutions were also observed.

Table 1 shows a summary of the polymorphisms for each population analyzed, excluding the regions with gaps. Populations of Lu. longipalpis were grouped according to the type of copulation song they produce: Pulse or Burst. The results showed that Lapinha was the least polymorphic among the Pulse song populations while Jacobina had the highest values of δ and ê. Among the Burst song populations, Marajó and Jaíba 2S were the least and most polymorphic samples, respectively. Tajimas D and Fu and Li's D* and F* tests of neutrality [52–53] were performed for each population. Although one value was significant at a 5% level, all values were non-significant after Bonferroni correction.

Molecular differentiation analysis was performed for all pairwise comparisons involving the Lu. longipalpis populations, except for the small sample of Marajó. Again, the populations were grouped according to the song type they produce, Pulse or Burst. Table 2 shows the fixation indexes (Fst) as well as the number of fixed differences (Sf) in each comparison. The lowest pairwise Fst values were obtained between populations producing the same song type, while very high values of differentiation were observed in the comparisons involving populations producing either Burst or Pulse-type copulation songs. Indeed, fixed differences were found in those latter comparisons, except for the Estrela 1S sample. However, when sequences of a single fly (sequences Est1S8A and Est1S8B) were excluded from the Fst analysis (Table 2) and those from the more distant sibling species as in Sobral [15,22,30,32–34,37]. However, para gene Fst values clearly confirm the presence of two sympatric species in Estrela, i.e. Estrela 1S and 2S (Table 2 and below).

Smaller sequence differences in para were observed between Burst-type populations than between Pulse-type populations. Indeed, the mean pairwise Fst value among Burst-type populations was 0.063 ± 0.067 compared with 0.147 ± 0.104 among Pulse-type populations. In contrast, the mean pairwise differentiation between populations with the two main song types was much higher (0.790 ± 0.044). These results were corroborated by AMOVA performed to examine the partition of para sequence variation within Lu. longipalpis (Table 3). Most of the molecular variation (64.95%) was observed between the two main song types (Burst × Pulse), reflecting a clear separation between these groups. In addition, the results revealed a small part of this variation (7.0%) distributed among populations within groups.

The same 383 bp para gene fragment studied in Lu. longipalpis was also amplified in Lu. cruzi from Corumbá, State of Mato Grosso do Sul. As shown in Table 1, Lu. cruzi showed levels of polymorphism in para that were similar to the lowest values observed among the Lu. longipalpis samples. Higher differentiation and fixed nucleotide differences between Lu. cruzi and all Lu. longipalpis populations with high Fst values (ranging from 0.7139 and 0.9271) were also observed (Table 2). However, a number of Fst values were smaller than comparisons between Burst-type and Pulse-type populations of Lu. longipalpis. Furthermore, Lu. cruzi that produces Burst-type songs showed two fixed differences compared with Burst-type populations and four to six differences compared with Pulse-type populations, whereas comparisons between the two song types of Lu. longipalpis displayed two to four fixed differences.

A Minimum Evolution tree including all Lu. longipalpis and Lu. cruzi sequences and those from the more distant sibling Lu. pseudolongipalpis (Figure 2) showed clear separation between the two main groups producing different copulation songs. In the tree, the two sequences (E1S8A and E1S8B) belonging to one Estrela de Alagoas 1S fly that were excluded from the Fst analysis (Table 2) clustered with the sequences corresponding to the Burst-type

### Table 1. Polymorphism summaries of the para gene fragment from populations of Lu. longipalpis and Lu. cruzi.

| Population | Song-type | n   | S   | δ    | ê    | D*, D* | D*   | F*   |
|------------|-----------|-----|-----|------|------|-------|------|------|
| Sobral 1S  | P         | 32  | 12  | 0.0033 (0.0032) | 0.0080 (0.0079) | −2.0334* | −1.2001 | −1.7165 |
| Lapinha    | P         | 28  | 2   | 0.0016 (0.0016) | 0.0014 (0.0014) | 0.3094  | −0.7144 | −0.4930 |
| Jacobina   | P         | 22  | 9   | 0.0051 (0.0050) | 0.0067 (0.0066) | −0.8129 | −1.2837 | 1.3111  |
| Teresina   | P         | 24  | 9   | 0.0037 (0.0036) | 0.0065 (0.0064) | −1.4200 | −2.4491 | −2.4955 |
| Jaíba 1S   | P         | 24  | 6   | 0.0023 (0.0023) | 0.0043 (0.0043) | −1.3944 | −0.9729 | −1.2699 |
| Estrela 1S | P         | 22  | 5   | 0.0019 (0.0019) | 0.0037 (0.0037) | −1.4525 | −0.4601 | −0.8577 |
| Sobral 25  | B         | 28  | 8   | 0.0040 (0.0057) | 0.0056 (0.0073) | −0.8846 | 0.0821  | 0.2423  |
| Estrela 25 | B         | 32  | 6   | 0.0038 (0.0056) | 0.0041 (0.0058) | −0.1481 | 1.2092  | 0.9335  |
| Jaíba 25   | B         | 24  | 11  | 0.0047 (0.0047) | 0.0080 (0.0080) | −1.3831 | −1.3688 | −1.5994 |
| Natal      | B         | 24  | 11  | 0.0047 (0.0049) | 0.0080 (0.0087) | −1.4081 | −1.8366 | −1.9906 |
| Pancas     | B         | 32  | 10  | 0.0047 (0.0051) | 0.0067 (0.0074) | −0.9295 | −1.3358 | −1.4167 |
| Marajó     | B         | 6   | 1   | 0.0016 (0.0048) | 0.0012 (0.0044) | −1.7188 | 1.0525  | 1.1577  |
| Lu. cruzi  | B         | 24  | 5   | 0.0019 (0.0018) | 0.0037 (0.0036) | −1.4315 | −2.1728 | −2.2703 |

B, Burst type song; P, Pulse type song; n, number of sequences; S, number of segregating sites; δ, nucleotide diversity; ê, neutral parameter based on the segregating sites; D*, Tajima test of neutrality. Numbers in parentheses represent the analysis of nucleotide diversity considering the regions with gaps. *p<0.05.

doi:10.1371/journal.pone.0044323.t001
populations indicating that this individual probably represents a case where the spot phenotype did not match the correct song type in this locality. Sequences of *Lu. pseudolongipalpis* that also produce Burst-type songs (light green circles) were grouped together with the sequences corresponding to H4 are from Estrela 1S, whose mutation between H11 and H4. H11 represents sequences of *Lu. cruzi* haplotypes appeared as a separate cluster more closely related to the Burst-type populations of *Lu. cruzi*. The two main haplotypes generated were H13 and H28. Haplotype 13 corresponds to Burst-type populations and was composed of sequences of Sobral 2S, Estrela 2S, Natal and Pancas. Haplotype 28 was the most frequent haplotype of Pulse song populations from Sobral 1S, Jaíba 1S, Lapinha and Teresina. There was clear separation between the two groups producing different song types. These groups were connected by a single mutation between H11 and H4, H11 represents sequences of Sobral 2S, Pancas, Estrela 2S and Marajo. Interestingly, most of the sequences corresponding to H4 are from Estrela 1S, whose males produced the same type of pheromone, Cembrene-1 [16] found in populations with the H11 haplotype. In addition, nearly all *Lu. cruzi* haplotypes appeared as a separate cluster more closely related to the Burst-type populations of *Lu. longipalpis*.

**Discussion**

Understanding the structure of sibling species complexes is a difficult task for evolutionary biologists and this is particularly true in the case of cryptic species [2]. The lack of diagnostic morphological changes coupled with incomplete reproductive isolation and introgression, a common phenomenon among very closely related siblings [54–55], makes the identification and delimitation of the different species a difficult assignment.

### Table 2. Pairwise differentiation between Pulse-type and Burst-type populations of *Lu. longipalpis* and *Lu. cruzi*.

|                  | [Pulse-type populations] | [Burst-type populations] |
|------------------|--------------------------|--------------------------|
|                  | S1S Lap Jac Ter J1S E1S  | S2S E2S J2S Natal Pancas  |
| **Pulse-type S1S** | 0.2077*** 0.1261*** 0.0315*** 0** 0.6171*** | 0.7819**** 0.8257**** 0.8103*** 0.8009**** 0.8003**** 0.8695**** |
|                  | (0.4914***)             |                          |
| **Lap**          | 0 0.2915*** 0.2633** 0.2007** 0.8079*** | 0.8285**** 0.8704**** 0.8504**** 0.8455**** 0.8443**** 0.9083*** |
|                  | (0.6491***)             |                          |
| **Jac**          | 0 0 0.1697** 0.1706** 0.3251** | 0.6899**** 0.7414**** 0.7335*** 0.7146**** 0.7152**** 0.8002** |
|                  | (0.2343**)              |                          |
| **Ter**          | 0 0 0 0 0.0050** | 0.6002*** 0.7701**** 0.8122*** 0.7987** 0.7886** 0.7883** 0.8585** |
|                  | (0.4938**)              |                          |
| **J1S**          | 0 0 0 0 0 | 0.7031*** 0.8026**** 0.8458**** 0.8282**** 0.8209**** 0.8200**** 0.8871**** |
|                  | (0.5603***)             |                          |
| **E1S**          | 0 (0) 1 (1) 0 (0) 0 (0) 1 (1) | 0.8356*** 0.8913*** 0.8627** 0.8579** 0.8561*** 0.9271** |
|                  | (0.7312***) (0.7922**) (0.7741***) (0.7581***) (0.7577***) (0.8504**) |
| **Burst-type S2S** | 3 4 2 3 4 3 | 0.6635** 0.2272** 0.0380ns 0.0914* 0.7139** |
| **E2S**          | 3 4 2 3 4 3 | 3 (0) 0.0744** 0** 0** 0.7761*** |
| **J2S**          | 3 4 2 3 4 3 | 3 (0) 0 0 0.0737** 0.0633* 0.7534*** |
| **Natal**        | 3 4 2 3 4 3 | 3 (0) 0 0 0 0** 0** 0.7389**** |
| **Pancas**       | 3 4 2 3 4 3 | 3 (0) 0 0 0 0 0.7306*** |
| **Lu. cruzi**    | 5 6 4 5 6 5 | 2 2 2 2 2 2 |

Upper right matrix – pairwise differentiation (Fst) and significance (P values were obtained with 10,000 random permutations). Lower left matrix – fixed differences between samples. S1S – Sobral 1S, Lap – Lapinha, Jac – Jacobina, Ter – Teresina, J1S – Jaíba 1S, S2S – Sobral 2S, E2S – Estrela 2S, J2S – Jaíba 2S, Natal – Sobral 1S, Jac – Jacobina, Ter – Teresina, J1S – Jaíba 1S, S2S – Sobral 2S, E2S – Estrela 2S, J2S – Jaíba 2S, Natal – Sobral 1S, Jac – Jacobina, Ter – Teresina, J1S – Jaíba 1S, S2S – Sobral 2S, Estrela 2S, J2S – Jaíba 2S, Natal – Sobral 1S, Jac – Jacobina, Ter – Teresina, J1S – Jaíba 1S, S2S – Sobral 2S, E2S – Estrela 2S, J2S – Jaíba 2S, Natal – Sobral 1S, Jac – Jacobina, Ter – Teresina, J1S – Jaíba 1S, S2S – Sobral 2S, E2S – Estrela 2S, J2S – Jaíba 2S, Natal – Sobral 1S, Jac – Jacobina, Ter – Teresina. Significance corresponding to the fixation indexes was obtained through 10,000 permutations. *p<0.001; **p<0.0001; ***p<0.00001; ****p<0.000001.

do:10.1371/journal.pone.0044323.t002

### Table 3. AMOVA statistics.

| Source of variation | Percentage of variation |
|---------------------|-------------------------|
| Among groups        | 64.95                   |
| Among populations within groups | 7.00               |
| Within populations  | 28.06                   |
| Fst (haplotypes/populations within groups) | 0.1996*** |
| Fst (haplotypes/populations/groups) | 0.7194*** |
| Fct (populations/groups) | 0.6495* |

**Copulation song groups: Burst-type:*** Sobral 2S, Estrela 2S, Jaíba 2S, Natal and Pancas; Pulse-type:*** Sobral 1S, Jaíba 1S, Estrela 1S, Lapinha, Jacobina and Teresina. Significance corresponding to the fixation indexes was obtained through 10,000 permutations. *p<0.01; **p<0.001; ***p<0.0001; ****p<0.00001.

do:10.1371/journal.pone.0044323.t003
Combined analyses using molecular markers, particularly the *per* gene [16] and microsatellites [34–35], and behavioral traits (songs and pheromones) strongly suggest that Brazilian *Lu. longipalpis* populations can be divided into two main groups according to the type of song (Burst vs. Pulse) males produce during copulation [16]. Fixed *para* gene differences between these two main lineages further support this notion. Indeed, the haplotype networks obtained with *per* [16] and *para* (Fig 3) showed a clear separation between the two population groups. In addition, although no fixed differences between the two lineages were observed in *per*, the pairwise divergence between *Lu. longipalpis* populations measured by Fst values in these two genes were highly correlated (Mantel test, r = 0.819, p < 0.01). Furthermore, both genes show a higher level of divergence among Pulse-type than among Burst-type song populations, consistent with the idea that the latter populations that produce the same song-type and the same pheromone (Cembrene-1) belong to a single species [16]. However, data from both genes indicate that the relationship among populations producing the different subtypes of Pulse-type song is more complex and heterogeneous. For example, males from Jacobina produce the P1 song and a combination of alpha-himachalene and 3-methyl-alpha-himachalene sex pheromones; Lapinha males

Figure 2. Minimum Evolution tree of sequences from Brazilian populations of *Lu. longipalpis* producing Burst-type (dark green circles) and Pulse-type songs (red circles), *Lu. cruzi* (light green circles) and the more distant sibling species *Lu. pseudolongipalpis* (open circles) used as outgroup. The sequences E1S8A and E1S8B are the only red circles that cluster with the Burst-type sequences. Bootstrap values based on 1000 replications (values below 50% are not shown).
doi:10.1371/journal.pone.0044323.g002
produce the P2 song and 9-methyl-germacrene-B, (9MGB), sex pheromone; and Sobral 1S and Teresina produce the same P3 song associated with 9MGB sex pheromone [16]. Jaı´ba 1S males produce the P4 song and Cembrene-2 sex pheromone whereas in Estrela, 1S males produce the P5 song and the Cembrene-1 sex pheromone. Thus, combined molecular and behavioral data strongly suggest that these populations belong to five different incipient sibling species [16]. Indeed, for at least one pair of Pulse-type song populations (Jacobina and Lapinha) crossing experiments [26] and cytogenetic analysis [56] support this hypothesis.

Comparative para and per data ([41], this study) also suggest that Lu. cruzi is another member of the Lu. longipalpis complex. However, per analysis indicated higher genetic differentiation between Lu. cruzi and Burst-type song populations where the present results with para showed a higher Fst value between the former and Pulse-type populations. Lu. cruzi males produce a variation of the Burst-type song with shorter inter-burst intervals [41] and the 9MGB sex pheromone [40] also found in many Pulse-type populations of Lu. longipalpis [16]. Considering that Lu. cruzi males produce Burst-type song, it is tempting to speculate that para might be an important genetic determinant of song type (Burst vs. Pulse) between the two groups of Lu. longipalpis populations. Alternatively, para and per might be linked, with different levels of linkage disequilibrium and/or ancestral polymorphisms, to other loci associated with the reproductive isolation between the Brazilian sibling species.

The D. melanogaster courtship song genes are involved in a number of different molecular functions (reviewed in [12]). The three song genes used so far to study the Lu. longipalpis complex, para, cac and per encode, respectively, a voltage-gated sodium channel, a voltage-gated calcium channel, and a transcriptional repressor primarily involved in the circadian clock. It is possible that future RNA interference experiments (e.g. [57]) will help to confirm the potential role of these and other song genes in controlling copulation song differences among Lu. longipalpis sibling species. In addition, playback experiments (e.g. [7–9,58]) should also be carried out to directly infer whether copulation songs are involved in mate choice and reproductive isolation.

Finally, our para data also confirm existence of three localities (Sobral, Jaı´ba and Estrela) where pairs of species carrying different spot phenotypes and producing either Burst-type or Pulse-type songs occur in sympatry [16]. The existence of fixed differences in para allowing easy genotyping of females of the different species, will be particularly useful in these three localities to investigate whether the Burst-type and Pulse-type song females show any differences in other aspects of behavior when they occur sympatrically. The study of such phenotypic differences among closely related or incipient vector species is necessary because of the evolutionary and epidemiological implications of traits such as host or habitat preferences that have potential roles in ecological speciation [59] and/or in vector capacity [20].

Supporting Information

Figure S1 Alignment of the paralytic gene whole fragment. Intron sequence is highlighted in grey and non-recombinant block used to construct the haplotype network is highlighted in yellow. Dots indicate the same nucleotide and dashes indicate gaps. (DOC)

Table S1 Distribution of the 40 haplotypes found among Lu. longipalpis and Lu. cruzi samples, segregating sites within a 251 bp non-recombinant fragment and number of sequences represented in each sample. (DOC)

Acknowledgments

We are grateful to Dora Feliciangeli (BIOMED, Maracay, Venezuela) and Jan Fish (Keele University) for Lu. pseudolongipalpis sand flies and para clones, respectively. We would also like to thank Robson Costa da Silva for technical assistance at FIOCRUZ, Brazil, Gabriel E. M. Ferreira for helping with the figures, and two anonymous reviewers and the editor William Etges for helpful comments on the manuscript.

Author Contributions

Conceived and designed the experiments: RL AP. Performed the experiments: RL. Analyzed the data: RL AP. Contributed reagents/materials/analysis tools: NS RB RM. Wrote the paper: RL RM AP.

Figure 3. Haplotype network of Brazilian populations of Lu. longipalpis and Lu. cruzi. Each population is represented by a different color and each node represents a unique haplotype. doi:10.1371/journal.pone.0044323.g003
References

1. Marie Curie SPECIATION Network, Bultin R, Debelle A, Kerth C, Snook RR, et al. (2012) What do we need to know about speciation? Trends Ecol Evol 27: 271–280.

2. Bickford D, Lohman DJ, Soluda NS, Ng PK, Meyer R, et al. (2007) Cryptic species as a window on diversity and conservation. Trends Ecol Evol 22: 148–155.

3. Ewing AW (1998) Arthropod Bioacoustics. Ithaca, New York: Cornell University Press.

4. Cato L, Arthur BJ, Harrington LC, Hoy RR (2009) Harmonic convergence in the sex pheromone of the sandfly Lutzomyia longipalpis (Diptera: Psychodidae). Insect Mol Biol 18: 619–627.

5. Warren B, Gibson G, Russell IJ (2009) Sex recognition through midflight mating duets in Culex mosquitoes is mediated by acoustic distortion. Curr Biol 19: 485–491.

6. Pennetier C, Warren B, Dahire KR, Russell JI, Gibson G (2010) "Singing on the wing" as a mechanism for species recognition in the malaria mosquito Anopheles gambiae. Curr Biol 20: 131–136.

7. Ritchie MG, Halsey EJ, Gleason JM (1999) Drosophila song as a species-specific mating signal and the behavioural importance of Kyriacou & Hall cycles in D. melanogaster. Anim Behav 58: 689–697.

8. Saarikettu M, Liimatainen JO, Hoikkala A (2005) The role of male courtship song in species recognition in Drosophila melanogaster. Behav Genet 35: 257–263.

9. Klappert K, Mazza D, Hoikkala A, Ritchie MG (2007) Male courtship song and female preference variation between phylogeographically distinct populations of Drosophila melanogaster. Evolution 61: 1481–1489.

10. Wen SY, Yamada H, Li YF, Kimura MT, Oguma Y, Sawamura K, Toda MJ (2007) Drosophila lini: a new gene involved in the courtship song of Drosophila. Behav Genet 37: 263–277.

11. Dayrat B (2005) Towards integrative taxonomy. Biol J Linnean Soc 85: 407–415.

12. de Souza NA, Ward RD, Hamilton JG, Kyriacou CP, Peixoto AA (2002) Analysis of the copulatory courtship songs of Drosophila. Behav Genet 32: 457–469.

13. Lainson R, Rangel TC (2006) Lutzomyia longipalpis and the ecobiology of American visceral leishmaniasis, with particular reference to Brazil. A review. Mem Inst Oswaldo Cruz 101: 811–827.

14. Lanzaro GC, Varini H, Norsini D, Alexandre B, et al. (2003) The taxonomic status of genetically divergent populations of Lutzomyia longipalpis (Diptera: Psychodidae) based on the distribution of mitochondrial and isozyme markers. J Med Entomol 40: 615–627.

15. Bahia-Neto EF, Ferraretto JR, Cardoso MA, Alves CR, Brazil RP, et al. (2008) The molecular forms of Anopheles gambiae complex. Mol Biol Evol 19: 1624–1627.

16. Lehmann T, Diabate A (2008) The molecular forms of Anopheles gambiae complex. Mol Biol Evol 19: 1624–1627.

17. Lainson R, Rangel TC (2006) Lutzomyia longipalpis and the ecobiology of American visceral leishmaniasis, with particular reference to Brazil. A review. Mem Inst Oswaldo Cruz 101: 811–827.

18. Lanzaro GC, Varini H, Norsini D, Alexandre B, et al. (2003) The taxonomic status of genetically divergent populations of Lutzomyia longipalpis (Diptera: Psychodidae) based on the distribution of mitochondrial and isozyme markers. J Med Entomol 40: 615–627.

19. Nair PK, Hamilton JG, Brazil RP, Noyes HA, Souza NA, et al. (2003) Male sex pheromones and the phylogeographic structure of the Lutzomyia longipalpis species complex (Diptera: Psychodidae). J Med Entomol 20: 734–743.

20. ENWONE T, Hamilton JG, Brazil RP, Noyes HA, Souza NA, et al. (2003) Male sex pheromones and the phylogeographic structure of the Lutzomyia longipalpis species complex (Diptera: Psychodidae). From Brazil and Venezuela. Am J Trop Med Hyg 73: 734–743.

21. PEIXOTO AA, Hamilton JG (2009) Analysis of temperature-sensitive mutants reveals new genes involved in the courtship song of Drosophila. Genetics 148: 827–838.

22. Lins RM, Souza NA, Peixoto AA (2008) Genetic divergence between two sympatric species of the Lutzomyia longipalpis complex in the paralytic genus, a focus associated with insecticide resistance and loxosceles production. Mem Inst Oswaldo Cruz 103: 736–740.

23. Sims JT, Monteiro AG, Silva-Sanabria D, Eiras MP, Alves CR, et al. (2008) Divergence with Gene Flow: Models and Data. Annu Rev Genet 42: 130–136.

24. Pinho IS, Filho JD, Santos CB, Fukutake A, Leite YL (2010) Phylogenetic relationships among species of Lutzomyia, subgenus Luizomyia (Diptera: Psychodidae). J Med Entomol 47: 16–21.

25. de Pita-Pereira D, Cardoso MA, Alves CR, Brazil RP, Britto C (2008) Detection of natural infection in Lutzomyia cruzi and Lutzomyia flaviscutu (Diptera: Psychodidae: Phlebotominae) by Leishmania infantum chagasi in an endemic area of visceral leishmaniasis in Brazil using a PCR multiplex assay. Trans R Soc Trop Med Hyg 107: 66–69.

26. Brasil RP, Hamilton JG (2002) Isolation and identification of 9-methylgermacrene-B as the putative sex pheromone of Lutzomyia cruzi (Mangabeira, 1938) (Diptera: Psychodidae). Mem Inst Oswaldo Cruz 97: 435–436.

27. Vigoder FM, Araki AS, Bauzer LG, Souza NA, Brazil RP, Peixoto AA (2010) Loxosceles and period gene polymorphisms indicate Lutzomyia cruzi (Mangabeira, 1938) as a sibling species of the Lutzomyia longipalpis (Lutz and Neiva, 1912) complex. Insect Genet Evol 10: 734–739.

28. Jowett T (1998) Preparation of nucleic acids. In: Roberts DR, editor. Drosophila. A practical approach. Oxford: IRL Press. 347–371.

29. Sambrock D, Russell JI (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor: CSIL Press. 2100 p.

30. Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41: 95–98.

31. Arribilaga J, Mustel JP, Pinango H, Norris D, Alexandre B, et al. (2003) The taxonomic status of genetically divergent populations of Lutzomyia longipalpis (Diptera: Psychodidae) based on the distribution of mitochondrial and isozyme variation. J Med Entomol 40: 615–627.

32. Bauzer LG, Souza NA, Maingon R, Peixoto AA (2007) Lutzomyia longipalpis in Brazil: a complex or a single species? A mini-review. Mem Inst Oswaldo Cruz 102: 1–12.

33. Souza NA, Andrade-Coelho CA, Viegas FM, Ward RD, Peixoto AA (2000) Reproductive isolation between sympatric and allopatric Brazilian populations of Lutzomyia longipalpis (Diptera: Psychodidae). Mem Inst Oswaldo Cruz 103: 216–219.

34. Hamilton JG, Hooper AM, Mori K, Pickard JA, Sano A (1999a) 9-Methylz-inhainachalane confirmed, and the relative stercorechemistry defined, by synthesis as the sex pheromone of the sandfly Lutzomyia longipalpis from Jacobina, Brazil. Chem Commun 355–356.

35. Hamilton JG, Bruce CJ, Ribot JB, Ribeiro HC, Hooper AM, Mori K, Pickard JA, et al. (1999b) 9-Methylgermacrene-B confirmed by synthesis as the sex pheromone of the sandfly Lutzomyia longipalpis from Latinpa, Brazil, and the absolute stereochemistry defined as 9S. Chem Commun 2353–2356.
57. Sant’Anna MR, Alexander B, Bates PA, Dillon RJ (2008) Gene silencing in phlebotomine sand flies: Xanthine dehydrogenase knock down by dsRNA microinjections. Insect Biochem Mol Biol 38: 652–660.

58. Ritchie MG, Townhill RM, Hoikkala A (1998) Female preference for fly song: playback experiments confirm the targets of sexual selection. Anim Behav 56: 713–717.

59. Schluter D (2009) Evidence for ecological speciation and its alternative. Science 323: 737–741.