# *Lutzomyia longipalpis* in Brazil: a complex or a single species? A mini-review

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Lutzomyia longipalpis *is the main vector of* Leishmania infantum chagasi*, the causative agent of American visceral leishmaniasis (AVL). Although there is strong evidence that* Lu. longipalpis *is a species complex, not all data concerning populations from Brazil support this hypothesis. The issue is still somewhat controversial for this large part of* Lu. longipalpis *distribution range even though that it is the Latin American region contributing to most of the cases of AVL. In this mini-review we consider in detail the current data for the Brazilian populations and conclude that* Lu. longipalpis *is a complex of incipient vector species with a complexity similar to* Anopheles gambiae s.s. *in Africa.*

Key words: visceral leishmaniasis - sand flies - *Lutzomyia longipalpis* species complex - introgression - Brazil

The primary vector of *Leishmania infantum chagasi* and hence of visceral leishmaniasis in Latin America, *Lutzomyia longipalpis* (Lutz & Neiva 1912) (Diptera: Psychodidae: Phlebotominae) is probably the best-studied sand fly species in the Neotropical region (Soares & Turco 2003, Lainson & Rangel 2005). There is a fairly large body of data pointing out to the existence of a species complex (reviewed by Uribe 1999). Despite of this, there is no consensus yet about the number of sibling species of *Lu. longipalpis* and what are their areas of distribution (Arrivillaga et al. 2003).

In this mini-review, we intend to give a general overview on the *Lu. longipalpis* species problem and to consider in detail the current data for the Brazilian populations.

## *Lu. longipalpis*, a species complex?

It has been more than 35 years since the first paper suggesting that *Lu. longipalpis* might be a species complex was published (*post mortem*) by Mangabeira (1969) (see also Sherlock & Sherlock 1961). Males collected from the state of Ceará (Northeast Brazil) were shown to have a pair of pale patches on the third and fourth abdominal tergites (2-spot phenotype called henceforth 2S), while those from the state of Pará (North Brazil) were shown to have pale patches only on the fourth abdominal tergite (1-spot phenotype called henceforth 1S). Mangabeira also mentioned that the two forms were found in different ecological conditions speculating that they might represent different species or varieties. Finally, considering that *Lu. longipalpis* could be a highly variable species, he suggested that *Lu. gaminarai* and *Lu. cruzi* could be either good species or regional variants of *Lu. longipalpis*. In this respect, it is worth noting that *Lu. cruzi* has been established as a *bona fide* vector for *Le. infantum chagasi* in the state of Mato Grosso do Sul, Brazil (Santos et al. 1998) and recent data supports the idea that *Lu. cruzi* might be another species of the *Lu. longipalpis* complex (Watts et al. 2005).

Mangabeira hypothesis was the starting point for subsequent three and half decades of discussion concerning the real status of the main vector of visceral leishmaniasis in Latin America (see Table). On the one hand, there are some authors that have defended the hypothesis of the existence of *Lu. longipalpis* as a single species. On the other hand, there are authors that have argued that *Lu. longipalpis* occurs as a complex of sibling species although, as mentioned before, there is no consensus on the number and distribution range of the different siblings (see Arrivillaga et al. 2003).

*Lu. longipalpis* may have once occupied a much larger area than at present and climatic changes may have resulted in the current focalised pattern of distribution (Ward et al. 1985). If true, this suggestion is in agreement, for example, with the now classic forest refugee theory of Haffer (1969). This theory proposes that during several dry climatic periods of the Pleistocene and post-Pleistocene much of South America consisted of tracts of open non-forested vegetation, interspersed with "islands" of humid forest in which speciation of many animal groups could have occurred. The isolated forests were re-united during humid climatic periods. This rupturing and rejoining of the various forests was repeated several times during the Quaternary period and led to rapid differentiation of the fauna in geologically and evolutionary relative very recent times. Based on the known habitat preferences of *Lu. longipalpis*, climatic conditions might have allowed these insects to attain the wide and discontinuous distribution currently seen (Alexander

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et al. 1998). During the dry period populations of *Lu. longipalpis* may have spread over a broader area and humid climatic periods may have separated them. Several other hypotheses have been postulated to explain species diversity in the Neotropical region, but no single model can by itself explain evolution in the tropics, (reviewed by Marroig & Cerqueira 1997).

At present, there is considerable geographical isolation among *Lu. longipalpis* populations. This may be attributed to their low mobility as well as to the extrinsic barriers that exist within their broad but discontinuous areas of distribution (Lanzaro et al. 1993, Young & Duncan 1994, Alexander et al. 1998). Isolation by distance is recognised as one of the conditions that eventually may cause species rising as a consequence of the low gene flow between the populations involved. Due to its importance as the main vector involved in the transmission of visceral leishmaniasis in Central and South America, *Lu. longipalpis* has been examined in terms of its morphological variability, male pheromones, sexual behaviour and population genetic structure (reviewed by Uribe 1999).

Table summarises all major research studies reported on the elucidation of the taxonomical status of this putative species complex. These studies collectively sampled populations distributed over a broad geographical range in Central and South America. The table shows the sample collection sites, the different study approaches employed and the main results and conclusion concerning the taxonomic status of the *Lu. longipalpis* populations (as either single species or species complex). It is interesting to observe that all studies carried out on samples from South America versus Central America have strongly suggested that *Lu. longipalpis* is a species complex (Lanzaro et al. 1993, Warburg et al. 1994, Morrison et al. 1995, Dujardin et al. 1997, Lanzaro et al. 1998, 1999, Yin et al. 1999, 2000, Soto et al. 2001, Arrivillaga et al. 2002, 2003, Watts et al. 2005). *Lutzomyia pseudolongipalpis* was formally recognized as the first cryptic species of the complex by virtue of morphological, genetic and behavioural differences when compared to several populations, including those separated by a very long distance (Arrivillaga & Feliciangeli 2001). Equally interesting from the data compiled in this table is that it is only within Brazil where broadly separated *Lu. longipalpis* populations have been considered either a single species (Mukhopadhyay et al. 1997, 1998a,b, Mutebi et al. 1999, Azevedo et al. 2000, Arrivillaga et al. 2003, de Queiroz Balbino et al. 2006) or a species complex (Ward et al. 1983, 1988, Yin et al. 1999, Souza et al. 2002, 2004, Bauzer et al. 2002a,b, Maingon et al. 2003, Bottecchia et al. 2004, Hamilton et al. 2004, 2005, Watts et al. 2005) depending on the markers used. Therefore the controversy concerning the existence of a complex is centred on data from Brazilian populations.

## *Lu. longipalpis* in Brazil, evidence for a species complex

Following the lead by Mangabeira (1969), Ward et al. (1983, 1988) published results of a number of crossing experiments that provided the first strong evidence that *Lu. longipalpis* was not a single species in Brazil. Even though this earlier work was somewhat contradicted by subsequent data from isoenzyme studies (Mukhopadhyay et al. 1998b, Mutebi et al. 1999, Azevedo et al. 2000), Ward et al. (1983) was the first study to show clear evidence that *Lu. longipalpis* was a species complex when reproductive isolation was found in crosses between some allopatric and sympatric populations (see below).

Ward et al. (1983) were, in fact, testing Mangabeira's hypothesis that the 1S and 2S phenotypes might represent different species. Studies on the distribution of *Lu. longipalpis* showed that males having pale patches only on the fourth abdominal tergite (1S phenotype) are found broadly and discontinuously distributed all over South and Central America whereas males having a pair of pale patches on the third and fourth abdominal tergites (2S phenotype) are more concentrated in the Northeast region of Brazil (Ward et al. 1985). Crossing experiments were performed between sympatric and allopatric Brazilian populations that differed by the number of tergal spots (Ward et al. 1983). Highly reduced insemination rates were observed in crosses between two colonies originated from Sobral, state of Ceará, one with 1S and the other with 2S males, suggesting that they represent different sympatric sibling species. In addition, low insemination rates were also observed between the 2S colony from Morada Nova, Ceará, and 1S sand flies from Lapinha cave and between 1S Marajó Island and 1S Lapinha. Later work on other populations (Ward et al. 1988) came to confirm and extend these earlier results and the conclusion was that *Lu. longipalpis* was in fact a species complex in Brazil.

The above studies showed that the spot morphology could not be used as a species-specific character. Indeed, an intermediate phenotype (a small spot on the third tergite in addition to the spot on the fourth tergite) was observed in high frequencies in some localities, especially around the Northeast coast, indicating an intraspecific polymorphism (Ward et al. 1988). Crosses between some 1S and 2S populations yielded mainly males carrying an intermediate phenotype strongly suggesting a semi-dominant genetic model of inheritance for this character (Ward et al. 1988). Further evidence regarding spot morphology as a genetic polymorphism was obtained by Mukhopadhyay et al. (1998a) in a visceral leishmaniasis endemic area near the city of Natal, state of Rio Grande do Norte. However, although this character is not species-specific, it may be useful to identify two sympatric species in localities where intermediates are very rare, such as Sobral (Ward et al. 1988, see below).

The existence of a complex of *Lu. longipalpis* in Brazil was substantiated by results of analysis of sex pheromones (Ward et al. 1988, Hamilton et al. 1996a,b) and male "lovesongs" that are produced during copulation (Souza et al. 2002, 2004). These two traits can have an important role in the reproductive isolation among closely related species. Interestingly, differences in both traits are strongly correlated in the Brazilian *Lu.*

TABLE

Summary of studies on the taxonomical status of *Lutzomyia longipalpis* listed in chronological order

| Reference | Location of samples | CC* | Methodologies | Results and conclusions | Taxonomic Status |
|---|---|---|---|---|---|
| Mangabeira 1969 | Russas & Icó, along the Jaguaribe river, Ceará | BR | Morphological and ecological observations carred out in 1940 | North and Northeast populations differed in their male tergal pale spot and female mouth/spermatheca phenotypes as well as in their ecology | First suggestion of a species complex |
| Ward et al. 1983 | Marajó, Sobral, Morada Nova, Lapinha | BR | Laboratory crossing experiments | At least two sexually isolated forms were identified. Insemination failure was found between two forms from allopatric and sympatric sites as well as between allopatric populations with similar tergal spot patterns | Species complex |
| Bonnefoy et al. 1986 | Yungas | BO | Isoenzyme eletrophoresis | No cryptic sibling species indication. Size variability and isoenzyme profile were not associated. Founder effect as putative cause for the low genetic variability | Single species |
| Ward et al. 1988 | São Luiz, Sobral, Morada Nova, Marajó, Jacobina, Lapinha | BR | Crossing experiments, sex pheromone analysis, and recordings of pre-mating acoustic signals | Tergal spots variation showed no relationship to pheromones types. Populations producing distinct pheromones were reproductively isolated. First report of acoustic communication in *Lu. longipalpis* | Species complex |
| Lanzaro et al. 1993 | Liberia<br>Melgar<br>Lapinha | CR<br>CO<br>BR | Isoenzyme electrophoresis and crossing experiments. | Very high level of genetic divergence among the colonies. Sterility was detected in male progeny from all inter-colony crosses | Species complex |
| Warburg et al. 1994 | Liberia<br>Melgar<br>Lapinha | CR<br>CO<br>BR | Vasodilator peptide maxadilan amount, activity and genetic polymorphism measurements | Populations differed in propensity to modulate the pathology of the disease they transmit. Females from CR produced relatively less maxadilan than samples from BR and CO. Maxadilan DNA sequences were different between population/species | Species complex |
| Morrison et al. 1995 | Lapinha, Abaetetuba<br>Northern Costa Rica<br>Melgar, El Callejón | BR<br>CR<br>CO | Isoenzyme electrophoresis | Field populations exhibited higher genetic heterogeneity than colonies. High genetic distances among populations from different countries. Little genetic differentiation among El Callejón field populations. Comparison of field and colonized individual from the same site indicated high level of divergence | Species complex over a large geographic range.<br>Single species in CO |
| Hamilton et al. 1996c | Pavana, San Juan Bautista, Orocuina, Tololar<br>Liberia | HN<br>CR | Male sex pheromone analyses. | The three different pheromones types found in Liberia population revealed the existence of two or three distinct sympatric populations | Species complex |
| Mukhopadhyay et al. 1997 | Abaetetuba Lapinha<br>Santarém, Sobral<br>Melgar | BR<br><br>CO | Isoenzyme electrophoresis | Polymorphism probably explains the observed variability among the populations analysed | Single species |
| Dujardin et al. 1997 | Apa Apa,Imanaco, Guayabal, Toro Toro<br>Minas Gerais<br>CIDEIM colony strain<br>Somotillo and Cico Pinos | BO<br><br>BR<br>CO<br>NI | Isoenzyme electrophoresis among Bolivian samples and wing morphometry among different countries samples | Low genetic distance among allopatric 1S Bolivian populations. Considerable genetic distance among 1S and 2S populations in BO. Geographic distance correlation suggests isolation by distance model and a simple intraspecific differentiation. Considerable wing morphometric divergence between two BO phenotypes and other countries populations | Species complex among different countries<br>Single species in BO |

| Reference | Location of samples | CC* | Methodologies | Results and conclusions | Taxonomic Status |
|---|---|---|---|---|---|
| Mutebi et al. 1998 | Liberia, Brasilito<br>Pavana, San Juan Bautista, Orocuina, San Francisco Coray, Isla El Tigre, Los Guatales, Rancho Grande<br>Pochomil, Las Huertas | CR<br>HN<br><br><br><br>NI | Isoenzyme electrophoresis | Analysed populations were not panmictic. Effective migration estimates indicated restricted gene flow among the populations that was high enough to prevent significant levels of genetic divergence. There was no indication of populations clustering by disease | Single species |
| Lanzaro et al. 1998 | Palo Gordo, Durania, Neiva, Melgar<br>Lapinha<br>Liberia | CO<br><br>BR<br>CR | Isoenzyme electrophoresis | Very low levels of genetic distance among field populations, in spite of being separated by geographic barriers. No significant genetic differentiation between field and colony samples. Additional support for earlier conclusions that *Lu. longipalpis* represents distinct species in CO, CR and BR | Single species in CO<br><br><br>Species complex among different countries |
| Mukhopadhyay et al. 1998a | Natal, Jacobina, Lapinha. | BR | Isoenzyme electrophoresis | No significant differences in isoenzymes frequency associated with morphological phenotype | Single species |
| Mukhopadhyay et al. 1998b | Santarém, Marajó, São Luis, Natal, Jacobina, Pancas, Lapinha | BR | Isoenzyme electrophoresis | Low genetic distances, diagnostic *loci* absence and allele's distribution in the geographic space indicated the existence of a single, but genetically heterogeneous, polymorphic species | Single species |
| Yin et al. 1999 | Jacobina, Lapinha<br>El Callejón<br>Liberia | BR<br>CO<br>CR | Metaphase karyotypes and G-banding | Different karyotype configurations between populations. Only CO and CR populations had the same karyotype formula but with different G banding pattern. Chromosome 4 homologues were heteromorphic in hybrid progeny from Lapinha and Liberia cross | Species complex |
| Lanzaro et al. 1999 | Lapinha, Marajó<br>Brasilito, Liberia<br>El Callejón, Neiva, Bucaramanga, Durania<br>Brasilito, Liberia | BR<br>CO<br><br>CR | Maxadilan gene/peptide sequence variation in colony populations. Single strand conformation polymorphism (SSCP) in natural populations | High variation in primary DNA and inferred amino acid sequence of maxadilan. SSCP analysis revealed high variation in maxadilan-encoding gene among individuals within natural populations. All maxadilan variants peptide had equivalent vasodilatory activities | Species complex |
| Mutebi et al. 1999 | Santarém, Bacabal, Camará, Salvaterra, Sobral, Itapipoca, Montes Claros, Fortaleza, Baturité, Jacobina, Lapinha | BR | Isoenzyme electrophoresis | Genotypic frequencies within populations suggested that there were no sympatric species among them. Frequency data indicated some degree of genetic substructuring | Single species |
| Lampo et al. 1999 | La Rinconada, El Paso, Altagracia, Mapire | VE | Isoenzyme electrophoresis | Fixed differences in two diagnostic *loci* revealed two sympatric reproductive isolated species. Large genetic distance between allopatric populations of different electromorphs | Species complex |
| Yin et al. 2000 | Lapinha, Jacobina<br>El Callejón<br>Liberia, Brasilito | BR<br>CO<br>CR | Quantitative RT-PCR for maxadilam mRNA | Colonies originating from South America had significantly more maxadilan mRNA than those from CR. Maxadilan mRNA polymorphism found in sibling species throughout Latin America | Species complex |

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| Azevedo et al. 2000 | Salvaterra,São José do Ribamar, Canindé, Natal, Lapinha | BR | Morphological and morphometric comparisons. Isoenzyme electrophoresis | No significant morphological differences among populations that could distinguish them. Genetic distances were within the range of intra-population differences | Single species |
| Arrivillaga et al. 2000 | Curarigua, Trujillo, Guayabita, Cojedes | VE | Isoenzyme electrophoresis | Strong genetic substructure indicating a complex of at least two subspecies | Species complex |
| Arrivillaga & Feliciangeli 2000 | Curarigua,Trujillo Guayabita, Cojedes | VE | Larval morphologic differentiation | Morphological differences between populations whose adults have shown different isoenzymatic profiles indicated genetic separation | Species complex |
| Arrivillaga & Feliciangeli 2001 | La Rinconada | VE | Analysis of morphometric characters | The first new sand fly species within the longipalpis complex (*L. pseudolongipalpis*) was described. Males were shown to be isomorphic when compared to *Lu. longipalpis s. l.*, females were shown to differ morphologically | Species complex |
| Soto et al. 2001 | Tulumajillo<br>Rancho Grande, San Francisco del Coray<br>Los Guatales, Isla El Tigre Pavana, Liberia.<br>Girón, El Callejón, Neiva<br>El Batatillo<br>Sobral, Jacobina, Lapinha | GT<br>HN<br><br><br>CR<br>CO<br>VE<br>BR | Analysis of nucleotide variation in the ND4 mitochondrial gene | Certain allopatric populations were sufficiently differentiated as to represent sibling species. Four clades were identified on specimens from northern South America, BR, Central America and an isolated CO population. Existence of sibling species within Central American and BR populations | Species complex throughout the species range |
| Souza et al. 2002 | Natal, Lapinha, Jacobina | BR | Analysis of male copulation songs | Striking differences found in copulation songs between males from three populations belonging to distinct isoenzyme groups and representing 3 kinds of pheromones | Species complex |
| Bauzer et al. 2002a | Natal, Lapinha, Jacobina | BR | Analysis of nucleotide variation in the *period* gene | Populations were highly differentiated with a very low level of gene flow between them. Evidence of sibling species in Brazil | Species complex |
| Bauzer et al. 2002b | Sobral | BR | Analysis of nucleotide variation in the *period* gene | High genetic distance between 1S and 2S phenotypes revealing two sympatric species. Comparison with Jacobina, Lapinha and Natal populations revealed high genetic distances among them with the exception of Sobral 2S and Natal. First molecular evidence of sympatric sibling species in Brazil | Species complex |
| Arrivillaga et al. 2002 | Liberia<br>Pavana<br>Curarigua, Trujillo<br>Bucaramanga, Neiva<br>Roraima, Santarém, Salvaterra,Baturite, Jacobina, Lapinha | CR<br>HN<br>VE<br>CO<br>BR | Screening for polymorphism by SSCP, using the mitochondrial cytochrome oxidase I gene | The majority of sequence variation was inter-population rather than intra-population. Phylogenetic analysis revealed four main clades. It was suggested that the speciation process began in the Pliocene during the East Andean Cordillera formation | Species complex throughout the species range<br><br>Single species in BR and Central America |
| Mutebi et al. 2002 | Bucaramanga,Palo Gordo, Neiva, Durania.<br>Brasilito, Liberia<br>Pochomil, Las Huertas<br>Orocuina, Los Guatales, | CO<br><br>CR<br>NI<br>HN | Isoenzyme electrophoresis | Allele frequencies for three *loci* discriminated three population groups (CO, Central America, and BR). Overall Fst indicated genetic substructure and restricted gene flow among populations. There was a significant negative correlation between $\log N_e m$ and geographical | Species complex throughout the species range |

| Reference | Location of samples | CC* | Methodologies | Results and conclusions | Taxonomic Status |
|---|---|---|---|---|---|
| | Pavana, San Juan Bautista, Rancho Verde, San Francisco del Coray, Tiger Island | | | distance when comparing all populations (isolation by distance scenario). Negative correlation did not hold when the data were split between comparisons made within regions and putative species. Allele frequency data for BR population were previously published (see Mutebi et al. 1999) | Single species in BR |
| | Fortaleza, Salvaterra, Bacabal, Camara, Sobral Itapipoca, Baturité Santarém, Lapinha, Montes Claros, Jacobina | BR | | | |
| Maingon et al. 2003 | Sobral | BR | Analysis of allele frequencies distribution using five microsatellite *loci* | Two phenotypically different populations overlapping the sampling site were genotyped. Samples with the same pheromone showed either no significant or low genetic differentiation. Highly significant genetic differentiation was shown between samples with different pheromones. $N_em$ estimates indicated little gene flow between the two sympatric populations | Species complex |
| Arrivillaga et al. 2003 | Brasilito, Liberia | CR | Isoenzyme electrophoresis analysis and SSCP screening using three mitochondrial genes (COI, 12S and 16S rRNA genes) | Independent sets of markers were largely concordant revealing four distinct clades. Species were defined as Brazilian Clade (represented by populations sampled throughout BR), Laran Clade (represented by populations from Northwest VE), the *cis*-Andean Clade (represented by populations in CO, VE and Northern BR) and the *trans*-Andean Clade (represented by populations from various regions of Central America) | Species complex throughout the species range |
| | Rancho Verde, Orocuina | HN | | | |
| | Tiger Island, Pavana, San Francisco del Coray, San Juan Bautista, Losguatales | | | | |
| | Pochomil, Las Huertas | NI | | | Single species in BR |
| | Bucaramanga, Palo Gordo | CO | | | |
| | Neiva, Durania, El Paso, Curarigua, Cojedes, Trujilllo. | VE | | | |
| | Pacaraima, Santarém, Baturité, Jacobina, Montes Claros, Lapinha, Fortaleza Sobral, Camara, Bacabal, Itapipoca | BR | | | |
| Hodgkinson et al. 2003 | Natal, Itamaracá, Juazeiro Monte Santo, Feira de Santana, Pancas | BR | Analysis of nucleotide variation in the mitochondrial cytochrome b gene | Statistically significant population structuring between Northern and Southern populations (and among the Southern populations). Sequence divergence was not sufficient to indicate cryptic species | Single species |
| Bottecchia et al. 2004 | Natal, Lapinha, Jacobina Sobral | BR | Analysis of nucleotide variation in the *cacophony* gene | High genetic distance among the populations. Divergence between two phenotypes from Sobral and no differentiation among Sobral 2S and Natal confirmed previous studies with the *per* gene (Bauzer et al. 2002b) | Species complex |
| Hamilton et al. 2004 | Jaiba | BR | Male sex pheromone analysis | *Lu. longipalpis* occurs as two sympatric sex pheromone chemotypes. One chemotype is the cembrene type previously recorded in Sobral 2S males and the other is a new cembrene isomer | Species complex |

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|---|---|---|---|---|---|
| Souza et al. 2004 | Natal, Lapinha, Jacobina, Sobral, Marajó | BR | Analysis of male copulation songs | Four copulatory courtship song types found in males from five localities from BR. Sobral siblings have different songs. Differences in songs correlate with genetic divergence in the *period* gene | Species complex |
| Watts et al. 2005 | Altagracia, El Layero, Guayabita, Las Cabreras, La Rinconada | VE | Analysis of allele frequencies distribution using five microsatellite *loci* | High genetic differentiation between samples. Sobral populations with different sex pheromones showed genetic distance as great as many of the comparisons between populations separated by considerable geographical distance. Weak relationship between genetic and geographic distances. Only populations with the same pheromone type showed a correlation between genetic and geographic distance. The type of sex pheromone shaped genetic structure. A phenotypically and genetically different population cluster was identified mapping to Northeastern BR | Species complex |
| | Jacobina, Lapinha, Natal, Corumba, Marajó, Sobral | BR | | | |
| Hamilton et al. 2005 | Estrela de Alagoas, Sol do Costa, Santarém, Lapinha, Montes Claros, Sobral | BR | Male sex pheromone analysis | Evidence of a fifth chemotype in BR | Species complex |
| de Queiroz Balbino et al. 2006 | São Luis, Teresina, Patos, João Pessoa, Itamaracá, Calumbi, Camaçari | BR | Random amplified polymorphic DNA - PCR (RAPD-PCR) | The expected mean heterozygosity was at least twice as high as values commonly obtained through the use of isoenzymes. Differences observed between populations were compatible with isolation by distance model | Single species |

CC: country code; BO: Bolivia, BR: Brazil, CO: Colombia, CR: Costa Rica, GT: Guatemala, HN: Honduras, NI: Nicaragua, VE: Venezuela.

*longipalpis* populations studied so far indicating the existence of at least four reproductive isolated populations in Brazil (Figure). Males from Jacobina, Lapinha, Natal, Sobral and Marajó island had both their pheromones (Ward et al. 1988, Hamilton et al. 1996a,b) and copulatory courtship songs (Souza et al. 2002, 2004) analysed. Remarkable differences among these populations were found. Males from Jacobina produce the 3-methyl-α-himachalene pheromone type and song with trains of pulses that resemble those produced by *Drosophila* ("pulse-song type 1") (e.g. Peixoto & Hall 1998) while Lapinha males produce the 9-methyl-germacrene-B pheromone type and songs with trains of essentially monocyclic pulses interrupted by highly polycyclic pulses ("pulse-song type 2") (Hamilton et al. 1996a,b, Souza et al. 2002, 2004).

Sobral 1S males also produce 9-methyl-germacrene-B that can be differentiated from Lapinha's pheromone by the amount of specific terpenes (Hamilton et al. 2005). Yet a third type of pulse-song is produced by Sobral 1S, different from Jacobina and Lapinha, in which high and low amplitude pulses alternate almost perfectly ("pulse-song type 3") (Souza et al. 2004). These three populations therefore produce different types of pulse-songs and 16C type pheromones (9-methyl-germacrene-B or 3-methyl-α-himachalene) (Figure). Males from Natal, Marajó, and Sobral 2S on the other hand showed a completely different acoustic signal made of highly polycyclic bursts that are modulated in frequency and amplitude ("burst-song"). These three populations also produce the same type of 20C pheromone (cembrene type 1) (Figure). As expected from the crossing experiment results (Ward et al. 1983, 1988), the two forms that co-




Map of Brazil with the approximate locations of *Lutzomyia longipalpis* populations for which there is data on copulatory courtship songs, pheromones, and molecular markers. The different song types (burst and pulse type 1, 2, and 3 songs) and pheromones (cemb 20C → cembrene; germ 16C → 9-methyl-germacrene-B; and hima 16 C → 3-methyl-α-himachalene) found in each population are indicated between brackets. See text for more details.

exist in Sobral were shown to be associated with different pheromone types and copulatory courtship songs.

Molecular studies using two sand fly homologues (Peixoto et al. 2001) of *Drosophila* courtship song genes, *period* and *cacophony* were also carried out to address the *Lu. longipalpis* "species problem" in Brazil. *period* is involved in the control of circadian and courtship song rhythms and it was used in a number of studies of closely related species of *Drosophila* (reviewed in Peixoto 2002). Because this gene controls song differences among *Drosophila* species that are important to their sexual isolation it is considered an example of a "speciation gene" (Coyne 1992). *cacophony* codes for an α-1 subunit of a voltage-gated calcium channel involved in the control of the *Drosophila* courtship song (Smith et al. 1998, Peixoto & Hall 1998).

Both genes were used to study the molecular divergence between the allopatric populations of Jacobina, Lapinha, and Natal (Bauzer et al. 2002a, Bottecchia et al. 2004). It was shown that these three broadly distributed populations are highly differentiated and the estimated number of migrants per generation was much smaller, particularly for the *period* gene ($N_em = 0.29$), than those estimated using isoenzymes (see below). This suggests that *Lu. longipalpis* in Brazil might be in an incipient speciation process and that isoenzymes do not evolve fast enough to detect genetic divergence among the Brazilian populations.

Bauzer et al. (2002b) and Bottecchia et al. (2004) also analysed the reproductively isolated sympatric populations of Sobral. Data from both genes confirm that the two sympatric populations were genetically distinct and very high and significant levels of differentiation were found, particularly for *period* (Fst = 0.395, $p < 0.001$; $N_em = 0.38$). Some evidence for introgression was found in the case of *cacophony*, probably due to the incomplete reproductive isolation observed in mating experiments (Ward et al. 1983, 1988). In fact, this evidence of introgression, associated with the lower evolutionary rates of isoenzymes could easily explain why these markers did not show a significant deviation from HWE in Sobral (Mutebi et al. 1999) when samples from this locality were analysed pooling together 1S and 2S males.

Highly variable microsatellite marker *loci* have been isolated from *Lu. longipalpis* by Watts et al. (2002). Maingon et al. (2003) used five of these microsatellite markers to genotype 190 field specimens captured at Sobral, in two separate years at different seasons. As mentioned, at Sobral, a 1S, 9-methyl-germacrene-B, pulse-song type 3 (with alternate high and low pulses) overlaps with a 2S, cembrene type 1 isomer, burst-song population. No temporal genetic differences were found within each of these populations. In contrast, divergent allelic frequencies (mainly at two of the five *loci*) indicated significant genetic differentiation ($\theta = 0.221$; $P < 0.05$) for comparisons between the two populations. Genetic differentiation remained high, ($\theta = 0.229$; $P < 0.001$), when temporal collections were pooled according to their spot/pheromone/song type. Estimated gene flow between the two sympatric populations was relatively low ($N_em = 0.84$), and comparable to that obtained from the analysis of polymorphisms in the *period* gene ($N_em = 0.38$), by Bauzer et al. (2002b).

Analyses of polymorphism in *period* and *cacophony* genes (Bauzer et al. 2002a,b, Bottecchia et al. 2004) also indicate that Natal and Sobral 2S are highly similar to each other and distinct from the other three populations. This is in agreement with the fact that these two populations produce the same type of pheromone and copulatory courtship song. Furthermore, a correlation seems to exist between the level of divergence in the *period* gene amongst Natal, Sobral 2S, Marajó, Lapinha, Jacobina, and Sobral 1S, and the level of phenotypic differences in the copulation songs of these six populations (Souza et al. 2004).

Watts et al. (2005) have extended their studies on the allele frequency distribution of the microsatellite markers used for Sobral to nine other Brazilian and Venezuelan populations. Temporal genetic differences were non-significant within populations collected at the same location. Geographic separation, however, influenced genetic differentiation, although the effect was relatively weak ($r^2 = 0.095$; $P = 0.0014$) when samples were pooled together irrespective of their spot/pheromone type. The correlation between genetic and geographic distances between samples increased when populations with different pheromone types were separately analysed ($r^2 = 0.541$, $P = 0.0028$ for 9-methyl-germacrene-B populations; and $r^2 = 0.965$, $P = 0.039$ for cembrene type 1 populations). A cluster analysis revealed, for the first time, a highly distinct group of Brazilian cembrene-1 populations mapping to the Northeast of the country, plus a geographically dispersed non-cembrene (9-methyl-germacrene-B and 3-methyl-α-himachalene) cluster comprising Brazilian and Venezuelan populations. Interestingly, this separation between cembrene and non-cembrene samples matchs that observed between populations with burst and pulse songs (Souza et al. 2004).

**Evidence against a complex in Brazil**

The technique most used, so far, to estimate genetic divergence among Brazilian *Lu. longipalpis* populations is the variation in isoenzyme *loci*. According to Mukhopadhyay et al. (1998b), Mutebi et al. (1999), and Azevedo et al. (2000), isoenzyme analysis failed to show a genetic distance large enough to indicate the existence of two or more species in Brazil. Nevertheless, the comparison of allele frequencies among Brazilian populations indicated a certain degree of genetic substructure. The estimated number of migrants per generation ranged from 2.0 to 3.6 in these three different studies (Mukhopadhyay et al. 1998b, Mutebi et al. 1999, Azevedo et al. 2000). As stated by the authors, these numbers suggested a level of gene flow between the Brazilian populations that would not have allowed a speciation process to occur. Although the isoenzyme data did not seem to support the existence of different sibling species in Brazil, on closer examination, the results are not inconsistent with the molecular studies mentioned above. For example, Azevedo

et al. (2000) showed that Natal and Salvaterra (Marajó island) were far more similar to each other than either is to Lapinha even though the geographical distance between the two Northern populations, Salvaterra and Natal (1550 km), is about the same as the distance between Natal and Lapinha (1700 km). These results confirmed similar observations of two other broader population analyses in Brazil (Mukhopadhyay et al. 1998b, Mutebi et al. 1999). In both studies, the populations occurring along the Northeast coast (down to the southeastern locality of Pancas, state of Espírito Santo) were shown to be genetically more homogeneous and distinct from the populations of Jacobina and Lapinha, even though the distance that separates some of the Northeastern populations is about the same as the distance that separates them from Jacobina or Lapinha. Therefore, the isoenzyme data does not agree with a simple isolation by distance model of genetic differentiation and the patterns observed are consistent with the results obtained with *period*, *cacophony,* and microsatellite *loci* (Bauzer et al. 2002a,b, Bottecchia et al. 2004, Maingon et al. 2003, Watts et al. 2005).

While the authors of the isoenzyme studies mentioned above were unanimous in their conclusion concerning the single species status of the Brazilian population of *Lu. longipalpis*, analysis of mitochondrial DNA in three different studies (Soto et al. 2001, Arrivillaga et al. 2002, Hodgkinson et al. 2003) gave somewhat ambiguous results. According to Soto et al. (2001), their results question the conspecific status of Brazilian populations. Arrivillaga et al. (2002) and Hodgkinson et al. (2003) reach a different conclusion, even though the latter study did find a significant differentiation between Northern and Southern populations in their analysis of mitochondrial cytochrome b gene sequences in six locations representing a geographic transect across Eastern Brazil.

Recently the genetic structure of seven populations from Brazil Northeastern region was described based on polymorphism analysis of 24 RAPD-PCR *loci* (de Queiroz Balbino et al. 2006). Although the detected levels of genetic variation were higher than those obtained with the use of isoenzyme markers, genetic distances were considered to be compatible with those found between members of a single species. However, inspection of the data indicates a few *loci* with very large, even fixed, differences in some comparisons. This suggests that a different conclusion would be reached if a more detailed analysis was carried out on populations where evidence for a complex was found with other molecular markers (Bauzer et al. 2002a,b, Bottecchia et al. 2004, Maingon et al. 2003, Watts et al. 2005).

### *Lu. longipalpis* in Brazil, a complex of incipient species?

It is becoming evident that gene flow and differential introgression among *loci* between closely related or incipient vector species is far more common than previously thought, as the studies on the *An. gambiae* complex and *An. gambiae s.s.* are showing (Black & Lanzaro 2001, della Torre et al. 2002, Besansky et al. 2003, Donnelly et al. 2004, Tripet et al. 2005, Slotman et al. 2005, Stump et al. 2005, Turner et al. 2005).

For *Lu. longipalpis*, it is fair to say that the available evidence for the occurrence of a complex in Brazil is overwhelming, particularly from the studies carried out in Sobral where two populations with distinct phenotypes overlap. Crossing experiments, pheromone and copulatory courtship song analysis, studies on the "lovesong" genes *period* and *cacophony*, and variation at microsatellite *loci*, all indicate the existence of at least two sympatric sibling species in this Brazilian locality (Ward et al. 1983, 1988, Bauzer et al. 2002b, Maingon et al. 2003, Bottecchia et al. 2004, Souza et al. 2004, Hamilton et al. 2005). The data for other Brazilian allopatric populations based on the same markers and types of analysis used in Sobral are also very convincing in our view (Ward et al. 1988, Bauzer et al. 2002a, Souza et al. 2002, 2004, Bottecchia et al. 2004, Hamilton et al. 2005, Watts et al. 2005). However, it is also fair to say that the differentiation among Brazilian populations, based on isoenzymes and mitochondrial *loci*, in general, is not as large as the divergence between the Brazilian and other South and Central American populations (Soto et al. 2001, Arrivillaga et al. 2003).

These inconsistencies between different markers may probably be attributed to a number of factors such as: (a) maintenance of ancestral polymorphisms caused by recent speciation events, (b) slow rates of evolution in some markers, such as isoenzymes, due to negative selection, and (c) the occurrence of introgression among these populations caused by incomplete reproductive isolation. Moreover, the levels of introgression are very likely quite different among the various *loci*, probably affecting much less those closely linked or directly involved in reproductive isolation (Stump et al. 2005, Turner et al. 2005).

There are a number of difficulties associated with the study of recently diverged species and populations in an incipient speciation process (Hey 2001, Coyne & Orr 2004) such as the members of the *Lu. longipalpis* complex within Brazil. However, in our view, the data reviewed in this paper is strong enough to indicate without any doubt, that the Brazilian populations do not belong to a single panmictic species given that there are strong reproductive barriers even among some sympatric populations. We conclude that *Lu. longipalpis* in Brazil is one example of a complex of incipient vector species showing perhaps a similar level of complexity presented by *An. gambiae s.s.* in Africa.

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