The Status of the *Lutzomyia longipalpis* Species Complex and Possible Implications for *Leishmania* Transmission

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The sand fly Lutzomyia longipalpis sensu latu *has been identified as the principal vector of American visceral leishmaniasis, a potentially fatal disease that primarily affects children in several countries of South and Central America. Over the past several years increases have occurred both in the number of reported cases and the population at risk: approximately 1.6 million people reside in highly endemic areas with 16,000 cases reported annually. Several studies have attempted to relate the epidemiology of this disease to variability in* Lu. longipalpis *that is now recognized to be a complex of at least three sibling species. Morphological variation in this species was first noted by Mangabeira (1969). Since then physiological and biochemical differences have been reported by several investigators. Recent reports in Costa Rica of the presence of* Lu. longipalpis *in a focus of cutaneous leishmaniasis caused by* Leishmania chagasi *may be an additional indication of variability in this species. While existing evidence indicates that the morphospecies* Lu. longipalpis *may represent a complex of sibling species, genetic, epidemiological and ecological distinctions have not been fully resolved. Thus, delimitation of systematic boundaries within the complex and corresponding to geographic distributions and roles in transmission remain unresolved. The purpose of this review is to summarize from the literature observations of polymorphism in this morphospecies and consider what significance this reported variability may have to the epidemiology of visceral leishmaniasis.*

Key words: leishmaniasis - *Lutzomyia longipalpis* - species complex

Lutzomyia longipalpis was originally described by Lutz and Neiva (1912) from specimens collected in the state of São Paulo and Benjamin Constant (Minas Gerais) Brazil. This species is a member of the subgenus *Lutzomyia*, which is characterized by annulated spermathecae in the female and the coxite of the male genitalia bearing simple or modified persistent setae at the base. Also, the paramere of the male genitalia often has one or more seta along the median dorsal margin (Young & Duncan 1994). *Lu. longipalpis* has an extensive distribution that extends from Mexico to Argentina. However, within this range its distribution is patchy, the species occur primarily in dry habitats in Central and northern South America although it is also associated with humid forest in the Amazon river basin (Lainson et al. 1985, Lanzaro et al. 1993, Dujardin et al. 1997). A considerable degree of geographical isolation exists between the various populations of *Lu. longipalpis*, which may be attributed to the interrelationship between its apparent limited flight range and geographical and climatic barriers (Lanzaro et al. 1993, Alexander et al. 1998, Munsterman et al. 1998). Lanzaro et al. (1993) postulated that genetic divergence caused by genetic drift and /or selection may affect vectorial capacity resulting in some populations being more efficient vectors than others.Variability among populations of *Lu. longipalpis* has been observed at several levels including morphological, isozymic, molecular and biochemical.

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MORPHOLOGICAL VARIABILITY

Morphological variation was first recognized by Mangabeira (1969) who observed that males collected from Ceará (northeastern Brazil) had a pair of pale patches on the third and fourth abdominal tergites (two-spot phenotype) while those from Pará had pale patches only on the fourth abdominal tergite (one-spot phenotype). These differences were also noted by Ward et al. (1985) in colonies established from Lapinha, near Belo Horizonte (Minas Gerais) and Morada Nova (Ceará), leading then to suggest that the two forms may represent separate taxa. Crosses between these two colonies indicated that significant reproductive isolation had occurred. An examination of males from several Central and South American countries indicated that

the one-spot phenotype was the most widely distributed, occurring in populations from Mexico to southern Brazil, while the two-spot phenotype was found only in Brazil, primarily in populations from the southern and northeastern regions of the country. Since approximately 87% of visceral leishmaniasis cases in the Americas occur in Brazil, with the majority from the northeastern states, Ward et al. (1983) suggested that the two-spot phenotype may be a more efficient vector than the one-spot form.

Ward et al. (1988) carried out a series of hybridization studies using colonies of *Lu. longipalpis* which had been established from several different localities in Brazil. These studies suggested that barriers to insemination existed between the two phenotypes in both allopatric and sympatric situations and consequently that two morphologically distinct species existed. However, additional studies failed to corroborate these findings and showed that fertile crosses could occur between populations characterized as having the one-spot and the two-spot phenotypes (Ward et al. 1988). Recent work by Dujardin et al. (1997) indicates that the "two spot phenotype" also occurs in Paraguay and Bolivia and that the pale patch character cannot be related to transmission efficiency. Also Mukhopadhyay et al. (1998a) using isozymes to study populations from Brazil, determined that the spot phenotype appears to be a polymorphic character not related to genetic isolation or differentiation at the species level.

MALE PHEROMONES AND SEXUAL BEHAVIOUR

Electron microscopy of the pale patches located on the third and fourth abdominal tergites in males revealed the presence of vacuolated glands which Lane and Ward (1984) interpreted as having a pheromone-secreting function. Analysis by gas chromatography of extracts from the vacuolated glands revealed the presence of two different molecules associated with sexual communication in *Lu. longipalpis*. The terpenoid pharnasene homopharnasene ($C_{16}H_{26}$) was found in some populations and the diterpenoid ($C_{20}H_{32}$) in others (Lane et al. 1985). Although there was no direct relationship between the type of pheromone secreted by males and the two different phenotypes, it was observed that specimens that produced the same pheromone were reproductively compatible, whereas those with different pheromones were reproductively isolated (Ward et al. 1988).

Complementary studies with the same *Lu. longipalpis* populations (Ward et al. 1988) showed additional mating behavioral differences including intensity of wing vibration and wingbeat frequency in males of different populations, which suggest that these aspects might act as reproductive isolation factors similar to those described in other insects (Ewing 1977, Ward et al. 1988). Courtship songs and displays have gained widespread use in taxonomy as reliable characters for separating closely related species and it has been observed that genetic isolation among subspecies or sibling species in insects may be accompanied by behavioral differences in species recognition (Salomon 1989, d'Winter & Rollenhagen 1990, Quicke 1993, Futuyma 1998).

Based on analysis of sexual pheromones secreted by males from Honduran and Costa Rican populations, Hamilton et al. (1996a) suggested that two or three different populations of *Lu. longipalpis* occur in this region. The terpene components analyzed by High Performance Liquid Chromatography (HPLC) showed that populations from Honduras produce 9-metylgermacrene-B while those from Costa Rica produce three different types of terpenes: 9-methyl germacrene B, a novel homosesquiterpene, and a small amount of diterpene.The populations in Hamilton's study were from areas endemic for atypical cutaneous leishmaniasis or mixed atypical cutaneous and visceral leishmaniasis caused by *L. chagasi*. However, no association was found between disease manifestation and the type of pheromones produced by different populations of *Lu. longipalpis*.

Recent studies have indicated that there are at least three different types of sex pheromone produced by Brazilian *Lu. longipalpis* (Hamilton et al. 1996 b,c). The results of laboratory experiments indicate that the females are only attracted to the pheromone type produced by conspecific males (Hamilton et al.1996 b,c, Alexander et al. 1998).

ISOZYMIC VARIABILITY

The technique most often used to estimate genetic divergence between populations of *Lu. longipalpis* is that of genetic variation loci which codify for isoenzymes. Initially, Bonnefoy et al. (1986) analyzed the genetic structure of some *Lu. longipalpis* populations from Bolivia using ten loci from a group of individuals with different body sizes. They found that only 2% of the enzyme loci were variable and that mean heterozygosity was 3.7%, thus revealing no evidence of speciation. Lanzaro et al. (1993) observed high genetic polymorphism levels between colonies that originated in Brazil (Lapinha), Colombia (Melgar), and Costa Rica (Liberia), showing that 14 of 27 loci studied had two or more polymorphic alleles and a characteristic allele was found in each one of the colonies. The genetic divergence levels between the different colonies estimated as genetic distance with Nei's method were high, ranging from 5.7 to 7.1;

similar results of genetic divergence were obtained by Morrison et al. (1995) and Mukhopadhyay et al. (1997b) for colonies with the same origins.

Based on results obtained in their experiments Lanzaro et al. (1993), Warburg et al. (1994) and Lanzaro and Warburg (1995) differentiated three distinct species with origins in Brazil, Colombia and Costa Rica. They also postulated that the different forms of the disease, atypical cutaneous in Costa Rica and visceral in other countries of South and Central America, might be transmitted by two different species of the *longipalpis* complex, because the *L. chagasi* strains isolated from these regions were indistinguishable by traditional means. In addition, *Lu. longipalpis* was considered to be the predominant vector in the cutaneous focus.

Morrison et al. (1995) examined possible genetic differences between *Lu. longipalpis* comparing specimens from four colonies (Lapinha and Abaetetuba in Brazil, northern Costa Rica and Colombia) and another five different field sites in Colombia which were located at between 1 and 25 km from the place where the Colombian colony originated. Although genetic divergence levels were low between the specimens, heterogeneity was higher in field populations than within individual colonies; of 15 loci studied, three were monomorphic in all the samples while 12 were polymorphic and some were diagnostic for only one population. Also it was determined that genetic distances between the populations of *Lu. longipalpis* sampled were positively correlated to geographical distance. These results were consistent with those obtained previously by Lanzaro et al. (1993) who proposed the existence of three sibling species from these countries.

An interesting finding in this study was the high genetic distance between those *Lu. longipalpis* specimens from the Colombian colony, which was established from specimens collected at El Callejon (near Melgar, Tolima) and those collected from same site four years later, suggesting rapid genetic homogenization in the colony (Morrison et al.1995).

In complementary studies with the Colombian populations from El Callejon, Munsterman et al. (1998b) analyzed the genetic variability of *Lu. longipalpis* from five different sites in order to infer population genetic structure and its dispersion. They found 12 out of 15 loci studied to be polymorphic, and the rate of polymorphic loci in the populations ranged from 4.8% to 7.3%. Genetic distance values among the populations were very low ranging from 0.001 to 0.0007 and gene flow estimates based on FST (coefficient of genetic variation) indicated high levels of gene flow among local populations and minimal population substructuring.

The influence of colonization on genetic structure, and how this may relate to comparisons made between laboratory colonies, has been the subject of considerable discussion (Lanzaro et al. 1993, Munsterman 1994, Lanzaro & Warburg 1995, Dujardin 1997).

Mukhopadhyay et al. (1997) compared the genetic profiles of 14 loci in five colonies of *Lu. longipalpis* from different geographical regions with those from several field populations in Brazil. They found genetic heterogeneity to be less in the laboratory colonies that in field collected specimens, and concluded that laboratory colonies represent just a sample of the genome present in field populations. In the study where they proposed that *Lu. longipalpis* was comprised of at least three sibling species, Lanzaro et al. (1993) took into consideration that divergence levels in laboratory populations might be affected by the colonization process; however, they discounted its importance since each colony had been established just a few years previously with eggs from over 100 field-collected females. Also, their estimates of heterogeneity were all greater than 0.037, which was the only known value published for natural populations of *Lu. longipalpis* (Bonnefoy et al. 1986).

Isozymic systems also were used by Dujardin et al. (1997) to assess genetic relationships between the one and two-spot phenotypes of *Lu. longipalpis* in Bolivia. Based on an analysis of ten isozymes, they concluded that genetic difference (Nei's method) was correlated with geographic distance, and therefore, an "isolation by distance " model best explained the population pattern of *Lu. longipalpis*. These authors also indicated that more studies were needed on genetic variability within and between natural populations (rather than on insectary colonies) to better interpret the results of isozyme electrophoresis data. In addition, Dujardin et al. (1997) and Tabachnick and Black (1995) argue that Nei's genetic distance cannot be used to determine species *per se*. Dujardin et al. (1997) also compared populations from Bolivia (both the one and two-spot phenotypes), Brazil, Colombia, Nicaragua, and Honduras on the basis of five morphometric characters associated with the wing. The resulting analysis placed the Colombian flies as a distinct group; placed the Bolivian flies in second group, which was further for subdivided into the one and two-spot phenotypes and placed the Nicaraguan and Brazilian flies in a third group that also was subdivided. The distinctiveness of the Colombian species was thought to be due, at least in part, to the fact that they were from a laboratory colony. However, additional analysis led them to conclude that the Colombian population could represent a distinct species as previously proposed

by Lanzaro et al. (1993). Also in agreement with Lanzaro et al. (1993), the Nicaraguan and Costa Rican specimens were considered to represent a distinct species.

Mutebi et al. (1998) also used isoenzymes to study eleven populations of *Lu. longipalpis* from Central America in an attempt to detect the presence of sibling species in that region. However, they found no evidence based on ten enzyme loci of speciation among the populations they sampled from Costa Rica, Honduras and Nicaragua. Also, they were unable to detect any isozyme profile linking disease manifestation (visceral or atypical cutaneous) and the multilocus isozyme profile in the flies. These findings differ from those of Hamilton et al. (1996 a) that differenciated two or three Central America populations on the basis of distinctive pheromone production. They also do not support the theory proposed by Lanzaro and Warburg (1995) that different clinical manifestations of *L. chagasi* are related to different species/subspecies of the *Lu. longipalpis* complex. Although Warburg et al. (1994) have shown that the saliva of *Lu. longipalpis* from different geographical regions of Central America differs in composition and its capacity to enhance *Leishmania* infections, the significance of this variation with regard to speciation in *Lu. longipalpis* and transmission of the different clinical manifestations of *L. chagasi* remains uncertain.

Also further studies are needed to resolve conflicting evidence based on isozyme analysis indicating one *Lu. longipalpis* species in Brazil, and sex pheromone analysis indicating three species in this country (Hamilton et al. 1996 b,c, Alexander et al.1998, Mukhopadhyay et al. 1998 b).

MOLECULAR DATA

Molecular based techniques are now beginning to be used to address the issue of speciation in *Lu. longipalpis* (Lanzaro et al. 1993, Warburg et al. 1994, Lanzaro & Warburg 1995). Also, the polytene chromosomes of larval salivary glands of *Lu. longipalpis* have been described, but their fragility has deterred cytogenetic analysis and comparisons (Lanzaro & Warburg 1995). Retrotransposons similar to those described in *Drosophila melanogaster* have been identified in phlebotomine sand flies, and their presence in *Lu. longipalpis* may provide a useful genetic marker for differentiating populations or sibling species (Booth et al. 1994, 1995).

Recent studies using various portions of the mitochondrial genome (Dotson et al. 1998 unpublished, Uribe et al. 1998 a,b) support the premise that the morphospecies *Lu. longipalpis* represents a complex of sibling species. The work of Dotson et al. confirms, on the basis of restriction fragment length polymorphism analysis (RLFP), earlier work by Lanzaro et al. (1993) indicating that high polymorphism exists between Colombian, Brazilian and Costa Rican populations. Thus, the restriction enzyme Alu I produced polymorphisms that differentiated all three of the populations, and RLFP analysis using Dra I and Hae III detected both within – and between – population differences.

Variation within mitochondrial haplotypes of *Lu. longipalpis* from Honduras, Guatemala, Costa Rica, Colombia, Venezuela and Brazil also corroborated differences between Central and South American populations that were previously detected by isozyme analysis. In addition, sequence divergence was found to be higher among Colombian and Brazilian populations than among Central American populations; thus supporting Mutebi's et al. (1998) study, which failed to detect significant variation between Central American populations. Mitochondrial haplotypes showed high levels of polymorphism among Brazilian populations indicating Sobral in Ceará and Jacobina as very distantly related populations. Groupings between Lapinha and Minas Gerais were also observed (Uribe 1998b). Results of mitochondrial DNA agree with those obtained through chemical analysis of male sex pheromones suggesting that in Brazil there are at least three distinct groups of *Lu. longipalpis* producing different types of sex pheromone (Hamilton et al. 1996 b,c, Alexander et al. 1998).

Mitochondrial (mt) DNA is maternally inherited and tends to evolve rapidly so that haplotype variations may reflect evolutionary processes leading to speciation such as dispersal and subsequent fragmentation of the environment (Beard et al. 1993). When this is taken into consideration along with the relative ease with which it can be amplified via PCR, mitochondrial DNA appears to be a useful source of phylogenetic information, especially for evaluating sibling species complexes, as recently shown by Esseghir et al. (1997).

The molecular studies currently carried out on the *Lu. longipalpis* complex and *Lu. evansi*, the alternate vector of visceral leishmaniasis (Montoya 1996) will be helpful in defining phylogenetic relationships among the distinctive populations already identified by isozyme analysis and in delimiting their distributions. Considering the expansive yet disjunct distribution of *Lu. longipalpis sensu lato*, additional comprehensive molecular based studies will likely result in the discovery of additional taxa in this complex. Also, molecular techniques may prove helpful in evaluating the various sibling species of *Lu. longipalpis* with regard to their ability to transmit *L. chagasi* and to produce the different clinical forms of the disease.

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