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Comparison of wing geometry data and genetic data for assessing the population structure of *Aedes aegypti*

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ABSTRACT

Aedes aegypti is the most important vector of dengue viruses in tropical and subtropical regions. Because vaccines are still under development, dengue prevention depends primarily on vector control. Population genetics is a common approach in research involving *Ae. aegypti*. In the context of medical entomology, wing morphometric analysis has been proposed as a strong and low-cost complementary tool for investigating population structure. Therefore, we comparatively evaluated the genetic and phenotypic variability of population samples of *Ae. aegypti* from four sampling sites in the metropolitan area of São Paulo city, Brazil. The distances between the sites ranged from 7.1 to 50 km. This area, where knowledge on the population genetics of this mosquito is incipient, was chosen due to the thousands of dengue cases registered yearly. The analysed *loci* were polymorphic, and they revealed population structure (global $F_{ST} = 0.062$; $p < 0.05$) and low levels of gene flow (Nm = 0.47) between the four locations. Principal component and discriminant analyses of wing shape variables (18 landmarks) demonstrated that wing polymorphisms were only slightly more common between populations than within populations. Whereas microsatellites allowed for geographic differentiation, wing geometry failed to distinguish the samples. These data suggest that microevolution in this species may affect genetic and morphological characters to different degrees. In this case, wing shape was not validated as a marker for assessing population structure. According to the interpretation of a previous report, the wing shape of *Ae. aegypti* does not vary significantly because it is stabilised by selective pressure.

1. Introduction

Aedes (*Stegomyia*) *aegypti* (L.) is native to afrotropical areas and has been spread worldwide by human activities (Belkin, 1962). Its presence and dispersal are considered a public health concern because this species is recognised as the main vector for yellow fever, chikungunya and dengue viruses. Approximately 50 million cases of dengue occur annually worldwide (WHO, 2011), and approximately 80% of the cases in the Americans were reported in Brazil during the 1990s (Schatzmayr, 2000).

Reducing *Ae. aegypti* population is the primary way to fight dengue viruses because no efficient therapies are available and a vaccine for dengue is still under development. Strategies to control mosquitoes have been developed for decades, including chemical and biological insecticides, and, more recently, transgenic mosquitoes (Speranca and Capurro, 2007; Yakob et al., 2008).

Although *Ae. aegypti* was eradicated in Brazil in the 1950s, it was re-introduced in the 1970s (SUCEN, 2011). Since then, it has become the principal vector for dengue viruses in this country. This mosquito is currently thoroughly distributed across the entire country (SVS, 2010). Further aggravating this situation in Brazil, cases of insecticide resistance and high vectorial capacity for dengue viruses have also been reported for these mosquitoes (Braga et al., 2004; Da-Cunha et al., 2005; Macoris et al., 2003, 2007). Moreover, disordered urbanisation and human transit facilitate the spread of vectors and the associated viruses (Gubler, 1998; Herrera et al., 2006).

Understanding the dispersal of *Ae. aegypti* is a central question in surveys focusing on controlling the vector because vector dispersal is a major determinant of the spread of pathogens (Wang et al., 2001; Costa-Ribeiro et al., 2006; Urdaneta-Marquez and Failloux, 2011). Equally important is the quantification of the exchange of individuals between demes. Studies carried out in Brazil have shown that imagoes do not disperse widely. The males and females usually travel up to 100 and 500 m, respectively, if sufficient blood sources and oviposition sites are available. However, in the absence of such resources, they are capable of flying longer distances (Maciel-de-Freitas and Lourenço-de-Oliveira, 2009).

Dispersal can also be indirectly estimated by population genetics approaches. Microevolutionary patterns may be informative for

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estimating gene flow between locations of epidemiological relevance (Lourenço-de-Oliveira et al., 2004; Costa-Ribeiro et al., 2007). The description of the population structure and genetic dissimilarities within this anthropophilic and synanthropic species permits the evaluation of the influence of human activities on the movement of mosquitoes (Costa-Ribeiro et al., 2006; Scarpassa et al., 2008).

Among the classical genetic markers used in population characterisation are SSRs (simple sequence repeats), which are also known as microsatellites. These microsatellites are often sensitive to genetic fluctuations that have occurred within short periods or over small geographical scales. Another promising biological marker is wing morphology. Wing shape in insects is heritable, is evolutionarily informative and can be described by morphometrics (Bitner-Mathé and Klaczko, 1999; Dujardin, 2008). Using wing morphologies to determine population structure has been encouraged by some authors who consider this method to be a low-cost alternative for the preliminary estimation of population structure (Dujardin, 2008; Morais et al., 2010).

The concomitant use of genetic data for validating the shape metrics in population structure investigations may be useful in preliminary surveys. Wing shape variation has been increasingly documented in *Ae. aegypti* (Dujardin et al., 2010; Henry et al., 2010); however, comparisons including genetic data obtained from the same sample sets are still scarce. Among the few attempts to decipher the association between morphological and molecular variations, Paupy et al. (2010) showed that body peculiarities of *Ae. aegypti formosus* do not reflect the population genetic structure. Morphological variability can be measured by the Q_{ST} index. In some cases, the values approximate the genetic F_{ST} index. Despite the usefulness of the Q_{ST} index, employment of this index is quite rare.

To evaluate the potential of wing morphometric analysis for population genetic studies of *Ae. aegypti* on a microgeographic scale, we compared the morphometric output to the genetic information yielded by analysis of five microsatellite *loci*. Both analyses were performed for the same population samples collected from four sites that ranged from 7.1 to 50 km away from each other in São Paulo, Brazil. This city is a large urban agglomeration with 8974 registered autochthonous dengue cases in 2010 (CVE, 2011).

2. Materials and methods

2.1. Mosquito samples

Brazilian samples of *Ae. aegypti* mosquitoes were collected from four sites in the greater São Paulo area (Fig. 1 and Table 1): Butantã (BUT), a district situated to the west of the city of São Paulo, and Guarulhos (GUA), Osasco (OSA) and Suzano (SUZ), which are three municipalities situated in the metropolitan area of São Paulo. These four locations were chosen due to their epidemiological relevance. Autochthonous cases of dengue in 2010 numbered 189, 1206, 316 and 3 at each site, respectively (SMS, 2011; CVE, 2011). The two closest sites are BUT and OSA, which are separated by a distance of 7.1 km. The maximal distance was 50 km between OSA and SUZ. Despite the long distances between some locations, all of them are placed in a single urban patch and are interconnected by a dense and homogeneous street network. To avoid sampling sibling individuals at the different sites, larvae and pupae were collected from houses that were least 200 m apart. In each home, at least two domestic water containers were sampled when available. Even without this precaution, the likelihood of collecting a sibling-enriched sample was low because females tend to avoid laying all of their eggs in the same container when others are available (Colton et al., 2003). Containers consisted of artificial water receptacles that were naturally filled with approximately 500 ml of rainwater. Immature stages of the mosquitoes were kept in the laboratory under standard conditions of temperature and humidity (25 ± 1 °C; 80 ± 10%), and the emerging adults were species-identified and stored in liquid nitrogen. All individuals subjected to genetic analysis ($n = 116$) were also subjected to morphometric analysis ($n = 210$; Table 1).

Fig. 1. Partial geographic map of Brazil (left) and the greater São Paulo (right) showing the borders of some municipalities. Black circles indicate locations where *Ae. aegypti* populations where collected and letters represent the respective toponyms: **B**utantã, **G**uarulhos, **O**sasco, **S**uzano.

2.2. Geometric morphometric analysis

Wings were detached from the thorax and mounted with Entellan (Sigma, St. Louis, MO, USA) on a microscope slide with a coverslip. Images of wings were captured with a Leica 320 digital camera coupled to a Leica S6 stereoscope with plain optics, which eliminated image aberrations. On these pictures of Brazilian mosquitoes, 18 landmarks (Fig. 2) were digitised using the TPSdig V.1.40 software (Rohlf, 2006). Additional wing pictures of *Ae. aegypti* specimens from other countries, including the United States of America (USA), Colombia (COL) and Thailand (THA), were taken from the CLIC image bank (http://www.mpl.ird.fr/morphometrics/; Henry et al., 2010), and their landmarks were digitised and included in some analyses.

Standard procedures for geometric morphometric analysis were employed as follows. Global wing size was assessed from the isometric estimator centroid size (Bookstein, 1991) that was derived from the coordinates, obtained using the TpsRelw 1.44 program (Rohlf, 2006). The sizes were then statistically compared between samples using a parametric ANOVA test. The generalised least-squares Procrustes superimposition algorithm (Rohlf, 1996) was used to produce shape variables (partial warps), and the principal components (relative warps; Bookstein, 1991) were used to compare population samples. To assess the degree of similarity between populations, pairwise Mahalanobis distances between populations were calculated using PAD software (Dujardin, 2002) and plotted in neighbour-joining trees using the PHYLIP package (Felsenstein, 2005). To statistically validate the comparisons, the significance of the metric disparity of the partial warps between populations (Brazilian and foreign samples) and the Q_{ST} (quantitative differentiation) estimates were tested by nonparametric permutation tests (2000 iterations each) using COV software (Dujardin and Slice, 2006).

Pooled individuals of Brazilian samples were reclassified according to their similarity to each group using the Mahalanobis distances as estimators of metric distance. Distances were computed on discriminant axes estimated without the individual (wing) to be classified. The individual was only introduced afterwards (validated classification, PAD software Dujardin, 2002). Voucher specimens were deposited in the Butantan Institute insect collection (São Paulo, Brazil), and wing images were deposited in the CLIC image bank.

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Table 1
Sampling information for *Aedes aegypti* in São Paulo.

Sampling site	Geographic coordinates	Homes sampled	Containers per home	Gender	(Morphometrics)		(Genetics)	Collection months (2009)
					Individuals	Wings	Individuals	
Butantã	23.566° S 46.719° W	2	5	M F	34 30	53 42	15 15	April to June
Guarulhos	23.463° S 46.521° W	3	4	M F	37 38	61 64	12 18	May
Osasco	23.523° S 46.806° W	6	5	M F	27 25	40 35	16 14	May
Suzano	23.501° S 46.340° W	3	3	M F	9 10	13 13	15 11	June and July

F: Females; M: Males.

2.3. Microsatellite analyses

DNA extractions were performed using imagoes according to the method from Jowett (1986), and DNA pellets were resuspended in 20 μL of MilliQ H_2O before being stored at −20 °C until the microsatellite analyses. Individual genotypes were scored at the five microsatellite *loci* that were described by Huber et al. (2001): 38/38, 34/72, C2A8, T3A7 and AED19. DNA amplification was performed using polymerase chain reactions in AG-22331 thermocycler (Eppendorf, Hamburg, Germany). A total volume of 20 μL for the final reaction contained 1× buffer (Thermo Scientific, Vilnius, Lithuania), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmol of each primer, 0.25 U Taq polymerase and 2 μL of DNA. The PCR conditions were as follows: 5 cycles of 2′ at 96 °C, 30″ at the annealing temperature (*Ta*) and a 1′15″ extension at 72 °C; 25 cycles of 30″ at 95 °C, 30″ at *Ta* and 1′15″ at 72 °C; and a final elongation step for 5′ at 72 °C.

The PCR products were labelled with the fluorescent dyes HEX, TAMRA and FAM (Bioneer, Alameda, CA, USA), and the allele size was read on an ABI 3730 automatic sequencer (Applied Biosystems, Foster, CA, USA) using Gene Scan 500 ROX (Applied Biosystems) as the molecular standard. Microsatellite alleles were scored using the GeneMarker software package (Softgenetics).

Population genetic parameters were computed according to the method described by Nei (1973). Genotypic differentiation was tested for each population by a parametric *t*-test. Deviations from Hardy–Weinberg equilibrium (HWE) and genetic differentiation indices were assessed using GENEPOP V4.0 software (Raymond and Rousset, 1995). Significance levels for multiple testing were corrected using the Bonferroni procedure (Rice, 1989). Frequencies of null alleles were estimated using GENEPOP V4.0 (according to Dempster et al., 1977). F_{IS} and F_{ST} coefficients were estimated as described by Weir and Cockerham (1984) and tested for statistical significance with exact tests using GENEPOP V4.0.

Gene flow was estimated from F_{ST} statistics, where $Nm = ((1/F_{ST}) - 1)/4$, assuming an infinite island model (Wright, 1965). Genetic isolation by geographic distance was tested by estimating correlations between $F_{ST}/(1 - F_{ST})$ and $\log_{10}$-transformed geographic distances.

3. Results

3.1. Morphology

Wing size is a plastic trait and may be influenced by either endogenous or environmental factors (Dujardin, 2008). Owing to their limited evolutionary informativeness, size parameters are shown only for descriptive purposes in the supplementary data (Fig. S1; Table S1).

The relative warps in the wing shape did not reveal clear differentiation between the BUT, GUA, OSA and SUZ population samples (Fig. 3). Some wing shape variation was detected within and between localities, and part of this variation occurred at landmarks 14–18, as exemplified in Fig. 4. Validated reclassification scores (Table 2) were only slightly higher than those expected by chance. Cluster analysis of pooled individuals from the four samples using Mahalanobis distances did not reveal a clear structure (not shown).

The shape metric disparity was not significant when the four Brazilian samples were compared pairwise. When compared with *Ae. aegypti* from the other three countries, only the USA-OSA pair exhibited significant disparity in wing shape ($p < 0.05$). The Mahalanobis distances of shape between the seven samples (Fig. 5A) showed that shape dissimilarity was higher internationally. The mean quantitative differentiation (Q_{ST}) calculated for all relative warps from all populations was 0.4103 for females and 0.4118 for males.

3.2. Genetics

All *loci* were polymorphic, showing a number of distinct alleles: six for 38/38; five for T3A7, 34/72 and C2A8; and four for AED19. In total, 25 alleles were visualised with a mean of five alleles per *locus* (Table 3). Some private alleles were detected, including allele 160 (*locus* AED19, GUA, frequency 3.3%), allele 237 (*locus* T3A7, GUA, frequency 1.7%), allele 90 (*locus* 38/38, SUZ, frequency 44.2%) and allele 88 (*locus* 38/38, OSA, frequency 6.7%).

Twenty-five per cent of the analysed *loci* showed significant deviations from HWE, as evidenced by chi-squared analysis (Table 3). Of these, 80.0% of the *loci* showed heterozygote deficits, whereas the remaining 20.0% showed heterozygote excesses. More detailed data are available at in the supplementary data (Table S2).

Population genetic differentiation estimated through F_{ST} reached a mean value of 0.0622, indicating population stratification ($p < 0.05$). A neighbour-joining tree of genetic distances between populations indicated that SUZ was the most divergent (Fig. 5B). Gene flow was low because $Nm = 0.47$ individuals per generation (Table S3).

Locus T3A7 deviated from HWE in two populations and has a high homozygote frequency, which might be due to the presence of null alleles. To minimise the influence of this type of possible artefact, genetic analyses were performed again without this *locus*. This round of analysis demonstrated very similar genetic parameters (distance, gene flow, differentiation; data not shown) as the analysis performed with five *loci*.

4. Discussion

4.1. Interpreting morphological and genetic data

When examining the wing shape, the relative warps, Mahalanobis distances and the reclassification tests indicated a slight population differentiation. However, as with the metric disparity

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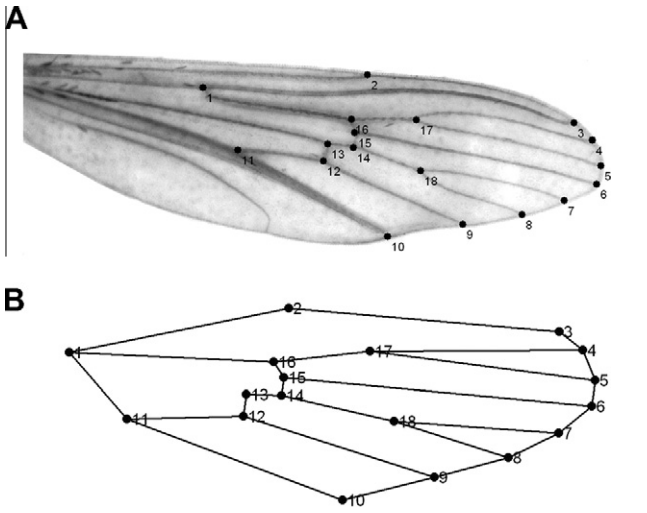

Fig. 2. (A) Wing of *Aedes aegypti* showing the chosen 18 landmarks; (B) Imaginary geometric diagram representing the portion of wing considered in this study.

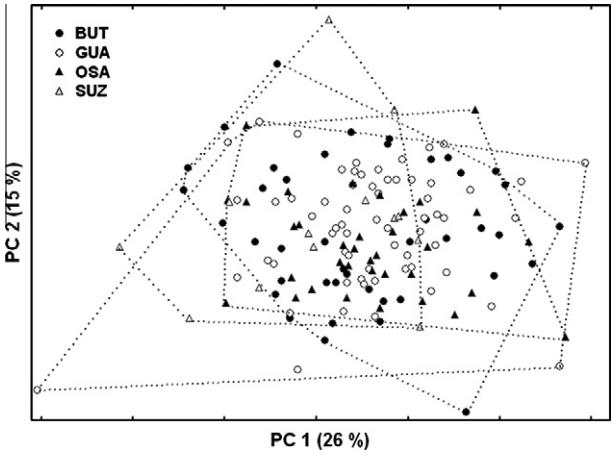

Fig. 3. Morphological space of 1st and 2nd principal components (PCs) derived from wing shape comparison among the four populations (females only). Between brackets, the relative contribution of each component.

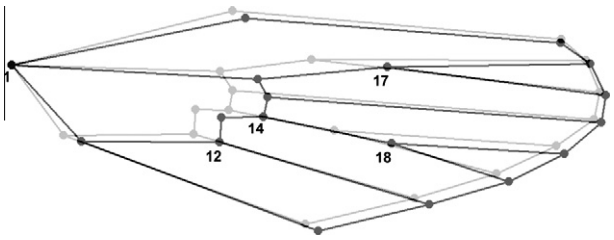

Fig. 4. Superimposition of wing diagrams of two individuals located at extreme values of the 2nd principal component (from Fig. 3). In black is BUT, in gray is SUZ. Isometric and rotation divergences were discarded and diagrams were superimposed on landmark 1.

Table 2
Scores of reclassification tests, after validation.

Sample	Females (%)	Males (%)
Butantã	38	50
Guarulhos	48	47
Osasco	68	60
Suzano	38	38

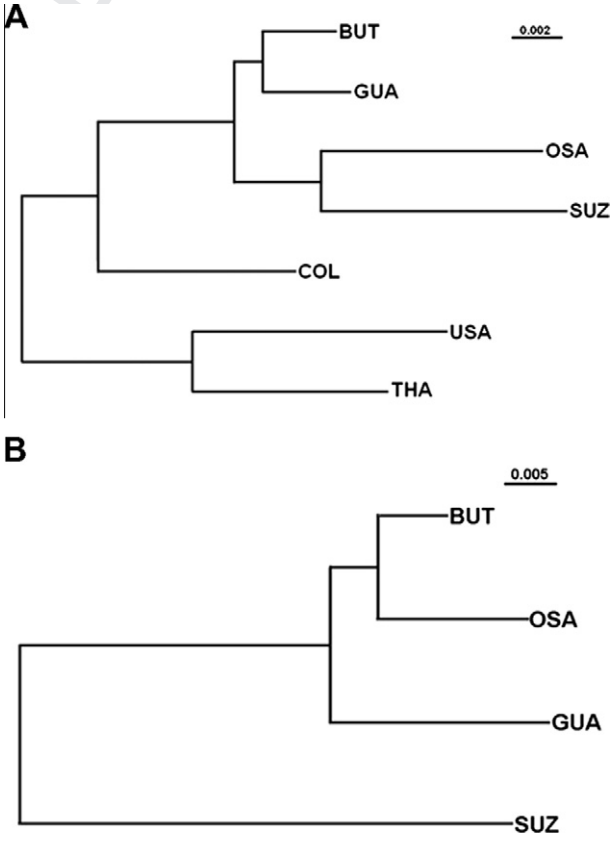

Fig. 5. Neighbour-joining dendrograms. (A) Mahalanobis pairwise distances among samples describing shape divergencies; (B) Genetic distances [$F_{ST}/(1 - F_{ST})$].

assay, these tests failed to detect any significant differences among the tested Brazilian samples.

When examining the microsatellites, the population structure was revealed by the moderate genetic differentiation (mean $F_{ST} = 0.0622$) between the BUT, GUA, OSA and SUZ populations, according to Wrights criterion (1978). Additionally, the low gene flow and the presence of population-private alleles indicate that the *Ae. aegypti* populations in São Paulo have diverged, a result that is consistent with those of similar survey that was conducted in other localities in Brazil (Costa-Ribeiro et al., 2006). Other molecular markers pointed to analogous results in larger geographical scale (Ayres et al., 2003; Bracco et al., 2007; Paduan and Ribolla, 2008; Scarpassa et al., 2008; Urdaneta-Marquez and Failloux, 2011).

Departures from HWE detected within each population were not all the result of the same effect. OSA, for instance, presented excess and mainly deficit of heterozygotes. Endogamy could not explain the deficit because such skewness was not observed in all *loci*. The deficit of heterozygotes may be suggestive of the Wahlund effect, as hypothesised by others (Costa Ribeiro et al., 2006; Bello and Becerra, 2009). This possibility is compatible with our method of collecting larvae across multiple breeding containers, which could have lead us to sample more than one single populational deme. Another possibility is that some *loci*, such as the T3A7 *locus*, contain null alleles (Costa-Ribeiro et al., 2006). In fact, high frequencies of null alleles were estimated for T3A7 in OSA (0.43) and BUT (0.38). In the particular case of the T3A7 *locus*, the possible null alleles did not influence the final interpretation, as demonstrated by the similarity in the analysis performed with or without

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Table 3
Genetic variability at five microsatellite *loci* of *Aedes aegypti* from São Paulo state.

Locus	Population	No. of alleles	Observed heterozygosity	Expected heterozygosity	F_{IS}	*P*
AED19	Butantã	3	20.0	18.8	−0.067	1.0000
	Guarulhos	4	36.7	37.3	0.0185	0.6580
	Osasco	3	60.0	51.3	−0.173	**0.0000**
	Suzano	3	23.0	33.5	0.317	0.0130
T3A7	Butantã	4	46.7	74.1	0.374	**0.0007**
	Guarulhos	5	53.3	76.1	0.303	0.0031
	Osasco	4	36.7	73.0	0.502	**0.0000**
	Suzano	4	69.2	65.4	−0.06	0.0460
38/38	Butantã	4	26.7	37.7	0.295	0.0050
	Guarulhos	2	0	6.5	1	0.0180
	Osasco	5	23.3	49.5	0.5333	**0.0005**
	Suzano	3	65.4	52.4	−0.2537	0.1710
34/72	Butantã	5	66.7	63.8	−0.046	1.0000
	Guarulhos	5	80.0	63.4	−0.267	0.0065
	Osasco	5	73.3	62.0	−0.187	0.5095
	Suzano	4	23.1	33.9	0.323	0.0343
C2A8	Butantã	4	83.3	69.7	−0.199	0.0449
	Guarulhos	4	83.3	72.7	−0.149	0.0093
	Osasco	4	83.3	68.7	−0.2175	0.0907
	Suzano	5	69.2	73.5	0.06	**0.0000**

F_{IS}: inbreeding coefficient. In bold, significant *P*-values (= 0.05) after Bonferroni correction rejecting Hardy–Weinberg equilibrium.

the inclusion of this *locus*. However, there is no discrepancy between the present genetic results and those reported elsewhere.

4.2. Comparing the two approaches

At first glance, the present results of the genetic and morphological markers could appear contradictory because the genetic markers revealed differentiation of the populations, whereas the morphological markers point to population similarity. Although both types of markers are useful for assessing microevolutionary patterns, the wing shape was quite conserved, and the microsatellites were more sensitive to microevolutionary processes.

In the present study, five microsatellite *loci* were used, which is less than what was used in some comprehensive surveys on population genetics of *Ae. aegypti* (Muturi et al., 2010; Brown et al., 2011). Although the use of only five *loci* limits the conclusions that can be drawn, our primary aim of comparing the resolution power of the two types of analysis was not jeopardised by the use of only five *loci*.

Wing shape has been shown to be diagnostic for populations of *Culex quinquefasciatus* in South America (Morais et al., 2010) on a macrogeographic scale (from 2100 km). The distances between our sampling sites in São Paulo (50 km maximum) may be too short for *Ae. aegypti* to exhibit significant shape distinctness. Taking into account that gene flow between these populations was not fully interrupted, occasional migrants could be responsible for sustaining the observed levels of similarity, as suggested for worldwide *Ae. aegypti* populations (Henry et al., 2010).

When the three exogeneous *Ae. aegypti* samples were included in the Mahalanobis distance analysis, wing geometry was found to diverge more between countries than within São Paulo, confirming the evolutionary informativeness of wing shape. However, the metric disparity was significant in only one comparison (OSA-USA), suggesting a low wing shape divergence in populations around the world. It is possible that wing geometry in *Ae. aegypti* evolves more slowly than microsatellites do. This conjecture should be investigated in the future.

Although *Ae. aegypti* is not native to Brazil, it exhibits high genetic variability, especially in the State of São Paulo (Bracco et al., 2007; Paduan and Ribolla, 2008). Thus, a possible founder effect resulting from the introduction of the species could not satisfactorily explain the observed data. The existence of morphologic polymorphisms unrelated to genetic variability may be simply due to the fact that molecular and phenotypic characteristics are determined by distinct genomic regions and may thus evolve differently than the examined microsatellites. One example is the case of *Ae. aegypti formosus* in Senegal, for which the diagnostic chaetotaxy for this´subspecieś is not correlated to the population groups defined by neutral genetic markers (Paupy et al., 2010).

The explanation of the decoupling between the observed genetic diversification and the morphological traits between populations may rely on the thesis of Henry et al. (2010), who also reported wing shape homogeneity in *Ae. aegypti* isolates found across several countries. That study proposed that wing shape in *Ae. aegypti* is stabilised by canalising mechanisms, which is possibly driven by selective pressure. Accordingly, in our results, the Q_{ST} values for shape were approximately 6.5 times higher than the F_{ST} values for the microsatellites, suggesting that wing shape is under selection. If wing shape were neutral, the two estimates should converge to the same value (Rogers and Harpending, 1983; Whitlock, 1999). Theoretically, wings may be subjected to evolutionary selection because they are important organs for both flight and mating behaviour (Cator et al., 2009).

It was not possible to validate wing shape as a marker for distinguishing populations of *Ae. aegypti* on this microgeographic scale. Nevertheless, for parasitology, wing geometry is still a useful tool for detecting subpopulations and the introduction of migrants (Dujardin et al., 2007; Dujardin, 2008; Morais et al., 2010).

4.3. Conclusions

To the best of our knowledge, this is the first examination of wing shape and microsatellites in the same sample set of *Ae. aegypti*. In this study population, wing geometry was not an adequate marker for assessing population structure. Wing shape canalisation and selective pressures on *Ae. aegypti* could explain why wing shape variability was lower than what has been previously observed in other populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.11.013.

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