

Genetic structure of *Aedes aegypti* in the city of Córdoba (Argentina), a recently reinfested area

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*To understand the transmission of a vector-borne disease, knowledge of the magnitude of dispersal among vector populations is essential because of its influence on pathogen transfer. The principal vector of dengue, the most common arboviral disease in the world, is the mosquito *Aedes aegypti* (L.). This tropical and subtropical species is native to Africa but has dispersed worldwide since the XV century. In Argentina, the species was declared eradicated in 1963, but has reinfested the country in recent years. In the present work, we used RAPD-PCR markers to assess the levels of genetic variability and differentiation among populations of *Ae. aegypti* (the vector of dengue and yellow fever) in Córdoba, the second largest city in Argentina. We detected similar levels of genetic variability (H_e between 0.351-0.404) across samples and significant genetic differentiation between most population pairs within the city (F_{ST} between 0.0013-0.0253). Genetic distances indicate that there are three distinct groups, formed predominantly by populations that are connected by, or near, main roads. This suggests that, in addition to other factors such as availability of oviposition sites or step-by-step migration, passive transport plays an important role in gene flow within the city.*

Key words: *Aedes aegypti* - genetic structure - gene flow - RAPD-PCR markers

The mosquito *Aedes aegypti* (L.), a tropical and subtropical species native to Africa, is the principal vector of dengue, the most common arboviral disease in the world. The mosquito has dispersed worldwide since the XV century, probably favoured by ever-increasing trans-continental trading activities. The species is currently found in all continents between latitudes 35°N-35°S and about two thirds of the world's population lives in areas infested with *Ae. aegypti* (Pinheiro & Corber 1997, Gibbons & Vaughn 2002). *Ae. aegypti* is a domestic species; females oviposit mostly in artificial containers in and around human dwellings and feed preferentially on humans (Gubler 1998).

Knowledge of dispersal among vector populations is essential for understanding the transmission of the disease among human populations because of its influence on pathogen transfer and the spread of genetically determined traits of epidemiological importance, such as insecticide resistance and vectorial competence. Dispersal is also one of the main forces that shape the spatial distribution of neutral genetic variation. Several factors influence the levels and patterns of dispersal of *Ae. aegypti*: climate conditions, type and availability of oviposition sites, contiguous areas of suitable habitat, human control

efforts, colonization history and passive transport by humans. The magnitude of active dispersal capacity of *Ae. aegypti* remains controversial because recorded dispersal distances have been variable. Honorio et al. (2003) observed dispersal at distances between 100-800 m in an urban area of the state of Rio de Janeiro (RJ), Brazil. However, Harrington et al. (2005), in experiments conducted in Puerto Rico and Thailand, found that most individuals disperse a few tens of meters and very few individuals disperse up to 500 m.

In Argentina, *Ae. aegypti* was declared eradicated in 1963 as a result of a campaign organized by the Pan American Health Organization, which succeeded in eliminating the species from most countries in the Americas. In subsequent decades, however, the species reinfested all of its former distribution areas, being detected again in the Northeastern end of Argentina in 1986 (Bejarano 1968, Pinheiro & Corber 1997, Gibbons & Vaughn 2002). During a surveillance program implemented by the Ministry of Health of Córdoba Province (Central Argentina), the presence of mosquitoes was detected in five localities between 1994-1996, including Córdoba, the capital city (Avilés et al. 1997).

The genetic structure of *Ae. aegypti* populations within cities have been extensively studied in Southeast Asian cities of Cambodia, Vietnam and Thailand (Tran Khanh et al. 1999, Huber et al. 2002, 2004, Paupy et al. 2004a, b, Bosio et al. 2005). In general, samples from different neighbourhoods showed significant genetic differentiation. The number, type and proximity of suitable breeding sites and human population density significantly influenced the dispersal of mosquitoes. The few studies of genetic structure conducted in South American cities showed similar trends (Ocampo & Wesson 2004, da Costa-Ribeiro et al. 2006). Córdoba (64°12'W 31°22'S;

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1,250,000 inhabitants), the second largest city in Argentina, is an important commercial and industrial centre, as it is located at the crossroads of many national and international routes. In the present work, levels of genetic variation and microgeographic differentiation among seven neighbourhoods in Córdoba were assessed using RAPD-PCR markers, with the aim of examining local patterns of genetic structure of *Ae. aegypti* populations.

MATERIALS AND METHODS

Sampling - In February 2002, 3rd and 4th instar larvae were collected from artificial containers located in seven neighbourhoods of Córdoba that were between 4–16 km apart (Fig. 1): Yofre (n = 16), Parque Liceo (n = 20), Las Flores (n = 23), San Lorenzo (n = 18), Cerro de las Rosas (n = 16), Observatorio (n = 25) and Los Robles (n = 20). In all cases, except the sample from Cerro de las Rosas, the artificial containers consisted of old tires accumulated outdoors in used tire shops. The sample from Cerro de las Rosas was obtained from a container placed *ad hoc* outside a house. The larvae were reared to maturity under laboratory conditions. Adults were frozen and stored at -30°C until processing.

RAPD-PCR procedures - DNA was extracted from mosquitoes as described by Levitan and Grosberg (1993). PCR were carried out as described by de Sousa et al. (2001). In each reaction, a negative control (including ddH₂O instead of mosquito DNA) was included to test for contamination with foreign DNA. To test for band repeatability, a positive control consisting of DNA of one individual (CR5) was included in each PCR reaction and electrophoresed. Additionally, DNA from approximately 25% of the individuals of each population was amplified

twice on different days. In both cases, we checked that the same bands were amplified.

The same arbitrary primers analyzed by de Sousa et al. (2001) were used for PCR amplifications: OP-A02 (5'-TGCCGAGCTG-3'), OP-B03 (5'-ACTTCGACAA-3') and OP-B13 (5'-TTCCCCCGCT-3') from Operon Technologies (Alameda, CA). The same 17 bands used by de Sousa et al. (2001) to analyze the genetic differentiation among *Ae. aegypti* from five cities in Argentina were screened. Additionally, another 20 arbitrary primers (kits A and B from Biodynamics SRL, Buenos Aires, Argentina) were screened to obtain more loci for the microgeographic study proposed in this work. For our analyses, we selected three RAPD primers that produced polymorphic bands that were repeatable, bright and well separated on gels: BD-A02 (5'-GGTGC GGGA A-3'), BD-A06 (5'-GAGTCTCAGG-3') and BD-A10 (5'-ACGGCGTATG-3').

PCR products were separated by electrophoresis on 1.8% agarose gels stained with ethidium bromide in Tris-borate-EDTA buffer (Sambrook et al. 1989). Gels were run for 6 h at 1.5 V/cm and photographed on a UV transilluminator.

Statistical analyses - Only bright and repeatable RAPD bands were used for analyses. Bands were scored as present (1) or absent (0). These data were used to estimate allele frequencies, assuming that each RAPD band of a given molecular size was the product of a dominant allele at a given locus and that genotype frequencies at RAPD loci were in Hardy-Weinberg equilibrium, as demonstrated by de Sousa et al. (2000). These calculations were performed with AFLPsurv (Vekemans et al. 2002) using the Bayesian method with non-uniform prior distribution of allele frequencies. Zhivotovsky (1999) demonstrated that this method gives the most accurate estimation of allele frequencies for dominant markers. These allele frequencies were used for all subsequent calculations.

Levels of genetic variability for each neighbourhood were estimated according to Lynch and Milligan (1994) as the percentage of polymorphic loci (95% criterion) and the unbiased expected heterozygosity (H) (Nei 1978). Ninety-five percent confidence intervals were calculated for H_e values. These calculations were performed using the program AFLPsurv (Vekemans et al. 2002).

Genetic differentiation among neighbourhoods was estimated by means of Wright's (1978) F_{ST} index. Calculations were performed with AFLPsurv. To test the hypothesis of isolation by distance, the correlation between genetic differentiation between pairs of populations and geographic distance was assessed according to Rousset (1997): pairwise F_{ST} values were calculated with AFLPsurv, transformed as $F_{ST}/(1-F_{ST})$ and their correlation with the ln of geographic distances between populations was calculated with a Mantel test using the IBD Web service (Jensen et al. 2005).

Reynolds et al. (1983) genetic distances among neighbourhoods were calculated using AFLPsurv and represented graphically in a multi-dimensional scaling graphic (MDS).

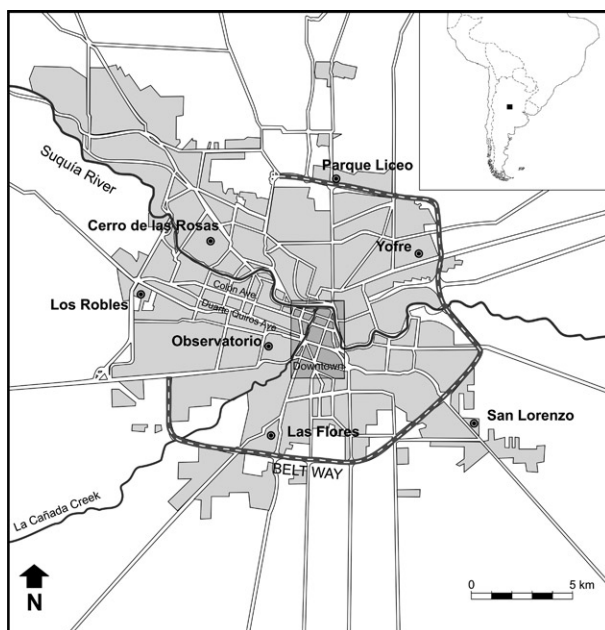


Fig. 1: map of Córdoba city showing the geographic location of the seven populations of *Aedes aegypti* analysed.

RESULTS

A total of 50 RAPD bands, ranging in size from 448-1,769 bp, were selected for analyses, 17 of which were the same fragments used by de Sousa et al. (2001) to analyse samples from different cities in Argentina. Examples of the amplification patterns obtained with the new RAPD primers are shown in Fig. 2.

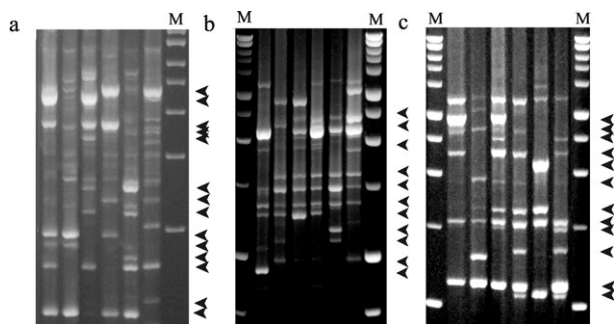


Fig. 2: example of the band patterns obtained for RAPD primers BD-A02 (a) BD-A06 (b) and BD-A10 (c). Arrows indicate the bands selected for analyses.

Table I shows the expected H values and proportion of polymorphic loci. There were no differences in levels of H among mosquito populations in the different neighbourhoods. Overall genetic differentiation among all populations was low but statistically significant ($F_{ST} = 0.0103$; $p < 0.05$); F_{ST} decreased one order of magnitude, but still remained significant when the furthestmost population was removed from the analysis ($F_{ST} = 0.007$; $p < 0.05$). F_{ST} values between pairs of populations ranged from 0.0013-0.0253 (Table II); $F_{ST}/(1-F_{ST})$ showed a non-

significant correlation with geographic distance (Mantel test: $r = 0.320$, $p = 0.113$) (Fig. 3A). When the furthestmost population (San Lorenzo) was removed from analysis, the correlation disappeared (Mantel test $r = -0.041$, $p = 0.540$). San Lorenzo was the most differentiated population and showed highly significant F_{ST} values in comparison with all other samples. This was also seen in the MDS graphic of Reynolds et al. (1983) distances (Fig. 4), where a large genetic distance was observed between San Lorenzo and the other neighbourhoods. The remaining populations clustered into two groups: Observatorio with Los Robles in one group and Yofre, Parque Liceo, Las Flores and Cerro de las Rosas in another group.

TABLE II

F_{ST} values between pairs of populations of *Aedes aegypti* in Córdoba city

	Y	PL	LF	SL	CR	O
Yofre (Y)						
Parque Liceo (PL)	0.0014					
Las Flores (LF)	0.0032	0.0073 ^a				
San Lorenzo (SL)	0.0222 ^c	0.0165 ^b	0.0217 ^c			
Cerro de las Rosas (CR)	0.0013	0.0032	0.0024	0.0112 ^a		
Observatorio (O)	0.0165 ^c	0.0099 ^b	0.0133 ^b	0.0184 ^c	0.0051 ^a	
Los Robles (LR)	0.0046 ^a	0.0122 ^b	0.0144 ^c	0.0253 ^c	0.0046	0.0032

a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$.

TABLE I

Levels of genetic variability in population samples of *Aedes aegypti* from different neighbourhoods in Córdoba city estimated as percentage of polymorphic loci (95% criterion; P95%) and expected mean heterozygosity (H_e)

Population	Specimens n	P _{95%}	H_e (95% CI) ^a
Yofre	16	100	0.404 (0.377-0.431)
Parque Liceo	18	100	0.394 (0.366-0.423)
Las Flores	22	100	0.372 (0.343-0.401)
San Lorenzo	17	92	0.351 (0.310-0.393)
Cerro de las Rosas	15	100	0.386 (0.355-0.417)
Observatorio	24	100	0.391 (0.358-0.424)
Los Robles	19	98	0.393 (0.363-0.432)

a: values were calculated on the basis of Bayesian estimation of allele frequencies considering a non-uniform prior distribution.

DISCUSSION

In this paper, we analysed the levels of genetic variation and microgeographic differentiation among seven neighbourhoods in Córdoba, using RAPD-PCR as genetic markers.

Populations of *Ae. aegypti* from Córdoba showed high levels of genetic variability, as revealed by the analysis of 50 RAPD loci. Values of expected H were similar to those found previously by de Sousa et al. (2001) in several Argentine cities using only 17 RAPD loci. Likewise, levels of variability were similar to those found in other populations from Brazil (Ayres et al. 2003, Paduan et al. 2006), Puerto Rico (Apostol et al. 1996) and Mexico (Gorrochotegui-Escalante et al. 2000), using the same molecular markers. Rondán Dueñas (2005) reported that haplotype diversity values at the mitochondrial control region in *Ae. aegypti* from Córdoba were among the highest in Argentina. He also found that the haplo-

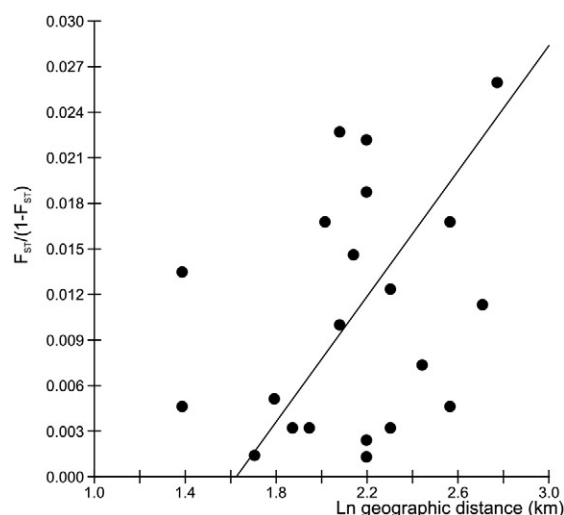


Fig. 3: scatterplot of genetic differentiation between pairs of populations of *Aedes aegypti* from Córdoba city against the ln of geographic distances separating them.

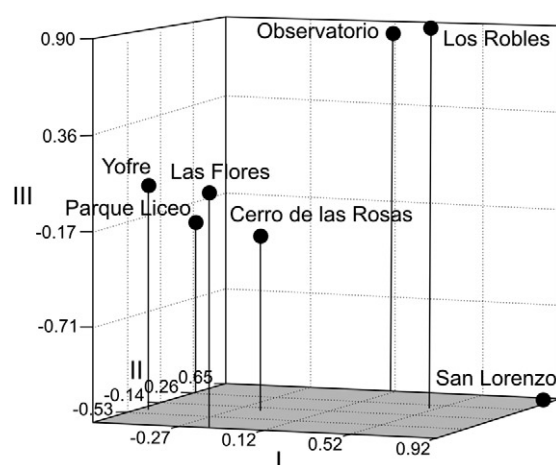


Fig. 4: multidimensional scaling plot of Reynolds et al. (1983) genetic distances among populations of *Aedes aegypti* from the city of Córdoba.

types detected in Argentine cities formed three phylogenetically distinct clusters. One cluster was restricted to populations from the northern part of the country and Bolivia, a second cluster included populations native to Northwestern Argentina (probably relicts of the eradication campaigns) and a third cluster included populations recently introduced by passive transportation from Brazil. The latter two haplogroups converged in Córdoba, explaining, at least in part, the high diversity found in this recently colonized area.

The level of genetic differentiation among *Ae. aegypti* populations from different neighbourhoods in densely populated cities seems to be related to factors such as population density (favouring feeding of female mosquitoes) and the increased availability of breeding sites in places without a piped water supply (because

water is stored in open containers). For example, Huber et al. (2004) found that in Ho Chi Minh City (Vietnam), the level of genetic differentiation among populations from the outskirts of the city, where human density is lower and water is stored in open containers, is twice as high as among populations in downtown areas, where human density is high and there is a piped water supply. Similar results were obtained in other Southeast Asian cities, such as Chiang Mai in Thailand (Mousson et al. 2002) and Phnom Penh in Cambodia (Paupy et al. 2004a, b). Climatic factors, such as seasonality of temperature and rainfall, also influence the level of genetic differentiation among mosquito populations. Paupy et al. (2004a) found more genetic differentiation among *Ae. aegypti* collected from temporary water containers than among those from permanent domestic water storage in a single, 400 metre-long street in Phnom Penh. In RJ, Brazil, differentiation among populations was lower during the dry season, when females needed to disperse farther to find suitable breeding sites. The periodicity of application of insecticide treatments was found to be another factor that determines the genetic structure of *Ae. aegypti* because treatments cause periodic population bottlenecks that enhance genetic differentiation, as reported for populations from Cali (Colombia) (Ocampo & Wesson 2004) and several Asian cities (Mousson et al. 2002, Paupy et al. 2005).

In the present work, we found an overall small but statistically significant genetic differentiation among mosquitoes from different neighbourhoods in Córdoba, which showed no correlation with geographic distance. Two conditions are necessary for a pattern of isolation by distance to occur: higher probability of short-distance than long-distance gene flow and enough time for the pattern to be established. Otherwise, a random distribution of allele frequencies will be found, with high differentiation among populations when gene flow is restricted, or with non-significant F_{ST} values when gene flow is common (Hutchison & Templeton 1999). Given that the reinfestation of Córdoba is a relatively recent event (10–15 years), the significant pairwise F_{ST} values would indicate that gene flow between the sampled populations is low. Sampling points were located more than 4 km apart, a distance much larger than the flight range of the species, but within residential neighbourhoods that had low human densities and consisted mostly of houses with gardens and backyards. In Buenos Aires (the capital city of Argentina), Carbajo et al. (2006) found that oviposition activity was higher in areas with a larger proportion of houses and lower human density, compared to more urbanized areas with high-rise buildings and higher human density. A positive association was also found between oviposition and industrial sites, possibly due to a high density of water containers. Vezzani et al. (2005) also found that, in Buenos Aires, the microhabitats most favourable for reproduction of *Ae. aegypti* were sites less exposed to sunlight, such as shaded and vegetated neighbourhoods, these environments are also frequent in the neighbourhoods sampled in Córdoba. Although a step-by-step migration of mosquitoes between

neighbourhoods is highly possible, had dispersion been active, it would have been reflected in lower or non-significant F_{ST} values. An explanation for our result is that short-distance gene flow would not be enough to balance genetic drift. In Córdoba, there have been neither extensive insecticide applications nor extensive mosquito eradication campaigns, such as those to which strong drift events have been attributed in other cities. However, unlike the climate of Southeast Asian cities and the Latin-American cities studied so far, Córdoba's climate is temperate semi-arid, with winter temperatures that can fall below 0°C and rainfall concentrated almost completely in the summer months. Domínguez et al. (2000) found that adults and larvae of *Ae. aegypti* do not survive through the winter in Córdoba. Oviposition significantly decreases during autumn, stopping when temperature falls to less than 17°C and the first spring cohort originates from overwintering eggs. Therefore, the climatic pattern would cause yearly genetic drift events that increase interpopulation differentiation.

On the other hand, genetic distances between population pairs show three clear groups (Fig. 4). In two of the groups, populations are connected by main roads: Observatorio and Los Robles by Colón and Duarte Quirós Avenues and Parque Liceo, Yofre and Las Flores by the beltway that surrounds most of the city (Fig. 1). At the spatial scale of the present study, passive transport would be the most influential means of dispersal. The third group includes only the sample from San Lorenzo, which showed the highest level of genetic differentiation (F_{ST}) of all samples. It is the most isolated population, located in the outskirts of the city and separated from other neighbourhoods by undeveloped areas and adjacent to several industrial complexes (Fig. 1). The sample was obtained in a tire shop on the route connecting Córdoba with Buenos Aires, where traffic is particularly intense. Mosquitoes from this population could have originated from a mixture of individuals of different origins and this fact, together with geographic distance and discontinuity of suitable habitat, might explain the high differentiation observed.

The genetic structure of *Ae. aegypti* populations in Córdoba would be determined by a combination of forces: large genetic drift events every winter that differentiate populations at random and low to moderate levels of gene flow, mediated mainly by passive transport along main roads. Some level of short-distance, active dispersal would also occur, but a more detailed sampling design would be necessary to confirm this assumption. Rotela et al. (2007) studied the spreading dynamics of a recent dengue outbreak in the city of Tartagal, in Northern Argentina. They reported that the outbreak originated in a town near the city and spread along the international Route 34, which has intense national and international traffic. Within Tartagal, the epidemic spread rapidly, with the simultaneous appearance of several cases throughout the city, the authors point out the importance of the movement of people in the introduction of the virus in many houses infested with the vector. After the initial introductions, the epidemic showed a clear spatio-temporal clustering, most likely determined by *Ae. aegypti* flight

capacity and its expected lifetime. The genetic structure of *Ae. aegypti* in Córdoba would favour dengue transmission dynamics similar to those found in Tartagal.

Most of the samples analysed in the present study were obtained from tire shops frequented by trucks from national and international commercial transport. In these places, tires (used, recapped or discarded) are usually stored outdoors, where they easily accumulate rainwater during the wet summer season. Several studies have addressed the idea that water-containing tires are good breeding sites for *Aedes* species and that desiccated eggs can be attached to them and transported large distances (Reiter & Sprenger 1987, Reiter 1998, Nathan & Knudsen 1991). Neighbourhoods situated at the periphery of the city could have acted as initial foci of reinfestation, from which active dispersion of females would have originated different populations within their flight ranges.

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