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Factors influencing the population structure of *Aedes aegypti* from the main cities in Cambodia

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A population genetic analysis was conducted on 47 *Aedes aegypti* collections from Cambodia. Genetic differentiation at seven polymorphic isoenzyme loci was analysed by starch gel electrophoresis. Low ($F_{ST} = 0.024$) but significant ($P < 10^{-6}$) differentiation was found when all samples were considered. Whatever the grouping of samples tested, differentiation remained significant but low. The role of human activities (ie insecticide treatments or water storage practices) and environmental factors (ie rainfall) in shaping mosquito differentiation are discussed.
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Introduction

Dengue fever (DF) and dengue haemorrhagic fever (DHF) are an important public health issue in tropical and subtropical areas. The frequency of DF has increased dramatically since the first description of DHF in the mid-1950s (Gubler, 2002). A total of 50–100 million cases of DF and several hundred thousand cases of DHF occur annually over 100 countries including many in South-East Asia (Lam, 1998).

The main vector of dengue viruses, *Aedes aegypti*, was probably inadvertently introduced from Africa into Asia at the end of the 19th Century, by the increased trade and shipping. The insects' establishment and spread in Asia coincided with the first dengue epidemics in urban areas (Smith, 1956). *Ae. Aegypti* is closely associated with humans. The species is well adapted to urban environments, which provide numerous opportunities for blood-meals (Scott *et al*, 1997) and larval breeding sites (Strickman and Kittayapong, 1993). Water storage containers (particularly concrete jars with a capacity of 100 l or more) represent the most frequent larval habitat in Cambodia. They sustain high larval infestation rates throughout the year (Ngan Chantha, personal communication). Indeed, in 1990, *Ae. aegypti* comprised more than 40% of indoor-resting mosquitoes in Phnom Penh (Kohn, 1990).

Cambodia is located in the Southwest of the Indochinese peninsula and bounded on the west by Thailand, on the north by Laos, on the east by Vietnam and to the south by the gulf of Thailand. Most Cambodians (85% of the 11.4 million inhabitants) live in rural areas on the fertile central plains of the Mekong-Tonlé basin. Cambodia's climate is governed by two monsoons: the cool, dry north-eastern monsoon from November to February, and the humid south-western monsoon from May to October.

Though Cambodia has regularly been confronted with dengue since 1962, more severe and recurrent outbreaks have been observed in the last 20 years (Rathavuth *et al*, 1997). In 1990 and 1995, Cambodia was subjected to two major epidemics with 7241 cases (331 deaths) and 10 208 cases (424 deaths), respectively. However, it was in 1998 that the worst epidemic was recorded: 16 216 DHF cases and 475 deaths (Ngan *et al*, 1998). In 2001, 10 264 DHF cases, 195 of them fatal, were reported (Ngan Chantha, personal communication). The National Dengue Control Programme of the Ministry of Health implemented insecticide spraying and mass applications of the larvicide temephos, in drinking water containers leading to a long-term larval control.

We analysed the genetic structure of *Ae. aegypti* populations in four cities differing in their level of urbanisation and insecticide treatments, both of which are known to shape mosquito genetic structure (Huber *et al*, 2002; Mousson *et al*, 2002). In the present study, we addressed the following questions: (1) is the genetic structure of *Ae. aegypti* populations similar in the different cities, (2) what are the main factors driving mosquito genetic differentiation and (3) what are the implications of our findings in the control of dengue transmission in Cambodia.

Materials and methods

Mosquito samples

In total, 47 *Ae. aegypti* samples were collected in different localities in the four main cities of Cambodia from February to April 2001 (Supplementary Table 1, Figure 1): 22 in Phnom Penh (Figure 1), nine in Kampong Cham, eight in Kampong Som (Sihanoukville), and eight in Siem Reap. Samples that consisted of larvae or pupae

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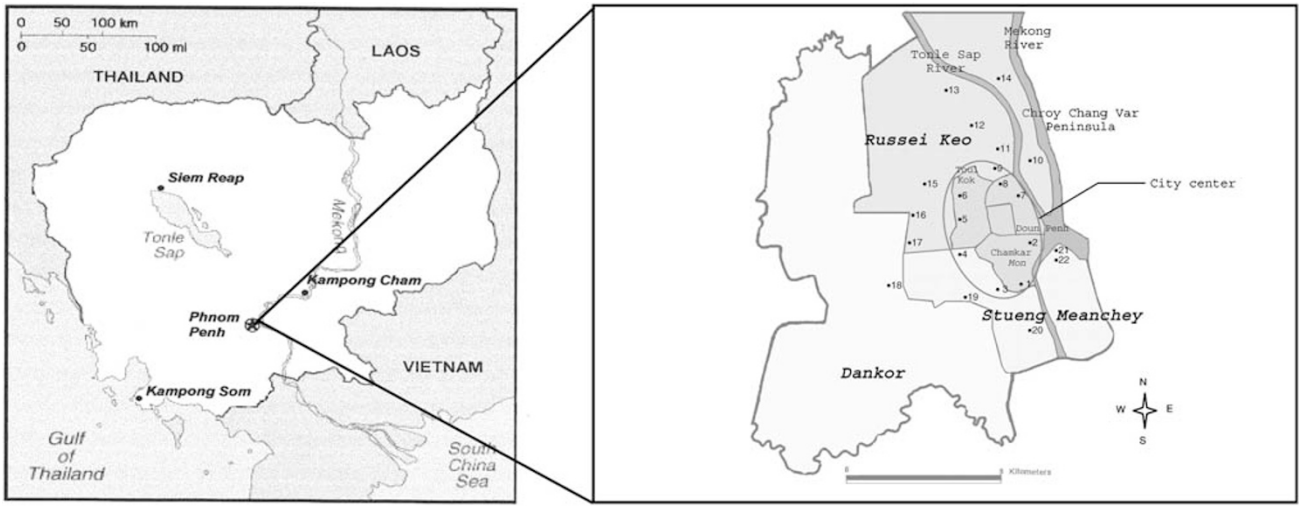

Figure 1 Map of Cambodia and Phnom Penh showing the location of *Aedes aegypti* samples collected in 2001.

were reared until the imago stage. Adults were kept for analysis of enzyme polymorphism.

Isoenzyme polymorphism

Each individual mosquito was ground in 25 µl of distilled water. The sample was centrifuged (15 000 rpm for 4 min at $+4^{\circ}$C) and the supernatant was loaded on a starch gel using the Tris-Maleate-EDTA (pH 7.4) buffer system. Seven enzyme systems were studied: glutamate oxaloacetate transaminase (Got-1 and Got-2), glycerol-phosphate dehydrogenase (G-3-pdh), hexokinase (Hk-1, Hk-2 and Hk-3), malate dehydrogenase (Mdh), malate dehydrogenase (NADP) (Mdhp-1), phosphoglucoisomerase (Pgi) and phosphoglucomutase (Pgm) (for more details, see Paupy *et al*, 2000). An *Ae. aegypti* strain developed from an isofemale lineage was used in control lanes as a marker for protein mobility. For field-collected samples, alleles were numbered according to their mobility compared to that of the most common allele obtained at each locus in the control strain.

Genetic analysis

Deviations from Hardy–Weinberg proportions, genotypic linkage disequilibrium and genetic differentiation were tested using the GENEPOP software (version 3.3). (Raymond and Rousset, 1995). Hardy–Weinberg proportions were tested by the probability test proposed by Haldane (1954). The overall significance of multiple tests for each locus or for each sample was estimated by Fisher's combined probability test (Fisher, 1970). Heterozygote deficit or excess were tested using an exact test procedure (Rousset and Raymond, 1995). Genotypic association between pairs of loci was tested for each sample using Fisher's test contingency tables. F_{IS} and F_{ST} were calculated according to the formula described by Weir and Cockerham (1984). Genetic differentiation across populations was assessed by calculating *P*-value for the F_{ST} estimate. The overall significance of multiple tests was estimated by Fisher's combined probability test (Fisher, 1970). The significance level for multiple tests was adjusted by the sequential method of Bonferroni (Holm, 1979).

Results

Hardy–Weinberg equilibrium

Out of 158 tests run, six significant deviations from Hardy–Weinberg equilibrium were detected after application of Bonferroni procedure: Hk-2/PP9 ($F_{IS} = +0.793$), Mdhp-1/PP8 ($F_{IS} = +1$), Mdhp-1/PP14 ($F_{IS} = +1$), Mdhp-1/PP15 ($F_{IS} = +1$), Pgi/PP2 ($F_{IS} = +0.850$) and Pgi/PP8 ($F_{IS} = +0.793$) (Supplementary Table 2). Hardy–Weinberg equilibrium was also tested assuming that the alternative hypothesis H1, was a heterozygote deficit (H0: random mating). All deviations detected were due to a heterozygote deficit. When considering global tests (ie all loci for each sample), three samples showed significant heterozygote deficit: PP2, PP8 and PP17.

Linkage disequilibrium

Genotypic disequilibrium was tested between pairs of loci for each sample. Out of 549 possible combinations, 42 significant nonrandom associations were detected (after Bonferroni's sequential test; $P<0.05$): 13 concerning the combination Hk-1/Hk-2, 13 for Hk-1/Hk-3, 13 for Hk-2/Hk-3, one for G-3-pdh/Hk-1, one for G-3-pdh/Hk-2 and one for G-3-pdh-Hk-3 (data not shown). Therefore, the loci Hk-1, Hk-3 and G-3-pdh were excluded from the data set.

Population differentiation

When considering all 47 samples (Table 1), significant differentiation was observed ($F_{ST} = 0.024$. and $P<10^{-6}$). Each locus showed significant F_{ST} values.

When comparing samples between cities (ie, Kampong Cham (KC), Kampong Som (KS), Phnom Penh (PP) and Siem Reap (SR)), all combinations were highly significant (F_{ST} ranging from 0.015 to 0.030) whatever the geographic distances separating the localities (Table 1).

Within cities significant genetic differentiation (Table 1; $P<10^{-6}$) was detected in all cases except for the eight samples collected in Kampong Som ($F_{ST} = -0.001$, $P>0.05$). It may be relevant that Kampong Som suffers the heaviest rainfalls in the country (eg 7210.5 mm for 1999–2000 compared to Phnom Penh with 3689 mm).

Table 1 *Aedes aegypti* differentiation in Cambodia

Comparison	*N*	F_{st}							
		Got-1	*Got-2*	*HK-2*	*Mdhp-1*	*Mdh*	*Pgm*	*Pgi*	*All loci*
All samples	47	**0.009**	**0.013*** **	**0.080*** **	**−0.002****	**0.021*** **	**0.022*** **	**0.019*** **	**0.024*** **
Between cities									
SR–KC	17	0	**0.043****	**0.103*** **	—	**0.023*** **	**0.026*** **	**0.015*** **	**0.030*** **
SR–PP	30	**0.006**	**0.012*** **	**0.090*** **	**−0.002***	**0.027*** **	**0.021*** **	**0.017*** **	**0.028*** **
SR–KS	16	0.009	**0.031*** **	**0.127*** **	—	**0.014*** **	**0.020*** **	**0.009**	**0.024*** **
PP–KC	31	**0.010***	**0.007****	**0.039*** **	**−0.003***	**0.025*** **	**0.024*** **	**0.018*** **	**0.024*** **
PP–KS	30	**0.011**	**0.082*** **	**0.045*** **	−0.002	**0.019*** **	**0.019*** **	**0.018*** **	**0.019*** **
KC–KS	17	**0.013**	0.009	**0.019****	—	**0.011*** **	**0.021*** **	**0.011***	**0.015*** **
Within cities									
Kampong Cham	9	—	—	**0.021****	—	**0.021*** **	**0.026*** **	**0.018****	**0.022*** **
Kampong Som	8	0.013	0.009	0.004	—	0.005	0.008	−0.005	−0.001
Phnom Penh	22	**0.010***	**0.007****	**0.046*** **	**−0.003***	**0.027*** **	**0.021*** **	**0.014*** **	**0.024*** **
Siem Reap	8	0	**0.043****	**0.115*** **	—	**0.029*** **	**0.021*** **	**0.014***	**0.036*** **
Phnom Penh									
City center	9	—	0.006	**0.036*** **	−0.001	**0.025***	**0.022*** **	**0.009****	**0.022*** **
Suburbs	13	**0.010**	**0.008****	**0.050*** **	−0.002	**0.028*** **	**0.022*** **	**0.018*** **	**0.026*** **
Within suburbs									
South	4	—	—	−0.002	—	0.007	0.002	0.005	0.005
West	4	—	**0.012**	**0.030**	0	**0.016**	0.087	**0.023****	**0.015*** **
North	5	**0.010**	−0.002	**0.055*** **	−0.001	**0.058*** **	**0.021****	0.013	**0.053*** **
North (without peninsula)	3	—	0.003	**0.055*** **	—	**0.013*** **	**0.033**	**0.016**	**0.024*** **
Between suburbs									
South—west	8	—	**0.016***	**0.026***	−0.002	**0.009**	0.009	**0.022***	**0.011*** **
South—north	9	**0.010**	**0.006**	**0.058*** **	−0.002	**0.037*** **	**0.028*** **	**0.012***	**0.032*** **
West—north	9	**0.011**	**0.004***	**0.049*** **	−0.002	**0.038*** **	**0.021*** **	**0.018*** **	**0.030*** **

N: sample size; PP: Phnom Penh; KC: Kampong Cham; KS: Kampong Som; SR: Siem Reap. In bold when *P* was significant (<0.05), *$P<0.01$, **$P<0.001$, ***$P<0.0001$.

There is no significant association between geographic distance and genetic differentiation.

Within Phnom Penh the samples from the city centre were highly and significantly differentiated ($F_{ST}=0.022$, $P<10^{-6}$) and isolation by distance was detected (slope $b=0.013$, $P<0.05$). In the suburbs, genetic differentiation was lower in the south ($F_{ST}=0.005$, $P>0.05$) and in the west ($F_{ST}=0.015$, $P<10^{-6}$) and highest in the north ($F_{ST}=0.053$, $P<10^{-6}$) mainly due to the two samples from Chroy Chang Var peninsula (PP10 and PP14). With these samples excluded, $F_{ST}=0.024$ ($P<10^{-6}$).

The highest differentiation between suburbs was that between the south and the north (Table 1: $F_{ST}=0.032$).

Discussion

The spatial survey of genetic variability of *Ae. aegypti* in Cambodia underlines the low level of genetic differentiation compared to that detected in Thailand (Mousson *et al*, 2002) or from Vietnam (Tran *et al*, 1999; Huber *et al*, 2002).

Climatic factors such as rainfall are important in generating breeding sites. The severe and periodic rainfalls in Kampong Som (Sihanoukville) could fill containers, which accumulate at the vicinity of houses (Trips, 1972) and are used by females to oviposite. The abundance of breeding sites could enhance *Ae. aegypti* movement and genetic exchange and reduce genetic differentiation. A similar explanation may apply in reverse in Siem Reap, where the high genetic differentiation coincides with low rainfall. Temporary breeding sites disappear and a population bottleneck may have led to a reduction of polymorphism. This same pattern was very marked in populations from Ho Chi Minh City (Huber *et al*, 2002).

In addition to climatic factors, human factors such as insecticide application and water-storage practice can affect mosquito genetic structure. In Seam Reap, a city famous for its Angkorian temples, there is massive insecticide use in the numerous hotels accommodating the flood of visitors. Mosquito populations were highly differentiated implicating insecticide treatments as a factor producing subdivision (McCauley, 1991). Conversely, Kampong Som, a city essentially visited by Cambodians, has less insecticide treatment and thus, *Ae. aegypti* is less genetically differentiated.

Phnom Penh is currently developing and making up for the time lost during the civil war. Out of more than one million people living in Phnom Penh province, 570 000 are settled in the urban area. The uncontrolled immigration and consequent urbanisation of the suburbs has led to severe shortages in the water supply, thus compelling people to store water. The water storage containers prove most of the larval breeding sites for *Ae. aegypti* (about 80%; Ngan Chantha, personal communication). The low genetic differentiation detected in all but the northern suburbs could be attributed to the high densities of *Ae. aegypti* and increased gene flow. The

higher differentiation in the northern suburbs can be explained by the Tonle Sap river, which may act as a barrier to gene flow. It isolates samples PP10 and PP14 (located in the Chroy Chang Var peninsula) from the others (see Figure 1).

Similarly the differentiation between northern and southern populations could be explained if the city centre is a barrier to gene flow. Piped water supply is available almost everywhere in the city centre reducing open water storage and hence the density of *Ae. aegypti* (Kohn, 1990).

The relatively low genetic differentiation observed amongst all Cambodian *Ae. aegypti* could indicate the rapid spreading of populations and thus, of genes of interest such as those controlling the susceptibility to insecticides. Such wide dispersal would dictate that vector control campaigns should be implemented simultaneously over large areas.

Insecticide treatments of breeding sites and source reduction are both recommended to limit dengue epidemics. However, these stategies could also potentially promote vector dispersal as a result of reducing available oviposition sites.

Our study gives us a general insight into of *Ae. aegypti* ecology in the main Cambodian cities. Future studies should address whether these patterns persist throughout the year and, in particular, if there are differences between the rainy and at the dry season. For such purposes, more population genetic studies must be supported.

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