GENETIC STRUCTURE, DEMOGRAPHIC HISTORY AND TAXONOMIC STATUS OF THE MALARIA VECTORS: *ANOPHELES ALBIMANUS*, *ANOPHELES DARLINGI* AND *ANOPHELES PUNCTIMACULA*, IN SOUTHERN CENTRAL AMERICA

by

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The Neotropical Environmental Option

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Abstract

Little research has been undertaken on malaria vectors in southern Central America, particularly in Panama, where a serious outbreak took place from 2001 to 2005. Here, I have confirmed the presence of fourteen *Anopheles* species, seven of which have the potential to transmit malaria in Panama. In addition, I collected *An. darlingi* for the first time in Panama, Darien, near the Colombian border. Sequence comparisons using the *white* gene confirmed the presence of the northern genotype, and a demographic analysis using the mtDNA *COI* gene indicated that *An. darlingi* has not been recently introduced from Colombia into eastern Panama. Three independent molecular markers (mtDNA *COI*, nDNA *white*, rDNA ITS2) supported the status of a single species of *An. albimanus*, and uncovered significant population structure across Nicaragua, Costa Rica, Panama, Colombia and Ecuador. The genetic structure of *An. albimanus* appears to be related to Pleistocene contraction, geographic fragmentation and subsequent expansion. Therefore, *An. albimanus* is not at mutation - drift equilibrium regionally. This pattern of population expansion is similar to the one depicted for *An. darlingi* in Amazonia South America. Phylogenetic analyses using the *COI* and the ITS2 markers detected lineage divergence in *An. punctimacula* s.l. Seven clades were well supported and clearly differentiated from *An. malefactor*. In contrast, the Folmer *COI* region did not support this divergence, perhaps due to the unequal mutation rate across the *COI* gene. My findings have provided a more complete figure of the population history and taxonomic status of three important malaria vectors; this information will be valuable to better understand the

epidemiology of malaria in the Neotropics, and it will contribute to the design of more efficient strategies of vector control.

Resume

Peu de recherches ont été effectuées sur les vecteurs de la malaria dans le sud de l'Amérique centrale, en particulier au Panama, où pourtant plusieurs épidémies ont eu lieu de 2001 à 2005. Dans cette présente étude, quatorze espèces d'*Anopheles* ont été inventoriés, dont sept qui ont le potentiel de transmettre la malaria au Panama. De plus, *An. darlingi* a été recueilli pour la première fois dans la région de Darien au Panama, près de la frontière colombienne. La comparaison des séquences du gène blanc a révélé la présence du génotype du nord, et l'analyse démographique utilisant le gène COI mtDNA a indiqué que la présence d'*An. darlingi,* à l'est du Panama, ne serait pas l'origine d'une migration récente en provenance de la Colombie. Trois marqueurs moléculaires indépendants (mtDNA COI, nDNA blanc, rDNA ITS2) supportent l'état d'une seule espèce d'*An. albimanus,* mais tout en ayant des distinctions significatifs entre les populations du Nicaragua, Costa Rica, Panama, Colombie et l'Équateur. La structure génétique d'*An. albimanus* semble être liée à la contraction du Pléistocène, à la fragmentation géographique et aux expansions ultérieures. Par conséquent, *An. albimanus* n'est pas en mutation - drift equilibrium regionally. Le modèle d'expansion de la population d'*An. albimanus* est semblable à celui représenté pour *An. darlingi* de l'Amazonie en Amérique du sud. L'analyse phylogénétique utilisant les marqueurs COI et ITS2 a détecté des lignées divergentes pour *An. punctimacula* s.l. Sept groupes ont bien été supportés et différenciées d'*An. malefactor*. Cependant, la région de Folmer COI ne support pas cette divergence, peut-être en raison d'un taux de mutation variable du gène

COI. Cette étude établi un meilleur portrait de l'histoire des populations et du statut taxonomique de trois importants vecteurs de la malaria. Cette précieuse information sera utile pour mieux comprendre l'épidémiologie de la malaria sur le territoire néo-tropical. De plus, cela contribuera à la conception future de stratégies efficaces pour le contrôle des vecteurs.

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Contribution of Authors

The present thesis consists of three original papers co-authored by me, my academic advisor Dr. Marilyn E. Scott, my co-supervisors, Dr. Eldredge Bermingham, Dr. Jan E. Conn, Dr. Manfred Rau and several collaborators. Chapters III, IV, and V were published in different peer – reviewed journals, and chapter VI was submitted to another peer – reviewed journal at the time of submission. I wrote the preliminary drafts of all the chapters and incorporated direct contributions and comments from my advisory committee. For each of the papers presented in this thesis, I planned the experimental designs, conducted fieldwork, identified most of the specimens collected and performed all of the population genetic analyses presented with feedback from my advisors. In addition, Sara Bickersmith helped me considerably to extract and amplify DNA from mosquitoes used in Chapters V and VI, and Oris Sanjur offered me valuable logistical support for fieldwork and laboratory assistance. Jose R. Rovira, Rufino Valdez, Urbano Arrocha and Dagoberto Atencio contributed largely to collect mosquitoes in several localities of Panama whereas Jordane Roy Leblanc did so in Costa Rica as well. Professor Carl D. Schlichting provided great academic input and helped me to choose the statistical analyses performed in this thesis as well as to interpret the results. Finally, Harilaos Lessios, Matthew Miller and Laura B. Geyer provided valuable comments on various manuscripts of the present doctoral thesis.

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List of Abbreviations

AMOVA	Analysis of molecular variance
ANAM	Autoridad Nacional del Ambiente de Panamá
ANOVA	Analysis of variance
am	Ante meridian
BC	Bocas del Toro
bp	Base pairs
b/p/nt	Bites per person per night
CDC-LTs	Centers for Disease and Control miniature light trap
CEPCO	Central-eastern Panama and Colombia
COI	Cytochrome oxidase sub-unit one gene
D_A	Net nuceotide divergence
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
DR	Darien
EIR	The entomological inoculation rate
FQRNT	Fonds Québécois de la recherche sur la nature et les technologies
GDP	Gross domestic product
GPS	Global positioning system
GIS	Geographical information software
HBR	Human biting rate

Hd	Haplotype diversity
HLC	Human landing catches
hrs	Hours
IBD	Isolation by distance
ICGES	Instituto Conmemorativo Gorgas para Estudios de la Salud
IFHARU	Instituto para la formacion y aprovechamiento del recurso humano of Panamá
IGS	Intergenic transcribed spacer
INBio	Instituto Nacional de Biodiversidad de Costa Rica
ITS1	Internal transcribed spacer one
ITS2	Internal transcribed spacer two
IRS	Insecticide residual spraying
KYA	The Kuna Yala Comarca
km	Kilometers
LC	Larval collections
LSD	Least significant difference
MBI	Mosquito barcoding initiative
MDIV	Isolation with migration analysis
MINSA	Ministerio Nacional de Salud de Panamá
MK	McDonald-Kreitman neutrality test
MJ	Median joining network
mm	Millimeters
mtDNA	Mitochondrial DNA

NBC	The Ngöbe Buglé Comarca
NCRWP	Nicaragua, Costa Rica and western Panama
Nd	Nucleotide diversity
N_e	Effective population size
ND5	NADH dehydrogenase subunit five gene
ND6	NADH dehydrogenase subunit six gene
NIH	National Institute of Health
NSERC	Natural Sciences and Engineering Research Council of Canada
NYSDOH	The New York state department of health
OAS	Organization of American States
PAHO	Pan American Health Organization
PCOLE	The Pacific coast of Colombia and Ecuador
PCR	Polymerase chain reaction
PCR – RFLP	Polymerase chain reaction – restriction fragment length polymorphism
pm	Post meridian
PROC GLM	General linear models
rDNA	Ribosomal DNA
SAMOVA	Spatial analysis of molecular variance
SAS	Statistical analysis software
SE	Standard error
SENACYT	Secretariat for Science, Technology and Innovation of

	Panama
SNE	The standard neutral equilibrium
SIVIGILA	The Epidemiological National System of Colombia
SP	Statistical parsimony network
SNEM	The National Malaria Eradication Service of Panama
s.l.	sensu lato
s.s.	sensu stricto
STRI	Smithsonian Tropical Research Institute
TMRCA	The time to the most recent common ancestor
USA	United State of America
WRBU	The Walter Reed Biosystematics Unit
WHO	The World Health Organization

Statements of Originality

- I collected fourteen *Anopheles* species using human landing catches (HLC), from 1970 to 2005, in malaria endemic areas of Panama. *Anopheles* (*Nyssorhynchus*) *albimanus* and *An.* (*Anopheles*) *punctimacula* were the most prevalent and widespread species, accounting for 72.2% and 17.9% of the total, respectively.

- I identified *Anopheles* (*Nyssorhynchus*) *darlingi*, considered the most important malaria vector in the Neotropics, for the first time in three localities of eastern Panama. *PCR - RFLP* profiles of the single copy nuclear *white* gene (scn*white*) confirmed the presence of 66 specimens of the northern genotype of *An. darlingi* in Darien.

- I found that *An. darlingi* has not been recently introduced into eastern Panama from neighboring Colombia, based on a population analysis using the mtDNA cytochrome oxidase subunit I gene (*COI*).

- I hypothesized that *An. albimanus* is a single species, based on analyses using three independent molecular markers (mtDNA *COI*, rDNA ITS2, scn*white*). Nonetheless, I detected three non - randomly distributed population demes and four discrete lineages of *An. albimanus* across Nicaragua, Costa Rica, Panama, Colombia and Ecuador using the *COI* gene.

- I discovered that *Anopheles albimanus* underwent historical population contraction, geographic fragmentation and expansion in eastern Panama; therefore, it is not at mutation - drift equilibrium regionally.

- I found that despite having different host feeding behaviours and primary breeding sites, both *An. albimanus* and *An. darlingi* have contracted and expanded demographically likely due to cyclical changes in temperature and precipitation that affected larval habitat availability during the Pleistocene.

- I detected lineage divergence in mosquitoes morphologically identified as *An. punctimacula* s.l. based on phylogenetic analyses using the *COI* gene and the ITS2 marker. I identified seven highly supported clades, all of which were clearly differentiated from *An.* (*Anopheles*) *malefactor*.

- I confirmed that two of these clades correspond to isomorphic species within the *An. punctimacula* Group; yet, one clade was mistakenly identified as *An. punctimacula* s.l., and may correspond to *An.* (*Anopheles*) *neomaculipalpus*.

Citations

Material from Chapter III has been published in the following form:

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Material from Chapter V has been published in the following form:

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Material from Chapter VI has been submitted for publication to *Molecular Phylogenetic and Evolution* on April the 24th of 2010, in the following form: Late Pleistocene environmental changes lead to unstable demography and population divergence of *Anopheles albimanus* in the northern Neotropics.

1. Chapter I: General Introduction

Every day, roughly 2,700 children die from an infection by the human malarial parasite *Plasmodium falciparum*. The vast majority of malaria cases caused by *P. falciparum* occur in sub-Saharan Africa, where, if the children live through their earlier infections, they might go on to fight up to 40 separate malarial infections in their lifetime. In contrast, *Plasmodium vivax* is rarely fatal, but it causes most of the malaria morbidity in tropical and sub-tropical areas worldwide (Mendis et al., 2001; Guerra et al., 2008; Hay et al., 2009). Malaria is the third leading global killer of humans, and increasing resistance of the parasite to current drugs and experimental vaccines, owing to its high genetic diversity, is alarming (Sheena et al., 2007). In addition, climate change is predicted to expand vector range and intensify human malaria globally (Patz & Olson, 2006). Therefore, more research to seek alternative solutions to mitigate the malaria burden is needed. Due to the epidemiological complexity of malaria, such solutions will only be effective if a thorough revision of potential control methods is considered, and adjusted to each local situation (WHO, 2009).

In 2006, there were an estimated 247 million malaria cases among 3.3 billion people at risk, causing nearly a million deaths, mostly of children under 5 years of age. Worldwide, 109 countries were endemic for malaria in 2008, 21 of these within the Americas (WHO, 2008). In the Americas, 902,373 malaria cases were reported in 2006, which in comparison to 2000, represented roughly 22% and 69% reduction in the morbidity and morality rates, respectively. The improvement in malaria control was evident in most of the endemic countries

such as Brazil, Colombia, Peru and Surinam; yet, 15.9% of the inhabitants were still at risk of transmission by 2006 (PAHO, 2006; WHO, 2008). Among the likely causes for the persistence of malaria transmission in the Americas are: intense human migration, poor housing conditions, high dropout rate of treatments, considerable number of asymptomatic carriers, extensive mosquito breeding sites in the proximities of the communities, socio-political problems, and inadequate access to health services (PAHO, 2006). Moreover, the lack of basic knowledge regarding distribution, vectorial capacity, taxonomic status and population structure of *Anopheles* species has also precluded a better understanding of the malaria transmission dynamics in this region.

Similar to most Central America countries, in Panama, 85% of malaria cases are *P. vivax*, 15% are *P. falciparum*. However, unique to Panama, the total number of cases has increased significantly (six-fold) since 2001 (Samudio et al., 2005). Most transmission has been reported in four rural areas: Bocas del Toro (BC), the Ngöbe Buglé Comarca (NBC), the Kuna Yala Comarca (KYC), and the Darien (DR). *Plasmodium vivax* is widespread across Panama, but *P. falciparum* appears to be restricted to the eastern part of the country, encountered mostly in KYC and DR (Figure 1), where a chloroquine - resistant strain has recently been detected (Calzada et al., 2008).

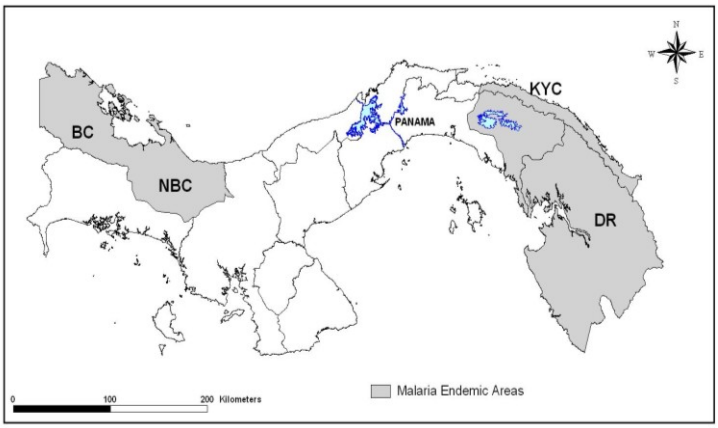

Figure 1. Malaria distribution in Panama (2001-2005)

Bocas Del Toro (BC)
Comarca Ngöbe Buglé (NBC)
Comarca Kuna Yala (KYC)
Darien (DR)

Factors associated with high malaria incidence in Panama include increased migration between endemic and non-endemic malaria regions, inadequate health care, and high humidity eight months of the year that enhances the prevalence of mosquito breeding sites and adult female longevity (PAHO, 1996; Service & Townson, 2002; World Bank, 2005). Recently there has also been an increase in urban cases in Panama (Ministerio de Salud – MINSA, 2007 / http://www.minsa.gob.pa), a new trend that is troubling and not understood. Although the number of malaria cases had returned to fewer than 300 by 2007 and malaria is no longer regarded as epidemic in Panama, there is a general concern that with more environmental perturbation and human migration, the drug resistant strain of *P. falciparum* could become widespread throughout Panama and invade other Central American countries (Samudio et al., 2005; Calzada et al., 2008).

One critical unknown factor that could influence the recent malaria epidemic in Panama is whether mosquito species have shifted temporally across the country. Such a shift could be caused by deforestation, and the subsequent alteration of breeding sites, and result in the need to change tactics for vector

control. Moreover, Panama has undergone significant changes in human demography and substantial human mediated changes during the last four decades (Contraloría, 2004). Yet, studies in Panama on the impact of these changes in the distribution of malaria vectors have not been conducted.

Future studies may discover that the vector species incriminated in neighboring countries such as *Anopheles* (*Nyssorhynchus*) *aquasalis* (Venezuela, Berti et al., 1993; Brazil, Póvoa et al., 2003), *An.* (*Nyssorhynchus*) *darlingi* (Belize, Achee et al., 2005; Colombia, Olano et al., 2001), *An.* (*Kerteszia*) *neivai* (Colombia, Carvajal et al., 1989; Solarte et al., 1996) and *An.* (*Anopheles*) *vestitipennis* (Belize, Grieco, 2001; Achee et al., 2007), are either locally or regionally established vectors in Panama. Additional studies may also find that in some localities species compositions have altered, prompting changes in relative malaria parasite abundances, and possibly increasing the frequency of malaria outbreaks. Such data will be invaluable for the vector control department, in focusing scarce resources for vector management in Panama.

Historically *Anopheles* (*Nyssorhynchus*) *albimanus* has been considered to be the only important vector of human malaria in Panama. However, *An.* (*Nyssorhynchus*) *bachmani* (Syn. *An. triannulatus*), *An.* (*Anopheles*) *punctimacula* s.l., *An.* (*Anopheles*) *apicimacula*, *An.* (*Anopheles*) *neomaculipalpus* and *An.* (*Anopheles*) *pseudopunctipennis* have also been found infected with *Plasmodium* parasites and incriminated as potential malaria vectors based on field and laboratory studies in Panama (Darling, 1910; Rozeboom, 1935; Simmons, 1936a,b, 1937). In addition, seven other *Anopheles* species involved in

malaria transmission elsewhere in the Americas have been reported from Panama (Wilkerson & Strickman, 1990), of which at least five are considered sibling species complexes (Wilkerson, 1990; Rosa-Freitas et al., 1998; Moreno et al., 2005; Marrelli et al., 2006; Quiñones et al., 2006; Bourke et al., 2010). Their current distributions and roles in malaria transmission are still unknown across Panama.

The existence of cryptic species in anopheline mosquitoes may compromise the successful application of malaria control methods that target the vectors. This is because some of these species may or may not be involved in malaria transmission, but still they are indistinguishable morphologically from each other (Mirabello & Conn, 2006; Marrelli et al., 2006). These facts are of vital importance to malaria control in Central America and Panama because high diversity of anopheline species involved in regional or local transmission with a range of breeding sites, biting times and vector competences may suggest that distinctive control strategies need to be seriously considered to effectively reduce the number of malaria cases regionally (Lounibos & Conn, 2000).

On the other hand, little research has been done to assess the population structure, and demographic history of *Anopheles* mosquitoes in the Americas, with investigations undertaken largely on primary malaria vectors at a continental scale, giving scarce attention to micro-regional patterns of genetic structure and to secondary malaria vectors (De Merida et al., 1995, 1999; Manguin et al., 1999; Molina-Cruz et al., 2004; Mirabello & Conn, 2006; Mirabello et al., 2008). Research on the population genetics of malaria vectors is required to identify

barriers to hybridization that would prevent gene flow between natural populations and genetically engineered mosquitoes (Walton et al., 2000, 2001; Lehmann et al., 2003; Mirabello, 2007; Mirabello et al., 2008; O'Loughlin et al., 2008). Similarly, such studies are needed to determine the extent of genetic exchange among populations and to predict the spread of genes conferring insecticide resistance (Lehmann et al., 2003; Mirabello et al., 2008).

Measures of differential gene flow and population boundaries in *Anopheles* species can be used to predict the potential spread of genes involved in insecticide resistance across Panama. Historically, Panama has used nationwide residual insecticide spraying (IRS) with DDT, propoxur, fenitrothion, deltamethrin, and cyflutrin since 1957 as the main control method for malaria vectors. Currently, control for malaria in Panama by the Ministerio de Salud (MINSA) is fenitrothion applied as a residual spray inside houses in endemic malaria areas every four months. In addition, during an outbreak, deltamethrin is applied twice a day as a fog for three consecutive days at the beginning and again at end of a 21-day period (Vector Control Department, Panama, unpublished data). The impact of historical and current vector control strategies on the population structure of anopheline mosquitoes in Panama is unknown.

To date, no studies about genetic diversity have been undertaken in disease vectors such as anopheline mosquitoes across southern Central America. Evidence for population fragmentation has been found in other groups of organisms across Panama. For instance, studies carried out in fresh water fishes, the pseudoscorpion *Cordylochernes scorpioides*, and the tungara frog

Physalaemus pustulosus, have supported a complex biogeographical history in the Isthmus of Panama where significant genetic differentiation is seen at short distances, and waves of colonization, extinctions and re-invasions appear to be the main forces shaping the distribution of genetic diversity. The geographic position of Panama and its complex biogeographical history (Bermingham & Martin, 1998; Weigt et al., 2005; Miller et al., 2008) represent an ideal opportunity to study intraspecific and interspecific levels of genetic divergence in malaria vectors.

The study of the demographic history of *Anopheles* species across this area will help to fill in the gap of information on vector biogeography in the northern Neotropics. These studies will facilitate the identification of historical processes (*i.e.* vicariance or expansion) that may affect the current patterns of disease transmission across Central and South America (Mirabello & Conn, 2008). A better understanding of the population history of *Anopheles* mosquitoes in Panama is also crucial to provide insights into how they colonized Central and northern South America; this information could be used to predict changes in mosquito biogeography due to ongoing and future climate changes in the Neotropics (Walton et al., 2000, 2001; Mirabello & Conn, 2008; O'Loughlin et al., 2008).

Anopheles albimanus is the most important malaria vector in Central America, the Caribbean islands and in coastal areas of Colombia and Ecuador (Faran, 1980). The role of *An. albimanus* as a primary malaria vector of *P. vivax* and *P. falciparum* has been historically acknowledged (Rubio-Palis &

Zimmerman, 1997; Lounibos & Conn, 2000); yet, its capacity to transmit malaria parasites varies across its range (Grieco et al., 2005; Achee et al., 2007; Gutiérrez et al., 2008). Evidence for cryptic species in *An. albimanus* has been unsubstantiated so far (Hobbs et al., 1986; Beach et al., 1989; Narang et al., 1991; De Merida et al., 1995), and population differentiation seems to be low overall (De Merida et al., 1999; Molina-Cruz et al., 2004). Previous work using the intergenic spacer (IGS) region of the nuclear ribosomal DNA supported barriers to gene flow for *An. albimanus* across the species range, but failed in rejecting the hypothesis of a single taxon (De Merida et al., 1995). Furthermore, the lack of fixed chromosomal inversions in samples of *An. albimanus* from Panama, Mexico and Colombia provided further support for a single species (Keppler et al., 1973; Conn, J. personal communication). The shallow population structure of *An. albimanus* in the Americas could be attributed to long-term effective population size due to high dispersal capability, opportunistic feeding behavior and substantial ecological plasticity (Breeland, 1972; Frederickson, 1993; González & Martinez, 2006). However, at the same time there is significant evidence for intraspecific variation in host feeding behavior, *Plasmodium* susceptibility, longevity and insecticide resistance (Collins et al., 1976; Frederickson, 1993; Grieco et al., 2005). Moreover, *An. albimanus* is believed to have originated in the Caribbean islands and then spread to the American continent and a bottleneck (*i.e.* founder effect) was suggested as the main factor shaping its continental population structure (Molina-Cruz et al., 2004).

An initial colonization path of *An. albimanus* throughout the Americas implies that a decrease in genetic diversity should be found, as this would be expected under a founder effect and sequential bottleneck in the colonizing front. However, this pattern has not been supported by previous studies across the species range (De Merida et al., 1999; Molina-Cruz et al., 2004). Furthermore, isolation by distance (IBD) was hypothesized to be the main force causing genetic structure in mainland populations of *An. albimanus*, whereas populations from Cuba were significantly differentiated from inland populations, likely due to the Atlantic Ocean, which may restrict gene flow (De Merida et al., 1999). In contrast, more recent studies rejected IBD and hypothesized barriers to dispersal for *An. albimanus* between Costa Rica and western Panama, and between eastern Panama and northern South America (Molina-Cruz et al., 2004). Because *An. albimanus* has a lowland-coastal distribution, mountain ranges are expected to restrict its dispersal and gene flow. Nevertheless, all these hypotheses have to be retested because these studies included only scattered populations from across Panama and Costa Rica and limited numbers of samples from the Caribbean and South America.

To date, few attempts have been made to assess the population history of *An. albimanus*. For example, demography (*i.e.* stability of the population size) and natural selection (*i.e.* neutrality), which are known to influence inferences of population structure in *Anopheles* mosquitoes, have not been explored fully (Besansky et al., 1997; Mirabello & Conn, 2006; O'Loughlin et al., 2008). Moreover, the taxonomic status of *An. albimanus* as a single species still needs to

be confirmed with other molecular markers and better sampling. Information on the population history of *An. albimanus* across southern Central America will help to understand and possibly predict the pattern of genetic structure of other similarly distributed malaria vectors. For instance, it has been demonstrated that population structure can differ sharply even between closely related *Anopheles* species (*An. albitarsis* species complex, Lehr et al., 2005, compared with *An. darlingi,* Mirabello & Conn, 2006) and this affects their roles in malaria transmission (Conn et al., 2002; Póvoa et al., 2003, 2006). On the other hand, there have also been instances where closely related species depict similar patterns of population structure. For instance, in Southeast Asia, *Anopheles* (*Cellia*) *dirus* and *Anopheles* (*Cellia*) *baimaii*, two important malaria vectors, have expanded demographically due to changes in forest structure and climatic conditions in the Pleistocene (300,000 years ago) (O'Loughlin et al., 2008).

Anopheles darlingi, considered the most important malaria vector in the Neotropics, is responsible for the transmission of *P. falciparum*, *P. vivax*, and *P. malariae* (summarized in Mirabello, 2007). This malaria vector has a broad distribution across Central and South America; yet, it has never been collected in Nicaragua, Costa Rica and Panama, showing a discontinuous geographic distribution. *Anopheles darlingi* has expanded in South America likely due to forest fragmentation and refugia isolation in the late Pleistocene (Mirabello & Conn, 2006). Although *An. albimanus* is quite different from *An. darlingi* in terms of larval habitat, the latter is more adapted to live primarily along warm lowland rivers and more stable larval habitat; they are closely related species that belong

to the same subgenus *Nyssorhynchus* (Sallum et al., 2002). Therefore, it is probable that despite having different primary breeding sites they have both expanded demographically in response to a common Pleistocene event. The impact of Pleistocene environmental changes on the population structure of *An. albimanus* and in relation to *An. darlingi* still needs to be investigated.

Anopheles punctimacula s.l., has been corroborated as a malaria vector in Panama (Simmons, 1936a,b; 1937). More recently, *An. punctimacula* s.l., has been the subject of morphological revision resulting in the re-description of two closely related cryptic species, *Anopheles (Anopheles) malefactor* and *Anopheles (Anopheles) calderoni* (Wilkerson, 1990, 1991). *Anopheles punctimacula* s.l., is found from Mexico to Argentina (Forattini, 1962), and also in the Caribbean Islands (Knight & Stone, 1977) whereas *An. malefactor* seems to be restricted to eastern Panama and northern Colombia (Wilkerson, 1990). Both *An. punctimacula* s.l., and *An. malefactor* have been collected biting humans and coexisting in the same larval breeding sites in Panama, but *An. calderoni* has not been reported from this country (Wilkerson & Strickman, 1990).

Unlike *An. albimanus*, which is straightforward to identify morphologically with standard keys, *An. punctimacula* s.l., can be easily confused with *An. malefactor*, or with other morphologically similar species within the Arribalzagia Series (Harbach, 2004). In this respect, seven other species have the potential to be found in Panama based on their geographical distribution: *An. calderoni*, *An.* (*Anopheles*) *costai*, *An.* (*Anopheles*) *forattinii*, *An.* (*Anopheles*) *intermedius*, *An.* (*Anopheles*) *mattogrossensis*, *An.* (*Anopheles*) *peryassui*, *An.*

(*Anopheles*) *pseudomaculipes* (Harbach, 2004). To date substantial variability in egg, larvae and adult morphology has been hypothesized in samples identified as *An. punctimacula* s.l., and ecological evidence for the existence of a species complex has been supported by several authors (Wilkerson, 1990, 1991; Harbach, 2004; Achee et al., 2005; Ulloa et al., 2006). Populations of *An. punctimacula* s.l., have shown to be mostly zoophagic in southern Mexico, feeding primarily on cattle, and displaying the highest vector competence during the dry season when this species is more abundant (Ulloa et al., 2006). In contrast, Achee et al. (2005) encountered *An. punctimacula* s.l., in Belize with one peak of density in January at the beginning of the dry season and another peak in August during the rainy season, perhaps reflecting differences in larval habitat distributions determined by local environmental conditions.

The feeding and resting behaviors of *An. punctimacula* s.l., are not fully understood yet, and may vary across the species geographic range. For instance, *An. punctimacula* s.l., may display a more anthropophagic tendency in areas where domestic animals are scarce or absent. On the other hand, it is also possible that *An. punctimacula* s.l., (in the past Syn. *An. malefactor* and *An. calderoni*) consists of more than three cryptic species, and this could have significant implications for vector control and malaria transmission in Panama. Population genetic studies on *An. punctimacula* s.l., may lead to the discovery of other members of the Arribalzagia Series, which could have been morphologically misidentified as *An. punctimacula* s.l., in samples from poorly studied areas of

Panama. The uncertain taxonomic status and the role of *An. punctimacula* s.l., as a malaria vector, needs to be further investigated.

1.1. Research Objectives

The present doctoral thesis focuses on the malaria problem from the point of view of the anopheline vector. I apply molecular techniques and population genetic tools to assess the genetic structure, demographic history and molecular taxonomic status of three important malaria vectors, *An. albimanus*, *An. darlingi* and *An. punctimacula* s.l. In addition, this thesis focuses locally on Panama where malaria has had a recent increase in case numbers, to investigate the species composition and distribution of malaria vectors in traditionally endemic areas. In chapters three and four, I used historical (1970-2005) and contemporary (2006-2009) information on mosquito collections to assess the species composition and distribution of adult *Anopheles* in Panama. Historical mosquito information was obtained partially from existing data sets gathered by members of the National Malaria Eradication Service (SNEM) and the Instituto Conmemorativo Gorgas de Estudios de la Salud (ICGES). Also, more recent collections were carried out in four main malaria endemic regions of Panama (Figure 1), including more than 35 localities and approximately 25 field trips during both the dry and rainy seasons. Analysis and comparison between the historical and contemporary data sets will serve as a starting point to assess whether malaria vectors differ by region (BC, NBC, KYC and DR), as these habitats are likely to be distinctive enough to warrant different control strategies in Panama. This information can be used for predictive models of malaria transmission, which are likely to vary regionally,

and will depend greatly on the accurate identification of vector species composition. I hypothesize that additional putative vector species exist in Panama and some of them may be involved locally as secondary vectors, or they may become more abundant in Panama with habitat changes.

In chapters five and six, I used partial sequences of the cytochrome oxidase subunit I gene (*COI*), and extensive sampling throughout Central America and northern South America to identify the pattern of micro-geographic genetic structure of *An. albimanus*. Additionally, I sequenced a subset of samples for the single copy nuclear *white* gene to corroborate the pattern depicted in the mitochondrial analyses, and to test the hypothesis of an initial colonization path of *An. albimanus* from northern Central America into South America as proposed by Molina-Cruz et al. (2004). I also used sequences of the ribosomal DNA ITS2 region to test for restricted gene flow or cryptic speciation in *An. albimanus*. A total of 612 sequences of the *COI*, 175 of the nuclear *white* gene and 173 of the rDNA ITS2 region of mosquitoes from Nicaragua, Costa Rica, Panama, Colombia and Ecuador were obtained and analyzed. I used the information from the *COI* and the *white* gene to test previous hypotheses of genetic differentiation in *An. albimanus* due to geographic distance, physical barriers to dispersal, natural selection and/or demographic phenomena. Specifically, I was interested in testing the hypothesis of historical geographic fragmentation due to Pleistocene environmental changes. I expected *An. albimanus* to have a similar pattern of population history as *An. darlingi* in South America, because despite having different primary breeding sites, climatic oscillations may have affected larval

habitat availability for both species during the Pleistocene. I hypothesize that *An. albimanus* is single, albeit polymorphic species, that is not at mutation-drift equilibrium likely due to Pleistocene geographic fragmentation and population expansion in southern Central America.

In chapter seven, I sequenced the mtDNA *COI* gene, and the ITS2 region of the ribosomal DNA in mosquitoes from Panama and Costa Rica to investigate the taxonomic status of the malaria vector *An. punctimacula* s.l. I included specimens of *An. malefactor* and other members of the Arribalzagia Series within the subgenus *Anopheles* to provide a hypothesis of the phylogenetic status of these closely related taxa. First, I used a variable region located at the 3' end of the *COI* gene to assess the initial genetic structure, and then I used the Barcoding region of the *COI* (Hebert et al., 2003) to corroborate the findings of the 3' *COI* region. The Barcode region or Folmer region, located at the five prime (5') end of the *COI* gene, has proven useful to tell cryptic mosquito species apart (Cywinska et al., 2006; Pradeep Kumar et al., 2007), but see also Foley et al. (2007). Finally, I used the ITS2 marker to test for cryptic speciation in *An. punctimacula* s.l. I hypothesize that *An. punctimacula* s.l. is a species complex that comprises more than three cryptic species in Panama.

1.2. Hypotheses

– I hypothesize that additional putative vector species exist in Panama and may be encountered with more intensive mosquito sampling across the country.

– I hypothesize that *Anopheles albimanus* is single, albeit polymorphic species, which is not at mutation - drift equilibrium in southern Central America.

– I hypothesize that *Anopheles albimanus* depicts a similar pattern of Pleistocene population expansion as *Anopheles darlingi* in South America.

– I hypothesize that *Anopheles punctimacula* s.l., is a species complex that comprises more than three cryptic species in Panama.

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2. Chapter II. Literature Review

2.1. Molecular population genetics of Anopheles *mosquitoes*

Anopheles mosquitoes, within the family Culicidae (Subfamily: Anophelinae), comprise the most economically and medically significant group of insects worldwide. They transmit important pathogens such as filarial worms, arboviruses, and malaria parasites, causing significant economic burden and human anxiety (Knight & Stone, 1977; Service, 1971, 1993). Malaria control through the management of *Anopheles* populations has been one of the core priorities of mankind during the last two centuries, yet this task has proven very difficult so far (WHO, 2004, 2006; Walker & Lynch, 2007). Molecular population genetics is one research avenue that could potentially contribute to achieve this goal. However, little research has been carried out to assess the overall patterns of *Anopheles* diversification, phylogenetic relationships and population structure. Only the primary vectors of malaria, such as the Africans *Anopheles* (*Cellia*) *gambiae* s.l., and *An.* (*Cellia*) *funestus* s.l.; the Neotropical *An.* (*Nyssorhynchus*) *darlingi*; and the Asians *An.* (*Cellia*) *dirus* s.l., and *An.* (*Cellia*) *sundaicus* s.l., have received much attention, and consequently, a considerable number of *Anopheles* species still remains poorly studied (Harbach, 2004, 2009; Hay et al., 2010).

Anopheles mosquitoes are characterized by large populations of active flyers that feed on vertebrate blood (only the female) and breed in aquatic habitats; most species are seasonal and depend primarily on local and regional patterns of rainfall to develop (Service, 1993; Service & Townson, 2002). Flight

range is low to moderate (1 – 5 km), but usually < 2 km (Costantini et al.,, 1996; Service, 1997; Achee et al., 2007) and ten generations per year were calculated for *An. dirus* s.s., in tropical southeast Asia (Walton et al., 2000). As a result of these characteristics, anophelines are expected to show abundant genetic variability at the DNA level, and low genetic differentiation among populations.

Theoretical expectations indicate that patterns of population structure are likely to differ between primary and secondary malaria vectors due to marked differences in ecology and demographic attributes (Donnelly et al., 2002). Most primary vectors are widely distributed; they have generally excellent colonizing abilities and have adapted to live under varying environmental conditions throughout their geographical ranges. Such species are expected to face few barriers to dispersal, so allopatric speciation is unlikely and population structure is expected to be shallow (Besansky et al., 1997; Donnelly et al., 2002; Lehmann et al., 2003; Djadid et al., 2005; Oshaghi et al., 2006), but see also Foley & Torres (2006). In contrast, secondary vectors are less locally abundant, and more patchily distributed, thus they are likely to be more diverged and geographically structured than primary vectors (Donnelly et al., 2002, 2004; Mirabello & Conn, 2008; O'Loughlin et al., 2007). While these generalizations seem logical in theory, they still need to be rigorously tested with many *Anopheles* species and across different geographic areas. The overall low representation of secondary vector in malaria endemic regions may partially reflect the outcome of a biased sampling protocol, since most mosquito collections occur usually around human habitations and these vectors are less anthropophilic than primary vectors. To my knowledge

there has not been any extensive direct comparison of genetic diversity between primary and secondary malaria vectors, although Lounibos & Conn's 2000 review did provide some comparisons based on data available at that time.

Recent advances in DNA sequencing have allowed researchers to obtain large amounts of data in the form of nucleotide sequences in a short period of time. Also advances in software development and population genetics theory, such as "the coalescent" have had a profound impact on the way DNA sequence data are analyzed currently (Kuhner, 2008). These improvements have permitted the study of each species' demographic history in a more comprehensive fashion, using most of the information encountered in the DNA nucleotide sequences, and therefore, providing additional insight into the historical factors causing genetic structure in *Anopheles* mosquitoes (Emerson et al., 2001; Hey & Machado, 2003; Wandeler et al., 2007; Kuhner, 2008).

The present review chapter aims primarily at summarizing the results from past studies on the molecular population genetics of malaria vectors. It focuses mostly on research regarding *Anopheles* demographic history, which was done with DNA nucleotide sequences (*i.e.* mitochondrial genes, the single copy nuclear *white* gene and the ribosomal internal transcribed spacer two), but it also briefly touches on other subjects (*i.e.* phylogenetic reconstruction, phylogeography and molecular taxonomic status) as well as other types of molecular markers (*i.e.* microsatellite loci). I present the findings obtained with two different approaches to investigate the population structure, and discuss general information about

DNA markers, methods and sampling strategies used to accurately investigate the demographic history of *Anopheles* mosquitoes.

2.2. Rationale

The primary goal of population genetics studies on malaria vectors is to define demes, lineages or molecular forms that may have differential involvement in malaria transmission caused by the lack of genetic connectivity. These genetic entities are defined in terms of a history of migration events and genetic exchange, and their limits may vary spatially and temporally (Walton et al., 2001; Mirabello et al., 2008; O'Loughlin et al., 2007, 2008). Furthermore, knowledge on intra-specific levels of genetic diversity of malaria vectors can be informative about the demographic cohesiveness (random mating) and the effective population size (N_e) and this information can be applied to reduce malaria through mosquito control (Temu et al., 2004; Molina-Cruz et al., 2004; Michel et al., 2005a,b; Scarpassa & Conn, 2007; Mirabello et al., 2008). For example, monitoring the N_e in populations of *Anopheles* at distinctive temporal points can be valuable in deducing effects of insecticide in comparisons before and after application (Wondji et al., 2005). Also, the identification of physical or ecological barriers to dispersal can be used to determine the number of release points for transgenic mosquitoes (Catteruccia, 2007). Such studies can also help to predict the potential spread of genes involved in parasite refractoriness and/or insecticide resistance (Lehmann et al., 2003; Mirabello et al., 2008).

Molecular markers can shed light on the phylogeographical pattern and phylogenetic relationships among closely related *Anopheles* species, and this

knowledge can help to clarify the taxonomic status and biogeographic origin of those lineages that have only recently diverged. This is crucial for future malaria control since correct species designation will be required for effective insecticide treatment, which is likely to be severely compromised by the increasing number of sibling *Anopheles* species complexes detected during the past ten years (Garros et al., 2005; Dusfour et al., 2004, 2007; Paredes-Esquivel et al., 2009; Bourke et al., 2010). Also, an understanding of the molecular demographic history of *Anopheles* mosquitoes is crucial to provide insights into how they responded to past geological and climatic changes and colonized their current geographic distribution. This information could also be used to predict changes in mosquito biogeography due to ongoing and future climate changes worldwide (Donnelly et al., 2004; Foley & Torres, 2006; Hasan et al., 2008; Morgan et al., 2009; Gutiérrez et al., 2009). Another motivation for establishing correct demographic history in malaria vectors is to accurately identify genes acting under natural selection, which may be especially important in *An. gambiae* s.s., where sites of host-pathogen coevolution could serve as targets for malaria control intervention (Lehmann et al., 2009; Crawford & Lazzaro, 2010).

2.3. Molecular markers

The successful application of genetic tools to investigate the population structure of *Anopheles* species will depend largely on the sampling scheme and the use of the appropriate molecular marker. In general, the optimal number of populations and samples per population will be determined by the species' ecology and its historical demography. As previously stated, ubiquitous and

widespread *Anopheles* species, capable of developing in a wide variety of aquatic habitats, should be more able to maintain higher N_e than less abundant and more patchily distributed species (Donnelly et al., 2002). This means that the former group will require more extensive sampling with more populations and individuals per population than more geographically restricted species (Walton et al., 2000; Donnelly et al., 2002, 2004; Lehmann et al., 2003). Furthermore, species that have undergone recent population expansion or bottleneck (*i.e.* founder effect) will required fewer individuals and populations, but different independent markers and more widespread sampling, so the geographic extent of any particular demographic event can be determined (Walton et al., 2001; Mirabello & Conn, 2006a,b; O'Loughlin et al., 2008; Hasan et al., 2008; Gutiérrez et al., 2009; Pedro & Sallum, 2009; Crawford & Lazzaro, 2010).

It is also important to choose the appropriate marker depending on the type of study, for example, molecular taxonomy (testing for monophyletic clades), population structure (assessing contemporary rates of gene flow), and phylogeography (identifying the geographical origin of a lineage) are all different in scope, and thus, they require different markers and sampling schemes (Kuhner, 2008). Contemporary levels of gene flow among *Anopheles* populations can be assessed using nuclear microsatellite loci because, due to their high mutation rate, these markers can detect weak differentiation even in species with high gene flow (Temu et al., 2004; Molina-Cruz et al., 2004; Scarpassa & Conn, 2007; Mirabello et al., 2008). In contrast, large - scale geographical differences are better detected using mitochondrial DNA polymorphism because, due to maternal inheritance, N_e

for mtDNA is a quarter of that of nuclear genes, and consequently genetic drift may produce strong geographic signal (De Merida et al., 1999; Chen et al., 2004; Mirabello & Conn, 2006a,b; Jung et al., 2007; O'Loughlin et al., 2007; Hasan et al., 2008; Gutiérrez et al., 2009; Paredes-Esquivel et al., 2009; Bora et al., 2009). Hence, mistaken estimates of contemporary rates of gene flow among *Anopheles* populations may be obtained, for example, when working under the phylogeographic framework, as mitochondrial DNA may reflect the signal of more ancient demographic processes rather than current genetic exchange (Foley & Torres, 2006; Mirabello & Conn, 2006a,b; Reiff et al., 2007; O'Loughlin et al., 2008; Hasan et al., 2008; Gutiérrez et al., 2009).

Different molecular markers can provide information at different genetic levels. The mutation rate for mitochondrial genes is usually higher than for single copy nuclear genes, but slower than for microsatellite loci, thus each marker offers a different time-frame in the demographic history of malaria vectors. Also, the recombination rate, mode of inheritance and genome location must be considered for adequate resolution of different species - population processes (Hey & Machado, 2003; Wandeler et al., 2007; Kuhner, 2008).

A combination of molecular markers that evolve at a fast enough rate to be informative for the taxa under study, and adequate sampling, are the best strategies for the correct identification of demes, lineages and species relationships when working with *Anopheles* mosquitoes (Krzywinski & Besansky, 2003; Goswami et al., 2005; Garros et al., 2005; Ma et al., 2006; Sallum et al., 2007; Reidenbach et al., 2009; Bora et al., 2009; Paredes-Esquivel et al., 2009).

Agreement between different markers may provide a robust perspective of cladogenesis, and/or population divergence, for example, congruent results of demographic history in *Anopheles* mosquitoes should be obtained by both nuclear and mtDNA genes when genetic structure is caused by past demographic processes, as these are genome wide forces, whereas selection is locus specific. Nevertheless, results could vary widely depending on species ecology, the markers and geographic areas (Donnelly et al., 2004; Ma et al., 2006; Dusfour et al., 2007a,b; Morgan et al., 2009; Bourke et al., 2010).

A multiple marker approach can also reduce bias in parameter estimation and genealogical uncertainty due to differential gene coalescence (Goswami et al., 2005; Reidenbach et al., 2009; Paredes-Esquivel et al., 2009; Crawford & Lazzaro, 2010). Nevertheless, caution should be exercised when using the total evidence approach (*i.e.* concatenating multiple loci), as different genes may depict different topologies or molecular signals, and this may be indicative of natural selection, lack of marker resolution or introgression. In these cases, a locus-by-locus assessment is recommended to recover correct phylogenetic relationships among closely related *Anopheles* species (Garros et al., 2005; Reidenbach et al., 2009; Bourke et al., 2010). Moreover, all these molecular markers are assumed to be evolving neutrally, so they can accurately represent the relationship between drift and genetic diversity (N_e) (Emerson et al., 2001; Hey & Machado, 2003; Kuhner, 2008).

In addition, the geographic context needs to be considered, as some areas may required more sampling due to a complex geological and environmental

history leading to cyclical isolation, greater loss of genetic diversity and higher speciation rate. Lack of proper sampling in an island system, for example, may lead to mistaken inferences of speciation and colonization pattern in some *Anopheles* species. This situation may also be seen in mainland *Anopheles* populations when genetic differentiation is largely due to sampling gaps, rather than lack of gene exchange (Foley & Torres, 2006; Dusfour et al., 2004, 2007a; Mirabello & Conn, 2008; Hasan et al., 2008; Bora et al., 2009)

2.3.1. Mitochondrial DNA

The mitochondrial DNA (mtDNA) has been used as the standard marker in population genetic studies of *Anopheles* mosquitoes. High copy numbers and the availability of conserved primers and PCR protocols, useful in a wide range of taxa, make the mtDNA an ideal starting point to investigate genetic relatedness in malaria vectors. In addition, mtDNA can be used inter-changeably to answer questions about molecular taxonomy, phylogenetic reconstruction and demographic history. For example, most of the work done during the past ten years in *Anopheles* phylogeography has been based on the protein coding genes and the rapidly evolving non-coding control region of the mtDNA (Besansky et al., 1997; De Merida et al., 1999; Walton et al., 2000; Fairley et al., 2002; Chen et al., 2004; Dusfour et al., 2004; Lehr et al., 2005; Foley & Torres, 2006; Mirabello & Conn, 2006a,b; Sallum et al., 2007; Oshaghi et al., 2006, 2007; Jung et al., 2007; O'Loughlin et al., 2008; Hasan et al., 2008; Gutiérrez et al., 2009; Paredes-Esquivel et al., 2009).

The outcome of population genetic studies obtained with mitochondrial genes generally matches those carried out with other molecular markers (*i.e.* microsatellites and nuclear genes), especially when the target groups have diverged a long time ago and differentiation is shared across loci (Garros et al., 2005; Goswami et al., 2005; Ma et al., 2006; Paredes-Esquivel et al., 2009). Nevertheless, sometimes discrepancies arise when mosquito populations and/or lineages defined by mtDNA analysis are not well resolved by other DNA regions (Di Luca et al., 2004; Oshaghi et al., 2006, 2007; Dusfour et al., 2007; Bora et al., 2009; Bourke et al., 2010). Among the likely reasons for this incongruence are: contemporary interspecific hybridization with sister taxa or incomplete lineage sorting due to retention of ancestral polymorphism (Besansky et al., 1997; Donnelly et al., 2004; Walton et al., 2000). In contrast, more recent restriction to gene flow may promote genetic divergence only in the mtDNA while other markers with slower mutation rates may still depict signals of substantial gene exchange (Donnelly et al., 2004; Dusfour et al., 2007a; Bora et al., 2009; Bourke et al., 2010; *McKeon et al., submitted to the Malaria Journal April 16, 2010*).

In addition, because all mitochondrial genes are inherited as a single block, they only provide a limited view of the species' demographic history, as selective pressures could confound the molecular signal. Studies undertaken with mitochondrial genes have been criticized due to nucleotide sequence saturation (multiple hits) and evidence for non-neutral evolution such as recurrent selective sweeps or background selection (Ballard & Rand, 2005; Bazin et al., 2006). Consequently, it is always desirable to cross-validate the mitochondrial results

with a nuclear marker, particularly when targeting taxonomically questionable *Anopheles* species (Walton et al., 2000, 2001; Molina-Cruz et al., 2004; Di Luca et al., 2004; Garros et al., 2005; Dusfour et al., 2007b; Gutiérrez et al., 2009; Reidenbach et al., 2009; Bora et al., 2009; Bourke et al., 2010). Another potential cause for a mistaken molecular signal by mtDNA genes is the occurrence of *Wolbachia* spp. in *Anopheles* mosquitoes. This endosymbiotic bacterium makes all individuals within a population have the same mitochondrial haplotype due to rapid horizontal gene transfer, thus obscuring the signal of gene exchange. Nonetheless, to my knowledge no information has been published about the occurrence of *Wolbachia* spp. in any *Anopheles* species (Kittayapong et al., 2000).

Despite all the drawbacks of the mtDNA, mitochondrial genes have proven to be extremely useful in identifying the population structure and demographic history of *Anopheles* species, and they have been used extensively to study malaria vectors worldwide, allowing for comparison across species and geographic areas (De Merida et al., 1999; Walton et al., 2000; Foley & Torres, 2006; Jung et al., 2007; Sallum et al., 2007; O'Loughlin et al., 2008; Hasan et al., 2008; Gutiérrez et al., 2009; Paredes-Esquivel et al., 2009; Bora et al., 2009).

Likewise, the Barcoding or Folmer region, located at the five prime (5') end of the mitochondrial cytochrome oxidase one gene (*COI*) (Hebert et al., 2003) has been successfully employed in molecular taxonomy of *Anopheles* mosquitoes. Fixed mutations and more than three percent (3%) divergence among *COI* sequences may indicate restricted gene flow and/or speciation (Dusfour et al.,

2004; Cywinska et al., 2006; Pradeep Kumar et al., 2008). More recently, Foley et al. (2007) suggested that the threshold level to define *Anopheles* species could be set lower than 3% to minimize false positives.

2.3.2. The single copy nuclear white *gene*

Nuclear genes offer an attractive alternative, combining the advantage of relative ease of alignment and unambiguous identification of rapidly evolving sites with the flexibility of choosing among gene fragments that evolve at appropriate rates for a given systematics question (Besansky & Fahey, 1997; Krzywinski & Besansky, 2003). Although using a nuclear gene seems to be a logical solution to the sex-biased representation of the mtDNA, nuclear genes might be too conserved to uncover genetic structure due to more recent ecological adaptations (Bora et al., 2009; *McKeon et al., submitted to the Malaria Journal April 16, 2010*). In addition, nuclear genes undergo recombination, which may compromise their use in population genetic studies because of non-unique signal of historical demography (Krzywinski & Besansky, 2003).

The *white* gene, a homolog of the *Drosophila melanogaster* eye color gene, is a single copy nuclear-coding gene that belongs to a superfamily of Traffic ATPase membrane transporters; the product of this gene helps transport eye pigment precursors, guanine and tryptophan, into pigment cells. Alignment of the inferred amino acid sequences is usually not problematic, and intron length is short, which permits PCR amplification directly from genomic DNA (Besansky & Fahey, 1997). The *white* gene has been used as molecular marker in several studies involving *Anopheles* mosquitoes (Besansky & Fahey, 1997; Merrit et al.,

2005; Brochero et al., 2007; Mirabello & Conn, 2008; Reidenbach et al., 2009; Bourke et al., 2010).

Besansky & Fahey (1997) investigated the phylogenetic relationships within the family Culicidae using the *white* locus. Overall the phylogeny recovered by the *white* gene supported the traditional view of monophyly of Culicidae and reinforced the idea that the subfamily Anophelinae occupies a basal position. Although, these findings are in agreement with other phylogenetic trees built with morphological characters and other molecular markers, differential weighting schemes of the *white* gene did not recover a uniform phylogenetic signal. Substantial homoplasy likely due to high levels of divergence in the third codon position and biased nucleotide composition appear to cause this pattern. More recently, Reidenbach et al. (2009) reassessed the mosquito phylogeny using the *white* gene, five other nuclear genes and morphological characters. The findings were in agreement with the ones previously obtained, and this reinforces the taxonomic value of this nuclear gene to study *Anopheles* mosquitoes.

Bourke et al. (2010) used both the *white* and the NADH dehydrogenase subunit six (*ND6*) genes to investigate phylogenetic relationships among 21 species of the epidemiologically significant subgenus *Nyssorhynchus*. This study depicted conflicting results between these two genes, and rendered no support for the monophyly of the three subsections within *Nyssorhynchus* (Albimanus, Argyritarsis, Myzorhynchella) using the concatenated data set. Moreover, cryptic speciation was hypothesized for *An.* (*Nyssorhynchus*) *antunesi*, *An.* (*Nyssorhynchus*) *deaneorum* and *An.* (*Nyssorhynchus*) *strodei*.

Although, it was corroborated that intron presence-absence in the *white* gene may not be a reliable character for phylogenetic reconstruction given the great number of independent losses and gains in several groups of Diptera (Besansky & Fahey, 1997), and afterwards within Culicidae (Merrit et al., 2005; Brochero et al., 2007; Reidenbach et al., 2009; Bourke et al., 2010). It appears that some introns may be useful to identify certain sibling species of *Anopheles*. For example, the exclusive absence of the fourth intron in the *white* locus of *An.* (*Nyssorhynchus*) *marajoara* differentiates this malaria vector from other isomorphic members of the *An.* (*Nyssorhynchus*) *albitarsis* Complex (Merrit et al., 2005).

The *white* locus has also been used to investigate the taxonomic status of some Neotropical malaria vectors through population genetic analysis. Mirabello & Conn (2008) used the *white* gene to investigate the population structure and lineage divergence of *An.* (*Nyssorhynchus*) *nuneztovari* s.l., in South America. Six lineages, after removing the intron, were recovered from Colombia, Venezuela and Brazil, and hypothesized to have diverged during the Pleistocene and Pliocene. Lineage divergence in this malaria vector was hypothesized to be the result of Miocene/Pliocene marine incursion and Pleistocene climatic changes leading to refugia isolation. Furthermore, the *white* gene was used in conjunction with the ribosomal DNA ITS2 region to identify a new species, *An.* (*Nyssorhynchus*) *albitarsis* F, within the *An. albitarsis* Complex, in Colombia (Brochero et al., 2007), and more recently, the northern genotype of *An. darlingi* was identified for the first time in Panama using partial sequences of this gene

(*see results from Chapter IV of the present doctoral thesis*).

2.3.3. The ribosomal DNA internal transcribed spacer 2

Ribosomal DNA (rDNA) has been mostly used to answer systematics questions in *Anopheles* mosquitoes. The functional regions that produce the ribosomes are highly conserved, while at the same time there are transcribed and non-transcribed spacer regions that have high interspecific and low intraspecific variability. Multiples copies of the rDNA are thought to evolve by concerted evolution and thus exhibit little intraspecific and intragenomic variability (Wilkerson et al., 2004; Li & Wilkerson, 2005, 2007; Walton et al., 2007a,b). These characteristics make the rDNA a useful marker to study the taxonomic status of closely related *Anopheles* species and as a basis for identification of isomorphic species complexes (Collins & Paskewitz, 1996; Sallum et al., 2002; Wilkerson et al., 2004; Marrelli et al., 2006; Paredes-Esquivel et al., 2009). The internal transcribed spacer two (ITS2) has become the gold standard as a source of species-specific PCR primers for uncovering cryptic *Anopheles* species. Length differences and/or fixed substitutions among ITS2 sequences are taken as proof of lineage splitting especially if the lineages are geographically co-distributed (Wilkerson et al., 2004; Li & Wilkerson, 2005, 2007; Marrelli et al., 2006; Walton et al., 2007a,b; Paredes-Esquivel et al., 2009).

Among some recent examples of studies that used the ITS2 region in molecular taxonomy of *Anopheles* species are those by Djadid et al. (2005), and Bezzhonova & Goryacheva (2008). The work done by the former authors provided strong support for the status of a single species of *An.* (*Cellia*) *stephensi*

across most of the Indian continent. The results showed only minor polymorphism between different populations, despite their vast geographical distances. Hence, this malaria vector could be considered a single molecular species in Iran, but with biological and ecological variants in different zoogeographical zones. Furthermore, Bezzhonova & Goryacheva (2008) found nine different unique ITS2 sequences in *An.* (*Anopheles*) *messeae* from Russia, an indication of the likely occurrence of isomorphic taxa. Yet, because ITS2 sequences that were considered specific to *An. messeae* and *An.* (*Anopheles*) *daciae* were simultaneously present in one individual, the taxonomic status of the latter as a different species was refuted.

In spite of the fact that multiple alignments of divergent ITS2 sequences may sometimes be problematic, this molecular marker has also been used to study the phylogenetic relationships among closely related *Anopheles* species (Ma & Xu, 2005; Marrelli et al., 2005; Ma et al., 2006; Djadid et al., 2005, 2006; Paredes-Esquivel et al., 2009). Ma & Xu (2005) investigated the phylogeny of the *Anopheles* Hyrcanus Group in China using the ITS2 region. Twelve species within this group were differentiated by 0.4% to 50.8% sequence divergences and comparison between different ITS2 profiles revealed two unknown taxa. Moreover, a close phylogenetic relationship was hypothesized among *An.* (*Anopheles*) *liangshanesis*, *An.* (*Anopheles*) *kunmingensis*, *An.* (*Anopheles*) *kweiyangensis*, *An.* (*Anopheles*) *lesteri* and *An. sinensis*. Similarly, Garros et al. (2005) used the ribosomal ITS2 and D3 regions plus the mtDNA *COI* gene to investigate the phylogenetic relationships between the Afrotropical Funestus and

the Oriental-African Minimus group of the Myzomyia Series of *Anopheles*. Although phylogenetic trees recovered four well-supported clades, the total evidence approach confirmed the monophyletic status of this assemblage and claim that these two groups should be classified as one, with Funestus, Minimus and Rivolorum clades recognized as part of the Aconitus Subgroup.

In South America, phylogenetic studies using ITS2 sequences strongly supported the monophyly of the members of the *An. albitarsis* Complex, although it was noticed that sequences from *An. marajoara*, *An. deaneorum*, and *An. albitarsis* s.s., were distributed in more than one group of the tree, and this could indicate large intraspecific variations, recent speciation, or morphological misidentification (Marrelli et al., 2006). More recently, Paredes-Esquivel et al. (2009) used both the ITS2 region and the *COI* gene to distinguish among cryptic species within the *An.* (*Anopheles*) *barbirostris* Sub-group in Southeast Asia. These authors reported significant variation in the length of the ITS2 of five major clades, including *An. barbirostris*, and *An.* (*Anopheles*) *campestri*, clades IV (anthropophilic) and V (zoophilic). These clades were found co-occurring in many localities and also corroborated by the *COI* gene, which clearly indicates that conventional taxonomic keys used to identify theses mosquitoes need to be revised.

Both failures in identifying putative cryptic *Anopheles* species as well as the recovery of correct phylogenetic relationships by the ITS2 have also been reported in the literature. This though may reflect the lack of cloning (intraindividual variation) and sampling gaps in some studies or an insufficient

molecular signal in the ITS2 marker (Di Luca et al., 2004; Bargues et al., 2006; Dusfour et al., 2007; Bezzhonova & Goryacheva, 2008; Bora et al., 2009)

Anopheles population structure has also been studied with the ITS2, though to a much less extent compared with molecular taxonomy and phylogenetic reconstruction. Di Luca et al. (2004) investigated intraspecific variation in the Paleartic *An.* (*Anopheles*) *maculipennis* Complex using samples from 12 countries and six species. All the species within this group had 100% identity for their corresponding ITS2 sequence, in addition *An. messeae* depicted significant intraspecific genetic structure. These authors identified five different ribotypes in *An. messeae* that were consistent with the geographical origin of populations. Furthermore, the ITS1 phylogeny of *An.* (*Cellia*) *farauti* s.s., was largely explained by geography, and this indicates that concerted evolution is acting at the intraspecific level despite significant intraindividual variation. The combined effect of a high rate of turnover and rapid divergence means that this marker is more likely to reflect the recent population structure of this coastal malaria vector (Bower et al., 2008).

In contrast, Fairly et al. (2005) used both the ITS1 and ITS2 to investigate the pattern of population structure in the coastal Neotropical malaria vector, *An.* (*Nyssorhynchus*) *aquasalis*. Although point mutations were common in both spacers, neither of them had a diagnostic distribution or were informative in distinguishing populations from Brazil and Venezuela, providing additional support for the status of *An. aquasalis* as a single species.

2.4. Summary statistics and the equilibrium approach

Initially, studies about genetic structure of *Anopheles* mosquitoes were mostly conducted using summary statistics such as the number of segregating sites, the nucleotide diversity and the fixation indexes. All these statistics are based on predetermined models of spatial genetic structure "the so-called null hypothesis" that harbor several assumptions. For example, the standard neutral equilibrium model (SNE), suggests that the N_e has remained stable for at least $4N_e\mu$ generations and random mating is occurring throughout the species' geographic range. This means that all populations within a species have identical historical demography, with a continuous rate of gene exchange and regardless of their geographic locations (Hey & Machado, 2003; Wandeler et al., 2007).

This approach was very useful at the outset to uncover the spatial genetic structure of malaria vectors, yet it has many limitations. For example, when gene flow is inferred indirectly from *F* statistics, the assumption between drift and migration is often reasonable, since migration is a relatively fast homogenizing force (Wright, 1951). However, the genetic signature of demographic processes such as colonizations (historical migration), expansions (demographic or spatial) and contractions (bottlenecks or founder effect) can remain for a long time obscuring the true spatial genetic structure that may exist (Templeton, 1998). In such cases inferring values of contemporary gene flow, and N_e from fixation indexes or other summary statistics can be misleading (Besansky et al., 1997, Walton et al., 2000, 2001; Donnelly et al., 2002).

Restricted gene flow due to geographic distance, the so-called IBD pattern, is an equilibrium model that has been hypothesized in many *Anopheles* species worldwide. The IBD model is based on the hypothesis that gene flow is operating among neighboring populations, and its goal is to track down the distance that make different samples belong to the same deme. Consequently, under IBD random mating is more likely to occur between individuals in nearby populations than those more distantly located (Wright, 1951; Jensen et al., 2005). Temu et al. (2004) hypothesized that geographic distance was the cause of genetic structure in *An. funestus* s.s., in the eastern-central and southern regions of Africa. These authors found evidence of restricted gene flow due to geographic distance across a 1,200 km transect using six microsatellite loci. *Anopheles funestus* s.s., is an important malaria vector that breeds in permanent bodies of water such as swamps and slow-moving streams, and thus, it has a patchy distribution (Michel et al., 2005a,b). In contrast, generally neither *An. gambiae* s.s., nor *An.* (*Cellia*) *arabiensis*, depict signals of IBD across Africa. The hypotheses used to explain this phenomenon are extensive gene exchange due to high dispersal capabilities or a high long-term and stable N_e. Although these claims are hard to reconcile with a limited flight range and the strong seasonality recorded in these malaria vectors (Lehmann et al., 1997; Besansky et al., 1997; Donnelly et al., 1999; Simard et al., 2000; Pinto et al., 2003; Temu & Yan, 2005). Significant IBD was also evidenced between mainland populations of *An.* (*Cellia*) *moucheti* in Africa. Antonio-Nkondjio et al. (2008) used ten microsatellite loci and hypothesized IBD as the main force driving population differentiation in samples of *An. moucheti* from

Cameroon, the Democratic Republic of Congo and an island on Lake Victoria, in Uganda. Nevertheless, this pattern was not depicted in previous studies that showed very low levels of genetic differentiation among populations positioned 65 - 400 km apart (Antonio-Nkondjio et al., 2006). A positive and significant relationship between genetic and geographic distance has been supported in *An. nuneztovari* s.l., in South America using the single copy nuclear *white* gene (Mirabello & Conn, 2008). Similarly, studies based on microsatellites have shown that IBD is one force shaping genetic differentiation in *An. darlingi* in Amazonian Brazil (Conn et al., 2006; Scarpassa & Conn, 2007; Mirabello et al., 2008). However, in most of these cases the correlation was weak, and not detected when different lineages and geographic regions were analyzed separately. IBD was not detected in *An. nuneztovari* s.l., with analysis of mtDNA RFLPs (Conn et al., 1998) or in *An. darlingi* using the *COI* gene (Mirabello & Conn, 2006). De Merida et al. (1999) used a fragment of the mitochondrial NADH dehydrogenase subunit five (*ND5*) gene and hypothesized restricted gene flow due to geographic distance in mainland populations of the Neotropical malaria vector, *An.* (*Nyssorhynchus*) *albimanus*. Yet, IBD was not detected in *An. albimanus* with analysis of the *ND5*, *COI* and four microsatellites across Central America and between coastal populations of Colombia (Molina-Cruz et al., 2004; Gutiérrez et al., 2009).

The lack of IBD seen in many malaria vectors may suggest significant long distance human-mediated dispersal, and/or physical barriers to gene flow, yet this pattern can also be the result of a recent population expansion from an already

large genetic pool, or the lack of proper sampling. In contrast, misleading signals of IBD can be produced by a recent range colonization with sequential founder effect along a colonizing path, thus mimicking the IBD pattern (*i.e.* bottleneck) or due to lack of karyotypic information (Coluzzi et al., 1979; Besansky et al., 1997; Fairley et al., 2000; Donnelly et al., 2001, 2002; Molina-Cruz et al., 2004; Michel et al., 2006; Matthews et al., 2007; O'Loughlin et al., 2008; Crawford & Lazzaro, 2010).

Another commonly described spatial pattern of genetic structure using summary statistics and the equilibrium approach is the genetic structure due to physical barriers to dispersal. Mountain ranges (cordilleras), rivers, oceans and forest have been identified as main barriers to gene flow for some malaria vectors. Because *Anopheles* mosquitoes are primarily distributed in lowland areas, moderate to high elevation mountain ranges are believed to restrict their dispersal and gene exchange. For example, the Great Rift Valley is a phenomenal geographic structure in the eastern part of Africa that is believed to restrict dispersal in several *Anopheles* species (Lehmann et al., 1999, 2000, 2003; Braginets et al., 2003; Michel et al., 2005 a,b). This mountain range has been demonstrated to be a true barrier to gene flow for *An. funestus* s.s. A clear division between eastern and western populations have been defined using microsatellite DNA markers; and rather similar results were obtained in an independent mtDNA analysis (Michel et al., 2005 a,b). Likewise, Lehmann et al. (1999, 2000) used mtDNA and reported high levels of genetic differentiation in populations of *An. gambiae* s.s., located < 700 km apart, but separated by the Rift Valley, in Africa.

These findings were later corroborated by analysis of 11 microsatellite loci and widespread continental sampling (Lehmann et al., 2003).

Mountains have also been hypothesized as the cause of population differentiation for some Asian malaria vectors. Population analysis using 238 bp of the mitochondrial control region suggested that the Taebaek and Sobaek mountains have promoted population sub-division of *An.* (*Anopheles*) *sinensis* in South Korea (Jung et al., 2007). Furthermore, studies based on seven microsatellite loci demonstrated that *An.* (*Cellia*) *maculatus* exhibited significant population differentiation in Thailand only when separated by the Phuket mountain ranges, but in the absence of such barriers, populations spanning 650 km are nearly panmictic (Rongnoparut et al., 2006). In the Americas, the Green Mountains in the eastern United States are believed to restrict dispersal in *An.* (*Anopheles*) *punctipennis*, and some lineages of *An. nuneztovari* s.l. were partially separated by the eastern Andean cordillera (Fairley et al., 2000; Mirabello & Conn, 2008). Molina-Cruz et al. (2004) hypothesized barriers to dispersal for *An. albimanus* between Costa Rica and western Panama, and between eastern Panama and northern South America. This was believed to be due to the many mountain ranges that cross southern Central America. Moreover, populations from Cuba were significantly differentiated from mainland populations, likely due to the Caribbean Ocean, which may also act as a barrier.

Oceans have also been hypothesized as barriers to dispersal for many malaria vectors. Moreno et al. (2007) found significant genetic differentiation among samples of *An. gambiae* s.s., from the island of Annobon and continental

populations from Equatorial Guinea and Gabon, in Africa. These authors used 11 microsatellite loci and hypothesized significant differentiation due to both physical (the ocean) and biological (the M – S form discontinuity) barriers to gene flow. Similarly, populations of *An. gambiae* s.s., from four Lake Victoria islands (20 – 50 km apart) were significantly differentiated from two mainland populations (96 km apart) based on microsatellite analysis. This was attributed to their separation by water, higher genetic drift in smaller demes and/or local ecological adaptation (Kayondo et al., 2005). A discrete geographic distribution was also hypothesized for different *ND4*/*ND5* mtDNA lineages of *An. farauti* s.s., in five islands of Vanuatu. This pattern may reflect either limited gene flow among islands or a paucity of passive human-mediated dispersal over long distances (Reiff et al., 2007).

In contrast, the Amazon River did not cause significant genetic differentiation in the saltwater tolerant malaria vector, *An. aquasalis*. Estimates of molecular divergence between localities from both sides of this river using the mtDNA *COI* gene were low and not significant. Moreover, phylogenetic trees did separate two population clusters, but they were poorly resolved, thus providing no geographical resolution and no support for the existence of a fresh water barrier to *An. aquasalis* (Fairley et al., 2002). On the other hand, Conn et al. (2006) did detect significant differentiation between populations of *An. darlingi* on either side of the Amazon River, but this signal was weaker for those localities located far from the mouth.

Forest environments also appear to restrict gene flow for certain malaria vectors. For example, populations of *An. funestus* s.s., from the east and the west coasts of Madagascar that are separated by dense vegetation were significantly differentiated based on ten microsatellite loci (Ayala et al., 2006). Because this malaria vector does not disperse far from its primary breeding site, woody areas may restrict its dispersal (Temu et al., 2004; Michel et al., 2005a,b). However, it was not clear from this study whether genetic differentiation was truly due to obstacles to dispersal or due to two radically different bio-climates in these areas. Other signals of genetic structure due to forest-restricted gene flow have been hypothesized for *An. moucheti* in mainland Africa. As in the case of *An. funestus* s.s., this malaria vector is usually restricted to forest environments occurring mainly in villages situated along slow moving streams and rivers, thus its movement is believed to be restricted by thick vegetation (Antonio-Nkondjio et al., 2006).

2.5. Coalescence and the non-equilibrium approach

The non-equilibrium approach assumes that population parameters are not stable, but can change as a function of time. The N_e might change over time, as might the number of populations and the rates of gene exchange. There is generally not a steady-state pattern of genetic variation in the non-equilibrium approach because the genetic structure is different at different points in time (Hey & Machado, 2003). More recently, coalescent theory and non-equilibrium approaches were incorporated into the study of the demographic history of malaria vectors. This has allowed for a better understanding of the various

historical processes, and past changes in the N_e influencing the history of migration among *Anopheles* populations. Unlike the equilibrium approach, genealogical analysis (*i.e.* coalescent-based methods) has been used to infer both historical and contemporary rates of gene flow and to explore whether barriers to dispersal are current or historical in nature (Walton et al., 2000, 2001; Mirabello & Conn, 2006; O'Loughlin et al., 2008; Morgan et al., 2009; Pedro & Sallum, 2009; Crawford & Lazzaro, 2010).

To date, non-equilibrium populations of *Anopheles* mosquitoes have been documented worldwide, with historical bottleneck, geographic fragmentation or population expansion, as the most likely causes of genetic structure. In general, closely related and co-distributed *Anopheles* species have responded similarly to historical demographic processes, thus they usually depict overlapping spatial and temporal patterns of genetic structure (Walton et al., 2000; Donnelly et al., 2001, 2002; Crawford & Lazzaro, 2010), but see also O'Loughlin et al. (2007). For example, phylogenetic research in the Neocellia Series of *Anopheles* from the Oriental region demonstrated that while speciation has been almost entirely due to climatic alteration in the Miocene period, most intra-specific divergence have been driven by later sea level changes during the Pleistocene (Morgan et al., 2009). Furthermore, members of the *Anopheles* Barbirostris Sub-group have expanded in population size likely due to increased farming and cattle ranching during the Agrarian revolution in Southeast Asia, roughly 13,000 – 20,000 years ago (Paredes-Esquivel et al., 2009).

The pattern of demographic history seen in many *Anopheles* species so far appears to be determined by their larval ecology and/or adult feeding behavior. Both larval habitat requirements and availability are key determinants of mosquito demography. Anthropogenic adapted mosquitoes are more likely to be transported via human activities, and this may promote genetic homogenization over long geographic distances, despite true physical barriers to dispersal. In contrast, forest loving mosquitoes are less likely to be carried by humans because they tend not to breed in human-made habitats. Furthermore, feeding behavior has been hypothesized as another important factor driving speciation and demographic expansion in the *An. gambiae* Complex (Della Torre et al., 2005). Because most members of this group are highly anthropophilic (they preferentially feed upon human blood, and breed in man-made habitats), an increase in human populations is likely to trigger a corresponding increase in mosquito populations (review in Cohuet et al., 2010).

Most primary malaria vectors depict signals of population growth and/or expansion, albeit at different times and likely in response to different factors. The shallow population structure in the African malaria vector *An. gambiae* s.s., is hypothesized to be the result of recent expanding human populations (5,000 - 10,000 ya) due to the development of agriculture (Coluzzi et al., 1979; Donnelly et al., 2001, 2002). In contrast, the late Pleistocene population expansion of *An. darlingi* in northeastern Brazil is proposed to be due to changes in climatic conditions leading to forest fragmentation and refugia isolation, roughly 25,000 ya (Mirabello & Conn, 2006a,b), but see Pedro & Sallum (2009) for disagreement

on the time of Pleistocene expansion. Similarly, forest refugia are believed to have triggered expansions in both *An. dirus* s.s., and *An.* (*Cellia*) *baimaii* (in the *Anopheles dirus* Complex) in Southeast Asia. The demographic history of the latter species was associated with cyclical changes in forest structure during the Pleistocene (300,000 ya), yet the expansion time for these two species and for populations within each species varied slightly among different geographic areas (O'Loughlin et al., 2008).

Anopheles species that preferentially exploit human blood for reproduction and that are capable of breeding in human-made habitats should reflect signals of population history in response to changes in their host demography. Hence, the expansion of *An. gambiae* s.s., may be well explained by expanding human populations (Mattews et al., 2007; Cohuet et al., 2010). In contrast, the causes of expansion for *An. darlingi* and the members of the *An. dirus* Complex are less clear; less human dependent mosquitoes may not change demographically due to changes in human demography because they preferentially breed in sylvatic areas and are less reliant on human blood for egg maturation. Consequently, population divergence in these mosquitoes may instead be the result of past changes in climatic conditions that affected larval habitat availability. This idea seems logical since all *Anopheles* species depend on temperature and water for larval development and survival (Service & Townson, 2002), and changes in climatic variables are generally considered as major drivers for *Anopheles* diversification (Garros et al., 2005; Morgan et al., 2009; Paredes-Esquivel et al., 2009; Simard et al., 2009). Moreover, Pleistocene environmental changes were substantial

worldwide, triggering cyclical changes in temperature and precipitation, all of which are likely to have affected the N_e of *Anopheles* mosquitoes through niche contraction and expansion (Costatini et al., 2009; Simard et al., 2009).

Similar and time-overlapping patterns of demographic history may support the view of a common response of *Anopheles* species, regardless of their geographic distributions, to changes in Pleistocene environmental conditions. This is consistence with evidence of population fragmentation and expansion in both *An. darlingi* and the members of the *An. dirus* Complex during the Pleistocene (Mirabello & Conn, 2006; O'Loughlin et al., 2008; Pedro & Sallum, 2009). To date, more than 20 *Anopheles* species depict signals of genetic divergence likely associated to Pleistocene environmental changes, and this list includes both M and S molecular forms of *An. gambiae* s.s., which have also expanded during this time frame. Nevertheless, this may not be related to population contraction triggered by Pleistocene climatic changes, as the bottleneck hypothesis has not been supported so far. Instead, past changes in the N_e of both M and S forms may be the result of earlier anthropogenic events such as the movement out of the ancestral East African range by early humans or subsequent human population expansion (Crawford & Lazzaro, 2010).

The strength of genetic divergence seen in *Anopheles* species due to Pleistocene environmental changes appears to vary depending on the geographic region. For example, higher population divergence and speciation rate should be expected for island *Anopheles* species than for continental ones. A lower N_e and higher genetic drift in islands in comparison to broader geographic areas may

explain this pattern (Pinto et al., 2003; Kayondo et al., 2005; Moreno et al., 2007; Paredes-Esquivel et al., 2009). This is consistent with strong signals of isolation and bottleneck found in the coastal *An. sundaicus* s.s. A combination of cyclical island and refugium creation during the Pleistocene have been hypothesized as the cause of allopatric speciation in this important malaria vector (Dusfour et al., 2004, 2007a). However, genetic structure in some island malaria vectors is also explained by human-aided introduction and founder effect, because in many cases divergences and/or expansion times are concordant with human arrival and establishment in these islands (Hasan et al., 2008; Paredes-Esquivel et al., 2009; Morgan et al., 2009).

Mainland populations of certain *Anopheles* species may also harbor less genetic diversity due to Pleistocene environmental changes, in particular when they inhabit narrow stretches of land. For example, stronger signals of population contraction and reduced genetic diversity have been recovered in *An. albimanus* from the Isthmus of Panama using several molecular markers (De Merida et al., 1995, 1999; Molina-Cruz et al., 2004; *see results from Chapters V and VI of the present doctoral thesis*). This may suggest that Pleistocene climatic changes had a stronger impact on mosquitoes distributed across smaller geographic areas. In contrast, it is also possible that the Isthmus Panama acts a filter of genetic diversity (*i.e.* bottleneck), as some haplotypes can have problems to cross it and get lost more easily stochastically. This view is consistent with the evidences of population fragmentation in different groups of organisms across southern Central America (Bermingham & Martin, 1998; Weigt et al., 2005; Miller et al., 2008).

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3. Chapter III. Species composition and distribution of adult *Anopheles* (Diptera: Culicidae) in Panama

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3.1. Abstract

Anopheles (Diptera: Culicidae) species composition and distribution were studied using human landing catch data over a 35-yr period in Panama. Mosquitoes were collected from 77 collection sites during 228 field trips carried out by members of the National Malaria Eradication Service. Fourteen *Anopheles* species were identified. The highest average human biting rates were recorded from *Anopheles* (*Nyssorhynchus*) *albimanus* (Wiedemann) (9.8 bites/person/night), and *Anopheles* (*Anopheles*) *punctimacula* (Dyar & Knab) (6.2 b/p/nt). These two species were also the most common, present in 99.1% and 74.9%, respectively, of the sites. *Anopheles* (*Nyssorhynchus*) *aquasalis* (Curry) was encountered mostly in the indigenous Kuna Yala Comarca along the eastern Atlantic coast where malaria case history and average human biting rate (9.3 b/p/nt) suggest a local role in malaria transmission. *An. albimanus*, *An. punctimacula*, and *Anopheles* (*Anopheles*) *vestitipennis* (Dyar & Knab) were more abundant during the rainy season (May – December) whereas *An. aquasalis* was more abundant in the dry season (January – April). Other vector species collected in the present study were *Anopheles* (*Kerteszia*) *neivai* (Howard, Dyar & Knab) and *Anopheles* (*Anopheles*) *pseudopunctipennis* s.l. (Theobald). High diversity of *Anopheles* species and six confirmed malaria vectors in endemic areas of Panama emphasize the need for more detailed investigations to better understand malaria transmission dynamics.

Key words: Anopheles, human biting rate, malaria, Panama.

3.2. Introduction

Entomologists from Instituto Conmemorativo Gorgas de Estudios de la Salud (ICGES) and the National Malaria Eradication Service (SNEM) have led field studies on *Anopheles* bionomics across Panama since 1921 and 1956, respectively. These studies, focused mostly on species distribution, infection rate, feeding behavior, and pesticide resistance, have resulted in few publications on *Anopheles* bionomics since 1960. Furthermore, before 1956 most studies on *Anopheles* ecology were undertaken nearly exclusively in the Canal Zone, which represents less than five percent of the country. Early attempts to describe the distribution of *Anopheles* in Panama were conducted by Baxter & Zetek (1944), Arnett (1947), Blanton et al. (1955) and Blanton & Peyton (1956). These surveys reported 19 species of *Anopheles* collected by light traps, horse bait traps, Shannon traps, and larval collections. Additional reports summarized in Wilkerson & Strickman (1990) elevated the total to 22 species within the *Anopheles* genus, and included *Anopheles* (*Anopheles*) *malefactor* (Dyar & Knab), which was recovered from synonymy with *Anopheles* (*Anopheles*) *punctimacula* (Dyar & Knab) in the Canal Zone (Wilkerson 1990).

Seven *Anopheles* species have been previously found infected with *Plasmodium sp.* in Panama (Darling 1910; Simmons 1936a,b, 1937). These studies were based on dissection of individual mosquitoes after being fed on humans with circulating gametocytes, but not all of the parasites reached the sporozoite stage in mosquito salivary glands (see summary in Table 3.1.). In addition, Darling (1910) and Rozeboom (1935) reported naturally infected

specimens of *An.* (*Nyssorhynchus*) *argyritarsis* and *An.* (*Nyssorhynchus*) *bachmanni* {Syn. *An.* (*Nyssorhynchus*) *triannulatus*}, adding two more species to the earlier list. Although the procedure employed in these experiments is now unethical, the results described the known complexity of malaria transmission during this time in Panama.

The human landing catch (HLC) technique is recommended as the most useful for collecting host-seeking anthropophilic mosquitoes (Service 1993). Its efficiency and low cost far surpass results obtained by any other mosquito collecting technique (WHO 1975), and it remains the only reliable method to assess the degree of human-vector contact, a crucial component of the entomological inoculation rate (EIR) (Macdonald 1957). SNEM has used HLC to survey *Anopheles* mosquitoes in Panama since 1970. To date, the distributions of sibling species within the *Anopheles* (*Nyssorhynchus*) *albitarsis* s.l. (Lynch-Arribálzaga) (Wilkerson et al. 1995; Lehr et al. 2005; Wilkerson et al. 2005), *Anopheles triannulatus* s.l. (Neiva & Pinto) (Silva Do-Nascimento et al. 2006) and *Anopheles* (*Nyssorhynchus*) *nuneztovari* s.l. (Gabaldon) (Kitzmiller et al. 1973; Conn et al. 1998) complexes, as well as other local malaria vectors such as *Anopheles* (*Kerteszia*) *neivai* (Howard, Dyar & Knab), are poorly known in Panama. Moreover, no systematic mosquito surveys have ever been carried out in the Darien Province, eastern Panama, near the Choco region in Colombia, an area where other *Anopheles* species are likely to be present (Wilkerson & Strickman 1990).

According to the Epidemiological National System (SIVIGILA), northwestern Colombia had the highest levels of malaria morbidity in the department of Antioquia in 2007 (Estadísticas de la vigilancia en Salud Pública 2007 / http://www.ins.gov). Both *Plasmodium vivax* (Grassi & Feletti) and *P. falciparum* (Welch) are registered throughout the year near the Panamanian border in Turbo, Colombia (Echeverri et al. 2003) where frequent migration of Kuna (Tule) people into Panama has been reported (Carmona-Fonseca et al. 2005). In this region, two primary malaria vectors, *An.* (*Nyssorhynchus*) *albimanus* (Wiedemann) and *Anopheles* (*Nyssorhynchus*) *darlingi* (Root) coexist (Lounibos & Conn 2000) and are likely to contribute to the high malaria endemicity. *Anopheles darlingi* occurs from southern Mexico to South America, but it has never been officially reported from Nicaragua, Costa Rica, or Panama (Linthicum 1988). As no obvious physical or ecological barriers seem to prevent *An. darlingi* from migrating into Panama, the possibility that this important malaria vector may occur in eastern Panama warrants further attention.

Panama reported 5,095 cases of malaria during 2004, a six-fold increase in incidence since 2001. Previously, Panama had never reported more than 2000 cases per year since 1970 (Boletín Epidemiológico, 2005 / http://www.minsa.gob.pa). The highest prevalence of malaria occurs in rural areas where indigenous people reside; however, recently, there has been an increase in case numbers in peri-urban and urban areas as well (Ministerio Nacional de Salud de Panamá MINSA, 2005 / http://www.minsa.gob.pa). Some of these urban epidemics may result from circular migration from both rural

(endemic) and urban (non-endemic) areas in Panama in recent decades (World Bank 2005). In addition, an increase in resistance to prophylaxis by *Plasmodium sp.* (Samudio et al. 2005) and to insecticides by vectors (Caceres 1999) is altering the epidemiological setting of malaria in unknown ways. Information on changes in vectorial systems, vector abundance and species succession is vital for planning effective transmission interventions and for monitoring the efficacy of vector control measures (Ndenga et al. 2006). The objective of this report is to provide up-to-date information on anopheline species collected in close association with human habitations over a 35-yr period in malaria endemic areas in Panama.

3.3. Materials and Methods

Study Site: The Isthmus of Panama (9^0 00' N, 80^0 00' W) is the geographical link between Central and South America. Panama borders both the Caribbean Sea and the North Pacific Ocean, between Colombia and Costa Rica, and it encompasses a population of 3,242,173 people (Contraloría General de la República 2004). The annual population growth rate is 5.6%, and its gross domestic product (GDP) is one of the fastest growing in Central America, with a per-capita GDP on par with a middle class nation. Despite this, more than 60% of the rural population lives in poverty (Ficher & Vasseru 2000), and is more vulnerable to malaria. Malaria, dengue fever, Chagas disease and leishmaniasis are among the most important vector-borne infections (Boletín Epidemiológico 2002 / http://www.minsa.gob.pa), found predominantly along the Atlantic coast, in Bocas Del Toro, the Ngöbe Buglé Comarca, the Kuna Yala Comarca and Darien, where up to forty-five percent of the intact tropical rain forest exists [Autoridad

Nacional del Ambiente (ANAM) 2003]. Some of these locales are currently undergoing extensive changes in landscape due to an increase in tourism, therefore, a potential risk of malaria infection is expected for the influx of susceptible travelers. The annual climate in Panama varies according to two seasons: the dry season, from January to late April (average monthly rainfall 34.2 mm), and a prolonged rainy season, from early May to December (average monthly rainfall 507 mm) (Condit et al. 2001). The rainy season triggers changes in relative humidity and human activities throughout the country, and it is when overall mosquito density reaches its maximum in Panama (Wolda & Galindo 1981).

Mosquito Sampling: Mosquito collections were carried out upon request from MINSA shortly after notification of malaria outbreaks. Therefore, entomologists from SNEM and ICGES visited virtually all localities in endemic malaria regions of Panama since 1970. Collection teams usually stayed 5 - 10 days at each specific locality. Mosquitoes were collected outdoors at no more than six meters away from the main entrance of human dwellings. A six-hour collection period (18:00 – 24:00 hr) was conducted daily, using manual aspirators (WHO 1975). Mosquitoes were morphologically identified to species based on available keys (anonymous document from ICGES; Wilkerson & Strickman 1990). Information was recorded by locality and collection time, and human biting rate (HBR) per species was calculated. Voucher specimens were deposited in entomological collections at ICGES, in Panama City. Two neighboring localities in Colombia (Capurgana and Acandi) were surveyed by SNEM in collaboration with its

Colombian counterpart Servicio de Erradicación de la Malaria (SEM) in 1977. The collecting procedure for these field trips was as described above.

Data Analyses: Overall average HBR were log transformed (x +1) before testing for differences among species using one-way analysis of variance (ANOVA). Means for significant main effects were separated using Scheffe's test, which makes unplanned comparisons among the means with unequal sample sizes. LSD was used to compare the average HBR between the dry and rainy season for each *Anopheles* species using PROC GLM function and mean comparison. To control for differences in the average HBR of prevalent species (*An. albimanus*, *An. punctimacula* and *An. aquasalis*) among only 31 of 77 collecting sites, a one-way ANOVA was performed with collecting site or locality as a fixed treatment (Table 3.2.). The localities for the latter statistical procedure were visited most frequently, have the best statistical representation, and highest degree of malaria endemicity in Panama. They were distributed as follows: seven sites in Bocas Del Toro (BC), nine in the Ngöbe Buglé Comarca (NBC), nine in Darien (DR), and six in the Kuna Yala Comarca (KYC) (Table 3.2.). The first two regions (BC and NBC) are located in western and the latter two (DR and KYC) in eastern Panama, respectively (Fig. 3.1.). We used SAS 9.0 version software package (SAS institute Inc., Cary NY) for statistical analysis, and $p < 0.05$ as a cut-off for statistical significance. We visited all sampling sites, obtained the geographic coordinates by using handheld global positioning system (GPS) units (Garmin International Inc., Olathe, KS), and imported them into ArcView GIS software (Environmental Systems Research Institute, Redlands, CA) to create maps of

Anopheles species distribution in relation to hydrology, vegetation type, and altitude. Updated GIS data sets were obtained from NAOS Molecular Biology and Evolution Laboratories of Smithsonian Tropical Research Institute (STRI), Panama City.

3.4. Results

Species composition and distribution: Fourteen *Anopheles* species in four subgenera and *Chagasia bathana* (Dyar) were caught using HLC, from 1970 to 2005, in malaria endemic areas of Panama (Table 3.3.). 33,917 specimens were gathered from 77 localities during 228 field trips and roughly 13,680 hours of sampling. Forty percent of collecting sites were visited more than ten times during the dry and the rainy seasons (Figs. 3.2.; 3.3.). *An. albimanus* was the most abundant, common and widespread species, accounting for 24,490 (72.2%) of the total, and it was collected in 76 localities (99.1%) and 216 (94.7%) of 228 field trips. *Anopheles punctimacula* was the second most common species, accounting for 6,081 (17.9%) of the total, collected in 57 localities (74.9%) and in 129 (56.8%) field trips. *Anopheles aquasalis* (Curry) represented 3.6% (1,240) of the total mosquitoes collected, found in 13 localities (16.8%), 11 of which were located in KYC along the eastern Atlantic coast (Fig. 3.2.).

The remaining 11 species accounted together for less than 10 % (2,334) of the total (Table 3.3.). *An. neivai* and *Anopheles* (*Anopheles*) *vestitipennis* (Dyar & Knab) were mostly recorded from western Panama. The former species was collected from seven (9.1%) localities of NBC near the Atlantic coast whereas *An. vestitipennis* was recorded more inland in seven (9.1%) localities of BC, near the

Costa Rican border (Fig. 3.3.). In July, 1977, in Colombia, *An. darlingi* was identified from Capurgana (n = 29) and Acandi (n = 6), no more than 15 km from the Panamanian border (data not shown). However, *An. darlingi* was not found on the Panamanian side of the border. *Anopheles* (*Anopheles*) *pseudopunctipennis* s.l. (Theobald) was collected in higher numbers from Uala, Nurra and Punuloso, all situated in eastern Panama (Fig. 3.2.) whereas *An. albitarsis* s.l. was caught in Zapallal, Meteti and Biroquera. Furthermore, *An. triannulatus* s.l., *Anopheles* (*Nyssorhynchus*) *oswaldoi* s.l. (Peryassú), *Anopheles* (*Nyssorhynchus*) *strodei* s.l. (Root), *Anopheles* (*Anopheles*) *neomaculipalpus* (Curry), *Anopheles* (*Anopheles*) *apicimacula* (Dyar & Knab), *An. malefactor*, and *Chagasia bathana* were caught in only a subset of localities (Figs. 3.2.; 3.3.). *Anopheles* (*Lophopodomyia*) *squamifemur* (Theobald) was collected only in Las Cumbres near Panama City (locality data not shown in Fig. 3.2.). Ninety eight percent of mosquitoes were collected in localities less than 100 meters above sea level, and 37 (48%) localities had more than two *Anopheles* species (Figs. 3.2.; 3.3.). In general, species within the subgenus *Anopheles* (*An. punctimacula*, *An. vestitipennis,* and *An. pseudopunctipennis* s.l.) were encountered in woody areas whereas *An. triannulatus* s.l. was collected in areas with permanent water bodies.

Human Biting Rate: Overall average HBR varied significantly among mosquito species ($F_{8,\ 2188}$ = 170.01; $P < 0.05$) with the greatest rates obtained in *An. albimanus* and *An. punctimacula* (Table 3.4.). The average HBR of *An. albimanus* ($F_{1,\ 438}$ = 299.9; $P < 0.05$), *An. punctimacula* ($F_{1,\ 438}$ = 9.1; $P < 0.05$), *An. vestitipennis* ($F_{1,\ 438}$ = 5.7; $P < 0.05$) and *An. aquasalis* ($F_{1,\ 438}$ = 4.49; $P < 0.05$)

varied significantly between seasons (Table 3.3.). The greatest HBR for *An. albimanus*, *An. punctimacula* and *An. vestitipennis* was recorded during the rainy season (May – December) ranging from 1.1 to 18.5 bites per person per night (b/p/nt), 1.3 to 10.1 b/p/nt, and 0.8 to 5.9 b/p/nt, respectively. In contrast, *An. aquasalis* demonstrated the highest HBR during the dry season (January – April), ranging from 2.2 to 11.1 b/p/nt. There were significant among site variations in the average HBR of *An. albimanus* ($F_{30,\ 184} = 2.6$; $P < 0.05$), *An. punctimacula* ($F_{30,\ 184} = 8.16$; $P < 0.05$) and *An. aquasalis* ($F_{30,\ 184} = 10.4$; $P < 0.05$). The highest HBR for *An. albimanus* was recorded from western Panama, in Barranco Montaña (11.9 b/p/nt), Chiriqui Grande (15.4 b/p/nt), and Rio Chiriqui (18.5 b/p/nt). The first two sites are located in BC; Rio Chiriqui is in NBC (Table 3.2.; Fig. 3.3.). On the contrary, *An. punctimacula's* highest HBR was recorded from Santa Fe (9.8 b/p/nt), and Los Monos (10.1 b/p/nt), both located in DR (Table 3.2.; Fig. 3.2.). The highest HBR for *An. aquasalis* was recorded from three sites in KYC in the Atlantic coast, near the Colombian border: Navagandi (9.1 b/p/nt), Isla Pino (8.2 b/p/nt), and Carreto (11.1 b/p/nt) (Table 3.2.; Fig. 3.2.).

3.5. Discussion

The fact that mosquito collections for this study were carried out on the occurrence of malaria outbreaks in Panama and not based on a more comprehensive seasonal or temporal collection protocol may have resulted in a lower HBR overall and by species with respect to other studies (Table 3.4.). However, the range of average HBR of *An. albimanus* in 31 Panamanian localities (1.8-18.5; Table 3.2.; this study) does not differ substantially from a study in three

villages in Chiapas, Mexico (monthly range, wet season, 1.0 -18.8, compared with dry season, 0-10.7; Bown et al. 1991) or from the highest biting rate (7.1) for *An. albimanus* in coastal urban Buenaventura, Colombia (Olano et al. 1997). In the present study, *An. albimanus* and *An. punctimacula* were the most abundant and prevalent species showing the highest overall HBR over 35-yr in Panama. *An. albimanus* is considered the most important malaria vector across its distribution (Rubio-Palis & Zimmerman 1997) whereas *An. punctimacula* is considered a secondary vector in Costa Rica (Kumm & Ruiz 1939) and Colombia (Olano et al. 2001). The fact that both species have been previously incriminated as vectors in Panama (Rozeboom 1935; Darling 1910) combined with their considerable degree of association with human dwellings is very suggestive of their involvement in malaria transmission. The highest HBR in *An. punctimacula* was in DR, which has over 70% forest cover (ANAM 2003). This finding supports Service (1989) who noted that the larval habitats of *An. punctimacula* occur in deep or partial shaded areas.

Malaria outbreaks have occurred in KYC, where we found most *An. aquasalis*, at times when this species was the most abundant or even the only anopheline present (SNEM unpublished data). Darling (1910) considered *An. tarsimaculatus (Syn. An. aquasalis)* as the second most important malaria vector in Panama based on salivary gland dissections, and *An. aquasalis* is a known regional malaria vector in eastern Venezuela and Brazil (Berti et al. 1993; Póvoa et al. 2003). However, its more restricted distribution in Panama indicates that it is likely important only in local malaria transmission. *Anopheles vestitipennis* and

An. neivai are confirmed malaria vectors in Belize (Grieco 2001) and Colombia (Carvajal et al. 1989), and *An. vestitipennis* in Chiapas, Mexico (Loyola et al. 1991). Both species were predominantly collected from western Panama where they seem to cluster with specific types of vegetation and land use practices (ANAM 2003). In the Pacific coast of Colombia, *An. neivai* is presumed to be involved in malaria transmission both during the daytime and the evening due to its multiple haemorrhagic peaks (Solarte et al. 1996). This species breeds along the seashore, mainly associated with lowland tropical forest where it develops in several species of epiphytes (Carvajal et al. 1989). *Anopheles neivai* was frequently collected from NBC where transportation between villages is mostly by foot, increasing the human contact with this species and perhaps the likelihood of malaria transmission. As our data consist only of evening collections, the lower HBR of *An. neivai* may underestimate its actual role as a vector in Panama. In contrast, *An. vestitipennis* was collected more inland in BC, overlapping with banana plantations and woody areas. In Belize, *An. vestitipennis* is considered a more efficient vector than *An. albimanus* even though it is usually less abundant (Grieco 2001). The proposed explanation is a marked preference for human blood (anthropophagic), endophagic habit and higher infection rate (Achee et al. 2000). The lower relative abundance of *An. vestitipennis* in Panama could suggest poor vectorial competence or it may reflect bias in our sampling protocol (*i.e.*, collecting mosquitoes only outdoors). It is not possible to discern the transmission role of *An. vestitipennis* and *An. neivai* based only on the present dataset. To date, *An. vestitipennis* and *An. neivai* have not been found naturally

infected with *Plasmodium sp.* in Panama, nor have vector competence evaluations been undertaken.

An. darlingi was collected biting just after sunset in the Colombian town of Capurgana, less than 15 km near the Panama – Colombia border. *An. darlingi* is found in part of Central America, but never officially reported from Nicaragua, Costa Rica, or Panama, resulting in an apparent discontinuity in its distribution (Linthicum 1988). One hypothesis to explain its distribution is an introduction event from Colombia into Central America (Manguin et al. 1999; Mirabello & Conn 2006). Another possibility is that unsolved geological factors related to the uplift of the Panamanian Isthmus (Bermingham & Martin 1998) may have precluded its successful establishment in eastern Panama. The high malaria incidence in this region requires a more comprehensive mosquito survey to evaluate the potential presence of *An. darlingi*.

Due to their low density and limited distribution it is unlikely that *An. pseudopunctipennis* s.l., *An. oswaldoi* s.l., *An. strodei* s.l., *An. triannulatus* s.l., *An. neomaculipalpus* and *An. apicimacula* contribute significantly to malaria transmission in Panama. However, four of these taxa are species complexes that include proven malaria vectors in other Latin American countries (Rosa-Freitas et al. 1998; Coetzee et al. 1999; Marrelli et al. 2006; Quiñones et al. 2006). Moreover, *An. neomaculipalpus* was recently reported as a potential secondary vector of *P. vivax* in southern Venezuela, with an overall sporozoite rate similar to that for *An. darlingi* and higher than that reported for *Anopheles* (*Nyssorhynchus*) *marajoara* (Galvao & Damesceno) (Moreno et al. 2005). Previous incriminations

of *An. neomaculipalpus* and *An. apicimacula* as vectors in Panama were supported by the presence of *Plasmodium* sporozoites in salivary glands (Table 3.1.). Even though only 41 specimens of *An. malefactor* (formerly *An. punctimacula*) were collected in four localities in eastern Panama: Aguas Claras, Canazas – Bayano, Ipeti Kuna and Biroquera (Fig. 3.2.), this species has been collected using human landing catches in Panama (Wilkerson 1990). However, it seems unlikely to be involved in malaria transmission because of its restricted distribution (Canal Zone and northwestern Colombia) (Wilkerson 1990). On the other hand, populations of *An. punctimacula* in southern Mexico have been shown to be mostly zoophagic, feeding primarily on cattle (Ulloa et al. 2006). These findings are in disagreement with our results where *An. punctimacula* was frequently caught feeding on people. Zimmerman et al. (2006) encountered significant differences in host selection patterns by several *Anopheles* species among adjacent villages, attributing this to host availability. Cattle farming is still an incipient activity in some areas of NBC, KYC and DR, thus the partial absence of large mammals as alternative blood sources may explain a more anthropophagic tendency by *An. punctimacula* in these Panamanian regions.

Anopheles albitarsis s.l. was collected biting people in areas where malaria cases have occurred in eastern Panama. *Anopheles marajoara*, a member of the *An. albitarsis* complex (Lehr et al. 2005; Wilkerson et al. 2005), has been identified in Panama previously (Wilkerson & Strickman 1990). This species is a malaria vector in eastern Amazonian Brazil (Póvoa et al. 2000; Conn et al. 2002) and Venezuela (as revised in Rubio-Palis et al. 2003, Moreno et al. 2005; 2007);

however, its role in Panama cannot be established until we determine the identity of our Panamanian specimens of *An. albitarsis* s.l. using molecular techniques (Li & Wilkerson 2005). Despite previous records in DR, Panama (Faran 1980), *An. nuneztovari* was not identified in the present study, even though this species is considered a primary regional malaria vector in nearby Colombia (Brochero et al. 2005) and in Venezuela (Gabaldón 1981). Although highly trained personnel did mosquito identifications based on morphological characters, molecular confirmation was not done at the time. We plan to use molecular approaches to determine whether species such as *An. nuneztovari* s.l., *An.* (*Nyssorhynchus*) *benarrochi* and *An.* (*Nyssorhynchus*) *albitarsis* F (a new putative species in the *An. albitarsis* Complex) (Brochero et al. 2007) are distributed in Panama, using voucher specimens stored at ICGES.

An. albimanus and *An. punctimacula* were more abundant during the rainy season implying that the highest risk of malaria transmission by these species in Panama occurs from May to December. This is not a novel finding for *An. albimanus*, but it is known to occur in places where alternative breeding sites are absent during the dry season (Frederickson 1993). On the contrary, the highest vector competence for *An. punctimacula* in southern Mexico has been proposed to occur during the dry season when this species is more abundant (Ulloa et al. 2006). Achee et al. (2005) encountered *An. punctimacula* in Belize with one peak of density in January at the beginning of the dry season and another peak in August during the rainy season, perhaps reflecting differences in larval habitat distributions determined by local climatic and ecological conditions. It is also

possible that *An. punctimacula* (in the past *Syn. An. malefactor*) consists of more than two cryptic species. We also determined that *An. vestitipennis* was more abundant during the rainy season in Panama. In Belize, this species peaks in density in August and September at the end of the rainy season (Achee et al. 2005); however, its seasonal fluctuation seems to be ultimately determined by the presence of specific vegetation, *Typha domingensis*, at its main breeding sites (Grieco et al. 2006).

In Central America the highest risk of malaria transmission by *An. albimanus* occurs from 6:00 – 10:00 pm when children and young adults gather outside houses (PAHO 1996), and our outdoor collections in localities where *An. albimanus* was the most prevalent species confirm this. Nevertheless, a high diversity of *Anopheles sp.*, and six confirmed malaria vectors, provides evidence for a more complex situation in Panama. Panama has used residual insecticide spraying (IRS) since 1957 as the main method of vector control. Four discrete chemical groups have been employed: dieldrin and DDT (organoclorate), propoxur (carbamate), fenitrothion (organophosphate) and deltamethrin and cyflutrin (pyrethroid) with insecticide shifts according to chronological appearance of resistance in populations of *An. albimanus* (Caceres 1999).

The effectiveness of IRS in controlling malaria is acknowledged (Roberts et al. 2002); however, the exophagic and exophilic behavior displayed by *An. albimanus* in Central America (PAHO 1996) makes its control uncertain even in highly susceptible populations. IRS disrupts malaria transmission by reducing female longevity, and depending on the insecticides, by irritating mosquito

mechanoreceptors (*i.e.*, pyrethroids) thus repelling them from inside houses and minimizing overall vector contact. However, avoidance behavioral responses against DDT displayed by Panamanian populations of *An. albimanus* may have lessened the successful contact between this species and sprayed surfaces (Trapido 1952). The effectiveness of IRS in controlling *An. albimanus* and other *Anopheles* species needs further assessment in Panama.

Recent studies in Thailand have demonstrated that the seasonal migration of cross-border laborers is a leading cause of malaria transmission (Zhou et al. 2005). In Panama malaria is largely endemic in rural areas, especially near the Panama-Costa Rica and Panama-Colombia borders where transmission could be influenced by human migration. Furthermore, the fact that *P. falciparum* has been recorded traditionally from KYC (Panama-Colombian border), where *An. albimanus* and *An. punctimacula* are not as prevalent as *An. aquasalis*, suggests that its incidence may be related to differential transmission capabilities among vector species or between populations of the same species across the country. Moreover, the absence of large mammals as alternative blood source for mosquitoes may enhance the transmission role of secondary and local vectors, especially in indigenous comarcas such as NBC and KYC.

These facts underscore the need for more in-depth studies to clarify the basic ecology of anophelines mosquitoes in western and eastern Panama. This report improves our understanding of malaria transmission in Panama, and also provides baseline information required for further research in *Anopheles* bionomics.

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Table 3.1. Summary of *Anopheles* species previously reported as vectors of *Plasmodium vivax* and *P. falciparum* in Panama based on studies conducted by Darling (1910), Simmons (1936a,b; 1937), and Rozeboom (1935).

Mosquito Species	*P. vivax*	*P. falciparum*
*An. albimanus**	1-2	1-2
An. pseudopunctipennis	1	1-2
An. tarsimaculatus (Syn An. aquasalis)	1-2	-
*An. punctimacula (Syn. An. malefactor)**	1-2	1-2
An. apicimacula	-	2
An. eiseni	1	-
An. neomaculipalpus	1-2	-
An. bachmanni (*Syn. An. triannulatus*)*	1	-
*An. argyritarsis**	1	-

* Found naturally infected with *Plasmodium sp.* in Panama

(1): Oocysts

(2): Sporozoites

(-): No infection

Table 3.2. Average human-biting rate (HBR) of *An. albimanus*, *An. punctimacula*, and *An. aquasalis* in 31 malaria endemic sites of Panama.

Province	Localities	*An. albimanus* (mean* ± SE)#	*An. punctimacula* (mean* ± SE)#	*An. aquasalis* (mean* ± SE)#
Bocas Del Toro (BC)	Barranco Montaña	11.9 ± 0.2a	2.8 ± 0.3a	-
	Barranco Adentro	9.9 ± 0.3b	2.7 ± 0.7a	-
	Guabito Centro	7.5 ± 2.1b	2.9 ± 1.1a	-
	Chiriqui Grande	15.4 ± 1.7a	4.1 ± 0.8a	-
	Finca Debora	5.0 ± 0.8b	1.2 ± 0.3b	-
	Las Delicias	8.3 ± 3.1b	1.8 ± 0.5b	-
	Barra Sixaola	5.4 ± 1.4b	1.3 ± 0.7b	5.2 ± 1.7a
Ngöbe Buglé Comarca (NBC)	Bisira	6.8 ± 2.7b	-	-
	Bahia Azul	7.3 ± 4.7b	4.5 ± 1.5b	6.4 ± 0.9a
	Tobobe	2.14 ± 0.8a	3.9 ± 0.7b	-
	Kusapin	4.1 ± 2.8a	3.7 ± 0.8b	-
	Kankintu	5.4 ± 1.3a	4.0 ± 0.9b	-
	Rio Chiriqui	18.5 ± 2.1b	-	-
	Playa Roja	3.7 ± 0.8a	-	-
	Rio Caña	3.2 ± 1.0a	1.1 ± 0.4a	-
	San Pedro	6.1 ± 0.6b	4.4 ± 0.6b	-
Darien (DR)	Los Monos	4.8 ± 0.9a	9.8 ± 1.1b	
	El Canglón	5.6 ± 2.8a	6.9 ± 1.4a	-
	Santa Fe	3.7 ± 0.6a	10.1 ± 0.9b	-
	Las Peñitas Jaque	9.2 ± 2.3b	5.3 ± 1.9a	-
	Cañazas-Bayano	7.1 ± 1.1b	8.3 ± 0.5a	-
	El Coco	6.9 ± 1.3b	3.6 ± 0.1b	-
	Biroquera	5.1 ± 0.6b	2.6 ± 1.7b	-
	Zapallal	4.1 ± 1.7b	3.1 ± 1.9b	-
	Punuloso	5.4 ± 0.8b	2.1 ± 1.1b	-
				-
Kuna Yala Comarca (KYC)	Navagandi	4.4 ± 1.4b	-	9.1 ± 1.0b
	Playón Grande	2.1 ± 2.8b	3.8 ± 0.7b	5.7 ± 0.4a
	Mansucun	2.6 ± 0.6b	-	6.2 ± 0.7a
	Anachucuna	2.8 ± 2.9b	3.1 ± 0.5b	6.3 ± 0.5a
	Isla Pino	1.8 ± 1.7b	-	8.2 ± 0.2b
	Carreto	2.1 ± 0.7b	2.8 ± 0.9b	11.1 ± 1.0a

* Average obtained from three collectors over 5 – 10 days / 6 hrs per day replicates
# Values followed by different letters are significantly different from each other, $P < 0.05$
(-): Species absence

Table 3.3. *Anopheles* species collected using (HLC) from 1970 – 2005 in Panama.

Anopheles species by Subgenera	Total number - % of mosquitoes by species	Number - % of localities were species was found	Number - % of collecting trips where species was found
Nyssorhynchus			
An. albimanus	24,490 – 72.2	76 – 99.1	216 – 94.7
An. aquasalis	1,240 – 3.6	13 – 16.8	37 – 16.2
An. triannulatus s.l.	496 – 1.5	< 5 – < 7	< 10 – < 5
An. albitarsis s.l.	112 – 0.3	< 5 – < 7	< 10 – < 5
An. oswaldoi s.l.	< 50 – 0.1	< 5 – < 7	< 10 – < 5
An. strodei s.l.	< 50 – 0.1	< 5 – < 7	< 10 – < 5
Anopheles			
An. punctimacula	6,081 – 17.9	57 – 74.9	129 – 56.8
An. vestitipennis	868 – 2.6	7 – 9.1	21 – 10.5
An. pseudopunctipennis	155 – 0.5	7 – 9.1	15 – 6.6
An. apicimacula	124 – 0.4	< 5 – < 7	< 10 – < 5
An. neomaculipalpus	< 50 – 0.1	< 5 – < 7	< 10 – < 5
An. malefactor	< 50 – 0.1	< 5 – < 7	< 10 – < 5
Kerteszia			
An. neivai	279 – 0.9	7 – 9.1	19 – 8.1
Lophopodomyia			
An. squamifemur	< 50 – 0.1	< 5 – < 7	< 10 – < 5
Chagasia bathana	< 50 – 0.1	< 5 – < 7	< 10 – < 5
Total	33,917 individuals	77 localities	228 field trips

Table 3.4. Overall and seasonal average human-biting rate (HBR ± SE) of *Anopheles* species collected in Panama.

Anopheles Species	HBR (mean* ± SE)# Dry season	HBR (mean* ± SE)# Rainy season	HBR (mean* ± SE)# Overall
1. *An. albimanus*	2.4 ± 1.11a	10.2 ± 0.91b	9.8 ± 0.08a
2. *An. punctimacula*	1.3 ± 0.70a	8.9 ± 1.12b	6.2 ± 0.13b
3. *An. aquasalis*	6.1 ± 0.23b	0.2 ± 0.10a	5.1 ± 0.03a
4. *An. vestitipennis*	1.2 ± 0.91a	6.1 ± 0.25b	2.3 ± 0.72b
5. *An. neivai*	3.9 ± 1.94a	5.4 ± 0.83b	1.3 ± 1.12b
6. *An. pseudopunctipennis*	1.3 ± 0.21a	1.2 ± 0.56a	1.5 ± 1.26b
7. *An. triannulatus* s.l.	3.1 ± 2.45a	3.3 ± 0.17a	1.1 ± 0.19b
8. *An. apicimacula*	0.8 ± 0.12a	0.6 ± 1.11a	0.8 ± 0.11b
9. *An. neomaculipalpus*	1.1 ± 0.30a	1.5 ± 0.72a	0.6 ± 0.09b
10. *An. albitarsis* s. l.	< 0.5	< 0.5	< 0.5
11. *An. oswaldoi* s.l.	< 0.5	< 0.5	< 0.5
12. *An. strodei* s.l.	< 0.5	< 0.5	< 0.5
13. *An. malefactor*	< 0.5	< 0.5	< 0.5
14. *An. squamifemur*	< 0.5	< 0.5	< 0.5
15. *Chagasia Bathana*	< 0.5	< 0.5	< 0.5

* Average obtained from three collectors in between 5 – 10 days / hrs per day replicates

Values followed by different letters are significantly different ($P < 0.05$; PROC GLM; Mean comparison by LSD)

HBR: Number of bites / per person / per night

Values of < 0.5 were not analyzed

Dry season: January to April (average monthly rainfall of 34.1 mm)

Rainy season: May to December (average monthly rainfall of 507 mm)

s.l. sensu lato

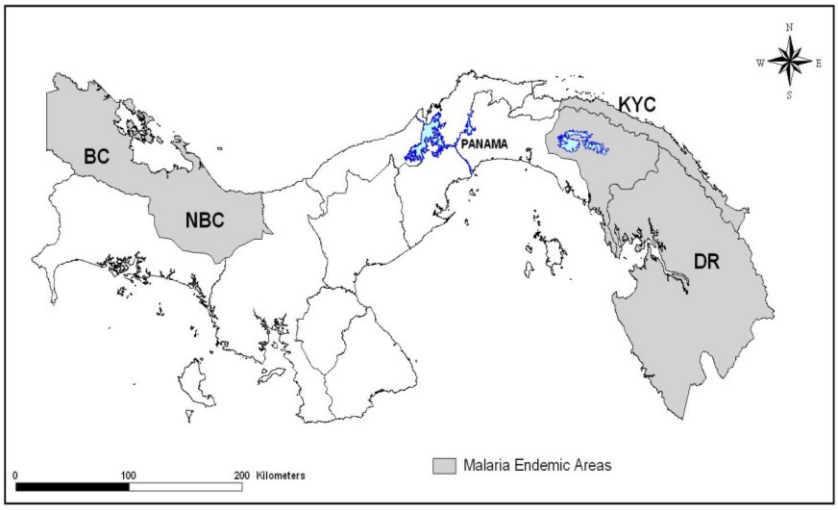

Figure 3.1. Malaria endemic areas of Panama: Bocas Del Toro (BC); Comarca Ngöbe Buglé (NBC); Comarca Kuna Yala (KYC) and Darien (DR).

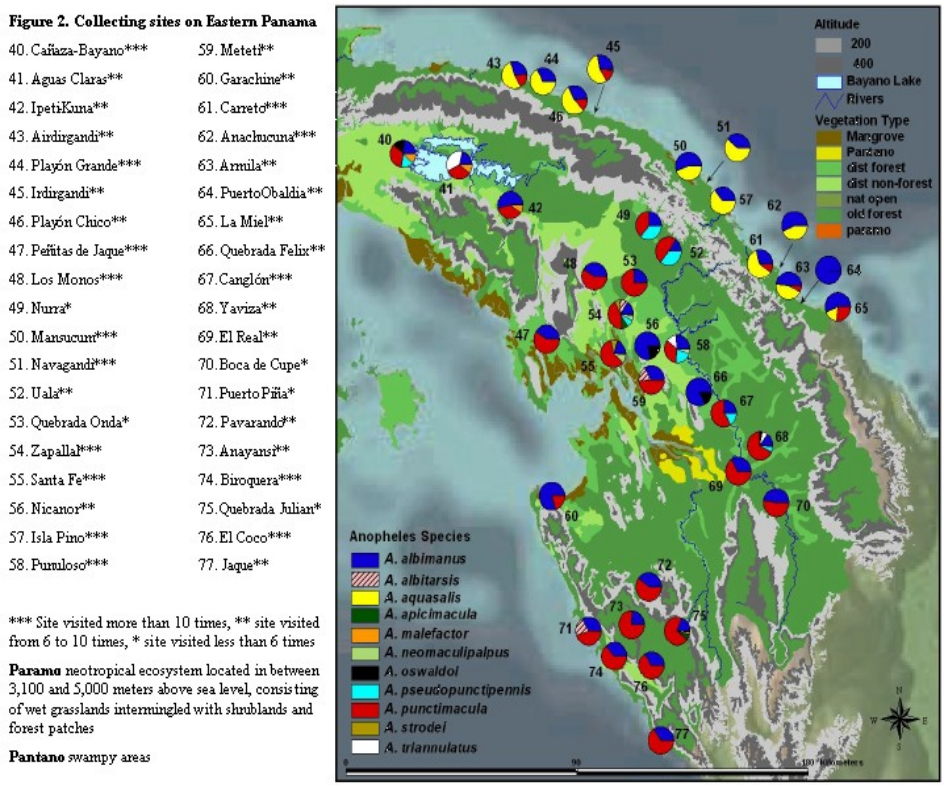

Figure 3.2. Collecting sites in Eastern Panama.

Figure 3. Collecting sites in Western Panama

1. Las Delicias***
2. Finca Debora***
3. Barra Sixaola***
4. Barranco Adentro***
5. Las Tablas**
6. Barranco Montãa***
7. San-San Pond*
8. Finca California**
9. Finca Once*
10. Guabito Centro***
11. Milla-11**
12. Almirante**
13. Pumuna*
14. Robalo*
15. Chiriqui Grande***
16. Manati*
17. Cricamola**
18. Bahia Azul***
19. Kusapin***
20. Tobobe***
21. PlayaRoja***
22. Bisira***
23. Kankintu***
24. PlayaChiriqui**
25. Vegay*
26. Guazaro**
27. Rio Caña***
28. Rio Chiriqui***
29. San Pedro***
30. Palo Blanco*
31. Santa Catalina**
32. Calmita*
33. Calobebora**
34. Concepcion**
35. Veraguas**
36. Belem**
37. Progreso*
38. Pto Armuelles*
39. PuntaBurica*

*** Site visited more than 10 times, ** site visited from 6 to 10 times, * site visited less than 6 times

Paramo neotropical ecosystem located in between 3,100 and 5,000 meters above sea level, consisting of wet grasslands intermingled with shrublands and forest patches

Pantano swampy areas

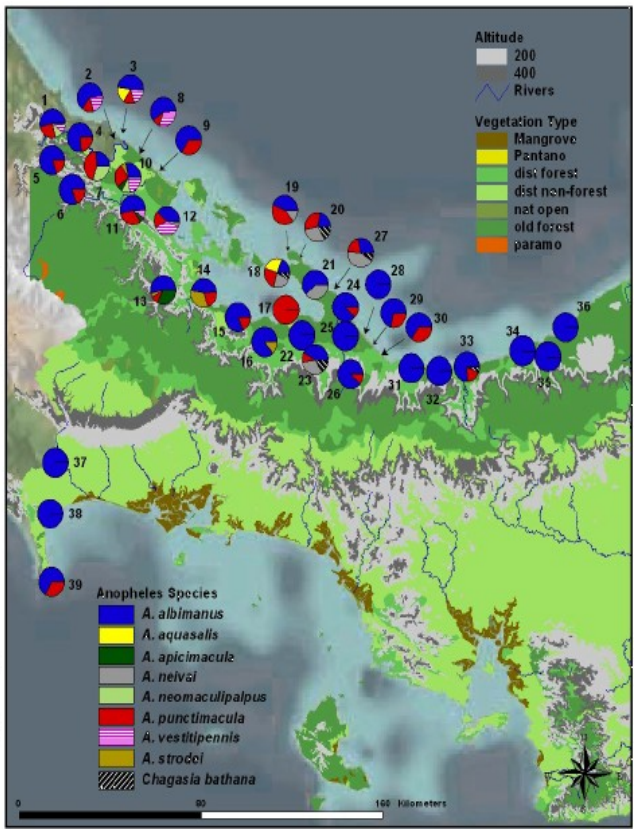

Figure 3.3. Collecting sites in Western Panama.

Connecting statement I

One hypothesis to explain the recent increase in malaria case numbers in Panama is a possible change in vector species composition due to colonization and adaptation of some *Anopheles* species to new settings, prompted by human ecological perturbation and changes in landscape features. This hypothesis has not been formally tested in Panama, despite significant anthropogenic change and human migration during the last three decades. *Anopheles* (*Nyssorhynchus*) *darlingi* is widely distributed in the Americas, but it is believed to be absent from Panama, Costa Rica and Nicaragua. This species is considered the most efficient vector of *Plasmodium falciparum* in the Neotropics. Recently, *An. darlingi* has undergone extensive colonization of western Amazonian Brazil and in the city of Belém; human mediated changes such as road construction and forest clearing have been shown to enhance its human biting rate due to increased density. Moreover, *An. darlingi* has expanded during the last ten years in the Peruvian Amazon, likely due to unplanned deforestation, an increase in fish farming providing novel habitat, and increased human migration. The high malaria prevalence and the almost exclusive occurrence of *P. falciparum* in eastern Panama are suggestive of the presence of a more efficient and possibly unidentified vector in this region. Furthermore, because no obvious physical or ecological barriers seem to prevent *An. darlingi* from migrating into eastern Panama from northern Colombia, the possibility that this malaria vector may have been recently introduced needed to be investigated. In the following chapter, I present the findings from a more comprehensive mosquito sampling across

Panama and use population genetic tools to investigate the demographic origin of *An. darlingi* in the Darien.

4. Chapter IV. Short report of *Anopheles darlingi* (Diptera: Culicidae) in Panama

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4.1. Abstract

We report *Anopheles darlingi* for the first time in the Darien Province, eastern Panama. *PCR-RFLP* profiles of the single copy nuclear *white* gene and sequence comparisons confirmed the presence of 66 specimens of the northern genotype of *An. darlingi*. The parsimony network depicted five *COI* haplotypes in forty individuals of *An. darlingi*, which connected through 7-8 mutational steps with sequences from Central and South America. Furthermore, the presence of haplotypes in Biroquera, Darien, identical to those previously published from northern Colombia suggests that Panamanian samples originated in Colombia. The neutrality tests (R_2 and Fu's F_S) were not significant and the mismatch distribution was multimodal and did not fit the model of sudden population growth, all of which may indicate a long and stable presence of *An. darlingi* in eastern Panama.

4.2. Introduction

Panama (9° 00' N, 80° 00' W) reported 5,095 malaria cases during 2004, which represented a six-fold increase in the incidence since 2001. The highest prevalence of malaria occurs in rural areas where more than 60% of the population is indigenous, living in extreme poverty and more vulnerable to malaria infection.[1] Four moderate-high risk areas for malaria transmission are clearly identified in Panama: Bocas Del Toro (BC), the Ngöbe Buglé Comarca (NBC), the Kuna Yala Comarca (KYC) and the Darien (DR), with some of these locales currently undergoing extensive changes in landscape due to an increase in tourism. Among the reasons proposed for the recent increase in Panama's malaria cases is a change in vector species composition due to colonization and possible adaptation of some anophelines to new settings, prompted by changes in landscape features. This scenario has taken place in other geographical contexts, i.e. *An. darlingi*, the main malaria vector in South America, has undergone extensive colonization of Iquitos, Peru, western Amazonian Brazil[2,3] and in the city of Belém,[4] whereas in parts of northeastern Amazon, *An. darlingi*, has been mostly replaced by *An. marajoara*.[5] Moreover, *An. darlingi* has increased in abundance during the last 10 years in the Peruvian Amazon most likely due to deforestation, and consequently, *Plasmodium falciparum* has reinvaded previous malaria eradicated areas.[2,6] In Panama studies on changes in *Anopheles* species composition have not been conducted yet, despite substantial anthropogenic perturbation during the last three decades. Loaiza and others[7] recently examined the distribution of *Anopheles* mosquitoes in Panama, reported 14 species and

suggested that *An. albimanus*, *An. aquasalis*, and *An. punctimacula* are each potential malaria vectors. Yet, *An. darlingi* was not collected during this survey.

Anopheles darlingi is broadly distributed, found from southern Mexico to northeastern Argentina, and in some parts of Central America, but never officially reported from Nicaragua, Costa Rica, or Panama, resulting in an apparent discontinuity in its distribution.[8] One hypothesis to explain finding *An. darling* in DR is a relatively recent invasion from Colombia into eastern Panama possibly enhanced by local changes in the landscape due to deforestation and other anthropogenic changes[9,10]. However, it is possible that *An. darling* has been in eastern Panama for a longer time, but never collected or perhaps misidentified. In the present study, we report *An. darlingi* for the first time in DR, the region in eastern Panama with the highest prevalence of drug-resistant *P. falciparum*. In addition, we identified which genotype of *An. darlingi* is present in Panama and investigated the genetic relatedness between our samples and previous published sequences of the mitochondrial *COI* gene.

4.3. Materials and Methods

We collected adult mosquitoes using the human landing catch technique (HLC) and CDC miniature light traps (CDC-LTs) for four uninterrupted hours (18:00 – 22:00 pm). Anti malarial drugs were taken by collectors based on recommendation by Instituto Conmemorativo Gorgas de Estudios de la Salud (ICGES) in Panama, and prior consent was obtained. The ICGES safety board approved the mosquito collecting protocol. Adult females were killed in the field with chloroform, placed individually in 1.5 milliliter tubes, and stored in plastic

bags with desiccant. Larval stages of *Anopheles* were caught using a standard dipping technique during three consecutive days per locality.[11] Third and fourth larval stages were retained for adult emergence and species confirmation. Mosquitoes were sorted by species using the morphological key of Wilkerson and Strickman.[12] They were subsequently hand carried to the US, where molecular identification and sequencing was carried out by the Wadsworth Center Molecular Genetics Core and the Griffin Laboratory, New York State Department of Health in Albany, New York.

Molecular confirmation of *An. darlingi* was carried out using two approaches: first, we PCR-amplified and sequenced a portion of the mitochondrial gene, cytochrome oxidase subunit I (*COI*), and confirmed the sequences as that of *An. darlingi COI* using Blast algorithm nucleotide search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Second, we used *PCR-RFLP* of the single copy nuclear *white* gene to distinguish between the northern and southern genotypes. These two lineages of *An. darlingi* are thought to be undergoing incipient speciation.[13] *PCR-RFLP* profiles were confirmed by sequencing 800 bp of the *white* gene where nucleotide sequences were compared with known sequences from these two lineages. [13] The procedures and primers employed for DNA isolation, PCR amplification and sequencing of the *COI* and *white* genes, were published elsewhere.[10,13] Genealogical relationships among *An. darlingi COI* haplotypes were investigated using a statistical parsimony network implemented in the program TCS version 1.12.[14] For this analysis, we used other previously published *COI* sequences from several countries encompassing *An. darlingi's*

geographical distribution range (Genebank accession no. DQ298209 to DQ298244).[10] Neutrality tests of Fu's F_S[15] and R_2[16] were used to test for population size changes, assuming neutrality[17] and calculated in Dna*SP*, version 4.50.02.[18] In addition, the mismatch distribution,[19] and the haplotype and nucleotide diversities were computed in Arlequin 3.11.[20]

4.4. Results and Discussion

A total of 1,056 female mosquitoes belonging to ten *Anopheles* species were collected in Yaviza, Jaque and Biroquera (Table 4.1., Figure 4.1.). *Anopheles punctimacula* was the most common species (51.5%), confirming previous findings in DR where it seems to be largely predominant.[7] In contrast, *An. albimanus* was less prevalent overall (26.7%), but more common than the former in Jaque. *An. darlingi* was more prevalent than *An. albimanus* in Biroquera, but proportionally less abundant than *An. punctimacula* and *An. apicimacula* (Table 4.1.). *Anopheles darlingi* was only collected in Jaque and Biroquera by human landing catch, and not recorded in either CDC-LTs or in larval collections. Furthermore, *An. albimanus* was the most common species in larval collections whereas *An. punctimacula*, *An. apicimacula* and *An. oswaldoi s.l.* were the only species collected in CDC-LTs. We also collected 8 specimens of *An. nuneztovari s.l.* in Biroquera where previous sampling has not identified this species (Table 4.1.).[7] The *COI* sequences from both Jaque and Biroquera showed 100% identity when compared with *An. darlingi COI* sequence accession numbers AF417698 and AF270932 reported in Sallum and others[21]. *PCR -RFLP* profiles of the single copy nuclear *white* gene and sequence comparison

confirmed the presence of 63 individuals of the northern *An. darlingi* genotype in Biroquera and three in Jaque.

Five *COI* haplotypes were detected in forty individuals of *An. darlingi*, three from Jaque and 37 seven from Biroquera (GenBank Accession numbers FJ550354 – FJ550358). All the haplotypes were separated in the parsimony network by 7-8 mutational steps from sequences of Central and South America. Moreover, six sequences from Biroquera were identical to haplotype M (D2 in Figure 4.2.), which had been previously found in Colombia, [10] thus suggesting a Colombian origin for the Panamanian samples (Figure 4.2.). The haplotype and nucleotide diversities for the *COI* gene were moderate to low (*Hd*: 0.58, *SD*: 0.07 and *Nd*: 0.0006, *SD*: 0.0003, respectively), which could suggest a past bottleneck and subsequent expansion with a rapid build ups of mutations.[22] Nevertheless, the neutrality tests $F_S = 2.23 > 0.05$[17] and $R_2 = 0.014 > 0.05$[18] were not significant and the mismatch distribution was multimodal and did not fit the model of sudden population expansion.

A history of malaria cases, plus the almost exclusive occurrence of *P. falciparum* in eastern Panama, where *An. albimanus* is not as prevalent as other *Anopheles* species, suggests that its incidence there may be related to the presence of a more efficient and possibly unidentified vector in this region.[7] In 2007, an outbreak of *P. vivax* and *P. falciparum* (97 cases) occurred in Puerto Piña, located approximately five kilometers from Jaque. Although no *Anopheles* survey was conducted at the time we hypothesize that *An. darlingi* could have been the main vector during this episode. Panama has undergone significant changes in land

use, urbanization, and human migration since 1960,[23] and all these changes may have altered the habitats for *Anopheles* larvae. However, we believe these changes have not yet impacted areas like Jaque and Biroquera, which are still geographically isolated, and where *An. darlingi* breeds in partially shaded and clean bodies of water, often associated with rivers and floating vegetation. In fact, the DR is still 70% forested, providing ample breeding sites for *An. darlingi* and other *Anopheles* species.[24] Our results, where we collected 45% of the total *Anopheles* species reported from Panama, in DR, support this.[7] Moreover, the balance in the proportion of low, intermediate, and high-frequency *COI* gene haplotypes, nonsignificant neutrality test results, and a multimodal mismatch distribution support a stable past effective population size for *An. darlingi* in eastern Panama and argue against a bottleneck caused by a possible recent invasion. Nevertheless, a larger sample size, more localities, other molecular markers, and a more sophisticated simulation analysis that does not depend on summary statistics to construct the mismatch distribution will be required to fully evaluate the demography of *An. darlingi* in eastern Panama.

In the present study, *An. darlingi* was not collected from Yaviza, which is located roughly 300 kilometers from Panama City, but it is possible that with future environmental perturbation in DR *An. darlingi* could expand towards central and western Panama, and contribute to a range expansion of *P. falciparum* into new areas, aggravating the malaria situation. Additional sampling throughout DR and KYC may reveal the presence of *An. darlingi* in other localities where *P. falciparum* has been previously encountered. At the local level, the influx of

either refugees escaping from armed conflict in Colombia or travelers visiting eastern Panama, plus the presence of *An. darlingi*, are likely to intensify malaria outbreaks by *P. falciparum* in DR. In the short term, it will be necessary to avoid unplanned deforestation through more sustainable methods of development for agriculture and human settlements in DR. This in combination with traditional malaria control measures will reduce the likelihood of a possible expansion of *An. darlingi* into western Panama.

4.5. Acknowledgments

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Table 4.1. *Anopheles* species collected from Yaviza, Jaque and Biroquera, in DR, Panama from the 11-29th of August 2008.

Anopheles species by Subgenera	Number of mosquitoes (%) by species	Number of mosquitoes by collecting method			Number of adult mosquitoes by locality		
		HLC	LT	LC	Yaviza	Jaque	Biroquera
Nyssorhynchus							
An. albimanus	281 (26.7%)	216	0	65	7	204	5
An. darlingi	66 (6.3%)	66	0	0	0	3	63
An. oswaldoi s.l.	7 (>1%)	6	1	0	5	0	2
An. triannulatus s.l.	9 (>1%)	6	0	3	6	0	0
An. nuneztovari s.l.	8 (>1%)	8	0	0	0	0	8
Anopheles							
An. punctimacula	541 (51.5%)	528	7	6	350	91	94
An. malefactor	7 (>1%)	7	0	0	1	1	5
An. apicimacula	112 (10.6%)	111	1	0	25	0	87
An. neomaculipalpus	21 (2%)	3	0	17	1	2	0
An. pseudopunctipennis	6 (>1%)	6	0	0	6	0	0
Total	1,056	956	9	91	401	302	256

Human landing catch (HLC), Light trap (LT), Larval collection (LC).

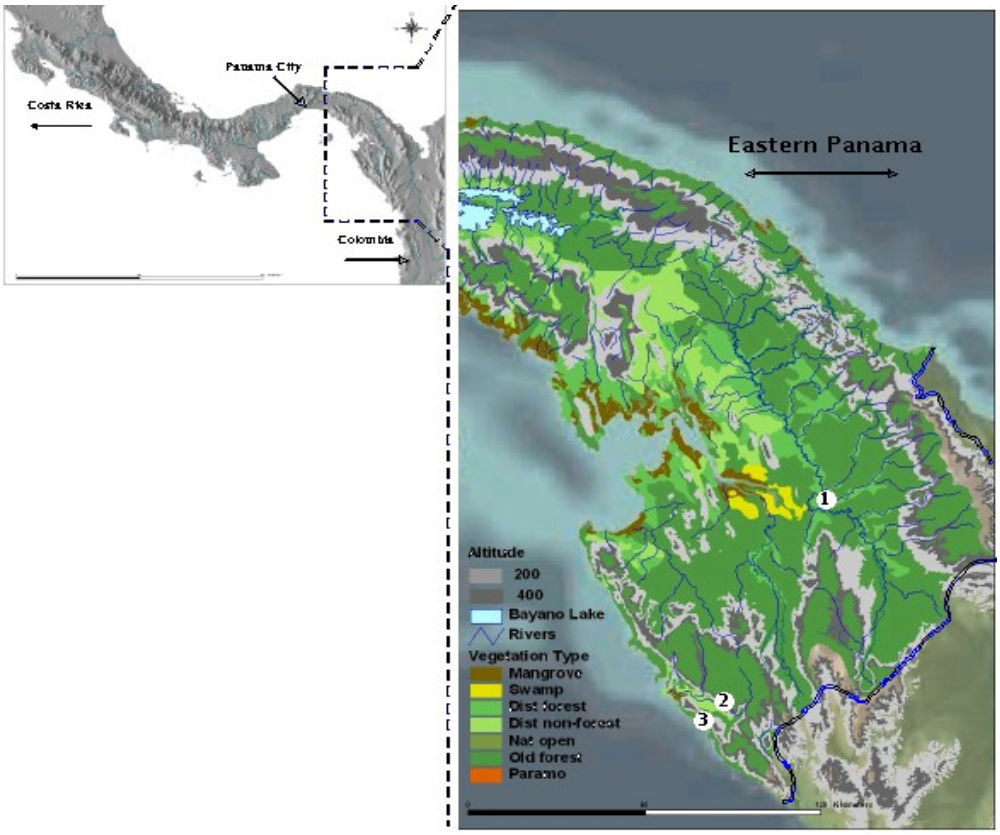

Figure 4.1. Geographic position of Yaviza (1), Biroquera (2) and Jaque (3). Altitude is in meters; Dist non-forest, Dist forest, and Nat open represent areas with high, medium and low deforestation, respectively. Old forest represents non-disrupted primary forest. Paramo is a neotropical ecosystem at elevations between 3100 and 5000 meters.

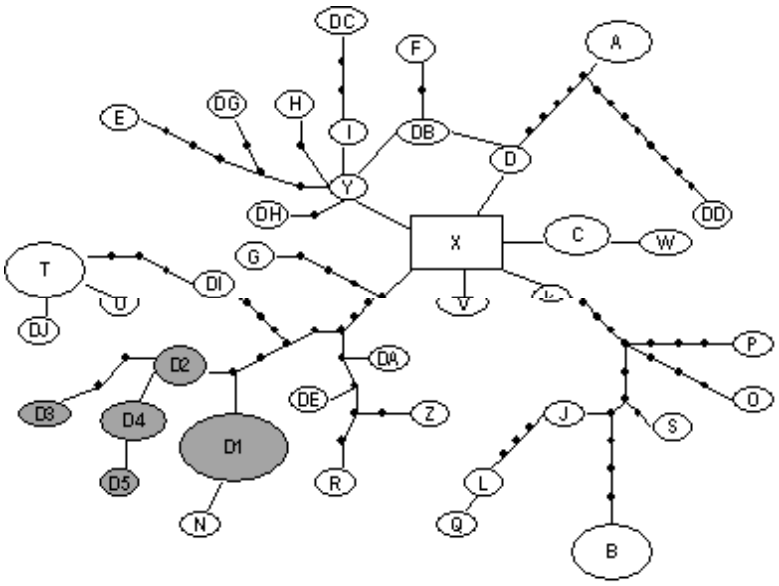

Figure 4.2. Statistical parsimony network re-drawn from Mirabello and Conn (2006). Shaded haplotypes (D1-D5) are recorded from Jaque and Biroquera in the Darien, eastern Panama. Haplotype D2 is identical to M of Mirabello and Conn (2006) from Nuqui, Colombia.

Connecting statement II

Little research has been carried out to assess the molecular population structure, demographic history and phylogeography of *Anopheles* species in the Americas, despite the fact that this information may be helpful to mitigate malaria through vector control. Most investigations have focused largely on primary malaria vectors at a continental geographic scale, giving scarce attention to local or regional patterns of genetic structure and to secondary vectors. *Anopheles* (*Nyssorhynchus*) *albimanus* is the most important malaria vector in the northern Neotropics. Significant genetic structure has been hypothesized for this species across southern Central America, yet the causes and the geographic extent of this divergence are still poorly understood. This new information may have important implications to malaria control, especially if divergence is associated with differences in the vectorial capacity and/or insecticide resistance of *An. albimanus*. Panama has used residual insecticide spraying (RIS) since 1957 as the main method for vector control; still no information exists about the impact of these measures to mitigate *Anopheles* mosquitoes in Panama. Information about the genetic diversity of Panamanian populations of *An. albimanus* may be useful to test the efficacy of past vector control strategies. Similarly, knowledge on gene flow can potentially help to track genes conferring insecticide resistance. In the following chapter I investigate the micro-geographic pattern of genetic structure of *An. albimanus*. Specifically, I use partial sequences of the mitochondrial gene, cytochrome oxidase subunit one (*COI*), and extensive sampling throughout southern Central

America, to test for geographic distance, physical barriers to dispersal, natural selection and/or demographic phenomena as the causes of genetic structure in *An. albimanus*.

5. Chapter V. Evidence for Pleistocene population divergence and expansion of *Anopheles albimanus* in southern Central America

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Running title: mtDNA COI DIVERGENCE OF ANOPHELES ALBIMANUS

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5.1. Abstract

The micro-geographic structure of *Anopheles albimanus* was investigated in southern Central America using partial sequences of the mtDNA cytochrome oxidase subunit I gene (COI). Analysis of molecular variance (AMOVA) supported significant genetic structure between populations from Costa Rica and western Panama versus those from central - eastern Panama ($\Phi_{CT} = 0.33$) whereas the within group divergence was shallow and statistically insignificant ($\Phi_{ST} = 0.08$). Furthermore, a statistical parsimony network depicted three divergent groups of haplotypes that were not evenly distributed across the study area. Our findings are in partial agreement with previous studies, yet they do not support physical barriers to gene flow or contemporary isolation by distance (IBD) in this region. Instead, three co-occurring groups of *An. albimanus* may be the result of multiple introductions, most likely due to historical fragmentation and subsequent secondary contact. In addition, the molecular signature of population expansion of *An. albimanus* was detected in central - eastern Panama approximately 22,000 years ago (95% CI 10,183 – 38,169). We hypothesize that the population structure of *An. albimanus* as determined by our COI locus analysis is the result of late Pleistocene climatic changes in northern South America.

Keywords: *Anopheles albimanus*, mtDNA COI gene, population structure, expansion

5.2. Introduction

Malaria prevalence has decreased overall during the last decade in Central America.[1] However, Panama suffered a major outbreak from 2001 to 2005, which represented 60% of the total number of malaria cases reported in the past 35 years.[2] The main vector in Panama is *An. albimanus*; nevertheless, six other *Anopheles* species involved in malaria transmission elsewhere in the Americas have been recorded in Panama.[3] Malaria is currently under control, but an influx of non - immune travelers into endemic areas, the presence of drug-resistant *Plasmodium falciparum*[4] and the recent discovery of *An. darlingi* in eastern Panama,[5] are expected to worsen the situation. Studies on the population structure of malaria vectors are important to determine the extent of genetic exchange among populations and to predict the spread of genes conferring insecticide resistance.[6, 7] In theory, focal control interventions against mosquitoes would only be effective if mosquito immigration from outside the target area is low. In addition, such studies may provide key information on lineage divergence, discrete populations and timing of demographic phenomena, all of which can influence the involvement of vectors in malaria transmission.[8-10] *An. albimanus* is a malaria vector in the subgenus *Nyssorhynchus* with considerable ecological adaptability and broad, mostly coastal, geographic distribution in the Neotropics.[11] Generally, *An. albimanus* is crepuscular, zoophilic, exophagic, exophilic and seasonally abundant.[12] However, heterogeneity in environmental factors, host availability and vector control strategies across the species range may lead to population differentiation. Although several studies have demonstrated

that *An. albimanus* is a single taxon[13-16] there is evidence for significant intra-species variation in host feeding behavior, *Plasmodium* susceptibility, longevity and insecticide resistance.[17-19] These differences may affect the ability to transmit *Plasmodium sp.* as well as the response to vector control strategies. For example, in Belize, *An. albimanus* is regarded as a secondary vector because it seldom enters human habitations, and prefers to feed on domestic animals.[19, 20] However, domestic animals are rarely encountered in some areas of Central America, where indigenous people reside and malaria is endemic. In these situations, high numbers of *An. albimanus* and exposure to mosquito bites due to poor housing construction may enhance its importance as a vector.[3, 5]

Previous research on the population genetics of *An. albimanus* using mitochondrial DNA (mtDNA) ND5 sequences found low to moderate differentiation between Central and South American populations, attributed mainly to isolation by distance (IBD).[21] In contrast, analysis of microsatellite loci using the same sample localities demonstrated a small effect of distance, but uncovered differences in allele frequencies between Costa Rica and eastern Panama.[22] The discrepancy in allele frequencies may be attributed either to the mountain range that crosses Costa Rica and western Panama, which may act as a barrier to gene flow, or to population contraction in Panama due to intense insecticide use.[22] Nonetheless, the analysis of only four populations in Costa Rica and Panama in the latter studies may have produced inaccurate results for the IBD analysis, and demographic history (*e.g.*, stability of the population size) and natural selection (*e.g.*, neutrality), which are known to influence inferences of

population structure in *Anopheles* mosquitoes,[8, 10] were not explored in these studies.

Intensive chemical and physical larval control (*e.g.*, source reduction) was carried out in Panama during and after the construction of the Panama Canal (1904 - 1914). Likewise, Panama has used nationwide residual insecticide spraying (IRS) with DDT, propoxur, fenitrothion, deltamethrin, and cyflutrin since 1957 as the main control method for malaria vectors. To date, little is known about the impact of these control strategies on the population structure of *An. albimanus* and other malaria vectors. MtDNA has proven to be extremely useful in identifying the population structure and demographic history in malaria vectors because of its lack of recombination and high levels of variability, and it has been used extensively to study anopheline mosquitoes in the Neotropics, allowing for comparison across species and geographic areas.[8,21-25] In the present study, we use partial sequences of the mitochondrial gene, cytochrome oxidase subunit I (COI), and extensive sampling throughout southern Central America, to address the following questions: (1) Is there genetic differentiation in *An. albimanus* at this micro-geographic scale?; and if so (2) What are the main factors driving this differentiation (*e.g.*, geographic distance, physical barriers to dispersal, natural selection and/or demographic phenomena)?

5.3. Materials and Methods

- *Mosquito collection*

Females of *An. albimanus* were collected outdoors by human landing catches and using CDC miniature light traps (model 2848, BioQuip Products,

Inc., Rancho Dominguez, CA, U.S.A) from 16 localities spanning roughly 1,100 km of a northwestern - southeastern transect across southern Central America (Figure 5.1., Table 5.1.). The landing catch protocol was reviewed and approved by a Human Ethics Review Board at McGill University, in Canada and the Instituto Commemorativo Gorgas de Estudios de la Salud (ICGES), in Panama City. All mosquitoes were killed with chloroform, identified to species level in the field with the morphological key of Wilkerson and Strickman [26] and stored in desiccant within 24 hrs of capture. Samples were hand carried to the U.S. and stored at -80˚C at the Griffin Laboratory until molecular work was performed.

- *Molecular procedures*

Eight to 25 mosquitoes were analyzed from each locality and samples from different days, collectors and sampling methods were randomly included in the DNA analysis. We followed the procedures employed by Mirabello and Conn[25] for DNA isolation and PCR amplification. We used the primers: UAE3/UAE10 (5' TATAGCATTCCCACGAATAAATAA-3' / 5' TCCAATGCACTAATCTGCCATATTA-3')[27] to amplify a 1,300 bp fragment of the COI gene. PCR products were cleaned using CentriSpin 40 columns (Princeton Separations, NJ, USA), and sequencing was carried out by the Wadsworth Center Genomics Core Facility (New York State Department of Health). Sequences were unambiguously aligned with Sequencher 3.0 (Gene Codes Corp, MI, USA), grouped together by site and trimmed in PAUP, version 4.0.[28] Sequences fragments of 824 bp (only the overlap between the two primers), spanning positions 1873 - 2698 of the mitochondrial genome of *An. gambiae*,

were used to determine unique haplotypes in MacClade, version 3.0.[29] The DNA sequences were checked for stop codons and confirmed as that of COI by translation into amino acid sequences using *An. quadrimaculatus* mtDNA genetic code in MEGA 4.0.[30]

- *Structure of genetic variation*

The program TCS version 1.12[31] was used to calculate the number of mutational steps necessary to link any two haplotypes at the 95% confidence level as determined in ParsProb 1.1.[32] A statistical parsimony network represented genealogical relationships among *An. albimanus* haplotypes, and homoplasy was resolved based on the algorithm estimation rules described in Crandall and Templeton[33] and summarized in Uthicke and Benzie.[34] Analysis of molecular variance (AMOVA) was carried out in Arlequin 3.11.[35] First, we grouped the sequences of *An. albimanus* as western (1-9) and central - eastern localities (10-16) (Figure 5.1.,Table 5.1.) to test for congruence with previous studies.[21, 22] Secondly, we grouped the sequences from localities in the Pacific (1,2,3,8,9,10,11 and 15) and the Atlantic coasts (4,5,6,7,12,13,14 and 16) (Figure 5.1., Table 5.1.) to test for genetic divergence due to potential ecological differences (*e.g.*, precipitation and/or tide regime). Hierarchical AMOVA tests using both haplotype frequencies and the number of mutations between haplotypes (Φ) partitioned the genetic variance within and among populations within groups (Φ_{SC}, Φ_{ST}) and among groups (Φ_{CT}). The Mantel analysis was used to test the null hypothesis of the independence of the geographic and genetic distance by a pairwise matrix of linearized genetic distances, estimated by $F_{ST,}$ and ln geographic distance among

all populations. We also assessed the effect of the Central American Cordillera on genetic differentiation by testing whether genetic distance between populations on either the Atlantic or the Pacific coast correlates better with geographic distance than with direct-line distances. This procedure was carried out using a third matrix that included a categorical variable in the partial Mantel test.[36] The significance of the Mantel test was determined by permutations of n = 10,000 through a non-parametric procedure implemented in the IBD web service version 3.15.[37]

- *Demographic history*

Neutrality tests of Tajima's D,[38] Fu and Li's F* and D*,[39] were used to test for strict neutrality (*e.g.*, the majority of mutations are selectively neutral) whereas Fu's F_S[40] and R_2 [41] were used to test for population size stability.[42] These statistics detect deviations from the pattern of polymorphism expected from a neutral model of evolution in a similar fashion, but use the information in the sample differently. Fu's F_S[40] and R_2 [41] are among the most powerful tests to detect population expansion and genetic hitchhiking, whereas Tajima's D,[38] Fu and Li's F* and D*,[39] are the most effective tests to detect background selection. Neutrality tests were calculated in DnaSP, version 4.50.02[43] for each population and for the haplotype groups defined in the parsimony network. Significance of neutrality tests was assessed by 10,000 coalescence simulations in DnaSP, version 4.50.02.[43] In addition, haplotype and nucleotide diversities[44] were computed in Arlequin 3.11[35] for each population and for the groups in the parsimony network.

The mismatch distribution was performed to distinguish between an exponentially growing population depicting a smooth unimodal distribution and a

stable population that has reached mutation drift-equilibrium depicting a multimodal, or erratic distribution, due to stochastic lineage loss.[45] To quantify the smoothness of the mismatch distribution,[46] we calculated the raggedness (*r*) statistic in DnaSP, 4.50.02.[43] The mismatch distribution was calculated in Arlequin 3.11[35] for the entire data set and for the haplotype groups in the parsimony network. Net divergence (Da)[44] among populations from western (1-9) and central - eastern Panama (10-16) (Figure 5.1., Table 5.1.) was estimated in MEGA 4.0[30] using the Tamura and Nei model.[47] Standard errors (SE) were estimated by the bootstrap method using 10,000 replicates. Divergence time (T) between groups of populations was estimated as ($Da = 2\mu t_D$) where 2μ is the divergence rate[8], and the 95% confidence intervals for the divergence times were calculated as ±1.96 SE of the net distances.

5.4. Results

In total, 265 female mosquitoes (Table 5.1.) from 16 localities were sequenced for an unambiguous alignment of 824 bp (both directions). Sequences were AT-rich (70.01% A and T bases) as encountered in the COI gene of other anopheline species.[8, 25] There were no nonfunctional genes (*e.g.*, pseudogenes) as shown by the absence of stop codons, the prevalence of synonymous substitutions, low pairwise divergence and clear electropherograms. Sequences were deposited into GenBank (accession numbers: FJ516463 – FJ516553). The total number of segregating sites was 85, with 85% transitions and 15% transversions, the majority of which were silent substitutions. Ninety-one

haplotypes were detected, 27 (29.6%) were shared among localities, and 64 (70.3%) were singletons (Figures 5.1., 5.2.).

- *Structure of genetic variation*

The statistical parsimony network illustrates the relationship among haplotypes of *An. albimanus*. All haplotypes differed by less than 12 mutational steps, so they could be connected parsimoniously. Three groups, each separated by 7 mutational steps, suggested substantial haplotype partition (Figure 5.2.). Group A was star-shaped with short branches and an excess of singleton mutations, the signal of a demographic expansion, background selection or selective sweep.[40,44,48] This group contained haplotype A1, which appears to be the most ancestral owing to its high frequency (75/265 sequences), wide geographic distribution (12/16 localities), and interior position in the network.[48] In addition, 82.4% of the haplotypes in group A were distributed exclusively in central - eastern Panama (Figure 5.1., Table 5.1.). In contrast, group B had longer branches, more missing haplotypes, fewer singleton mutations, and 90.2% of its haplotypes were encountered exclusively in Costa Rica and western Panama (Figure 5.1., Table 5.1.). Group C included only 9.8% of the total haplotypes in the parsimony network, is star-shaped with short branches, and only two missing haplotypes (Figure 5.2.). Similar to group B, 88.8% of the haplotypes in group C were restricted to Costa Rica and western Panama (Figure 5.1., Table 5.1.). These three haplotype groups represent a substantial genetic division in the data set, showing roughly 0.8% sequence divergence and an average number of 7.5 nucleotide differences (*k*) among them.

Hierarchical AMOVA tests attributed 33.99% (P = 0.0005) of the total genetic variance to the western *vs* eastern group comparison, thus partially supporting previous findings,[21, 22] whereas the Pacific *vs* Atlantic group comparison accounted only for 6.93% (P = 0.711) of the total variance and was not statistically significant (Table 5.2.). Furthermore, the Mantel analysis to test for IBD among all populations was statistically insignificant (R^2 = 0.0214, P = 0.577) suggesting little input of direct-line geographic distance as an explanatory factor of the observed spatial pattern of genetic diversity. The correlation coefficient using a third categorical variable to test the effect of geographic distance on the genetic distance after controlling for the effect of the Central American Cordillera was not statistically significant (R^2 = 0.0714, P = 0.377).

- *Demographic history*

Overall values of haplotype and nucleotide diversities were higher (ranging from 0.77 - 0.98 and 0.008 - 0.01, respectively) for populations in Costa Rica and western Panama than for populations in central - eastern Panama (ranging from 0.45 - 0.96 and 0.001 - 0.006, respectively) (Table 5.3.). By group, nucleotide diversity was highest in B, followed by lower and similar values for A and C (Table 5.4.). The mismatch distributions for the entire data set (all populations) and for B alone depicted multimodal curves, and did not fit a model of sudden population expansion, thus suggesting a stationary population at equilibrium. In contrast, A and C were unimodal and fit the model of sudden population expansion (Figure 5.3.). In support of these findings, the mismatch distribution for A was strongly biased towards low divergence values with a mode

of 3 nucleotide changes, suggesting a relatively recent expansion event (Figure 5.3.). The average number of nucleotide differences (*k*) for A, B and C was 1.31, 4.83 and 1.10, respectively; suggesting higher diversity and perhaps equilibrium only in B. The raggedness statistic for the entire data set and for A and B was low and not significant providing further support for population expansion. In contrast, C showed a higher and significant value for the raggedness statistic, therefore rejecting the model of sudden population expansion (Figure 5.3.).

Neutrality tests of Tajima's D,[38] Fu and Li's F* and D*,[39] and Fu's F_S[40] demonstrated negative and significant values for the entire data set, and for A (Table 5.4.), thus allowing the rejection of either strict neutrality or population size stability, due to either background selection, selective sweep or demographic expansion. The R_2 test[41] was also low and significant for A further supporting the departure from equilibrium most likely due to demographic expansion (Table 5.4.). The time since expansion for A was calculated using $t = \tau/2u$, where $u = \mu$ x number of base pairs sequenced x generation time, and μ is the mutation rate.[44, 45] The *Drosophila* mutation rate of 10^{-8}/site/year[49] and 10 generations/year[8] were used in this calculation. The estimate of τ, from the raggedness calculation, is 3.39 for A. Therefore, the time since expansion for *An. albimanus* in central - eastern Panama is approximately 22,000 years ago (95% CI 10,183 – 38,169), in the late Pleistocene.

5.5. Discussion

- *Pattern of genetic structure*

The role of *An. albimanus* as a primary malaria vector of *Plasmodium vivax* and *P. falciparum* has been historically acknowledged; yet, its capacity to transmit malaria parasites varies across its range.[3,19,20] Furthermore, evidence for cryptic species in *An. albimanus* have been unsubstantiated so far[13-16] and population differentiation seems to be low overall.[19,20] The shallow population structure of *An. albimanus* in the Americas may be attributed to long-term effective population size due to high dispersal capability, opportunistic feeding behavior and ecological plasticity. Nevertheless, this study demonstrates considerable geographic structure for *An. albimanus* across Costa Rica and the Isthmus of Panama, with a genetic discontinuity within less than 300 km.

Our results partially support previous findings on the population structure of *An. albimanus* using the mtDNA ND5 gene and microsatellites, which depicted differences in allele and haplotype frequencies between Costa Rica and eastern Panama.[21, 22] The authors in these studies invoked isolation by distance, physical barriers to gene flow, and/or population contraction in Panama due to intense insecticide use as the likely causes of population differentiation. However, neither IBD nor barriers to dispersal across Costa Rica and Panama were supported by our results. The correlation coefficient and the Mantel test remained low and statistically insignificant despite several analyses, and 27 (29.6%) haplotypes were shared among populations and 11 (13.5%) were shared between the Atlantic and Pacific coasts of Costa Rica and western Panama where the

mountain range reaches its maximum altitude (roughly 3,000 m). These findings indicate that the Central American Cordillera has not restricted the dispersal of *An. albimanus* in the past. They also demonstrate that the spatial pattern of haplotype diversity depicted by the COI gene is not due to restricted gene flow as a result of geographic distance.

Mountain ranges (cordilleras) have been identified as barriers to gene flow for some American malaria vectors. The Green Mountains in the eastern United States are believed to restrict dispersal of *An. punctipennis*, and some lineages of *An. nuneztovari* s.l. were partially separated by the eastern Andean Cordillera.[24, 50] Because these two *Anopheles* species have specific ecological requirements, and limited geographic distributions, mountain ranges are likely to restrict their dispersal. In contrast, *An. albimanus* is capable of flying up to 32 km, and has been found as high as at 1,941 meters[12, 18] thus, low mountain ranges are unlikely to play a substantial role in its geographic structure. A positive and significant relationship between geographic and genetic distances was reported for *An. nuneztovari* s.l. and *An. darlingi* in South America using nuclear markers: the *white* gene [7, 9] and microsatellites.[7, 51, 52] However, in most of these cases the correlation was weak, and not detected when different lineages and geographic regions were analyzed separately.[7, 9, 50] Furthermore, IBD was not detected in *An. nuneztovari* s.l. with analysis of mtDNA RFLPs [53] or in *An. darlingi* using the COI gene.[25] Given that neither *An. darlingi* nor *An. nuneztovari* s.l. are at mutation - drift equilibrium,[9] misleading IBD may be the result of historical

fragmentation, initial colonization pathway mimicking the IBD pattern or inappropriate sampling.[54]

Mosquito control during the construction of the Panama Canal was carried out in the former Canal Zone and few control measures were undertaken in the surrounding areas. However, we observed reduced diversity in several localities 95 to 410 km from the former Canal Zone (Figure 5.1.). Even though IRS is efficient in controlling endophilic *Anopheles* species, the exophagic and exophilic behavior displayed by *An. albimanus* in Central America[19, 20] make the efficacy of IRS uncertain even in highly susceptible populations. Moreover, avoidance behavioral responses against DDT exhibited by Panamanian populations of *An. albimanus* may have lessened the physical contact between this species and sprayed surfaces.[55] For the above reasons, we believe that intensive mosquito control alone does not explain the lower diversity and different haplotype composition seen in localities of central - eastern Panama as hypothesized by Molina-Cruz and others.[22] Nevertheless, a lower mutation rate of the COI gene indicates that this marker is not as sensitive as microsatellites to detect more recent demographic events.[22] However, the larger effective population size and longer coalescent time of microsatellites (*e.g.*, nuclear genome), suggest that population contraction in Panama due to mosquito control is unlikely, as insecticide treatment is a recent phenomenon and microsatellites are lagging indicators of population processes.[56] The general agreement of previous microsatellite and mtDNA ND5 gene studies,[21, 22] and rather similar results in our COI gene analysis could indicate that the processes causing genetic differentiation

in *An. albimanus* are genome-wide effects, and that they occurred at a much earlier time frame than mosquito control in Panama. The high prevalence and wide distribution of *An. albimanus* during the last 35 years in Panama also argue against a recent bottleneck due to insecticide treatment.[3, 5]

- *Demographic history*

The unimodal and statistically insignificant mismatch distribution under the demographic expansion model and 4 of 5 negative and significant neutrality tests in group A, indicate that *An. albimanus* is not at equilibrium, due to a past bottleneck, background selection, population expansion, or selective sweep in central - eastern Panama. The neutrality tests cannot distinguish between demographic phenomena and selection as the causes of departure from equilibrium. Therefore, we performed the McDonald-Kreitman neutrality test (MK) in DnaSP, version 4.50.02[43] using all the sequences of *An. albimanus* from central - eastern Panama and 52 COI sequences of *An. triannulatus* from the same localities as an outgroup (unpublished data). MK considers the evolution of a protein-coding gene in two closely related species in which nucleotide differences can be classified as either synonymous or non-synonymous, and it was used to investigate the influence of natural selection on the COI sequences. The MK test yielded a statistically insignificant *P*-value of 0.870 in the Fisher's exact test; therefore the departure from equilibrium observed in *An. albimanus* from eastern Panama is not due to directional selection, but more likely to an expansion. The observed haplotype partition seems to be more related to the demography of *An. albimanus* and a complex history of dispersal events throughout southern Central

America. The genetic signature of population historical processes can remain for long periods of time, obscuring the true geographical population structure that may exist.[57] In general, populations from Costa Rica and western Panama showed higher diversity, with a decreasing trend in the nucleotide diversity from Costa Rica towards eastern Panama. Although this may be interpreted as a single founder event followed by sequential population bottlenecks in the colonizing front, and range expansion from Costa Rica into eastern Panama, an alternate interpretation is the co-occurrence and haplotype mixing of three maternal lineages (A, B and C) in populations of Costa Rica and western Panama, and the predominance of only A in populations of central - eastern Panama. Furthermore, B and C depicted a decreasing trend in haplotype diversity from Costa Rica towards eastern Panama whereas A also decreased in haplotype diversity, but in the opposite direction (Figure 5.1.). This pattern may represent historical geographic fragmentation in eastern Panama, and a subsequent re-invasion by two different source populations (B and C) from the north and one source population from Colombia (A). The fact that old and predominant haplotypes from Costa Rica and western Panama gave rise to haplotypes found in eastern Panama and vice-versa, further supports a pattern of recent regional secondary contact.

Taken together, these findings suggest a discontinuous colonization pattern of *An. albimanus* throughout Costa Rica and the Isthmus of Panama as the most likely explanation for the observed distribution of genetic diversity. The dispersal of different groups of mosquitoes (multiple introductions) could explain three co-occurring and genetically differentiated maternal lineages in Costa Rica and

Panama, which may have arrived at different times, with a separate demographic history and therefore an already significant genetic structure between them.[58] The divergence time (t_D) from the net nucleotide substitution per site (Da = 0.004 ± 0.001, 95% CI) between western and central - eastern populations of *An. albimanus* placed the time since divergence around 200,000 years ago (235,000 ± 165,000, 95% CI) in the late Pleistocene.

Anopheles albimanus is believed to have originated in the Caribbean islands and subsequently invaded the American continent, but no definitive information on the colonization path exists.[22] Group B seems to be the oldest owing to its long branches and missing haplotypes in the parsimony network, and higher nucleotide diversity, which may support a north - south colonization path throughout Costa Rica and Panama; however, this disagrees with the coalescent theory, which predicts that group A is the oldest because of haplotype A1.[48] Nevertheless, given the non-equilibrium scenario and likely past geographic fragmentation of *An. albimanus* in the present study, it is possible that the COI gene does not have enough resolution to infer the original colonization pathway of *An. albimanus* throughout southern Central America. Due to the haploid nature and maternal mode of inheritance, mtDNA genes have four-fold smaller effective population size than nuclear markers, and therefore the COI gene may have been affected by more recent demographic phenomena during the Pleistocene, thus erasing more ancient signals of colonization events.

Pollen data from marine records of the Panama Basin and the Pacific slope of the northern Andes show cold and humid conditions between 39,410 and

28,120 years ago with moderately high sea levels and less precipitation. In contrast, the coldest and driest conditions and the lowest sea levels occurred between 28,120 and 14,500 years ago.[59] These environmental changes extend through the population expansion time proposed for *An. albimanus* in this study. *Anopheles albimanus* is highly opportunistic, able to develop in a wide variety of breeding habitats;[18] however, it is more abundant in coastal plains, close to the shore and at less than 100 m.[11] Therefore, a combination of Pleistocene climatic oscillation, most likely involving temperature, precipitation, and sea level changes in eastern Panama, seems to be a plausible explanation for the population expansion of *An. albimanus*.

The entire data set also concurs with population expansion, yet B and C showed a less clear signal. These two groups are distributed mostly in Costa Rica and western Panama where mountains and upland plains might have served as a refuge providing alternative and more stable breeding sites during glacial periods. It is noteworthy that haplotype A1 was not recorded from the Atlantic coast of Costa Rica and western Panama. Pleistocene glaciations exposed most of the Gulf of Panama, located in the narrowest strip of land of central Panama,[60] and this may have facilitated re-invasion of this expanding haplotype through the Pacific coast whereas re-invasion through the Atlantic coast might have been more irregular.

5.6. Conclusions

In the present study, the COI gene uncovered significant genetic structure at a micro-geographic scale. The genetic differentiation in *An. albimanus* appears

to be due to Pleistocene historical fragmentation, re-colonization and population expansion, rather than to contemporary geographic structure, insecticide treatment and selective pressures on the COI locus. Other studies carried out in fresh water fishes, the pseudoscorpion *Cordylochernes scorpioides* and the tungara frog *Physalaemus pustulosus* have supported a complex biogeographical history in the Isthmus of Panama where significant genetic differentiation is seen at short distances, and waves of colonization, extinctions and re-invasions appear to be the main forces shaping the distribution of genetic diversity.[61-63] Our results have to be viewed with caution as they may represent divergence only in the mitochondrial genome and therefore a nuclear marker will be required to test these findings. In addition, more research will be needed to determine whether or not these three maternal lineages may have different potential roles in malaria transmission.

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Table 5.1. *An. albimanus* collection localities and haplotype frequency.

Locality	n	Latitude/longitude	Frequency
1. Soley	21	10.98 N, -85.68 W	**A1**(2), **A4**, A41, **B4**(2), **B5**, **B6**, **B10**, **B11**, **B15**, **B19**, B33, B35, B37, **C1**(4), **C2**, **C3**
2. Palo Verde	21	10.36 N, -85.13 W	**A1**, **A4**, **B2**, **B4**(3), **B5**, **B6**, **B10**, **B11**, **B15**, **B19**, B31, **C1**(4), **C2**(2), **C3**
3. Dominical	11	9.31 N, -83.95 W	**A2**, **B1**(4), **B12**, **B17**(3), **B18**, B21, **C2**
4. Matina	19	10.25 N, -83.51 W	**A2**(2), **B1**(2), **B3**, **B6**, **B8**, **B12**(2), **B16**, **B17**, **B18**, B29, B39, **C2**, C4, C5
5. P. Blanco	15	9.45 N, -82.66 W	**A2**(2), A27, **B1**(3), **B2**(3), **B3**(3), **B8**, B24, C8
6. California	17	9.48 N, -82.48 W	**A2**, **B1**(8), **B3**, B7(2), B23, B25, B26, **C1**(2)
7. Rio Diablo	10	9.06 N, -81.75 W	**A2**, A28, **B1**(2), **B14**, B20 (2), B27, B30, B40
8. Barqueta	22	8.35 N, -82.48W	**A1**(3), **A2**, **A4**, **A5**, A36, A40, A10, A11, A12, A13, **B2**, **B13**, **B14**, **B16**, B28, B36, B32, **C1**, C6, C7
9. Las Lajas	12	8.11 N, -81.73 W	**A1**(4), A37, A38, A39, **B13**, B34, B38, **C1**(3)
10. Colomas	9	7.68 N, -80.93 W	**A1**(6), **A4**, A26
11. Santa Clara	16	8.46 N, -80.31 W	**A1**(8), **A3**, **A5**, A6, A7, A8, A9
12. Rio Indio	24	9.15 N, -80.44 W	**A1**(17), **A3**(2), A29, A30, A31, A33
13. Bayano	25	9.13 N, -78.33 W	**A1**(18), **A3**, **A4**, **A5**, A23, **B2**, B9(5)
14. Ukupa	17	9.11 N, -77.93 W	**A1**(5), **A3**(2), **A4**(4), **A5**, A14, A15, A16, **B2**, C9
15. Yaviza	8	7.98 N, -77.51 W	**A1**(2), **A3**, A17, A25, A32, **B2**, **B4**
16. Zapsurro	18	8.61 N, -77.31 W	**A1**(9), **A3**, A34, A35, A18, A19, A20, A21, A22, A24

Frequency = haplotype frequency; only those with n = 2 or more are in parentheses following haplotype designation; underlined bold letters are shared haplotypes, and plain letters are private haplotypes in that population; n = mosquitoes per locality. Haplotypes labeled A1-A41; B1-B41 and C1-C9 belong to groups A, B and C in the network, respectively. Costa Rica and western Panama (localities 1-9), central – eastern Panama (10-16).

Table 5.2. Comparative results of a hierarchical analysis of molecular variance (AMOVA), using Φ statistics from haplotype frequencies and genetic divergence among populations of *An. albimanus*. (a) Costa Rica and western Panama (localities 1-9) vs central – eastern Panama (10-16); (b) Pacific (1-3,8-11,13,15) vs. Atlantic (4-7,12,14,16) coasts.

	Source of variation	Total variation (%)	Fixation index	*P*
(a)	- Within populations	60.45	$\Phi_{SC} = 0.39$	< 0.0001***
	- Among populations within groups	5.57	$\Phi_{ST} = 0.08$	< 0.121
	- Among groups	33.99	$\Phi_{CT} = 0.33$	< 0.0005***
(b)	- Within populations	73.07	$\Phi_{SC} = 0.26$	< 0.00021***
	- Among populations within groups	30.24	$\Phi_{ST} = 0.29$	< 0.0011**
	- Among groups	6.93	$\Phi_{CT} = - 0.003$	< 0.711

*P<0.05; **P<0.02; ***P<0.001

Table 5.3. Summary statistics for polymorphisms of *An. albimanus*.

By locality	H/n	h (SD)	p (SD)
1. Soley	16/21	0.96 (0.031)	0.01 (0.002)
2. Palo Verde	15/21	0.95 (0.032)	0.01 (0.001)
3. Dominical	7/11	0.87 (0.089)	0.01 (0.001)
4. Matina	15/19	0.97 (0.020)	0.01 (0.002)
5. Puente Blanco	8/15	0.90 (0.046)	0.01 (0.001)
6. California	8/17	0.77 (0.099)	0.008 (0.001)
7. Rio Diablo	8/10	0.95 (0.053)	0.01 (0.001)
8. La Barqueta	20/22	0.98 (0.020)	0.01 (0.001)
9. Las Lajas	8/12	0.89 (0.071)	0.01 (0.001)
10. Las Colomas	3/9	0.55 (0.016)	0.001 (0.001)
11. Santa Clara	9/16	0.76 (0.011)	0.004 (0.001)
12. Rio Indio	6/24	0.49 (0.111)	0.001 (0.001)
13. Bayano	4/25	0.45 (0.105)	0.004 (0.003)
14. Ukupa	9/17	0.87 (0.051)	0.004 (0.001)
15. Yaviza	7/8	0.96 (0.077)	0.006 (0.001)
16. Zapsurro	10/18	0.76 (0.108)	0.002 (0.002)

H, unique haplotypes; n, number of individuals; h, haplotype diversity; SD, standard deviation; π = nucleotide diversity. Costa Rica and western Panama (localities 1-9), central – eastern Panama (10-16).

Table 5.4. Summary statistics for polymorphisms and neutrality tests of *An. albimanus*.

By groups	H/n	h (SD)	p (SD)	F_s	D_T	F*	D*	R_2	r
Group A	41/139	0.70 (0.043)	0.001 (0.0002)	-53.61***	-2.5**	-6.30*	-7.22*	0.018***	0.017
Group B	41/99	0.94 (0.014)	0.005 (0.0002)	-26.36	-1.36	-2.30	-2.29	0.072	0.012
Group C	9/27	0.71 (0.085)	0.001 (0.0003)	-4.57	-1.69	-2.42	-2.24	0.062	0.066
Total	91/265	0.907 (0.015)	0.008 (0.0002)	-73.26***	-1.42**	-4.07*	-6.26*	0.041**	0.011

*P<0.05; **P<0.02; ***P<0.001

H, unique haplotypes; n, number of individuals; h, haplotype diversity; SD, standard deviation; p = nucleotide diversity. F_S = Fu's F_S statistic; D_T = Tajima's D; F* = Fu and Li's F test; D* = Fu and Li's D test; R_2 = Ramos-Onsins & Rozas R_2 test; r = raggedness statistic. See the statistical parsimony network of *An. albimanus* for group definitions.

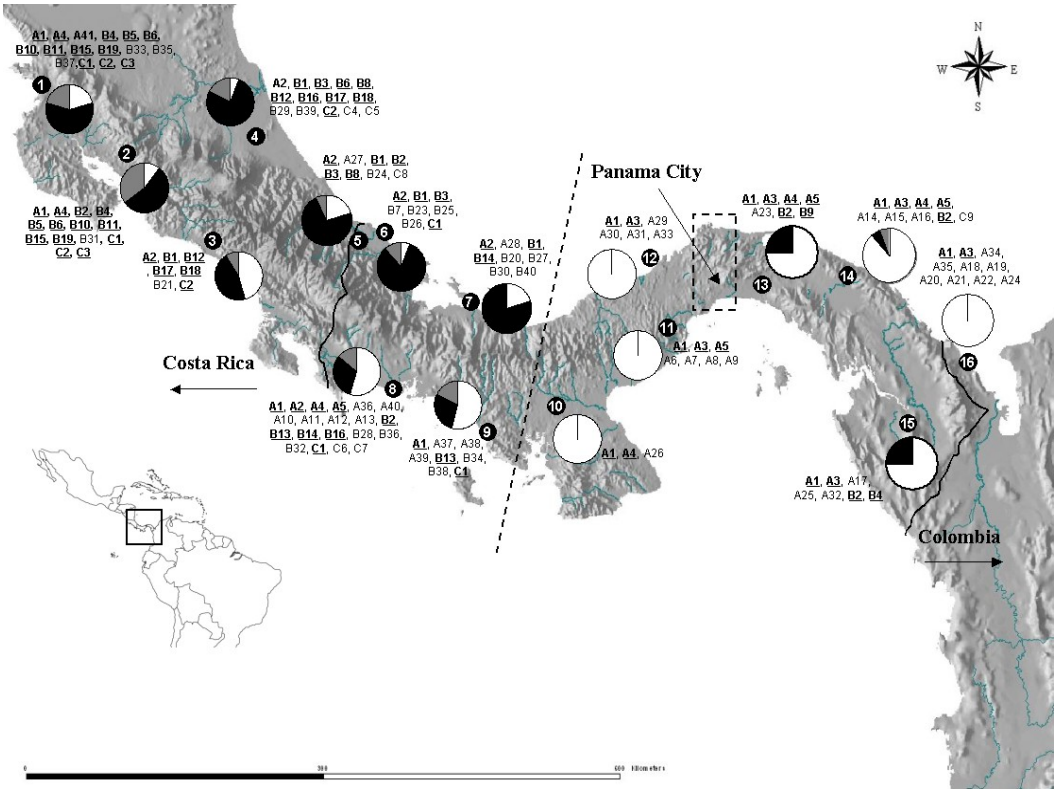

Figure 5.1. Geographic distribution of the COI haplotypes in Central America. The letters correspond to the haplotype(s) observed for each locality. The circled numbers correspond to the localities in Table 5.1., positioned on the map according to the longitude and latitude of the site. Underlined bold letters depict shared haplotypes, and plain letters depict private haplotypes. Circles represent the distribution of groups A (white), B (black) and C (shaded) in the network. The dotted rectangle shows the area of the former Panama Canal Zone. Dashed line divides Costa Rica and western Panama (localities 1-19), from central – eastern Panama (10-16). Inset map depicts the geographic position of the study area.

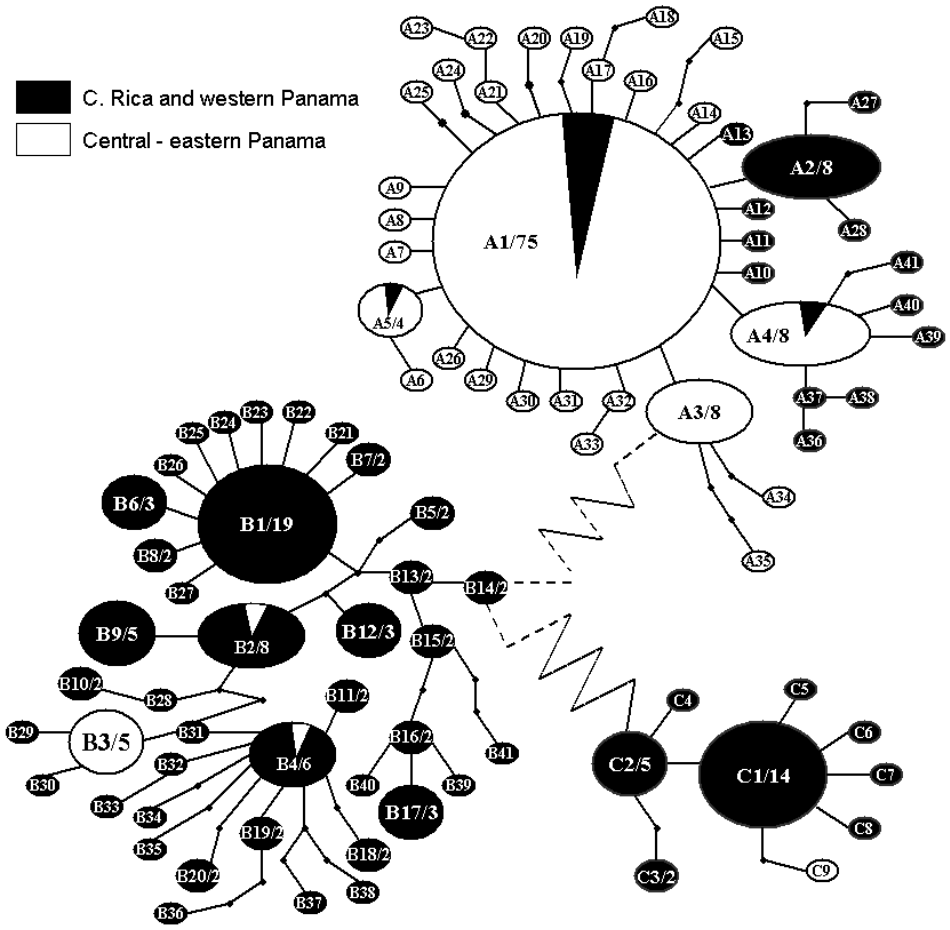

Figure 5.2. Statistical parsimony network of 91 COI haplotypes of *An. albimanus*. The letters correspond to the haplotypes observed for the 16 localities in Table 5.1. The solid black dots represent missing haplotypes. Dotted lines depict 7 mutational steps between groups A (labeled A1-A41); B (B1-B41); and C (C1-C9). The size of the circle is proportional to the frequency of the haplotype, which is indicated following the haplotype code, and the color indicates the geographic distribution.

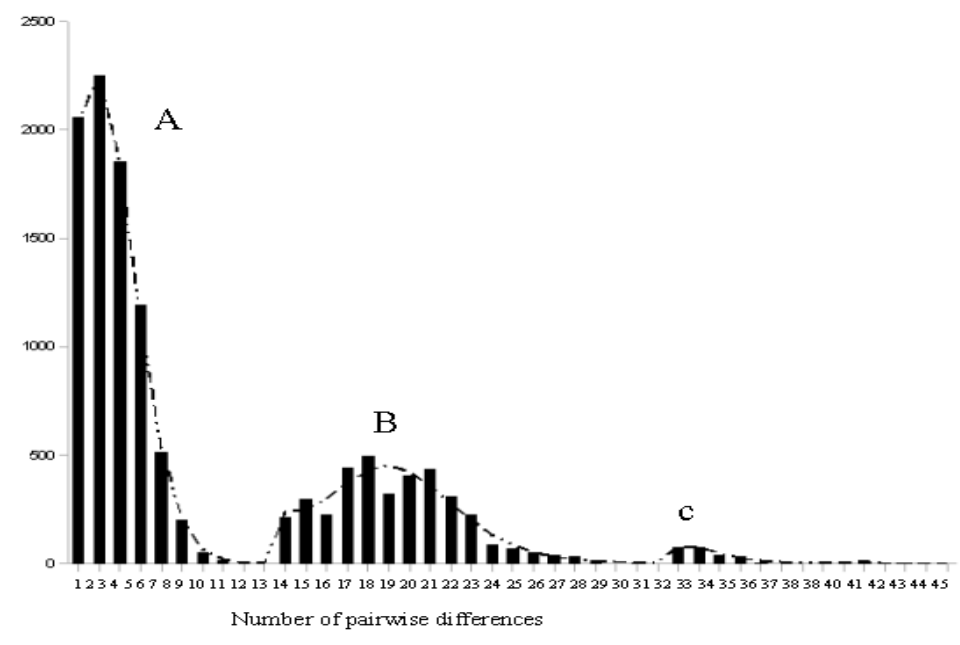

Figure 5.3. Mismatch distribution for groups A, B and C of *An. albimanus*. Bars are observed distribution, and the dashed line shows the distribution simulated under a model of sudden expansion.

Connecting statement III

Anopheles (*Nyssorhynchus*) *albimanus* is the most important malaria vector in Central America, the Caribbean islands and in coastal areas of Colombia and Ecuador. Significant genetic structure has been hypothesized for this malaria vector in southern Central America, likely due to historical fragmentation and population expansion. The geographic pattern of demographic history of *An. albimanus* as depicted by my previous *COI* gene analysis appears to have been driven by Pleistocene climatic oscillation. Such environmental changes were hypothesized to be mostly in temperature and rainfall, and they may have affected larval habitat availability and the effective population size of this malaria vector at a regional scale. Nevertheless, this pattern may also be due to selective pressures in the *COI* gene, and therefore, it needs to be confirmed with an independent nuclear molecular marker. Agreement between my previous *COI* analysis and a nuclear gene may help to identify whether genetic structure is due to demographic processes or natural selection. Moreover, the taxonomic status of a single species of *An. albimanus* still needs to be corroborated, with more extensive sampling and different molecular markers. In the following chapter, I use additional mtDNA *COI* sequences from Nicaragua, eastern Panama and Ecuador plus two other independent molecular markers, the ribosomal DNA ITS2 and the single copy nuclear *white* gene. I employ the information from these markers to investigate more comprehensively the demographic history of *An. albimanus* across the northern Neotropics.

6. Chapter VI. Late Pleistocene Environmental Changes lead to Unstable Demography and Population Divergence of *Anopheles albimanus* in the northern Neotropics

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Running title: POPULATION HISTORY OF ANOPHELES ALBIMANUS

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6.1. Abstract

We investigated the historical demography of *Anopheles albimanus* using mosquitoes from five countries and three different DNA regions, the mitochondrial cytochrome oxidase subunit I gene (*COI*), the single copy nuclear *white* gene and the ribosomal internal transcribed spacer two (ITS2). All the molecular markers supported the taxonomic status of a single species of *An. albimanus*. Furthermore, agreement between the *COI* and the *white* genes suggested a scenario of Pleistocene geographic fragmentation (i.e., population contraction) and subsequent range expansion across southern Central America. Our findings are contrasted with those of *Anopheles darlingi*, which has also expanded demographically in South America.

Keywords: *Anopheles albimanus*; *COI*; *white*; *ITS2*; Pleistocene environmental changes; geographic fragmentation; population expansion.

6.2. Introduction

Several phylogeographic studies have depicted a complex biogeographical history across the Isthmus of Panama and northern Colombia where significant genetic differentiation is seen at short distances, and waves of colonization, extinctions and re-invasions appear to be the main forces shaping the distribution of genetic diversity. Most of these studies have placed diversification events in a time frame earlier than the complete formation of the Isthmus of Panama (3– 3.5 mya) (Bermingham and Martin, 1998; Zeh et al., 2003; Weigt et al., 2005; Miller et al., 2008), and therefore, much less is known about the impact of more recent environmental changes across this region during the Pleistocene (0.01– 1.8 mya). Pleistocene climatic oscillations have had a profound effect on the genetic structure of vectors of pathogens, such as *Anopheles* mosquitoes, worldwide. For instance, in Southeast Asia, both *Anopheles dirus* and *Anopheles baimaii*, two important malaria vectors, have expanded demographically due to changes in forest structure and climatic conditions in the Pleistocene (300,000 years ago) (O'Loughlin et al., 2008). Likewise, *Anopheles darlingi*, an important malaria vector in most of the Neotropics, has undergone Pleistocene population expansion in parts of South America likely due to changes in climatic conditions leading to forest fragmentation and refugia isolation, although in a much more recent time frame when compared with the Asian mosquitoes (25,312 ya) (Mirabello and Conn, 2006a,b). These and other studies have uncovered historical changes in demography that have ultimately shaped the geographical distribution and relative abundance of *Anopheles* species, and perhaps their capabilities to vector malarial

parasites at a regional scale (Walton et al., 2000; Mirabello and Conn, 2008). Similar studies are scarce in southern Central America, despite the acknowledged geological and environmental complexity of this region during the Pleistocene (Crawford, 2003; Cortes-Oritz et al., 2003; Nettel et al., 2008; Miller et al., 2008).

Anopheles albimanus is a primary malaria vector throughout the northern Neotropics, yet its population history with respect to Pleistocene environmental changes has not been examined fully. Furthermore, no accurate information exists on the geographical origin of *An. albimanus*, and its likely initial colonization path throughout the Americas. Recent research on *An. albimanus* uncovered considerable geographic structuring across the Isthmus of Panama and northern Colombia. Several divergent groups of mtDNA *COI* haplotypes were found co-occurring across this region, and were hypothesized to be the result of late Pleistocene geographic fragmentation and multiple re-introductions via demographic expansion. The population expansion, in central-eastern Panama and the Caribbean coast of Colombia, was believed to be due to climatic oscillation around 22,000 ya, and thus *An. albimanus* is not at equilibrium regionally (Gutiérrez et al., 2009; Loaiza et al., 2010). The co-occurrence of several maternal lineages of *An. albimanus* across Panama and northern Colombia may have important implications for malaria control, especially if they are differentially involved in malaria transmission. Nevertheless, three hypotheses remain to be tested: (1) Is the population divergence in recent studies of *An. albimanus* due to cryptic speciation?; (2) Is this divergence associated with Pleistocene environmental changes?; and (3) Is the population expansion of *An. albimanus*

supported by a single copy nuclear gene?. To answer these questions, we combined the data from Gutiérrez et al. (2009) and Loaiza et al. (2010), with 123 additional mtDNA *COI* sequences from Nicaragua, eastern Panama and Ecuador, expanding the sampling scheme and augmenting the statistical power of the analyses. We also looked for fixed substitutional changes and length differences in the ribosomal DNA ITS2 marker as this may indicate the existence of cryptic species or restricted gene flow in *An. albimanus* (Collins and Paskewitz, 1996). Additionally, we sequenced a subset of individuals for the single copy nuclear *white* gene to test the demographic pattern depicted by the *COI* and to infer the initial colonization path of *An. albimanus* across southern Central America. Finally, we sought to compare the demographic history of *An. albimanus* with that of *An. darlingi* in the Neotropics.

6.3. Materials and methods

Sample processing and laboratory procedures

Information on mosquito sampling, collection sites, laboratory procedures and GenBank accession numbers for the *COI* gene can be found in Gutiérrez et al. (2009) and Loaiza et al. (2010). Additional samples were obtained either as adults or in larval collections. The latter were reared to adulthood and processed as in Loaiza et al. (2010). A total of 612 sequences of the *COI*, all shortened to 776bp, were analyzed in this study (new *COI* sequence submission GenBank accession nos. HM030881 – HM030907). We sequenced the w*hite* gene in 175 individuals (HM042289 – HM042297) and the ITS2 in 173 (HM042298 – HM042301) from a subset of randomly selected samples (Table 6.1.). Information on PCR-

amplification conditions, and sequencing reactions for the ITS2 region and the w*hite* gene can be found in Linton et al. (2002) and Mirabello and Conn (2008), respectively.

Neutral expectation and genetic diversity

The program DNASP v4.50.02 (Rozas et al., 2003) was used to calculate Tajima's *D* to determine whether or not the *COI* and the w*hite* gene sequences conformed to neutral expectations. Because the w*hite* gene is nuclear we allowed for recombination while testing for significant deviation from neutrality, thus accounting for overestimation of directional selection. We computed basic sequence statistics for all the gene markers in ARLEQUIN v3.11 (Excoffier et al., 2005) (Table 6.1.).

Population divergence and historical demography

- *COI*

We employed the spatial analysis of molecular variance (SAMOVA v1.0) (Dupanloup et al., 2002) to define aggregates of collection sites that are geographically homogeneous, but genetically differentiated from other similar aggregates. We ran SAMOVA from K = 2 to 30 and implemented 10,000 independent simulated annealing steps each starting from 100 random sets of initial conditions. The Mantel analysis was used to test for the isolation by distance (IBD) pattern, by using a pairwise matrix of linearized genetic distances, estimated by Φ_{ST}, and the natural log-transformed geographic distance. Because *An. albimanus* has a coastal distribution we used the shortest geographic distances among populations along the shore instead of straight-line distances. The

significance of the Mantel test was determined by n = 10,000 permutations using the IBD web service v3.15 (http://ibdws.sdsu.edu; Jensen et al., 2005). Genetic structure within population demes as defined by SAMOVA was further explored using the Mantel test as explained above. The program MDIV was used to distinguish between (1) a scenario of past isolation with no subsequent migration from (2) a scenario with no isolation, but contemporary gene flow or secondary contact among populations using MDIV@BioHPC (http://cbsuapps.tc.cornell.edu/mdiv.aspx; Nielsen and Wakeley, 2001). We ran MDIV under the HKY model, each simulation 6 x10^6 times and assumed a 10% burn-in period with priors for Max T = 5 – 10, and Max M = 5 – 12. Estimates of θ, M and T were taken from the highest posterior probability. The net divergence D_A (Nei, 1987), the between population distance minus the within population distance, was calculated using the Tamura and Nei model in MEGA v4.0 (Tamura et al., 2007). To assess the long-term stability of *An. albimanus* we used the mismatch distribution and the raggedness (r) statistic (Rogers and Harpending, 1992), both calculated in ARLEQUIN v3.11 (Excoffier et al., 2005). Neutrality tests of Fu's F_S (1997) and R_2 (Ramos-Onsins and Rosas, 2002) were obtained in DNASP v4.50.02 (Rozas et al., 2003), and 10,000 coalescence simulations assessed significance. Dates of population expansion were estimated with the formula $T = \tau/2u$ (Rogers and Harpending, 1992) using 10 generations per year (Walton et al., 2000) and the mutation rate of *Drosophila* estimated at 1.2 x10^{-8} substitutions per site per year (Powell et al., 1986). We examined changes in the effective population size through time implementing the Bayesian skyline plot

(BSKP). The software BEAST v.1.4.2. (Drummond and Rambaut, 2007) was used to generate the BSKP with the SRD06 model of sequence evolution as suggested when analyzing mitochondrial protein coding genes. The analysis was implemented under a relaxed clock with rate for each branch drawn from a log normal distribution. The Markov Chain Monte Carlo algorithm was iterated for 10 x 10^7generations with a burn-in of 2 x10^5 generations. We assessed the genealogical relationship among *COI* haplotypes using the statistical parsimony (SP), and the median-joining (MJ) network methods. These analyses were implemented in the programs TCS v1.12 (Clement et al., 2000) and NETWORK v4.2.0.1 (http://www.fluxus-engineering; Bandelt et al., 1999), respectively, and *Anopheles triannulatus*, in the same sub-genera *Nyssorhynchus*, was used as the outgroup in the MJ network to provide directionality.

White

For the *white* gene data set we computed the SP network (Clement et al., 2000), the neutrality test Fu's F_S (1997), the mismatch distribution (Rogers and Harpending, 1992) and the BSKP (Drummond and Rambaut, 2007). We used the *Drosophila* substitution rate of 0.004 – 0.008 per site million years, 10 generations per year and 759bp of the *white* gene to calculate time of population growth or decline in the BSKP.

6.4. Results

Neutral expectation and genetic diversity

In total, 191 *COI* hapotypes and eighteen *white* alleles were recovered in this study (Table 6.1.; Fig. 6.1.). Sequence variation in the *COI* was considerably

higher than in the *white* gene. The average number of nucleotide differences among sequences was 11.23 in the *COI* and 0.66 for the w*hite* gene (Table 6.1.). Both markers conformed to neutral expectations, as estimates of Tajima's *D* were not significantly different from zero (Tajima's *D*, *COI* = - 1.38, $P > 0.05$ and *white* *D* = - 0.59, $P > 0.05$). Two short insertions were found in the ITS2 region of *An. albimanus*. These insertions were located in positions 281-283 (ATG) and 291-292 (AG), respectively and were found in 9 (5.2%) of 173 individuals. Furthermore, one base pair ambiguity in position 234 (e.g., multiple copies of the ITS2) separated 38 (21.9%) individuals from Nicaragua (4), Costa Rica (13) and Panama (21) from the remaining 135, all of which had a single peak for that nucleotide and were distributed over the entire geographic area (Table 6.1.).

Population divergence and historical demography

In agreement with previous studies the *COI* showed considerable population structure in *An. albimanus*. SAMOVA defined three population demes with the highest among group variation at 64.75% (F_{CT} = 0.6475, $P > 0.0001$). These population demes were clearly segregated geographically indicating either the existence of specific barriers to gene flow or separate demographic origins. To further analyze the data set, we assumed the population definition obtained by SAMOVA (see Table 6.1.; Fig. 6.1.). The Mantel analyses to test for IBD among all populations, and within each population deme were all statistically insignificant (all populations, R^2 = 0.0152, P = 0.813; NCRWP, R^2 = 0.0134, P = 0.931; CEPCO, R^2 = 0.0612, P = 0.429; PCOLE, R^2 = 0.0074, P = 0.701), suggesting no association between geographic and genetic distances overall, and

shallow genetic structure within population demes. MDIV analysis supported recent gene flow or secondary contact as a better model to explain the *COI* geographical pattern versus historical isolation without subsequent gene flow among population demes (Fig. 6.2.). The net nucleotide substitution per site between NCRWP and CEPCO (D_A = 0.005 ± 0.002, 95% CI) placed the time of divergence between these population demes around 250,000 (285,000 ± 215,000) years ago in the late Pleistocene. Furthermore, divergence times between NCRWP vs. PCOLE (D_A = 0.017 ± 0.004, 95% CI) and CEPCO vs. PCOLE (D_A = 0.016 ± 0.003, 95% CI) were around 850,000 (975,000 ± 725,000) and 827,000 (952,000 ± 702,000) years ago, respectively, in the middle Pleistocene. Fu's F_S (1997) neutrality test was negative and highly significant in all of the population demes (NCRWP *Fs* = - 24.444, $P < 0.0001$; CEPCO *Fs* = - 25.719, $P < 0.0001$; PCOLE *Fs* = - 24.633, $P < 0.0001$), therefore rejecting the equilibrium assumption in *An. albimanus* and strongly favoring the interpretation of population expansion. Similarly, the R_2 test (Ramos-Onsins and Rosas, 2002) was low, positive and significant in all population demes (NCRWP R_2 = 0.0628, P = 0.0053; CEPCO R_2 = 0.0224, P = 0.0012; PCOLE R_2 = 0.0634, P = 0.0032), thus reinforcing a scenario of population expansion. It is noteworthy that the mismatch distribution for the entire *COI* data statistically fit the model of sudden expansion, but showed three clearly defined peaks reflecting the existence of three main population demes (NCRWP, CEPCO and PCOLE) each of them expanding, albeit at different times (Fig. S6.1. Supplementary data).

The SP and the MJ networks further supported the population divergence defined by SAMOVA, and provided deeper resolution of the geographic structure of *An. albimanus* uncovering four different *COI* haplogroups (Fig. 6.1.; Fig. S6.2. Supplementary data). These four maternal lineages were separated by 6 – 18 mutational steps and segregated among the three geographical regions defined by SAMOVA. Haplogroups (B) and (C) co-occurred and were more prevalent in NCRWP; (A) was prevalent in CEPCO, and (D) was largely predominant in PCOLE (Fig. 1). The net nucleotide substitution per site among these haplogroups (D_A = 0.008 – 0.026) placed the time of divergence between them around (1,300,000–400,000) years ago, in the middle and late Pleistocene. We therefore re-calculated the mismatch and neutrality tests for each haplogroup separately, because the nucleotide diversity and the coalescence signal of the mismatch distribution are likely to become inflated (e.g., Type II error) when divergent haplotype groups come into secondary contact. Both the Fu's F_S and the R_2 tests rejected the equilibrium assumption in all the haplogroups, likely due to population expansion, and this was corroborated by the non-significant raggedness indexes and unimodal mismatch distributions (Fig. S6.3. Supplementary data). The expansion times for A (τ = 3.658), B (τ = 5.132), C (τ = 2.929) and D (τ = 4.765) were 19,641 (95% CI 9,324 – 39,641), 27,555 (95% CI 14,177 – 47,533), 15,727 (95% CI 6,324 – 35,641) and 25,585 (95% CI 10,077 – 46,533), respectively, all in the late Pleistocene. The BSKPs further supported significant population growth and showed an increase in effective population size

in each of these four *COI* haplogroups, all within 150,000 – 550,000 years before the present (Fig. 6.3.).

In contrast to the *COI* gene, genetic diversity was lower for the *white* gene. Eighteen alleles were joined by 1 – 3 nucleotide differences in a SP network (Fig. 6.1.). These alleles were very closely related and not segregated geographically as were the four *COI* haplogroups. Instead, one ancestral allele (I) was present in all the sample locations, but more frequently in localities across NCRWP, and CEPCO, and almost fixed for those in Costa Rica and Panama. Other three alleles, of intermediate-frequency (II, III, and IV), were shared among regions whereas the rest were less frequent and more restricted geographically (Table 6.1.; Fig. 6.1.). It is noteworthy that seven out of ten *white* gene singletons were recovered exclusively from CEPCO, and this may suggest a recent pattern of population growth across this region (Fig. 1). Furthermore, Fu's F_S (1997) neutrality test was negative and significant (Fs = - 7.65, $P < 0.0001$) and the mismatch distribution fit the model of sudden population expansion (Fig. S6.3. Supplementary data). The expansion time for the w*hite* alleles based on $\tau = 2.55$ from the mismatch distribution, was between 20,586 – 41,996 years ago, in the late Pleistocene. Nevertheless, the BSKP for the nuclear marker depicted a recent decrease in the effective population size of *An. albimanus* around 50,000 years before the present, although it was not statistically significant (Fig. 6.3.).

6.5. Discussion

The present study provides further evidence that late Pleistocene environmental changes had a profound effect on the demography and regional

geographic structure of *An. albimanus* in the northern Neotropics. The *COI* findings appear to be the result of historical fragmentation leading to four *COI* haplogroups that were geographically isolated during the middle Pleistocene, but reconnected across southern Central America via more recent secondary contact and a common late Pleistocene expansion event. Although the mismatch distributions for NCRWP, CEPCO and PCOLE were not statistically different from a model of sudden population expansion, they were all visually bimodal, perhaps reflecting the co-occurrence of more than one haplogroup per region (i.e., B and C in NCRWP) or the admixing of haplogroups among different regions, and this trend agrees with the pattern of secondary contact estimated by MDIV. In addition, MDIV estimated higher secondary contact between NCRWP and CEPCO, and this is supported by more haplotype sharing between these two population demes, as well as less divergence between haplogroups (A), (B) and (C) (6 – 13 mutations) than between (A, B, C) and (D) (18 – 25 mutations). Clearly, this pattern reflects the fact that haplogroup D is the most geographically restricted because none of its haplotypes were encountered outside PCOLE (Fig. 6.1.). Alternatively, the *COI* divergence may be due to directional selection in the mitochondrial genome, for instance, an advantageous mutation sweeping to fixation across southern Central America. However, selection is generally a locus specific force, whereas demographic changes affect the entire genome. The latter is more consistent with the evidence of population expansion in both the *COI* and *white* genes. Furthermore, all the *COI* haplogroups showed an excess of low frequency haplotypes indicating a common pattern of recent population growth

regardless of their geographical distributions. This would make a selective sweep unlikely because the same advantageous mutation would have to appear independently in each of NCRWP, CEPCO and PCOLE. On the other hand, two short insertions, found in the ITS2 of *An. albimanus*, may support the presence of unidentified cryptic taxa; however, very few individuals harbored these indels, and only one of these insertions was found exclusively in PCOLE (Table 6.1.). Beside these indels, no fixed substitutional changes or length differences were encountered in the ITS2 sequences of 52, 59, and 52 individuals from NCRWP, CEPCO and PCOLE, respectively. Based on our ITS2 analysis, we cannot reject substantial historical gene flow among populations of *An. albimanus*. The restricted occurrence of intragenomic variability in the ITS2 of samples from NCRWP and CEPCO, and an exclusive insertion in three samples from PCOLE, may suggest past geographical fragmentation as proposed for the *COI* data (Bower et al., 2008).

Anopheles albimanus is believed to have originated in the Caribbean islands and then colonized the American continent, and a founder effect may have been a main factor shaping its continental population structure (Molina-Cruz et al., 2004). The colonization routes suggested by these authors hypothesize that *An. albimanus* invaded South America from northern Central America. In the present analysis, *Anopheles triannulatus* joined through 52 mutations to haplogroup B in the *COI* MJ network (Fig. 6.1.) indicating that haplogroups (C) and (A) originated from (B), and then (D) originated from (A). This pattern is consistent with the scenario suggested by Molina-Cruz et al. (2004). The co-

occurrence of two distinct *COI* haplogroups (B and C) in NCRWP supports this view and indicates that successful initial colonization of South America was achieved by two separate introductions of *An. albimanus* from Nicaragua into Colombia and Ecuador. Nevertheless, genetic diversity as measured by both the *COI* and *white* genes does not decrease progressively from north to south, as would be expected under a founder effect and sequential bottleneck in the colonizing front. Instead genetic diversity is lower in CEPCO than in NCRWP and PCOLE (Table 6.1.). Coalescent theory predicts that the most frequent and widely distributed gene variants are ancestral, because they have had more time to disperse. In our data, both of these variants, the *COI* haplotype (A1) and the *white* allele (I), were prevalent across Panama, implying a possible Panamanian geographical origin for *An. albimanus* (Fig. 6.1.; Fig. S6.2. Supplementary data). This view is unlikely though given the recent geological origin of the Isthmus of Panama, dated approximately 3 – 3.5 mya (Bermingham and Martin, 1998). Due to the non-equilibrium frequencies of variants in both markers and our incomplete sampling of the geographic range of *An. albimanus*, we do not have enough evidence to draw firm conclusions about its geographical origin and initial colonization path across southern Central America.

The observed geographic structure seems to be the result of population contraction of an ancestral and perhaps stable population of *An. albimanus* across the Isthmus of Panama (i.e., the four *COI* haplogroups diverged and expanded within the same time frame). This view is supported by lower diversity in the *COI*, *white* gene and the ITS2 marker across Panama (CEPCO), which may

indicate that *An. albimanus* went through a severe population bottleneck followed by a subsequent demographic expansion toward Nicaragua and Ecuador (Table 6.1.). Previous work using four microsatellites loci and the mtDNA *ND5* gene also indicated significantly reduced diversity for one locality in central Panama, suggesting genome wide trends (Molina-Cruz et al., 2004). Our results from two independent molecular markers may represent two temporal pictures of the demography of *An. albimanus* in the study area, with the *white* gene showing a bottleneck and the *COI* depicting a subsequent demographic expansion, and this may be caused by different mutation rate, and effective population sizes in these genes. The bottleneck hypothesis is supported by a drop of 35% precipitation and changes in vegetation and faunal structure in central - eastern Panama in the late Pleistocene (Piperno and Jones, 2003; Gonzales et al., 2006). Additional signals of Pleistocene geographic fragmentation across Panama have been demonstrated in howler monkeys, black mangroves and dirt frogs, and hypothesized to be caused by forest fragmentation, sea level changes, and climatic refugia, respectively (Crawford, 2003; Cortes-Oritz et al., 2003; Nettel et al., 2008).

A common population history for An. albimanus and An. darlingi in the Neotropics

Another malaria vector, *An. darlingi*, has also undergone population expansion in South America within the same time frame as *An. albimanus* (Mirabello and Conn, 2006a,b). Both species show evidence of late Pleistocene geographic fragmentation and subsequent re-colonization through population expansion, although in somewhat different fashions. *Anopheles darlingi* was

hypothesized to follow a refugia - expansion - refugia trend, across South America in response to forest contraction and expansion. In contrast, the expansion of *An. albimanus* seems to have little to do with changes in forest structure, but more with climatic oscillations, sea level changes and an expansion path along the coastline. Studies in other insect taxa have demonstrated that different dispersal rates induced by habitat type and stability (e.g., temporal persistence and spatial heterogeneity of habitat) may explain the phylogeographic pattern among co-distributed lineages. Those authors predicted that stable habitats persist for longer geological time promoting less dispersal and therefore higher phylogeographic structure, genetic divergence, and speciation rate, whereas short-lived and temporally unstable habitats would enhance gene flow, thus resulting in a shallow genealogy (Papadopoulou et al., 2009). In this respect, *An. albimanus* is quite distinct from *An. darlingi*, as the latter is adapted to live in more stable larval habitats, primarily along warm lowland rivers, whereas the mainly coastal distribution and short-lived and temporal sunlit habitats of *An. albimanus* make it more likely to be affected by sea level changes and climatic oscillation than by forest fragmentation. Nevertheless, *An. albimanus* and *An. darlingi* are closely related species that belong to the same subgenus *Nyssorhynchus*, and therefore it is probable that they have responded similarly to a common Pleistocene event. Because the occurrence of late Pleistocene forest fragmentation in northern Colombia and South America has not been well supported in recent studies (Mayle et al., 2004; Gonzales et al., 2006), we argue here that the population expansion of these two malaria vectors is the result of Neotropical climatic

changes, mostly in temperature and precipitation, that might have affected larval habitat availability during the late Pleistocene. It is noteworthy that the geographic fragmentation of *An. albimanus* described in this study coincides to some extent with the geographic discontinuity of *An. darlingi* in Central America (Mirabello and Conn, 2006a). This may indicate that *An. darlingi* and other *Anopheles* species went through the same history of population contraction due to late Pleistocene climatic changes in central - eastern Panama, with *An. albimanus*, but not *An. darlingi* able to survive and successfully re-colonize this region. However, without additional evidence this view remains a working hypothesis.

6.6. Conclusions

Anopheles albimanus is a single, albeit polymorphic, species that is not at equilibrium due to past geographic fragmentation and regional fluctuation in its effective population size. There is a strong geographic component in its genetic structure with three population demes and an admixture zone across eastern Panama and northern Colombia. The *COI* gene suggests a common pattern of historical isolation, subsequent haplotype mixing, and population expansion of four mtDNA *COI* lineages in the late Pleistocene. Data from the *white* gene does not reflect the high genetic diversity of the *COI*, but it is consistent with the scenario of late Pleistocene population expansion, thus supporting demographic phenomena as the cause of structure rather than a selective sweep. Furthermore, lower genetic diversity by both the *COI* and *white* genes across Panama suggests that *An. albimanus* underwent population contraction followed by a subsequent range expansion toward Nicaragua and Ecuador. Finally, despite having different

primary breeding sites and host feeding behaviour, *An. albimanus* and *An. darlingi* responded similarly to Pleistocene environmental changes, suggesting that ongoing climate change will play a key role in vector distribution and perhaps future malaria transmission patterns in the Neotropics.

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Table 6.1. Summary of diversity measures for *Anopheles albimanus*

Gene marker	N	No. of *COI* haplotypes	*K*	$H_d \pm SD$	$\pi \pm SD$
COI	612	191 (131)	11.23	0.91 ± 0.008	0.014 ± 0.0003
NCRWP	135	55 (30)	6.76	0.95 ± 0.011	0.008 ± 0.0003
CEPCO	309	93 (65)	3.41	0.79 ± 0.024	0.004 ± 0.0004
PCOLE	168	53 (36)	7.42	0.94 ± 0.009	0.009 ± 0.0005

Gene marker	N	*white* alleles	*K*	$H_d \pm SD$	$\pi \pm SD$
White	175	I - XVIII	0.66	0.50 ± 0.001	0.0008 ± 0.0005
NCRWP	56	**I**, **II**, **III**, **IV**, **V**, XIII, XVIII	0.57	0.39 ± 0.001	0.0007 ± 0.0001
CEPCO	61	**I**, **II**, **III,** X, XI, XII, **XIII**, XIV, XV, XVI, XVII,	0.12	0.12 ± 0.003	0.0001 ± 0.0002
PCOLE	58	**I**, **II**, **III**, **IV**, VI, VII, VIII, IX	1.06	0.73 ± 0.004	0.0014 ± 0.0002

Gene marker	N	ITS2 variants	*K*	$H_d \pm SD$	$\pi \pm SD$
ITS2	173	a, b	0.52	0.54 ± 0.00	0.0011 ± 0.00
NCRWP	56	**a** - *2;* <u>17</u>	0.42	0.33 ± 0.00	0.0014 ± 0.00
CEPCO	61	**a** - *2*; <u>21</u>	0.11	0.11 ± 0.00	0.0003 ± 0.00
PCOLE	56	**a** - *2*, b - *3;* <u>0</u>	0.03	0.04 ± 0.00	0.0001 ± 0.00

N = number of individuals of *An. albimanus* sequenced per gene marker and population deme as define by SAMOVA (see Fig. 1). The number in parentheses indicates the number of *COI* singletons. Bold letters are shared *white* alleles or ITS2 variants and plain letters are unique in that population deme. (a) and (b) are insertions in positions 281-283 (ATG) and 291-292 (AG) of the ITS2; these are followed by their frequencies shown by the number in italics. Underlined numbers are the number of individuals with multiple gene copies of the ITS2. *K* is the average number of nucleotide differences among sequences. H_d, haplotype diversity ± standard deviation; π, nucleotide diversity ± standard deviation.

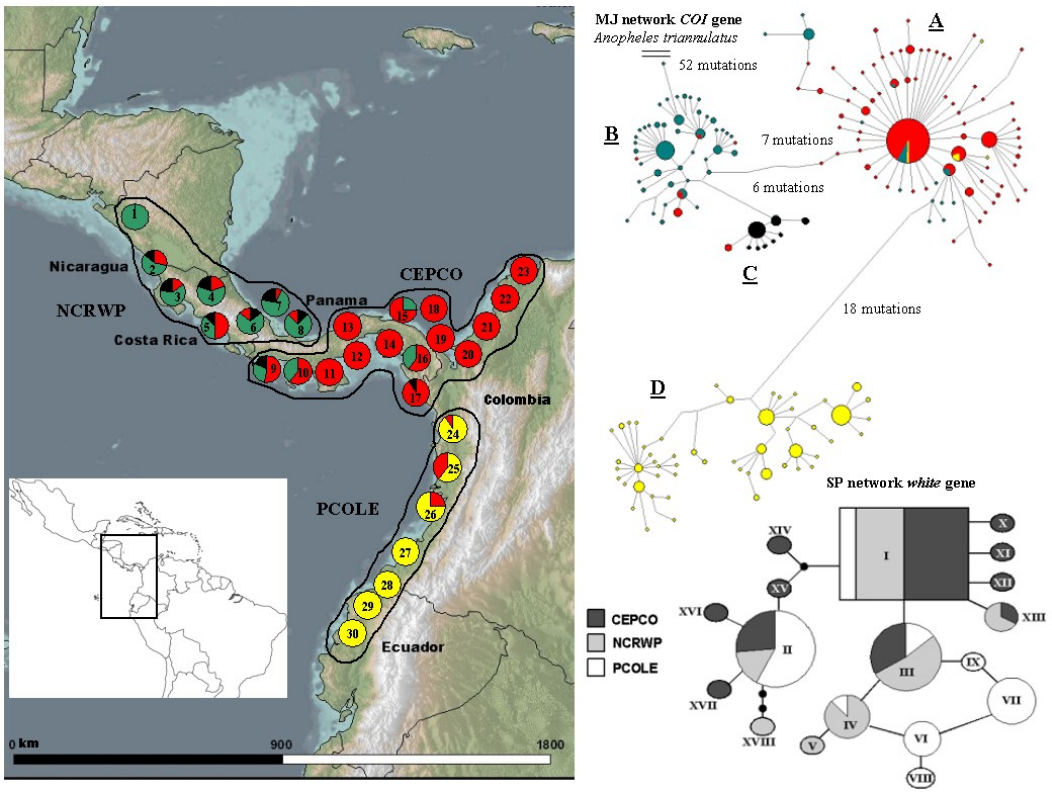


Figure 6.1. The map shows the geographic distribution of four *COI* haplogroups (A, red), (B, green), (C, black) and (D, yellow) from the MJ haplotype network of *Anopheles albimanus*, located in the upper right corner. The circled numbers correspond to 30 localities, positioned on the map according to the longitude and latitude of each site. Three population demes defined by SAMOVA are outlined: Nicaragua, Costa Rica and the Atlantic coast of western Panama (NCRWP = localities 1– 8); the Pacific coast of western Panama, central – eastern Panama and the Caribbean coast of Colombia (CEPCO = localities 9 – 23); and the Pacific coast of Colombia and Ecuador (PCOLE = localities 24 – 30). The statistical parsimony network of nine *white* gene alleles (I – XVIII) and their geographical distributions are shown in the lower right corner. The inset map depicts the geographic position of the study area.

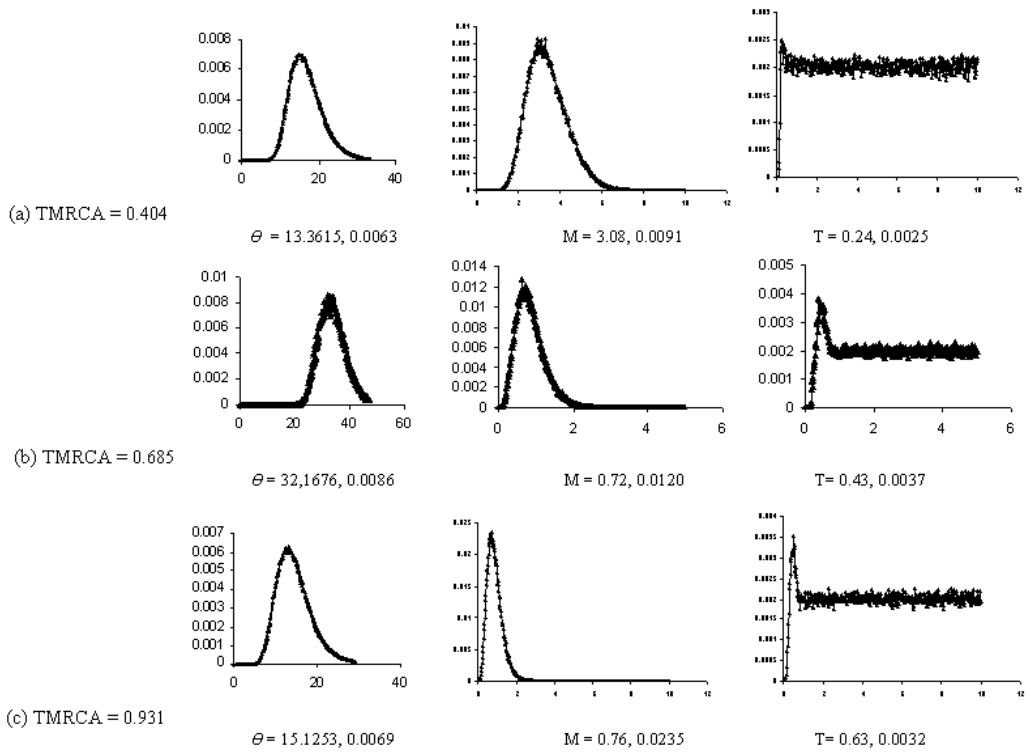


Figure 6.2. Pairwise estimate of migration rates and divergence times among population demes using MDIV analysis: (a) NCRWP vs. CEPCO; (b) CEPCO vs. PCOLE and (c) NCRWP vs. PCOLE. (TMRCA) is the time to the most recent common ancestor, (Θ) is the scaled parameter for the effective population size between population demes, (M) represents values of migration rates and (T) is the scaled parameter for divergence times between pair of population demes.

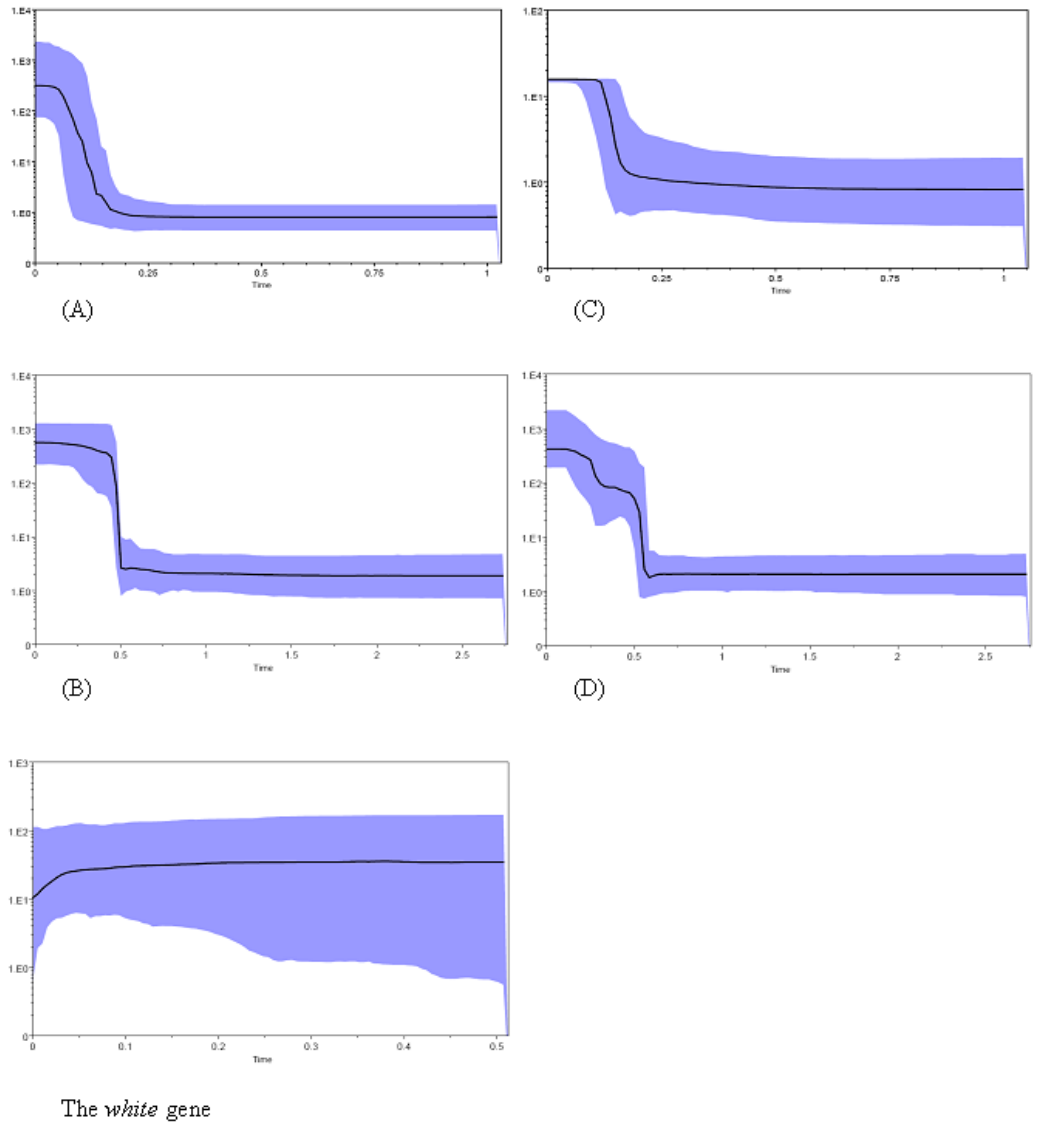
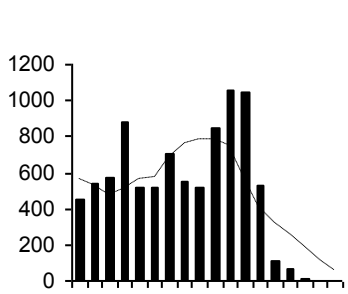
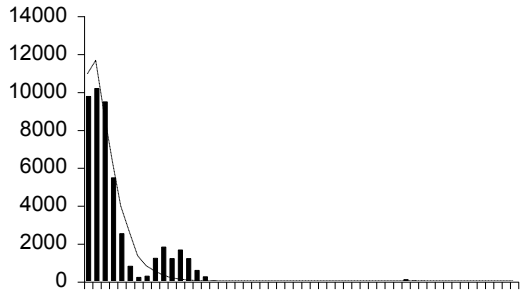
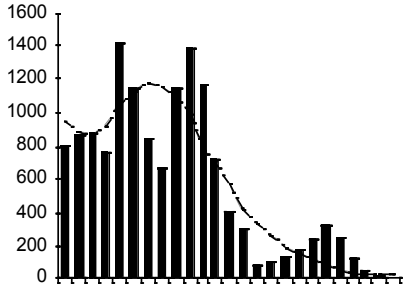


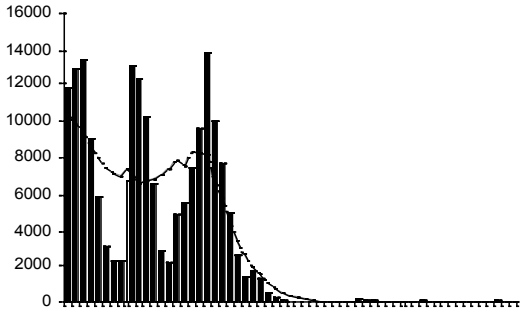
Figure 6.3. Bayesian Skyline Plots (BSKPs) for the four *COI* haplogroups (A), (B), (C) and (D) and the *white* gene alleles. (*x*) axis: time in 10^6 years before present (substitution/sites/my), divided by 10 generations; (*y*) axis: estimated population size [units = N_em the product of the effective female population size and the mutation rate (log transformed)]. The shaded area represents the upper and lower 95% credible intervals.





(PCOLE); $r = 0.006$, $P = 0.90$ (CEPCO); $r = 0.01$, $P = 0.98$





(NCRWP); $r = 0.01$, $P = 0.66$ Total *COI* data set; $r = 0.005$, $P = 0.83$

Fig. S6.1. Supplementary data: The mismatch distribution for the population demes defined by SAMOVA. The Pacific coast of Colombia and Ecuador (PCOLE); Central-eastern Panama and the Caribbean coast of Colombia (CEPCO); Nicaragua, Costa Rica and western Panama (NCRWP); and the total data set. The *x* axes show the nucleotide differences between pairs of sequences, and the *y* axes depict the frequency of each sequence in the sample. The (*r*) = the raggedness index and the associated *P* values.

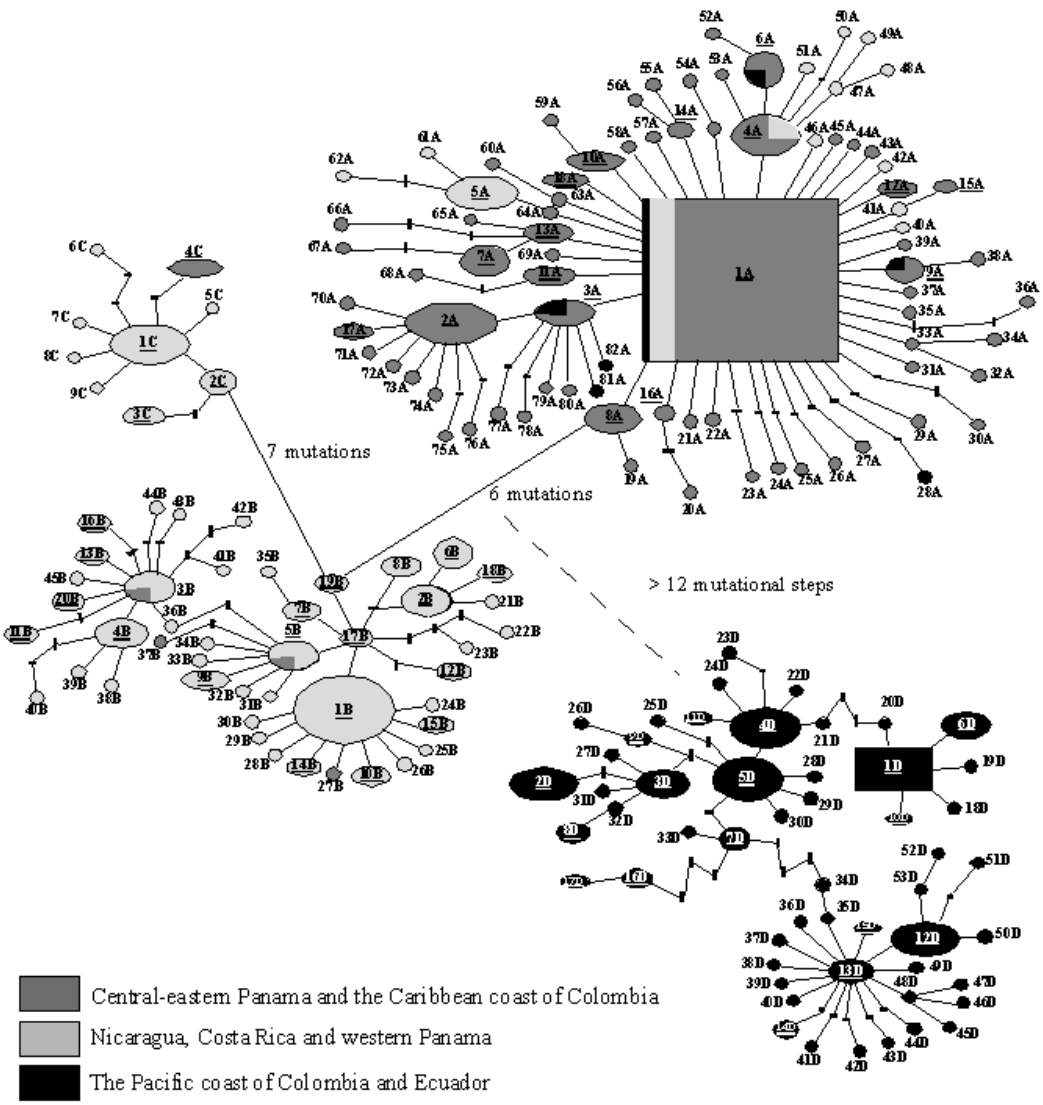
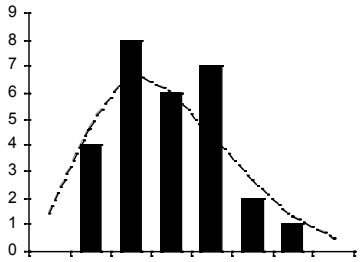
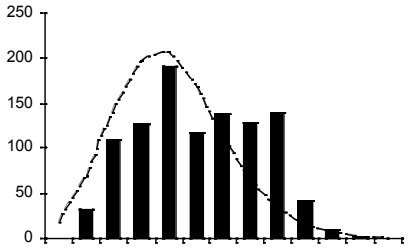
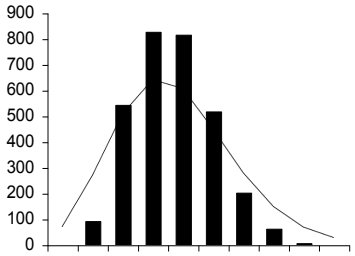


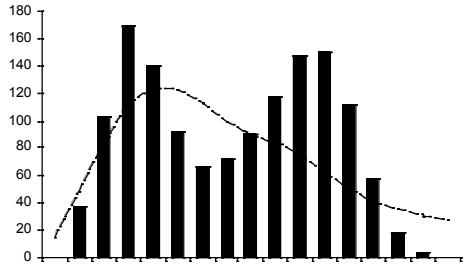
Fig. S6.2. Supplementary data: Statistical parsimony network of 191 *COI* haplotypes of *Anopheles albimanus*.



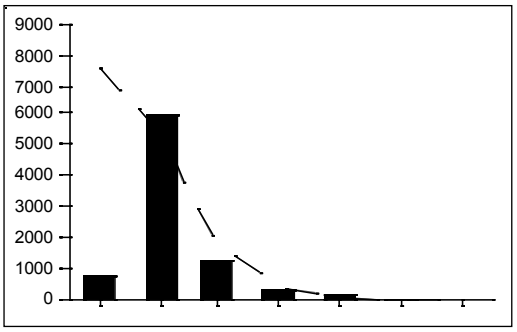


Haplogroup (A); r = 0.081, P = 0.56 (B); r = 0.026, P = 0.29





(C); r = 0.053, P = 0.11 (D); r = 0.011, P = 0.34



The *white* gene; r = 0.023, P = 0.21

Fig. S6.3. Supplementary data: The mismatch distribution for the haplogroups (A, B, C, and D) and the *white* gene alleles defined by the SP and MJ networks. The *x* axes show the nucleotide differences between pairs of sequences, and the *y* axes depict the frequency of each sequence in the sample. The (*r*) = the raggedness index and the associated *P* values.

Connecting statement IV

Anopheles (Anopheles) punctimacula s.l. has been hypothesized to be a secondary malaria vector in Panama and in other parts of Central America. This species is found from Mexico to Argentina, and also in the Caribbean Islands. Unlike *Anopheles (Nyssorhynchus) albimanus*, which is straightforward to identify morphologically with standard systematics keys, *An. punctimacula* s.l. can be easily confused with other morphologically similar species within the Arribalzagia Series of *Anopheles*. Furthermore, more recently *An. punctimacula* s.l. was the subject of a morphological revision resulting in the re-description of two closely related cryptic species, *An. (Anopheles) malefactor* and *An. (Anopheles) calderoni.* Both *An. punctimacula* s.l. and *An. malefactor* have been collected biting humans and coexisting in the same larval breeding sites in Panama, but *An. calderoni* has never been reported from this country. Moreover, substantial variability in egg, larvae and adult morphology has been found in samples under the categorical name of *An. punctimacula* s.l., and several authors have found ecological evidence for the existence of a species complex. It may be possible that *An. punctimacula* s.l. (in the past Syn. *An. malefactor* and *An. calderoni*) consists of more than three cryptic species, and this could have significant implications for vector control and malaria transmission in Panama. In the following chapter, I use a variable fragment in the three prime (3') end of the *COI* gene to investigate the pattern of phylogenetic, spatial, and demographic genetic structure in *An. punctimacula* s.l. The information from the Folmer region of the *COI* and the ITS2 marker was used to test for cryptic speciation using

samples of *An. punctimacula* s.l. and *An. malefactor* from Panama and Costa Rica.

7. Chapter VII. Molecular taxonomic status of *Anopheles punctimacula* s.l., of the Arribalzagia Series (Root, 1922), in Panama

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7.1. Abstract

Background

Anopheles punctimacula s.l., is a secondary malaria vector in parts of Central America, yet its molecular taxonomic status has never been comprehensively examined. Three isomorphic species have been described in this taxon (*An. punctimacula s.s.*, *An. malefactor* and *An. calderoni*), and evidence for the existence of a species complex has been demonstrated. It is possible that *An. punctimacula* s.l. consists of more than three cryptic species, and this could have significant implications for vector control across the species range, and especially in Panama, where it is believed to be an important regional vector.

Methods

We collected adult female mosquitoes from several localities across Panama and one in Costa Rica. We used DNA sequences from two independent molecular markers, the mitochondrial cytochrome oxidase subunit one gene (*COI*) and the internal transcribed spacer two (ITS2). Initially, we sequenced 310 samples for a more variable fragment of the *COI* (*3'* prime end) to investigate the pattern of phylogenetic structure. Then we used the information from the Folmer region (*5' COI*) and the ITS2 marker to test for cryptic speciation in *An. punctimacula* s.l.

Results

Phylogenetic analyses using the *3' COI* region depicted seven clades in mosquitoes morphologically identified as *An. punctimacula* s.l., all of which were very differentiated from *An. malefactor*. In contrast, analysis with the *5' COI* region demonstrated paraphyly among some of these clades. A Neighbor-joining

tree of ITS2 sequences further supported the same seven clades as the *3′ COI*. One of these clades was mistakenly identified as *An. punctimacula* s.l., but may instead correspond to *An. neomaculipalpus*. Time to the most recent common ancestor among the nodes of these clades, based on the combined *COI* data (*5' COI* and *3' COI)*, was estimated to the early – late Pleistocene.

Conclusion

Conflicting phylogenetic signal between the *5' COI* region and a more variable portion of the *COI* could be due to the unequal mutation rate across this gene. Evidence from the ITS2 and the combined *COI* regions suggest that the *An. punctimacula* Group comprised two geographically isolated lineages (*An. punctimacula s.s.* and clade B). Yet, it is not clear whether these lineages are true species or just divergent populations. We cannot make any firm conclusions about the taxonomic status of clades C, D, E and G. They could be either other unidentified species within the Arribalzagia Series or true genetic diversification within *An. punctimacula* s.l. We report *An. malefactor* for the first time in Costa Rica, but our data don't support the presence of *An. calderoni* in Panama.

7.2. Introduction

Incorrect species identification is a serious issue when dealing with malaria vectors (Diptera: *Anopheles*), because choosing the appropriate mosquito control methods would strictly depend on having accurate knowledge of the species' taxonomic status, ecology and behavior. Traditionally, female "*adult*" morphology has been used to tell *Anopheles* species apart, and this approach has proven highly valuable in some groups. Nevertheless, morphological characters are not always discrete and have been shown to overlook key genetic divergence, which may be related to differences in vectorial capacity and/or insecticide resistance [1]. The increasing number of cryptic species complexes in *Anopheles* mosquitoes also indicates that reliance on morphology alone is likely to continue compromising vector control strategies [2-5].

Two DNA regions have been used to assist with species identification in the genus *Anopheles*: the nuclear internal transcribed spacer two (ITS2) and the Barcoding or Folmer region of the mitochondrial cytochrome oxidase one gene (*COI*) [2, 6]. Both DNA markers have been successfully employed in molecular taxonomy of *Anopheles* species either through phylogenetic or population genetics analysis. The ITS2 is recognized as the gold standard for species identification in most *Anopheles* sibling complexes; length differences and/or fixed substitutional changes between ITS2 sequences are taken as proof of lineage splitting especially if the lineages are geographically co-distributed [2-4, 7]. Similarly, fixed mutations and more than three percent (3%) divergence among *COI* sequences may indicate cession of gene flow and speciation [8, 9]. More

recently, Foley *et al.* [10] suggested that the threshold level to define *Anopheles* species with the Barcode region could be set lower than 3% to minimize false positives. While agreement between the ITS2 and the *COI* should provide a robust demonstration of the species' taxonomic status, discrepancies may provide evidence about different evolutionary processes acting at different genetic levels [4, 5]. Conflicting results between mitochondrial genes and the ITS2 are relatively common in the literature; and this could be caused by unequal mutation rates at different portion of the former, which may disrupt its molecular signal [11, 12].

The Arribalzagia Series (Root, 1922) of the subgenus *Anopheles* comprises 24 recognized species (Table 7.1.). Of these, *Anopheles punctimacula* s.l., (Dyar and Knab) and *An. neomaculipalpus* (Curry), were originally described from Panama, with three other species being reported from the country as follows: *An. apicimacula* (Dyar and Knab), *An. mediopunctatus* (Lutz) and *An. vestitipennis* (Dyar and Knab). In addition, seven other morphologically similar species within this Series have the potential to be in Panama due to their reported geographical distributions (Table 7.1.).

Anopheles punctimacula s.l., ranges from Mexico throughout Argentina [13], and also in the Caribbean Islands [14]. This species was found infected with *Plasmodium* parasites in Panama and corroborated afterwards as a potential malaria vector in laboratory experiments [15-17]. More recently, *An. punctimacula* s.l., has been the subject of morphological revision resulting in the re-description of two closely related cryptic species, *An. malefactor* (Dyar & Knab) and *An. calderoni* (Wilkerson) [18, 19]. Both *An. punctimacula* s.l., and

An. malefactor have been collected biting humans and coexisting in the same larval breeding sites in Panama, but *An. calderoni* has not been reported from this country [18, 20, 21]. Unlike some other Neotropical malaria vectors, which are straightforward to identify morphologically with standard keys, *An. punctimacula* s.l., can be easily confused with *An. malefactor*, or with other morphologically similar species within the Arribalzagia Series [1, 18, 19]. To date substantial variability in egg, larvae and adult morphology has been reported from samples identified as *An. punctimacula* s.l., and several authors have hypothesized ecological evidence for the existence of a species complex [1, 18, 19, 22, 23]. It is possible that *An. punctimacula* s.l., (in the past syn. *An. malefactor* and *An. calderoni*) consists of more than three cryptic species, and this could have significant implications for vector control and malaria transmission across the species range, and especially in Panama, where it is believed to be an important vector [15-17, 20, 21].

Our goal is to decipher the molecular taxonomic status of *An. punctimacula* s.l., using adult females mosquitoes and two independent DNA markers. Initially, we used a more variable fragment in the three prime (3') end of the *COI* gene to investigate the pattern of phylogenetic structure. Then we used the information from the Folmer region (*5′ COI*) and the ITS2 marker to test for cryptic speciation and to corroborate the genetic divergence defined by the *3' COI* sequences. Furthermore, we investigated the presence of *An. calderoni* in Panama, which could have been misidentified as *An. punctimacula* s.l., in samples from poorly studied areas. We hypothesize that *An. punctimacula* s.l., is a species

complex that comprises more than three cryptic species in Panama.

7.3. Materials and Methods

Mosquito source and DNA extraction

Samples were collected from 10 localities across Panama and one from Costa Rica (Table 7.2.). Female *Anopheles* mosquitoes were collected and processed as previously described in Loaiza *et al.* [24]. All the specimens were identified morphologically following the key of Wilkerson and Strickman [25] and another anonymous entomological key from the Instituto Conmemorativo Gorgas de Estudios de la Salud (ICGES), in Panama City. Genomic DNA was extracted from mosquito abdomens using the DNeasy Blood & Tissue extraction kit (Qiagen, Hilden, Germany), following the procedures of the manufacturers, and stored at -80°C until needed. Three hundred and ten mosquitoes, roughly 30 per site, were selected for sequencing of the three prime (3') variable region of the *COI* gene (hereafter the *3' COI*). Upon initial analysis a subset of individuals representing the most likely genetic divergence (*i.e.,* groups of populations, haplotypes or phylogenetic clades defined with the 3' *COI* sequences) was then sequenced for the Folmer region (hereafter the *5' COI*) and the ITS2 marker.

PCR amplification

The mitochondrial cytochrome oxidase one (COI)

The *3' COI* region was amplified following the protocol and thermocycler conditions described in Mirabello and Conn [26]. A 1300 bp fragment of the *COI* gene was amplified using the forward UEA3 and reverse primer UEA10 [27].

Individual PCR reactions were preformed using Ready-To-Go-PCR beads (Amersham Pharmacia/Biotech, NJ, USA). The amplification of 648 bp of the *5' COI* region was made using the primer pair LCO 1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G- 3') and HCO 2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') [28]. Samples were amplified using the following thermocycler parameters: 95°C for 5 minutes, then 34 cycles of 95°C for 30 seconds, 48°C for 30 minutes and 72°C for 45 seconds, followed by 72°C for 5 minutes and a 10°C hold. All amplification products were purified with the Exo-SAP-IT kit (Amersham Biosciences) and sequenced directly either on a PTC 100 (MJ Research, Inc.) or on a 200 series thermal cyclers (Biorad, Inc.).

Nuclear internal transcribed spacer two (ITS2)

Amplification of the ITS2 region was achieved using the 5.8SF (5'-TGTGAACTGCAGGACACATG-3') and 28SR (5'-ATGCTTAAATTTAGGG GGTAGTC-3') primers listed in Collins and Paskewitz [29]. Each PCR contained 5 µl of 10x NH_4 BioLine Buffer, 5 µl of dNTPs (2 mM), 5 µl of each primer (5 µM), 2.5 µl of MgCL (25 mM), 0.1 µl of Taq polymerase (BioLine) and 2 µl of DNA template. The mixture was made up to a total volume of 50 µl by adding ddH_20. The PCR products were amplified using the following thermocycler parameters: 94°C for 2 minutes, then 35 cycles of 94°C for 30 seconds, 57°C for 1 minute and 72°C for 30 seconds, followed by 72°C for 10 minutes and a 10°C hold [30].

Sequence processing

The Applied Genomics Technology Core (Wadsworth Center) carried out the sequencing of the *3' COI* region using an ABI 3700 DNA automated sequencer. The *5' COI* and the ITS2 sequences were obtained from the Sequencing Facility of the Natural History Museum, in London, using a Big Dye® Terminator kit (PE applied Biosystems, Warrington, England). Forward and reverse sequences were edited using Sequencher™ version 4.8 (Genes Codes Corporation, Ann Arbor, MI). Complete consensus sequences for both the *3' COI* and the *5' COI* regions were aligned using the ClustalW application in Bioedit v 7.0 [31]. The alignment of the ITS2 sequences was done manually in MEGA v 4.0 [32]. Unique sequences were deposited in GenBank using new features of Bankit (http://www.ncbi.nlm.nih.gov/WebSub/).

Phylogenetic structure and sample identity

Samples of *An. punctimacula* s.l. were also molecularly identified by sequence comparisons using FASTA searches (http://blact.ncbi.njm.nih.gov/) to those *COI* and ITS2 sequences available in GenBank, most of which were extracted from Sallum *et al*. 2002 [33], Marrelli *et al.* 2005 [2] and Brelsfoard *et al.* 2006 [34]. We also compared our sequences with the *5' COI* sequences of other members of the Arribalzagia Series from the public records of the Mosquito Barcoding Initiative (MBI) section of Barcode of Life Data Systems v. 2.5 (BOLD; www.boldssystems.org) (unpublished data). Two of these sequences belong to *An. calderoni* from Colombia and Ecuador, considered one of the cryptic species previously included in *An. punctimacula* s.l. In addition, we sequenced both the *5' COI* and the ITS2 regions of other species that coexist with

An. punctimacula s.l., in Panama, but have currently no sequences deposited in either GenBank or in the MBI data sets (*i.e., An. neomaculipalpus*, *An. apicimacula*, and *An. vestitipennis*). Also, samples of *An. malefactor* were sequenced for the same *3' COI*, *5' COI* and ITS2 fragments, and this species was used as the outgroup in all phylogenetic analysis due to its close systematic relationship and overlapping distribution with *An. punctimacula* s.l., in Panama [18].

Maximum parsimony (MP) trees were conducted using the *3' COI* sequences of *An. punctimacula* s.l., (287 specimens) and *An. malefactor* (18). One hundred replicates of a heuristic search were performed with an initial random stepwise addition of sequences and TBR branch swapping. Bootstrap values were calculated with 1,000 replicates using PAUP v 4.0b10 [35]. Bayesian inference (BI) was performed in MrBayes v 3.1.1. [36], partitioned by codon position, and using the model of nucleotide substitution calculated with jModelTest [37]. The settings were two simultaneous runs of the Markov Chain Monte Carlo (MCMC) for 15 million generations, sampling every 1000 and discarding the first 25% as burn-in. MP and BI trees were also constructed for a subset of samples using only the *5' COI* region and also with the combined *COI* regions (*5' COI* and *3' COI*) (*i.e.,* total evidence approach). In addition, we performed Neighbor-joining (NJ) analyses using the ITS2 sequences from the same subset of samples as for the *5' COI*. NJ trees were unrooted with all characters equally weighted, treated as unordered, and calculated in MEGA v 4.0 [32]. The subset of samples for the

ITS2 and *5' COI* regions was randomly selected from the *3' COI* clades initially defined in the MP and BI trees.

Time to the most recent common ancestor (TMRCA)

A Bayesian Markov Chain Monte Carlo approach, (MCMC) available in the software BEAST v.1.4.6 [38] was used to infer the time to the most recent common ancestor (TMRCA) among the nodes of the clades obtained with the combined *COI* regions. We applied an uncorrelated relaxed lognormal clock and the SRD06 model for the partition as suggested when working with protein coding genes [39]. The partition was run with the model of nucleotide substitution estimated for both the *5' COI* and the *3' COI* separately, and also together, calculated in jModelTest [37]. We used a uniform tree prior and input the substitution rate of *Drosophila* estimated at 0.0115 per site per million years [40]. Final analyses consisted of four separate MCMC runs of 40 million generations sampled every 1000 generations. Tracer v1.4 software (available at http://beast.bio.ed.ac.uk/Tracer) was used to confirm the adequate mixing of the MCMC chains upon independent runs. We used LogCombiner v1.4.7 to merge separate runs into one file and FigTree v1.2.1 for visualization of branch topologies. We used ten generations per year to calculate the ages of the nodes in the topology (*i.e.,* TMRCA for all the haplotypes included in that node) as suggested when studying tropical *Anopheles* mosquitoes [41].

7.4. Results and Discussion

Two hundred and forty nine (88%) of the samples initially identified as *An. punctimacula* s.l., based on morphological characters in Wilkerson and

Strickman [25] matched with a 100% similarity the *COI* sequence of *An. punctimacula* s.l., from Nicaragua (accession number [AF417719]), reported in Sallum *et al.* [33]). These sequences also matched the ITS2 of *An. punctimacula* s.l., from Nechi, Colombia (MBI data set). We refer to these sequences hereafter as the *An. punctimacula* Group. On the other hand, 41 samples (14%) had less than 40% similarity with these sequences. This could mean either that a substantial portion of our samples have been mistakenly identified as *An. punctimacula* s.l., and may belong to other species within the Arribalzagia Series, or that divergence actually corresponds to unidentified cryptic taxa within *An. punctimacula* sensu lato.

Both the MP and the BI trees using the *3' COI* haplotypes and the combined (*5' COI* plus *3' COI*) haplotypes depicted the same topology, and therefore we only present the results from the BI majority-rule consensus tree of the combined data. Seven distinct and highly supported phylogenetic clades (A-G) were clearly differentiated from samples of *An. malefactor* (Figure 7.1.). These clades have no shared haplotypes and some of them appear to occur mostly in different geographic areas. In contrast, the *5' COI* region did not support the *3' COI* divergence and grouped the sequences in six different clades. Although the *5' COI* clades were also strongly supported, some of them included mixed individuals from clades A, B, C and D, thus depicting paraphyly (Figure 7.2.). This suggests conflicting results between these two regions, which could be due to the unequal mutation rates across the *COI* gene. Sequence divergences among these clades were not calculated given the uncertain molecular signal of the

Folmer region. In addition, an unrooted NJ tree constructed with ITS2 sequences and the same subset of samples used in the *5' COI* analysis, strongly supported the same seven clades (Figure 7.3.). All of these clades had either length differences and/or fixed substitutions among their ITS2 sequences.

Comparison of the sequences generated in this study with others available in GenBank and in the MBI data sets revealed conflicting results. For example, all our samples belonging to clade D matched the *5' COI* sequence of *An. apicimacula* from the MBI data sets with 97% sequence similarity. Nevertheless, the ITS2 fragments of the same group of sequences differ in length from another sample of *An. apicimacula* identified from Panama. The latter cluster instead with those sequences in clade C (Figure 7.3.). This may indicate either a mistaken identification for our Panamanian samples of *An. apicimacula* or low resolution in the *5' COI* region to discriminate among different members of the Arribalzagia Series in Panama.

We did not have problems distinguishing *An. malefactor* from the *An. punctimacula* Group (clades A and B). Only three specimens out of 18 *An. malefactor* were mistakenly assigned to the *An. punctimacula* Group. The fact that these two species are more similar morphologically to each other than to any of the other species of Arribalzagia present in Panama, supports the idea that clade D is different from *An. apicimacula*, *An. neomaculipalpus* and *An. vestitipennis,* and has never been identified in Panama. Moreover, the uncertain taxonomic value of the *5' COI* region makes it hard to draw firm conclusions

about species identity based only on this portion of the *COI* gene. The taxonomic status of clade D remains unknown based on the present data set.

Because of the congruency between the combined *COI* regions and the ITS2 marker, we rely more on the taxonomic value of the latter to distinguish among species of the Arribalzagia Series of *Anopheles*. All the ITS2 sequences in clade F had the same length and matched with high similarity the ITS2 sequences of two samples identified as *An. neomaculipalpus* from Panama, and therefore, they have been mistakenly assigned to *An. punctimacula* s.l. The remaining seven sequences representing clades E and G remain unknown and do not match any of the following sequences: the ITS2 of *An. fluminensis* from Bolivia [DQ328638], the *COI* of *An. intermedius* from Brazil [AF417718], the ITS2 of *An. mattogrossensis* from Brazil [AF461754], the ITS2 of *An. mediopunctatus* from Brazil [AF462379], the ITS2 of *An. peryassui* from Brazil [AF461755], and the ITS2 of *An. eiseni* from Brazil [AF462380]. In addition, sequences from clades E and G did not matched any of the *5' COI* sequences from *An. calderoni*, *An. mattogrossensis, An. intermedius, An. costai*, *An. fluminensi* or *An. peryassui* in the MBI data set.

Anopheles punctimacula s.s., *An. malefactor* and *An. calderoni* are closely related isomorphic species that have been morphologically described [18, 19]. The former two species occur in Panama, but *An. calderoni* has not been reported from this country. The recent discovery of *An. calderoni* in Colombia (*Gonzales Ranulfo et al., submitted to Malaria Journal by April 2010* [42]) may suggest that this species occurs in Panama, but could be mistakenly identified as *An.*

punctimacula s.l., due to marked morphological similarities. However none of our clades (C, D, E, F and G), including *An. malefactor*, matched the *5' COI* sequence of *An. calderoni* from Colombia and Ecuador (MBI data sets). Because we do not have samples of *An. calderoni*, it was not possible to obtain its ITS2 sequence, and therefore we cannot make a definitive conclusion about the presence of this species in Panama.

Due to the uncertain taxonomic status of clades C, D, E, and G in the present study we will focuse on discussing the findings within the *An. punctimacula* Group. This group comprises two different clades, both of which were clearly distinct from *An. malefactor*, but closer genetically to this species than to other clades. We will name the members of clade A hereafter as *An. punctimacula s.s.*, because haplotypes from this group were recovered from Gamboa, the type locality. These two clades received high levels of support for the ITS2, *3' COI* alone and the combined *COI* regions (Figures 7.1, 7.2, and 7.3). The phylogenetic structure in the *An. punctimacula* Group appears to be influenced by geography as haplotypes from *An. punctimacula s.s.*, were recovered from across the sampling area, but only in the Pacific coast of western Panama and Costa Rica. In contrast, individuals belonging to clade B were only recovered from the Atlantic coast of western Panama. It is noteworthy that all the clades in the tree appear to have discrete geographic distributions; clade D was found exclusively in the Atlantic coast of western Panama, where it co-occurs with *An. punctimacula* B, and this may suggest significant intraspecific variability in *An. apicimacula* that mimics morphological characters of *An. punctimacula* s.l.,

(*i.e., An. apicimacula* is actually another species complex). In contrast, clades E and G were only recovered from eastern Panama. Moreover, clade C is also subdivided into western and eastern haplotypes.

Sequence analysis of the ITS2 region from *An. punctimacula s.s.*, and clade B showed no differences in length (both were 396 base pairs long), but uncovered four fixed substitutional changes in positions 62, 131, 181, and 330, respectively. This inter-lineage divergence is more consistent with historical, perhaps allopatric fragmentation than with species diversification. Future studies are needed to confirm sympatry without heterozygotes between *An. punctimacula s.s.*, and clade B [43, 44].

Recent studies uncovered considerable genetic structuring in *Anopheles albimanus* (Wiedemann) across southern Central America. Four divergent and not randomly distributed *COI* haplogroups were found between populations from Costa Rica and western Panama and those from central-eastern Panama. The genetic structure of *An. albimanus* was hypothesized to be the result of Pleistocene geographic fragmentation (850,000 - 250,000 years ago) and subsequent secondary contact via demographic expansion (22,000 ya). In general, the estimated 95% confidence intervals for the TMRCA of samples of *An. punctimacula* s.l. were very large, ranging from 1,900,000 to 700,000 ya for the node separating clades A, B, from clades C, D, E; and from 2,100,000 to 800,000 ya for the node separating the latter group from *An. malefactor*, and clades G, F, respectively (Figure 7.4.). All these estimates were during the early and late Pleistocene. Moreover, the node separating *An. punctimacula s.s.*, and clade B

was slightly more recent, around 700,000 and 150,000 ya, but also within the Pleistocene.

These findings may suggest that both *An. albimanus* and *An. punctimacula* s.l., species have gone through the same historical process of Pleistocene geographic fragmentation across the Isthmus of Panama. The fact that the same geographic pattern was found across clades and by different molecular markers strongly indicates that lineage divergence in *An. punctimacula* s.l., has been driven by geographic fragmentation. Yet, at this point it is uncertain whether or not this is due to past unstable demography (*i.e.,* temporal fluctuation in the effective population size) or due to currently restricted gene flow across the study area (*i.e.,* physical barriers to gene flow). More studies using other molecular markers, and a population approach, are required to distinguish between these hypotheses.

Recent studies have confirmed that traditional *Anopheles* identification based on morphological characters frequently fails in representing the true intra-inter specific extent of genetic variability. This may indicate that vector control can be severely compromised in malaria endemic areas where mosquitoes are being identified only morphologically, as it is the case in Panama [20, 21]. The problem of incorrect *Anopheles* species designation may be even more prominent for those taxa with slightly less medical relevance, such as those in the Arribalzagia Series of the Subgenus *Anopheles* [4, 5]. Our results clearly support this view and indicate that more studies need to be undertaken to fully assess the taxonomic status of *An. punctimacula* s.l., in Central America. Furthermore, the

lack of phylogenetic resolution of the *5' COI* region in the present study suggests that caution should be exercised when using this fragment alone to discriminate among poorly studied species. Our results support the use of the combined *COI* regions plus the ITS2 as the most robust evidence of cladogenesis in *An. punctimacula* s.l.

Abbreviations

The abbreviations used in this work were: *COI*, cytochrome oxidase subunit one; ITS2, the internal transcribed spacer two.

Authors' contributions

JRL designed the study, performed most of the molecular and genetic analysis and wrote the initial manuscript. MES, EB, OIS, YL, SB and JEC provided advice on the analysis and assisted in preparing the manuscript. JR and JEC helped to obtain valuable samples of *Anopheles punctimacula* s.l. from various regions of Panama. MES, EB, YL and JEC conceived and supervised the study, and assisted in the writing of the manuscript. All authors read and approved the final manuscript.

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Arribalzagia Series	Authors	Geographic Records
An. anchietai	Correa & Ramalho	Sao Paulo, Brazil.
An. apicimacula *	Dyar & Knab	Belize, Bolivia, Colombia, Costa Rica, Ecuador, El Salvador, Guatemala, Guyana, Honduras, Mexico, Panama, Suriname, Trinidad and Tobago, Venezuela.
An. bustamentei	Galvao	Brazil (Santa Caterina).
An. calderoni	Wilkerson	Peru, Venezuela.
An. costai	Fonseca & Ramos	Brazil, Suriname
An. evandroi	Da Costa Lima	Argentina, Brazil.
An. fluminensis	Root	Argentina, Bolivia, Brazil, Peru.
An. forattinii	Wilkerson & Sallum	Brazil, Colombia, French Guiana, Peru.
An. gabaldoni	Vargas	Guatemala, Mexico.
An. guarao	Anduze & Capdevielle	Venezuela, (Monangas).
An. intermedius	Peryassu	Belize, Brazil, French Guiana, Guatemala, Guyana, Mexico, Peru, Suriname, Trinidad and Tobago.
An. maculipes	Theobald	Argentina, Brazil, French Guiana, Trinidad and Tobago Uruguay.
An. malefactor *	Dyar & Knab	Panama, (Chagres River)
An. mattogrossensis	Lutz & Neiva	Bolivia, Brazil, Colombia, Peru, Venezuela.
An. mediopunctatus *	Lutz	Argentina, Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Panama, Peru, Suriname, Trinidad and Tobago, Venezuela.
An. minor	Da Costa Lima	Argentina, Brazil, French Guiana, Paraguay, Suriname.
*An. neomaculipalpus**	Curry	Argentina, Belize, Brazil, Colombia, Costa Rica, El Salvador, Mexico, Panama, Paraguay, Peru, Trinidad and Tobago, Venezuela.
An. peryassui	Dyar & Knab	Bolivia, Brazil, Colombia, Peru, French Guiana, Guyana, Peru, Suriname, Venezuela.
An. punctimacula *	Dyar & Knab	Argentina, Belize, Bolivia, Brazil, Colombia, Costa Rica, Ecuador, Mexico, Panama, Peru, Uruguay, Suriname, Trinidad and Tobago, Venezuela.
An. rachoui	Galvao	Brazil, (Paraná).
An. shanoni	Davis	Bolivia, Brazil, Guyana, Peru, Suriname.
An. veruslanei	Vargas	Mexico, (Yucatan)
*An. vestitipennis**	Dyar & Knab	Belize, Colombia, Costa Rica, Cuba, Dominican Republic, El Salvador, Guatemala, Haiti, Honduras, Jamaica, Mexico, Nicaragua, Panama, Puerto Rico.

** Species reported from Panama. The type locality by country for each species is underlined.*

Table 7.1. Members of the Arribalzagia Series (Root, 1922).

Locality	Code	Coordinates	Taxa	N
Parrita	PAR	*9°17'N, 85°55'W*	*An. punctimacula* s.l.	34
			An. malefactor	6
Diablo River	DR	*9°10' N, 81°54' W*	*An. punctimacula* s.l.	30
			An. malefactor	1
White Bridge	WB	*9°27' N, 82°37' W*	*An. punctimacula* s.l.	34
			An. vestitipennis	2
			An. neomaculipalpus	1
Hilo Creek	HC	*9°09'N, 81°53'W*	*An. punctimacula* s.l.	30
			An. apicimacula	1
Gamboa	GAM	*9°14'N, 79°32'W*	*An. punctimacula* s.l.	8
Guayabo	GYO	*8°24'N, 82°52'W*	*An. punctimacula* s.l.	4
Playon Chico	PC	*9°18'N, 78°18'W*	*An. punctimacula* s.l.	27
			An. malefactor	3
Jaque	JAQ	*7°30'N, 78°08'W*	*An. punctimacula* s.l.	30
Yaviza	YAV	*8°07' N, 77°39' W*	*An. punctimacula* s.l.	30
			An. malefactor	8
Biroquera	BI	*7°29'N, 77°58'W*	*An. punctimacula* s.l.	30
			An. neomaculipalpus	1
Puerto Obaldia	PO	*8°40'N, 77°25'W*	*An. punctimacula* s.l.	30
			Total	310

Table 7.2. Collection sites and sample size of *Anopheles punctimacula* s.l., and other taxa from the Arribalzagia Series of the Subgenus *Anopheles*.

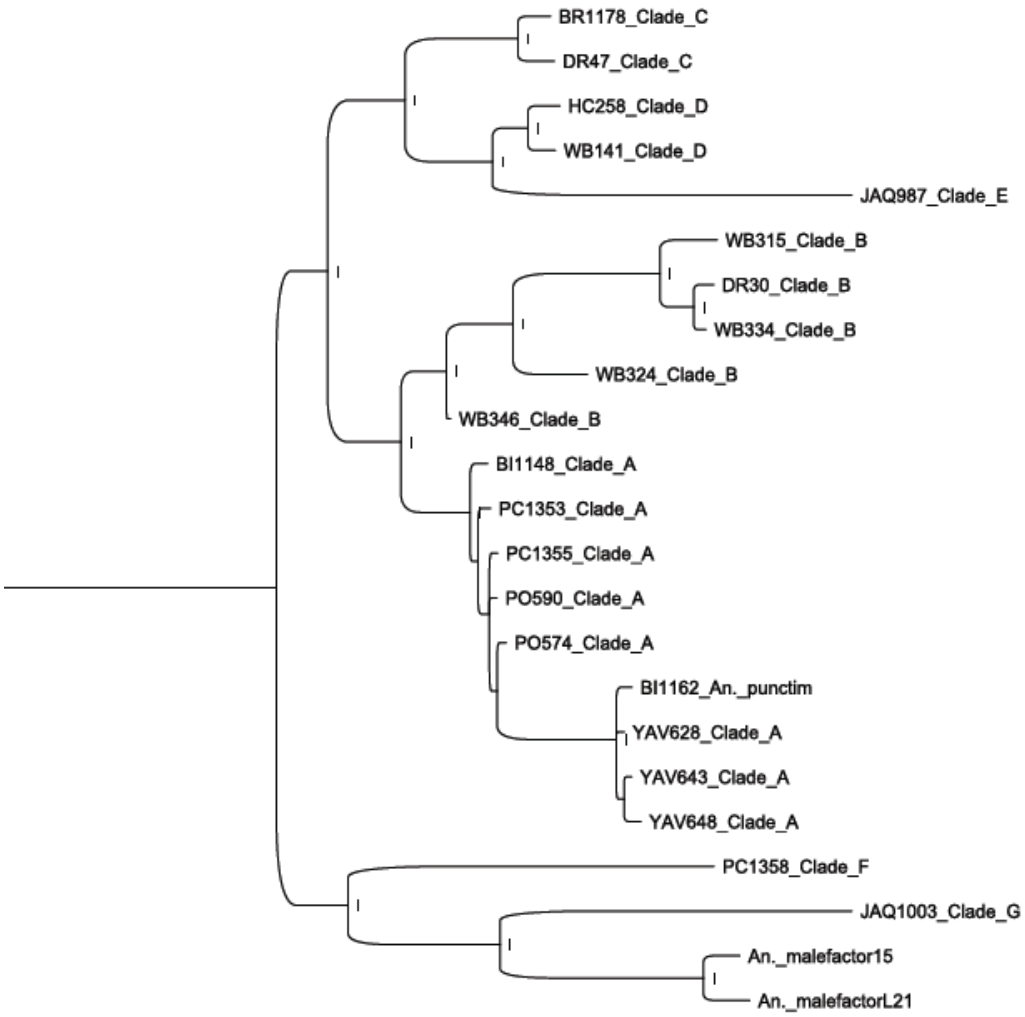

Figure 7.1. Phylogenetic tree based on Bayesian inference of 23 mtDNA haplotypes of *An. punctimacula* s.l., showing seven distinct clades (A, B, C, D, E, F, and G) and *An. malefactor*. The majority-rule consensus tree was obtained under a GTR + I + C model of substitution, using 1,258 bp of the combined *5' COI* and *3' COI* regions. Bayesian posterior probabilities are shown at the nodes.

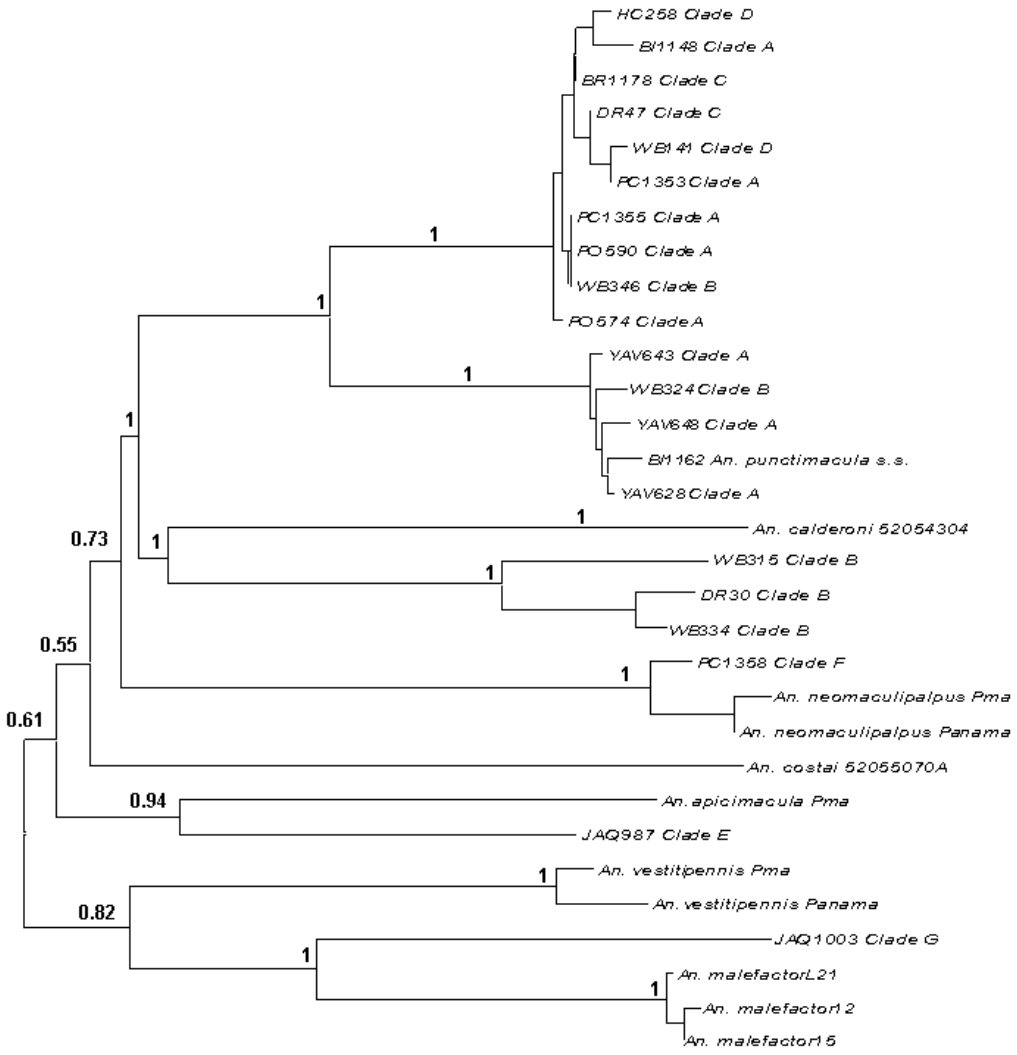

Figure 7.2. Phylogenetic tree based on Bayesian inference for samples of *An. punctimacula* s.l., showing six distinct clades and specimens of *An. malefactor*, *An. apicimacula*, *An. neomaculipalpus*, *An. vestitipennis* identified from Panama plus *An. calderoni* and *An. costai* from the MBI data sets. The majority-rule consensus tree obtained under a GTR + I + C model of substitution, using a 648 bp of the 5*' COI* region. Bayesian posterior probabilities are shown at the nodes.

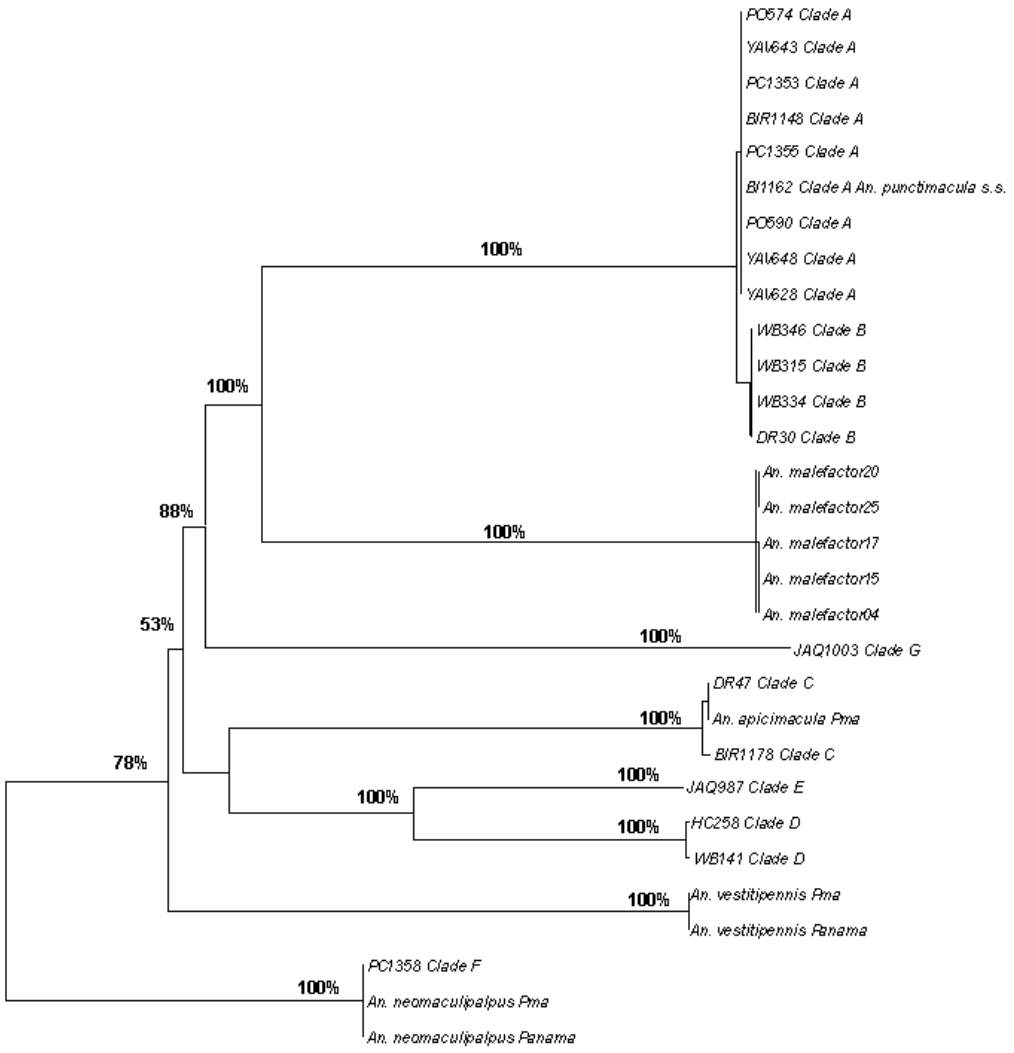

Figure 7.3. Unrooted NJ tree using unique sequences of the ITS2 region for clades A (*An. punctimacula* s.s.), B, C, D, E, F, and samples of *An. malefactor, An. apicimacula, An. neomaculipalpus and An. vestitipennis* from Panama. Branch support was estimated from 1000 replicates of a bootstrap search.

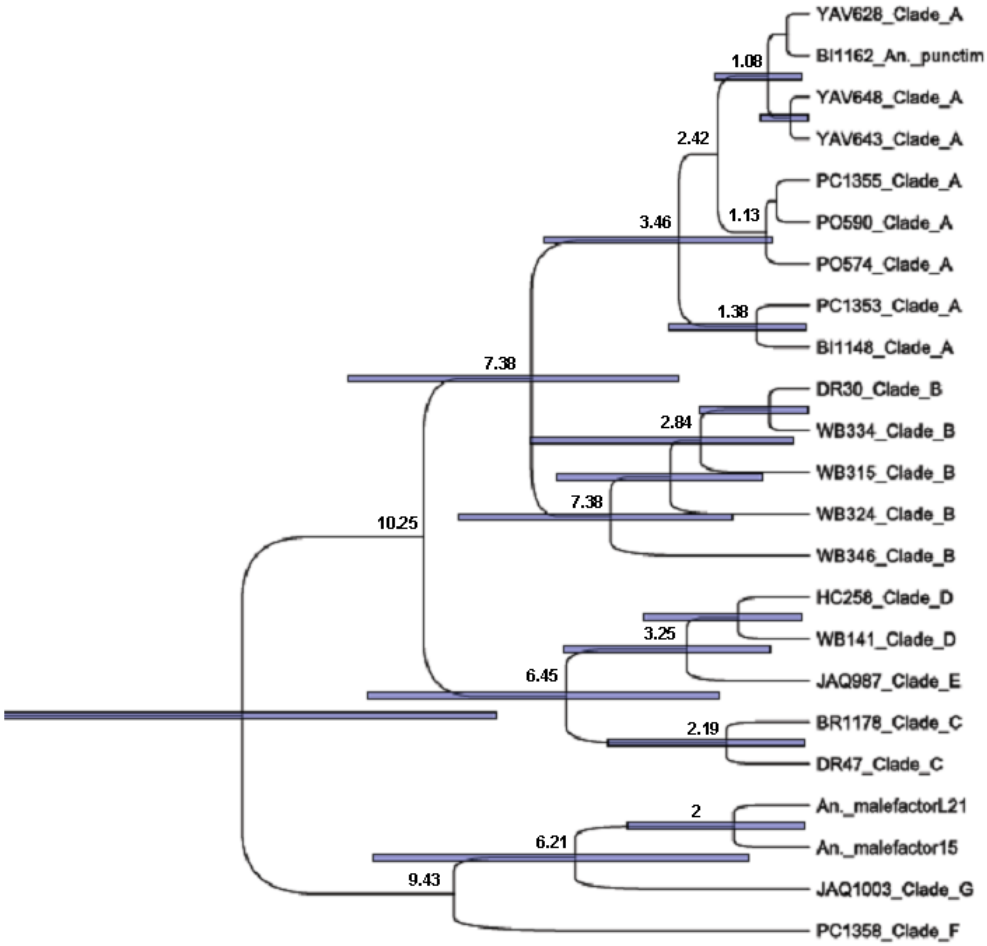

Figure 7.4. Time to the most recent common ancestor for the nodes represented in the tree topology. Numbers on the branches represent the node age for clades A (*An. punctimacula* s.s.), B, C, D, E, F, G and *An. malefactor*. Bars represent the HPD 95% confidence intervals.

8. Summary and Conclusions

Malaria was very prevalent in Panama at the beginning of the last century during the construction of the inter-oceanic Canal. During the main period of French construction (1881–1889), it was estimated that as many as 22,000 workers died, and another 10,000 died during the American construction phase (1903–1914). High incidence of malaria was also recorded from rural areas of Panama, between 1920 and 1960, when health care was extremely inefficient and the number of underreported cases was significant. The disease was then fairly well controlled for more than 30 years with less than 500 cases recorded annually (PAHO, 1996; Caceres, 1999). Nevertheless, from 2001 to 2005, a significant increase in malaria incidence was recorded again in Panama, primarily from indigenous territories. The number of cases during this outbreak went up dramatically for both *Plamodium vivax* (five-fold) and *P. falciparum* (three-fold), and roughly 20 people died (Contraloría, 2004; World Bank, 2005; Samudio et al., 2005; PAHO, 2006; Ministerio de Salud – MINSA, 2007 / http://www.minsa.gob.pa; Calzada et al., 2008).

The striking resurgence of malaria in Panama stressed the need for more research to better understand the epidemiology of this disease and to identify the likely causes that triggered past epidemics. However, at present there are not any firm conclusions as to the factors that provoked this substantial outbreak. Scarce treatments in remote areas, increased human migration and significant drug resistance by *Plasmodium spp*., might all have contributed to this epidemic (PAHO, 2006; WHO, 2008; World Bank, 2005; Samudio et al., 2005; Calzada

et al., 2008). Similarly, ongoing ecological and demographic changes in Panama may have modified the regional distribution and relative abundance of malaria vectors, thus increasing the risk of transmission in non-traditionally endemic areas (ANAM, 2003; Contraloría, 2004).

The potential for the occurrence of future outbreaks is high in Panama. For example, the numbers of Colombian refugees are likely to have tripled across the eastern Panamanian border within the last decade (Samudio et al., 2005). This rise in human traffic may enhance the introduction of new strains of *P. falciparum* from Colombia into areas of Panama where malaria treatment is very inefficient. Moreover, a new chloroquine-resistant strain of *P. falciparum* has recently been identified from eastern Panama (Calzada et al., 2008), where *Anopheles* species are still poorly known, and therefore, vector control might be hindered. All these factors are likely to worsen the future malaria situation in Panama, so more research is urgently needed to better understand the transmission dynamic of this disease. The information generated in the present doctoral thesis will contribute to this goal, and also to the design of more efficient tactics for vector control in Central America.

In chapter three, I hypothesized that additional putative vector species may be encountered with more comprehensive mosquito sampling across Panama. To test this hypothesis, I first evaluated the species composition and distribution of adult *Anopheles* in Panama using existing data gathered by members of SNEM and ICGES (1970 – 2004). In total, 33,917 specimens belonging to fourteen *Anopheles* species in four subgenera were collected from

77 localities of Panama. Although these historical data sets provided baseline knowledge about potential malaria vectors, this information remained incomplete. Therefore, from September 2005 to January 2010, I collected roughly 13,000 mosquitoes from another 35 previously un-sampled localities, representing 12 *Anopheles* species. All these species have been previously reported (Darling, 1910; Rozeboom, 1935; Simmons, 1936a,b, 1937; Wilkerson, 1990; Wilkerson & Strickman, 1990), except for *Anopheles darlingi*, which myself and colleagues identified for the first time in Panama. These two mosquito data sets represent the only information available about malaria vectors since 1954, and therefore, most records are new within Panama.

Anopheles albimanus and *An. punctimacula* s.l., are the most abundant species in malaria endemic regions of Panama. The former is prevalent in lowland open areas, close to the coastline where it breeds in a great variety of temporary aquatic habitats. In contrast, *An. punctimacula* s.l., seems to be more predominant in woody areas, such as Bocas del Toro and Darien, where sometimes its abundance can exceed that of *An. albimanus*. The higher prevalence of *An. punctimacula* s.l., in forest environments may be the result of specific requirements, as larvae of this species develop primarily in shady aquatic habitats (Service, 1989). Overall, these mosquito distribution and relative abundance data sets also suggest a possible seasonal component to the risk of acquiring malaria in Panama, as I found that most anopheline species, including *An. albimanus* and *An. punctimacula* s.l., are more abundant during the rainy season. These findings suggest that malaria control would be more

effective if it is intensified in Panama from May to December. Active surveillance of *Plasmodium* spp. should start shortly before the beginning of the rainy season, so localities at high transmission risk can be identified and epidemics prevented through appropriate timing of vector management.

Although no scientific information has been published since the 1930s to conclusively implicate any species as a malaria vector in Panama, both historical and more recent mosquito data sets strongly suggest that *An. albimanus* is the primary vector and *An. punctimacula* s.l., is the secondary vector in most of Panama. Data on *Plasmodium* infection rate in Panamanian mosquitoes using the enzyme-linked immune-absorbent assay (ELISA) agree with these historical findings, and indicate that *An. albimanus* is the primary vector. Six pools out of 750 (five individuals per pool) of *An. albimanus* have been found infected with *P. vivax* (both VK210 and VK247 variants) and *P. falciparum* from three localities across Panama. In contrast, none of 1,020 mosquitoes out of 206 pools of *An. punctimacula* s.l., analyzed so far have been found infected with any *Plasmodium* species (unpublished data). Both *An. albimanus* and *An. punctimacula* s.l., have been previously incriminated as vectors in Panama (Darling, 1910; Simmons, 1936a,b, 1937; Rozeboom, 1935), which further suggests their involvement in malaria transmission. Nonetheless, by the time *An. punctimacula* s.l., was incriminated as a vector, no comprehensive studies about its taxonomic status had been carried out. Later work revealed that *An. punctimacula* s.l., is a group of at least three isomorphic species, two of which co-occur in Panama (Wilkerson, 1990, 1991). Given the uncertain taxonomic

status of *An. punctimacula* s.l., it might be premature to validate its role of secondary malaria vector in Panama (Ulloa et al., 2006). In addition, a potential role of *An. aquasalis* (Berti et al., 1993), *An. neivai* (Carvajal et al., 1989; Solarte et al., 1996) and *An. vestitipennis* (Grieco, 2001; Achee et al., 2007) as secondary malaria vectors in Panama is also partially supported by these data, yet these vectors seem to be of local importance only, because they are restricted to specific geographic areas of eastern and western Panama.

Among other potential malaria transmitters collected in Panama are *An. darlingi*, *An. nuneztovari* s.l., *An. neomaculipalpus* s.l., *An. triannulatus* s.l., and *An. pseudopunctipennis* s.l., thus making a total of at least eight proven malaria vectors (Olano et al., 2001; Rubio-Palis & Zimmerman, 1997; Lounibos & Conn, 2000). These findings disagree noticeably with previous beliefs that identified *An. albimanus* as the only important malaria vector, and envisioned a very simple transmission dynamics throughout the country. Moreover, the malaria situation may be even more complex because *An nuneztovari* s.l., *An. triannulatus* s.l., *An. strodei* s.l. and *An. albitarsis* s.l., are all species complexes present in Panama whose taxonomic status remain unknown, and therefore, their transmission roles may be underestimated (Rosa-Freitas et al., 1998; Moreno et al., 2005; Marrelli et al., 2006; Quiñones et al., 2006; Bourke et al., 2010). The existence of isomorphic species in *Anopheles* mosquitoes may severely compromise vector control strategies in Panama as these efforts may mistakenly target non-vector species within these complexes (Garros et al., 2005; Lehr et al., 2005; Dusfour et al., 2004, 2007; Paredes-Esquivel et al., 2009; Bourke et

al., 2010). It is also very likely that many other species of *Anopheles* from South America occur in Panama, but have not yet been identified.

It is clear now that more fine scale sampling, larger collecting efforts and new methodologies such as molecular taxonomy must be implemented in Panama to fully assess the diversity and transmission roles of *Anopheles* species. This will be crucial for the near term of malaria control as correct species designation is required for effective insecticide treatment. Overall my results indicate that insecticide control is likely to have been historically compromised by either the uncertain taxonomic status of several cryptic *Anopheles* complexes or by the presence of unidentified malaria vectors in Panama.

An example of this situation is my recent finding of *An. darlingi* in Panama, which is considered the most important malaria vector in the Neotropics (Aramburu et al., 1999; Conn et al., 2002; Soares et al., 2003; Póvoa et al., 2003; Achee et al., 2005; Vittor et al., 2006). In chapter four, I reported *An. darlingi* for the first time in Darien, the region of eastern Panama with the highest prevalence of chloroquine-resistant *P. falciparum* in the country. A history of high numbers of malaria cases and the almost exclusive occurrence of *P. falciparum* in eastern Panama suggested the presence of an efficient malaria vector in this region. However, previous work undertaken by entomologists at MINSA, SNEM and ICGES had consistently failed to detect this species. I collected adult female specimens of *An. darlingi* in Biroquera and Jaque using human landing catches, and molecularly confirmed them, as the northern lineage using *PCR-RFLP* and 800 bp of the single copy nuclear w*hite* gene.

The epidemiological implications of finding *An. darlingi* in Panama are to be further investigated. The government of Panama, through MINSA and ICGES, should initiate systematic studies about the ecology and vector competence of *An. darlingi* to establish its role in malaria transmission. Past outbreaks of *P. vivax* and *P. falciparum* in Darien strongly suggest that *An. darlingi* is already an established malaria vector in eastern Panama. Therefore, key points for this species need to be investigated, for example, rapid assessment of the effectiveness of IRS in controlling *An. darlingi* in Darien is required, so malaria can be effectively controlled through vector suppression. One way to achieve this goal indirectly is using microsatellite loci because these markers mutate at fast enough rates to provide information about current values of genetic diversity (N_e). MINSA could monitor the values of intraspecific diversity in samples of *An. darlingi* at distinctive temporal points and deduce the effects of insecticide in comparisons before and after application (Wondji et al., 2005). This could also help to decipher key behavioral characteristics of *An. darlingi*, for example, high values of N_e after a period of IRS treatment could be related to insecticide resistance and/or to a more exophilic tendency in Panamanian populations. In areas under intense IRS treatment *An. darlingi* is able to switch from an endophagic (capable of feeding indoors) behavior to feed more preferentially outdoors, thus avoiding the contact with sprayed surfaces (Suarez et al., 1990). Such studies could also help to predict the spread of genes involved in parasite refractoriness and/or insecticide resistance (Molina-Cruz et al., 2004; Scarpassa & Conn, 2007; Mirabello et al., 2008).

Studies about population differentiation are also required to understand the demographic origin of the Panamanian samples of *An. darlingi*. To date substantial genetic variability and significant geographic structure has been reported in samples of *An. darlingi* from Central and South America (summarized in Mirabello, 2007). Two genotypes or incipient lineages were hypothesized based on a phylogeographic analysis using partial sequences of the *COI* gene. The divergence in *An. darlingi* was attributed to either a founder effect due to an introduction from Colombia into Central America or to physical barriers to dispersal across the northern Neotropics (Mirabello & Conn, 2006). Only the northern genotype of *An. darlingi* was found in samples from eastern Panama, and this agrees with the results of Mirabello (2007) who hypothesized that the probably porous barrier between these two lineages is located in south central Colombia and across the Guyana highlands of Venezuela. Panama could then be used as a site for eradication trials, if genetic diversity is reduced in isolated populations from Darien. My population analysis with the *COI* gene suggested a Colombian origin for the Panamanian samples of *An. darlingi*, but the neutrality tests were not significant and the mismatch distribution did not fit the model of sudden population expansion. All of these test results provided little support for a bottleneck in the recent history of *An. darlingi*, thus rejecting a possible recent introduction from northern Colombia.

These results clearly indicate that the changes in land use, urbanization, and human migration that have occurred in Panama since 1960 (Contraloría, 2004) have not altered the habitats for *An. darlingi* in remote areas of Darien

where it still breeds in partially shaded and clean bodies of water. MINSA and ICGES should also study the extent of geographic distribution of *An. darlingi* in DR, as this species has demonstrated significant ecological plasticity, being able to adapt very rapidly to new environments. A clear example of this situation was seen in Brazil where *An. darlingi* underwent extensive colonization of western Amazonia and in the city of Belém, likely due to road construction and forest clearing. Moreover, *An. darlingi* also expanded during the last ten years in the Peruvian Amazon, perhaps due to unplanned deforestation and increased human migration (Póvoa et al., 2003, 2006). Therefore, it is possible that with future environmental perturbation in eastern Panama, *An. darlingi* could expand towards central and western Panama, and contribute to a range expansion of the chloroquine-resistant *P. falciparum* into new areas, aggravating the malaria situation.

Inferring the population history of *Anopheles* species is of practical relevance to malaria control because any attempt to estimate current patterns of gene flow will first require a clear understanding of population history. This will allow for the distinction of migration, natural selection and historical demographic processes as the causes of genetic structure (Walton et al., 2000, 2001). *Anopheles albimanus* is one of the most important malaria vectors in the Americas (Faran, 1980). Evidence for significant intra-specific divergence has been reported in *An. albimanus* (Breeland et al., 1972; Collins et al., 1976; Frederickson, 1993; Grieco et al., 2005; González & Martinez, 2006; Gutiérrez et al., 2008). Also, considerable molecular geographic structure was hypothesized

across its geographic range, yet all these studies failed to reject the hypothesis of a single species (Narang et al., 1991; De Merida et al., 1995, 1999; Molina-Cruz et al., 2004; Gutiérrez et al., 2009).

In chapter five, I hypothesized that *An. albimanus* is a single, albeit polymorphic species, that is not at mutation - drift equilibrium in southern Central America. My results, based on three independent molecular markers (mtDNA *COI*, rDNA ITS2, *white*), supported the status of a single species, and provided strong evidence of genetic divergence that seems to be regionally allocated across southern Central America and northern South America. A signal of multiple introductions (*i.e.* secondary contact) was initially supported by my *COI* analysis in samples from eastern Panama and Costa Rica. Moreover, three non - randomly distributed population demes and four discrete lineages of *An. albimanus* were identified across Nicaragua, Costa Rica, Panama, Colombia and Ecuador using a more comprehensive data set. The geographical division is also partially supported by the ribosomal DNA ITS2 marker; the occurrence of ITS2 intragenomic variability only in samples from Nicaragua, Costa Rica and Panama may suggest higher gene exchange among these countries. In contrast, an exclusive insertion in three samples from Ecuador agrees with past geographical fragmentation as proposed by the *COI* gene. This information could be used as a starting point to study potential differences in the vector competence (*i.e*. parasite susceptibility) and/or insecticide resistance among different strains of *An. albimanus*.

The *white* gene was not as variable as the *COI* and did not corroborate its spatial geographic pattern, but provided support for population contraction and subsequent expansion across Panama and Costa Rica. Similarly, the *COI* gene suggested population expansion at a rather similar time as the nuclear gene, therefore rejecting a selective sweep as the possible cause of genetic structure in *An. albimanus*. The reduced diversity found in samples from Panama and Costa Rica confirmed the findings from previous studies that also indicated significantly reduced diversity in central Panama based on four microsatellites and the *ND5* gene (Molina-Cruz et al., 2004). However, contrary to their hypotheses my results do not seem to be caused by any of the following: contemporary IBD, barriers to dispersal or more intense IRS treatment in Panama. Instead, the phylogeographic pattern of *An. albimanus* appears to be related to Pleistocene environmental changes that affected its N_e and contracted its geographic range in southern Central America.

Many other malaria vectors around the world have depicted signals of unstable demography likely due to population expansion and/or growth (Mirabello & Conn, 2006; O'Loughlin et al., 2008; Crawford & Lazzaro, 2010). In general, it appears that *Anopheles* species adapted to humans would change demographically in response to changes in their host demography, and therefore, do not retain signals of more ancient demographic processes (Coluzzi et al., 1979; Donnelly et al., 2001; Crawford & Lazzaro, 2010). In contrast, those that are less anthropophilic may have changed demographically in response to earlier climatic

changes, as they do not strongly rely on humans to develop (Mirabello & Conn, 2006; O'Loughlin et al., 2008).

In chapter six, I hypothesized that *An. albimanus* displays a similar pattern of Pleistocene population expansion as *An. darlingi* in South America. Both the *COI* and the *white* gene supported a scenario of population expansion in *An. albimanus* within the same time frame as proposed for *An. darlingi* in Brazil (Mirabello & Conn, 2006). This suggests that the two most important Neotropical malaria vectors responded similarly to the same Pleistocene climatic changes. Moreover, my results would fit with *a priori* predictions, as these two species have rather opportunistic feeding behaviors and are less dependent upon humans for larval breeding (Donnelly et al., 2002; Achee et al., 2007; Zimmerman et al., 2006). Nevertheless, while both species showed signals of unstable demography their expansion patterns are somewhat different. *An. darlingi* appears to have followed a refugia - expansion - refugia trend in response to forest contraction and expansion. In contrast, the expansion of *An. albimanus* seems to have little to do with changes in forest structure, but more with climatic oscillations, sea level changes and an expansion path along the coastline. In addition, the population contraction of *An. albimanus* appears to be more drastic, perhaps due to its narrow geographic range across the Isthmus of Panama and Costa Rica, whereas *An. darlingi* expanded across a broader geographic area probably from the center of its ancestral genetic pool, and therefore, it may have a higher N_e than the former (Pedro & Sallum, 2009).

Anopheles punctimacula s.l., is a secondary malaria vector in parts of Central America, yet its molecular taxonomic status has never been comprehensively examined. In chapter seven, I hypothesized that *An. punctimacula* s.l. is a species complex that comprises more than three cryptic species. Phylogenetic analyses using the combined *5' COI* and *3' COI* regions and also the ITS2 marker suggested lineage divergence and possible cryptic speciation in *An. punctimacula* s.l. These analyses supported the existence of seven distinct clades in mosquitoes morphologically identified as *An. punctimacula* s.l., all of which were differentiated from *An. malefactor* and *An. calderoni*, and also from other species within the Arribalzagia Series. In contrast, the same type of analysis using only the Folmer or Barcoding region (*5' COI*) suggested paraphyly. Conflicting phylogenetic signals between different regions of the *COI* could be due to the unequal mutation rate across this gene. Evidence from the ITS2 and the combined *COI* regions suggested that *An. punctimacula* s.s., comprises two geographically isolated lineages. Yet, based on the present data sets, it is not clear whether these lineages are true species or just divergent populations, since they were not found in sympatry. One clade was mistakenly identified as *An. punctimacula* s.l., but instead, it corresponded to *An. neomaculipalpus*. The remaining clades have an uncertain taxonomic status, and they could either be other unidentified species within the Arribalzagia Series or true genetic diversification within *An. punctimacula* s.l. My results demonstrated that the taxonomic status of this secondary malaria vector is still uncertain. Also, they indicated that despite the fact that the Barcoding region has been successfully

used in mosquito molecular taxonmony, this region might not be a good predictor of species boundaries within the Arribalzagia Series of *Anopheles* (Hebert et al., 2003; Cywinska et al., 2006; Foley et al., 2007; Pradeep Kumar et al., 2008). It is noteworthy that the divergence of the members of *An. punctimacula* s.l., as defined by my analyses, occurred roughly during the same time frame as the geographic fragmentation hypothesized for *An. albimanus*, in the early – late Pleistocene. This may suggest a common pattern of geographic fragmentation and diversification among several *Anopheles* species across the Isthmus of Panama due to Pleistocene climatic changes.

Overall my findings have provided a more complete picture of the genetic population structure and taxonomic status of three important Neotropical malaria vectors, *An. albimanus*, *An. darlingi* and *An. punctimacula* s.l. Future studies will have to address some limitations encountered during my investigations. For example, the overall scarcity of microsatellite loci has precluded a better assessment of the rates of contemporary gene flow among populations of *An. albimanus*. The physical location of microsatellite markers with respect to polymorphic inversions can be potentially informative for interpreting population genetic structure, yet few of these markers are currently available for *An. albimanus*. Another limitation for my studies is the lack of alternative nuclear genes that can be used to accurately investigate the demographic history of *Anopheles* mosquitoes. For example, the use of additional single copy nuclear genes will significantly improve my estimates of the timing of diversification and population expansion of malaria vectors across the Isthmus of Panama. This

imformation could be useful to predict the spatial pattern of genetic diversity in other malaria vectors that have also been affected by Pleistocene climatic changes around the world. The results of the present doctoral thesis will be valuable to better understand the epidemiology of malaria in the Neotropics, and they will contribute to the design of more efficient strategies of vector control.

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