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Historical and contemporary forces combine to shape patterns of genetic differentiation in two species of Mesoamerican *Anopheles* mosquitoes

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Pleistocene environmental changes were important drivers of species- and population-level diversification in *Anopheles* mosquitoes. However, *Anopheles* species have different ecologies, so their response to Pleistocene climate oscillations should have differed. We investigate whether genetic diversification in *Anopheles punctimacula s.s.* (a forest specialist and secondary vector of *Plasmodium vivax*) and *Anopheles albimanus* (a habitat colonist and primary vector of *P. vivax* and *P. falciparum*) is due to: (1) historical population processes, (2) contemporary population processes or (3) a combination of both. Differences in the degree of refugial isolation during the Last Glacial Maximum (LGM) and the degree to which isolated populations evolved habitat niche differences appear to explain differences in the phylogeographical patterns between *A. punctimacula s.s.* and *A. albimanus* in Lower Middle America (Mesoamerica). Refugial isolation during the LGM and subsequent niche diversification shaped the phylogeographical history of *A. punctimacula s.s.* During the LGM, the genetic pool of this species was fragmented into extremely narrow and scattered habitat refugia, resulting in two discrete mitochondrial lineages. Subsequently, these lineages appear to have further evolved distinct niche preferences and diversified due to different climatic conditions between populations, which may have contributed to the lack of introgression or range overlap among mitochondrial lineages. While *A. albimanus* also experienced range contraction, recovery was more rapid, and we find no evidence of niche evolution among lineages. This appears to explain the broad mitochondrial introgression in this species. Greater resilience to climatic instability by *A. albimanus* might contribute to its principal transmission role for human *Plasmodium* parasites across the Neotropics.

ADDITIONAL KEYWORDS: *Anopheles* – colonist – habitat specialist – malaria vectors – niche dynamic – Pleistocene refugia.

INTRODUCTION

Understanding the evolutionary dynamics of disease vectors is critical for designing effective control strategies against life-threatening vector-borne pathogens such as *Plasmodium falciparum* and dengue virus (Donnelly *et al.*, 2001; Walton *et al.*, 2001; Messina *et al.*, 2014). Knowledge of a vector's evolutionary history can shed light on systematics, identify cryptic species, estimate levels of genetic diversity and infer transmission capability (Loaiza *et al.*, 2012; Zhou *et al.*, 2014; Bennett *et al.*, 2016). Comparative phylogeography of spatially co-distributed and ecologically distinct vector species can help to further untangle the role of past and contemporary forces in shaping their evolutionary trajectories (O'Loughlin *et al.*, 2008; Morgan *et al.*, 2011; Zarowiecki *et al.*, 2011).

Phylogeographical studies from a variety of *Anopheles* mosquitoes – the principal vector of human malaria – share a consistent signal of historical demographic

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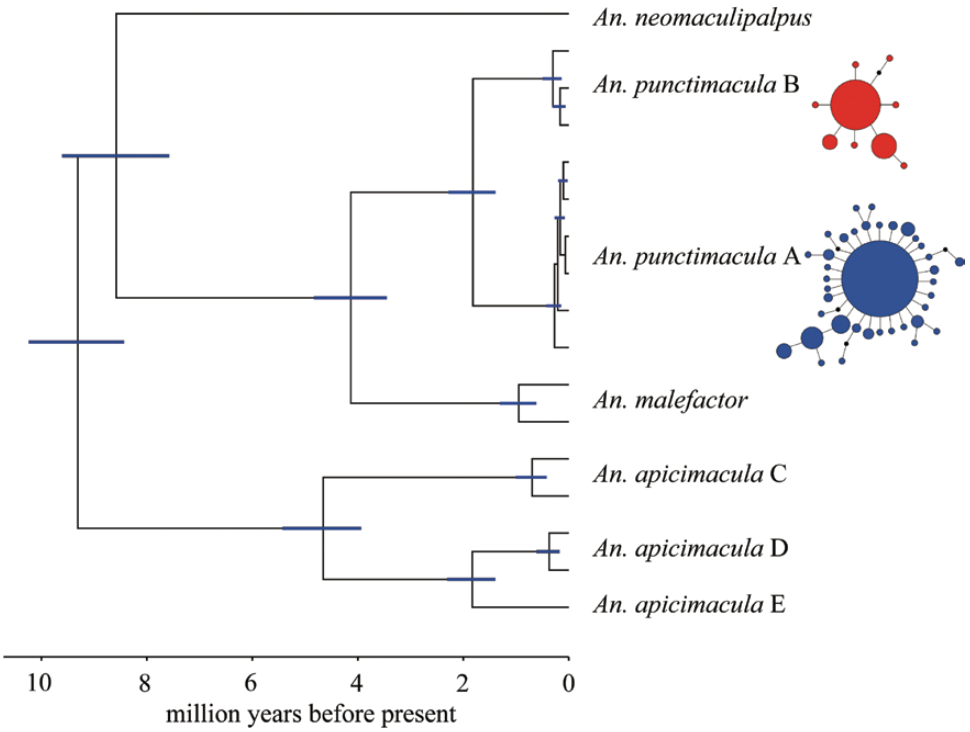

Figure 1. Time to the most recent common ancestor (TMRCA) for nodes represented on the topology of the Arribalzagia Series of *Anopheles*. Bars represent the 95% highest posterior density interval (HPD) for the age of nodes between *Anopheles punctimacula* lineages A and B and other representative species of this Series (i.e. *Anopheles neomaculipalpus*, *Anopheles malefactor* and *Anopheles apicimacula* C, D, E). Statistical parsimony networks of *COI* haplotypes belonging to *A. punctimacula* A and B are depicted on the right side next to each particular lineage. The size of the circle is proportional to the frequency of the haplotype, and the colour indicates the geographical distribution range of that haplogroup across Mesoamerica as depicted in Fig. 2 (i.e. *A. punctimacula* A in blue and B in red).

instability, which is generally believed to be due to environmental variation during the Pleistocene. Temperature, rainfall and sea level oscillated remarkably during this climatic period, probably affecting availability of larval breeding habitats for many *Anopheles* species around the world, including vectors and non-vectors of human plasmodia (Dusfour *et al.*, 2004, 2007; Mirabello & Conn, 2006; O'Loughlin *et al.*, 2008; Loaiza *et al.*, 2010a, b; Morgan *et al.*, 2011). The consensus from this body of work suggests that geographically overlapping *Anopheles* species suffered similar and drastic variation in effective population sizes due to past environmental instability, ultimately resulting in shared signatures of phylogeographical history (Dusfour *et al.*, 2004, 2007; O'Loughlin *et al.*, 2007, 2008; Mirabello *et al.*, 2008; Morgan *et al.*, 2010, 2011; Loaiza *et al.*, 2013).

Not all *Anopheles* mosquitoes share the same ecological niche, however, and therefore have different demographic and population trends under contemporary circumstances. For instance, *Anopheles* species that are forest habitat specialists are typically patchily distributed because they have stringent ecological requirements for larval development. In contrast, non-forest-inhabiting *Anopheles* are generally colonist species with plastic breeding behaviour and broader breeding habit tolerances (Donnelly *et al.*, 2002). Furthermore, forest habitat specialists normally use permanent aquatic breeding habitats that are less affected by climatic fluctuation and persist for longer ecological time, promoting higher population fragmentation than the short-lived and unstable water breeding sites of colonist *Anopheles* species (Donnelly *et al.*, 2002; Zarowiecki *et al.*, 2011; Loaiza *et al.*, 2012). These ecological differences may explain contemporary differences in spatial patterns of genetic variation. However, few if any studies have compared current patterns of genetic variation to historical predictions of population extent during the Pleistocene, and none of them has compared the response of ecologically different *Anopheles* species.

In this study, we use a combination of phylogeographical and ecological niche modelling approaches to predict the demographic history of two human malaria vector species, *Anopheles* (*Anopheles*) *punctimacula s.s.* and *Anopheles* (*Nyssorhynchus*) *albimanus*, and to evaluate the role of historical and

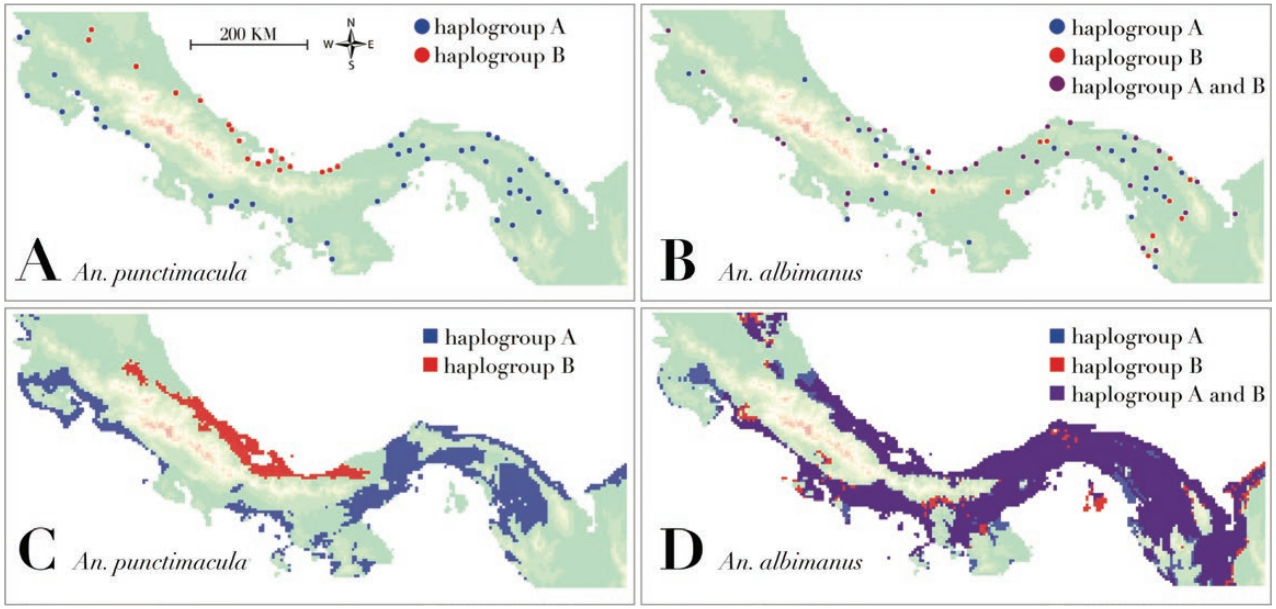

Figure 2. A, occurrence points for *Anopheles punctimacula* haplogroups A and B; B, occurrence points for *Anopheles albimanus* haplogroups A and B. For *A. punctimacula*, haplogroup assignment was based on the *COI* sequences in Loaiza *et al.* (2013) while for *A. albimanus* we used the *COI* sequences in Loaiza *et al.* (2010a, b). C, contemporary species distribution model for halogroups A and B of *A. punctimacula*; D, contemporary species distribution model for halogroups A and B of *A. albimanus*.

contemporary forces in shaping the differences in mitochondrial genetic variation observed between these two species in Mesoamerica (Fig. 2). *Anopheles punctimacula s.l.* is a cryptic species complex consisting of *Anopheles punctimacula s.s.*, *Anopheles calderoni*, *Anopheles malecfactor* and *Anopheles shanonni*; it prefers woody environments where it breeds in permanent and heavily shaded aquatic breeding habitats (Wilkerson, 1990) whereas *A. albimanus* develops in a wide variety of aquatic breeding sites, including shaded sites, but typically develops in ephemeral sunlit habitats (Faran, 1980). Recent studies identified significant population genetic structure in both species across the Isthmus of Panama and Costa Rica (Loaiza *et al.*, 2010a, b, 2013). The phylogeographical history of *A. albimanus* is thought to be the result of Pleistocene population bottleneck (50 000 years ago; Loaiza *et al.*, 2010a, b), with subsequent geographical fragmentation, and more recent secondary contact via demographic expansion across the region in the Last Glacial Maximum (LGM, ~ 22 000 years BP). Likewise, Loaiza *et al.* (2013) suggested that the phylogeographical history of *A. punctimacula s.s.* was driven by past geographical fragmentation similar to that observed in *A. albimanus*. However, in contrast to the pattern of broad mitochondrial haplogroup overlap observed in *A. albimanus*, no haplotype mixing was observed between western and eastern mitochondrial lineages of *A. punctimacula s.s.* across this region.

Here, we investigate whether genetic diversification in the forest-specialist and secondary malaria vector *A. punctimacula s.s.* is due to: (1) historical population processes (i.e. Pleistocene bottleneck, geographical fragmentation and demographic expansion in the LGM), (2) contemporary population processes (i.e. gene pool partitioning and niche diversification due to adaptation to different contemporary climatic conditions) or (3) a combination of both historical and contemporary population processes.

Owing to a specialized and spatially scattered type of larval habitat, we expect *A. punctimacula s.s.* (i.e. a forest specialist) to show signals of severe refugial isolation during the LGM plus ongoing restricted gene flow due to adaptation to different climatic regimes (i.e. niche diversification). In contrast, due to higher ecological plasticity at the larval stage and wider geographical distribution, we expect *A. albimanus* (i.e. a habitat colonist) to show signals of more continuous suitable habitat at the LGM and contemporary niche homogenization due to extensive gene flow more recently (i.e. lack of niche diversification). These expectations are not just worth reviewing from an evolutionary standpoint; rather, they should also be evaluated from an epidemiological perspective. For example, *A. punctimacula s.s.* is a secondary vector of *Plasmodium vivax* in Costa Rica, Panama and Colombia, and *A. albimanus* is the primary vector of *P. vivax* and *P. falciparum* throughout most of Central

America, Colombia, Ecuador and Peru (Simmons, 1936a, b, 1937; Faran, 1980). In the Neotropics, principal malaria vectors such as *A. albimanus* are mainly habitat colonists, whereas secondary malaria vectors such as *A. punctimacula s.s.* are typically forest habitat specialists (Simmons, 1936a, b, 1937; Faran, 1980; Donnelly *et al.*, 2002; Loaiza *et al.*, 2017). As a result, comparative phylogeographical studies involving habitat specialist and colonist *Anopheles* species might ultimately help to forecast the potential roles of evolutionary history and ecology in shaping regional patterns of malaria transmission.

MATERIAL AND METHODS

Molecular data

We used previously published molecular data for *A. punctimacula s.s.* from ten Panamanian localities and one from Costa Rica, including 273 sequences of the cytochrome C oxidase subunit one gene (*COI*) and 73 of the second internal transcribed spacer (ITS2) (GenBank accession numbers: JX212783–JX212823, in Loaiza *et al.*, 2013). To improve the geographical sampling of this species we genotyped 30 females from additional localities in Costa Rica and Panama (Supporting Information, Table S1) using PCR plus restriction fragment length polymorphism (RFLP), which discriminated *A. punctimacula* lineages A from lineage B, as validated in Loaiza *et al.* (2013) (Supporting Information, Table S2). Sequencing and phylogeographical analysis of *A. albimanus* was undertaken by Loaiza *et al.* (2010a, b).

Phylogeographical approaches

We generated a statistical parsimony network using the program TCS v.1.21 to depict spatial and temporal connections among *COI* haplotypes of *A. punctimacula s.s.* (Clement *et al.*, 2000). We assessed statistical significance of the molecular partition in the TCS analysis using 10 000 coalescence simulations in DNASP 4.50 (Rozas *et al.*, 2003). The R_2 (Ramos-Onsins & Rozas, 2002) and Fu's F_S (Fu, 1997) neutrality tests were used to detect signals of demographic bottlenecks and/or expansion using the whole data set of *A. punctimacula s.s.* and the molecular partition defined in the TCS analysis (e.g. sequences belonging to two molecular lineages within *A. punctimacula s.s.*). These tests were calculated independently for each molecular lineage using the *COI* and ITS2 molecular markers.

BEAST v.1.4.8 (Drummond & Rambaut, 2007) was used to infer the time to the most recent common ancestor (TMRCA) among molecular lineages of *A. punctimacula s.s.*, and other closely related species within the Arribalzagia Series of *Anopheles*, which were also collected from Panama and Costa Rica (Loaiza *et al.*, 2013). We applied the Bayesian Markov chain Monte Carlo (MCMC) algorithm, an uncorrelated relaxed lognormal clock, and the model of nucleotide substitution estimated for the *COI* and ITS2 partitions in JMODELTEST (Posada, 2008). We used a uniform tree prior and input the *Drosophila* per-site substitution rate estimated at 0.0115 per site per million years (Powell *et al.*, 1986). Final analyses consisted of four separate MCMC runs of 50 million generations sampled every 1000 generations. The software TRACER v.1.4 was used to confirm adequate mixing of the MCMC chains upon independent runs (http://tree.bio.ed.ac.uk/software/tracer/), LOGCOMBINER v.1.4.7 (http://beast.community/logcombiner) to merge separate runs into one file, and FIGTREE v.1.2.1 (http://tree.bio.ed.ac.uk/soft- ware/figtree/) to visualize the topology. We used ten generations per year to calculate the age of nodes in the tree (i.e. TMRCA for all the haplotypes included in a particular node) as suggested when studying tropical *Anopheles* mosquitoes (Walton *et al.*, 2000).

Ecological niche modelling approaches

To generate a dataset of species occurrence points, we began with geo-referenced samples of *A. punctimacula s.s.* and *A. albimanus* from previous studies (Loaiza *et al.*, 2010a, b, 2013). We then added samples of both species that we had collected across Panama and Costa Rica between 2011 and 2013. Mosquitoes were preserved as voucher specimens in the reference collection of the University of Panama's Master Program of Entomology, in Panama City, Republic of Panama.

We generated species distribution models (SDMs) to test the hypothesis that phylogeographical differences between *A. punctimacula s.s.* and *A. albimanus* are due to differences in contemporary habitat specificity. SDMs were generated in Maxent v.3.3 (Phillips *et al.*, 2006) using the statistical test developed by Warren *et al.* (2008), as implemented in the ENMTools v.1.3 software package (Warren *et al.*, 2010). We started with the entire set of sampling points for each species as occurrence points for the models. Subsequently, we analysed the two major haplogroups/lineages described herein for *A. punctimacula s.s.* and the A and B haplogroups described previously for *A. albimanus* in Costa Rica and Panama (Loaiza *et al.*, 2010a, b), respectively, as separate occurrence points datasets.

Two studies, Dormann *et al.* (2007) and Kramer-Schadt *et al.* (2013) have demonstrated that uneven geographical sampling of occurrence points may bias SDMs; therefore, we created a distance matrix of all points in QGIS v.2.6.1 (Quantum GIS Development

Team, 2014), and filtered points, so that no two points were within 10 km of each other, using an R script generated by the filter_points_rcode shell script (available at http://github.com/mjmillerlab/Batch_ENM). This resulted in a final geo-referenced dataset of 62 occurrence points for *A. punctimacula s.s.* and 70 occurrence points for *A. albimanus* (Table S1). Sampling points for *A. punctimacula s.s.* and *A. albimanus* are shown in Figure 2A and B, respectively.

SDMs based on contemporary conditions were generated using the standard bioclimatic data layers sampled at 2.5 arc-minute resolution downloaded from the WorldClim database v.14 (Hijmans *et al.*, 2005; http://www.worldclim.org/), trimmed to the extreme maximum and minimum latitude and longitude of Costa Rica and Panama. Following Miller *et al.*, (2014), we found that seven layers were highly correlated with other layers (Spearman's $r > 0.90$), and thus they were excluded from the analysis (Warren *et al.*, 2008; Kramer-Schadt *et al.*, 2013). SDMs for each species and lineage (i.e. haplogroups) within species were generated as the average of ten runs with cross-validation.

To test whether the two haplogroups/lineages from each species had significantly non-overlapping estimated climatic niches, we calculated three statistics: Schoener's *D*, *I* (a standardized measure of Hellinger distance) and *RR* (relative rank) (Warren *et al.*, 2008). We used ENMTools to calculate the observed value of these statistics from a pairwise comparison of the two haplogroups for each species. We then compared the observed statistics to null distributions of these statistics from 100 pairs of pseudo-replicated datasets generated by combining occurrence points from both haplogroups/lineages A and B, from a given species and randomly dividing them into two pools. The observed statistic is considered statistically significant if it is lower than 95% of the null distribution.

To assess how historical climate variation may have contributed to the origin and maintenance of geographical structuring of mitochondrial variation in *A. punctimacula s.s.* and *A. albimanus*, we generated palaeo-ecological SDMs by projecting current suitable habitat onto bioclimate layers simulated for three palaeo-geographical time periods available on the WorldClim site: Last Interglacial [LIG, ~120 000–140 000 years before the present (ybp)], LGM (~22 000 ybp) and Mid-Holocene (MIDH, ~6000 ybp). For MIDH, nine simulations of general circulation models (GCMs) are available; for LGM, three GCMs are available; and for LIG, only one GCM is available. We were interested in evaluating whether historical climatic conditions generated currently observed phylogeographical patterns, so we combined all occurrence points for each species into a single dataset and geographicalally filtered it as described above (Table S1). We then generated a contemporary SDM for both species. For a given GCM, we generated ten contemporary SDM replicates with cross-validation, which we projected onto the GCM, and took the average of the output as the model for that GCM. For a given palaeo-period, using QGIS raster mathematics, we averaged the model outputs for all GCMs, and applied the contemporary 10% suitability threshold to generate a presence–absence model for each palaeo-period.

RESULTS

Phylogeographical history of *Anopheles punctimacula s.s.* in Mesoamerica

Haplotypes in the TCS statistical parsimony network of *A. punctimacula s.s.* formed two lineages that differed by more than 12 mutational steps (i.e. haplogroups A and B), and so they could not be connected parsimoniously (Fig. 1). Representative haplotypes of these two haplogroups were geographically isolated; those belonging to *A. punctimacula* A were found exclusively along the Pacific coast of Panama and Costa Rica, whereas those belonging to *A. punctimacula* B were encountered exclusively along the Atlantic coast of Costa Rica and western Panama (Figs 1, 2). The presence of molecularly divergent and geographically isolated *A. punctimacula* A and B confirms a significant partition in the gene pool of this mosquito species that has been acknowledged in previous studies (Loaiza *et al.*, 2013).

Both A and B lineages of *A. punctimacula s.s.* were star-shaped, depicting an interior highly frequent haplotype, with short branches and an excess of singleton mutations, which could be the signal of a bottleneck followed by a demographic expansion. In support of these outcomes, neutrality tests R_2 and Fu's F_S showed negative values for the entire data set and for lineages A and B, respectively, although they were only statistically significant in lineage A (Table 1). This therefore allows for the rejection of historical population size stability due to either demographic bottlenecks or expansion on this lineage. It is important to note that the outcomes of neutrality tests could also mean a lack of strict neutrality due to either background or positive selection (i.e. selective sweep).

The estimated 95% confidence intervals for the TMRCA of *Anopheles* species within the Arribalzagia Series ranged from 8.2–10.2 million years before present (Mybp) for the node separating *Anopheles apicimacula s.l.* (i.e. C, D and E) from *Anopheles neomaculipalpus*, *A. punctimacula s.s.* (i.e. A and B) and *Anopheles malefactor* to 7.8–9.3 Mybp for the node dividing *A. neomaculipalpus* from *A. punctimacula* A, B and *A. malefactor*. The estimated node age for the

Table 1. Genetic diversity metrics and neutrality tests for lineages A and B of *Anopheles punctimacula s.s.*

Lineage	h (SD)	π (SD)	R_2	F_s
Anopheles punctimacula A	0.70 (0.043)	0.001 (0.0002)	0.018***	–53.61***
Anopheles punctimacula B	0.94 (0.014)	0.005 (0.0002)	0.072	–26.36
Total	0.91 (0.015)	0.008 (0.0002)	0.041**	–73.26***

h = haplotype diversity; SD = standard deviation; π = nucleotide diversity; R_2 = Ramos-Onsins & Rozas test of neutrality; F_S = Fu's F_S statistic.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

split between *A. punctimacula* A and B was during the early Pleistocene (1.7–2.2 Mybp) (Fig. 1).

Niche specificity and temporal dynamics of *Anopheles* in Mesoamerica

We found that Bioclim layers 1, 7, 8, 9, 10, 11 and 17 were correlated with other biolayers, so we removed them from further analyses. SDMs generated in Maxent v.3.3 (Phillips *et al.*, 2006) were better than random predictions, although models for *A. punctimacula s.s.* had a better fit to the data [area under the receiver operating characteristic curve (AUC): 0.841 and 0.949] than models for *A. albimanus* (AUC: 0.701 and 0.727). SDMs for *A. punctimacula s.s.* suggest that distinct climatic conditions occur between the range of haplogroups A and B (Fig. 2C), while SDMs for *A. albimanus* suggest that most of its range in Costa Rica and Panama is suitable habitat for both haplogroups (Fig. 2D).

These observations are corroborated by identity tests, which found significant niche identity differences between *A. punctimacula* A and B using all test metrics [$I_{obs} = 0.418$, 95% confidence interval (CI): $I_{exp} = 0.910$–0.981, $P < 0.01$; $D_{obs} = 0.418$, 95% CI: $D_{exp} = 0.697$–0.859, $P < 0.01$; $RR_{obs} = 0.460$, 95% CI: $RR_{exp} = 0.701$–0.851, $P < 0.01$]. However, identity tests failed to find a significant difference in niche identity between the two haplogroups of *A. albimanus* ($I_{obs} = 0.993$, 95% CI: $I_{exp} = 0.959$–0.992, $P > 0.99$; $D_{obs} = 0.912$, 95% CI: $D_{exp} = 0.871$–0.907, $P > 0.99$; $RR_{obs} = 0.895$, 95% CI: $RR_{exp} = 0.765$–0.893; $P = 0.98$). Removing 10% of observation points with the lowest modelled suitability values gave a suitability threshold of 0.36 for *A. punctimacula s.s.* and 0.29 for *A. albimanus*. Using these thresholds, the results of our species distribution modelling suggest that there is currently 11 771 km^2 of climatologically suitable habitat for *A. punctimacula s.s.* and 15 070 km^2 for *A. albimanus* (Table 2).

Our models further suggest that the range size and distribution of both *Anopheles* species have varied considerably at several points between the LIG and present time (Fig. 3). Specifically, the LGM is modelled to have been a time of severe range contraction with *A. albimanus* reduced to just 3% of its current range while suitable habitat for *A. punctimacula s.s.* during the LGM is modelled to be just 23 km^2, or less than 0.2% of its current range in Costa Rica and Panama (Fig. 3; Table 2).

Table 2. Modelled range size for two species of *Anopheles* in Lower Middle America (Mesoamerica) during contemporary and palaeontological periods based on ecological niche models

	Area (km^2)
Anopheles albimanus	
Currently suitable	15070
MIDH suitable	18395
LGM suitable	501
LIG suitable	4894
% change MIDH to current	0%
% of MIDH that is also current*	0%
% of LGM relative to current	3.3%
Anopheles punctimacula s.s.	
Currently suitable	11771
MIDH suitable	8034
LGM suitable	23
LIG suitable	13943
% change MIDH to current	0%
% of MIDH that is also current *	0%
% of LGM relative to current	0.2%

LIG, Last Interglacial; LGM, Last Glacial Maximum; MIDH, Mid-Holocene.
*It is also a measure of recent stability.

The expansion of suitable habitat between the LGM and the MIDH was more pronounced for *A. albimanus* than for *A. punctimacula s.s.* Our model suggests that suitable habitat during the MIDH for the former was 18.395 km^2, or 122% of the range size from the current model, whereas the MIDH-modelled habitat was estimated to be 8034 km^2, or 69% of the current range (Table 2). Overall, our models suggest that suitable habitat for *A. punctimacula s.s.* has been more stable than that of *A. albimanus*. For example, 83% of the MIDH range of *A. punctimacula s.s.* is also modelled to be currently suitable whereas only 74% of the MIDH range of *A. albimanus* is also modelled as currently suitable (Fig. 4; Table 2).

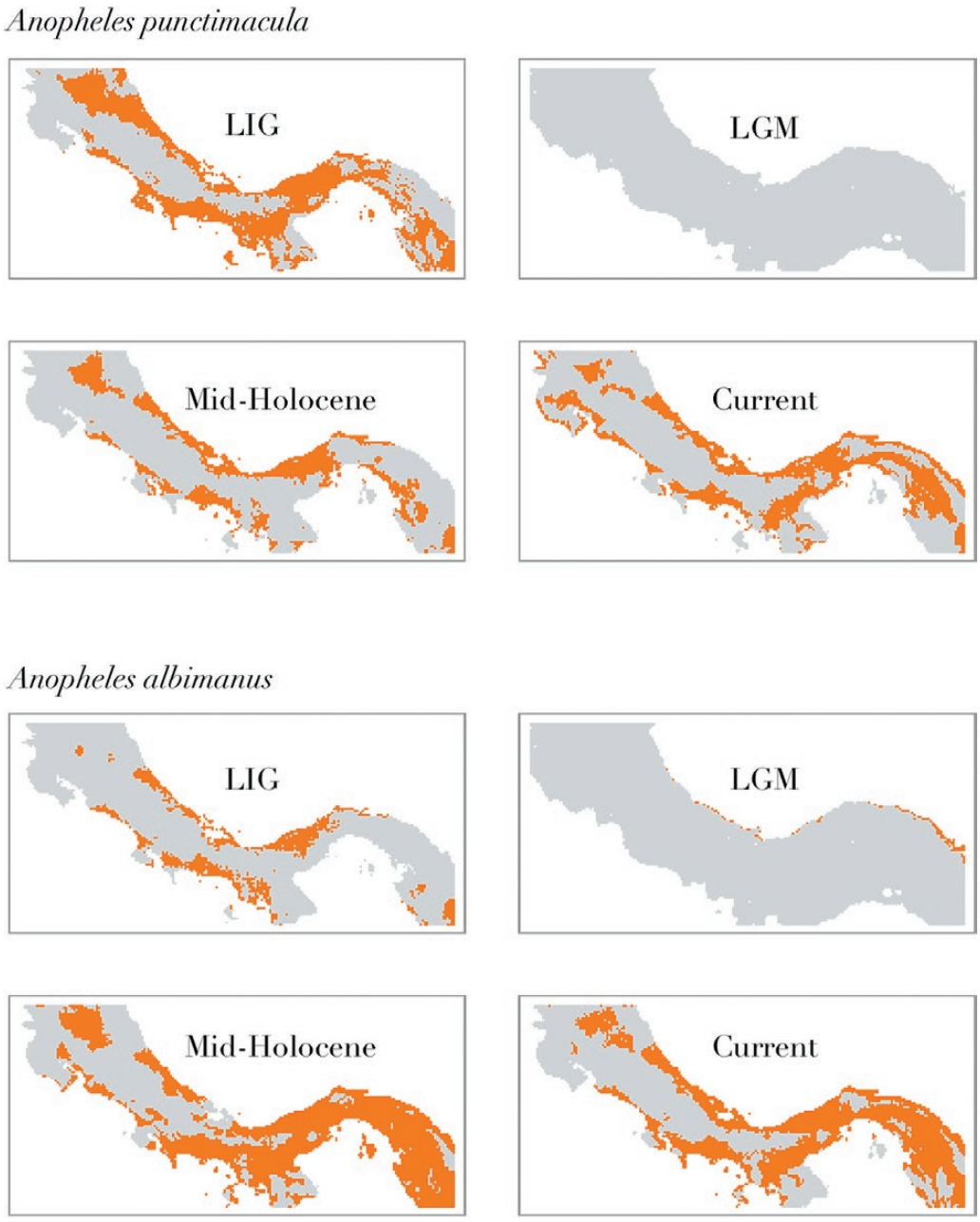

Figure 3. Species distribution models for *Anopheles punctimacula* (top panel) and *Anopheles albimanus* (bottom panel) during three palaeo-geographical time periods available on the WorldClim site plus current conditions: Last Interglacial [LIG, ~120 000–140 000 years before present (ybp)], Last Glacial Maximum (LGM, ~22 000 ybp) and Mid-Holocene (MIDH, ~6000 ybp). For MIDH, nine general circulation model (GCM) simulations are available; for LGM, three GCMs are available; and for LIG, only one GCM is available. Predicted niche ranges across time for these species are represented in orange colour.

DISCUSSION

Phylogeographical studies of the terrestrial fauna of Mesoamerica have suggested a major role for Pleistocene climate oscillations as a driving force for shaping regional biogeographical patterns (Bagley & Johnson, 2014). During the Pleistocene in Mesoamerica, more-or-less continuous lowland tropical forest habitat contracted, resulting in a fragmented mosaic of non-forested and savanna habitats (Piperno & Jones, 2003). These shifting environmental conditions were probably the driving forces of the initial

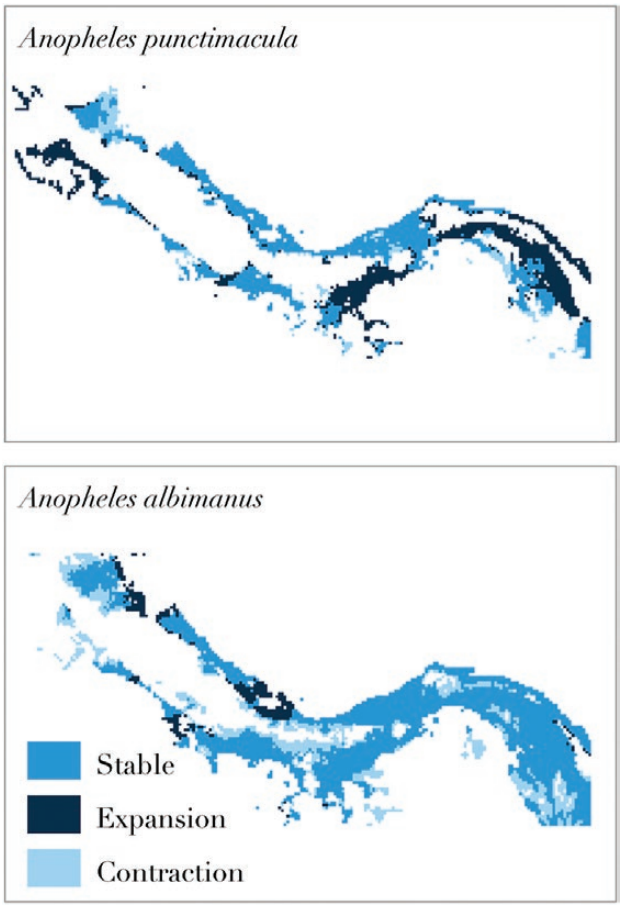

Figure 4. Modelled range size for Mesoamerica *Anopheles punctimacula s.s.* (top panel) and *Anopheles albimanus* (bottom panel) during palaeontological and contemporary periods based on species distribution models (SDMs). Niche dynamics for both *Anopheles* species are generated using averaged model outputs for all GCMs plus the contemporary 10% suitability threshold.

fragmentation of the gene pool for *A. apicimacula* and *A. albimanus*, which is supported by previous studies in *A. albimanus* (Loaiza *et al*., 2010a, b, 2013) and also by the divergence reported here between *A. punctimacula* A and B. Our data further support this notion, as the node separating *A. apicimacula* lineages D and E has a similar age to the node splitting *A. punctimacula* A from B, suggesting a shared period of splitting during the late Pleistocene for potential incipient species within these two ecologically similar taxa.

The impact of the Pleistocene can also be seen in the shared pattern of population structure across Mesoamerica for both *A. punctimacula s.s.* and *A. albimanus*, two important vectors of human malaria parasites. Furthermore, both species also show statistically significant signals of recent population expansion. However, patterns of mitochondrial admixture differ between the two species. Molecular lineages within *A. albimanus* mix broadly in a north-west to south-east gradient over 1 000 km across Costa Rica and Panama, whereas two discrete molecular lineages within *A. punctimacula s.s.* do not mix across this region. Studied independently, the mitochondrial phylogeographical patterns for both species can be explained by Pleistocene climate oscillations that caused fragmented forest refugia in the region. In fact, Loaiza *et al*. (2010a, b) make this argument to explain the phylogeographical pattern for *A. albimanus*.

Are the differences in mitochondrial admixture between *A. albimanus* and *A. punctimacula s.s.* simply the result of stochastic response to Pleistocene climate change? Our model of contemporary and historical distributions of these species suggests not. Although both species encountered reduced habitat availability during the LGM, the extent of range reduction was substantially more severe for *A. punctimacula s.s.* Similarly, recovery from that severe range LGM contraction was not the same: our models indicate that MIDH ecological conditions were less favourable for *A. punctimacula s.s.* than for *A. albimanus*, especially

given that the latter species is a habitat colonist. *Anopheles albimanus* would probably have been able to expand its range rapidly from LGM refugia, relative to *A. punctimacula s.s.*, both because less habitat for expansion was available and also because its forest-inhabiting ecology may make geographical expansion less likely. Our data confirm previous suggestions that Pleistocene climate oscillations resulted in range contractions for *Anopheles* mosquito species around the world (Dusfour *et al.*, 2004, 2007; Mirabello & Conn, 2006; O'Loughlin *et al.*, 2008; Loaiza *et al.*, 2010a, b; Morgan *et al.*, 2011), while suggesting that the magnitude of the effect, as well as the impact on present-day patterns of genetic variation, may depend on the particular ecology of the species in question.

At the same time, our results suggest that the patterns of genetic variation observed in *Anopheles* from Mesoamerica are not solely due to historical effects. We found significant ecological differences between the predicted suitable ranges of lineages A and B of *A. punctimacula s.s.*, whereas we found that both lineage A and lineage B of *A. albimanus* have sufficiently broad habitat tolerances that nearly all the occupied range of this species in Costa Rica and Panama is considered suitable for both lineages. Therefore, we posit that the resulting genetic diversification in Mesoamerican *Anopheles* species is due to a combination of past (i.e. population bottlenecks and geographical fragmentation during the LGM) and contemporary forces (i.e. subsequent niche diversification due to adaptation to different climatic conditions more recently). More specifically, the disparities in the evolutionary trajectories between *A. punctimacula s.s.* and *A. albimanus* are probably due to differences in their larval ecologies. Because the former is more specific in its breeding requirements (i.e. it prefers woody environments where it breeds in permanent and heavily shaded aquatic breeding sites), it needs forest to be able to colonize new areas, whereas *A. albimanus* is more plastic in its larval breeding requirements (i.e. it develops in a wider variety of aquatic breeding sites, including shaded ones, but it does so more generally in short-lived and sunlit habitats), and therefore it can quickly colonize savanna-type vegetation as long as standing water accumulates on the ground.

Another key ecological attribute that might have contributed to the observed evolutionary differences between *A. punctimacula s.s.* and *A. albimanus* is their flight range. For example, the flight range of *A. albimanus* in one night has been estimated to be 1.5–18 km, although studies of this ecological aspect are still inconclusive (Frederickson, 1993). In contrast, the flight range of *A. punctimacula s.s.* is anticipated to be much more restricted, although it has not yet been estimated in the wild. Future studies will need to test for differences in dispersal capabilities between *A. punctimacula s.s.* and *A. albimanus* as another factor probably explaining their distinct evolutionary histories across Mesoamerica.

Our findings have implications not only for understanding the evolutionary trajectory of Mesoamerican *Anopheles*, but also for our understanding of biogeographical patterns in the region. For example, the Talamanca mountain range, which creates a montane barrier separating Caribbean and Pacific lowland habitats in southern Costa Rica and western Panama, has often been suggested as a vicariant barrier responsible for genetic diversification in both vertebrate and invertebrate taxa in Mesoamerica (e.g. snakes: Zamudio & Greene, 1997, Daza *et al.*, 2010; freshwater fish: Bermingham & Martin, 1998; scorpions: Zeh *et al.*, 2003; frogs: Weigt *et al.*, 2005, Crawford *et al.*, 2007, Wang *et al.*, 2008; birds: Miller *et al.*, 2008, 2011). Here we confirm that *A. punctimacula* lineages A and B are separated by the Talamanca mountain range in most of western Panama and southern Costa Rica, suggesting a potential role for this range as a physical barrier to gene flow driving genetic diversification. Surprisingly, however, our results indicate no direct role for this mountain range in keeping *A. punctimacula* A and B from mixing or re-colonizing with their original geographical range. Instead, the lack of genetic mixing appears to be related to the fundamental habitat unsuitability between these lineages in these two areas, as demonstrated in the area across central Panama where no highland habitat interrupts lineages A and B. While the Talamancas' rain shadow may be responsible for the strong seasonality of Pacific lowland habitats, it is fundamentally this seasonal drynessrather than the height of the mountains that is responsible for the lack of haplotype mixing among different geographical regions. We recommend a more thorough evaluation of the role of contemporary population processes versus historical drivers of genetic variation in Mesoamerica, as supported by more recent studies that indicate a profound role of cryptic ecotones for shaping species distributions and biogeographical patterns in this region (Miller, 2014; Miller *et al.*, 2014).

Finally, our findings may have implications for efforts to control malaria in Mesoamerica. First, *A. albimanus* is recognized as the principal malaria vector in this region, and this has historically been attributed to its preference for non-forested habitats with clear, non-flowing breeding sites (Sinka *et al.*, 2010), which typically accompany human settlements. Our results suggest that ecological plasticity and resilience to climatic instability may contribute to its role as the principal vector of *Plasmodium*. Furthermore, it is possible that the two clades of *A. punctimacula s.s.* are at least partially reproductively isolated, and may be better considered as incipient species.

Adaptive introgression across the species boundary has recently been demonstrated for African *Anopheles* species responsible for malaria transmission (Norris *et al.*, 2015). Recently, several studies have shown a primary role of human-assisted movements in explaining *Aedes* mosquitoes range expansions in Mesoamerica (Miller & Loaiza, 2015; Eskildsen *et al.*, 2018). While these are introduced mosquitoes that favour highly anthropogenically modified habitats, and while *A. punctimacula s.s.* is only considered a secondary vector of malaria in Mesoamerica, care should be taken that human movements do not unnecessarily facilitate genomic introgression among *A. punctimacula* lineages, and genome-wide studies of both *Anopheles* species should be conducted across the region.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. 'Species-level occurrence points, geographically trimmed'. Sampling geo-referenced points (latitude and longitude) from Costa Rica and Panama used to construct the species distribution models (SDMs) of *Anopheles punctimacula s.s.* and *Anopheles albimanus*.

Table S2. 'Haplogroup-specific occurrence points, geographically trimmed'.