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# Understanding *Anopheles* Diversity in Southeast Asia and Its Applications for Malaria Control

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Additional information is available at the end of the chapter

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## 1. Introduction

### 1.1. Why study *Anopheles* diversity: Relevance for malaria control

The need to understand diversity in *Anopheles* mosquitoes to win the fight against malaria first became apparent with the paradox of ‘anophelism without malaria’, as it became evident that there is a vast diversity of *Anopheles* species and that not all species transmit malaria [1]. For example, in Europe it was eventually deduced that the mosquito *Anopheles maculipennis* existed as a species complex comprising several species that differed in their breeding, feeding and resting habitats, which resulted not only in differences in malaria epidemiology but also the success or failure of malaria control efforts [2]. This realisation resulted in countless studies around the world to distinguish and characterise *Anopheles* species, often using molecular or chromosomal characters in the absence of reliable morphological characters [3-4]. Such studies have played an invaluable role in improving malaria control and have, in turn, revealed another layer of complexity. This is exemplified most clearly in the *Anopheles gambiae* Complex, which includes several important African malaria vectors. Taxa within the *An. gambiae* Complex can exist as recently diverged species such as *An. gambiae* and *An. arabiensis*, which still have the potential to exchange genes [5]; as incipient species such as the S and M molecular forms, or as genetically divergent locally adapted forms, e.g. adapted to forest or savannah [6]. Recent genomic studies of the *An. gambiae* Complex are revealing patterns of differential divergence and introgression across the genome between species [7-8]; such phenomena are likely to further complicate the definition of species boundaries within *Anopheles* complexes. Differences in characteristics relevant to malaria control may be present at even the subspecific level (e.g. larval habitat and insecticide resistance both within and between the S and M

molecular forms [9-11]), demonstrating the need to understand the generation and maintenance of *Anopheles* diversity at all levels.

This chapter focuses on the need to not only characterise species boundaries, ecology and distributions, but also to understand the potential for divergence and the extent of gene flow within and between species of *Anopheles* in Southeast Asia. Southeast Asia is characterised by having numerous vector taxa and epidemiological settings, and though there has been great progress in reducing malaria in Southeast Asia, it has proved difficult or impossible to completely eradicate in many places, e.g. [12-13]. A complete understanding of transmission dynamics in Southeast Asia and the best approach to interrupt them is complicated by several factors, including intraspecific variation in ecology and vector status across species distributions, potential interactions between species in malaria transmission (i.e. the fact that the vectorial capacity of one species may vary depending on the presence of a second vector species), and by the potential for ongoing gene flow between species. In this chapter, we argue that understanding the complexity and diversity of *Anopheles* species in this region and the nature of isolation, ecological variation and gene flow in driving divergence or homogenising variation within and between them is key to a complete understanding of malaria transmission dynamics and our attempts to interrupt it via vector control. This involves determining the historical processes that have driven diversification to understand both current intraspecific and interspecific variation and the potential for future change (e.g. in adaptation to environmental change) that could affect malaria transmission and/or vector control efforts.

## 2. Diversity of *Anopheles* species across Southeast Asia

This chapter primarily focuses on the diversity of *Anopheles* species in Southeast Asia, which encompasses the geographical area east of India, south of China and west of New Guinea. Southeast Asia is further subdivided into two sub regions: mainland Southeast Asia, comprised of Myanmar, Thailand, Cambodia, Lao People's Democratic Republic, Vietnam and peninsular Malaysia; and insular Southeast Asia, comprised of Indonesia, East Timor, Singapore, East Malaysia, Brunei and the Philippines. However, as many of the vector species found within Southeast Asia, e.g. members of the *An. minimus*, *An. dirus* and *An. subpictus* Complexes, and *Funestus* and *Maculatus* Groups, also overlap into India (particularly northeast India), Sri Lanka and China we have included these regions where relevant in order to achieve a more complete understanding of *Anopheles* diversity in Southeast Asia.

The diversity of Anopheline fauna that exists within Southeast Asia is richer than in any other region of the world [14], and at least 19 species, some of which comprise cryptic species complexes, are known to play some role in malaria transmission [15]. Exactly 50% of the 24 currently recognised *Anopheles* species complexes are found within Asia, which when compared with the 21%, 13%, 13% and 4% found in the Americas, Africa, Australia-Pacific and Europe, respectively, emphasises the complexity of diversity found within the Asian continent [14]. The considerable variation that exists between species in terms of habitat preference and feeding behaviour makes the characterisation of species distributions highly relevant to

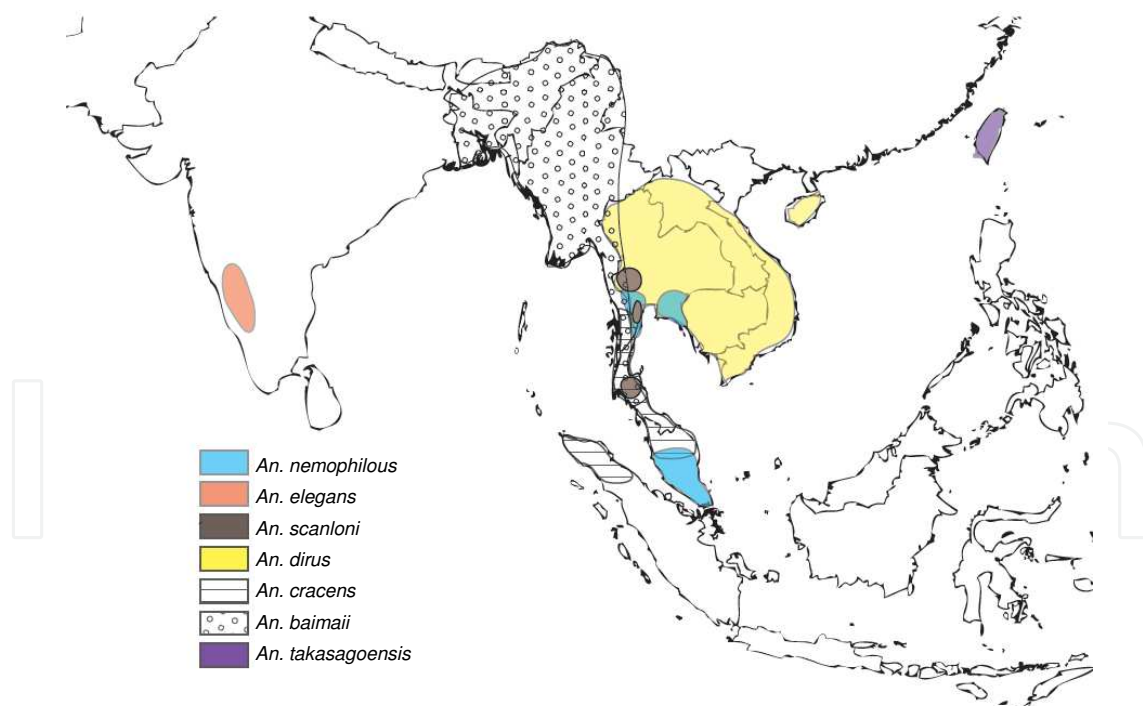
malaria control efforts. Malaria transmission characteristics and the effectiveness of control efforts such as insecticide treated bednets (ITNs), larvicides, and indoor residual spraying (IRS), will depend to a large extent on the vector species present in a given area [14], and since the effectiveness of a given vector species can be influenced by other species present in the region, malaria transmission dynamics also depend on species composition. Hence considerable effort has been focussed on the stratification of malaria units for effectively targeted malaria control, with the ecological characteristics and geographical distributions of species having particular relevance [16]. In this section we discuss the geographical features that appear to define and limit species distributions, and the relevance of this information for malaria control.

Early attempts for a geographical stratification of malaria units [17] were based on the biogeographical realms of Wallace (1876). However, Wallace's Oriental Realm is largely inappropriate for South Asia and Southeast Asia due to the exceptionally high biodiversity and high heterogeneity of spatial distribution of vectors in this region [14-15]. On a smaller spatial scale there are multiple biogeographical subregions within Southeast Asia, including the biodiversity hotspot regions of IndoBurma, Sundaland, the Philippines and Wallacea ([18]; see figure 1). These hotspots were defined in part on the basis of endemism so it is not surprising that they appear to define the distributions of many malaria vectors, with clear patterns of species turnover apparent at each of the biogeographical boundaries.



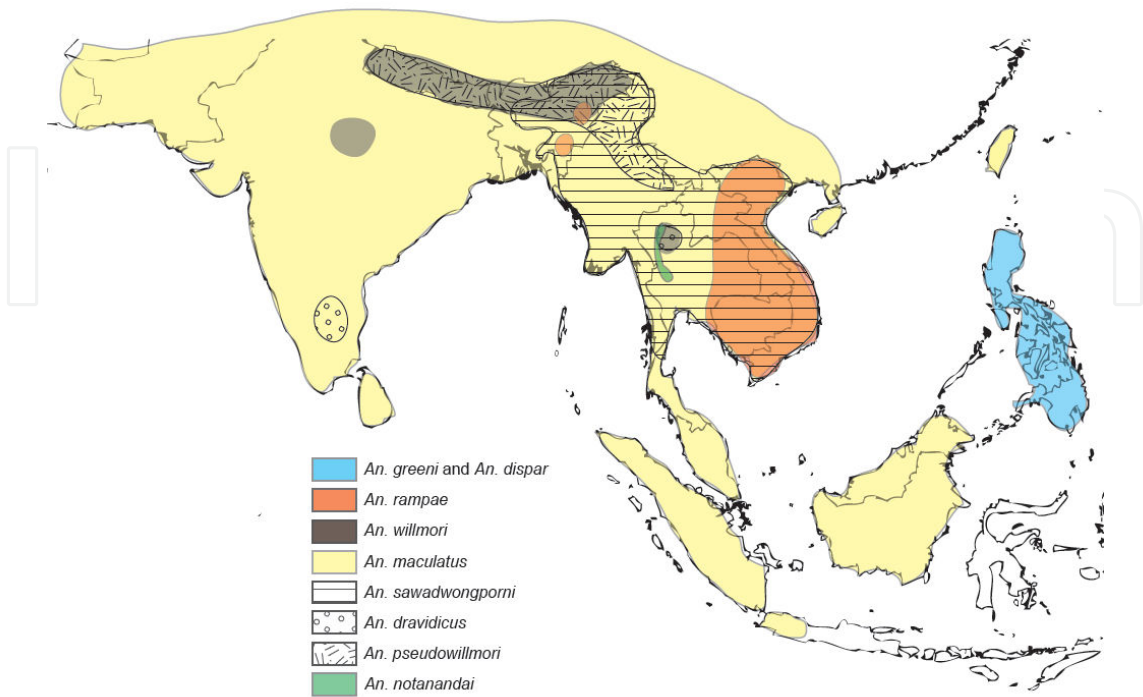
**Figure 1.** Topological map of Southeast Asia, indicating the four main biogeographical zones as defined by Myers *et al.* (2000) [17].

The first biogeographical boundary that shows a clear association with species distributions is that separating IndoBurma from southwestern Asia (Figure 1). It should be noted that northeast India, although politically part of India, is biogeographically and ecologically aligned with IndoBurma rather than southwestern Asia. The *Anopheles* fauna on either side of this boundary is generally distinct, for example several vector species that are distributed across IndoBurma, including *An. baimaii*, *An. sawadwongporni* and *An. maculatus* (Figures 2 and 3), have distributions that extend little further than this western border. The closely related *An. minimus* and *An. fluviatilis* Complexes show largely parapatric distributions that overlap along the western border of IndoBurma, with the distribution of the *An. minimus* Complex being primarily restricted to IndoBurma and that of the *An. fluviatilis* Complex being mostly limited to southwestern Asia (Figure 4).

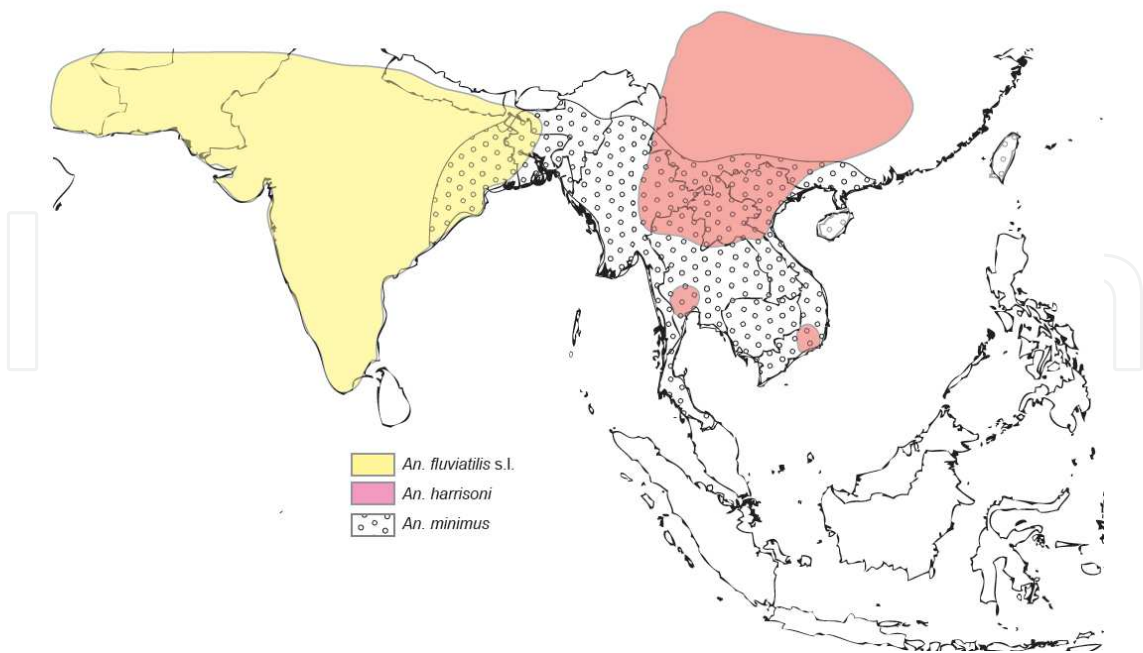


**Figure 2.** The distribution of species within the *Anopheles dirus* Complex.





**Figure 3.** The distribution of species within the Maculatus Group.

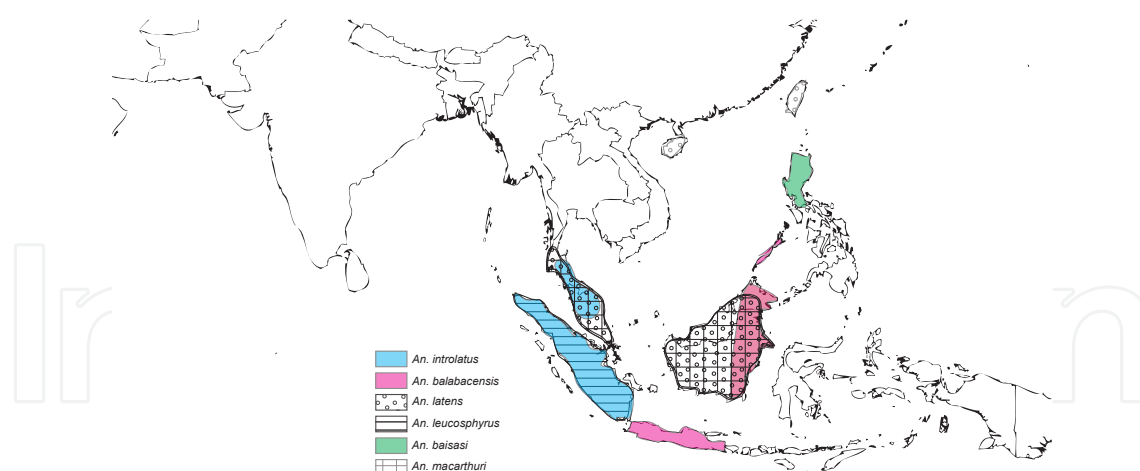


**Figure 4.** The distribution of species within the Minimus Subgroup (which encompasses the *An. minimus* and *An. fluviatilis* Complexes).

The boundary between the biodiversity hotspot regions of IndoBurma and Sundaland (Figure 1) represents a second major biogeographic transition in Southeast Asia, and is characterised by high species turnover in a number of taxonomic groups (e.g. birds, mammals and reptiles [19-21]). This long-recognised biogeographic transition was first noted by Wallace in 1869, and though its exact position along the Thai-Malay Peninsula is debated, with some dispute as to whether the transition occurs at the Isthmus of Kra (10°30'N) or the Kangar-Pattani line (6-7°N) further south [22], its biogeographical significance is unquestioned. The transition is associated with dramatic climate and phytological changes. IndoBurma has a very seasonal climate in terms of both temperature and rainfall, whereas that of Sundaland is much more stable, with precipitation levels remaining high throughout the year. Whereas mixed moist deciduous forest is the dominant forest habitat type of IndoBurma, that of Sundaland is perhumid evergreen forest [23-24]. Thus it seems unsurprising that this is a region of high species turnover, as the selective pressures on either side of the Isthmus of Kra biogeographic transition would differ considerably, potentially driving rapid adaptive change and subsequent ecological speciation following the dispersal of taxa from one side to the other.

Again, the majority of *Anopheles* species are limited in distribution to either side of the IndoBurma-Sundaland biogeographical transition. Within the Leucosphyrus Group (which encompasses both the *An. dirus* and *An. leucosphyrus* Complexes), for example, *An. baimaii* and *An. dirus* are found to the north of this biogeographical boundary whereas many other species in the Leucosphyrus Group occur only to the south, with many species spanning from the mainland of peninsular Malaysia into the major islands e.g. *An. macarthurii*, *An. cracens*, *An. introlatus* and *An. latens* (Figures 2 and 5). Again, the major vector species of the *An. minimus* Complex, *An. minimus* and *An. harrisoni*, are limited in distribution to IndoBurma, as are the majority of species within the Maculatus Group (Figures 3 and 4). Although there does appear to be species turnover between the mainland and each of the islands (e.g. *An. nemophilous* is found within peninsular Malaysia but on none of the islands (Figure 2); *An. leucosphyrus* is found only on Sumatra (Figure 5)), several species are found on more than one of the major landmasses but are limited to only one of the biogeographical zones (e.g. *An. balabacensis* is found on both Borneo and Java). This suggests that whilst sea barriers play a role in limiting dispersal, the mainland biogeographical transition is clearly important in limiting species distributions despite the lack of such an obvious physical barrier.

The final distinct biodiversity hotspot regions of Southeast Asia are those of Wallacea and the Philippines, each of which harbours a unique assemblage of *Anopheles* species. Although separated from Borneo by only a narrow sea barrier, the Philippines are thought to share few of the major vector species of Southeast Asia. The Minimus Subgroup (which comprises the *An. minimus* and *An. fluviatilis* Complexes) appears not to have colonised the Philippines, and the species within both the *An. leucosphyrus* Complex and the Maculatus Group found in the Philippines (*An. baisasi*, and *An. greeni* and *An. dispar*, respectively) are limited in distribution to these islands (Figures 3 and 5). *An. balabacensis* provides somewhat of an exception, being found on both Borneo and within the Philippines, although its distribution within the Philippines is limited to the small, western islands between Borneo and the major Philippine Island of Luzon (Figure 5). *Anopheles annularis* s.l., on the other hand, is distributed within the Philippines as well



**Figure 5.** The distribution of species within the *Anopheles leucosphyrus* Complex and *Anopheles macarthurii* of the Leucosphyrus Group

as throughout mainland and insular Southeast Asia, although the limited available evidence suggests that the Philippine populations of this species show strong differentiation from those in other regions of Southeast Asia [25]. As a result of the described species turnover patterns, the subregions differ in terms of major malaria vectors, with the *An. dirus* and *An. minimus* Complexes, and Maculatus Group dominating throughout IndoBurma, the *An. leucosphyrus* Complex dominating within the Sundaic Region, and *An. flavirostris* being the main malaria vector within the Philippines and a major malaria vector within Indonesia [15].

In addition to the divisions between the biogeographic regions discussed above, there are some apparent transitions within biogeographic regions. As previously discussed, there is some distinction between the species composition of each of the major Sundaic Islands and the mainland, although several species within the *An. dirus* and *An. leucosphyrus* Complexes are found on more than one of the landmasses. An apparent distinction in species composition between the landmasses is seen in other taxa from shrike babblers [26] to macaques [27]. Besides this pattern, there is also an apparent distinction within IndoBurma, between the distribution of genetic diversity east and west of the Thai-Myanmar border. The closely related sister species *An. dirus* and *An. baimaii* have parapatric distributions within Southeast Asia, which overlap along this border region (Figure 2). *An. sawadwongporni* and *An. rampae* are a second pair of sister species that show a similar pattern, with *An. rampae* having a primarily easterly distribution, which extends from eastern Thailand towards Vietnam and does not overlap the Thai-Myanmar border (Figure 4). *An. rampae* has, however, recently been recorded at low frequency within northeastern India, suggesting the distribution and population structure of this species warrant further attention [28]. The Thai-Myanmar border region is also the site of a suture zone between highly divergent intraspecific lineages within species including *An. splendidus*, *An. minimus* and *An. annularis* [29]. The patterns in species distribution discussed throughout this section, with closely related species often falling on either side of biogeographical divisions that lack obvious geographical barriers, clearly indicate a role for vicariance and/or ecology in generating biodiversity within Southeast Asia, as will be discussed later in this chapter.



Although the distributions of the majority of *Anopheles* taxa appear to be defined by biogeographical boundaries, there are some taxa with relatively wide distributions that span many of the biogeographic subregions discussed above. For example, *An. maculatus* is distributed throughout Nepal, Pakistan, Bhutan and India and throughout the IndoBurma (including Taiwan) and Sundaic Regions of Southeast Asia, and *An. vagus* has a similar distribution throughout India, IndoBurma and the Sundaic Region. These species appear to be largely panmictic throughout their distributions [29-30], suggesting an ability to combine high dispersal capacities with generalist habitat requirements.

The distinctiveness of the Anopheline fauna of each of the major biogeographic regions of Southeast Asia, which occurs despite the continuity of landmass between these regions, suggests that ecological factors, such as climate and dominant habitat type, play a key role in defining species distributions. Malaria stratifications based on ecological biomes, such as forest, foothill and urban regions, are therefore especially useful in designating control efforts [16]. The clear ecological similarity between many closely related vector species also suggests a strong conservation of ecological niche. Species within the *An. dirus* and *leucosphyrus* Complexes, for example, show a strong association with forest habitat [31-33]. Thus in the IndoBurma and Sundaic Regions, where species within these complexes are distributed, malaria is often most prevalent in villages that are in close proximity to the forest fringe, and people involved in forest activities are often most at risk [16]. Species within the Minimus Complex, on the other hand, are prevalent within foothill regions and generally breed in slow running streams [31, 33-34], leading to the designation of a 'foothill' malaria stratification. The brackish water tolerant species *An. sundaicus* and *An. epiroticus*, which are also major vectors of malaria throughout Southeast Asia, dominate malaria transmission in coastal regions [35-37]. Thus the characterisation of species relationships, ecology and distributions has clearly facilitated great improvements to malaria control efforts. However, understanding of malaria transmission dynamics is still complicated by the potential for interactions between vector species, variation in vector capacity across a species range, and remaining taxonomical confusion in some groups (e.g. the *An. culicifacies* Complex) (reviewed in [33]). Thus the previously discussed high diversity of cryptic species within Southeast Asia may be one of the factors making malaria difficult to eliminate in parts of Southeast Asia.

### 3. Processes driving the diversification of the Anopheline fauna of Southeast Asia

#### 3.1. The role of historical environmental change

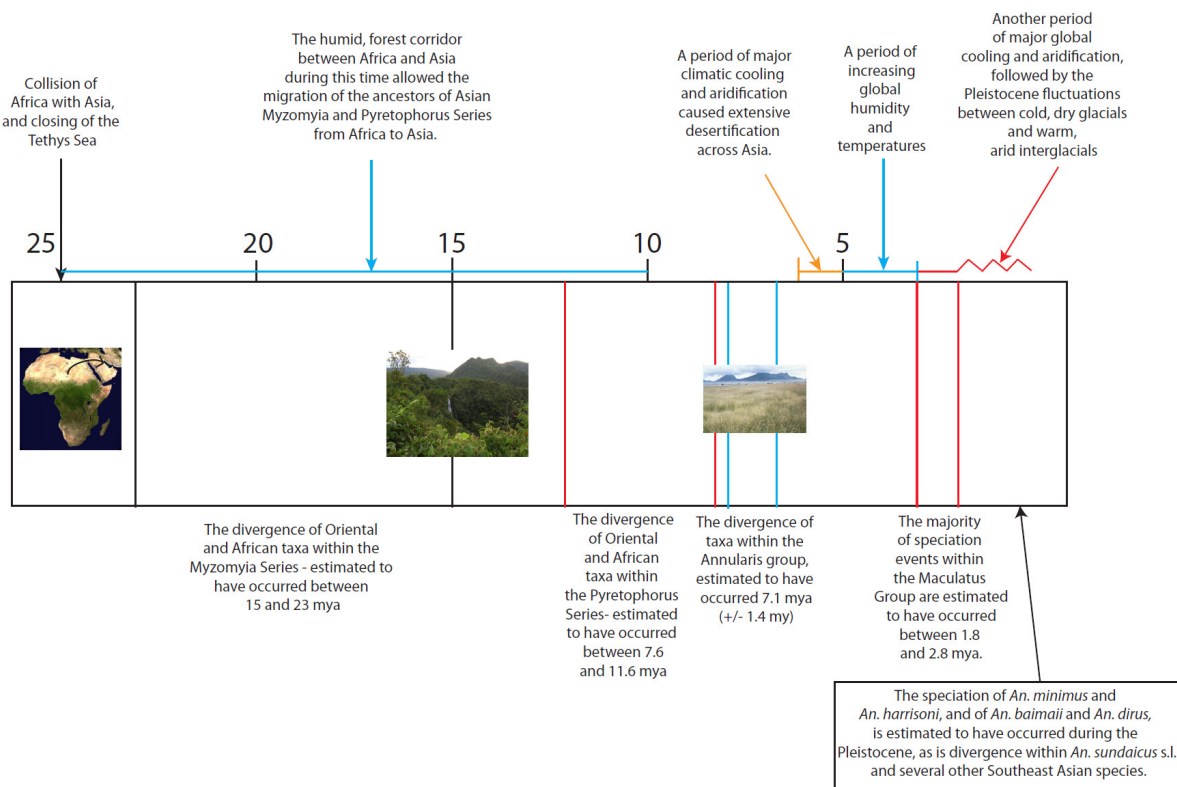
As discussed in the first section of this chapter, as well as an understanding of extant species distribution and ecology, the characterisation of population dynamics and levels and patterns of gene flow both within and between species is essential, as the effective size and connectivity of populations will influence the speed at which traits relevant to malaria control evolve and spread between them [38]. The release of genetically modified mosquitoes has been proposed for the control of vector populations in Africa [39]; if such approaches were developed for

Southeast Asia, population genetic studies would be necessary to determine the number of genetically modified individuals and release sites needed for a successful program [39-40]. The estimation of levels of contemporary gene flow is greatly complicated, however, by the historical genetic structuring of mosquito populations [41-42]. In order to reliably infer patterns of contemporary gene flow, it is therefore essential that we first gain a thorough understanding of the population history of the *Anopheles* fauna.

As with all organisms, the genetic structuring of *Anopheles* populations through time is likely to have been greatly impacted by the influence of geographical features on patterns of gene flow and dispersal. Geographical barriers such as mountains, rivers or sea can restrict or prevent gene flow between populations, so causing them to become increasingly differentiated from one another due to the processes of neutral genetic drift and differential natural selection [38]. Many of the *Anopheles* taxa of Southeast Asia, including those within the *Minimus* and the *Leucosphyrus* subgroups and the *Maculatus* Group, are forest associated [31]. Hence for these taxa, expanses of open habitat such as grassland or savannah can constitute an important barrier to gene flow and dispersal. In the absence of gene flow, reproductive barriers may accumulate between isolated populations and cause allopatric speciation [43]. Geographical barriers can shift over time, leading to patterns of repeated expansion and contraction in the ranges of species constrained by them. The biogeographical history of Southeast Asia is especially dynamic, featuring tectonic activity [44], substantial sea-level fluctuations, large shifts in the region's landmass configuration [45], and climate-associated fluctuations in the distribution and extent of forest habitat [46-47]. The time-line below indicates the major biogeographic events inferred to have influenced Anopheline diversification from the mid-Miocene onwards (see figure 6).

### 3.1.1. Miocene (23.0 – 5.3 mya): Dispersal of *Pyrethrophorus* series and *Myzomyia* series from Africa to Asia

The collisions of the Indian, African and Australian plates with Eurasia all had substantial impacts on the landscape and fauna of Southeast Asia. India initially collided with Southeast Asia approximately 50 million years ago (mya), and the subsequent northwards push of the Indian plate resulted in the formation and uplift of the Himalayas [44], forming a geographical barrier between Southeast Asia and the rest of the Asian continent. The second major period of tectonic activity, which involved the uplift of the Himalayas approximately 25mya, coincided with the collision of the African and Eurasian plates. This latter event resulted in the closure of the Tethys Sea and so created a land connection between the continents of Africa and Asia [48]. Although this region is now characterised by arid desert habitat, a corridor of tropical forest is thought to have persisted during the humid periods of the early and mid-Miocene [48]. Combined with low sea-levels, this allowed forest taxa such as the ancestors of the Oriental *Myzomyia* and *Pyrethrophorus* Series to disperse from their African origins into Southeast Asia [49-50]. Increasingly arid conditions and the consequent desertification of East Asia during the late Miocene (6.2 – 5mya) restricted this exchange [48, 51], effectively isolating the forest fauna of Asia and Africa. The Oriental and African taxa within the *Myzomyia* and *Pyrethrophorus* Series form monophyletic groups in both cases (with the exception of the



**Figure 6.** Timeline showing the major biogeographic events inferred to have driven speciation and divergence in the Anopheline fauna of Southeast Asia.

placement of the African species *An. lesoni* within the Oriental Myzomyia clade), and are estimated to have diverged during the late Miocene [49-50]. This suggests that dispersal from Africa to Asia occurred during the humid mid Miocene in both cases, and was followed by the isolation of Asian and African lineages after the late-Miocene expansion of desert across East Asia (Figure 6). As *Anopheles* species rely on water bodies for their larval habitats, desert habitat is likely to pose an extremely effective barrier to dispersal. The close relationship of the African species *An. lesoni* with the Oriental Myzomyia species, from which it is estimated to have diverged just 2-3 mya, is somewhat of a mystery, and suggests some faunal exchange during the mid Pliocene despite the dominance of desert habitat throughout East Asia [49].

### 3.1.2. Late Miocene and Pliocene (6 – 2mya): Forest fragmentation drives allopatric speciation

The increasingly cool and arid climate responsible for extensive desertification across East Asia during the late Miocene also resulted in the expansion of grassland and savannah habitat across Southeast Asia [52]. The consequent reduction in available *Anopheles* larval habitats likely to have occurred during this time, and the potential consequent fragmentation and isolation of populations in allopatry, is hypothesised to have driven late Miocene speciation (dated to 7.1 mya +/- 1.4 my) within the Neocellia Series Annularis Group [25] (Figure 6). This trend of increasing aridification was reversed during the early Pliocene (5-2.8 mya), which was characterised by increasingly warm and humid conditions, with global temperatures reaching approximately 3°C above current temperatures [53-54]. Tropical forest would have expanded

across Southeast Asia during this period, and *Anopheles* habitats would have been more abundant and widespread. A subsequent major climatic transition towards a substantially cooler and more arid climate began approximately 2.8 mya, and culminated in the first of the Pleistocene glacial maxima, 1.8 mya [55]. Once again, tropical forest habitat would have been replaced by large areas of grassland and savannah, fragmenting and isolating populations of forest-dependent *Anopheles* species across Southeast Asia. The consequent divergence of populations in allopatry is thought to have driven speciation within the forest-associated Maculatus Group [25], with contemporary species distributions in this group being fairly distinct (although exhibiting large areas of overlap), and the majority of speciation events dating to within the 2.8-1.8 mya period of major climatic cooling (Figure 6).

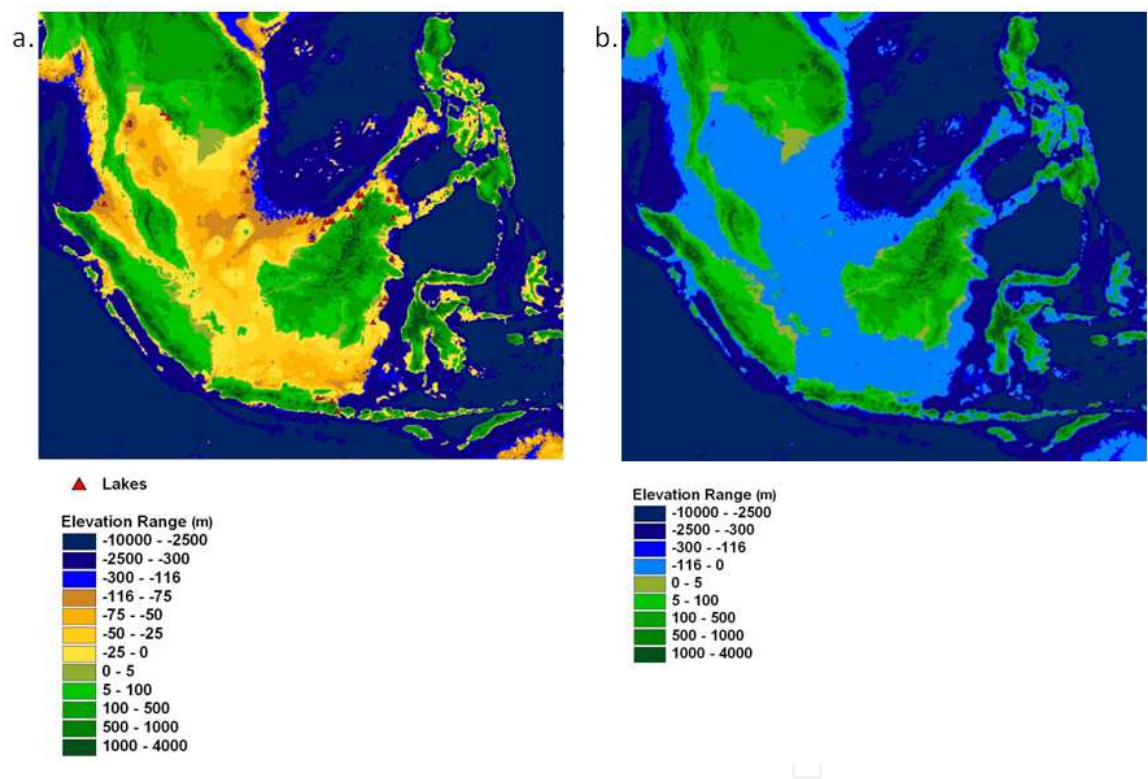
### *3.1.3. Pleistocene (1.8 mya – 11,000 ya): Changes in landmass configuration drive dispersal and divergence within species*

During the Pleistocene, the ongoing fluctuations in the extent of forest cover across Southeast Asia were exacerbated by the dramatic impact of glacio-eustatic sea level change on the region's climate [45-46]. These sea-level fluctuations, which involved drops of between 50 and 200 meters during each of the Pleistocene glaciations [56], had a more dramatic effect on the climate and habitats of Southeast Asia than those of any other tropical region [46]. Sea level regressions of 60 meters or more result in the exposure of the Gulf of Thailand, and dramatically reduce the surface area of the South China Sea [45] (Figure 7). This reduction in the surface area of ocean across Southeast Asia would have reduced evaporation from the ocean's surface, and consequently the levels of moisture carried across the mainland by the monsoon rains. Due to the coincidence of periods of reduced sea level with glacial maxima, the reduction in the monsoon moisture content would have been exacerbated by the cool temperature and consequently reduced moisture-carrying capacity of the air [46]. The distribution of forest across Southeast Asia was in turn affected by the reduced precipitation levels, as regions with sufficient moisture to support them shrank [47, 57]. Reconstructions of the dominant habitat types across Southeast Asia during the Last Glacial Maximum (LGM), which are based on palynological and sedimentological data, indicate that tropical forest became restricted to small and isolated pockets, often at intermediate altitudes and at the base of mountains, where precipitation runoff ensured moisture levels remained high enough to support it [58-59]. Substantial areas of forest habitat were replaced by grassland and savannah, although larger areas of forest are thought to have persisted in insular relative to mainland Southeast Asia [47, 57].

The reduction of forest habitat to small and isolated patches would have resulted in the fragmentation of forest-associated *Anopheles* populations, and their subsequent divergence in allopatry through genetic drift and differential local adaptation (see figure 8). The repeated climatic fluctuations during the Pleistocene are thought to have led to repeated cycles of forest fragmentation during the cool and arid glacial periods, and expansion during the warm and humid interglacials. This would have caused associated repeated cycles of *Anopheles* population range reduction and fragmentation, and subsequent divergence of populations in allopatry, followed by range expansion and secondary contact between the now genetically differentiated populations. The 'refuge hypothesis' of Haffer [52] was originally put forward to



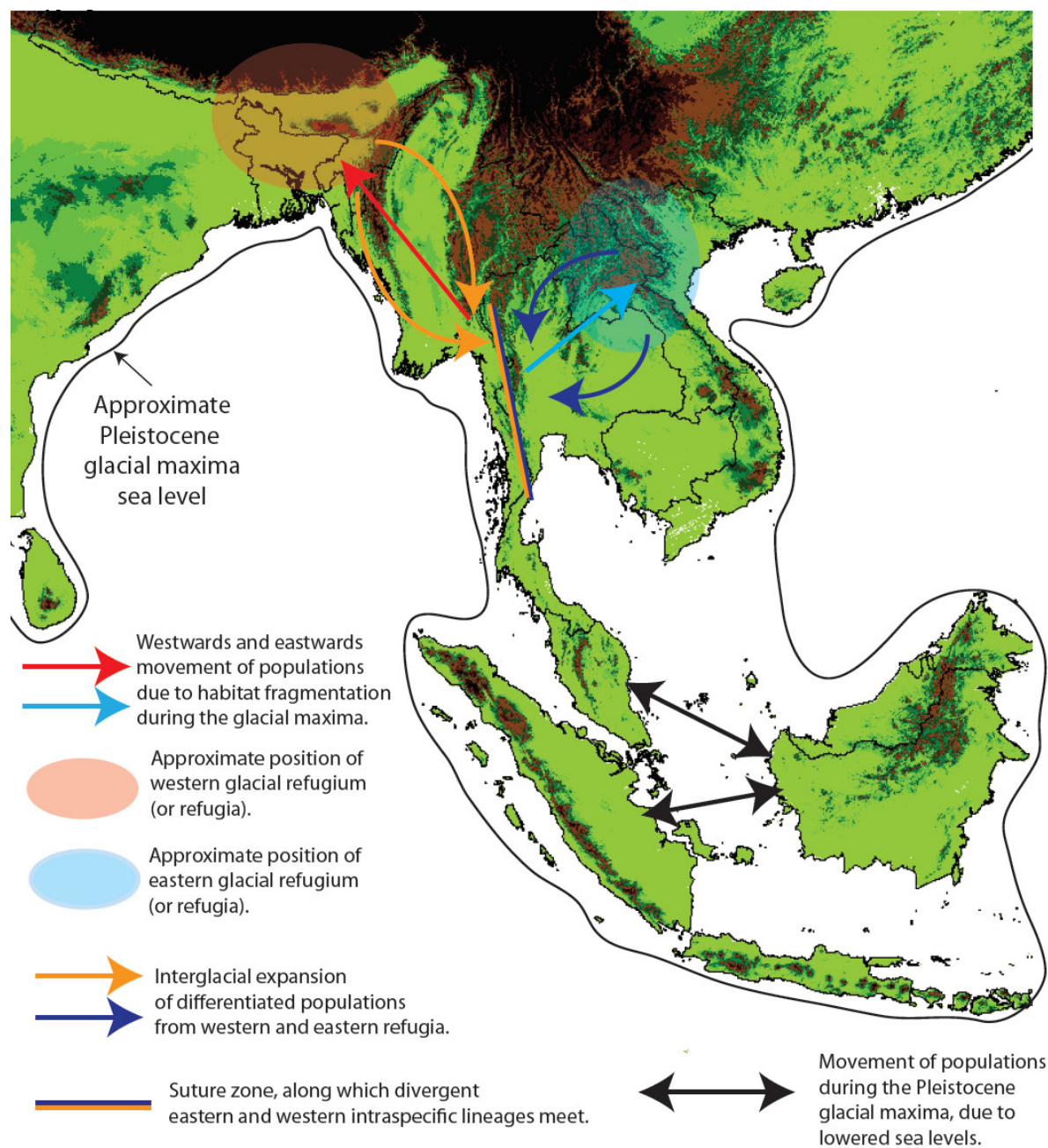
propose a scenario of increased allopatric speciation driven by such repeated cycles of population divergence during periods of major climatic fluctuation such as that characterising the Pleistocene. This hypothesis has since been frequently discussed in the literature and often contested as an explanation for Pleistocene tropical diversification events, due to evidence that speciation in tropical taxa generally predates the Pleistocene, and that forest habitat was not reduced in tropical regions to the extent originally thought [60-62]. As previously discussed, however, the biogeographical changes within Southeast Asia during the Pleistocene were more severe than in other tropical regions, due to the substantial impact of the sea level changes on the region's climate [45]. The likelihood of allopatric speciation driven by such biogeographical change could therefore be expected to be greater. Indeed, speciation dated to within the Pleistocene has been inferred in both the forest-dependent *Leucosphyrus* Group [63-64] and the *Minimus* Subgroup [49], as well as the coastal *An. sundaicus* Complex [65], and has been attributed to the repeated isolation of populations following the reduction of forest habitat and on sea-level fluctuations, respectively, across mainland Southeast Asia during glacial periods [25, 49].



**Figure 7.** Maps showing the IndoBurma and Sundaic Regions of Southeast Asia, a. 21 kya, the Last Glacial Maximum (LGM), when sea levels were 116 m below the current level, and b. 6.07 kya, when sea levels were the same as at present. Figures taken from [66]).

The evidence for allopatric speciation associated with Pleistocene environmental change is especially strong between the cryptic sister species *An. dirus* and *An. baimaii*, which are classified within the *An. dirus* Complex of the *Leucosphyrus* Subgroup. As discussed in the previous section, these species are major malaria vectors throughout mainland Southeast Asia, and have a parapatric distribution that overlaps along the Thai-Myanmar border. Although





**Figure 8.** The influence of Pleistocene climatic change on *Anopheles* diversity within Southeast Asia.

characterisation of their divergence is complicated by mitochondrial introgression and consequent widespread haplotype sharing between the species [42, 67], application of an isolation-with-migration model to data from three nuclear genes supported their divergence within the last 1.5 my of the Pleistocene [63]. The east-west divide between the distributions of these species suggests that their common ancestor was restricted to habitat fragments in the west and east of the Southeast Asian mainland, and that the subsequently differentiated lineages expanded from these restricted distributions during the warm and moist interglacials to meet along the Thai-Myanmar border (figure 8) [63].

Although the above examples provide exceptions, the majority of speciation events within the Anopheline fauna of Southeast Asia are estimated to pre-date the Pleistocene [25, 30, 49], and the environmental fluctuations of the Pleistocene appear to have been much more influential in driving divergence and shaping population structure within, rather than between, *Anopheles* species. Patterns of genetic divergence between largely allopatric eastern and western lineages, and signals of Pleistocene population expansion, have been reported within several *Anopheles* species (e.g. *An. minimus* [68]); *An. annularis* and *An. splendidus* [25]). These patterns have generally been attributed to the restriction of populations to isolated forest 'refugia' during the glacial periods, and expansion from these regions during the interglacials (Figure 8). Chen *et al.* [68] investigated this hypothesis further in the forest-associated *An. minimus*, using a modelling approach to compare the hypotheses of a single panmictic population, a stable but spatially structured population, and past fragmentation into eastern and western refugia followed by growth and range expansion. The latter hypothesis was strongly supported, providing further evidence for an evolutionary history shaped by Pleistocene climatic change [68].

Such an influence of Pleistocene climatic change might be expected to be shared across multiple forest-dependent taxa. This hypothesis has been statistically evaluated in several *Anopheles* species, which exhibit varying degrees of forest-dependency, using a comparative phylogeographical approach [29]. Simultaneous divergence of eastern and western lineages within four *Anopheles* species (*An. annularis*, *An. splendidus*, *An. minimus* and *An. maculatus*), dated to the mid-Pleistocene and attributed to the similarly-timed restriction of populations to allopatric forest refugia, was strongly supported. Patterns of isolation in allopatry followed by secondary contact across the ranges of these species resulted in the formation of a common suture-zone along the Thai-Myanmar border [29]. Various hypotheses of Pleistocene demographic history were further evaluated using a spatially explicit modelling approach, in which the simulation of demographic and spatial expansions, incorporating environmental information, is followed by the generation of simulated genetic datasets through coalescent theory [69]. Comparison of real to simulated datasets best supported scenarios in which populations were restricted to allopatric eastern and western refugia, before expanding their ranges during the warm and moist interglacials, in all seven species examined (*An. aconitus*, *An. philippinensis*, *An. maculatus*, *An. sawadwongporni*, *An. annularis*, *An. baimaii*, and *An. minimus*). Similarly timed population expansions dating to the mid-Pleistocene were inferred in all species, further supporting this scenario [29]. Hence there is substantial evidence supporting a common role of historical environmental change in driving vicariance, and shaping the intraspecific population structure that we see today.

Besides driving divergence between isolated populations, the restriction of populations to refugial regions is also likely to have influenced patterns of genetic diversity across the landscape. The long-term persistence of populations within refugial regions leads to the accumulation of high genetic diversity and population structure. Since only a fraction of the gene pool is generally involved in range expansion, regions that are repeatedly re-colonised following local extinction are expected to harbour substantially lower genetic diversity [70-71]. These predicted patterns can be used to identify potential refugial regions, and in Southeast

Asia have led to the identification of the mountainous regions of northeastern India, northern Myanmar, northern Thailand, southern China and northern Vietnam as potential Pleistocene glacial refugia for *Anopheles* mosquitoes [25, 29, 42, 68, 72]. Indeed, mountain foothills are the most likely regions to support the persistence of forest habitat during cool and arid climatic periods, due to the interception of precipitation by the mountains surrounding them [46]. The prediction and characterisation of these historically driven patterns, of high diversity and spatially structured populations within formal refugial regions and more homogeneous populations in more recently colonised regions, is important if contemporary levels of gene flow are to be reliably estimated and used to predict malaria transmission dynamics.

Although the majority of main *Anopheles* malaria vectors within Southeast Asia show a strong association with forest habitat, this is not true of all species. The influence of historical environmental change on species such as *An. vagus* and *An. sundaicus*, which typically inhabit open habitat and coastal habitat [31, 37, 73], respectively, are likely to have differed substantially from the effects on forest-associated species discussed above. Relative to the majority of forest-associated species, *An. vagus* shows relatively little population structure, and appears to be a single, widespread and highly diverse species that is distributed throughout the biogeographic realms of IndoBurma, Sundaland and the Philippines. The expanse of the open grassland habitat favoured by this species throughout much of the Pleistocene is thought to have facilitated gene flow and dispersal, maintaining population connectivity and homogenising population genetic structure [30]. The Pleistocene evolutionary history of the coastal species *An. sundaicus*, meanwhile, is likely to have been influenced by changes to the landmass configuration, as is discussed below. This illustrates the importance of taking species ecology into account when predicting patterns of historical intraspecific genetic structure across a landscape.

#### 3.1.4. The formation of land-bridges and consequent creation and destruction of dispersal routes during the Pleistocene

Besides substantially influencing climatic conditions across Southeast Asia, the alterations in landmass configuration during the Pleistocene also had a considerable effect on the availability of migration routes across Southeast Asia. The Sunda Shelf is thought to have been dominated by grassland and savannah habitats during periods of exposure, and thus was important in allowing the exchange of open-habitat species such as early hominins and hoofed mammals between the mainland and the Sundaic Islands [56, 74]. Although the open habitat is thought to have acted as a barrier to dispersal of forest-associated taxa between Borneo and Sumatra, the persistence of gallery forests along the major river systems of the Sunda Shelf is thought to have provided narrow dispersal corridors for such taxa [74]. The repeated exposure and submergence of the Sunda Shelf is thought to have promoted allopatric speciation in a number of Sundaic taxa, with periods of dispersal facilitated by the exposure of the Sundaland bridge being followed by the isolation of populations on different landmasses as sea levels rose, e.g. [26, 75]. Although as previously mentioned, there is some species turnover within *Anopheles* between each of the islands and the mainland, several species of the *An. leucosphyrus* Complex are found on more than one land mass. This suggests that the intermittent presence of forest



corridors between the mainland and insular regions during the Pleistocene was sufficient to allow some dispersal and gene flow between current land masses [64].

Inferred speciation events within the *An. sundaicus* Complex have also been attributed to patterns of dispersal and isolation driven by the Pleistocene exposure and submergence of sea barriers, with the subsequent isolation and divergence of the nominal species *An. sundaicus*, *An. sundaicus* E and *An. epiroticus* within Borneo, Sumatra and Java, and mainland Southeast Asia, respectively [65]. These species designations have since been disputed, however, and evidence supporting the existence of only a single, widespread species within the *An. sundaicus* species Complex was presented after more intensive sampling, sequencing of additional markers, and more comprehensive analysis [50]. An alternative scenario of Pleistocene evolutionary history was also presented for this littoral species. Although the current species distribution extends along the coast of mainland Southeast Asia, with the Thai-Malay Peninsula coast connecting that of southern Thailand with Cambodia and Vietnam [31, 37], the exposure of the Sunda Shelf would have eliminated habitat availability through the Gulf of Thailand and isolated populations on the east and west of the glacial insular landmass (Figure 7). This would have limited gene flow between the current coastal regions of Thailand, Cambodia and Vietnam, and facilitated dispersal between the mainland and insular regions. The detection of allopatric eastern and western mitochondrial and nuclear genetic lineages within *An. sundaicus* s.l., the closer relationship of Vietnamese populations with populations from Borneo and Indonesia than with those from Thailand and Myanmar, and the detection of Pleistocene gene flow between Borneo and Vietnam, and between Indonesia and the mainland, strongly support the influence of sea-level changes on the dispersal and population genetics of *An. sundaicus* s.l. [37, 50], although evidence suggests speciation has not resulted in this case.

### 3.2. Ecological factors

The rich diversity of habitat types and host species available within Southeast Asia is likely to have driven differential local adaptation leading to divergence between ecologically isolated populations and consequent ecological speciation [43]. Characterisation of the bionomics, habitat and feeding preferences of vector species, and of interspecific and intraspecific variation in these traits, is an important step in defining appropriate vector control strategies. Additionally, through the relation of species biology and ecology to phylogenetic relationships we may infer the ecological adaptations that are likely to have driven divergence and speciation, and given rise to the most effective malaria vectors within Southeast Asia. This may also give an indication of the characters that are evolutionarily labile and those that show niche conservatism, which may allow the prediction of how species may respond to anthropogenic change such as urbanisation and an expansion of agriculture. The *Leucosphyrus* Group provides one example of ecological differentiation between closely related species. This group includes several important vectors of both human and simian malaria, and due to its medical importance, has been well characterised in terms of taxonomy, phylogeny and ecology ([76]; reviewed in [33] and [32]). The mapping of species feeding preferences onto a phylogenetic tree supported two independent host-switching events, each leading to the evolution of

anthropophilic taxa from their zoophilic ancestors, which fed on non-human primates in the forest canopy [64]. This switch in host preference is likely to have involved a change in behaviour, from feeding in the forest canopy to feeding on the forest floor, as well as changes in host detection. This host switch was estimated to have occurred during the late Pliocene/early Pleistocene, which has important implications for human evolution, suggesting that hominins were present within Southeast Asia as early as 2.2 million years ago (mya), and that their arrival shaped the evolution of malaria vectors [64].

As well as the change in host preference, several other ecological adaptations are likely to have driven divergence within the Leucosphyrus Group. The distribution of the group overlaps the biogeographical transition zone that lies between IndoBurma and Sundaland (figure 1;[21]), with the majority of species being limited in distribution to the region either south, or north, of this divide. All basal species are limited in distribution to insular Southeast Asia, suggesting that this region represents the group's ancestral origin [64]. Despite the existence of several species within peninsular Malaysia only two northwards dispersal events into IndoBurma were supported, suggesting that this dispersal required some kind of ecological adaptation. It has been suggested that this may have involved an adaptation specific to the more seasonal climate of Southeast Asia, such as the increased resistance of larvae to desiccation observed in *An. dirus* and *An. baimaii* [32, 64]. Whatever the nature of the ecological adaptation, it is likely to have driven divergence between Indo-Burmese and Sundaic taxa, facilitated the spread of the Leucosphyrus Group throughout mainland IndoBurma, and maintained the distinction between Indo-Burmese and Sundaic species assemblages.

All species within the Leucosphyrus Group show a strong association with tropical forest habitat and are remarkably similar in terms of habitat preference; however *An. scanloni* and *An. nemophilous* do show a unique specialisation to specific habitat types. *An. scanloni* is found in association with limestone karst habitats, whereas *An. nemophilous* is found within mangrove swamp habitats [31], thus specialisation and ecological divergence is likely to have played a role in the history of these species. The divergence of *An. scanloni* from its sister species *An. dirus* occurred despite inferred uni-directional gene flow from *An. scanloni* into *An. dirus* [63]. The uni-directional nature of this gene flow is thought to have resulted from a unique ecological adaptation of *An. scanloni* to limestone karst habitat, which confers a fitness advantage to this species in regions of sympatry with *An. dirus*, reducing hybrid fitness. The accumulation and maintenance of reproductive isolation between *An. scanloni* and *An. dirus* is therefore likely to have been driven by ecological adaptation [63].

The likely involvement of ecological variation in species divergence has also been assessed within the Maculatus Group, within which the phylogenetic mapping of species' altitudinal distribution supported a scenario of ecological speciation through altitudinal replacement[25]. This is a phenomenon in which the distribution of one species replaces that of its sister species along an altitudinal gradient, as populations become adapted to the environmental conditions within their altitudinal zone [77-78]. Species within the Maculatus Group typically lay their eggs within streams or the rock pools associated with them. Various characteristics of these typical larval habitats, such as the water temperature and the speed of water flow, are likely



to vary with altitude. Adaptation to these specific larval habitats may therefore have played a role in the ecological divergence of populations at higher altitudes [25].

Whilst ecological differences between species may provide clues as to the factors driving past speciation events, investigation of intraspecific ecological variation within a species range may give an indication of the processes involved in the early stages of ecological divergence and speciation. Variation in traits such as anthropophilic vs. zoophilic, or exophagic vs. endophagic feeding preferences have the potential to greatly influence vector status, and there are several species in which vector status is reported to vary across the range. *Anopheles minimus*, for example, is reported to show strong anthropophily within central Vietnam and Laos, but is more attracted to cattle in northern Vietnam and Cambodia [79]. This behavioural variation is thought to be related to the availability of cattle hosts in a region, and will considerably impact the role of *An. minimus* in malaria transmission. Variation in anthropophily, endophagy, biting cycle and endophily in both *An. dirus* and *An. minimus* across the species' ranges have been related to regional variation in human land-use and habits [79], and may be driving intraspecific adaptive divergence between vector populations. Although it is not currently known whether this variation is the result of phenotypic plasticity or genetic adaptation, any rapid ecological diversification may affect patterns of disease transmission. Thus uncovering the processes involved in the generation of ecological divergence within a species may have considerable relevance for malaria control.

Although several examples of species-specific differences in ecology can be found, there does seem to be considerable ecological similarity between species within each of the major groups, as was discussed earlier in this chapter. All species within the Leucosphyrus Group, for example, show an extremely strong association with forest habitat, laying their eggs within temporary forest pools [31-32]. Although species vary in their feeding preferences, and *An. scanloni* and *An. nemophilous* show previously discussed unique habitat specialism, a number of species within the group show no apparent ecological differentiation from one another. This pattern of apparent 'niche conservatism' is also the case within the Maculatus Group and Minimus Subgroup, with the majority of species within showing preferences for disturbed habitat within forest clearings, and for hilly forest habitats, respectively [31, 80]. It seems surprising that so many apparently ecologically similar species coexist, often with large areas of distributional overlap, and it seems likely that there are subtle ecological differences between species that we are yet to uncover. These ecological differences may involve the bionomics or feeding behaviour of species, and may therefore be of considerable interest in terms of malaria control. The probability of undiscovered ecological differences between species seems especially likely given the fact that methods of cryptic species identification have only recently been developed (e.g. [81-86]), and that early studies of species biology and ecology were marred by incorrect species identifications. Besides the clear direct applications of studies into the biology of *Anopheles* species within Southeast Asia, such studies may shed further light on the role of ecological speciation in the evolutionary history of the region's Anopheline fauna.

## 4. Gene flow within and between species

The absence or presence of gene flow between populations and species has a considerable impact on the dynamics of malaria transmission, and on the measures used for vector control. In the absence of gene flow, genetic drift and local adaptation result in the genetic differentiation of populations, and potentially in divergence at ecological traits likely to influence malaria transmission [38, 43]. The presence of gene flow, on the other hand, homogenises genetic variation and may lead to the exchange of adaptive and potentially medically relevant alleles between populations. Although the accumulation of reproductive barriers generally restricts gene flow between species, gene flow may still continue across certain genomic regions, creating patterns of differential divergence and introgression across the genome [7, 87-89]. Numerous cases of mitochondrial introgression between *Anopheles* species, including the Southeast Asian malaria vectors *An. dirus* and *An. baimaii* [63, 67], reveal that gene flow between species may be fairly common. The adaptive exchange of the 2La inversion between *An. arabiensis* and *An. gambiae* provides evidence of the phenomenon of gene flow across certain regions of the genome [5, 8, 90-91], and recent advances in next generation sequencing and population genomics have enabled more detailed examination, providing comprehensive examples of interspecific gene flow such as between the purported species *An. gambiae* M and S [92-93], and between the diverged species *An. gambiae* and *An. arabiensis* [7]. An understanding of patterns of contemporary gene flow both within and between species, and of the landscape features that facilitate or restrict this exchange, is of great importance for malaria control efforts. Characterisation of gene flow within and between species will also be relevant to the design of control efforts involving the release of genetically modified mosquitoes, as it will enable prediction of spread of relevant alleles (such as those influencing vectorial capacity) throughout *Anopheles* populations [39].

The dynamic demographic histories of the major malaria vector species, as discussed previously in this chapter, complicate the inference of contemporary gene flow. For example, population bottlenecks and subsequent expansions, which appear to be common in the Anopheline fauna of Southeast Asia (e.g. [29, 42]), can homogenise genetic variation and thus eliminate accumulated genetic diversity between isolated populations, giving false signal of ongoing gene flow [94]. Knowledge of the historical patterns of divergence, range restriction and expansion in *Anopheles* populations, as discussed in previously in the chapter, may provide a baseline from which to study contemporary gene flow. Additionally, whereas to date studies of population structure and gene flow within and between species has been primarily restricted to neutral markers, the increasing availability of next generation sequencing (NGS) data will provide the opportunity to study the exchange of adaptive alleles across landscapes (e.g. [8], see below).

## 5. Future directions

Despite the wealth of knowledge of *Anopheles* diversity within Southeast Asia, there are many directions that remain to be explored. Firstly, although much is known of the historical

dynamics of gene flow and divergence and the climatic and landscape features that have been important in defining those patterns, little is known of the impact of contemporary landscape features on dispersal and gene flow. Such questions may be addressed using a landscape genetics approach, which involves the combination of fine-scale, dense spatial sampling with spatial and environmental information [95-96]. This approach has been successful, for example, in revealing the impact of urbanisation and forest corridors on connectivity in amphibian populations [97], and the impact of major roads on the genetic structure of caribou populations [98]. Such an approach may reveal the impact of phenomena such as deforestation and increased urbanisation on the demography of *Anopheles* populations, information which would be beneficial for predicting the impact of future landscape changes on the origin and spread of adaptive alleles relevant to vector control.

Secondly, the investigation of patterns of population structure at a genomic level remains to be performed in the *Anopheles* taxa of Southeast Asia, and will have many potential applications. As previously discussed in this chapter, intraspecific phenotypic variation such as that reported within *An. dirus* and *An. minimus* [79] may be due to phenotypic plasticity, or may have an underlying genetic adaptive basis. Patterns of divergence at small numbers of neutral loci, while useful in identifying general population genetic patterns, are insufficient to address such issues comprehensively. Genome-wide approaches can, however, facilitate the identification of loci involved in adaptive response to environmental variation, and may reveal associations between adaptive loci and phenotypic traits (e.g.[99-101]). The availability of the *Anopheles gambiae* reference genome [102] provides additional scope for genomic studies using NGS data, enabling annotation of any identified adaptive loci, and the future availability of 13 additional *Anopheles* genomes, including those of several Southeast Asian species, will aid genomic studies even further [103].

Besides gene flow between populations within a species, the possibility of contemporary interspecific gene flow should also be considered. The identification and characterisation of such contemporary gene flow between species will be vitally important in determining whether medically important traits may spread between them. Again, this issue will benefit from a genome-wide approach, as patterns of introgression and divergence will vary across the genome due to the differential influence of selection [7, 87-89]. Genomic studies have been invaluable in characterising divergence and introgression across the genome, and identifying the targets of selection within the genomes of *An. gambiae* M and S forms [8]. For example, in contrast to the *kdr* mutation, which is responsible for pyrethroid resistance to insecticide and is thought to have spread from the S to the M form of *An. gambiae* through introgression [104], different resistance substitutions within the resistance to dieldrin (*rdl*) gene are thought to have evolved independently within *An. gambiae* M and S forms [8]. Genome-wide approaches will enable similar issues to be addressed within recently diverged species pairs such as *An. baimaii* and *An. dirus*.

The possibility of ongoing gene flow or historic introgression between species is also important for the reliable delineation of species boundaries, particularly within complexes of closely related and morphologically identical *Anopheles* species. The importance of selecting appropriate markers for species delineation, and of considering levels of interspecific gene flow has

been recently reviewed [105], and highlights the potential benefits of a genome-wide approach. Questions relating to Anopheline taxonomy and ecology remain to be answered within several of the medically important *Anopheles* groups (including the *An. sundaicus*, *An. subpictus*, *An. culicifacies* and *An. fluviatilis* Complexes, for example [33]), and the delineation of species boundaries, resolution of species relationships, development of species identification methods and characterisation of species ecology are still vitally important for the design of more traditional methods of vector control. The usefulness of bed nets in reducing malaria, the identification and control of potential larval habitats within a region, and informing of residents of how to reduce exposure, all rely on detailed information of the species present within a region and of their ecology. Zarowiecki [50] has illustrated the importance of taking a systematic approach to delineating and identifying species and resolving taxonomic relationships, and such an approach should be followed for potentially cryptic species complexes in which taxonomy is still uncertain. Thus taken together, the development of NGS technologies and population genomic analytical methods provides great scope for studies into *Anopheles* diversity in Southeast Asia, which are likely to considerably benefit both the understanding of malaria transmission dynamics and the effectiveness of vector control.

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