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# Variation Under Nature: A Sesquicentennial DNA Barcoding Perspective

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## Abstract

Darwin's classic reference to the "tangled bank" acknowledges the living biodiversity. Traditionally, biodiversity assessment has been the realm of the classical taxonomist. However, times have changed swiftly and now biodiversity may be assessed by high throughput sequencing of short regions of DNA. Commonly, this is called DNA "barcoding". In this study, we investigate the genetic diversity within and between four morphological mosquito species that are vectors of medically important viral pathogens. We obtained 627 bp of sequence information from the N-terminus of the mitochondrially encoded cytochrome *c* oxidase I (COI) gene from 12 *Aedes vigilax* and 10 *Ae. camptorhynchus* representing five localities in Australia. We also downloaded GenBank COI sequence data from 18 *Ae. albopictus* and 14 *Ae. aegypti*. Maximum likelihood phylogenetic analyses recovered the four distinct morphological species, as well as a distinct lineage within what is currently described as *Ae. vigilax*. We termed this lineage 'A'. Intra- and interspecific variation quantified for *Ae. vigilax* and *Ae. vigilax* lineage 'A' suggest that this morphospecies may include distinct genetic or cryptic species. *Ae. aegypti* also had high intraspecific variation, however, we concluded that this is most likely due to the large effective population size of this species and its broad distribution through the tropics. Phylogenetic analyses and analyses of genetic diversity at COI suggests that *Ae. camptorhynchus* and *Ae. albopictus* are robust species. The results of this study highlight the efficiency, utility and ease of barcoding. The study also documents specific limitations including the importance of taxon sampling and the necessity for this tool to be used in conjunction with additional taxonomic techniques, ecological studies and physiological data.

**Keywords:** Barcode, Mosquito, Diversity, *Aedes vigilax*.

## 1. Introduction

"*It is interesting to contemplate a tangled bank, clothed with many plants of many kinds, with birds singing on the bushes, with various insects flitting about, and with worms crawling through the damp earth, and to reflect that these elaborately constructed forms, so different from each other, and dependent upon each other in so complex a manner, have all been produced by laws acting around us.*"

Charles Darwin [1]

One of the most remarkable features of nature is its enormous diversity of life, or

biodiversity. To date, the number of described species approximates 300,000 for plants, 1.3 million for animals, 120,000 for fungi and 60,000 for lichens with an estimated ten to 30 million species still awaiting discovery [2-4]. The astonishing scale of biological diversity has long been subject to scientific scrutiny trying to identify the driving forces and rationale leading to the observed variability.

Today we know that biodiversity does not only promote greater productivity in plant communities and nutrient preservation of ecosystems but even more importantly, is linked to ecosystem stability [5, 6]. Biodiversity is therefore of central importance to climate stabilization, maintenance of air, soil and water quality, food security and disease control. Considering this fundamental role of biodiversity, it is invaluable to understand the functioning and dynamics of biodiversity and to develop a tool to measure biodiversity to install protective and conservational measures [7].

The assessment of biodiversity, *i.e.* the identification, description and classification of species has traditionally been the sphere of taxonomists. However, considering the species richness of whole ecosystems and the global biodiversity, traditional assessment of species diversity based on morphology constitutes a major challenge [8]. The traditional approach is time- and labor-intensive and, in some situations, it may be hampered by difficulties linking distinct life-history stages of a single species while, in other cases, distinct genetic species may go unnoticed if they are morphologically indistinguishable to a given taxonomist [9-11].

An alternative to the traditional approach is DNA-based taxonomy. Due to the paucity of morphological variation it comes as no surprise that this method was initially developed for the identification and classification of prokaryotes [12]. Exploiting sequence diversity of 16S rRNA, DNA-based taxonomy allowed for reliable and time-efficient identification of morphologically similar organisms [11]. Following the success of the prokaryote identification system, a similar strategy was later developed and applied to animals [13,14]. Working on lepidopteron phylogeny, Hebert and colleagues [15] proposed the analysis of one universal short mitochondrial sequence of cytochrome *c* oxidase I (COI) as a global bioidentification system for animals.

The process of employing a short stretch (or more recently multiple short regions) of DNA for identification purposes is now commonly referred to as DNA barcoding. This method aims to identify and assign a single specimen to a certain animal species based on sequence diversity within one or more DNA barcode sequence(s). Central to this strategy are the assumptions that species have a unique DNA barcode and that genetic variation between barcodes is greater between species than within them. Although still contentious, the definition of species boundaries, i.e. the minimum sequence difference defining a new species has been proposed to be around 2 to 3% or 10 times the mean intraspecific sequence variation of the group under study [15-17]. Once the appropriate threshold is determined, DNA barcoding is easily accessible to non-specialists and holds promise for rapid and cost-efficient species identification.

Methodologies facilitating rapid turnover from large-scale sampling strategies to species identification will prove indispensable during the upcoming years if they are accurate, robust and repeatable. In times of global warming, rapid species assessment will be essential to examine and understand the impact of climate change on single species, on biodiversity and subsequently on ecosystems stability. Environmental change is not only expected

to lead to the extinction of single species and to the collapse/reorganization of ecosystems, but also to a change in global distribution of organisms and associated diseases [7, 18, 19].

Insects are one group of organisms that are particularly susceptible to global climatic changes [20]. They are a highly diverse and abundant assemblage that inhabits a wide spectrum of habitats. Within the Class Insecta, the distribution and abundance of mosquitoes holds particular significance as they are important vectors of multiple disease-causing agents including malarias, certain filarias and a range of arboviruses.

In a recent study, Kumar et al. [21] obtained COI DNA barcodes for 111 specimens belonging to 63 species and 15 genera. The DNA barcode approach identified 62 of the 63 morphological species. Unfortunately, this study included few examples of multiple specimens within a species and as such it was not possible to determine if significant genetic subdivision occurred within any morphological species. This is important because genetic variants may differ in their vector competence, and in the extreme case may be genetically distinct species with distinct ecologies and biting preferences.

The mosquito *Anopheles gambiae* is an example of the strategic and evolutionary importance of understanding genetic subdivision in biology. *Anopheles gambiae* refers to a complex of morphologically indistinguishable but genetically identifiable mosquito species. Members of this species complex are the most important vectors of malaria in sub-Saharan Africa and the most efficient vectors of the parasites in the world. Despite being morphologically indistinguishable, six species are now recognized, each with specific behavioural traits [22]. For example, *An. gambiae sensu stricto* is considered to be anthropophilic and therefore an important vector of malaria to humans. In contrast, *An. quadriannulatus* is generally considered to be zoophilic and typically takes blood from non-human animals.

In this study we employ the COI barcode region to provide a preliminary assessment of the genetic subdivision within four species of mosquitoes in the genus *Aedes*. *Aedes aegypti* and *Ae. albopictus* are important vectors of dengue viruses while *Ae. vigilax* and *Ae. camptorhynchus* are the major coastal vectors of Ross River virus in Australia.

Dengue viruses (DENV) are members of the genus *Flavivirus* and include four distinct but antigenically related serotypes (DENV-1, -2, -3, and -4). Dengue infects 50–100 million people each year [23]. Of these infections, 200,000–500,000 occur as the severe, life-threatening form of the disease, dengue haemorrhagic fever [24]. The incidence of dengue haemorrhagic fever in Thailand varies widely from year to year, showing as much as a tenfold difference between years. Generally, a wave of infection emanates from Bangkok and moves radially at a speed of about 150km per month [25]. In Australia, dengue only occurs in north Queensland and more than 600 cases have been reported in 2009 alone [26]. In Australia, *Ae. aegypti* is the only known vector of this disease.

Ross River virus does not occur in Thailand. It is the most common mosquito-borne pathogen in Australia, and about 5,000 human cases are reported annually. The infection is not fatal, but there is considerable morbidity associated with a debilitating polyarthritis that is the major symptom of concern. The virus is annually active in most regions of Australia and exists as distinct genotypes that vary in virulence to mosquito vectors and human hosts [27]. Macropods are thought to be the native vertebrate hosts, although humans and horses may be involved during epidemic activity. Vertical transmission of the virus may also occur through the eggs

of mosquitoes. The saltmash mosquitoes *Ae. vigilax* and *Ae. camptorhynchus* are the most important coastal vectors [27].

## 2. MATERIAL AND METHODS

### 2.1 Taxon Sampling

Four species of *Aedes* mosquitoes are included in this study. Data for *Ae. aegypti* and *Ae. albopictus* were downloaded from GenBank (Table 1). Data for *Ae. vigilax* and *Ae. camptorhynchus* were generated in the Ballard laboratory for this study.

*Aedes aegypti* probably originated in Africa but now is distributed throughout the tropics and semi-tropics. Adults are typically found within or close-by human environments, utilising artificial containers as larval habitat, and biting indoors or in sheltered areas near the house [28, 29]. We downloaded 13 sequences and generated one (Table 1).

*Aedes albopictus* is native to the tropical and subtropical areas of Southeast Asia, and can occur in urban, suburban and rural regions, in domestic and peri-domestic environments, utilising both natural and artificial containers as larval habitat [28]. This mosquito is not currently present on the Australian mainland but there is a serious and increasing risk of its introduction since it has become established in the Torres Strait. We downloaded 15 sequences and generated one (Table 1).

*Aedes vigilax* breeds in saline habitats on mudflats usually behind mangroves. Its eggs are resistant to desiccation and large numbers of eggs may hatch following inundation of mudflats following extremely high tides. It is absent from Tasmania, the Victorian coastline, and the far south of Western Australia but, within the habitat limitations, it is present along the rest of the Australian coastline, including the Torres Strait Islands, but is active only in the warmer months in southern regions [29 - 31]. It can disperse many kilometres and is a major nuisance pest as well as a virus vector. We have sampled 13 individuals from four localities (Table 1).

*Aedes camptorhynchus* is essentially the counterpart of the *Ae. vigilax* along the southern coastline of Australia, and in Tasmania. Adults can be active throughout the year in southern NSW and Victoria, and can disperse widely from larval habitats. They can be vicious biters readily attacking humans and have significant pest as well as vector status [29 - 31]. We have sampled 8 individuals from five localities (Table 1).

### 2.2 DNA Extraction, Amplification and Sequencing

Individual *Ae. vigilax* and *Ae. camptorhynchus* were preserved in ice-cold 99% ethanol. Immediately prior to extraction, animals were blotted onto filter paper until completely dry. Total DNA was extracted using the Puregene® Genomic DNA Purification Kit following the Isolation from Solid Tissue protocol (Gentra Systems Inc., Minneapolis, USA). Extracted DNA was resuspended in DNase- and RNase-free water and stored at -20$^{o}$C.

A 627 bp sequence from the N-terminus ('barcode' region) of the COI gene was amplified by PCR in 25 µL reactions containing 25 ng template DNA, 1x polymerase buffer, 4 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U AmpliTaq® DNA Polymerase (Applied Biosystems Inc., Foster City, USA) and 10 pmol of each universal primer LCO1490 and HCO2198 [32]. PCRs were run on a Bio-Rad® Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, USA).

PCR products were purified by treatment with ExoSAP-IT® (USB Amersham, Buckinghamshire, UK) in a 5:1 amplicon: enzyme ratio and incubated at 37$^{o}$C for 45 min followed by 80$^{o}$C for 15 min to inactivate the enzyme. Purified PCR product was used to sequence in both directions using the

**Table 1.** List of taxa used in this study, with accession numbers and locality data.

| Genus | Species | Locality | GenBank Accession No. |
|---|---|---|---|
| *Aedes* | *vigilax* | Shellharbour, NSW, Australia<br>Sydney, NSW, Australia<br>Adelaide, SA, Australia<br>Black Lake, WA, Australia | GQ143720-21, 27-28<br>GQ143722-23, 29-30<br>GQ143724-26<br>GQ143739-40 |
| *Aedes* | *camptorhynchus* | Shellharbour, NSW, Australia<br>Sydney, NSW, Australia<br>Meningie, SA, Australia<br>Wellington, SA, Australia<br>Black Lake, WA, Australia | GQ143733<br>GQ143734<br>GQ143737-38<br>GQ143739-40<br>GQ143735-36 |
| *Aedes* | *aegypti* | Townsville, Qld, Australia<br>Boa Vista, Brazil<br>Bouake, Cote d'Ivoire<br>Boulbinet, Guinea<br>Europa Island<br>Foz Iguacu, Brazil | GQ143718<br>AJ970961<br>AJ970962<br>AJ970963<br>AJ970964<br>AJ970965 |
| | | Guadeloupe, France<br>Hanoi, Vietnam<br>Ho Chi Minh City, Vietnam<br>Riviere salee, Martinique<br>Nha Trang, Vietnam<br>Bouake, Cote d'Ivoire<br>Phnom Penh, Cambodia<br>Quixeramobim, Brazil | AJ970966<br>AJ970967<br>AJ970968<br>AJ970970<br>AJ970971<br>AJ970972<br>AJ970973<br>AJ970974 |
| *Aedes* | *albopictus* | Represa do Cigano, Brazil<br>Hanoi, Viet Nam<br>Jacksonville, USA<br>Seam Reap, Cambodia<br>Diego Suarez, Madagascar<br>Montsecret, France<br>Naintre, France<br>Nha Trang, Viet Nam<br>Oahu, Hawaii<br>La Possession, Reunion<br>Sao Luis, Brazil<br>Chiang Mai, Thailand<br>York Island, Torres Strait<br>Mananthawadi, India<br>Mumbai, India | AJ971003<br>AJ971004<br>AJ971005<br>AJ971006<br>AJ971007<br>AJ971008<br>AJ971009<br>AJ971010<br>AJ971011<br>AJ971012- 13<br>AJ971014<br>AJ971015<br>GQ143719<br>EU259306<br>DQ424959 |
| *Anopheles* | *hyrcanus* | Ardebil Province, Iran | FJ210895 |
| *Anopheles* | *maculipennis* | Schinias, Greece | DQ118177 |
| *Anopheles* | *gambiae* | Nigeria | DQ465336 |

BigDye® Terminator v3.1 sequencing kit (Applied Biosystems Inc., Foster City, USA) in a 10 μL volume, containing 15 - 20 ng purified product and 3 pmol primer. Sequences were edited and manually aligned using Sequencher 4.5 (Gene Codes, Ann Arbor USA).

**2.3 Phylogenetic and Sequence Analysis**

All sequences generated in our lab and those from GenBank (see Table 1) were aligned using Clustal W [33]. To initially examine genetic subdivision we reconstructed phylogenies using a maximum likelihood as implemented in PAUP* 4.08b [34]. A Kimura-2-parameter model of nucleotide substitution was applied, with rates across the sites being equal. The proportion of invariable sites was estimated from the data. Maximum likelihood searches were heuristic with 10 random addition replicates and TBR branch swapping. Any identical sequences were removed prior to phylogenetic analyses. *Anopheles hyrcanus*, *An. gambiae* and *An. maculipennis* were used as outgroup taxa [35].

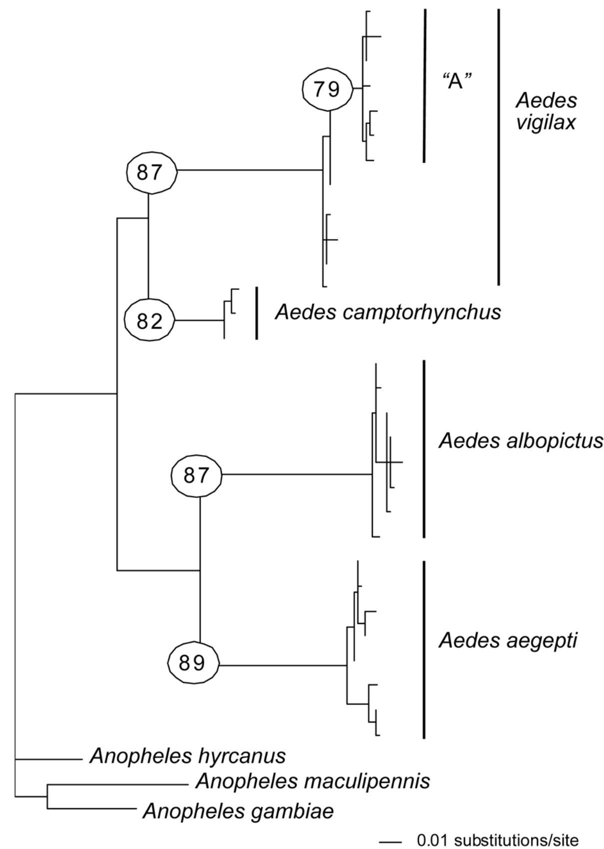


**Figure 1.** Maximum likelihood phylogenetic reconstruction as implemented in PAUP* 4.08b [34]. A Kimura-2-parameter model of nucleotide substitution was applied, with rates across the sites being equal. The proportion of invariable sites, estimated from the data, was 0.73. Maximum likelihood searches were heuristic with 10 random addition replicates and TBR branch swapping. Bootstrap support from 150 replicates is shown in circles where it is above 70%. *Anopheles hyrcanus*, *An. gambiae* and *An. maculipennis* were used as outgroup taxa.

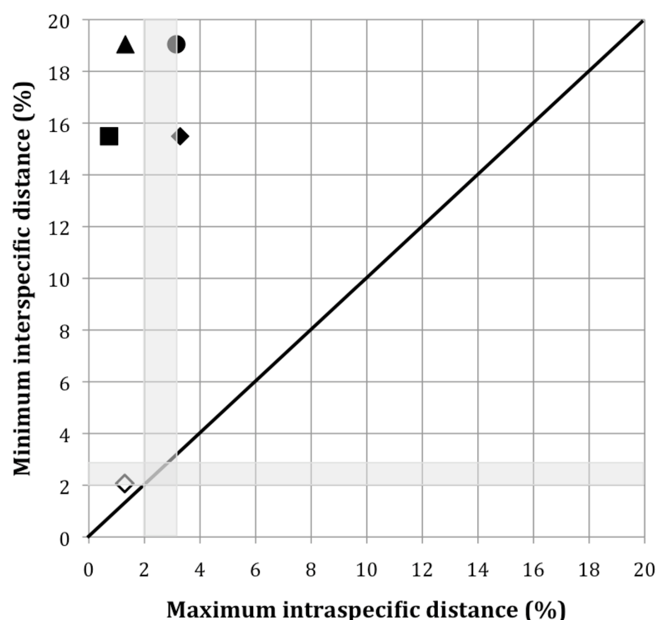


**Figure 2.** Interspecific distances versus intraspecific distances of COI sequences for morphospecies *Aedes aegypti* (●), *Ae. albopictus* (▲), *Ae. vigilax* (◆), *Ae. camptorhynchus* (■) and genetic lineage 'A' of *Ae. vigilax* (◇). Grey bars represent thresholds for within and between species variation [14, 16, 36]. Species in the top left hand quadrant are considered well-defined species, while species in the in the top right are thought to be composite species. Species in the bottom left hand quadrant represent recently diverged groups, and those in the bottom right hand quadrant represent misidentifications [15, 16]. Additionally, only species that lie above the diagonal line, i.e. where divergence between species is greater than divergence within species, are thought to be suitable for identification using barcoding.

In order to examine the applicability of the barcoding methodology to this mosquito taxonomy we assessed the inter- and intra-specific divergence of the four morphologically well known species; *Ae. albopictus, Ae. aegypti, Ae. camptorhynchus,* and *Ae. vigilax*. The phylogenetic analyses showed that *Ae. vigilax* has a distinct monophyletic lineage that we have termed *Ae. vigilax* lineage 'A'. As a result we performed three pairwise comparisons: *Ae. albopictus* with *Ae. aegypti*, *Ae. camptorhynchus* with *Ae. vigilax*, and *Ae. vigilax* lineage 'A' with closest *Ae. vigilax* taxa, to examine interspecific variation. Currently it is not clear what level of divergence designates a significant difference within and among lineages and we employ a 2 - 3% threshold [15, 17, 36, 37]. The maximum of intraspecific distance and the minimum interspecific distance were calculated using the same Kimura-2-parameter model as applied for the phylogenetic analyses [34].

## 3. RESULTS

The alignment of all mosquito COI gene sequences resulted in a consensus length of 559bp, of which 149 were variable. The maximum likelihood analysis resolved the four species being examined as distinct monophyletic groups (Figure 1). Furthermore, a distinct lineage within *Ae. vigilax* was identified: we termed this lineage "A" (Figure 1).

Specimens belonging to *Ae. vigilax* lineage "A" were not detected in South Australia but were collected from both Western Australia and New South Wales.

We then mapped the intra- and inter-specific divergence of COI to assess the ability of the barcoding methodology to identify the four well known medically important mosquitoes. Using a 2 to 3% threshold, Figure 2 is divided into four quadrants, with each quadrant making specific predictions as to the status of taxa being examined [15, 16]. The morphological species *Ae. vigilax* has an intraspecific variation of 3.3 % (Figure 2). Within this morphological species *Ae. vigilax* lineage "A" is genetically distinct and has low intraspecific (1.3%) and low interspecific variation (2.1%; Figure 2). Taxa with this set of variation are generally considered to represent recently diverged groups [15, 16].

*Aedes aegypti* has 3.1% intraspecific variation (Figure 2). However, phylogenetic analyses do not suggest that distinct monophyletic lineages occur. This result implies that the species has a very large effective population size. Additional sampling is needed to determine if locally adapted populations are monophyletic.

*Aedes albopictus* and *Ae. camptorhynchus,* exhibit low intraspecific (1.3 and 0.7%, respectively) and high interspecific variation (19.0 and 15.4 %, respectively; Figure 2).

## 4. Discussion

DNA Barcoding has been hailed as a quick and easy tool in the identification of species and species limits [15,16], and could have important medical implications for understanding mosquito diversity. Results of our phylogenetic study show that *Ae. vigilax* may represent a number of distinct lineages that could be distinct sibling species, while *Ae. albopictus*, *Ae. aegypti* and *Ae. camptorhynchus* appear to be distinct species by the COI barcode criterion.

This study illustrates several strengths of the COI barcode. Universal primers to amplify the barcode region were readily available, and experiments including *Ae. vigilax* and *Ae. camptorhynchus* were designed and conducted in a time-efficient manner. The barcode region was easily amplifiable and alignable for both species. Further, selecting this region facilitated comparison with GenBank data.

Phylogenetic analyses identified the four morphological species and a distinct lineage within *Ae. vigilax*. Based on the minimum interspecific divergence and the maximum intraspecific diversity, this lineage could be considered either a recently diverged species or perhaps a distinct species (Figures 1 and 2). *Aedes vigilax* has a wide distribution throughout the South Pacific and Australia [29], but samples from only four sites were included. Additional samples are clearly required. Further, sequence data from the nuclear genome, and also morphological and breeding studies, are needed before it can be determined whether *Ae. vigilax*, as it is currently described, is composed of a number of lineages or distinct species that differ in their Ross River virus vector competence. Interestingly, morphological variation has been detected between eastern and western Australian populations (JWOB unpublished data).

*Aedes aegypti* also has a high level of intraspecific variance. This may indicate that this morphological species is diverging genetically. However, phylogenetic analysis does not indicate any monophyletic assemblages within *Ae. aegypti*. As such, it seems more likely that the high intraspecific variation reflects the large effective population size and global tropical distribution of this species. Such variation can be employed to determine the source of a specific incursion.

For example, Beebe et al. [38] employed COI sequence data to show that an incursion of *Ae. aegypti* into the town of Tennant Creek in the Northern Territory of Australia originated from Cairns or Camooweal in the state of Queensland.

The two species, *Ae. albopictus* and *Ae. camporhynchus* were identified as having high interspecific and low intraspecific variation. These data suggest that both morphological species are robust.

The study also illustrates several concerns in using the COI barcode. The most obvious concern is the influence of taxon sampling on the results. Collections from four sites suggest that distinct lineages are arising within *Ae. vigilax* but not *Ae. camptorhynchus*. However, it is not known if this conclusion is biased because neither species was sampled from throughout its range. This potential for bias may have significant implications for understanding the distributions of medically important diseases. For example, Hemmerter and colleagues [39, 40] broadly sampled the mosquito *Culex annulirostris* from Australia and Papua New Guinea and showed that the morphological species contained divergent genetic lineages. Notably, the southern limit of one genetic lineage coincides exactly with the current southern limit of Japanese encephalitis virus activity in Australasia.

A second taxon sampling issue in this study is that the sister taxon of each morphological species was not included. *Aedes aegypti* was paired with *Ae. albopictus* and *Ae. vigilax* was paired with *Ae. camptorhynchus*. As neither pair are likely to be sister taxa, interspecific distance is expected to be inflated in Figure 1 [36]. The addition of appropriate sister taxa may cause a decrease in minimum interspecific distance, but is not expected to significantly influence the quadrant position nor the intraspecific distance in our study.

More generally, biological limitations of DNA barcoding may also bias our results in potentially unexpected ways. Most significantly, not all genetic species are clearly identified by a single mitochondrial sequence [41]. As a consequence, underlying variation and potentially genetic subdivision within nuclear encoded genes may not be detected [42]. Further, the influence of specific selective forces that may influence the evolution of the region, including genetic hitchhiking in locally adapted populations and protein-protein interactions in complex IV of the electron transport chain, have not been well studied.

It is essential to understand the ability of a mosquito to transmit diseases if we are to robustly predict how the distribution of a disease may alter with climate change. Understanding the parameters that influence vector competence is a complex issue, which is underpinned by the knowledge of the genetic diversity within a species. What underlies differences in vectorial capacity, such as attraction to alternative hosts? What underlies ecological differences, such as the saltwater tolerance of *An. merus*, the ability of the *An. gambiae* M molecular form to breed during the dry season, or the ability of the *An. gambiae* Bamako chromosomal form to breed in flowing water of the Niger River? What underlies premating isolation among species and forms, and what drives such rapid diversification? Can the answers to these questions provide a window into the speciation process as well as a set of practical tools to differentiate the vector from non-vector species? To begin to address these questions, what is needed is a tool for surveying genetic variation. DNA barcoding may well be one tool for rapid screening; however, it needs to be incorporated with other genetic, phenotypic and ecological studies to arrive at a globally robust and definitive conclusion.

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