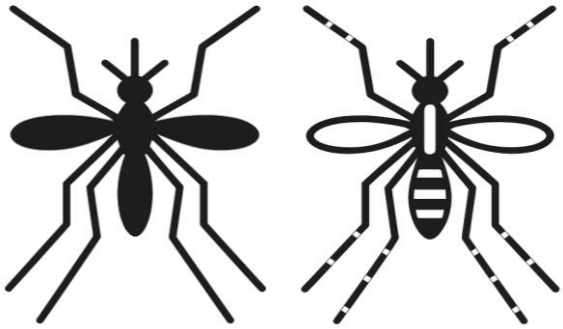
CHANGES IN OUTDOOR MALARIA AND DENGUE VECTORS FOLLOWING HUMAN ACTIVITIES



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Contributions

Boni Sebayang was the primary author of all thesis chapters

Chapter	Contributions
1. Introduction to the thesis	Boni Sebayang wrote the first draft, which was reviewed by Tanya Russell and Tom Burkot
2. Human-mediated activities driving changes in the distributions and vectorial capacities of vector populations	Boni Sebayang, Tanya Russell and Tom Burkot conceived and designed the review and critically revised the manuscript. All authors read and approved the final manuscript.
3. Identification of *Anopheles dirus sensu stricto* and *Anopheles scanloni* in Indonesia: potential zoonotic, enzootic and human malarias vectors	Boni Sebayang, Tom Burkot, Tanya Russell and Matt Grigg conceived and designed the study. Tom Burkot, Tanya Russell, Triwibowo Garjito, Inke Lubis and Matt Grigg advised on fieldwork. Boni Sebayang undertook the fieldwork with assistance from Bram van de Straat, and Ahadi Kurniawan. Boni Sebayang and Adzkia Haq conducted the molecular analyses with Sylvie Manguin advising on mosquito species molecular identification. Boni Sebayang and Tanya Russell statistically analysed the data. Boni Sebayang wrote the original draft of the manuscript. Tom Burkot and Tanya Russell provided supervision. All authors read and approved the final manuscript.
4. *Anopheles maculatus sensu stricto* is a potential vector of *Plasmodium knowlesi* and *Plasmodium inui*	Boni Sebayang, Tom Burkot, Tanya Russell and Matt Grigg conceived and designed the study. Tom Burkot, Tanya Russell, Triwibowo Garjito, Inke Lubis and Matt Grigg advised on fieldwork. Boni Sebayang, did the fieldwork with help from Bram van de Straat, and Ahadi Kurniawan. Boni Sebayang and Adzkia Haq conducted the laboratory analyses. Boni Sebayang and Tanya Russell statistically analysed the data. Boni Sebayang and Jacob Westaway conducted the phylogenetic analyses with useful input from Triwibowo Garjito, Inke Lubis, Matt Grigg, Nigel Beebe (University of Queensland) and Neil Lobo (University of Notre Dame (USA). Boni Sebayang wrote the original draft of the manuscript. Tom Burkot and Tanya Russell provided supervision. All authors read and approved the final manuscript.

Chapter	Contributions
5. Larval habitat preferences of *Anopheles dirus sensu stricto* and *Anopheles maculatus sensu stricto* in North Sumatra, Indonesia	Boni Sebayang, Tom Burkot and Tanya Russell conceived and designed the study. Boni Sebayang conducted the field studies and laboratory analyses with Adzkia Haq. Statistical analyses were by Boni Sebayang and Tanya Russell. Boni Sebayang wrote the original draft of the manuscript. Tom Burkot and Tanya Russell provided supervision. All authors read and approved the final manuscript.
6. Sugar feeding by *Aedes albopictus* in the Torres Strait, Australia	Boni Sebayang, Tom Burkot and Tanya Russell conceived and designed the study. Boni Sebayang, Susannah Mosby, Richard Gela, Darcy Roeger, Bram van de Straat, Kyran Staunton, and Tom Burkot conducted the fieldwork with laboratory analyses by Boni Sebayang. Darcy Roeger identified the plants in the study sites. Boni Sebayang and Tanya Russell analysed the field and lab data. Boni Sebayang wrote the original draft of the manuscript. Tom Burkot and Tanya Russell provided supervision. All authors read and approved the final manuscript.
7. General discussion	Boni Sebayang wrote the first draft, which was reviewed by Tanya Russell and Tom Burkot

Personal note

The scope of my research evolved along the PhD journey, especially in response to restrictions imposed during the COVID-19 pandemic that prevented access to the Indonesian field sites. The original thesis research design was adapted from the initial study design described and supported by ACIAR as part of the "Evaluating Zoonotic Malaria Transmission and Agricultural Land Use in Indonesia (ZOOMAL)" project. The aim of this multidisciplinary research program was to strengthen surveillance of enzootic malarias in Indonesia, including evaluating the disease burden, agricultural practices, and mosquito vectors associated with transmission to inform public health control efforts and sustainable agricultural development. The vector component on which this thesis is based focused on incriminating the outdoor enzootic and zoonotic vectors in North Sumatra and defining their behaviours to identify potential vulnerabilities to control strategies. However, when the COVID-19 pandemic began in March 2020, international borders closed and fieldwork in Indonesia was postponed indefinitely.

However, during the pandemic, it was possible to begin tackling the threat of dengue in mainland Australia posed by the potential invasion of the outdoor biting *Aedes albopictus* from the Torres Strait as travel to the Torres Strait Islands was permissible within the COVID-19 policies of the Australian and Queensland Governments. Hence, the scope of the research question was broadened to encompass understanding the behaviours of the outdoor dengue vector, *Ae. albopictus*, in the Torres Strait Islands.

Thus, studies on *Ae. albopictus* in the Torres Strait commenced while waiting for international travel restrictions to be lifted. When the international border of Australia reopened in early 2022, the project in Indonesia started albeit with some modifications due to the time lost during lockdown. Hence, the aims of this thesis focused on outdoor dengue and zoonotic malaria vectors and their behaviours in Australia and Indonesia.

The challenges to this PhD thesis were not only from the COVID-19 pandemic. During the first two years of my PhD studies, I had two major surgeries. This situation put me in my lowest physical and mental states. However, the endless support from my family, both of my supervisors, colleagues, and friends encouraged me to survive and believe in myself to complete my PhD project.

Thesis abstract

Mosquito-borne diseases such as malaria and dengue remain persistent global health challenges, particularly in the context of accelerating environmental change. Human activities – including deforestation, agricultural expansion, and urbanisation – continue to shape mosquito habitats and behaviours in ways that undermine traditional control efforts. While insecticide-treated nets (ITNs) and indoor residual spraying (IRS) have been instrumental in reducing human malaria transmission, they are largely ineffective against mosquito species that bite and rest outdoors. This outdoor transmission – often occurring beyond the reach of conventional tools – is now recognised as a major barrier to disease control and elimination. Chapter 2 of this thesis explores how anthropogenic pressures are reshaping mosquito ecology and argues for a renewed focus on outdoor-biting vectors as one of the key drivers of persistent transmission.

Preventing malaria and dengue transmission relies on two fundamental strategies: (1) vector incrimination – identifying mosquito species and detecting the pathogens they carry; and (2) understanding the ecological and behavioural traits of these vectors to inform targeted interventions. This thesis applies these principles in two geographic settings: North Sumatra, Indonesia, and the Torres Strait Islands, Australia. In chapters 3 and 4, molecular analyses were used to incriminate vectors in North Sumatra, where both human and zoonotic malaria are present. *Anopheles dirus sensu sricto* and *Anopheles scanloni* were confirmed in Indonesia for the first time, and *An. dirus s.s.* was found to harbour DNA from multiple malaria parasites, including *Plasmodium vivax*, *Plasmodium knowlesi*, *Plasmodium inui*, and *Plasmodium cynomolgi*. *Anopheles maculatus sensu sricto*, previously overlooked as a zoonotic vector, also tested positive for *P. knowlesi* and *P. inui*, expanding the list of potentially important vectors in the region.

Chapters 5 and 6 explore the behavioural ecology of outdoor-biting vectors as a basis for control. In North Sumatra, larval surveys showed that *An. dirus s.s.* and *An. maculatus s.s.* occupied distinct habitat classes. *Anopheles dirus* s.s. frequently utilised small, human-altered water bodies such as tyre tracks – habitats that are accessible and potentially suitable for larval source management (LSM). In contrast, *An. maculatus s.s.*

was associated with natural stream margins, which are less stable, more dispersed, and harder to manage. These differences highlight that vector control strategies must be tailored to local species ecology and landscape features. In the Torres Strait Islands, sugar-feeding behaviours of the invasive dengue vector *Aedes albopictus* were assessed to explore the feasibility of using attractive targeted sugar baits (ATSBs). Sugar detection in both male and female mosquitoes indicated that ATSBs have the potential to be a viable effective outdoor control tool to reduce dengue transmission risk and limit the risk of *Ae. albopictus* spreading to mainland Australia.

Together, these findings highlight the complexity and adaptability of outdoor-biting mosquito vectors. They show that effective vector control requires more than simply deploying existing tools – it requires understanding which species are responsible for transmission, the habitats where their larvae are found, and how they behave. This thesis supports a shift toward flexible, evidence-based strategies that respond to local ecological conditions and vector behaviours. By combining molecular vector incrimination with ecological field studies, it provides new insights into how control programs can better address the challenge of outdoor transmission in an era of environmental change.

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Chapter 1

Thesis scope and objectives

The hypothesis of this thesis is changes in vector distributions and bionomics are altering the risk of pathogen transmission. This thesis hypothesis is focused on vectors that bite and rest outdoors. Increases in transmission by such outdoor biting mosquitoes is of growing concern as there are only a limited number of World Health Organization (WHO) recommended interventions available to control these vectors. This research focuses on outdoor enzootic and human malaria vectors in North Sumatra, Indonesia, and an outdoor dengue vector in the Torres Strait, Australia. The primary objective is to characterise specific bionomic aspects of outdoor biting vectors that could be exploited by current or emerging vector control strategies. To do so required first identifying the anophelines present and then incriminating those species by detecting malaria parasites in them in the Sumatra study site. The thesis findings will contribute to strengthening the evidence base for future control efforts directed at malaria and dengue vectors in Sumatra and the Torres Strait (Figure 1.1). Evaluating vector control tools falls beyond the scope of this study.

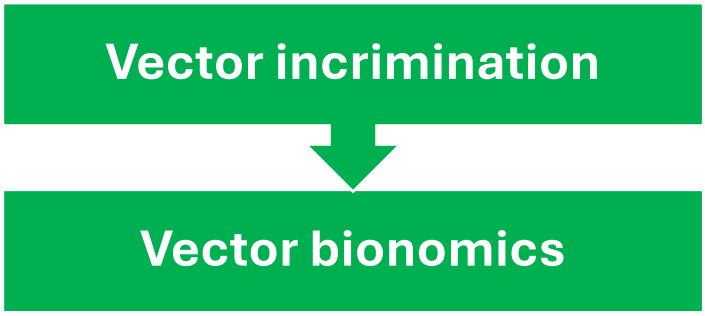


Figure 1.1. The thesis objectives

Background

Malaria and dengue as global health issues

Malaria and dengue are ongoing, significant global health threats. Despite concerted global efforts resulting in the certification of 45 countries and one territory as malaria-free [1] and with active elimination programs underway in 25 other countries, global malaria incidence has increased over the past five years [2]. In 2023, the World Health Organization (WHO) estimated that there were 263 million malaria cases globally, with an incidence rate of 60.4 cases per 1,000 population at risk [3]. Malaria was responsible for approximately 597,000 deaths, translating to a mortality rate of 13.7 per 100,000 population [3]. This highlights ongoing challenges in malaria control, particularly in regions like sub-Saharan Africa, Southeast Asia (SEA), and the Eastern Mediterranean [3]. Adding to the challenge, enzootic malaria, particularly that caused by *Plasmodium knowlesi*, has emerged as a significant public health concern in the SEA region. Malaysia reported 2,872 indigenous cases in 2023, followed by Thailand and Indonesia with 239 and 170 cases, respectively [4].

Dengue has similarly escalated in recent years, becoming one of the fastest-growing mosquito-borne viral infections worldwide. In 2023, global dengue cases reached 6.43 million, resulting in 6,892 deaths (e.g., 56,672 cases and 28.45 deaths per million population) [5, 6]. In Australia, 962 dengue cases were recorded in 2023, representing a 21.4% increase compared to the same period in 2022 [7]

The rising incidence of both malaria and dengue, along with the emergence of enzootic transmission, underscores the need for species-specific vector incrimination. Identifying the mosquito species responsible for transmission is essential for designing targeted interventions [8]. This thesis addresses these gaps by determining the presence and vector status of anopheline mosquitoes in North Sumatra and evaluating the control potential for outdoor-biting malaria and dengue vectors in Indonesia (North Sumatra) and Australia (the Torres Strait Islands), respectively.

The role of mosquito vectors in malaria and dengue transmission

Vector incrimination, identifying which mosquito species are responsible for malaria and dengue transmission, is critical for effective vector and transmission control. Human

and enzootic malarias are transmitted by *Anopheles* mosquitoes. The *Anopheles* genus has over 500 identified species; however, only 41 species are implicated as primary vectors [8, 9]. In SEA, the vector status of many *Anopheles* species remains uncertain, requiring in-depth entomological studies to determine which species are responsible for transmission [10]. The enzootic malaria, *Plasmodium knowlesi* transmission, presents unique challenges to malaria control when this simian malaria spills over to humans [9]. There are eight confirmed vectors of *P. knowlesi* in SEA, including five species from the Leucosphyrus Group of *Anopheles* [11-15], one species from the Barbirostris Group [16, 17] and two species in the Umbrosus Group [18, 19]. However, on Sumatra Island, Indonesia, *Anopheles balabacencis* from the Leucosphyrus Group is the only species that has been incriminated as a vector of *P. knowlesi* [15].

Similarly, dengue transmission in tropical and subtropical regions is vectored by *Aedes* mosquitoes, particularly *Aedes aegypti* and *Aedes albopictus* [20]. While *Ae. aegypt*i is the dominant vector of dengue, the spread of the rural, outdoor and more opportunistic biting *Ae. albopictus* complicates vector control efforts in both urban and rural areas [21]. Although other *Aedes* species can transmit dengue, their distribution is often geographically restricted. For instance, *Aedes polynesiensis* is an important vector, but only in some Pacific Islands [22]. Accurate species identification and vector incrimination are essential to clarify which mosquito species are most responsible for transmission. As every mosquito has a unique set of behaviours, accurate species identification coupled with knowledge of their behaviours better ensures that the interventions most likely to be effective are selected for deployment.

Vector incrimination: a critical first step in control

Vector incrimination is the process of determining which mosquito species are naturally infected with pathogens. This process often involves analyses of entomological surveillance by morphological and molecular techniques to identify both the mosquito and any pathogen that it may be harbouring. Following incrimination, understanding the bionomics including the biology and ecological behaviours, of these vectors is essential for selecting effective, control strategies [23].

Accurate species identification is particularly challenging when cryptic species complexes exist within a group [23]. By definition, a group consists of closely related species with similar morphologies, ecologies and behaviours but with substantial genetic divergence, while a subgroup is a division within a group, consisting of species more closely related to each other than to other members of the broader group and a complex is a group of cryptic species that are morphologically similar but genetically distinct [24]. For example, *Anopheles maculatus* and *Anopheles dirus*, two species within the Maculatus Subgroup and Dirus Complex may share similar morphological traits with other species within their subgroup and complexes, respectively, but exhibit distinct ecological behaviours from these other species [25]. Differences in ecological preferences (such as landscape type where found) and behaviours (such as larval habitat and host blood meal sources), can influence vectorial capacity and the effectiveness of different vector control interventions. Accurate species identification is, therefore, vital for effective control.

Mosquito vector biology and ecology

The family Culicidae includes over 3,500 mosquito species living in diverse habitats from tropical forests and savannas to urban environments [26, 27]. The mosquito life cycle has four stages: egg, larva, pupa and adult. The immature stages are aquatic, and the adult stage is terrestrial. Some species utilise both natural and artificial aquatic habitats, while others are more restricted to specific ecological niches. Both male and female mosquitoes feed on nectar and plant sugars, but only female mosquitoes are hematophagous, feeding on blood to obtain the necessary nutrients for egg development and their reproduction cycle [26, 27]. During blood-feeding, female mosquitoes can acquire pathogens from an infected host and later transmit them to another susceptible host through subsequent bites.

The vectorial capacity of mosquitoes, their ability to transmit pathogens, is influenced by several factors, including mosquito population density, survival rates, biting frequency, feeding preferences (anthropophagic vs. zoophagic), resting behaviours (endophilic vs. exophilic), and aquatic habitat preferences and competence (the ability to acquire and support the development and multiplication of pathogens until transmitted) [26, 27].

For example, species like *Ae*. *aegypti* are anthropophilic (preferring human blood) and endophilic (resting indoors), making them more susceptible to control measures like insecticide-treated nets (ITNs) and indoor residual spraying (IRS) [28]. Conversely, species like *Aedes albopictus* and members of the Leucosphyrus Group exhibit exophagic (outdoor-biting) and exophilic (outdoor-resting) behaviours, reducing the effectiveness of indoor-based control methods [27]. These behavioural variations influence the effectiveness of control strategies and highlight the need for a more comprehensive approach to mosquito control.

Vector control interventions: targeting mosquito behaviour

Vector control remains the cornerstone of malaria and dengue prevention. Current WHO recommendations focus on reducing human-vector contact, primarily through the use ITNs and IRS [29, 30], which are highly effective against indoor-biting and resting mosquitoes, respectively. Mosquito species that exhibit exophagic and exophilic behaviours, minimise their contact with ITNs and IRS by biting and resting outdoors, reducing the effectiveness of these tools, highlighting the need for complementary vector control interventions targeting other behaviours of such vectors.

Insecticide-treated nets are particularly effective for controlling nocturnal vectors such as *Anopheles* mosquitoes. Specifically, ITNs target species that seek blood meals mostly from humans (anthropophilic) and indoors (endophagic), meaning that they are more likely to encounter the insecticidal nets [29]. Indoor residual spraying is a highly effective tool for controlling endophilic *Anopheles* mosquitoes that rest indoors after feeding [30, 31]. Indoor residual spraying is also effective against the endophilic *Ae*. *aegypti* when applied to surfaces where *Ae*. *aegypti* is more likely to rest [3].

For outdoor mosquitoes like *Ae*. *albopictus* and members of the Leucosphyrus Group, outdoor-targeted interventions have promise for vector control. For example, attractive targeted sugar baits (ATSBs) can be deployed outside of houses to target the sugar feeding behaviour of *Ae*. *albopictus* while co-delivering as a promising alternative to traditional control methods [32, 33]. As mosquito behaviours shift through behavioural

resistance or through behavioural plasticity, vector control strategies must also shift to remain effective, requiring continuous monitoring and innovative approaches.

Malaria vectors in North Sumatra, Indonesia

North Sumatra Province, Indonesia, is classified as having low human malaria endemicity (1.19 annual malaria parasite incidence), with 18,361 cases reported in 2022 [34]. On the other hand, the caseload of enzootic malaria caused by *P. knowlesi* was 377 cases in 2017, the highest of any province in Indonesia [35]. Within the province, several *Anopheles* species have been identified as vectors in malaria transmission, including *Anopheles kochi*, *Anopheles nigerrimus*, *Anopheles sundaicus s.l.* and *Anopheles tesselatus s.l.* [36, 37]. Other species, such as *Anopheles barbirostris*, *Anopheles flavirostris*, *Anopheles karwari*, *Anopheles maculatus s.l.*, *Anopheles subpictus s.l.*, and *Anopheles vagus* were identified in the province, but their vector status remains undetermined [36, 37]. Despite the presence of enzootic malaria in North Sumatra, the *P. knowlesi* vectors have not been identified within the province [9, 38]. Given the emergence of enzootic malaria and the continued transmission of human malaria in Indonesia, it is essential to incriminate the mosquitoes responsible for human and zoonotic malaria transmission.

Dengue vectors in Torres Strait Islands, Australia

In the Torres Strait Islands (TSI), the displacement of *Ae. aegypti* by *Ae. albopictus* has complicated dengue control efforts. *Aedes albopictus*, a more opportunistic feeder, exhibits distinctly more outdoor-biting behaviours relative to *Ae. aegypti*, which has created additional vector control challenges [39, 40]. Efforts to eliminate *Ae. albopictus* in the TSI (through adult and larval control) were hindered by high human resource and operational costs, coupled with the technical difficulty of finding cryptic larval sites [41]. In response, in 2009, a *cordon sanitaire* was established to prevent the species spread to mainland Australia by maintaining an *Ae. albopictus*-free zone on Horn and Thursday Islands through intensive vector surveillance and control [41]. However, continued incursions of *Ae. albopictus* into the *cordon sanitaire*, highlight the need for effective, locally adapted, and easily deployable control strategies, particularly in the remote outer

islands with limited infrastructure [40]. Recently, ATSBs have shown promise to control outdoor vectors by delivering a toxicant during sugar feeding on baits [32, 33].

Thesis outline

This thesis addresses two fundamental requirements for vector control: (1) vector incrimination, which involves identifying the mosquito species and the pathogens they harbour, and (2) understanding the behaviours of these vectors to identify potential vulnerabilities to control strategies.

The rising incidence of both malaria and dengue, along with the emergence of zoonotic transmission, underscores the need for species-specific vector and pathogen identification to inform selection of targeted control measures. This thesis addresses these gaps by identifying and incriminating the species responsible for malaria transmission in North Sumatra. The research described in chapters 3 and 4 took place in Ujung Bandar Village, Salapian Subdistrict, Langkat Regency, North Sumatra Province, Indonesia, where enzootic and human malaria cases in the human population were detected. Chapters 3 and 4 each have two specific aims essential to establishing a foundation for malaria vector control in North Sumatra:

a. *Identifying mosquito species*: The first aim was to determine what anopheline species were present at the study site.
b. *Incriminating malaria vectors:* The second aim was to determine what anopheline species in the study site were potential vectors of malaria (whether enzootic or human)

Encompassing these aims, chapter 3 specifically examined species from the Leucosphyrus Group (the predominant Group responsible for enzootic malaria transmission) for their role as vectors in both enzootic and human malaria transmission, while chapter 4 identified other anopheline species not in the Leucosphyrus Group for their roles in both enzootic and human malaria transmission.

Understanding the behavioural traits of the target mosquito vectors is crucial for deploying effective vector control packages that target vectors that evade traditional

tools that target indoor biting and resting behaviours (i.e. ITNs and IRS). Chapter 5 builds on the results of chapters 3 and 4 to ascertain the potential vulnerability of the malaria vectors in North Sumatra to larval control, a strategy recommended by the WHO as a supplement to ITNs and IRS. Specifically: the third aim was to quantify the abundance and distribution of larval habitats of the zoonotic and human malaria vectors (incriminated in chapters 3 and 4) to define the potential of larval control for the enzootic and human malaria vectors in Ujung Bandar Village, North Sumatra.

The fourth aim addressed in chapter 6 was to quantify fructose feeding prevalence in the dengue vector, *Ae. albopictus*, in different habitats in two Torres Strait Islands to ascertain the potential of attractive targeted sugar baits to control this vector.

Before delving into the research chapters (chapters 3, 4, 5, and 6), chapter 2 explores in a literature review the hypothesis that human activities during the Anthropocene (climate, transport mechanisms, landscape changes, house structure, and vector control) are reshaping mosquito distributions and behaviours, directly affecting their vectorial capacity. Due to the limited understanding of many vector species, predicting these impacts remains a challenge. The review emphasizes the critical role of local knowledge of vector behaviour in developing and implementing effective control measures.

This thesis concludes with chapter 7, a general discussion that synthesizes the findings from the research chapters and offers an outlook on the future control of outdoor biting mosquitoes.

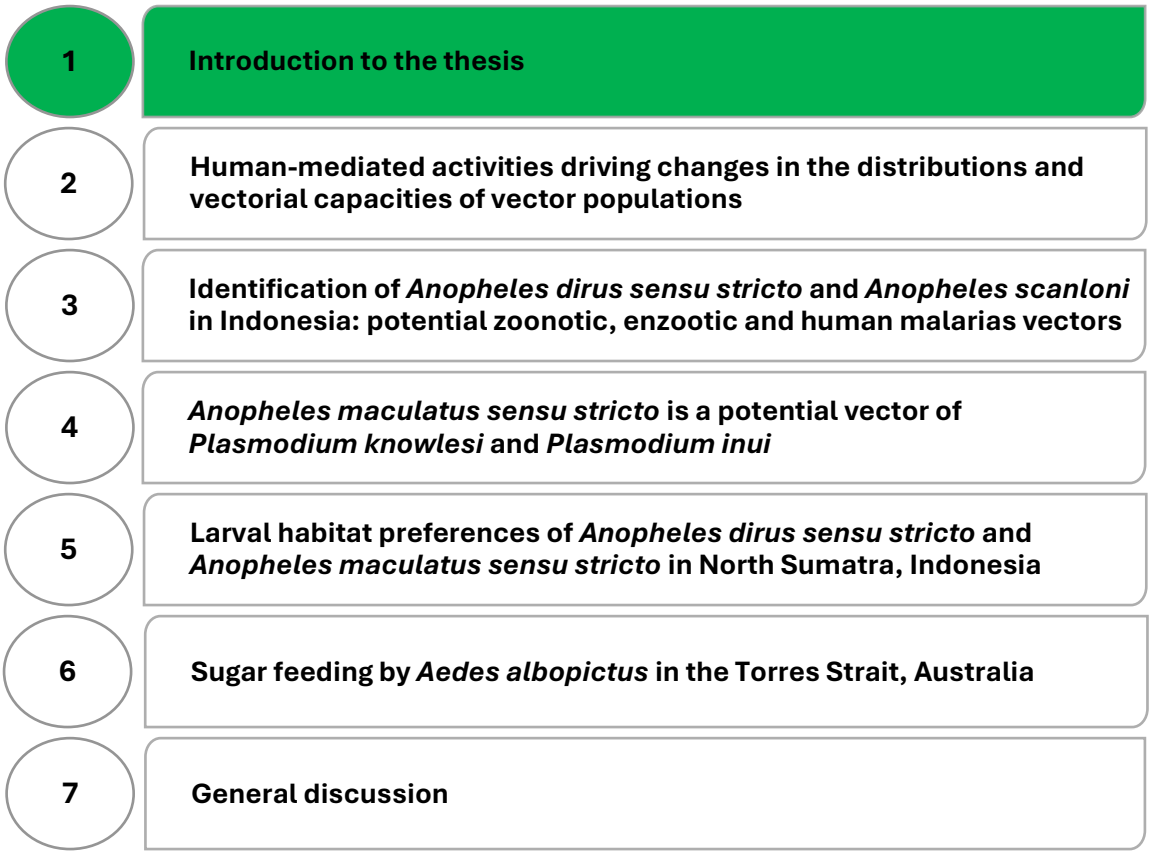
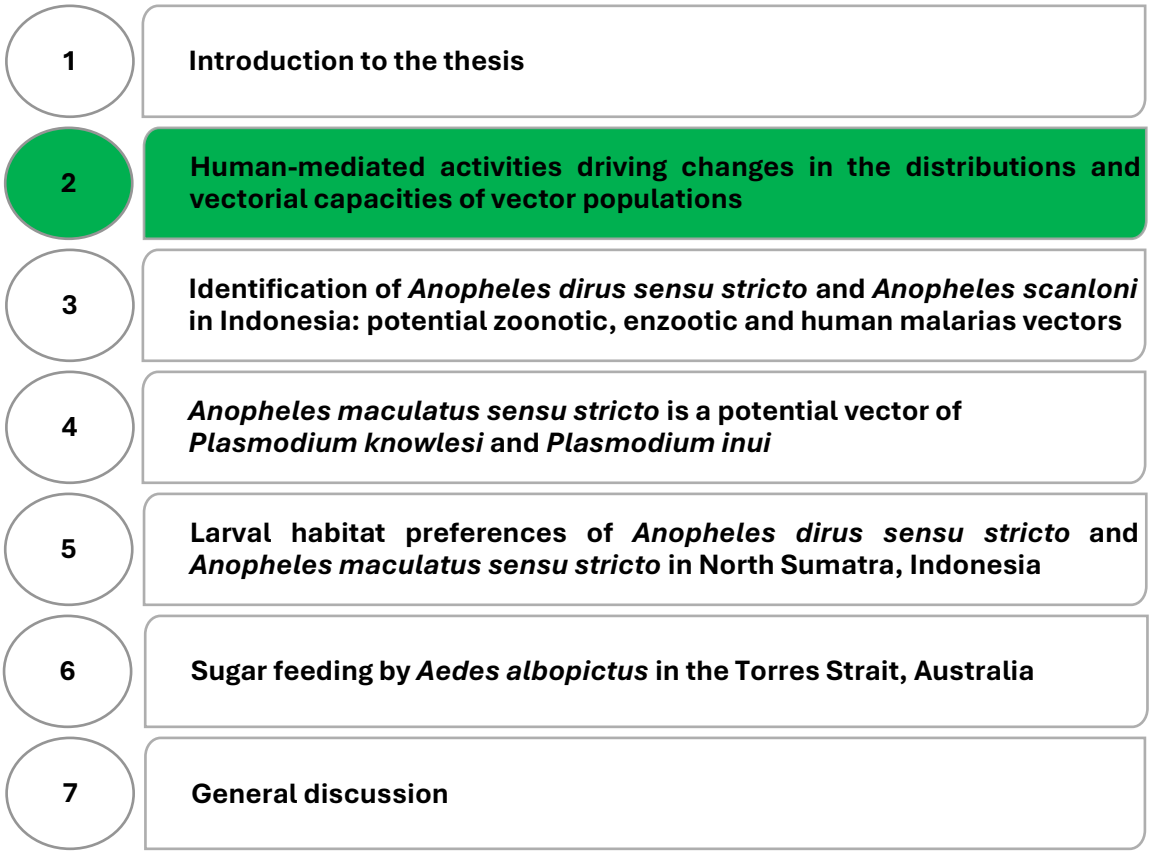


Figure 1.2. Thesis structure

Chapter 2

Chapter 2 presents the background for the thesis hypothesis: that human activities have changed vector distributions and bionomics, and, in so doing, change the risk of pathogen transmission, including the increasing risk posed by vectors that bite and rest outdoors. This literature review examines the impacts human-mediated climate change, passive vector transportation, land use changes (deforestation, agricultural development and urbanization), house improvements and programmatic vector control, have had on components of vectorial capacity (mosquito survival, behaviour, and distribution). Case studies illustrate how these human activities limit or enhance the capacity of mosquitos to transmit human pathogens.



Human-mediated activities driving changes in the distributions and vectorial capacities of vector populations

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Abstract

The Anthropocene is the proposed present geological epoch when human activities are significantly impacting the Earth's climate and ecosystems. During this time, human activities are creating selection pressures that are changing vector behaviours, and in so doing, change the distributions and vectorial capacities of mosquitoes, which will have effects on habitat receptivity. These human activities have changed land use patterns through urbanization, deforestation and agricultural developments, driven climate change and accelerated the movement of mosquitoes through human-mediated transportation by ships and planes resulting in changes in vector distributions. In addition, vector controls (both insecticide-based programmatic control and mosquito-proofing of houses) are driving changes in the receptivity and vectorial capacity of mosquitoes. Here, the complex relationships between mosquitoes and their environment in response to human activities are reviewed.

Keywords: Anthropocene, mosquitoes, receptivity, vectorial capacity, vector competence, climate change, urbanization, deforestation, landscape change

Background

Pathogens transmitted by mosquitoes cause important diseases in humans. There are over 3500 mosquito species worldwide, but only 331 species (< 10%) are implicated in human disease pathogen transmission [42, 43]. These mosquito vectors mainly belong to three genera, *Anopheles*, *Aedes,* and *Culex*. This review will focus on *Anopheles* spp., vectors of malaria and lymphatic filariasis (LF) and *Aedes*, that vector dengue, Zika and chikungunya [44].

Biological pathogen transmission by mosquitoes is complex with dependencies on vector competence and vectorial capacity [45]. To be competent means that the vector, after ingestion of a pathogen, can maintain the pathogen until transmitted. This requires the pathogen to overcome barriers to its development, multiplication and/or dissemination within the vector. The barriers to be circumvented depend on the route (e.g., oral, fecal) by which a pathogen is transferred from host to vector and from vector to host. The second requirement of a vector is described by vectorial capacity, which is closely tied to the concept of receptivity. Receptivity describes the potential of a spatial-temporally defined ecosystem to enable the transmission of pathogens. While originally describing the potential of an ecosystem to support vector populations, it also includes the susceptibility of the host population to a pathogen and the capacity of the health system to implement vector control as well as to diagnose and treat pathogens [46]. In this review, receptivity, defined as the capacity of an area to support vector populations, will be treated as synonymous with vectorial capacity.

Many insects and related arthropods live in close association with humans and animals. This characteristic enhances the opportunities for contact between hosts (humans/animals) and vectors (insects). Blood-feeding patterns can enhance the probability of an insect vector ingesting blood borne pathogens and subsequently transferring these pathogens to vertebrate hosts, either by mechanical or biological transmission [47].

In biological transmission, the pathogen may either multiply in the vector, develop but not multiply or multiply and undergo development. Dengue and Zika are well-known examples of one form of biological transmission by *Aedes aegypti* and *Aedes albopictus*

in which the viral pathogen is ingested during blood feeding and then multiplies and disseminates throughout the vector's body including into the salivary glands from which it can be transmitted when the vector next blood feeds. The time from the uptake of the pathogen, through its multiplication and dissemination and subsequent transmission via salivation during blood feeding is known as the extrinsic incubation period (EIP). Some arboviruses also invade the mosquito ovaries and can be transmitted during oviposition to the next generation, a process known as transovarial transmission.

A third form of biological transmission is when the pathogen both multiplies and develops inside the vector's body. Both *Trypanosoma cruzi*, the pathogen that causes Chagas disease, and the *Plasmodium* parasites of malaria develop and multiple inside the triatomine and mosquito vectors, respectively. Malaria is transmitted via the salivary glands when the infectious sporozoite stage is injected into the vertebrate host during blood feeding [48].

As mentioned earlier, for a vector to be competent, the pathogen needs to overcome internal mosquito barriers, including those associated with the stomach and salivary glands to be transmitted [49]. Vector competence is a function of the pathogen as well as the genetics of the vector which varies by species, strain and even individual mosquitoes. Vector competence can be strongly influenced by environmental factors including temperature, mosquito diet or microbial flora in the mosquito. Thus, “vector competence is a (function) of parasite infectivity and vector susceptibility and thus encompasses both host resistance mechanisms used to fight the infection and parasite infective mechanisms used to overcome the host’s defences” [50].

Vectorial capacity is an estimation of the potential for transmission and is thus estimated independent of the presence or absence of any pathogen. The components of vectorial capacity are vector density (abundance), frequency of feeding on pathogen susceptible hosts, and survivorship through the extrinsic incubation period [51, 52]. Vectorial capacity encompasses the behavioural characteristics of a species to find and blood feed on reservoir hosts and to survive long enough to compete the EIP. Hence, vectorial capacity is influenced by ecological factors. Many of the environmental factors that affect vectorial capacity, also affect vector competence, and those interactions can be

synergistic or offsetting in their impacts on the potential for transmission. The abundance of a vector species is an essential component of both the potential for transmission, the vectorial capacity, and as a component of actual transmission, the entomological inoculation rate, a product of the pathogen infection rate in the salivary glands (for malaria and dengue) and the biting rate. Hence, vector biting rates can serve as a proxy for estimating transmission (either potential or actual) [53].

The frequency of vectors feeding on susceptible hosts is a function of vector abundance and the relative host abundances and their distributions, often described by the host blood index or the host feeding preference. The host blood index being the proportion of blood meals on a species, whereas the host feeding preference is the comparative ratio of the number of blood meals on two species to the relative number of hosts of each of those two species in an area. These indices will be impacted by factors affecting access to hosts such as shelter/house construction and mosquito activity patterns including relative indoor and outdoor biting rates and time of peak biting.

Both vector competence and vectorial capacity can be influenced by environmental factors. For example, the availability of water is crucial to a mosquito's life cycle, starting from oviposition through larval development to emergence of the adult stage. The abundance of aquatic habitats for the immature mosquito stages will affect the abundance of adult mosquitoes as will survivorship of the adults which will be impacted by temperature and humidity. The behaviours of mosquitoes, and hence their vectorial capacity, are influenced by the local ecology and environment.

This review is focused on how human activities during the Anthropocene (climate, transport mechanisms, landscape changes, house structure, and vector control) drive changes in anopheline and *Aedes* mosquito behaviours that underly the components of vectorial capacity. In so doing, these mosquito behavioural changes can impact the potential for transmission. This review is limited to mechanisms by which human activities affect the potential for transmission with a focus on the components of vectorial capacity (but recognising that many of the environmental factors that affect vectorial capacity, such as temperature and rainfall, also affect vector competence and those interactions can be synergistic or offsetting in their potential impacts on

transmission. This review will focus on the biological vectors of malaria (species in the genus *Anopheles*) and dengue (*Aedes* species) with limited examples from *Culex* mosquitoes.

References were identified using the PubMed, Scopus and Google Scholar databases. The references of selected papers were also screened to identify relevant literature, including review articles, publications from the Centers for Diseases Control and Prevention (CDC) and the World Health Organization (WHO) were also examined**.** The body of this narrative will review how exogenous factors that have changed significantly during the Anthropocene are impacting vectorial capacity. Each of the anthropogenic drivers (changes in climate, transportation, landscapes, housing and vector control), are introduced separately before considering their combined influences on vectorial capacity (Figure 2.1).

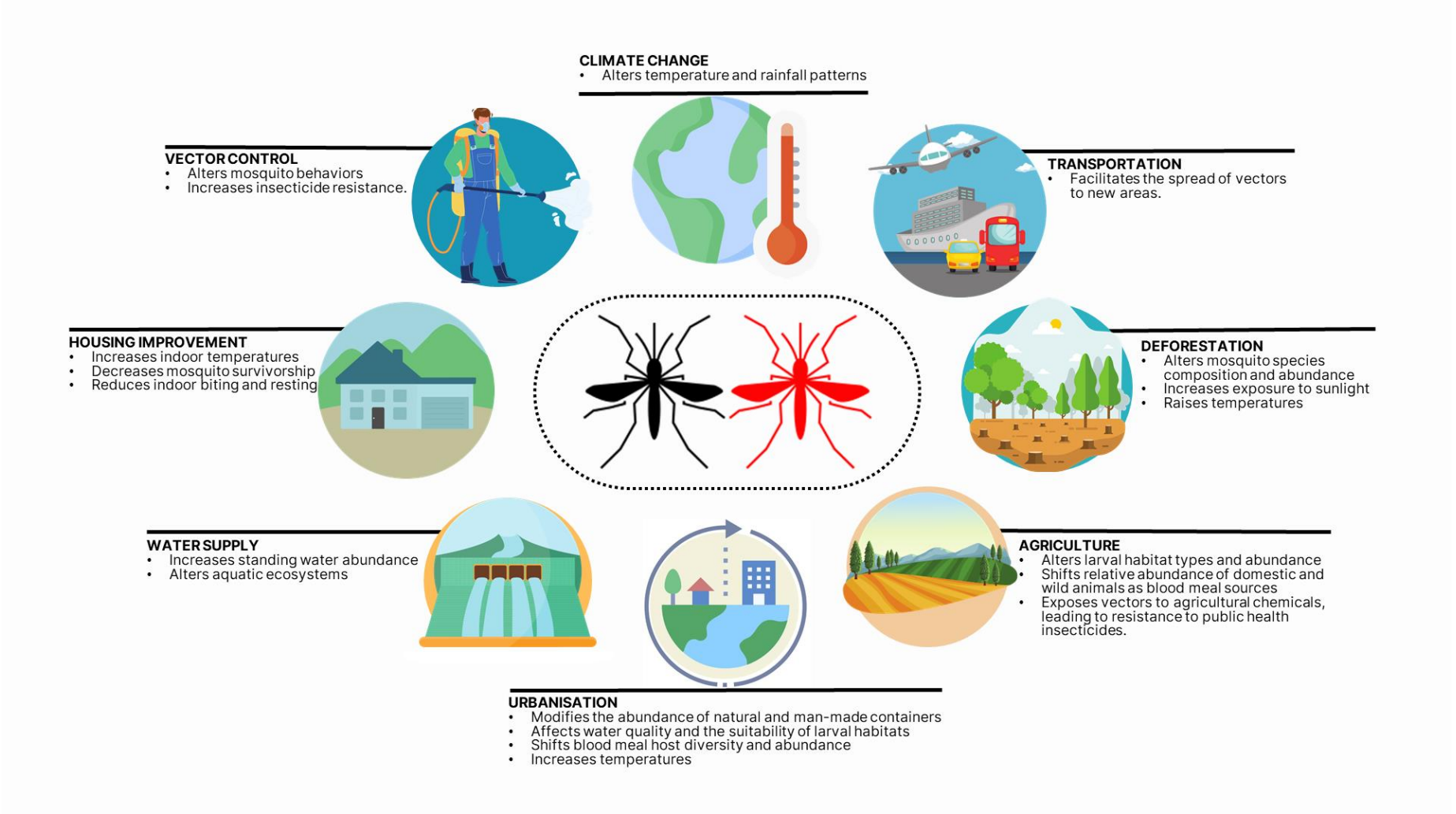

Figure 2.1. Human activities influencing the vectorial capacities and distributions of mosquitoes in the Anthropocene. All images are licensed under Canva.

Anthropogenic impacts on *Anopheles* and *Aedes* mosquitoes

Climate change

Climate change describes the variation over an extended time (decades or longer) in the weather. While nature can cause changes in climate, such as by volcanic eruptions and solar radiation, human activities are recognized as playing a significant role in causing climate change [54]. The anthropogenic factors are largely due to human activities that release greenhouse gases such as carbon dioxide and methane along with soot absorbing sunlight, leading to latitudinal and altitudinal temperature changes [54, 55]. Climate is becoming increasingly unpredictable with warmer shorter winters and hotter and longer summers.

The effects of human activity led climate change are being felt across the world and are intensifying. Resulting ecosystem changes will likely alter species composition and the structure of ecological communities [55-57]. Climate is more than temperature: it is a function of interrelated factors including rainfall patterns and humidity [57-61]. These three factors affect mosquito abundance [62-66], biting rates [65], and survival [67], all components of vectorial capacity. As well, climatic factors impact vector competence and the extrinsic incubation period [65, 68]). It is well documented that seasonal shifts in climate (summer/winter or wet/dry seasons) can drastically alter the abundance of mosquito populations. As the climate warms, the geographic range of mosquitoes will change with some places becoming more suitable for mosquito introduction and establishment, such as in Europe [69] and North America [70] for tropical species. On the other hand, some areas that are currently suitable for mosquitoes may become inhospitable [57].

In addition, environmental temperature (both average temperature and fluctuations in temperature) affects developmental times (generation times), fitness and thus vectorial capacity [59, 64]. Temperature acts on all mosquito species and their physiological processes to determine their rate of development and survival and, thus, their abundance [57, 59, 61]. The optimal temperature range for mosquito species varies greatly. While *Ae. aegypti* is primarily adapted to tropical and subtropical climates, *Ae.*

albopictus populations are well adapted to a broader range of environmental conditions, including tropical, subtropical, and temperate regions.

The optimal survival rate for immature *Ae. aegypti* is at 26°C [65]. The development time of *Ae. aegypti* from egg to adult ranges from 7.2 days at 35°C to 39.7 days at 15°C [66]. However, developmental rates can vary considerably among populations; for example, an Australian strain of *Ae. aegypti* developed 19 days faster at 15°C than a strain from Louisiana [66]. Such variation highlights the importance of considering population-specific thermal responses when interpreting laboratory findings. Furthermore, the temperature during immature stage development also affects adult traits such as body size, fecundity, and survivorship [71]. For *Aedes albopictus*, adult survival was inversely correlated with temperature, with the highest survival rate at 15°C and the lowest at 35°C [72].

Rainfall is another important climatic driver of species distributions and vectorial capacity. Total annual rainfall determines the abundance of larval habitats, but the relationship can be more complicated when examined at a finer scale. For mosquitoes that use groundwater pools as larval habitats, including many anopheline species, increased rainfall can create more potential larval habitats for mosquitoes. However, heavy rains can flush such larval habitats of their immature mosquitoes when they overflow [57, 73]. While droughts may eliminate standing water, droughts can also create standing water as streams levels drop to create groundwater pools [57], as was seen for the members of the Punctulatus Group in Indonesia [74], and similarly by creating *An. farauti* habitats in stagnant lagoons in the Solomon Islands [75]. Droughts may also stimulate humans to store water in barrels and tanks which serve as immature habitats for *Aedes* [57]. Precipitation also affects habitat productivity, as rainfall can increase the number and quality of water-filled sites that support larval development. However, excessive rain may flush out larvae, while drought may reduce natural habitats but increase artificial ones through water storage practices [76].

Temperature may change the distributions of adults. A modelling study of Africa in 1998 examined the redistribution of malaria during the previous 20 years when the average

temperature increased by 0.5°C and concluded that temperature significantly changed the altitude at which malaria occurred [77].

Transportation

Vector abundance and distribution are key components defining receptivity of an area for mosquito populations. When an exotic vector species becomes established in a virgin area, the potential transmission risk profile for that area changes dramatically by becoming receptive to the transmission of pathogens not previously documented there or by an increase in the number of vector species present in the area. Mosquito movement can be active or passive. Active mosquito flight can expand the distribution of a species into neighbouring areas where the microclimate is suitable to support vector populations [78]. There is variability in mosquito flight patterns: usually quite short, but varying by species [79, 80].

In contrast, passive transportation of mosquitoes by wind enables large numbers of mosquitoes to move rapidly across long distances as demonstrated in Africa [81] and hypothesized to be the mechanism by which *An. stephensi* crossed Saudi Arabia and into and across Africa [82]. Of interest to this review, is passive transport of mosquitoes by human activities, sometimes over great distances. During the Anthropocene, human assisted movement of mosquitoes by airplanes, ships, and land vehicles has increased in importance [83, 84]. Human aided transportation enabled the global expansion of *Ae. aegypti* in the 16th and 18th centuries[83], *Cx. quinquefasciatus* in the 19th and 20th centuries [85] and *Ae. albopictus* in the 20th and 21st centuries [84].

Aedes aegypti originated in African forests. The expanded distribution of *Ae. aegypti* started with its introduction to the New World between 1500 – 1650 on sailing ships with the slave trade from West Africa [83, 86, 87] during the early period of European colonization. *Aedes aegypti* is now found in 167 countries [8]. The opening of the Suez Canal in 1987 is believed to have facilitated the spread of *Ae. aegypti* to Asia, Australia and the Pacific countries [86]. *Aedes albopictus* originated in subtropical and tropical Asian regions but is now found in 126 countries [8]. The first reports of the expanded range of *Ae. albopictus* was the invasion into Albania (Europe) in 1979 [88] and then in 1985 to Texas (USA) [89]. *Aedes albopictus* dispersal was largely via used tyres and lucky

bamboo on ships [90]. There are 197 and 188 countries potentially suitable for *Ae. aegypti* and/or *Ae. albopictus* establishment, respectively, so the dispersal of these species may not be over [91]. The expanded range of the primary *Aedes* vectors has massively changed the dengue transmission landscape with the number of countries experiencing dengue outbreaks increasing 30-fold over five decades [92].

The *Aedes* arbovirus vectors have some key characteristics that make them well adapted to human assisted movement. The dissemination of *Aedes* is often via their desiccation-resistant eggs (e.g., the eggs can survive prolonged dry periods when the embryoes remain dormant until the eggs are flooded and hatch) [93, 94]. Up to 40% of *Ae. aegypti* eggs can hatch after 120-days of desiccation [95]. *Aedes albopictus* eggs can diapause to survive more temperate winter seasons than *Ae. aegypti* with eggs hatching up to six months after being oviposited [96, 97]. Hence, eggs oviposited in used tyres and other containers remain viable during prolonged transport on ships, planes and vehicles.

Human-mediated transport, particularly via ships, plays a critical role in shaping the vectorial capacity of invasive mosquito species by enabling their survival, reproduction, and eventual establishment in new environments. During long-distance maritime travel, multiple mosquito life cycles may be completed, especially when larvae inhabit water storage containers onboard [83]. For instance, *Ae. aegypti* and *Ae. albopictus*, whose life cycle from egg to adult spans approximately 8–10 days, can complete several generations during transcontinental voyages if suitable larval habitats (e.g., water-holding containers) and blood meal sources (e.g., passengers or crew) are available. Even in the absence of blood meals, female *Ae. albopictus* can survive for up to 12 days indoors by feeding on sugar sources, such as those found in ornamental plants like lucky bamboo [98], increasing the likelihood of successful transport.

Upon arrival, the ability of invasive mosquitoes to establish and transmit pathogens in a new region is influenced by their ecological competitiveness and reproductive interactions with native species. Both *Ae. aegypti* and *Ae. albopictus* are known to be superior larval competitors compared to many native mosquito species [99], allowing them to colonize new environments rapidly. Notably, *Ae. albopictus* has been associated with the displacement of *Ae. aegypti* in regions such as the southeastern United States

(e.g., Alabama [100] and Florida [101]) and Bermuda [102]. This displacement may result not only from larval competition but also from satyrization, a form of reproductive interference in which *Ae. albopictus* males mate with *Ae. aegypti* females, leading to infertile eggs and reduced reproductive success of *Ae. aegypti* [103-105].

These dynamics directly affect vectorial capacity, as the dominant vector species in an area influences the risk and efficiency of pathogen transmission. Interestingly, the outcome of interspecies competition is context-dependent. In some Asian cities (e.g., Kuala Lumpur, Manila, Bangkok, Taiwan) and in Colombia, *Ae. aegypti* has successfully displaced *Ae. albopictus*, demonstrating that local ecological and urban factors also mediate these interactions [104, 106].

Although anopheline species have limited tolerance to drying environments and can only persist for short periods in damp substrates, there are numerous examples of their introduction into new areas through human-mediated passive transport. One of the most well-known examples is the introduction of *Anopheles gambiae s.l.* into northeastern Brazil in the 1930s, most likely via shipping from West Africa, which resulted in severe malaria epidemics before the species was successfully eliminated in 1940 through an intensive eradication program [107]. More recently, *An. stephensi* has, in the Anthropocene, spread from the Indian subcontinent across the Arabian Peninsula and the Horn of Africa to Nigeria [108] increasing the risk of malaria transmission in urban environments [109-111]. A modelling study estimated that *An. stephensi* could put an additional 126 million people in Africa at risk of malaria if the vector were to spread unchecked [111]. The human-assisted movement of *An. bancrofti* from Australia to New Caledonia [112], most probably by aircraft (*An. bancrofti* was first found near the Noumea airport), raised the vectorial capacity in New Caledonia for malaria by transforming the previously malaria non-endemic Pacific Island territory to one where transmission could potentially occur. In Guam, *An. indefinitus, An. subpictus, An. sinensis, An. vagus, An. baezl, An. lesteri, An. tessellatus, An. litorialis* and *An. barbirostris* were introduced and became established after World War II [113]. Most invading mosquitoes reached Guam in association with increases in international air traffic. In Guam, local malaria transmission was documented following malaria parasite introductions via infected humans [103, 113, 114]. The introduction of *Anopheles*

species by human assisted transport into formerly non-malarious areas (e.g., Guam and New Caledonia) or into urban areas of limited receptivity for endemic species (e.g., *An. stephensi* in Africa) has increased the receptivity and potential for malaria transmission.

Anthropogenic landscapes

Anthropogenic landscapes are areas where human activities directly alter the physical environment to service the needs of human populations. These human activities include deforestation, introduction and expansion of agriculture, urbanisation, and water project developments [115, 116]. Anthropogenic landscapes benefit the human population through economic growth and productivity. However, anthropogenic landscapes are often highly fragmented fine-scale landscape mosaics that disturb the ecological processes affecting communities of vertebrate and invertebrate organisms, including mosquito species [117, 118]. The changing ecologies resulting from human activities have cascading impacts on mosquito species vectorial capacity due to the combined influence of interacting exogenous factors, including changes in habitat availability, blood meal host distributions and abundance and resting habitats that can impact mosquito survivorship [119, 120].

Deforestation

Deforestation is the removal of trees by human activities, often by harvesting logs for revenue or clearing land for agricultural development, with road construction and transmigration programs driving urbanisation [121-124]. Deforestation affects every occupant of an ecosystem [121]. Deforestation can either increase or decrease malaria receptivity and vectorial capacity, depending on the bionomics of mosquitoes in an area. Deforestation directly reduces or eliminates mosquitoes such as *Anopheles minimus* that use shaded water bodies as larval habitats [125]. Removing trees reduces the canopy creating favourable conditions for mosquitoes that prefer temporary ground pools exposed to full sunlight, such as *Anopheles gambiae s.l.*, *An. arabiensis* and *An. funestus* in Africa, particularly in African highlands [121, 126, 127] and *An. maculatus* in Malaysia [128]. Reductions in canopy cover by deforestation can increase the indoor mean temperature by 1.8°C and outdoor temperature by 1.4°C, while reducing mean

indoor humidity by approximately 22.6% [55, 129], these microclimatic changes can shorten the mosquito gonotrophic cycle by an estimated 1.7 days [129] and accelerate sporozoite development, reducing the average duration from 14 days to 12.6 days [130].

Deforestation has been associated with increased vector-host interactions [61]. Following deforestation in Southeast Asia, the Leucosphyrus Group mosquitoes blood-fed on long-tailed and pig-tailed macaques in undisturbed forests [122, 131]. As a consequence of logging, the macaque populations spend more time travelling and foraging on the forest floor [122]. The activities of forest workers, loggers and farmers in deforested and forest-fringe areas has escalated increasing in spatial overlap between humans and wildlife disease reservoirs, most notably macaques, in deforested areas [68, 122, 132]. These ecological shifts elevate host contact for Leucosphyrus Group mosquitoes; when macaques are unavailable, humans may serve as alternative blood sources. By feeding on both macaques and humans in altered landscapes, these vectors enhance their vectorial capacity for *Plasmodium* transmission from macaques to humans [133, 134]. To date, human *P. knowlesi* cases are mostly reported from individuals who have a history of exposure by travel to forests [134], underscoring the role of deforestation in promoting zoonotic disease transmission.

Agriculture

More than half of the habitable land is used for agriculture, with 77% of that land dedicated to livestock farming and the remaining 23% used for crop cultivation [135]. The conversion to agricultural lands following deforestation greatly influences mosquito species distribution and abundance [68, 136, 137] by changes to the available larval habitat types created by human activities, introduction of domesticated animals as blood meal hosts, and invasion of anthropophilic mosquitoes, all of which change vectorial capacity [137, 138]. A notable example is the conversion of forest to agro-forestry systems, such as oil palm plantations in Sarawak, Malaysia, which created new larval habitats for *Ae. albopictus* and increased opportunities for human–vector contact, thereby raising the risk of *Aedes*-borne diseases such as dengue [139].

Agricultural irrigation, including irrigated paddy fields, also creates ideal larval habitats for many malaria vector mosquitoes [140, 141]. *Anopheles gambiae s.l.* (most likely *An.*

arabiensis) and *An. funestus* populations are generally associated with irrigated paddy fields [142]. In the Rusizi Valley, Burundi, the vectorial capacity of *An. gambiae* s.l was 150 times higher in a paddy field irrigation scheme than in an adjacent area growing cotton [143]. Livestock, as documented in Ethiopia, create numerous water-filled hoof prints, ideal habitats for mosquito larvae such as *An. arabiensis* [57, 144]. Due to the increased presence of humans, agricultural activities are also associated with increases in the availability of artificial (man-made) containers that become larval habitats [68].

Agricultural pesticides contribute to insecticide resistance (IR) in mosquitoes [145-147]. The intensive and uncontrolled use of pesticides, particularly in small-scale agriculture holdings may affect mosquitoes, particularly those that bite and/or rest outside of houses [148, 149]. As most agricultural insecticides are of the same chemical classes and share the same targets and modes of action as those used for vector control, they can directly select for cross-resistance to the public health insecticides used to control mosquitoes [149]. The repeated exposure of *An. gambiae* larvae to deltamethrin, DDT and bendiocarb used in agriculture selected for resistance against the insecticides used for adult vector control [149]. In addition, mosquito larvae exposed to pesticides frequently display a higher tolerance to insecticides through the induction of their detoxifying systems and other mechanisms [150].

Urbanisation

Over half of humans now live in cities with predictions that this will increase to over three-quarters by 2050 [151]. Urbanisation replaces natural landscapes with vegetation with man-made surfaces which affect mosquito populations [152]. The types of aquatic habitats available changes with increases in man-made artificial water containers and with more polluted water in ground pools and drains. Urbanisation also reduces blood meal host diversity [153, 154]. For mosquitoes that readily use containers for aquatic habitats such as *Ae. aegypti* [155-159], urbanisation provides abundant larval habitats capable of generating large populations.

Aedes aegypti, in particular, successfully adapted to human habitats (domestication) following its migration to the New World (see section on transportation) [83, 84, 90]. As a result, adult *Ae. aegypti* numbers are higher in urban than in rural areas because of the

greater density of artificial containers (e.g., water storage jars and drums, cemetery urns, abandoned tyres, buckets, flowerpots, rain catch basins and discarded tins) available as larval habitats [157]. A study in China also showed that urbanisation substantially increased the mosquito density (adults) and adult survival of *Ae. albopictus*, which increased its vectorial capacity [160].

Many urban characteristics such as high human population density, areas with dense construction and lack of natural vegetation contribute to increases in surface temperature, known as urban heat islands (UHIs) [161]. The UHIs significantly influence both water quality and mosquito vector densities [161-163] by increasing the temperature of standing water in urban environments, mosquito larval development is accelerated, shortening both gonotrophic and extrinsic incubation periods, and thereby enhancing vectorial capacity [161-163].

In Africa, increases in urban malaria incidence involving *An. gambiae s.l.* are linked to their ability to exploit suitable natural breeding sites in urban and peri-urban settings, such as temporary sunlit pools, wells, and rice fields, rather than adaptation to artificial containers [164]. In contrast, *An. stephensi* readily breeds in human-made water containers, enabling it to persist in densely populated urban areas and potentially expand the range of urban malaria transmission [108-110].

Water Supply

Water supply and storage capacity systems (including dams, irrigation, water gates, piped water supply, guttering, drains and water storage such as water tanks) promotes economic growth and food security and alleviates poverty [165-167]. Water supply systems such as piped water to households contributes to reductions in the number of artificial containers used to store water (and which serve as *Aedes* larvae habitats) [167]. However, when water supply systems are not maintained, new challenges for vector control develop. For example, dams increase the abundance of standing water and drastically change aquatic ecosystems and the ecology of rivers [168]. Vegetation at the margins of dam-impounded waters can create potential larval sites, resulting in the proliferation of adult mosquitoes, particularly *Anopheles* [57]. In addition, dams are often associated with an increase in agriculture with irrigation creating numerous larvae

habitats for mosquito vectors [144]. Furthermore, by creating year-round water storage, dams often provide year-round suitable larval habitats in areas where larval habitats were previously seasonal, enabling mosquitoes to maintain their populations throughout the year. In 2015, 28.8 % of dams built in sub-Saharan Africa were in unstable malaria transmission areas [168], which could intensify vectorial capacity and malaria transmission or change transmission from seasonal to year-round and perennial [165].

The human population density close to irrigation schemes and dams is always high compared to area without a reliable water supply. High human density coupled with an associated abundance of potential mosquito larvae habitats near dams can increase the risk of malaria four-fold [169], contributing to more than 1.1 million malaria cases each year in Africa [165, 170]. At the Koka dam of Ethiopia, the malaria incidence in communities living close to the dam was nearly 20-times higher than in communities living more than 6 km away [169]. However, the association of dams and increased vectorial capacity is not universal. In some areas of Mali [171] and Mauritania [172], *An. gambiae s.s* and *An. funestus* were replaced after construction of dams by less anthropophilic species such as *An. arabiensis* and *An. pharoensis* that used the shoreline as larval habitats [171, 172]. While the vectorial capacity of *An. arabiensis* and *An. pharoensis* increased, there was no impact on malaria as the vectorial capacity of *An. gambiae* s.s. and *An. funestus* was diminished. The slope of the water body was the strongest predictor of malaria incidence around large dams [168]. In Zambia [173] and western Kenya [174], steeper slopes were associated with fewer transient mosquito habitats in shallow ground water pools.

Housing improvement (mosquito-proof houses)

Mosquito-proof houses target indoor anthropophagic and nocturnal biting vectors, such as *An. gambiae* [175, 176]. This species is highly endophagic (indoor biting), endophilic (indoor resting) and anthropophagic (feeding primarily on humans) [177]. House-screening against malaria mosquito bites was pioneered by Angelo Celli in 1899 [178]. Since then, houses have evolved in both construction and materials that reduce mosquito entry [179], a collateral benefit that often occurs with improvements in housing quality and standards of living. In many settings, features such as closed eaves,

screened windows and doors, brick walls, tiled or metal roofs, and ceilings installed to improve comfort, durability, and overall household well-being also reduce exposure to mosquitoes [175, 176, 179-181]. In this way, house improvements can be viewed both as part of the wider socio-economic development process that shapes healthier living environments, that also acts as a malaria control measure.

Traditional houses usually have an open construction, and this condition allows mosquitoes to easily detect humans and to enter the houses to bite [182-184]. When detecting humans inside a house, *An. gambiae* fly up and enter the house through the eave at the top of the walls [182] entering houses through wall gaps as small as 1-2 cm wide [185, 186]. Counterintuitively, housing with fewer doors and windows attract more mosquitoes [187, 188] because houses with less ventilation accumulate human odours, and CO_2 indoors (the primary long-range mosquito attractant), and elevate indoor temperatures, all of which increase the attractiveness of such houses to mosquitoes [187]. Similarly, smaller houses or houses with more residents increase the concentration of human odours, attracting mosquitoes and raising the vectorial capacity [180, 189]. When the number and size of screened windows of a house were increased, fewer mosquitoes entered, reducing human-vector exposure and vectorial capacity. Housing improvements, including screening, limit human biting by mosquitoes and thus lower vectorial capacity [176].

Vector control

Vector control strategies aim to reduce human–mosquito contact and interrupt disease transmission. These interventions lower vectorial capacity mainly by decreasing mosquito survival. As a result, fewer mosquitoes live long enough to become infectious. The most efficient vector control approaches for malaria and dengue vectors involves chemical insecticides, including the organochlorine, DDT, organophosphates, carbamates and pyrethroids [148]. Synthetic pyrethroids are the most widely used insecticides for public health applications today due to their relatively low cost, low mammalian toxicity and high invertebrate toxicity that rapidly immobilizes (‘knockdown’) and kills mosquitoes [190]. Insecticide-treated nets (ITNs) and indoor residual spraying (IRS) are the primary WHO-recommended malaria vector control tools. Larval control,

including the use of larvicides and focal IRS against adults, are recommended by WHO for A*edes* vector control [155, 191].

The success of these interventions is based on exploiting innate vector behaviours that make them vulnerable to the interventions. For example, *An. gambiae s.s.* is highly anthropophagic, endophagic, endophilic and late night-biting [192, 193]. Hence, IRS on walls targeted where *An. gambiae s.s.* would rest after blood feeding on humans late at night, targeting the endophilic (indoor resting) nocturnal and anthropophilic behaviours of *An. gambiae*.

Anopheles gambiae and *An. funestus* are generally more anthropophagic and endophilic, than *An. arabiensis* which is more opportunistic in biting both livestock and humans while feeding and resting both outside and inside houses [194, 195]. In Tanzania, after the introduction of IRS in 1955-1959, the highly endophilic *An. funestus* disappeared leaving only an extremely exophilic *An. gambiae s.l.* (most likely *An arabiensis*) [196]. Similarly, IRS eliminated the predominantly indoor resting and human biting *An. koliensis* in the Solomon Islands [197]**.** The use of IRS successfully reduced malaria transmission by *An. minimus* as this species strongly tended to bite indoors in some areas. However, this vector has shown marked variations in its behaviour from endophilic to exophilic and anthropophilic to zoophilic: in northern Vietnam, *An. minimus* was more attracted to cattle and other domestic animals kept near the house [198, 199].

Widespread access and use of ITNs resulted in a decline in abundance of *An. gambiae s.s.* and *An. funestus* in Tanzania and Kenya leaving the more behaviourally plastic *An. arabiensis* as the dominant vector in East Africa [200-202]. Insecticide treated nets and IRS changed the vector species composition and thereby impacted the vectorial capacity of the targeted malaria vectors that were not resistant to the insecticides used in ITNs and IRS. The intensive use of insecticides for both vector control and agriculture; however, had the unintended consequence of driving the development of resistance to insecticides in mosquito populations and reducing the effectiveness of control measures, particularly in Africa [148, 203, 204]. Resistance to insecticides has steadily increased in malaria, dengue and filariasis vectors since insecticides were first

introduced for vector control [203]. Furthermore, the uncontrolled use of insecticides in agriculture, including small-scale vegetable farming, played a significant role in selecting for insecticide resistance in mosquitoes [148, 205]. In addition, pollutants in urban and industrial areas such as heavy metals, hydrocarbons, and chemical residues can induce the upregulation of detoxification enzymes in mosquitoes, including cytochrome P450 monooxygenases, glutathione S-transferases (GSTs), and carboxylesterases [206]. This enzymatic upregulation enhances the mosquitoes' ability to metabolize and eliminate toxic substances, including insecticides, leading to increased physiological tolerance or resistance [207].

Vectors exhibit a range of resistance mechanisms against insecticides, including behavioural plasticity as well as behavioural and physiological resistance which increases their vectorial capacity by increasing their survivorship [208-210]. Behavioural resistance and plasticity are manifested as avoiding contact with insecticides by avoiding contact with insecticides and insecticide-treated surfaces, and thus not being killed [208, 211]. Behavioural resistance is based on a genetic change in the population such that the behavioural change remains after removal of the selective pressure to avoid insecticides whereas behavioural plasticity is a temporary change in behaviour with reversion to the original behavioural phenotype when then selective pressure (the insecticide) is removed. These behavioural adaptations include changes in host preference, peak biting time, biting location (endophagy or exophagic) and resting location (endophily and exophily) [208, 209, 212], thereby increasing their vectorial capacity by increasing mosquito survivorship and abundance. To avoid insecticides in ITNs, some mosquito species bite earlier in the evening before people enter houses or later in the morning after people exit houses. For example, *An. funestus* bites later in the morning in Benin [213] and even into daylight hours in Senegal [214]. To avoid the insecticides applied by IRS, *An. arabiensis* in Tanzania shifted from resting indoors to outdoors [215]. Overcoming, the challenge of behavioural change (whether resistance or plasticity) requires changing the intervention strategy [216].

Extensive insecticide use also results in physiological resistance in mosquito populations. Physiological resistance is the ability of an insect to survive exposure to an insecticide concentration that normally kills [210]. In general, the mechanism of IR in

mosquitoes is target-site mediated resistance which involves non-synonymous mutations affecting the proteins targeted by insecticides [149]. One or more mechanisms may be involved in physiological resistance, including alteration of target site nerve receptors (e.g., kdr, Rdl and Ace.1R genes) and detoxification via increased enzyme activity of nonspecific esterases, GSTs and P-450 mediated monooxygenases (mixed function oxidases) [217]. Other mechanisms of metabolic resistance in mosquitoes include improved biodegradation of the insecticide through metabolic processes.

Conclusion

Each mosquito species is found within a range of climatic (temperature, rainfall and humidity), ecologic (such as blood meal host species and availability) and environmental (including larval habitats and adult resting sites) parameters. The habitat characteristics (climatic, ecologic and environmental parameter) determine the receptivity of an area and collectively impact a mosquito species vectorial capacity. Changes to any of the climatic, ecologic and environmental parameters will affect its vectorial capacity.

This review examined the impact of human activities on the vectorial capacity of some malaria and dengue vectors. Human activities are changing the patterns of mosquito-borne diseases. Man-made climate change, human-mediated transportation, particularly shipping and air travel, landscapes modified by humans, mosquito-proof houses and vector control programs have had pronounced impacts on the receptivity of areas and on the vectorial capacity of mosquito vectors by changing vector survival, abundance, feeding behaviours and distributions. Where the immediate impact on human activities may be diminished vectorial capacity, many examples exist of vectors adapting their behaviours to their environment, and often result in a subsequent elevated vectorial capacity.

Anthropogenic landscapes from deforestation, agriculture development, urbanisation and water control projects are directly impacting mosquito abundance, species diversity, biting and resting behaviours. The relationship between mosquito species and their environment is complex and unique for each species. Human induced

perturbations of any of the climatic, ecologic and environmental characteristics of a landscape may enhance or reduce the vectorial capacity for a species. For most vector species, detailed knowledge of their distributions and behaviours is limited, making predictions of the impacts of anthropogenic drivers of vectorial capacity challenging.

Supplementary Materials

Additional files

Not applicable

Abbreviations

Ace.1R: acetylcholinesterase; *Ae.*: *Aedes*; *An.*: *Anopheles*; *Cx.*: *Culex*; GSTs: glutathione S-transferases; IR: insecticide resistance; IRS: indoor residual spraying; ITNs: insecticide-treated bed nets; JEV: Japanese encephalitis virus; Kdr: knockdown resistance; LF: lymphatic filariasis; Rdl: Resistant to dieldrin; SSA: sub-Saharan Africa; *T.*: Trypanosoma; UHIs: urban heat islands; USA: United States of America; *W.*: *Wuchereria*; WHO: World Health Organization; WNV: West Nile virus.

Declarations

Acknowledgements

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Availability of data and materials

Datasets were not generated nor analysed in this review.

Authors' contributions

BFS, TLR and TRB conceived and designed the review and critically revised the manuscript. All authors read and approved the final manuscript

Ethics approval and consent to participate

Not applicable

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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Chapter 3

The previous chapter supported the thesis hypothesis that human activities have altered vector distributions and bionomics, thereby influencing pathogen transmission risk, including the increasing threat by vectors that bite and rest outdoors. As reviewed, mosquito behaviours are continuously adapting to dynamic environmental conditions, highlighting the critical need for ongoing local knowledge of vectors to implement effective and targeted vector control. This chapter (and chapter 4) examines human and zoonotic malaria transmission in an area that had undergone significant land use change in which the forest was replaced by establishing an oil palm plantation and other agricultural areas and villages. Chapter 3 identified and incriminated species in the Leucosphyrus Group, which are known *P. knowlesi* vectors in other regions in Ujung Bandar Village, Salapian Subdistrict, Langkat Regency, North Sumatra Province, Indonesia. The research includes molecular confirmation of mosquito species present and an assessment of their vector status by analysing for the presence of malaria DNAs in the heads and thoraces of mosquitoes.

Identification of *Anopheles dirus sensu* stricto and *Anopheles scanloni* in Indonesia: potential zoonotic, enzootic and human malarias vectors

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Abstract

Background:

Despite North Sumatra having the most reported *Plasmodium knowlesi* malaria cases in humans in Indonesia, the vector species are unknown. This study aimed to identify the Leucosphyrus Group species present in Ujung Bandar Village, Salapian Subdistrict, Langkat Regency, North Sumatra and to screen for evidence of malaria infections.

Methods:

Leucosphyrus Group mosquitoes collected by human landing catch in two areas of Ujung Bandar village were identified morphologically to group with species identification confirmed with ITS2 gene sequencing, Dirus Complex species identification PCR and a Scanloni-specific PCR. Reverse-transcription real-time and nested PCR assays targeting the 18S rRNA gene were used to detect and identify zoonotic and human malaria parasites.

Results:

Two species of the *Anopheles dirus* complex were confirmed from an analysis of 597 *Anopheles leucosphyrus* group mosquitos. *Anopheles dirus sensu stricto* was identified in 97.8% of samples and 2.2% were *Anopheles scanloni*. A total of seven *An. dirus s.s.* samples (2.2%; 95% CI: 1.2–3.7%) tested positive for *Plasmodium* by genus-level RT-qPCR, while six samples (1.0%; 95% CI: 0.4–2.2%) yielded equivocal results. Among the positive samples, five were mixed infections involving *P. inui*, *P. knowlesi*, and/or *P. vivax* with one *Plasmodium* equivocal sample being a mixed infection with *P. coatneyi* and *P. knowlesi*. BLAST results for *P. fieldi* PCR-positive samples suggest possible primer cross-reactivity with *P. inui*.

Conclusions:

This study reports the first molecularly confirmed *An. dirus s.s.* and *An. scanloni* in North Sumatra. Macaque and human *Plasmodium* species were identified in *An. dirus s.s.* suggesting that this species may play a role in transmitting zoonotic, enzootic, and human malarias in North Sumatra.

Keywords: *Plasmodium knowlesi*, Leucosphyrus Group, *Anopheles dirus sensu stricto*, *An. scanloni*, North Sumatra, enzootic malaria.

Background

The incidence of human malarias with their associated morbidity and mortality has diminished significantly with 44 countries and one territory certified as having eliminated malaria since 1962, including 18 countries since 2000 [1, 218]. However, *Plasmodium knowlesi*, historically recognized as a enzootic malaria parasite in natural long-tailed and pig-tailed macaques, has emerged as a significant zoonotic public health threat in Southeast Asia (SEA), where human infections are increasingly being reported [218, 219]. In Indonesia, the first human case of *P. knowlesi* was documented in South Kalimantan in 2010, and since then, cases were reported in Central and South Kalimantan, Aceh, and North Sumatra [35, 220-225]. Despite 377 reported human *P. knowlesi* cases in North Sumatra in 2017, the vector(s) responsible remain unknown [35, 226, 227].

There are eight confirmed vectors of *P. knowlesi* in SEA, including five species from the Leucosphyrus Group of *Anopheles* (*Anopheles balabacencis*, *Anopheles latens*, *Anopheles dirus* (*s.s.* and *s.l.*), *Anopheles cracens,* and *Anopheles introlatus*) [11-15], one species from the Barbirostris Group (*Anopheles donaldi*) [16, 17] and two species in the Umbrosus Group (*Anopheles collessi* and *Anopheles roperi*) [18, 19]. *Anopheles hackeri* was reported as a vector of *P. knowlesi* and other enzootic malarias in the 1960s [228, 229], but there have been no subsequent reports of *An. hackeri* as a *P. knowlesi* vector. *Anopheles sundaicus* is a suspected *P. knowlesi* vector based on a finding of a single positive pool of whole mosquitoes in the Andaman and Nicobar Islands, India [230].

There are limited data on the distributions, behaviours, and vector competences of the Leucosphyrus Group members for zoonotic malaria in SEA [226]. North Sumatra, Indonesia is an understudied site for investigating the *Anopheles* vectors of zoonotic malaria [226, 227]. On Sabang Island in neighbouring Aceh Province, *An. dirus s.l.* and *An. cracens* were reported [231-233]. In the Langkat Regency, North Sumatra, Leucosphyrus Group mosquitoes were the most prevalent *Anopheles* mosquitoes collected [B. v. d. Straat et al., unpublished]. Understanding the species composition and malaria vector status of the Leucosphyrus Group is critical for developing targeted control strategies for both zoonotic and human malarias, as even closely related

Anopheles species may vary in feeding preferences, resting habits, and susceptibility to insecticides, which will impact the effectiveness of vector control strategies. This study analysed samples of morphologically identified Leucosphyrus Group mosquitoes from North Sumatra by PCR to identify the species present. It also determined their malaria vector status for Plasmodium species infecting humans and non-human simians through molecular analyses.

Methods

Study site and mosquito collection

Sites for mosquito sampling were based on a *P. knowlesi* regional transmission predictive risk map [234, 235] using estimates of the distributions of Leucosphyrus Group mosquitoes, macaques and human population densities, and environmental land types present to classify high-risk health facility catchment areas. The study was carried out in two of nine hamlets (dusuns), 'Dusun II' (3.371720, 98.328547) and 'Dusun V' (3.341947, 98.335406) of Ujung Bandar Village, Salapian Subdistrict, Langkat Regency, North Sumatra, Indonesia, where *P. knowlesi* and human malaria infections were previously identified by surveillance of humans cases in 2022-2023 [I. N. D. Lubis et al., unpublished]. The village is located in a hilly area at an elevation of 300-600 meters above sea level, surrounded by oil palm plantations, small-scale mixed agriculture areas, and patches of disturbed forest. The area receives over 3000 mm of rainfall annually, with daytime temperatures typically ranging from 29-34°C and nighttime temperatures from 19-24°C.

Mosquitoes analysed had been sampled by human landing catch (HLC) at six sampling stations in each of the two Dusuns from July 2022 to June 2023 [B. v. d. Straat et al., unpublished]. Mosquitoes were morphologically identified in a field laboratory to genus, species group or species [236, 237]. Adult mosquitoes were then stored in 1.5 ml Eppendorf tubes with cotton wool and silica gel. All samples were stored at room temperature until molecularly analysed to identify mosquito and *Plasmodium* species (Figure 3.1).

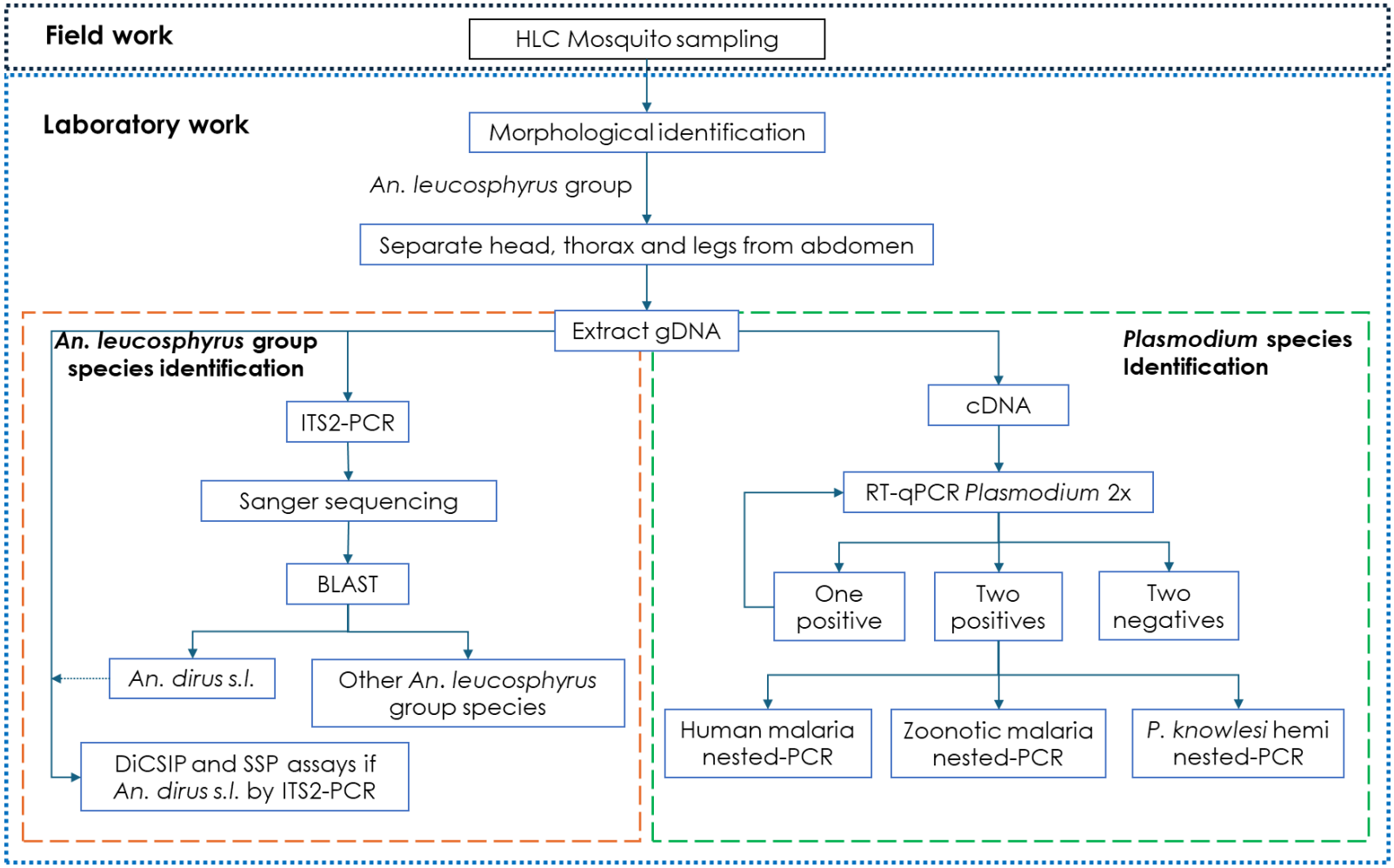

Figure 3.1. Workflow for mosquito collection, species identification of Dirus Complex mosquitoes and malaria species detection and identification.

DNA extraction and reverse transcription

The DNA of the heads and thoraces of individual adult Leucosphyrus Group mosquitoes were extracted separately using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A 10 µL aliquot of genomic DNA (gDNA) from each extracted sample was transformed into complementary DNA (cDNA) using a high-capacity cDNA reverse transcription PCR kit (Applied Biosystems by Thermo Fisher Scientific, Vilnius, Lithuania) for *Plasmodium* detection assays.

Identification of species in the Leucosphyrus Group

All morphologically identified specimens of the Leucosphyrus Group were analysed for mosquito species identification using gDNA PCR amplification of the ribosomal internal transcribed spacer 2 (ITS2) region [238, 239]. Initially, a single point ITS2 PCR using primers ITS2A and ITS2B confirmed which specimens belonged to the Dirus Complex. The ITS2 amplification products of thirty Dirus Complex specimens were subsequently sequenced at the Macrogen facility (Macrogen Inc., Seoul, Korea) and analysed through the Basic Local Alignment Search Tool (BLAST) in the NCBI database.

Specimens identified as *An. dirus* complex from ITS2 sequences were then analysed using Dirus Complex species identification PCR (DiCSIP) and a Scanloni-specific PCR (SSP) [239]. Five positive controls of species in the Dirus complex (i.e., *An. dirus*, *An. cracens*, *An. scanloni*, *An. baimaii,* and *An. nemophilous)* were provided by the Department of Entomology, Faculty of Agriculture, Kasetsart University (KU) and National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. Detailed assay protocols for the Leucosphyrus Group identifications are available in Additional file 1.

Plasmodium detection and identification

The *Plasmodium* genus 18 rRNA gene from the small ribosomal subunit was amplified from mosquito cDNA samples run in replicate using a real-time quantitative polymerase chain reaction (RT-qPCR) assay [240]. Samples generating discrepant replicant results, such a >3 difference in Ct values, or when one of the duplicates yielded an equivocal or negative result while the other duplicate was positive, were repeated. DNA from a clinical

blood patient infected with a *P. knowlesi* mono-infection served as the *Plasmodium* genus control for all samples. Positive results were defined as having a cycle threshold (Ct) value of <35. Negative results were defined as having a Ct value of >40 in duplicate. Equivocal results referred to ambiguous findings, with Ct value between 35-40. These equivocal samples were retested using cDNA template diluted 1:10 in the RT-qPCR assay. If the repeated assay still yielded a Ct value <40, the samples proceeded to species-specific *Plasmodium* identification.

Positive and equivocal samples for the *Plasmodium* genus were then analysed using reverse transcriptase nested polymerase chain reaction (RT-nPCR) with the same gene target (18S rRNA) to detect both human (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*) and macaque malaria parasites (*P. knowlesi, P. inui*, *P. cynomolgi*, *P. coatneyi and P. fieldi*) [131, 241, 242] with results visualised by 1.5% agarose gel electrophoresis. Clinical isolates for each human *Plasmodium* species and *P. knowlesi* were used as positive DNA controls, with validated gBlocks™ synthetic controls for other enzootic *Plasmodium* species such as *P. inui*, *P. cynomolgi*, *P. coatneyi and P. fieldi* [243]. Detailed assay protocols for *Plasmodium* detection and identification are available in Additional file 2.

Results

Members of the Dirus Complex

Of 597 morphologically identified *An. leucosphyrus* Group mosquitoes, 584 (97.8%) were identified by DiCSIP as *Anopheles dirus sensu stricto*, with 13 (2.2%) being *Anopheles scanloni* by the SSP assay. Both species were found in both Dusun II and V, Ujung Bandar Village, Salapian Subdistrict, Langkat Regency, North Sumatra. Nine specimens of *An. dirus s.s.*, with 729 bp ITS2 gene sequences, were submitted to GenBank as accession numbers: PQ589932 - PQ589940. The BLAST results for all specimens showed a minimum 99.9% sequence identity match with *An. dirus* in the reference database.

Plasmodium genus and species identification

Seven *An. dirus s.s.* samples (1.2%; 95% CI: 0.5-2.4%) tested positive for *Plasmodium* by RT-qPCR, while six samples (1%; 95% CI: 0.4-2.2%) yielded equivocal results (Table 3.1). *Plasmodium* cDNA was not detected in any *An. scanloni* specimens.

Five of the seven *Plasmodium* genus positive RT-qPCR samples had mean Ct values less than 31 (Table 3.1; Figure 3.2). These five *Plasmodium*-positive *An. dirus s.s.* samples. and one of the equivocal samples (Ct: 39.2 ±0.5) were identified to *Plasmodium* species (Table 3.1; Figure 3.2). Six mosquito specimens identified as positive for a *Plasmodium* species were co-infected with other *Plasmodium* species (Table 3.1; Figure 3.2). Of the three *An. dirus s.s.* cDNA specimens positive for *P. knowlesi*, one sample was also positive with *P. inui*, *P. fieldi* and *P. vivax*, one with *P. inui* alone, and one with *P. coatneyi*. Thus, the *P. knowlesi* infection prevalence in *An. dirus s.s.* in this area was 0.5% (3/597; 95% CI 0.1-1.5%). Four specimens were positive for both the enzootic macaque malaria, *P. inui,* and *P. vivax,* the human malaria.

PCR product identities were confirmed by comparing to GenBank database references for *P. vivax*, *P. inui*, *P. coatneyi, P. fieldi*, and *P. knowlesi*. The BLAST results matched the RT-nPCR results, except for RT-qPCR positives for *P. fieldi* which were identified as *P. inui* by BLAST.

Table 3.1. Identification of human and zoonotic *Plasmodium* species in *An. dirus s.s.*

No.	Ct value[1] x̄ (±SD)	RT-qPCR results	Human *Plasmodium* species[2]		Zoonotic *Plasmodium* species[3]				
			Pv*	Pf, Pm, Po	Pin*	Pcy	Pct	Pfiel	Pk[4]
1	24.7 (±0.1)	Positive	+	-	+	-	-	+/-	-
2	25.5 (±0.4)	Positive	+	-	+	-	-	+/-	-
3	27.8 (±0.1)	Positive	+	-	+	-	-	+/-	-
4	28.0 (±0.4)	Positive	-	-	+	-	-	-	+
5	30.1 (±0.8)	Positive	+	-	+	-	-	+/-	+
6	33.0 (±0.5)	Positive	-	-	-	-	-	-	-
7	34.2 (±0.4)	Positive	-	-	-	-	-	-	-
8	35.1 (±0.4)	Equivocal	-	-	-	-	-	-	-
9	35.2 (±0.9)	Equivocal	-	-	-	-	-	-	-
10	36.2 (±1.8)	Equivocal	-	-	-	-	-	-	-
11	37.3 (±1.4)	Equivocal	-	-	-	-	-	-	-
12	38.4 (±0.7)	Equivocal	-	-	-	-	-	-	-
13	39.2 (±0.5)	Equivocal	-	-	-	-	+	-	+

[1]RT-qPCR assay by Kamau et al., 2011
[2]RT-nPCR assay by Snounou et al., 1993
[3]RT-nPCR assay by Lee et al., 2011
[4]RT-hemi nPCR PCR assay by Imwong et al., 2009
Pf: *Plasmodium falciparum*; Pv: *P. vivax*; Pm*: P. malariae*; Po: *P. ovale*; Pk: *P. knowlesi*;
Pin: *P. inui*; Pcy: *P. cynomologi*; P. ct: *P. coatneyi*; Pfiel: *P. fieldi*. +: positive result; -: negative result

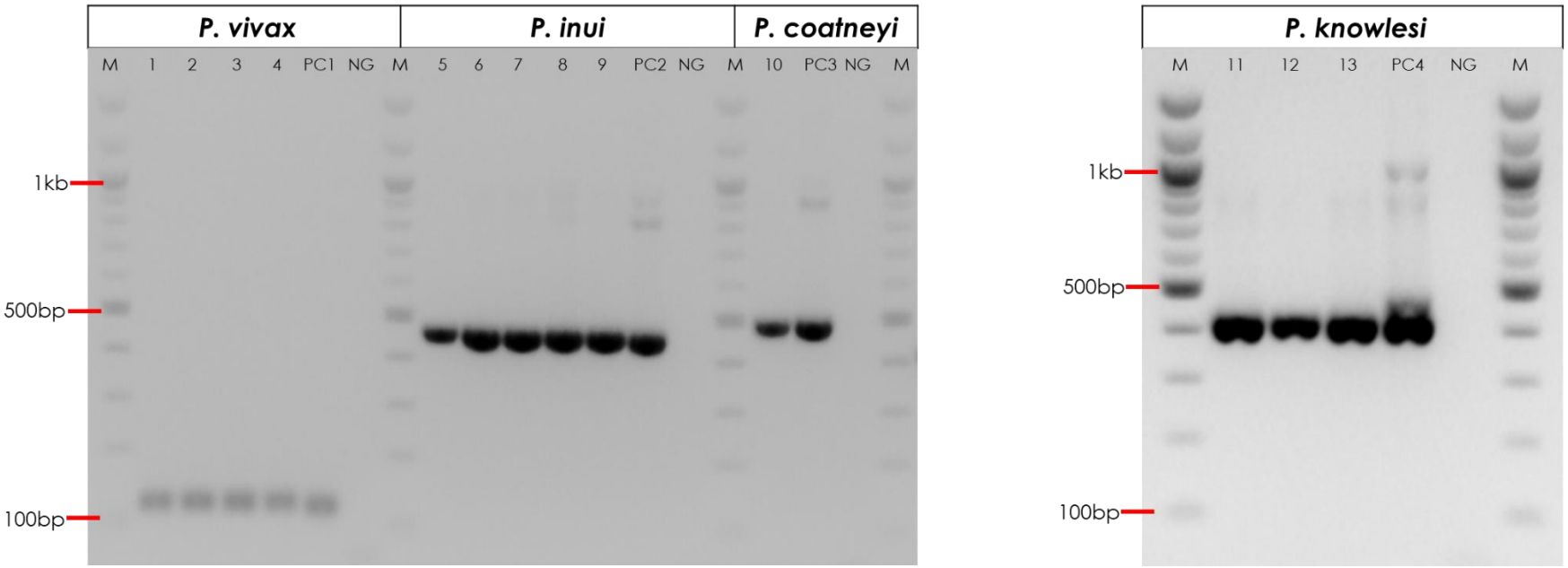

Figure 3.2. Positive *Plasmodium* species from RT-nPCR results. Lanes 1-4: positive *P. vivax* (120 bp); Lanes 5-9: positive *P. inui* (479 bp); Lane 10: positive *P. coatneyi* (503 bp); Lane 11-13: positive *P. knowlesi* (410 bp). PCs: positive controls; NG: negative control: M: 100 bp ladder marker. The 1.5 % agarose gel (EtBr 10mg/ml) was run at 100 volts for 85 minutes.

Discussion

Understanding and responding to the emerging threat of zoonotic and human malarias in the SEA region requires detailed comprehensive knowledge of vector species distributions, their ecological preferences, and the behaviours influencing their interactions with macaques and humans and the *Plasmodium* parasites that they harbour [226, 244]. Major knowledge gaps on the vectors of zoonotic, enzootic and human malarias were addressed in North Sumatra, Indonesia from this study. With a population of at least 15.6 million people in 2023, over 50% of whom live in rural areas, the at-risk population in North Sumatra, Indonesia depends on the distribution of the known vectors.

The spatial distribution of mosquito species within the Leucosphyrus Group remains poorly defined across South and SEA including North Sumatra. Because of potential shifts in distributions, occurrence mapping requires ongoing surveillance to understand transmission risk [235, 236, 245, 246]. *Anopheles dirus s.s.* and *An. scanloni* both belong to the Dirus Complex within the Leucosphyrus Group (*Cellia*) in the Neomyzomyia Series [236, 247]. The Dirus Complex consists of five additional known species: *An. cracens*, *An. baimaii*, and *An. nemophilous* (all found in mainland SEA), *An. elegans* (Sri Lanka and southwestern India), and *An. takasagoensis* (Taiwan) [236, 248-250]. In addition, a potential member of the Dirus Complex, *An. aff. takasagoensis*, refers to morphologically similar populations found in northern Vietnam that are genetically distinct from *An. takasagoensis* from Taiwan [251].

Anopheles dirus (*s.s.* and *s.l.*), formerly known as *An. dirus* A, has been confirmed in Thailand, Myanmar, Cambodia, Lao PDR, Vietnam, India, Bangladesh, and southern China (Hainan Island) [38, 226, 252-255]. In Indonesia, *An. dirus s.l.* has only been reported based on morphology and only in Sabang Island (Aceh) in 2013 and 2023 [231, 256]. In addition, *An. scanloni* had not been reported in Indonesia and had only been previously identified as *An. dirus* C along the Myanmar-Thailand [257, 258].

Species-specific identifications within the Dirus Complex are challenging due to overlapping morphological characteristics among complex members [236, 248]. Until recently, species differentiation relied primarily on allele-specific multiplex PCR [259].

However, this method often produced nonspecific and inefficient amplification, raising uncertainty as to identification accuracy [239]. The recent development of species-specific PCRs for *An. dirus s.s.* (DiCSIP) and *An. scanloni* (SSP) [239] has improved the reliability of distinguishing members of the Dirus Complex. Since *An. dirus s.s.* and *An. scanloni* are morphologically identical and previous conventional allele-targeting PCR assays were non-specific, reports of *An. dirus s.l.* in some areas may include *An. dirus s.s.* and *An. scanloni* in sympatry.

The likely presence of *An. dirus s.s.* and *An. scanloni* in North Sumatra were strengthened in this study by the use of the species specific DiCSIP and SSP assays and targeted sequencing. However, sequence data is resource-intensive, requiring specialised laboratory equipment, skilled personnel, and substantial computational resources, and its accuracy is highly dependent on the availability of a comprehensive and well-curated reference database. The absence of complete ITS2 sequence data for *An. scanloni* and *An. nemophilous* creates uncertainty in identifying the members of the Dirus Complex [239].

Anopheles dirus (*s.s.* and *s.l.*) is an important enzootic, zoonotic and human malaria vector species. Sporozoites of *P. falciparum* and *P. vivax* were detected by CSP-ELISA and PCR (18S rRNA) in *An. dirus* (*s.s.* and *s.l.*) in Thailand [260-265], Cambodia [266], Lao PDR [267, 268], Vietnam [269], Myanmar [270, 271], India [272] and southern China (Hainan Island) [273]. *Anopheles dirus* (*s.s.* and *s.l.*) was confirmed as a vector of *P. knowlesi* using molecular assays including PCR targeting the CSP and 18S rRNA genes, RT-PCR for CSP mRNA and sporozoite surface protein 2 (SSP2) mRNA and Sanger sequencing based on the CSP gene [274-276]. In previous studies, both *An. dirus* (*s.s.* and *s.l.*) salivary glands and human blood samples were found to be positive both for *P. knowlesi* and co-infected with *P. falciparum* and/or *P. vivax* [274-276]. In this study in North Sumatra, evidence of infections (e.g., DNA) of the zoonotic malaria, *P. knowlesi,* in *An. dirus s.s.* were found with an estimated prevalence of 0.5%.

In laboratory experiments, *An. dirus s.l.* supported *P. cynomolgi*, *P. inui*, *P. fieldi* and *P. coatneyi* development to the sporozoite stage [277-280]. The transmission of additional natural macaque *Plasmodium* species in SEA has yet to be confirmed in *An. dirus*

populations [233]. In this study, the DNAs of the enzootic malarias *P. inui* and *P.coatneyi* were detected in *An. dirus* with *P. vivax* co-infections, echoing findings from Vietnam where co-infections of *P. falciparum, P. vivax* and *P. knowlesi* were reported [274, 276].

Because macaque malaria parasite co-infections are commonly reported [274, 276], (including in this study in North Sumatra), concerns have been raised as to their veracity. *Anopheles balabacensis*, a member of the Leucosphyrus Complex, was reported in Sabah and Sarawak, Malaysia, to be co-infected with combinations of *P. knowlesi*, *P. cynomolgi*, *P. fieldi*, *P. inui,* and *P. vivax* as detected by PCR [16, 281]. *Plasmodium knowlesi* is predominantly a mono-infection in humans [282]. It is possible that some of these co-infections may reflect, a degree of primer cross-reactivity among closely genetically related *Plasmodium* species in macaque hosts, as was suggested in this study by *P. fieldi* specimens identified by PCR later identified as *P. inui* by BLAST. Furthermore, primer cross-reactivity between *P. cynomolgi* and *P. vivax* has also been reported [282, 283]. However, it must be noted that other studies failed to find *P. cynomolgi* primer amplification and the robust design and validation of the separate *P. knowlesi* hemi-nested assay for specificity against other macaque malarias [241, 242] support the major infection prevalence findings in this study.

In this study, seven *An. dirus s.s.* specimens tested positive for *Plasmodium*, with five specimens identified to species. Several factors may explain these results. Firstly, the RT-qPCR assay to detect the genus *Plasmodium* is more sensitive than the species identification nested PCR assays [240, 243]. Secondly, the species-specific identification assays used in this study were designed primarily for detecting human and macaque *Plasmodium* species [131, 240, 242]. This raises the possibility that *An. dirus* (*s.s.* and *s.l.*) may harbor other enzoonic malaria parasites from other hosts. While *An. dirus* (*s.s.* and *s.l.*) is typically known for its anthropophilic behaviour, variations in host feeding preferences observed in this study, along with reports of less anthropophilic tendencies in certain locations, suggest that its role as a malaria vector may vary depending on ecological factors [253].

In this study, samples with equivocal qPCR results, defined by borderline Ct values (in the range of 35–40) were further analysed at the species level. These results were

considered inconclusive as they may present either low level infections or false positives arising from technical artifacts such as non-specific amplification, degraded or low-concentration DNA, cross-contamination, or amplification near the limit of detection of the assay [284-286]. Importantly, high Ct values may indicate a low quantity of *Plasmodium* DNA in *Anopheles* vectors, potentially reflecting low parasitemias in vectors (and humans), which is often observed in the early stages of zoonotic malaria transmission [287]. Consistent with this, one of the equivocal samples in this study was confirmed as a mixed infection with *P. knowlesi* and *P. coatneyi*, underscoring the importance of analysis for weak positives, particularly in the context of zoonotic surveillance.

Although *An. scanloni* were not positive for *Plasmodium* DNA in this study, *An. scanloni* is known as a secondary vector of *P. falciparum* and *P. vivax* in Thailand [38, 288] suggesting that its involvement in malaria transmission in North Sumatra cannot be excluded. The absence of *Plasmodium* infections in *An. scanloni* here may be due to the limited sample size rather than a lack of vector potential. Future studies with larger sample sizes will be crucial to elucidate the vector status of *An. dirus s.s.* and *An. scanloni* in this region.

Conclusion

Anopheles dirus s.s. and *An. scanloni* were identified in North Sumatra, Indonesia. *Anopheles dirus s.s.* harboured the DNA of both macaque and human malaria parasites, including *P. knowlesi*, *P. inui*, *P. coatneyi*, and *P. vivax*. *Plasmodium* DNA was not detected in *An. scanloni*. These findings suggest that *An. dirus s.s.* may play an important role as a vector of zoonotic, enzootic and human malarias in North Sumatra, justifying intensified surveillance to better understand both its distribution and its role in malaria transmission, and its amenability to public health control measures in North Sumatra.

Supplementary Information

Additional file 1. Identification of *An. leucosphyrus* members.

Additional file 2. Detection of *Plasmodium* malaria parasites.

Additional file 3: Figure S3.1. Amplification of *An. dirus* and *An. scanloni* by PCR assay.

Abbreviations

An.: *Anopheles*; cDNA: complementary deoxyribonucleic acid; CSP-ELISA: circumsporozoite protein enzyme linked immunosorbent assay; Ct: cycle threshold; DNA: deoxyribonucleic acid; gDNA: genomic deoxyribonucleic acid; ITS2: internal transcribed spacer 2; *P.*: *Plasmodium*; PCR: polymerase chain reaction; RT- nPCR: reverse transcriptase nested polymerase chain reaction; RT-qPCR: reverse transcriptase real-time polymerase chain reaction; SEA: Southeast Asia; s.l.: *sensu lato*; s.s.: *sensu sricto*.

Declarations

Ethical approval

This work was approved by the Universitas Sumatera Utara, Faculty of Medicine (application number 723/KEP/USU/2021) on 14 July 2021 and James Cook University (application number H8583) on 29 September 2021.

Availability of data and materials

Not applicable

Competing interest

The authors declare that they have no competing interests.

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Foreign Affairs and Trade, Australian Government. BS was supported by a James Cook University Postgraduate Research Scholarship. MJG is supported by a NHMRC EL2 Investigator grant.

Authors' contributions

BFS, TRB, TLR and MJG conceived and designed the study. TRB, TLR, TAG, INDL and MJG advised on fieldwork. BFS, AH, and BvdS conducted field studies. BFS and AMH conducted the laboratory analyses with input from TAG, MS, SM, INDL and MJG. BFS drafted the original draft of the manuscript, and TRB and TLR provided supervision. All authors improved the manuscript and then reviewed and approved the final manuscript.

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Supplementary information

Additional file 1: Identification of the Leucosphyrus Group members

A. Single-point ITS2 PCR for Sanger sequencing

A single-point PCR targeted the ITS2 gene using ITS2A and ITS2B (table S3.1), as described by Beebe and Saul (1995). The amplification master mix consisted of 1x GoTaq® flexi green buffer (Promega, Madison, WI, USA), 3.0 mM MgCl2 (Promega), 0.2 mM dNTP mix (Promega), 0.5 µM of each ITS2A and ITSB primers in a total volume of 30 µL (Table S3.1), 1.25 U GoTaq® Flexi DNA polymerase (Promega), 1µL of DNA template and filled u with nuclease-free water (NFW) [25]. The cycling conditions were an initial denaturation step at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 51 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. The amplification products of single-point ITS2 PCR were purified using ExoSAP-ITTM (Applied Biosystems by Thermo Fisher Scientific, Vilnius, Lithuania) and sequenced using the forward primer and reverse with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems by Thermo Fisher Scientific) at the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea).

B. Dirus complex species identification PCR (DiCSIP) assay

The DiCSIP assay identified five members of the Dirus complex, as described by Saeung et al. (2024). The reaction master mix containing 1x GoTaq green buffer (Promega), 2.0 mM of $MgCl_2$ (Promega), DMSO 4%, 0.2 mM of dNTP mix (Promega), 0.1 µM of each primer (DiCSIP-Uni-Fwd, DiCSIP-Rev-AC, D-B, D-D and DiCSIP-Rev-F) (Table S3.1), 1.25 U GoTaq® Flexi DNA polymerase (Promega), 0.5 µL of template and NFW to 12.5 µL. The cycling parameters were an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 20 sec, annealing at 62 °C for 20 sec and extension at 72 °C for 1 min, with a final extension step at 72 °C for 5 min. The amplification products were visualised through an electrophoresis process.

C. Scanloni-specific PCR (SSP) assay

The SSP assay as described by Saeung et al. (2024) was used to differentiate *An. dirus* and *An. scanloni* when the PCR products of DiCSIP assay showed band sizes similar to *An. dirus* (521 bp) and *An. scanloni* (528 bp). The 12.5 µL master mix reaction was 1x GoTaq green buffer (Promega), 2.0 mM of MgCl2 (Promega), DMSO 4%, 0.2 mM of dNTP mix (Promega), 0.2 µM of primer DiCSIP-Fwd-C and DiCSIP-Rev-AC (Table S3.1), 1.25 U GoTaq® Flexi DNA polymerase (Promega), 0.5 µL of gDNA template and topped up with NFW. The cycling conditions were as follows: 94 °C for 2 min; followed by 35 cycles of denaturation at 94 °C for 20 sec, annealing at 62 °C for 20 sec and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. The amplified PCR products were separated by 1.5% agarose gel electrophoresis and visualised.

Table S3.1. List of primer sequences and modified annealing temperatures of the Leucosphyrus Group and Dirus Complex

References	PCR method	Gene target	Primer	Sequence (5'-3')	Annealing temp. (°C)	Targeted species	DNA size (bp)
Beebe and Saul, 1995	Single-point PCR	ITS2	ITS2A	TGT GAA CTG CAG GAC ACAT	51	Dirus Complex	880
			ITS2B	TAT GCT TAA ATT CAG GGG GT			
Saeung et al., 2024	DiCSIP	ITS2	DiCSIP-Uni-Fwd	GAG TGA TGG ATA CAG AGC GGG	62	-	-
			DiCSIP-Rev-AC	ATC ACT CCA CCT GAC CGG CAA C		*An. dirus* *An. scanloni*	521-528
			D-B	CGG GAT ATG GGT CGG CC		*An. cracens*	435
			D-D	GCG CGG GAC CGT CCG TT		*An. baimai*	225
			DiCSIP-Rev-F	TCC GCA GCG CAG AGC G		*An. nemophilous*	305
	SSP		DiCSIP-Fwd-C	GCT CCC ACA CAC ACA CAC	62	*An. scanloni*	300
			DiCSIP-Rev-AC	ATC ACT CCA CCT GAC CGG CAA C			

Additional file 2: Detection of *Plasmodium* malaria parasites

A. Detection of *Plasmodium* spp.

Plasmodium genus infection was detected using highly sensitive reverse transcriptase real-time polymerase chain reaction (RT-qPCR), as described by Kamau et al. (2011) and Braima et al. (2024). The master mix reaction consisted of 1x Taqman-PCR master mix (Applied Biosystems by Thermo Fisher Scientific, Warrington, UK), 0.4 µM of each forward and reverse KamG primers, 0.2 µM of KamGP probe (Table S3.2), 1 µL of cDNA template and NFW to 10 µL total volume[240]. PCR cycling reactions of duplicate samples, including positive and negative controls, were 2 min at 60°C, 10 min at 95°C, 45 cycles of 15 s at 95°C, and 1 min at 60°C. The RT-qPCR assay was performed using Rotor-Gene Q (Qiagen, Hilden, Germany).

B. Identification of human malaria parasites

The cDNA of *Plasmodium* genus positive specimens was analysed for species-specific *Plasmodium* infection by reverse transcriptase nested PCR (RT-nested PCR), using human primers as described in Snounou et al. (1993) and validated by Braima et al. (2024). The nest-1 PCR reaction mixture contained 1x GoTaq green buffer (Promega), 3 mM of $MgCl_2$ (Promega), 0.2 mM of dNTP mix (Promega), 0.25 µM of each primer (rPLU1 and rPLU5) (Table S3.2), 1.25 U GoTaq® Flexi DNA polymerase (Promega), 4 µL of cDNA template to 50 µL with NFW. The cycling conditions were an initial denaturation for 4 min at 94°C; 35 cycles of 30 sec at 94°C, 1 min at 58°C and 2 min at 72°C; followed by 4 min at 72°C for final extension. The PCR products from nest-1 were the template for nest-2. Nest-1 PCR products were diluted with NFW 1:20. The 20 µL reaction mixture contained 1x GoTaq green buffer (Promega), 3 mM of $MgCl_2$ (Promega), 0.2 mM of dNTP mix (Promega), 0.25 µM of each species-specific *Plasmodium* primer (Table S3.2), 1 U GoTaq® Flexi DNA polymerase (Promega), 2 µL of diluted nest-1 PCR product and NFW. The PCR conditions were an initial denaturation for 4 min at 94°C, 35 cycles of 30 sec at 94°C, 1 min with annealing temperature on the specific primer (Table S3.2) and 1 min at 72°C, followed by 5 min at 72°C for final extension. The amplified PCR products were separated by 1.5% agarose gel electrophoresis and visualised.

C. Identification of macaque malaria parasites

A separate RT-nPCR assay was conducted to identify four zoonotic *Plasmodium* species, including *Plasmodium inui*, *Plasmodium cynomolgi*, *Plasmodium coatneyi*, and *Plasmodium fieldi*, using the protocols outlined by Lee et al. (2011) and Braima et al. (2024). In nest-1, the master mix contained 1x GoTaq green buffer (Promega), 3 mM $MgCl_2$ (Promega), 0.2 mM dNTPs (Promega), 0.25 µM of each rPLU1 and rPLU5 primers (Table S3.2), 1.25 U of GoTaq® Flexi DNA polymerase (Promega), and 4 µL of cDNA template, with nuclease-free water (NFW) added to reach a final volume of 50 µL. The thermal cycling protocol involved an initial denaturation at 94°C for 4 minutes, followed by 35 cycles of 30 seconds at 94°C, 1 minute at 58°C, and 2 minutes at 72°C, with a final 4-minute extension at 72°C.

In nest-2, the PCR products from nest-1 were diluted with NFW at a 1:10 ratio and used as a template. The nest-2 master mix included 1x GoTaq green buffer (Promega), 3 mM $MgCl_2$ (Promega), 0.2 mM dNTPs (Promega), 0.25 µM of species-specific Plasmodium primers (Table S3.2), 1U GoTaq® Flexi DNA polymerase (Promega), 2 µL of the diluted nest-1 product, and NFW to reach a total volume of 20 µL. The thermal cycling steps started with an initial denaturation at 94°C for 4 minutes, followed by 35 cycles of 30 seconds at 94°C, a 1-minute annealing phase (primer-specific; see Table S3.2), and 1 minute at 72°C, with a final extension of 5 minutes at 72°C.

D. Detection of *Plasmodium knowlesi*

For identifying *P. knowlesi*, a RT-hemi nPCR was followed, as described by Imwong et al. (2009) and Braima et al. (2024), the master mix for the nest-1 consisted of 1x GoTaq green buffer (Promega), 2.5 mM of $MgCl_2$ (Promega), 0.5 mM of dNTP mix (Promega), 0.25 µM of each PKF1160 and PKR1150 primers (Table S3.2), 1.25 U GoTaq® Flexi DNA polymerase (Promega), 1 µL of cDNA template to 25 µL with NFW. The cycling conditions started with an initial denaturation for 5 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C; followed by 5 min at 72°C for final extension. Twenty µL of nest 2 master mix reaction contained 1x GoTaq green buffer (Promega), 3 mM of $MgCl_2$ (Promega), 0.5 mM of dNTP mix (Promega), 0.25 µM of

each primer PKF1140 and PKR1150 primers, 1 U GoTaq® Flexi DNA polymerase (Promega), 1 µL of 1-nest PCR product and NFW. The thermal conditions were an initial denaturation for 5 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C; followed by 5 min at 72°C for final extension. The amplified PCR products were separated by 1.5% agarose gel electrophoresis and visualised.

Table S3.2. List of primer sequences and modified annealing temperatures of *Plasmodium* genus and species-specific PCR assays

References	PCR method	Gene target	Primer/ Probe	Sequence (5'-3')	Annealing temp. (°C)	Targeted species	DNA size (bp)
Kamau et al., 2011	RT-qPCR	18S rRNA	KamGF	GCT CTT TCT TGA TTT CTT GGA	60	*Plasmodium* spp.	-
			KamGR	AGC AGG TTA AGA TCT CGT TCG			
			KamGP	FAM-ATG GCC GTT TTT AGT TCG TG-BHQ1			
Snounou et al., 1993	Nested PCR	18S rRNA	rPLU 1	TCA AAG ATT AAG CCA TGC AAG TGA	58	-	-
			rPLU 5	CCT GTT GTT GCC TTA AAC TCC			
			rFAL1	TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT	58	*P. falciparum*	205
			rFAL2	ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC			
			rVIV1	CGA CTT CCA AGC CGA AGC AAA GAA AG	58	*P. vivax*	120
			rVIV2	TCC TTA CTT CTA GCT TAA TCC ACA TAA CTG ATA C			
			rOVA1	ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA	58	*P. ovale*	880
			rOVA2	GGA AAA GGA CAC ATT ATT CIG TAT CCT AGT G			
			rMAL1	ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC	58	*P. malariae*	144
			rMAL2	AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA			
Lee et al., 2011	Nested PCR	18S rRNA	rPLU 1	TCA AAG ATT AAG CCA TGC AAG TGA	58	-	-
			rPLU 5	CCT GTT GTT GCC TTA AAC TCC			
			PctF1	CGC TTT TAG CTT AAA TCC ACA TAA CAG AC	60	*P. coatneyi*	503
			PctR1	GAG TCC TAA CCC CGA AGG GAA AGG			
			CY2F	GAT TTG CTA AAT TGC GGT CG	60	*P. cynomolgi*	137
			CY4R	CGG TAT GAT AAG CCA GGG AAG T			
			PfldF1	GGT CT TTT TTT TGC TTC GGT AAT TA	63	*P. fieldi*	421
			PfldR2	AGG CAC TGA AGG AAG CAA TCTA AGA GTT TC			
			PinF2	CGT ATC GAC TTT GTG GCA TTT TTC TAC	58	*P. inui*	479
			INAR3	GCA ATC TAA GAG TTT TAA CTC CTC			
Imwong et al., 2009	Hemi-nested PCR	18S rRNA	PKF1160	GAT GCC TCC GCG TAT CGA C	55	-	-
			PKR1150	GAG TTC TAA TCT CCG GAG AGA AAA GA			
			Pkf1140	GAT TCA TCT ATT AAA AAT TTG CTT C	50	*P. knowlesi*	410
			Pkr1150	GAG TTC TAA TCT CCG GAG AGA AAA GA			

Additional file 3

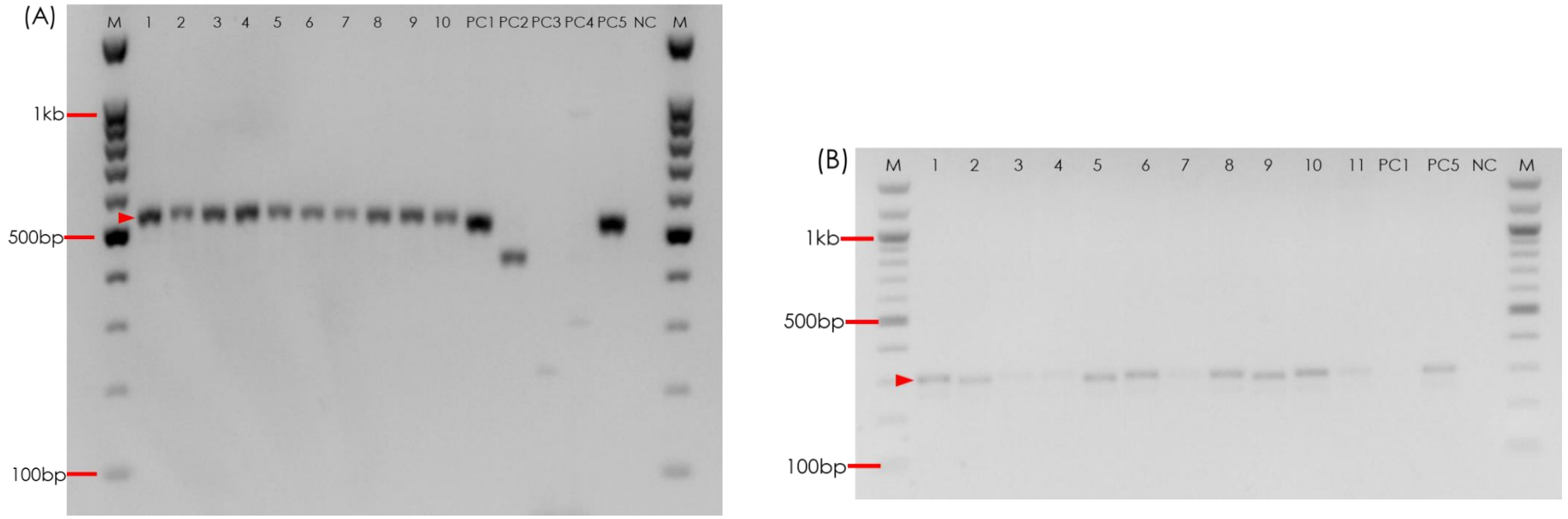

Figure S3.1. Amplification of *An. dirus* and *An. scanloni* by PCR assay. (A) ITS2 DiCSIP assay results. No1-10: collected specimens; PC1: *An. dirus* (521 bp); PC2: *An. cracens* (435 bp): PC3: *An. baimaii* (225 bp); PC4: *An. nemophilous* (301 bp); PC5: *An. scanloni* (528 bp); NC: Negative control; M: 100bp marker. (B) ITS2 SSP assay results. No.1-10: collected specimens; PC1: *An. dirus* (no amplification product); PC5: *An. scanloni* (300 bp); NC: negative control; M: 100 bp marker.

Chapter 4

The preceding chapter documented the first confirmed records of *An. dirus s.s.* and *Anopheles scanloni* in Indonesia, both members of the Dirus Complex. Molecular analysis conducted in Chapter 3, detected both macaque (*P. knowlesi, P. inui* and *P. coetneyi*) and human malaria parasite DNA (*P. vivax*) from *An. dirus s.s.* heads and thoraces. These findings suggest that *An. dirus s.s.* may play an important role as a vector of zoonotic, enzootic and human malarias in North Sumatra.

The vectors responsible for *P. knowlesi* transmission are predominantly in the Leucosphyrus Group, but species in the Barbirostris Group and the Umbrosus Group have recently been incriminated. This chapter, correspondingly, expands the investigation of enzootic, zoonotic and human malaria vectors to *Anopheles maculatus s.l.*, the most abundant *Anopheles* Group captured outside the Leucosphyrus Group in the study site. This study assesses its potential role in the transmission of *P. knowlesi* and other zoonotic and human malarias.

Anopheles maculatus sensu stricto is a potential vector of *Plasmodium knowlesi* and *Plasmodium inui*

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Abstract

Background:

In Indonesia, 31 anopheline species are vectors of human malarias. Many of these morphologically indistinguishable vectors are in species complexes requiring molecular analysis to identify. There is limited information on the malaria vectors in North Sumatra. *Anopheles dirus s.s.* and *An. scanloni,* two isomorphic species in the Leucosphyrus Group, are present in North Sumatra with *An. dirus s.s.* harbouring the DNAs of enzootic, zoonotic and human malarias. This study identified the species of mosquitoes in the Maculatus Subgroup in Ujung Bandar Village, Salapian Subdistrict, Langkat Regency, North Sumatra, Indonesia and investigated its potential as a vector of zoonotic, enzootic and human malarias.

Methods:

Mosquitoes collected by human landing catches in Ujung Bandar Village, from July 2022 to June 2023 were morphologically identified to species complex through a single-point PCR targeting the internal transcribed spacer region 2 (ITS2) of the ribosomal DNA (rDNA). Specimens identified as *Anopheles maculatus s.l.* were then analysed with a species-specific multiplex ITS2 PCR assay. Specimens from North Sumatra were phylogenetically compared with specimens from other parts of Indonesia and Southeast Asia. The cDNA of mosquito heads and thoraces were then screened for *Plasmodium* genus DNA using a RT-qPCR targeting the 18S rRNA. Positive samples were analysed for species-specific zoonotic, enzootic and human malaria parasites through an RT-nested PCR.

Results:

All 234 morphologically identified *An. maculatus s.l* specimens were identified as *An. maculatus sensu stricto*. The *An. maculatus s.s.* in North Sumatra were genetically identical to populations from other parts of Indonesia and mainland Southeast Asia. The heads and thoraces of four *An. maculatus* specimens were positive for *Plasmodium* spp. DNA with one specimen positive for the DNA of *Plasmodium knowlesi* and *Plasmodium inui*.

Conclusion:

Anopheles maculatus s.s. was identified in Langkat regency, North Sumatra, Indonesia, and for the first time, DNA of *P. knowlesi* and *P. inui* were detected in wild-caught *An. maculatus s.s*.

Keywords: *Anopheles maculatus sensu stricto*, North Sumatra, zoonotic and non-zoonotic malaria parasites

Background

Understanding the distribution of vector species is essential for implementing effective vector control as even closely related species can exhibit significant variations in larval habitats, host biting preferences, insecticide resistance and other behaviours which can impact their susceptibility to different control measures [155]. However, vector distributions are not static, but change in response to changes in insecticide exposure, rainfall patterns and land use changes (e.g., the conversion of forests into agricultural plantations and urban developments) [137], thus requiring ongoing surveillance.

Critical to vector surveillance is the incrimination of mosquito species as vectors which requires both identifying the mosquito species and the detection of malaria parasites in their heads and thoraces. The challenge of accurately identifying mosquitoes can be particularly challenging in Indonesia, which has the second-highest number of mosquito species of any country with 439 known mosquito species [289, 290]. At least 31 of these species transmit zoonotic and human malaria parasites [36, 38, 289, 291]. The majority of these malaria vectors belong to one of eight groups and four complexes within the *Anopheles* genus [36, 289]. The large number of species and the presence of morphologically similar sibling species requires precise morphological examination, followed by molecular analyses and sequencing for accurate identifications [292-296].

Sixteen percent of the malaria cases and 22% of malaria deaths in the Southeast Asia (SEA) region in 2023 occurred in Indonesia [218]. Malaria transmission intensity varies greatly across the country and achieving malaria elimination is complicated by the presence of *P. knowlesi* infections in humans. While North Sumatra may be classified as having low human malaria endemicity, with 18,361 suspected cases and 914 confirmed cases reported in 2022 [34, 297], it also recorded the highest number of *Plasmodium knowlesi* cases in Indonesia, with 377 cases reported between 2010 and 2021 [35, 227]. The vectors responsible for *P. knowlesi* transmission are predominantly in the Leucosphyrus Group [38, 258, 298], but *Anopheles donaldi* of the Barbirostris Group [16, 17], as well as *Anopheles collessi* and *Anopheles roperi* of the Umbrosus Group were recently incriminated as *P. knowlesi* vectors [18, 19]. In addition, *Anopheles hackeri* was incriminated once as a vector for *P. knowlesi* and other macaque malaria parasites in the

1960s but has not since been documented as a vector [228, 229]. *Anopheles sundaicus* is also suspected to be a *P. knowlesi* vector in India's Andaman and Nicobar Islands [230]. This manuscript examined the vector status of mosquitoes in the Maculatus Group in an area of North Sumatra with zoonotic *P. knowlesi* transmission.

Methods

Study site

The study was carried out in Dusun II (3.371720, 98.328547) and Dusun V (3.341947, 98.335406) of Ujung Bandar village, within the Salapian subdistrict of Langkat Regency, North Sumatra, Indonesia. The site was selected using a multi-criteria disease surveillance model [234, 235] incorporating predictive risk maps for *P. knowlesi* transmission using estimated distributions of macaques and humans, and environmental land classifications to identify and categorize high-risk health facility catchment areas for *P. knowlesi* transmission [234, 235]. Four confirmed human cases of *P. knowlesi* occurred in Ujung Bandar Village from March 2022 to March 2023 [I. N. D. Lubis et al., unpublished]. The study village has a tropical climate, receiving over 3,000 mm of rainfall annually, with daytime temperatures averaging between 29-34°C and nighttime temperatures between 19-24°C. The topography around Ujung Bandar village is hilly, ranging from 350 to 600 meters above sea level with extensive oil palm plantations, areas of small-scale mixed agriculture and patches of disturbed forest.

Mosquitoes sampled outdoors by human landing catches (HLC) between July 2022 and June 2023 [237] were morphologically identified before stored in 1.5 ml Eppendorf tubes with cotton wool and silica gel at room temperature with a maximum of 5 specimens per vial [B. v. d. Straat et al., unpublished].

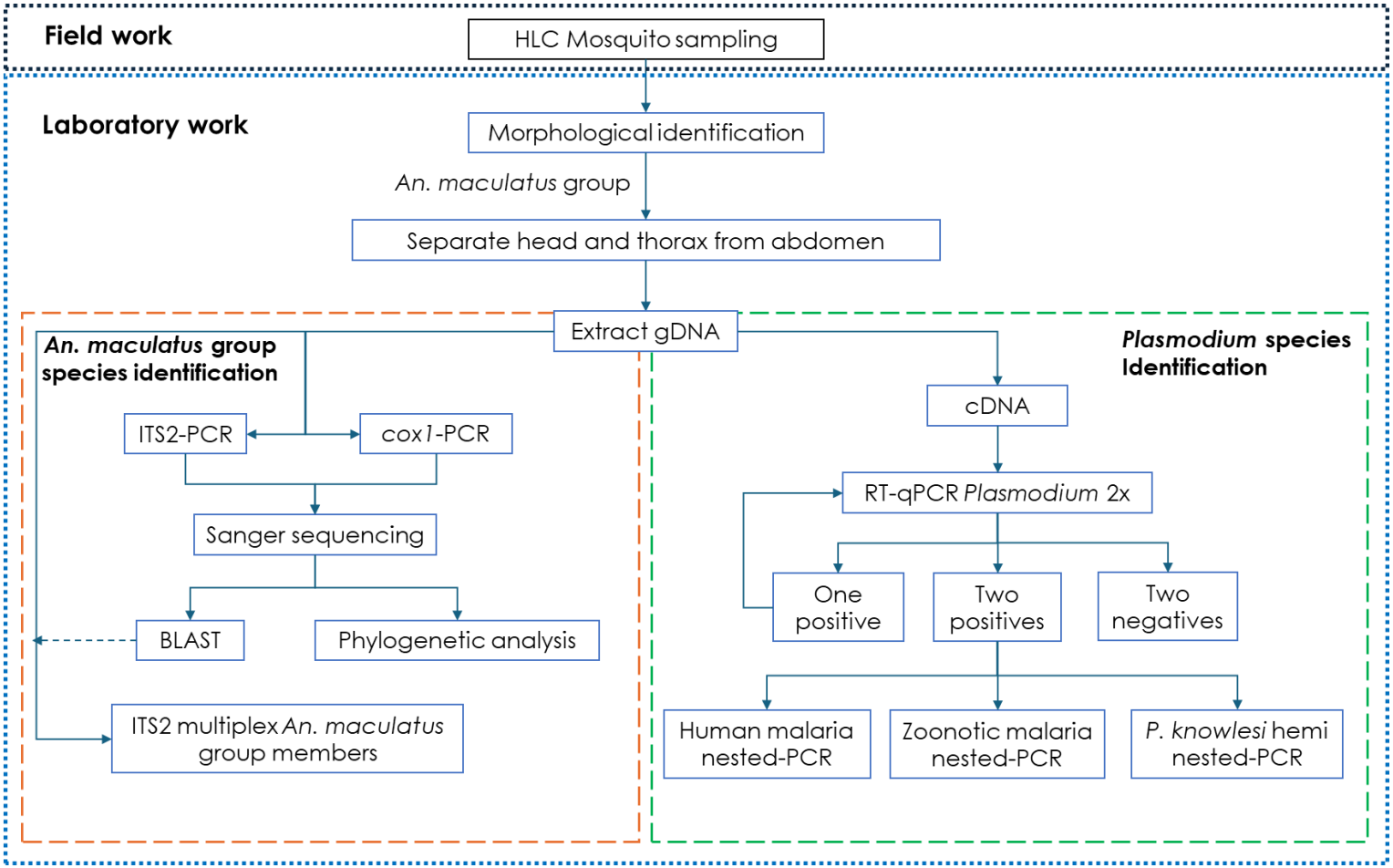


Figure 4.1. Overview of the methods for *Anopheles* and *Plasmodium* species identification

Isolation of DNA and reverse transcription

The methods for mosquito collection and mosquito species and *Plasmodium* identifications are provided in a flow chart in Figure 4.1. DNA from the head and thorax of *An*. *maculatus* group specimens were extracted using the DNeasy Blood & Tissue Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). A 10 µL aliquot of individual mosquito genomic DNA (gDNA) was transformed into complementary DNA (cDNA) by cDNA reverse transcription PCR (Applied Biosystems by Thermo Fisher Scientific, Lithuania) for *Plasmodium* detection. Molecular assays for identifying and sequencing of PCR amplicons for the Maculatus Group, as well as *Plasmodium* detection, are described in detail in Supplementary Information 1.

Anopheles maculatus species identifications

A single-point PCR targeting the internal transcribed spacer region 2 (ITS2) of the ribosomal DNA from the gDNA of all morphologically identified *An*. *maculatus* group specimens using the ITS2A (5'-TGTGAACTGCAGGACACAT-'3) and ITS2B (5'-TATGCTTAA ATTCAGGGGGT-'3) primers were used to identify specimens as *An*. *maculatus s.l.* [238]. All *An*. *maculatus s.l.* specimens were then identified to species using a species-specific multiplex ITS2 PCR assay [299]. The PCR master mix used the GoTaq® flexi DNA Polymerase kit (Promega, WI, USA) with previously published cycling conditions [238, 299]. The positive control was *An*. *maculatus* Javanese form/var. menorah from Balai Besar Litbang Vektor dan Reservoir Penyakit (B2P2VRP), Salatiga, Central Java, with the negative control being without template were also included. The PCR products were visualised on 1.5% agarose gel with a 100 bp DNA ladder (Biolabs Inc., New England, United States)

Samples of multiplex ITS2 amplified products of all *Plasmodium* DNA-positive *An*. *maculatus* samples and at least one other specimen from each collection station were sequenced. In addition, the *cox1* gene was PCR amplified using the CI-N-2087 (5'-AATTTCGGTCAGTTAATAATATAG-3') and TY-J-1460 (5'-TACAATTTATCGCCTAAACTTCAG CC-3') primers and sequenced [292].

The amplification products of the ITS2 and *cox1* gene PCRs were purified using ExoSAP-ITTM (Applied Biosystems by Thermo Fisher Scientific, Vilnius, Lithuania) and sequenced using the forward primer with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems by Thermo Fisher).

Phylogenetic tree analyses

Genetic distance and evolutionary relationships between subsamples and reference *An. maculatus* from different parts of Indonesia and Southeast Asia were assessed using the Mega X (version 10.2.2) software. References samples were selected based on the similarity of the length of the amplified band size and the position of the ITS2 and *cox1* DNA sequence with *An. maculatus* from North Sumatra. A non-Maculatus Group member was selected as the outgroup taxon for the *An. maculatus* phylogenetic construction. Nucleotide sequences of the ITS2 and *cox1* genes were aligned and compared using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) database website (https://blast.ncbi.nmm.nih.gov/Blast). Sequences were aligned using Clustal Omega with default parameters and ragged ends removed. The phylogenetic tree was constructed using the Maximum Likelihood Method and Kimura-2 (K80) evolutionary model with the General Time Reversible (GTR)+1 model. The reliability of the ML tree was assessed through 1000 bootstrap replicates.

Plasmodium detection and identification

A reverse transcriptase real-time PCR (RT-qPCR) targeting the cDNA of the18S rRNA gene was used to detect *Plasmodium* genus DNA [240]. *Plasmodium* DNA was amplified in the Taqman-Universal PCR master mix (Applied Biosystems by Thermo Fisher Scientific, Warrington, UK) using KamGF (5'-GCTCTTTCT TGATTTCTTGGA-'3) and KamGR (5'-AGCAGGTTAAGATCTCGTTCG-'3) primers with the KamGP probe (FAM-ATGGCCGTTTTT AGTTCGTG-BHQ1) [240]. Reactions were run in duplicate, along with positive and negative controls using the Rotor-Gene Q PCR machine (Qiagen, Hilden, Germany). Positive results had had Ct values under 35 for both replicates with replicates differing by fewer than three cycles. Results were classified as negative if both replicates showed Ct values greater than 40. Ct values between 35 and 40 were deemed equivocal and

retested using a 1:10 dilution of the cDNA template. If the repeated RT-qPCR yielded a Ct value below 40, the sample was processed for *Plasmodium* species-specific identification.

The cDNA of *Plasmodium* positive specimens were then analysed for species-specific *Plasmodium* DNA by reverse transcriptase nested PCR (RT-nPCR) using primers targeting human (*Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*) [241] and macaque malaria parasites (*Plasmodium inui*, *P. cynomolgi*, *P. coatneyi*, *P. fieldi* and *P. knowlesi*) [131, 242]. RT-nPCR amplification products were visualised by electrophoresis on a 1.5% agarose gel with positive DNA controls for all human *Plasmodium* species and *P. knowlesi* (from a human clinical isolates), while validated gBlocks™ synthetic controls were used for other enzootic *Plasmodium* species [243].

Results

Anopheles maculatus identifications

All 234 *An. maculatus s.l*, specimens that were analysed were confirmed as *An. maculatus sensu stricto* based on amplification of the ITS2 gene from the multiplex ITS2 gene PCR (Figure 4.2). The consensus ITS2 and *cox1* sample sequences also identified all samples as *An. maculatus s.s.* with >99.9% accuracy against the NCBI GenBank database using BLAST.

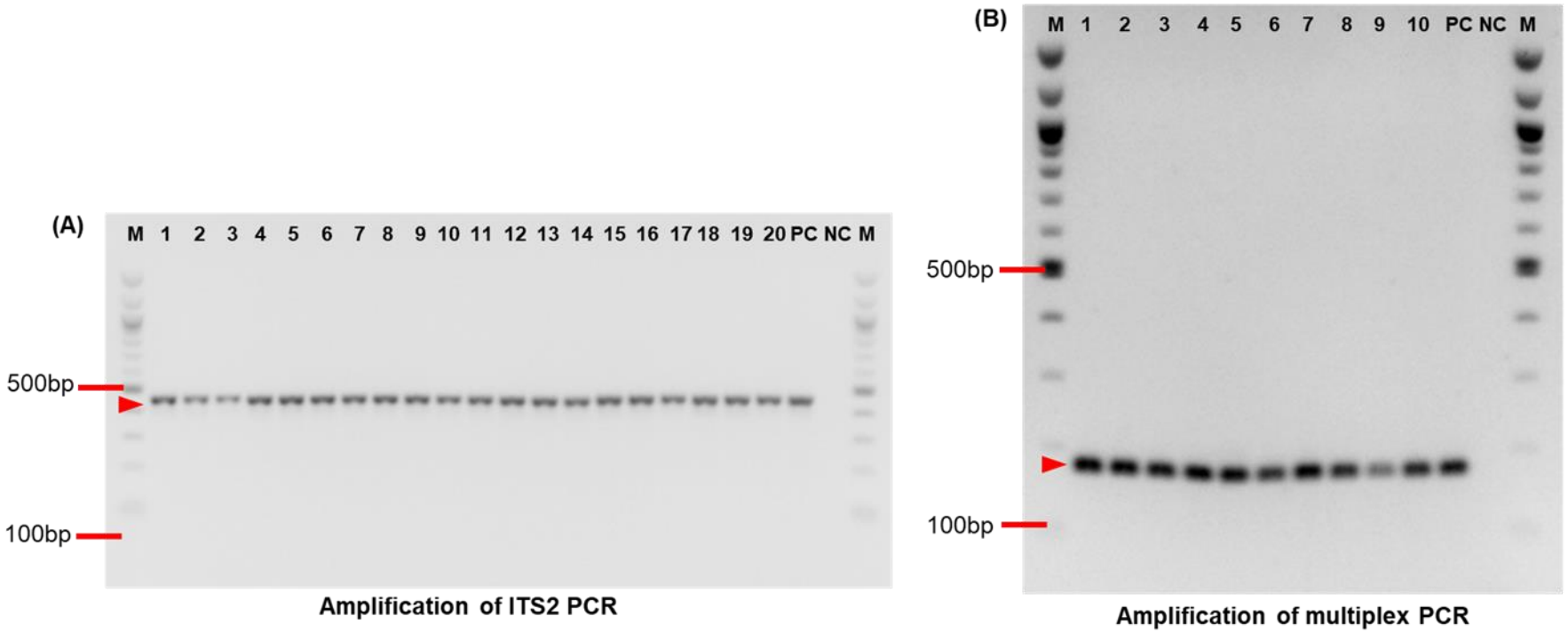


Figure 4.2. Polymerase chain reaction amplification results of the ITS2 region run on 1.5 % agarose gel (EtBr 10mg/ml) at 100 volts for 85 minutes. (A) Single point ITS2 PCR results. Amplification products of 460 bp correspond to *An. maculatus s.l* Lanes 1-20: individual adult mosquitoes. (B) Multiplex ITS2 PCR results. Amplification products of 180 bp corresponds to *An. maculatus s.s.* Lanes 1-10: Individual mosquitoes. PC: positive control; NC: negative control; Lane M: 100-bp marker.

ITS2 gene phylogenetic analysis

The *An. maculatus s.s.* sample ITS2 sequences from North Sumatra (accession numbers: PQ601060 - PQ601066) ranged from 372 to 403 base pairs (bp), with a GC content of 56.4% - 58%; polymorphisms were not observed among the samples (n=7). *Anopheles maculatus s.s.* from North Sumatra displayed 100% genetic similarity with *An. maculatus* from Thailand (FJ526582, MK204649), Vietnam (AY803346), Cambodia (DQ518618), India (JQ446435), Laos (FJ526584), Myanmar (PP339963), China (MF535219), Malaysia (MT623064, AY491974), and Indonesia (MK504456, MK504461, MK504464, MK504466, MK659793, MK656095, MK659797) (Figures 4.3 and 4.5A). High conservation and 99.41% - 99.44% genetic identity were also found with *An. maculatus* from Peninsular Malaysia (AY491974.1) (Figures 4.3 and 4.5A). The *An. maculatus s.s.* samples from North Sumatra differed from *An. greeni* from Philippines (FJ526585, FJ526586), and North Kalimantan-Indonesia (MK504467) by 6.51% - 6.86%; and also displayed 5.44% - 5.72% genetic distance with *An. dispar* from the Philippines (FJS26586) (Figure 4.3). The distance between *An. maculatus s.s.* samples from North Sumatra and *An. maculatus s.l.* (Sulawesi and Javanese form/var. menoreh) were 6.43% – 6.76%. The ITS2 sequences from North Sumatra *An. maculatus* samples showed distances of 10.92% - 11.52%, 2.41% – 2.55%, 2.32% - 2.44%, 2.72% - 2.87%, and 3.41% - 3.89% with *An. dravidicus, An. willmori, An. sawadwongporni, An. rampae,* and *An. pseudowillmori,* respectively.

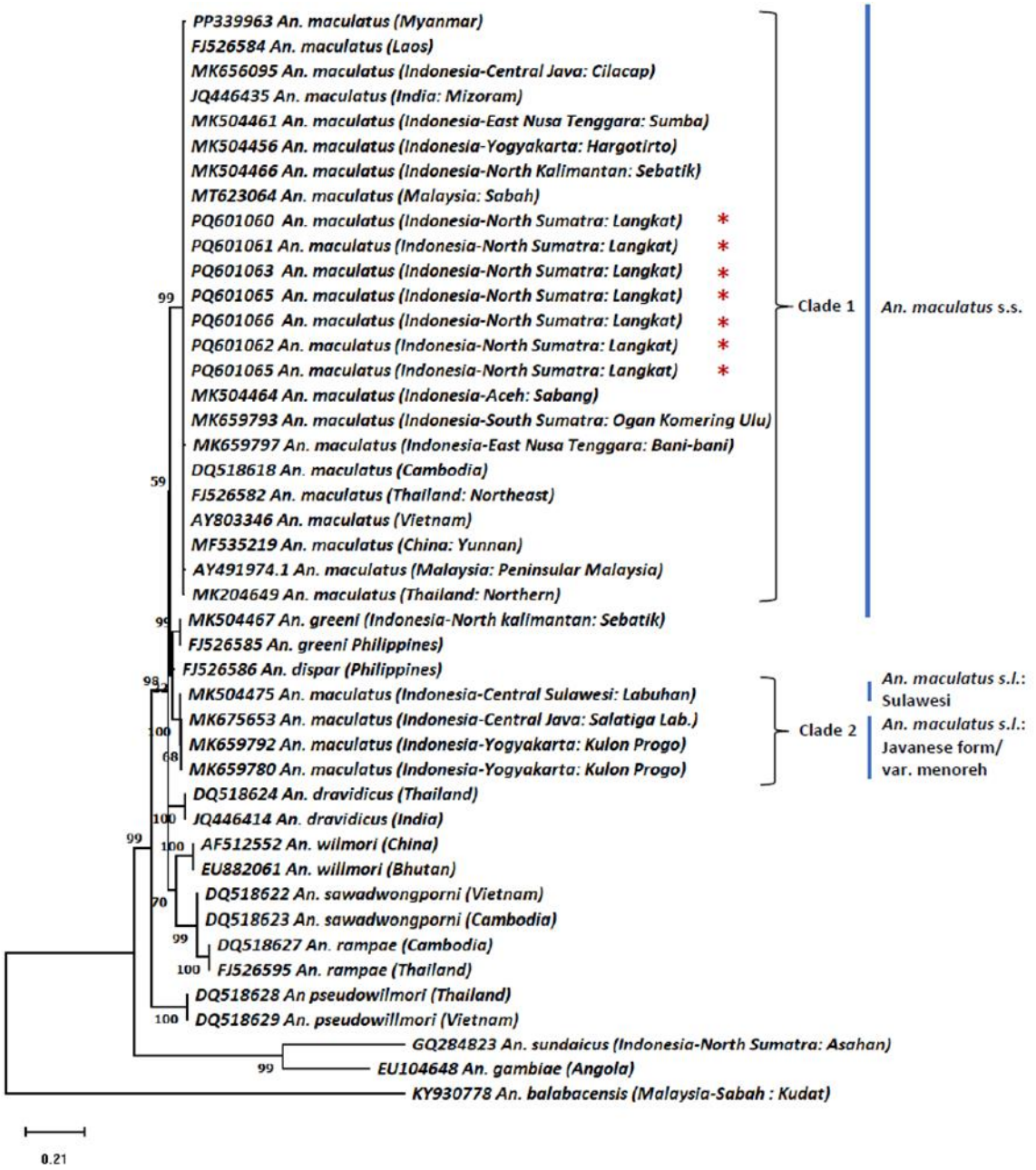


Figure 4.3. Phylogenetic analysis based on ITS2 sequences of the Maculatus Group. The phylogenetic tree was constructed using the maximum likelihood (ML) method and the Kimura-2 evolutionary model, with 1000 bootstrap replicates in MEGA X. Tree rooted using *An. balabacensis* from Malaysia (KY930778) as an outgroup. North Sumatra samples were identified as red stars (*).

cox1 gene phylogenetic analysis

The *cox1* sequences of North Sumatra *An. maculatus s.s.* samples had sequence lengths ranging from 563 to 576 bp with GC content of 32% (accession numbers PQ596587 - PQ596590) and belonged to five lineages. Lineage 1 consists of two sub-lineages (i.e., Ia and Ib), where sublineage 1a is comprised of four clades (Figures 4.4 and 4.5B). Clade 1 contained four *An. maculatus* from Thailand (MK579208, OL742870, OL742871, MK579209), two from India (MN276050, MT863706), and two from Myanmar (PP976502, PP976493), Clade 2 had three sequences from Purworejo, Indonesia (MK507451, MK507452, and MK507453), and two sequences from Thailand (MK507477, MK507478). Clade three was composed of four sequences of *An. maculatus s.s.* from this study in North Sumatra (PQ596587 - PQ596590) and two sequences from Sabang-Indonesia (MK507460, MK507459) (Figure 4.4). Sublineage1b comprised two sequences from Sri Lanka (KM669758, MK507459). Furthermore, lineage 2 consisted of two sequences of *An. maculatus* from Sri Lanka (KM669758, KM669759) and two sequences from Sebatik, Indonesia (MK507470, MK507469). Whereas six *An. maculatus s.l.* from Sulawesi, Indonesia (MK507471 - MK507476) formed lineage 3.

Lineage 4 had one *An. greeni* from Sebatik, Indonesia (MK507468) and two *An. dravidicus* from Myanmar (PP373008, PP976518). While one *An. rampae* from Cambodia (MW603586), and two *An. sawadwongporni* from Thailand (OQ363187- OQ363188) formed lineage 5. *Anopheles sundaicus* from Cambodia (MW603593) and *An. gambiae* (DQ465336) from Nigeria were used as outgroups.

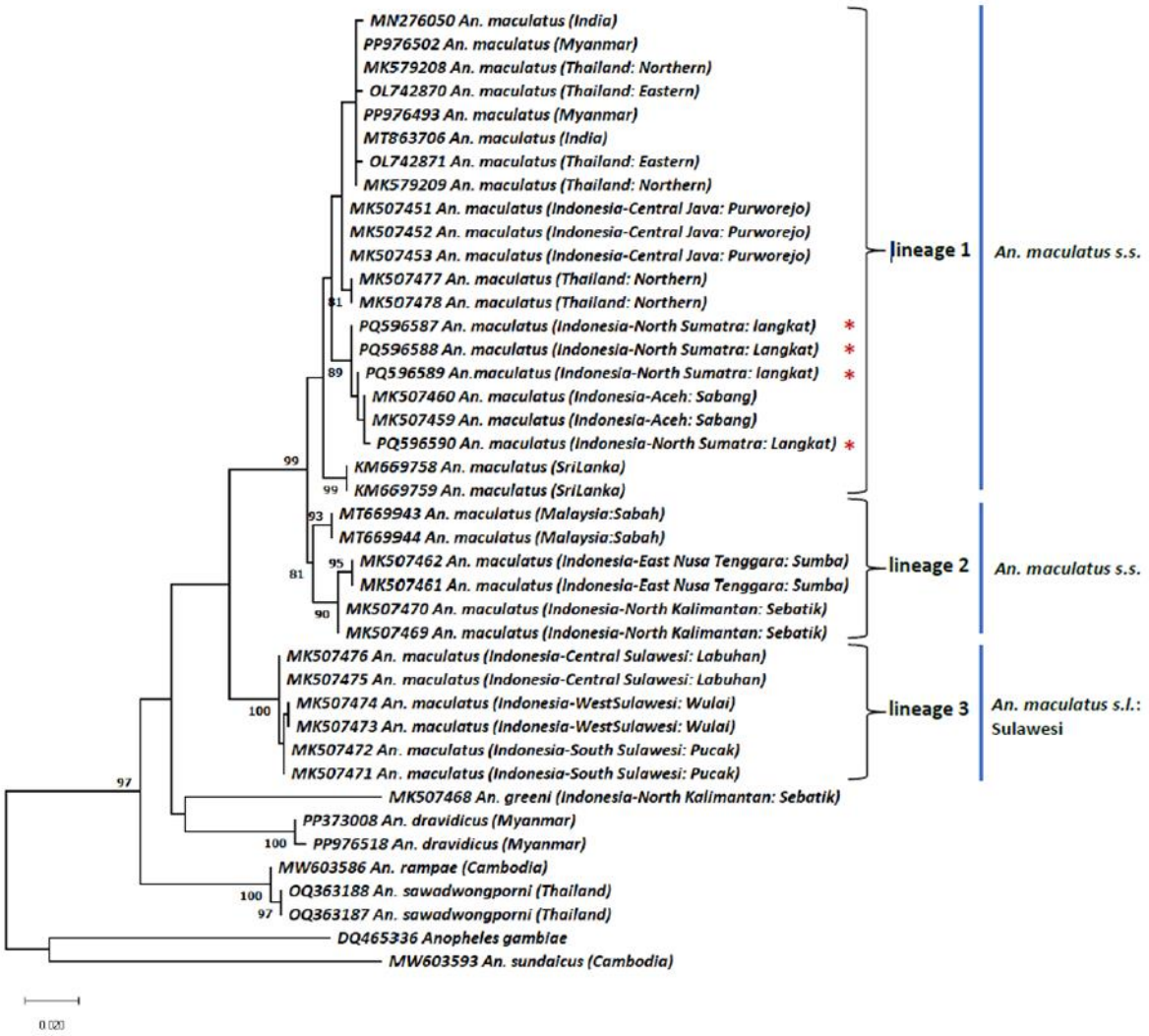
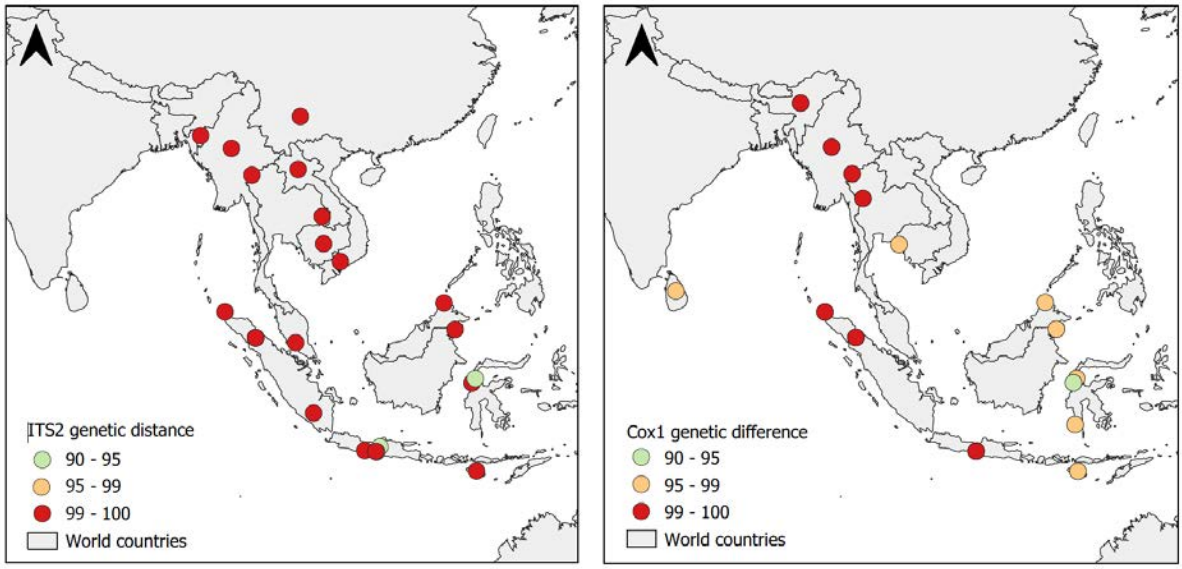


Figure 4.4. Phylogenetic analysis based on *cox1* sequences of the Maculatus Group. The phylogenetic tree was constructed with the maximum likelihood (ML) method and the Kimura-2 evolutionary model, with 1000 bootstrap replicates in MEGA X. Tree rooted using *Anopheles sundaicus* and *An. gambiae* as outgroup. North Sumatra samples were identified as red stars (*).



(A) (B)

Figure 4.5. The genetic distance of *Anopheles maculatus s.s.* from North Sumatra compared to populations from other regions of Indonesia and neighbouring countries. Based on (A) ITS2 gene; (B) *cox1* gene.

Plasmodium species detection

Four *An. maculatus s.s.* specimens yielded equivocal results for *Plasmodium* spp. 18S rRNA, with Ct values ranging between 35 and 40 in the RT-qPCR assay (Table 4.1). Human malaria parasite DNA was not detected. One specimen was DNA positive for both *P. knowlesi* and *P. inui* (Figure 4.6).

Table 4.1. *Plasmodium genus* and species-specific *An. maculatus s.s.* from North Sumatra

Sample ID	*Plasmodium* spp.[1] Ct (±SD)	RT-qPCR Results	Human malaria[2]	Simian malaria [3]		
			Pf, Pv, Pm, Po	Pk[4]	Pin	Pcy, Pct, Pfiel
1	37.13 (±1.09)	Equivocal	-	+	+	-
2	36.89 (±0.43)	Equivocal	-	-	-	-
3	36.92 (±1.54)	Equivocal	-	-	-	-
4	39.95 (±0.26)	Equivocal	-	-	-	-

[1]RT-qPCR by Kamau et al., 2011

[2]RT-nPCR by Snounou et al., 1993

[3]RT-nPCR by Lee et al., 2011

[4]RT-hemi nPCR Imwong et al., 2009

Pf: *Plasmodium falciparum*; Pv: *P. vivax*; Pm: *P. malariae*; Po: *P. ovale*

Pk: *P. knowlesi*; Pin: *P. inui*; Pcy: *P. cynomologi*; Pct: *P. coatneyi*; Pfiel: *P. fieldi*

+: positive result; -: negative result

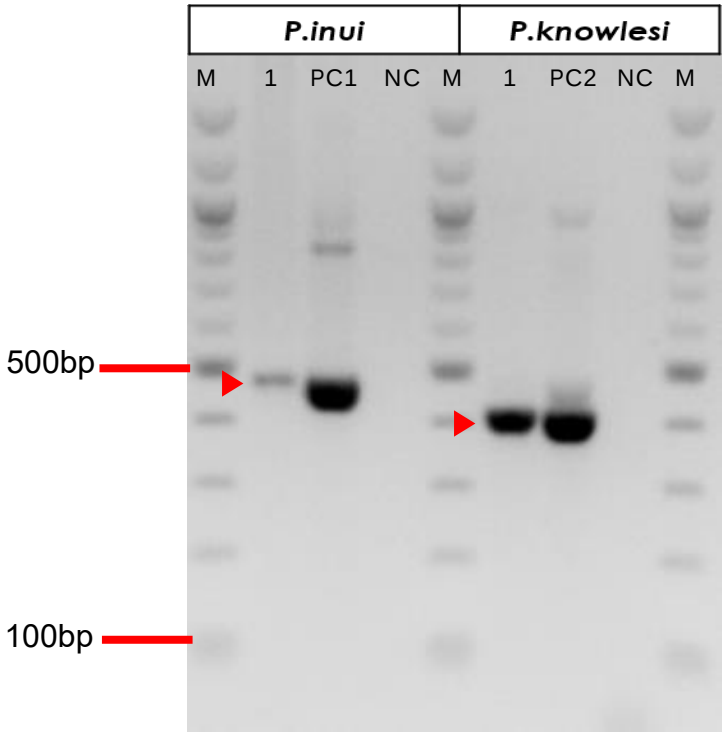


Figure 4.6. Amplification products of *Plasmodium* species by RT-nested PCR run on 1.5 % agarose gel (EtBr 10mg/ml) at 100 volts for 85 minutes. 1: Mosquito sample ID 1 (see table 4.1); PC1: positive control of *P. inui* (479 bp); PC2: positive control of *P. knowlesi* (479 bp); NC: negative control; Lane M: 100-bp marker.

Discussion

The Maculatus Group includes nine species distributed across SEA, with *An. maculatus s.s.* having the widest range [25, 300, 301]. In Indonesia, three species (*An. maculatus s.s.*, *An. maculatus s.l* (Javanese form/var. menoreh), and *Anopheles greeni*) are documented as endemic [292, 293]. Morphologically identified *Anopheles maculatus s.l.* was previously reported in North Sumatra [36]. The present study reports for the first time the presence of *An. maculatus s.s.* in Ujung Bandar Village, Salapian Subdistrict, Langkat Regency, North Sumatra by molecular analyses.

The ITS2 gene is a non-coding nuclear spacer region that is biparentally inherited and useful for distinguishing closely related mosquito species across various genera, including *Anopheles* [301-303]. Two distinct *An. maculatus* clades were previously reported: *An. maculatus s.s.* (Clade I) and *An. maculatus s.l.* (Sulawesi and Javanese form/var. menoreh) (Clade II) [292, 293]. This divergence is thought to have resulted from geographic isolation or environmental pressures, such as from insecticide exposure [9,

292]. The ITS2 sequence analyses of *An. maculatus* revealed a monophyletic grouping, indicative of a shared unique evolutionary ancestor [301] with all *An. maculatus s.s.* specimens from this study in North Sumatra belonged to Clade I (Figure 4.3). The genetic homogeneity of *An. maculatus s.s.* seen from ITS2 analysis from North Sumatra found no evidence for novel cryptic species in the region. However, due to the rapid evolutionary rate of the ITS2 region, phylogenetic analyses of the ITS2 region often cannot resolve deeper evolutionary relationships without additional genetic analyses, such as from mtDNA genes to provide a more comprehensive understanding of phylogenetic relationships, linage divergence and evolutionary history [299].

Unlike the ITS2 phylogeny, the faster rate of maternally inherited mtDNA, such as in the cox1 gene [304], can provide additional insights into the evolution of *An. maculatus* populations [303, 305, 306]. Here, evidence indicated that *An. maculatus s.s.* populations from North Sumatra share genetic ancestry with *An. maculatus* from mainland SEA and other areas of western Indonesia. Single nucleotide polymorphisms (SNPs) with genetic distances less than 0.1% suggest minimal genetic variation. However, the phylogenetic tree also showed that *An. maculatus s.l.* in Indonesia is divided into three distinct lineages (i.e. lineage 1, 2 and 3), confirming genetic diversity among *An. maculatus* in Indonesia (Figure 4.4). *Anopheles maculatus s.s.* from North Sumatra Province showed the closest genetic similarity to a reference population from Aceh Province (Sabang), and the least similarity to populations from Sulawesi Island. Similar to the ITS2 phylogeny, the high level of polymorphism observed in *An. maculatus s.l.* across Indonesia is likely influenced by both genetic and geographical distances [292, 293].

Anopheles maculatus (*s.s.* and *s.l.*) is a vector of human malarias (*P. falciparum* and *P. vivax*) across Indonesia (Central Java, Yogyakarta and southern Sumatra) [307, 308], Thailand [265], Lao PDR [268], Vietnam [198], peninsular Malaysia [309] and Bangladesh [310, 311]. Studies from the 1930s suggested that *An. maculatus s.l.* was a human malaria vector in the Kisaran and Londut areas of North Sumatra [312, 313]. Although human malaria parasites were not detected in *An. maculatus s.s.* in this study, this does not imply that *An. maculatus s.s.* is not a vector of human malaria in North Sumatra. As *An. maculatus* tends to bite animals rather than humans [53], human malaria sporozoite

rates would be expected to be very low and the limited number of samples analysed in the study makes the failure to detect human malaria DNA in the heads and thoraces of *An. maculatus s.s.* not surprising.

This study provides the first evidence for possible natural infections with zoonotic and enzootic *Plasmodium* infections, specifically the DNA of *P. knowlesi* and *P. inui*, in *An. maculatus s.s.*, suggesting that *An. maculatus s.s.* may play a role in enzootic and zoonotic malaria transmission. Previous laboratory studies established *An. maculatus s.l.* as competent for *P. knowlesi*, *P. inui* and *P. fieldi* [277, 314-316]. The detection of *Plasmodium* DNA in the head and thorax of *An. maculatus s.s.* demonstrates that this species could be a potential natural vector of enzootic and zoonotic malarias in North Sumatra. Finding a *P. knowlesi* vector species outside the Leucosphyrus Group was previously reported from other studies that detected *P. knowlesi* in *An. donaldi*, *An. collessi, An. roperi and An. sundaicus* [16-19, 228, 230]. Hence, zoonotic malaria transmission may potentially involve a broader array of vector species than previously acknowledged.

Plasmodium DNA-positive samples were not detected with Ct values below 35. However, four *Plasmodium*-positive *An. maculatus s.s.* specimens with Ct values between 35 and 40 were classified as equivocal. This range represents a diagnostic grey zone, where amplification is neither definitively positive nor negative. Several factors may contribute to such borderline outcomes, including non-specific amplification, degraded or low-concentration DNA, cross-contamination, or amplification occurring near the limit of detection of the assay [284-286]. Despite the uncertainty typically associated with equivocal Ct values, one specimen (Ct= 37.13 ± 1.09) was identified as a mixed infection with *P. knowlesi* and *P. inui*. This observation mirrors findings from *An. dirus s.s.* collected in the same location (Chapter 3), where an *An. dirus s.s.* sample with a Ct value of 39.2 ± 0.5 was confirmed as having a mixed infection of *P. knowlesi* and *P. coatneyi*. These results underscore the importance of analysing equivocal samples in zoonotic malaria surveillance, as they may reveal low parasitemia infections that would otherwise be missed.

It is possible that the three *Plasmodium* DNA-positive *An. maculatus* specimens with equivocal Ct values were infected with non-primate *Plasmodium* species, which could not be identified due to limitations in the species-specific assays or low parasite DNA concentrations. A study in Thailand found salivary glands of *An. maculatus* infected with *Plasmodium caprae*, a malaria parasite of goats [19], suggesting that other enzootic malaria parasites may potentially have been responsible for the *Plasmodium* DNA positive *An. maculatus* specimens in North Sumatra.

The *Plasmodium* 18S rRNA gene is known for its high degree of conservation, particularly in the AT-rich region among *Plasmodium* species. This conservation can lead to cross-reactivity among PCR primers, significantly impacting the specificity and sensitivity of diagnostic methods [242, 317, 318]. For instance, *P. knowlesi* primers (Pmk8/Pmkr9) have shown cross-reactivity with *P. vivax* [242]; another set of *P. knowlesi* primers described by Singh et al. (2004) demonstrated cross-reactivity with *P. vivax* and with macaque malaria parasites, including *P. cynomolgi, P. inui*, and *P. hylobati* [283, 320]. Similarly, primers for *P. inui* can cross-react with other macaque malaria parasites, such as *P. cynomolgi*, *P. coatneyi* and *P. hylobati* [320]. Additionally, some *Plasmodium* primers amplified non-specific bands due to cross-reactivity between human and parasite small subunit rRNAs [317, 321, 322]. In this study, *P. knowlesi* and *P. inui* were identified in a single specimen of *An. maculatus s.s*. Cross-reactivity has not been reported between *P. knowlesi* and *P. inui* primers [287] providing some support that the positive *An. maculatus s.s.* sample in this study was a dual *P. inui* and *P. knowlesi* infection.

Detection of *P. knowlesi* and *P. inui* DNA in a single *An. maculatus s.s.* in North Sumatra could result from blood stage primate malaria parasite contamination in the mosquito. While this cannot be dismissed entirely, it would be unlikely given that the mosquitoes were collected by HLC from malaria negative individuals and any residual malaria parasites contaminating the mouth or oesophagus would have to have been acquired during blood feeding during a previous blood meal, generally 2-4 days previously. The detection of *P. knowlesi* and *P. inui* DNA in the head and thorax of *An. maculatus s.s.*is insufficient to incriminate it as a vector. Determining whether *An. maculatus s.s.* is a zoonotic or macaque malaria vector that will require finding sporozoites in the salivary

glands, demonstrating vector competence, and obtaining robust entomological inoculation rate data from the field. *Anopheles maculatus* (*s.s.* and *s.l.*) is recognized for its catholic host-seeking behaviour which will be influenced by the availability of hosts [323]. Although this species tends to bite animals, including macaques, it bites humans when other host options are limited, suggesting a potential role in zoonotic malaria spillover to humans.

The role of *An. maculatus* (*s.s.* and *s.l.*) as potential vectors of simian as well as human malarias in Southeast Asia introduces new challenges for malaria elimination, particularly in regions of mixed enzootic and human malaria transmission, such as in North Sumatra. WHO recommends that malaria control strategies focus heavily on targeting the indoor biting and resting primary human malaria vectors [324]. Complementary strategies to address zoonotic, exophagic and exophilic vectors like *An. maculatus* (*s.s.* and *s.l.*) are needed. Understanding the distribution, behaviour and ecological adaptations of vectors is essential for the development or implementation of effective malaria control strategies, as vector control efforts that overlook any vectors may face unexpected barriers in reducing transmission.

Conclusion

This study expands the understanding of malaria transmission dynamics in North Sumatra suggesting that *An. maculatus s.s.* may play a role in enzootic and zoonotic malaria transmission. These findings challenge previous assumptions that enzootic malaria transmission is limited to the Leucosphyrus Group. The adaptability of *An. maculatus s.s.* to bite both macaques and humans highlights the importance of an integrated malaria control approach that considers transmission of both zoonotic and human malarias if malaria is to be successfully controlled or eliminated.

Supplementary Information

Additional file 1: Molecular assays to Identify species in the Maculatus Group

Additional file 2: Molecular assays to Identify *Plasmodium*

Additional file 3: Table S4.1. Pairwise genetic distance of ITS2 sequences.

Additional file 4: Table S4.2. Pairwise genetic distance of *cox1* sequences.

Abbreviations

An.: *Anopheles*; cDNA: complementary deoxyribonucleic acid; Ct: cycle threshold; DNA: deoxyribonucleic acid; gDNA: genomic deoxyribonucleic acid; ITS2: internal transcribed spacer 2; *P.*: *Plasmodium*; PCR: polymerase chain reaction; RT-nPCR: reverse transcriptase nested polymerase chain reaction; RT-qPCR: reverse transcriptase real-time polymerase chain reaction; SEA: Southeast Asia; s.l.: *sensu lato*; s.s.: *sensu sricto*.

Declarations

Ethical approval

This study was approved by the Universitas Sumatera Utara, Faculty of Medicine (application number 723/KEP/USU/2021) on 14 July 2021 and James Cook University (application number H8583) on 29 September 2021.

Availability of data and materials

Not applicable

Completing interest

The authors declare that they have no competing interests.

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Authors' contributions

BFS, TRB, TLR and MJG conceived and designed the study. T.R.B., T.L.R., T.A.G., I.N.D.L. and M.J.G. advised on fieldwork. B.vdS., B.F.S. and A.H. conducted field studies. B.F.S.

and A.M.H. conducted the laboratory analyses with input from I.N.D.L. and M.J.G. B.F.S., T.A.G. and J.W. conducted the phylogenetic analyses. B.F.S. wrote the original draft of the manuscript. T.R.B. and T.L.R. provided supervision and extensive editing of the manuscript. All authors contributed to improving the manuscript, reviewed it, and approved the final version.

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Supplementary information

Additional file 1: Molecular assays to identify species in the Maculatus Group

A. Identification of *Anopheles maculatus s.l.* by single-point ITS2 PCR

A single-point ITS2 PCR assay (Beebe and Saul, 1995), was used to confirm samples as *An. maculatus s.l* following morphological identification. The ITS2 gene was amplified using the ITS2A (5'-TGTGAACTGCAGGACACAT-'3) and ITSB (5'-TATGCTTAAATTCAGGGGGT-'3) primers. The amplification master mix consisted of 1x GoTaq® flexi green buffer (Promega, WI, USA), 3.0 mM MgCl2 (Promega), 0.2 mM dNTP mix (Promega), 0.5 µM of each ITS2A and ITS2B, 1.25 U GoTaq® Flexi DNA polymerase (Promega), 1µL of DNA template and nuclease-free water (NFW) to a total volume of 30 µL.

The cycling conditions were an initial denaturation step at 95 °C for 2 min, followed by 35 denaturation cycles at 95 °C for 1 min, annealing at 51 °C for 1 min and extension at 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The PCR products were visualised on a 1.5% agarose gel via electrophoresis at 100 volts for 85 minutes. *Anopheles maculatus s.l* generated an amplification product of approximately 460 bp.

B. Identification of *An. maculatus s.s.* species

A multiplex ITS2 PCR assay was conducted to identify species in the *An. maculatus* group, as described by Walton et al. (2007). One microliter of gDNA template was added to a master mix containing 1x GoTaq green buffer (Promega), 2.5 mM of $MgCl_2$ (Promega), 0.2 mM of dNTP mix (Promega), 0.2 µM of primer 5.8F, MAC, PSEU and K, 0.1 µM of primer SAW and DRAV (Table S4.1), and 1 U GoTaq® Flexi DNA polymerase (Promega), to a final volume of 30 µL using NFW. The cycling conditions were: 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 61 °C for 40 sec and extension at 72 °C for 40 sec, followed by a final extension step at 72 °C for 5 min.

Table S4.1. Primers to identify the species in the Maculatus Group (Walton et al.,2007)

Primer	Sequence (5'-3')	Targeted species	Size (bp)
5.8F	ATC ACT CGG CTC GTG GAT CG	universal forward primer	
MAC	GAC GGT CAG TCT GGT AAA GT-3	*An. maculatus s.s*	180
PSEU	GCC CCC GGG TGT CAA ACA G	*An. pseudowillmori*	203
SAW	ACG GTC CCG CAT CAG GTG C	*An. sawadwongporni*	242
K	TTC ATC GCT CGC CCT TAC AA	*Form K*	301
DRAV	GCC TAC TTT GAG CGA GAC CA	*An. dravidicus*	477

C. Identification of *Anopheles* species based on *cox1* gene

The *cox1* gene was amplified using the primers CI-N-2087 (5'-AAT TTC GGT CAG TTA ATA ATA TAG-3') and TY-J-1460 (5'-TAC AAT TTA TCG CCT AAA CTT CAG CC-3'), as described by Garjito et al. (2019). A 30 µL master mix was prepared by mixing 1x GoTaq® flexi green buffer (Promega), 2.5 mM MgCl2 (Promega), 0.2 mM dNTP mix (Promega), 0.1 µM of each CI-N-2087 and TY-J-1460 primers, 1.25 U GoTaq® Flexi DNA polymerase (Promega), 2 µL of DNA template and NFW. The PCR amplification conditions were an initial denaturation at 95 °C for 3 min, followed by 5 cycles of denaturation at 94 °C for 30 sec, annealing at 45 °C for 40 sec and extension at 72 °C for 1 min, and then followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 40 sec and extension at 72 °C for 1 min, and followed by a final extension step at 72 °C for 10 min. The amplification products were visualized using 1.5% agarose gel electrophoresis

Additional file 2: Molecular assays to identify *Plasmodium*

A. *Plasmodium* Detection

Reverse transcriptase real-time polymerase chain reaction (RT-qPCR), as described by Kamau et al. (2011) Braima et al. (2024), was run to detect *Plasmodium* from *Anopheles* samples. The amplification master mix reaction was prepared in a 10 µL duplicate reaction containing 1x Taqman-PCR master mix (Applied Biosystems by Thermo Fisher Scientific, Warrington, UK), 0.4 µM of each KamGF (5'- GCT CTT TCT TGA TTT CTT GGA-'3) and KamGR (5'-AGC AGG TTA AGA TCT CGT TCG-'3) primers, 0.2 µM of KamGP probe (FAM-ATG GCC GTT TTT AGT TCG TG-BHQ1), 1 µL of cDNA template to xx µL with NFW. The master mix samples, including positive and negative controls, were performed under the following cycling conditions: 2 min at 60°C, 10 min at 95°C, 45 cycles of 15 sec at 95°C, and 1 min at 60°C. The RT-qPCR assay was performed using Rotor-Gene Q (Qiagen, Hilden, Germany).

B. Identification of human malaria parasites

Reverse transcriptase nested PCR (RT-nPCR) assay, as described by Snounou et al. (1993) and Braima et al. (2024), was conducted to identify the human malaria parasites, *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale*. This assay consisted of two PCR reactions. The nest-1 amplification master-mix contained 1x GoTaq green buffer (Promega), 3 mM of MgCl2 (Promega), 0.2 mM of dNTP mix (Promega), 0.25 µM of each primer rPLU1 and rPLU5 (Table S4.2), 1.25 U GoTaq® Flexi DNA polymerase (Promega), 4 µL of cDNA template to a final volume to 50 µL with NFW. The cycling conditions were an initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 30 sec, 58°C for 1 min and 72°C for 2 min, with a final extension at 72°C for 4 min.

The amplification products from nest-1 were used as the template for the nest-2 PCR. The master-mix for nest-2 PCR contained 1x GoTaq green buffer (Promega), 3 mM of MgCl2 (Promega), 0.2 mM of dNTP mix (Promega), 0.25 µM of each species-specific *Plasmodium* primers (Table S4.2), 1 U GoTaq® Flexi DNA polymerase (Promega), 2 µL of diluted nest-1 PCR product (1:20) and NFW to 20ul. The PCR conditions were an initial denaturation for 4 min at 94°C, 35 cycles of 30 sec at 94°C,

1 min of annealing at 58°C and 1 min at 72°C, followed by 5 min at 72°C for final extension. 1.5% agarose gel electrophoresis was used to separate and visual the amplified PCR products.

C. Identification of simian malaria parasites

Following the protocols of Lee et al. (2011), an RT-nPCR assay identified the simian malaria species *Plasmodium inui*, *Plasmodium cynomolgi*, *Plasmodium coatneyi*, and *Plasmodium fieldi*. In the first step (nest-1), the master mix included 1x GoTaq green buffer (Promega), 3 mM $MgCl_2$ (Promega), 0.2 mM dNTPs (Promega), 0.25 µM of each primer (rPLU1 and rPLU5, see Table S4.2), 1.25 U GoTaq® Flexi DNA polymerase (Promega), and 4 µL of cDNA template, with nuclease-free water (NFW) added to a total volume of 50 µL. The thermal cycling protocol began with denaturation at 94°C for 4 min, followed by 35 cycles of 30 sec at 94°C, 1 min at 58°C, and 2 min at 72°C, concluding with a final 4-minute extension at 72°C.

The second PCR (nest-2) used the first PCR product as the template. The nest-2 master mix contained 1x GoTaq green buffer (Promega), 3 mM $MgCl_2$ (Promega), 0.2 mM dNTPs (Promega), 0.25 µM of species-specific *Plasmodium* primers (see Table S4.2), 1 U GoTaq® Flexi DNA polymerase (Promega), 2 µL of diluted nest-1 product (1:10), and NFW to a total volume of 20 µL. PCR cycling conditions began with denaturation at 94°C for 4 min, followed by 35 cycles of 30 sec at 94°C, 1 min of primer-specific annealing (Table S4.2), and 1 minute at 72°C, ending with a 5 min final extension at 72°C. To analyse and visualise the amplified PCR product,

D. Identification of *Plasmodium knowlesi*

A real time-hemi nested PCR assay, as described by Imwong et al. (2009), identified *P. knowlesi*. The master mix for the 1-nest was 1x GoTaq green buffer (Promega), 2.5 mM of MgCl2 (Promega), 0.5 mM of dNTP mix (Promega), 0.25 µM of each PKF1160 and PKR1150 primers (Table S4.2), 1.25 U GoTaq® Flexi DNA polymerase (Promega), 2 µL of cDNA template to a final volume of 25 µL with NFW. The cycling conditions started with an initial denaturation for 5 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and followed by 5 min at 72°C for a final extension.

For the 2nd-nested PCR, the master mix contained 1x GoTaq green buffer (Promega), 3 mM of MgCl2 (Promega), 0.5 mM of dNTP mix (Promega), 0.25 µM of each primer PKF1140 and PKR1150 primers (Table S4.2), 1 U GoTaq® Flexi DNA polymerase (Promega), 2 µL of 1-nest PCR product with NFW added to 20 µL. The thermal conditions were an initial denaturation for 5 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, with final extension at 72°C for 5 min. The PCR products were separated and visualised by 1.5% agarose gel electrophoresis.

Table S4.2. List of primer sequences and modified annealing temperatures of *Plasmodium* species-specific PCR assays.

References	PCR method	Gene target	Primer/ Probe	Sequence (5'-3')	Annealing temp. (°C)	Targeted species	DNA size (bp)
Snounou et al., 1993	Nested PCR	18S rRNA	rPLU 1	TCA AAG ATT AAG CCA TGC AAG TGA	58	-	-
			rPLU 5	CCT GTT GTT GCC TTA AAC TCC			
			rFAL1	TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT	58	*P. falciparum*	205
			rFAL2	ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC			
			rVIV1	CGA CTT CCA AGC CGA AGC AAA GAA AG	58	*P. vivax*	120
			rVIV2	TCC TTA CTT CTA GCT TAA TCC ACA TAA CTG ATA C			
			rOVA1	ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA	58	*P. ovale*	880
			rOVA2	GGA AAA GGA CAC ATT ATT CIG TAT CCT AGT G			
			rMAL1	ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC	58	*P. malariae*	144
			rMAL2	AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA			
Lee et al., 2011	Nested PCR	18S rRNA	rPLU 1	TCA AAG ATT AAG CCA TGC AAG TGA	58	-	-
			rPLU 5	CCT GTT GTT GCC TTA AAC TCC			
			PctF1	CGC TTT TAG CTT AAA TCC ACA TAA CAG AC	60	*P. coatneyi*	503
			PctR1	GAG TCC TAA CCC CGA AGG GAA AGG			
			CY2F	GAT TTG CTA AAT TGC GGT CG	60	*P. cynomolgi*	137
			CY4R	CGG TAT GAT AAG CCA GGG AAG T			
			PfldF1	GGT CT TTT TTT TGC TTC GGT AAT TA	63	*P. fieldi*	421
			PfldR2	AGG CAC TGA AGG AAG CAA TCTA AGA GTT TC			
			PinF2	CGT ATC GAC TTT GTG GCA TTT TTC TAC	58	*P. inui*	479
			INAR3	GCA ATC TAA GAG TTT TAA CTC CTC			
Imwong et al., 2009	Hemi-nested PCR	18S rRNA	PKF1160	GAT GCC TCC GCG TAT CGA C	55	-	-
			PKR1150	GAG TTC TAA TCT CCG GAG AGA AAA GA			
			Pkf1140	GAT TCA TCT ATT AAA AAT TTG CTT C	50	*P. knowlesi*	410
			Pkr1550	GAG TTC TAA TCT CCG GAG AGA AAA GA			

Additional file 3:

Table S4.1. Pairwise genetic distance of ITS2 sequences.

Additional Information: Table S1: Pairwise genetic distance of ITS2 sequences. Genetic distances were analysed with Kimura-2 (K80) using Mega X.

No	Species ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
1	GQ284823_An._sundaicus_(Indonesia:_North_Sumatra)																																												
2	DQ518628_An_pseudowilmori_(Thailand)	0.796																																											
3	DQ518629_An._pseudowilmori_(Vietnam)	0.796	0.000																																										
4	DQ518622_An._sawadwongporni_(Vietnam)	0.790	0.211	0.211																																									
5	DQ518623_An._sawadwongporni_(Cambodia)	0.790	0.211	0.211	0.000																																								
6	DQ518627_An._rampae_(Cambodia)	0.783	0.211	0.211	0.043	0.043																																							
7	FJ526595_An._rampae_(Thailand)	0.783	0.211	0.211	0.043	0.043	0.000																																							
8	AF512562_An._wilmori_(China)	0.805	0.216	0.216	0.127	0.127	0.147	0.147																																						
9	EU882061_An._willmori_(Bhutan)	0.805	0.216	0.216	0.127	0.127	0.147	0.147	0.000																																					
10	PQ601060_An._maculatus_(Indonesia-North_Sumatra:_Langkat)	0.820	0.197	0.197	0.121	0.121	0.146	0.146	0.122	0.122																																				
11	PQ601061_An._maculatus_(Indonesia-North_Sumatra:_Langkat)	0.757	0.187	0.187	0.119	0.119	0.143	0.143	0.116	0.116	0.000																																			
12	PQ601062_An._maculatus_(Indonesia-North_Sumatra:_Langkat)	0.820	0.197	0.197	0.121	0.121	0.146	0.146	0.122	0.122	0.000	0.000																																		
13	PQ601063_An._maculatus_(Indonesia-North_Sumatra:_Langkat)	0.754	0.190	0.190	0.119	0.119	0.142	0.142	0.119	0.119	0.000	0.000	0.000																																	
14	PQ601064_An._maculatus_(Indonesia-North_Sumatra:_Langkat)	0.769	0.189	0.189	0.117	0.117	0.140	0.140	0.117	0.117	0.000	0.000	0.000	0.000																																
15	PQ601065_An._maculatus_(Indonesia-North_Sumatra:_Langkat)	0.777	0.190	0.190	0.118	0.118	0.141	0.141	0.118	0.118	0.000	0.000	0.000	0.000	0.000																																
16	PQ601066_An._maculatus_(Indonesia-North_Sumatra:_Langkat)	0.777	0.190	0.190	0.118	0.118	0.141	0.141	0.118	0.118	0.000	0.000	0.000	0.000	0.000	0.000																																
17	MT623064_An._maculatus_(Malaysia:_Sabah)	0.754	0.190	0.190	0.119	0.119	0.142	0.142	0.119	0.119	0.000	0.000	0.000	0.000	0.000	0.000	0.000																																
18	MF535219_An._maculatus_(China:_Yunnan)	0.918	0.210	0.210	0.129	0.129	0.156	0.156	0.130	0.130	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000																															
19	PP339963_An._maculatus_(Myanmar)	0.924	0.211	0.211	0.130	0.130	0.156	0.156	0.130	0.130	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000																															
20	AY491974.1_An._maculatus_(Malaysia:_Peninsular_Malaysia)	0.771	0.198	0.198	0.126	0.126	0.149	0.149	0.126	0.126	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.003	0.003																												
21	MK504466_An._maculatus_(Indonesia-North_Kalimantan:_Sebatik)	0.741	0.187	0.187	0.116	0.116	0.143	0.143	0.117	0.117	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006																											
22	MK504456_An._maculatus_(Indonesia-Yogyakarta:_Hargotirto)	0.741	0.187	0.187	0.116	0.116	0.143	0.143	0.117	0.117	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000																									
23	MK504461_An._maculatus_(Indonesia-East_Nusa_Tenggara:_Sumba)	0.741	0.187	0.187	0.116	0.116	0.143	0.143	0.117	0.117	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000																								
24	MK504464_An._maculatus_(Indonesia-Aceh:_Sabang)	0.741	0.187	0.187	0.116	0.116	0.143	0.143	0.117	0.117	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000																							
25	MK204649_An._maculatus_(Thailand:_Northern)	0.735	0.173	0.173	0.105	0.105	0.132	0.132	0.101	0.101	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.009	0.000	0.000	0.000	0.000																						
26	FJ526584_An._maculatus_(Laos)	0.757	0.190	0.190	0.116	0.116	0.139	0.139	0.116	0.116	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.003																					
27	JQ446435_An._maculatus_(India:_Mizoram)	0.754	0.190	0.190	0.119	0.119	0.142	0.142	0.119	0.119	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.003	0.000																				
28	MK659793_An._maculatus_(Indonesia-South_Sumatra:_Ogan_Komering_Ulu)	0.754	0.190	0.190	0.119	0.119	0.142	0.142	0.119	0.119	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.003	0.000	0.000																		
29	MK656095_An._maculatus_(Indonesia-Central_Java:_Cilacap)	0.754	0.190	0.190	0.119	0.119	0.142	0.142	0.119	0.119	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000																	
30	MK659797_An._maculatus_(Indonesia-East_Nusa_Tenggara:_Bani-bani)	0.774	0.194	0.194	0.126	0.126	0.150	0.150	0.123	0.123	0.000	0.006	0.000	0.006	0.003	0.000	0.000	0.006	0.000	0.000	0.011	0.006	0.006	0.006	0.006	0.009	0.006	0.006	0.006	0.006																
31	DQ518618_An._maculatus_(Cambodia)	0.754	0.190	0.190	0.119	0.119	0.142	0.142	0.119	0.119	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.006															
32	DQ518624_An._dravidicus_(Thailand)	0.767	0.209	0.209	0.141	0.141	0.162	0.162	0.130	0.130	0.107	0.102	0.107	0.102	0.103	0.104	0.104	0.102	0.114	0.114	0.108	0.099	0.099	0.099	0.099	0.081	0.099	0.102	0.102	0.102	0.109	0.102														
33	FJ526582_An._maculatus_(Thailand:_Northeast)	0.754	0.190	0.190	0.119	0.119	0.142	0.142	0.119	0.119	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.006	0.000	0.102													
34	MK504467_An._greeni_(Indonesia-North_kalimantan:_Sebatik)	0.763	0.196	0.196	0.104	0.104	0.123	0.123	0.107	0.107	0.068	0.065	0.068	0.065	0.066	0.066	0.066	0.065	0.073	0.073	0.071	0.065	0.065	0.065	0.065	0.053	0.065	0.065	0.065	0.065	0.072	0.065	0.081	0.065											
35	GQ284823_An._sundaicus_(Indonesia-North_Sumatra:_Asahan)	0.000	0.796	0.796	0.790	0.790	0.783	0.783	0.805	0.805	0.820	0.757	0.820	0.754	0.769	0.777	0.777	0.754	0.918	0.924	0.771	0.741	0.741	0.741	0.741	0.735	0.757	0.754	0.754	0.754	0.774	0.754	0.767	0.754	0.763										
36	EU104648_An._gambiae_(Angola)	0.808	0.739	0.739	0.777	0.777	0.763	0.763	0.742	0.742	0.735	0.696	0.735	0.693	0.699	0.699	0.699	0.693	0.805	0.809	0.709	0.695	0.695	0.695	0.695	0.685	0.696	0.693	0.693	0.693	0.703	0.693	0.742	0.693	0.757	0.608									
37	JQ446414_An._dravidicus_(India)	0.767	0.209	0.209	0.141	0.141	0.162	0.162	0.130	0.130	0.107	0.102	0.107	0.102	0.103	0.104	0.104	0.102	0.114	0.114	0.108	0.099	0.099	0.099	0.099	0.081	0.099	0.102	0.102	0.102	0.109	0.102	0.000	0.102	0.081	0.767	0.742								
38	KY930778_An._balabacensis_(Malaysia-Sabah_:_Kudat)	1.481	1.236	1.236	1.177	1.177	1.191	1.191	1.148	1.148	1.235	1.227	1.235	1.218	1.214	1.206	1.206	1.218	1.273	1.284	1.218	1.214	1.214	1.214	1.214	1.221	1.210	1.218	1.218	1.218	1.214	1.218	1.194	1.218	1.160	1.481	1.546	1.194							
39	FJ526585_An._greeni_Philippines)	0.763	0.196	0.196	0.107	0.107	0.126	0.126	0.104	0.104	0.065	0.062	0.065	0.062	0.063	0.063	0.063	0.062	0.069	0.070	0.069	0.062	0.062	0.062	0.062	0.053	0.062	0.062	0.062	0.062	0.069	0.062	0.081	0.062	0.000	0.763	0.757	0.081	1.160						
40	AY803346_An._maculatus_(Vietnam)	0.754	0.190	0.190	0.119	0.119	0.142	0.142	0.119	0.119	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.006	0.000	0.102	0.000	0.065	0.754	0.693	0.102	1.218	0.062					
41	FJ526586_An._dispar_(Philippines)	0.773	0.183	0.183	0.103	0.103	0.136	0.136	0.099	0.099	0.055	0.053	0.055	0.052	0.053	0.053	0.053	0.052	0.058	0.059	0.059	0.050	0.050	0.050	0.050	0.040	0.053	0.052	0.052	0.052	0.059	0.052	0.064	0.052	0.032	0.773	0.730	0.064	1.114	0.032	0.052				
42	MK675653_An._maculatus_(Indonesia-Central_Java:_Salatiga_Lab.)	0.775	0.194	0.194	0.106	0.106	0.136	0.136	0.113	0.113	0.065	0.062	0.065	0.062	0.062	0.063	0.063	0.062	0.069	0.069	0.068	0.059	0.059	0.059	0.059	0.052	0.062	0.062	0.062	0.062	0.068	0.062	0.087	0.062	0.056	0.775	0.730	0.087	1.143	0.053	0.062	0.032			
43	MK659792_An._maculatus_(Indonesia-Yogyakarta:_Kulon_Progo)	0.775	0.194	0.194	0.106	0.106	0.136	0.136	0.113	0.113	0.065	0.062	0.065	0.062	0.062	0.063	0.063	0.062	0.069	0.069	0.068	0.059	0.059	0.059	0.059	0.052	0.062	0.062	0.062	0.062	0.068	0.062	0.087	0.062	0.056	0.775	0.730	0.087	1.143	0.053	0.062	0.032	0.000		
44	MK659780_An._maculatus_(Indonesia-Yogyakarta:_Kulon_Progo)	0.775	0.194	0.194	0.106	0.106	0.136	0.136	0.113	0.113	0.065	0.062	0.065	0.062	0.062	0.063	0.063	0.062	0.069	0.069	0.068	0.059	0.059	0.059	0.059	0.052	0.062	0.062	0.062	0.062	0.068	0.062	0.087	0.062	0.056	0.775	0.730	0.087	1.143	0.053	0.062	0.032	0.000	0.000	
45	MK504475_An._maculatus_(Indonesia-Central_Sulawesi:_Labuhan)	0.771	0.187	0.187	0.100	0.100	0.129	0.129	0.110	0.110	0.061	0.059	0.061	0.058	0.059	0.060	0.060	0.058	0.065	0.065	0.065	0.056	0.056	0.056	0.056	0.049	0.059	0.058	0.058	0.058	0.065	0.058	0.084	0.058	0.053	0.771	0.718	0.084	1.139	0.050	0.058	0.029	0.003	0.003	0.003

Additional file 4:

Table S4.2. Pairwise genetic distance of *cox1* sequences.

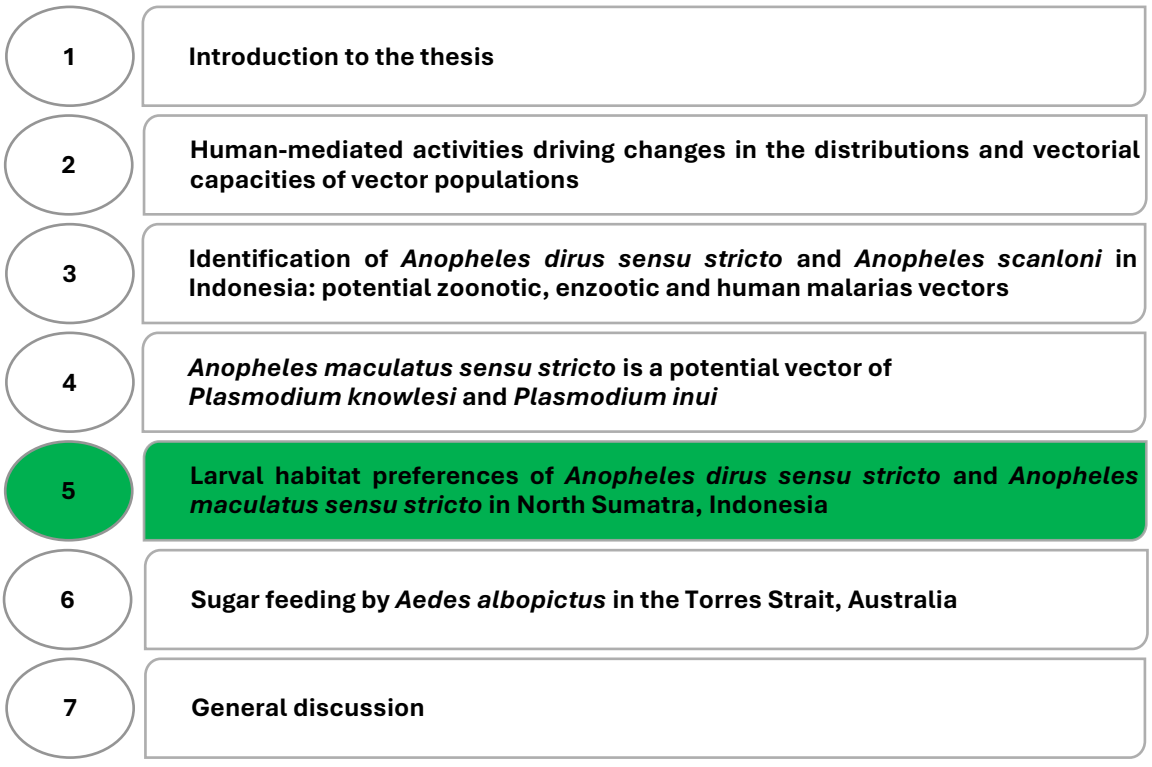
Additional Information: Table 2: Pairwise genetic distance of *cox1* sequences. Genetic distances were analysed with Kimura-2 (K80) using Mega X.

No	Species ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1	DQ465336.1_Anopheles_gambiae_isolate_GM84																																								
2	MK507462_An._maculatus_(Indonesia-East_Nusa_Tenggara:_Sumba)	0.1348																																							
3	MK507461_An._maculatus_(Indonesia-East_Nusa_Tenggara:_Sumba)	0.1348	0.0000																																						
4	MK507470_An._maculatus_(Indonesia-North_Kalimantan:_Sebatik)	0.1348	0.0049	0.0049																																					
5	MK507469_An._maculatus_(Indonesia-North_Kalimantan:_Sebatik)	0.1348	0.0049	0.0049	0.0000																																				
6	MT863706_An._maculatus_(India)	0.1300	0.0294	0.0294	0.0294	0.0294																																			
7	MK579208_An._maculatus_(Thailand:_Northern)	0.1230	0.0272	0.0272	0.0272	0.0272	0.0000																																		
8	MK579209_An._maculatus_(Thailand:_Northern)	0.1230	0.0272	0.0272	0.0272	0.0272	0.0000	0.0000																																	
9	MT669943_An._maculatus_(Malaysia:Sabah)	0.1308	0.0202	0.0202	0.0150	0.0150	0.0143	0.0150	0.0150																																	
10	MT669944_An._maculatus_(Malaysia:Sabah)	0.1308	0.0202	0.0202	0.0150	0.0150	0.0143	0.0150	0.0150	0.0000																																
11	OQ363188_An._sawadwongporni_(Thailand)	0.1393	0.0816	0.0816	0.0858	0.0858	0.0688	0.0715	0.0715	0.0736	0.0736																															
12	OQ363187_An._sawadwongporni_(Thailand)	0.1393	0.0816	0.0816	0.0858	0.0858	0.0688	0.0715	0.0715	0.0736	0.0736	0.0000																														
13	MW603586_An._rampae_(Cambodia)	0.1347	0.0789	0.0789	0.0789	0.0789	0.0658	0.0658	0.0658	0.0680	0.0680	0.0036	0.0036																													
14	MW603593_An._sundaicus_(Cambodia)	0.1253	0.1392	0.1392	0.1392	0.1392	0.1243	0.1243	0.1243	0.1195	0.1195	0.1269	0.1269	0.1220																												
15	OL742870_An._maculatus_(Thailand:_Eastern)	0.1226	0.0318	0.0318	0.0318	0.0318	0.0024	0.0022	0.0022	0.0224	0.0224	0.0790	0.0790	0.0750	0.1449																												
16	OL742871_An._maculatus_(Thailand:_Eastern)	0.1186	0.0318	0.0318	0.0318	0.0318	0.0000	0.0022	0.0022	0.0224	0.0224	0.0736	0.0736	0.0720	0.1414	0.0043																												
17	MN276050_An._maculatus_(India	0.1267	0.0302	0.0302	0.0329	0.0329	0.0000	0.0024	0.0024	0.0224	0.0224	0.0771	0.0771	0.0690	0.1398	0.0048	0.0048																											
18	PP976493_An._maculatus_(Myanmar)	0.1226	0.0272	0.0272	0.0272	0.0272	0.0000	0.0000	0.0000	0.0150	0.0150	0.0715	0.0715	0.0658	0.1243	0.0022	0.0022	0.0024																										
19	PP976502_An._maculatus_(Myanmar)	0.1226	0.0223	0.0223	0.0223	0.0223	0.0000	0.0000	0.0000	0.0153	0.0153	0.0710	0.0710	0.0651	0.1247	0.0022	0.0022	0.0024	0.0000																							
20	KM669758_An._maculatus_(SriLanka)	0.1150	0.0292	0.0292	0.0261	0.0261	0.0184	0.0172	0.0172	0.0172	0.0172	0.0732	0.0732	0.0699	0.1496	0.0202	0.0202	0.0172	0.0172	0.0172																						
21	KM669759_An._maculatus_(SriLanka)	0.1150	0.0292	0.0292	0.0261	0.0261	0.0184	0.0172	0.0172	0.0172	0.0172	0.0732	0.0732	0.0699	0.1496	0.0202	0.0202	0.0172	0.0172	0.0172	0.0000																					
22	MK507468_An._greeni_(Indonesia-North_Kalimantan:_Sebatik)	0.1474	0.0968	0.0968	0.0925	0.0925	0.0913	0.0968	0.0968	0.0946	0.0946	0.1074	0.1074	0.0995	0.1528	0.1019	0.1019	0.0955	0.0966	0.0902	0.0982	0.0982																				
23	PP373008_An._dravidicus_(Myanmar)	0.1482	0.0752	0.0752	0.0690	0.0690	0.0597	0.0671	0.0671	0.0650	0.0650	0.0768	0.0768	0.0669	0.1236	0.0767	0.0713	0.0834	0.0669	0.0676	0.0877	0.0877	0.0791																			
24	PP976518_An._dravidicus_(Myanmar)	0.1482	0.0782	0.0782	0.0762	0.0762	0.0632	0.0703	0.0703	0.0662	0.0662	0.0740	0.0740	0.0683	0.1246	0.0795	0.0740	0.0865	0.0701	0.0696	0.0841	0.0841	0.0887	0.0033																		
25	MK507476_An._maculatus_(Indonesia-Central_Sulawesi:_Labuhan)	0.1264	0.0547	0.0547	0.0508	0.0508	0.0468	0.0470	0.0470	0.0489	0.0489	0.0775	0.0775	0.0701	0.1243	0.0485	0.0485	0.0515	0.0469	0.0422	0.0539	0.0539	0.0782	0.0607	0.0718																	
26	MK507475_An._maculatus_(Indonesia-Central_Sulawesi:_Labuhan)	0.1264	0.0547	0.0547	0.0508	0.0508	0.0468	0.0470	0.0470	0.0489	0.0489	0.0775	0.0775	0.0701	0.1243	0.0485	0.0485	0.0515	0.0469	0.0422	0.0539	0.0539	0.0782	0.0607	0.0718	0.0000																
27	MK507474_An._maculatus_(Indonesia-WestSulawesi:_Wulai)	0.1305	0.0586	0.0586	0.0547	0.0547	0.0489	0.0508	0.0508	0.0528	0.0528	0.0816	0.0816	0.0723	0.1267	0.0535	0.0535	0.0572	0.0507	0.0460	0.0606	0.0606	0.0741	0.0567	0.0678	0.0033	0.0033															
28	MK507473_An._maculatus_(Indonesia-WestSulawesi:_Wulai)	0.1305	0.0586	0.0586	0.0547	0.0547	0.0489	0.0508	0.0508	0.0528	0.0528	0.0816	0.0816	0.0723	0.1267	0.0535	0.0535	0.0572	0.0507	0.0460	0.0606	0.0606	0.0741	0.0567	0.0678	0.0033	0.0033	0.0000														
29	MK507472_An._maculatus_(Indonesia-South_Sulawesi:_Pucak)	0.1305	0.0567	0.0567	0.0528	0.0528	0.0468	0.0489	0.0489	0.0508	0.0508	0.0795	0.0795	0.0701	0.1243	0.0510	0.0510	0.0544	0.0488	0.0441	0.0572	0.0572	0.0762	0.0587	0.0698	0.0016	0.0016	0.0016	0.0016													
30	MK507471_An._maculatus_(Indonesia-South_Sulawesi:_Pucak)	0.1305	0.0567	0.0567	0.0528	0.0528	0.0468	0.0489	0.0489	0.0508	0.0508	0.0795	0.0795	0.0701	0.1243	0.0510	0.0510	0.0544	0.0488	0.0441	0.0572	0.0572	0.0762	0.0587	0.0698	0.0016	0.0016	0.0016	0.0016	0.0000												
31	PQ596587__An._maculatus_(Indonesia-North_Sumatra:_Langkat)	0.1306	0.0182	0.0182	0.0220	0.0220	0.0073	0.0090	0.0090	0.0164	0.0164	0.0720	0.0720	0.0656	0.1233	0.0144	0.0144	0.0107	0.0090	0.0090	0.0180	0.0180	0.0951	0.0729	0.0706	0.0516	0.0516	0.0558	0.0558	0.0537	0.0537											
32	PQ596588__An._maculatus_(Indonesia-North_Sumatra:_Langkat)	0.1306	0.0183	0.0183	0.0221	0.0221	0.0073	0.0090	0.0090	0.0165	0.0165	0.0723	0.0723	0.0659	0.1213	0.0144	0.0144	0.0107	0.0090	0.0090	0.0180	0.0180	0.0955	0.0732	0.0709	0.0518	0.0518	0.0560	0.0560	0.0539	0.0539	0.0000										
33	MK507460_An._maculatus_(Indonesia-Aceh:_Sabang)	0.1350	0.0219	0.0219	0.0254	0.0254	0.0162	0.0202	0.0202	0.0237	0.0237	0.0755	0.0755	0.0722	0.1316	0.0225	0.0225	0.0225	0.0202	0.0153	0.0202	0.0202	0.0925	0.0731	0.0782	0.0508	0.0508	0.0547	0.0547	0.0528	0.0528	0.0036	0.0036									
34	MK507459_An._maculatus_(Indonesia-Aceh:_Sabang)	0.1350	0.0219	0.0219	0.0254	0.0254	0.0162	0.0202	0.0202	0.0237	0.0237	0.0755	0.0755	0.0722	0.1316	0.0225	0.0225	0.0225	0.0202	0.0153	0.0202	0.0202	0.0925	0.0731	0.0782	0.0508	0.0508	0.0547	0.0547	0.0528	0.0528	0.0036	0.0036	0.0000							
35	PQ596589__An._maculatus_(Indonesia-North_Sumatra:_Langkat)	0.1353	0.0216	0.0216	0.0254	0.0254	0.0108	0.0125	0.0125	0.0179	0.0179	0.0726	0.0726	0.0663	0.1253	0.0194	0.0194	0.0162	0.0125	0.0126	0.0211	0.0211	0.0975	0.0716	0.0712	0.0547	0.0547	0.0589	0.0589	0.0568	0.0568	0.0036	0.0036	0.0017	0.0017						
36	PQ596590__An._maculatus_(Indonesia-North_Sumatra:_Langkat)	0.1353	0.0198	0.0198	0.0235	0.0235	0.0090	0.0107	0.0107	0.0180	0.0180	0.0727	0.0727	0.0665	0.1256	0.0169	0.0169	0.0134	0.0107	0.0108	0.0211	0.0211	0.0953	0.0716	0.0713	0.0527	0.0527	0.0569	0.0569	0.0548	0.0548	0.0018	0.0018	0.0017	0.0017	0.0017					
37	MK507451_An._maculatus_(Indonesia-Central_Java:_Purworejo)	0.1226	0.0219	0.0219	0.0219	0.0219	0.0053	0.0049	0.0049	0.0202	0.0202	0.0775	0.0775	0.0722	0.1316	0.0022	0.0022	0.0024	0.0049	0.0000	0.0172	0.0172	0.0903	0.0669	0.0762	0.0414	0.0414	0.0451	0.0451	0.0433	0.0433	0.0090	0.0090	0.0151	0.0151	0.0143	0.0107				
38	MK507452_An._maculatus_(Indonesia-Central_Java:_Purworejo)	0.1226	0.0219	0.0219	0.0219	0.0219	0.0053	0.0049	0.0049	0.0202	0.0202	0.0775	0.0775	0.0722	0.1316	0.0022	0.0022	0.0024	0.0049	0.0000	0.0172	0.0172	0.0903	0.0669	0.0762	0.0414	0.0414	0.0451	0.0451	0.0433	0.0433	0.0090	0.0090	0.0151	0.0151	0.0143	0.0107	0.0000			
39	MK507453_An._maculatus_(Indonesia-Central_Java:_Purworejo)	0.1226	0.0219	0.0219	0.0219	0.0219	0.0053	0.0049	0.0049	0.0202	0.0202	0.0775	0.0775	0.0722	0.1316	0.0022	0.0022	0.0024	0.0049	0.0000	0.0172	0.0172	0.0903	0.0669	0.0762	0.0414	0.0414	0.0451	0.0451	0.0433	0.0433	0.0090	0.0090	0.0151	0.0151	0.0143	0.0107	0.0000	0.0000		
40	MK507477_An._maculatus_(Thailand:_Northern)	0.1267	0.0237	0.0237	0.0237	0.0237	0.0071	0.0082	0.0082	0.0219	0.0219	0.0795	0.0795	0.0722	0.1316	0.0065	0.0065	0.0073	0.0082	0.0033	0.0202	0.0202	0.0903	0.0669	0.0762	0.0414	0.0414	0.0451	0.0451	0.0433	0.0433	0.0090	0.0090	0.0116	0.0116	0.0106	0.0107	0.0033	0.0033	0.0033	
41	MK507478_An._maculatus_(Thailand:_Northern)	0.1267	0.0237	0.0237	0.0237	0.0237	0.0071	0.0082	0.0082	0.0219	0.0219	0.0795	0.0795	0.0722	0.1316	0.0065	0.0065	0.0073	0.0082	0.0033	0.0202	0.0202	0.0903	0.0669	0.0762	0.0414	0.0414	0.0451	0.0451	0.0433	0.0433	0.0090	0.0090	0.0116	0.0116	0.0106	0.0107	0.0033	0.0033	0.0033	0.0000

Chapter 5

The preceding chapters focused on identifying and incriminating the mosquito species responsible for malaria transmission in North Sumatra. Chapters 3 and 4 provided the entomological evidence needed to confirm that *Anopheles dirus s.s.* and *Anopheles maculatus s.s.* are likely involved in the local transmission of zoonotic, enzootic and human malarias, highlighting the importance of site-specific vector surveillance in areas where zoonotic spillover is a growing concern. These findings laid the foundation for understanding which mosquito species matter most and why they need to be the focus of control strategies.

Building on this foundation, the next two chapters shift from vector identification and incrimination to vector behaviours. This chapter examines the larval habitats, ecological preferences, and spatial distributions of *Anopheles* species in Langkat Regency, with a focus on how these behaviours can be exploited for control. In areas where outdoor-biting mosquitoes are difficult to manage with traditional indoor tools, approaches like larval source management (LSM) offer a promising alternative. By mapping and characterising larval habitats, this chapter explores how local environmental interventions could reduce adult mosquito densities and, ultimately, disease risk.



Larval habitat preferences of *Anopheles dirus sensu stricto* and *Anopheles maculatus sensu stricto* in North Sumatra, Indonesia

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Abstract

Background:

While human malaria transmission in Indonesia has declined, transmission of zoonotic *Plasmodium knowlesi* is increasing. This study examined the larval habitats of the malaria vectors in North Sumatra to assess the potential of larval source management (LSM) as a control strategy for both human and zoonotic malaria vectors.

Methods:

Larval habitat surveys were conducted over two years in Ujung Bandar Village, Salapian Subdistrict, Langkat Regency, North Sumatra, an area with both human and zoonotic (*P. knowlesi*) malaria cases. Larval habitats were characterised by larval presence and density, land use type, aquatic habitat class (i.e. naturally occurring, man-made from natural materials or man-man from artificial materials) and habitat subclass (the different specific habitats (i.e., streams, ditches, etc), characterising them by a range of abiotic and biotic characteristics.

Results:

A total of 1,413 mosquito larvae and 98 pupae were collected, with *Anopheles* larvae comprising 20.6% of immatures sampled. *Anopheles maculatus sensu stricto* was the most common species sampled, followed by *Anopheles dirus sensu stricto*, *Anopheles scanloni, Anopheles kochi*, and *Anopheles vagus*. Habitat class was a significant predictor of anopheline presence and density. *Anopheles maculatus s.s.* was predominantly associated with natural habitats (e.g., stream margins), whereas *An. dirus s.s.* was more frequently found in habitats created from natural substrates by human activities (e.g., tyre tracks). Four species (*An. maculatus s.s.*, *An. dirus s.s.*, *An. scanloni* and *An. kochi*) were also detected, albeit infrequently, in habitats man-made from artificial materials (e.g., small plastic containers).

Conclusion:

In North Sumatra, anopheline vector species utilise a diverse range of larval habitats, including natural, human-modified from natural and artificial containers. The species-specific habitat preferences observed in this study suggest that targeted larval control strategies should prioritise natural habitats and human-modified from natural substrates habitats. These findings support the potential utility of larval source

management for species like *An. dirus* s.s., while highlighting challenges for controlling species like *An. maculatus* s.s. that prefer more dispersed and inaccessible natural sites.

Keywords: North Sumatra, *Anopheles* larval habitats, *Anopheles maculatus sensu stricto*, *Anopheles dirus sensu stricto, Anopheles scanloni, Anopheles kochi*, *Anopheles vagus*

Background

Human malaria transmission in Indonesia has decreased and become increasingly localized, leading to a commitment to eliminate malaria by 2030 [3, 325]. However, human infections with zoonotic malarias, particularly those caused by *Plasmodium knowlesi*, a macaque malaria parasite, are increasingly being reported. Over the past decade, *P. knowlesi* infections were reported in the provinces of Aceh [224, 225, 232], North Sumatra [35, 326], Jambi [327], Central Kalimantan [222, 328] and South Kalimantan [221].

Knowledge of the potential vector species responsible for transmitting zoonotic malaria remains limited. Generally, species within the Leucosphyrus Group are associated with *P. knowlesi* transmission to humans. Several species within this Leucosphyrus Group are reported in Indonesia, including *Anopheles leucosphyrus*, *Anopheles balabacencis, Anopheles dirus s.l.,* (including *Anopheles dirus s.s.* [see Chapter 3]), *Anopheles scanloni, Anopheles cracens, Anopheles latens* [15, 226, 236, 256, 329]. However, evidence of *P. knowlesi* (e.g., DNA) in mosquitoes was only found in *Anopheles balabacencis*, *Anopheles dirus s.s.* (see Chapter 3) and *Anopheles maculatus s.s.* (see Chapter 4) in Indonesia. In addition, the circumsporozoite proteins of the human malarias (*Plasmodium falciparum* and *Plasmodium vivax*), were detected in *An. leucosphyrus* and *An. balabacensis* [329].

The World Health Organisation (WHO) recommended malaria vector control strategies, insecticide treated nets (ITNs) and indoor residual spraying (IRS), have significantly reduced human malaria transmission globally [3, 155, 324]. Insecticide treated nets primarily target endophagic (indoor biting) vectors and IRS primarily targets endophilic (indoor resting) vectors [324]. While ITNs and IRS can reduce transmission by exophagic and exophilic vectors, supplemental and complementary strategies are likely needed for effective control [3, 216, 330]. As many malaria vector species in Southeast Asia (SEA), including both the human and enzootic vectors, rarely bite or rest inside houses [220], understanding the other behaviours of the malaria vectors is critical for selecting vector control strategies with the potential to be effective. Larval source management (LSM), recommended by the WHO as a supplemental malaria vector strategy, has the potential

to control both indoor and outdoor mosquitoes [331]. Larval source management is most effective where the larval habitats are few in number, fixed in location and accessible, including where transmission is urban or seasonal [331].

However, there is limited information about the larval habitats of the human and zoonotic malaria vectors [226] and their vulnerability to LSM. The potential of LSM will be affected by land-use changes that can significantly impact the classes and abundance of mosquito larval habitats, directly influencing vector distributions and receptivity, and in so doing affect the transmission potential of both human and zoonotic malarias [332-334]. Hence, this study in North Sumatra in an area with both zoonotic and human malarias that had undergone dramatic land use changes over the past decade with deforestation and the introduction and expansion of oil palm plantations [234, 235] aimed to define the distribution and abundance of different anopheline larval habitats to understand the potential of LSM in North Sumatra.

Materials and Methods

Study site

The study was undertaken in 2 of 9 Dusuns (hamlets) of Ujung Bandar Village in the Salapian sub-district of Langkat Regency, North Sumatra Province, Indonesia. The study site was selected based on two key criteria: (1) a predictive model incorporating environmental data on macaques, mosquito vectors and land-use types to identify areas of high *P. knowlesi* transmission risk [234, 235]; and (2) confirmed transmission of both zoonotic and human malarias to humans through passive case detection [I.N.D Lubis et al., unpublished].

The main residential areas in both Dusuns were surrounded by agricultural land, including oil palm and mixed plantations. In 2023, 38.3% of the Salapian sub-district (22,173 Ha) was agricultural land, comprised of oil palm (7,846 Ha), rubber (595 H), coconut (31 Ha) and cacao (11 Ha) [335]. Ujung Bandar Village, with a population of over 1,500 people, is 357 m above sea level and had an annual rainfall of 3,493 mm in 2022 and 3,352 mm in 2023 [336, 337]. Rainfall is relatively less from January to June

(Additional file1: Figure S1) [336, 337]. The mean annual outdoor temperature is 27.3 °C, with an 84.9% average humidity and 47% sunlight exposure (Additional file1: Figure S5.1) [336, 337].

Landscape classifications

The landscapes in which larval habitats were surveyed were categorised by visual inspection into four land use types based on predominant human activity and vegetation. These included: (1) residential areas: where most residents' houses are located; (2) gardens: where food plants including vegetables are cultivated, usually near landowner houses; (3) oil palm plantations: monoculture cultivation of oil palm trees; and (4) mixed plantations: agricultural areas where two or more plant species are cultivated including young oil palm trees. The size of each land use type was estimated by recording GPS coordinates along the boundary during the survey. These coordinates were then used to create polygons in QGIS (ver.3.34.14), which allowed the calculation of the area for each land use class.

Larval habitat surveys

After receiving verbal permission from landowners or village representatives, larval habitat surveys were conducted up to 1 km from residential areas with the assistance of two residents in July-August 2022 and March-April 2023 in both Dusun II (Pondok Cengkeh; 3.371720, 98.328547) and Dusun V (Deleng Payung; 3.341947, 98.335406). The geolocation of each habitat was recorded using GPS (latitude and longitude).

All water bodies encountered during the surveys were sampled for mosquito immature stages by dipping using 250 ml (125 mm diameter) standard dippers. Small habitats were sampled with up to 10 dips. In larger habitats, sampling was performed at multiple sites, (e.g., in fishponds, up to 25 dips were taken from each of 4 sites, while in streams, sites were sampled at 5-meter intervals with up to 10 dips taken per site along the stream margins). Habitats with anopheline larvae were defined as positive habitats, while habitats without anopheline larvae were defined as negative habitats. The GPS coordinates of all potential (whether positive or negative) habitats were recorded.

The number of mosquito immature stages and the total number of dips per habitat were recorded. The density of immature mosquitoes was calculated by dividing the total number of larvae and pupae collected by the total number of dips taken at each habitat and expressed as the average number of immature mosquitoes per dip.

Larval habitat classifications

Larval habitats were categorised into three primary classes: naturally occurring, man-made from natural materials or man-man from artificial materials. Within each of these three types, habitat subclasses were used to further categorise the specific forms or examples of aquatic environments in which mosquito larvae were found.

Naturally occurring habitats were defined as water bodies formed without direct human or domestic animal input. Subclasses in this category included groundwater pools, rock holes, plant axils and stream margins. Man-made habitats from natural materials were those created by human or domestic animal activity that altered the landscape using natural substrates. Subclasses included road or agricultural ditches, tyre tracks, excavated ponds, footprints of humans or livestock, and modified coconut shells used for harvesting copra or rubber sap. Man-made habitats from artificial materials were defined as those formed with synthetic or industrial materials, such as plastics, cement, rubber, metal and glass. Subclasses in this category included discarded plastic containers, metal buckets, tyres, and ditches lined with concrete. A full list of the habitat subclasses, with detailed definitions and examples, is provided in Additional file 8: Table S5.1.

Each larval habitat was further characterised by a range of physical (abiotic) and biological (biotic) features, measured in the field to understand habitat variation and its potential influence on mosquito presence. Abiotic parameters included:

- Substrate type, referring to the material composition of the habitat floor (e.g., mud, rock, gravel, sand, concrete, metal, or plastic);
- Size, based on the approximate length of the water body and was classified as <0.5 m, 0.5-1 m, 1-2 m, 2-5 m, 5-10 m, and >10m;

- Water depth, categorised as very shallow (<10 cm), shallow (10-30 cm), medium (30-60 cm) and deep (>60 cm);
- Water flow, recorded as stagnant (no visible movement) and flowing (by observation of surface turbulence);
- Turbidity, assessed using the tube method, where 50 mL of water was placed in a clear tube, left to settle and visually classified as clear, cloudy or opaque;
- Water temperature, pH and salinity, recorded at the time of collection using a thermometer, pH meter and refractometer, respectively.

Biotic parameters included:

- Canopy cover, defined as the percentage of overhead vegetation shading the habitat and categorised as none, low (<50% cover), or high (≥ 50% cover);
- Aquatic vegetation, including algae, classified by the percent of the water surface covered (none, low <50% and high ≥ 50%);
- Aquatic fauna, including the presence or absence of potential predators such as fish or aquatic insects.

Field laboratory processing of samples

Mosquito larvae and pupae samples were transported to a field laboratory in 50 ml labelled plastic containers where larvae were identified to genus based on morphology. *Anopheles* larva stages were classified and recorded as early (stages 1 and 2), late (stages 3 and 4) and early-late (mixed) stages. Pupae and larvae were then preserved in 70% ethanol at room temperature.

Identification of *Anopheles* species

Initially, species identification was by PCR amplification of the internal transcribed spacer region II (ITS2) [238]. Representative PCR products from up to five mosquito immature samples with the same band size were Sanger sequenced to determine species using the basic local alignment search tool (BLAST). To confirm members of the Dirus Complex, the Dirus Complex species identification PCR (DiCSIP) and Scanloni specific PCR (SSP) assays were conducted [239], while multiplex PCR was used for

species identification within the Maculatus Group [299]. Detailed procedures are provided in chapters 3 and 4 (Additional file 2: Figure S5.2).

Statistical analysis

All field data were digitally recorded using the Ona platform (https://ona.io/) and the ODK application on an Android operating system. All data were exported to MS Excel (Microsoft Crop., Redmond, WA, USA) for statistical analyses with the R statistical environment (ver.4.4.2) and all graphs were visualised by GraphPad (ver.10.3.1). Generalized Linear Mixed Models (GLMMs) were used to compare the presence or absence of *Anopheles* larvae (binary model) and larval densities (negative binomial model) across different survey years, locations, land use types, and habitat classes. To account for potential clustering of similar environmental characteristics within specific habitat classes, habitat subclass was included as a random effect in all GLMMs. Abiotic and biotic parameters of potential larval habitats, including perimeter, vegetation, canopy cover, water depth, turbidity, presence of predators, substrate type, water flow rate, temperature, and pH, were also analysed in relation to the presence and density of *Anopheles* larvae. These analyses were performed using the *glmmTMB* package in R [338]. The random factor included variations in habitat subclasses observed during the survey. Distribution maps of *Anopheles spp*. larval habitats were created using QGIS (ver.3.34.14).

Results

Survey overview: distribution of *Anopheles* larval habitats and land use

A total of 338 aquatic habitats were found and characterised in a 1.348 km^2 area during two surveys conducted in 2022 and 2023 across Dusuns II and V in Ujung Bandar Village, Salapan Subdistrict, Langkat Regency, North Sumatra (Figure 5.1). The largest land use type surveyed was oil palm plantations, covering 0.805 km^2 (59.7% of the surveyed area), followed by mixed plantations (0.477 km^2, 35.4%), residential areas (0.06 km^2, 4.5%), and gardens (0.006 km^2, 0.4%) (Additional files 3 and 4: Figures S5.3 and S5.4). The majority of aquatic habitats (positive and negative) were found in mixed plantations (n =

195), followed by oil palm plantations (n = 107), main residential areas (n = 32), and gardens (n = 4). Some areas, particularly in the northeast of the main residential area in Dusun V were not surveyed due to limited accessibility or a lack of landowner permission (Additional file 4: Figure S5.4). All surveyed larval habitats had freshwater (e.g., 0 p.p.m salt), thus salinity was excluded from further analysis.

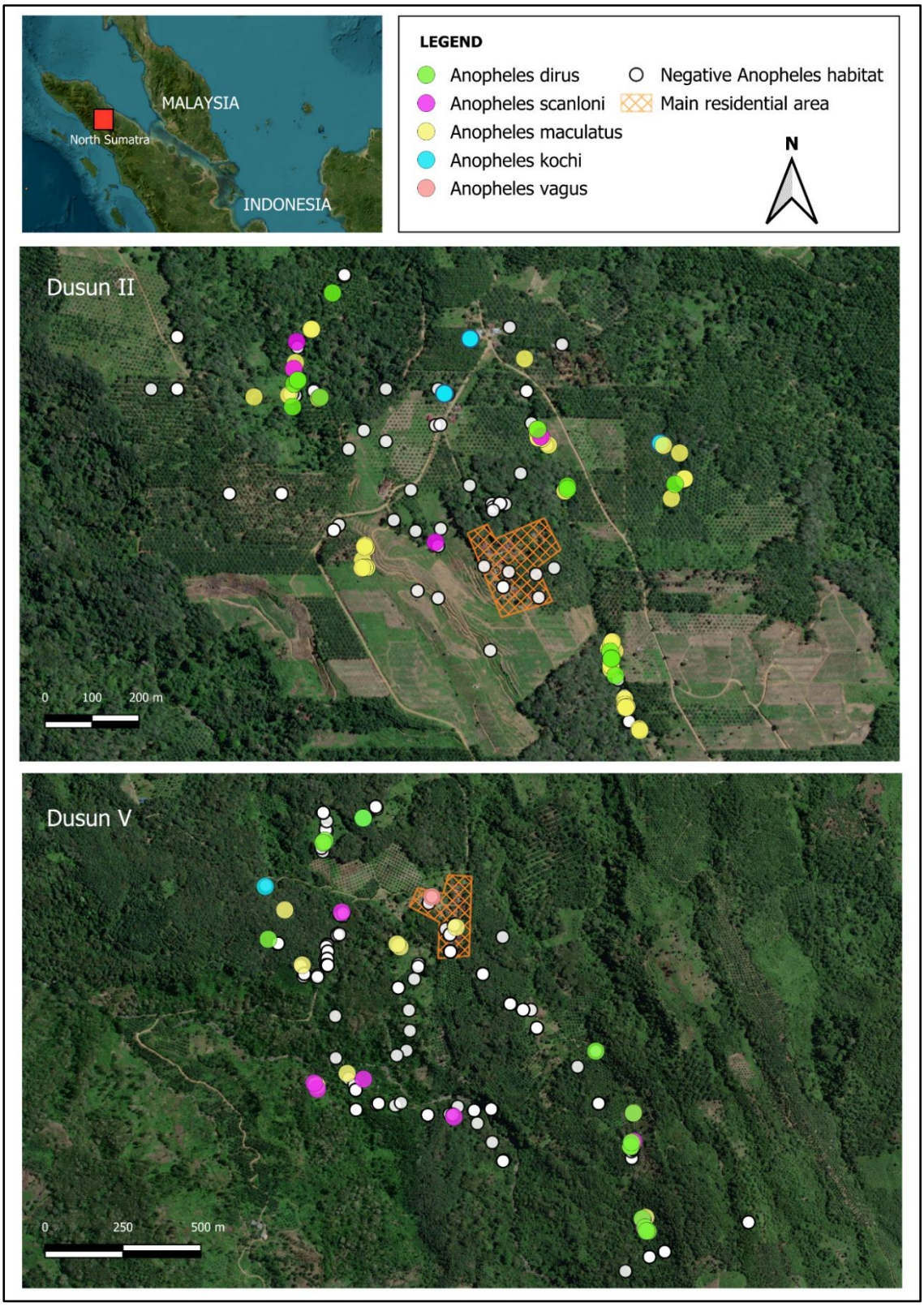

Figure 5.1. Spatial distribution of *Anopheles* larval habitats in Dusun II and V, Ujung Bandar Village, Salapian Subdistrict, Langkat Regency, North Sumatra. The basemap was created with EsriWorld Imagery (WGS84)
(source:https://www.arcgis.com/home/item.html?id=52bdc7ab7fb044d98add148764eaa30a)

Presence and density of *Anopheles* larvae

A total of 1,413 mosquito larvae were collected during the surveys with 291 (20.6%) larvae identified morphologically as anophelines, while 1,122 (79.4%) were culicines. Overall, 114 (33.7%) potential larval habitats were positive for anopheline larvae. Ninety-eight pupae were also collected. *Anopheles*-positive habitats were primarily located in mixed plantations (*n* = 68) and oil palm plantations (*n* = 42), with only two positive habitats each found in gardens and residential areas (Figure 5.2). Land use type did not significantly affect presence (GLMM, β = 0.217, P = 0.433) and density (GLMM, β = 0.329, P = 0.129) of *Anopheles* larvae (Table 5.1).

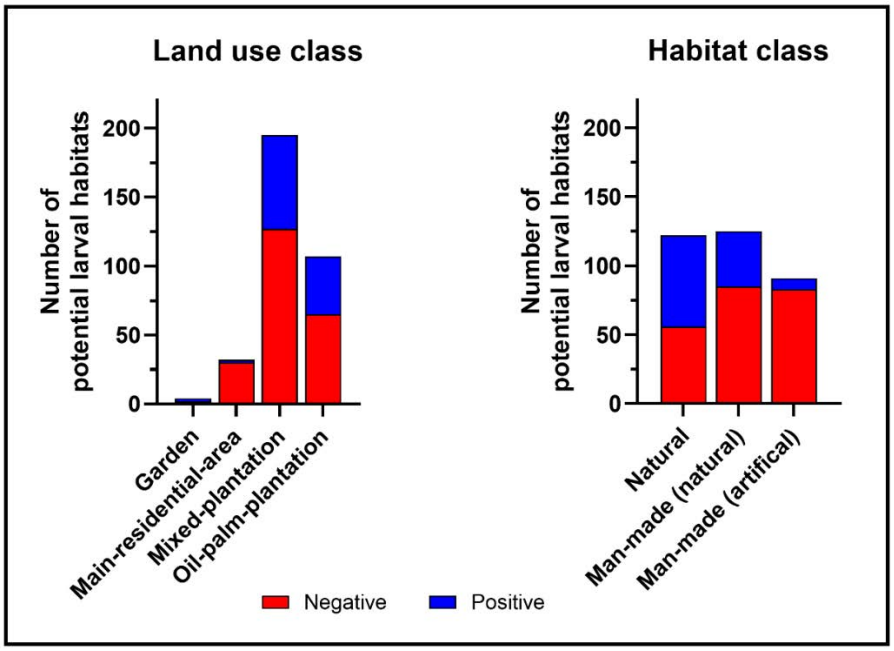

Figure 5.2. Number of potential larval habitats by land use type and habitat class.

Habitat class had a strong significant association with both presence (GLMM, β = -1.450, *P* = 0.006) and density (GLMM, β = -1.421, P = 0.001) of *Anopheles* larvae. Noting a reduced occurrence and abundance in artificial man-made habitats (Table 5.1), habitats that were naturally occurring and man-made from natural materials accounted for the majority of positive detections (Figure 5.2). A detailed breakdown of larval density and positivity rates across specific habitat subclasses is presented in Table 5.2. Notably, stream margins (77.4% positive, 0.74 larvae/dip) and rock holes (60% positive, 0.81 larvae/dip) were among the most productive habitats, while artificial containers such as tyres and large water drums yielded no anopheline larvae. These patterns further support

the reduced suitability of artificial man-made habitats for *Anopheles* larvae, as highlighted by the GLMM results (where subclass was included as a random variable).

Table 5.1. Association of survey year, hamlets, land use type and habitat class with the presence and density of *Anopheles* larvae. Data were compared with GLMMs with the habitat class as a random variable. The data was analysed with two different distributions: binary model (presence/absence) and negative binominal model (density). The asterisks indicate statistical significance.

Parameters	Binary (presence/absence) model			Negative binomial (density) model		
	β	***SE***	***P* value**	***β***	***SE***	***P* value**
***Anopheles* genus**						
(Intercept)	1.499	1.160	0.196	0.952	0.953	0.318
Year of survey	-0.345	0.292	0.236	-0.249	0.261	0.340
Hamlets	0.058	0.449	0.897	0.503	0.400	0.208
Land use type	0.217	0.277	0.433	0.329	0.217	0.129
Habitat class	-1.450	0.531	0.006*	-1.421	0.409	0.001*
***Anopheles maculatus* s.s. larvae only**						
(Intercept)	0.613	1.170	0.600	1.056	1.159	0.362
Year of survey	-0.054	0.371	0.884	-0.211	0.433	0.627
Hamlets	-0.386	0.570	0.498	0.913	0.648	0.159
Land use type	0.333	0.331	0.313	0.428	0.342	0.211
Habitat class	-1.452	0.508	0.004*	-2.168	0.449	<0.001*
***Anopheles dirus* s.s. larvae only**						
(Intercept)	-0.982	1.144	0.391	-0.628	1.172	0.592
Year of survey	-0.089	0.404	0.827	0.106	0.433	0.806
Hamlets	0.379	0.614	0.538	2.180	0.760	0.004*
Land use type	-0.070	0.408	0.864	0.314	0.438	0.473
Habitat class	-1.084	0.517	0.036*	-2.027	0.625	0.001*

Table 5.2. Larval habitats of anophelines in Dusun II and V in Ujung Bandar Village, Salapian Subdistrict, Langkat Regency, North Sumatra, Indonesia.

Habitat Class	Habitat subclass		Positive *Anopheles* habitats		Larval density per dip
		Containers (No)	(No)	(%)	(mean ± SE)
Natural	Groundwater pools	38	9	23.7	0.46 ± 0.05
	Rock holes	15	9	60.0	0.81 ± 0.05
	Stream margins	62	48	77.4	0.74 ± 0.1
	Tree holes or plant axils	7	0	0.0	na
Man-made from natural materials	Coconut shells	9	1	11.1	0.05 ± 0.00
	Ditches	8	5	62.5	0.31 ± 0.06
	Fishponds	16	4	25.0	0.07 ± 0.01
	Footprints and hoof prints	1	0	0.0	na
	Dug holes	22	3	13.6	1.18 ± 0.03
	Tyre tracks	69	27	39.1	0.32 ± 0.02
Man-made from artificial materials	Drain	3	0	0.0	na
	Large containers (>25 L) (plastic and metal drums, plastic and concrete water containers)	16	0	0.0	na
	Small containers (<10 L) (buckets, jerrycans, plastic sheets, plastic food containers)	65	8	12.3	0.85 ± 0.04
	Tyres	7	0	0.0	na

Larval presence was positively associated with aquatic vegetation (GLMM, $\beta = 0.583$, $P = 0.020$), specific substrate types such as mud, rock or gravel (GLMM, $\beta = 0.354$, $P = 0.024$), flowing water (GLMM, $\beta = 1.800$, $P < 0.001$), and alkaline pH (GLMM, $\beta = 1.169$, $P < 0.001$) (Table 5.3). In contrast, deeper water bodies were negatively association with larval presence (GLMM, $\beta = -0.422$, $P = 0.034$) (Table 5.3).

Larval density for *Anopheles* species followed a similar pattern. Higher larval densities were associated with aquatic vegetation (GLMM, β = 0.464, P = 0.024), muddy or organic substrates (GLMM, β = 0.283, P = 0.025), flowing water (GLMM, β = 0.794, P = 0.019), and higher pH (GLMM, β = 0.988, P < 0.001). In contrast, turbid water (GLMM, β = -0.672, P = 0.036) and warmer water temperatures (GLMM, β = -0.152, P = 0.036) were negatively associated with larval density (Table 5.3).

Table 5.3. Association of abiotic and biotic parameters with the presence and density of *Anopheles* larvae. Data were compared with GLMMs with the habitat class as a random variable. The data was analysed with two different distributions: binary model (presence/absence) and negative binominal model (density). The asterisks indicate statistical significance.

Parameters	Binary (presence/absence) model			Negative binomial (density) model		
	β	***SE***	***P* value**	***β***	***SE***	***P* value**
All *Anopheles* larvae						
(Intercept)	-10.232	3.238	0.002*	-6.080	2.640	0.021*
Perimeter	0.140	0.171	0.414	0.093	0.130	0.476
Vegetation	0.583	0.250	0.020*	0.464	0.205	0.024*
Canopy cover	0.266	0.183	0.146	0.196	0.156	0.208
Water depth	-0.422	0.199	0.034*	-0.054	0.132	0.680
Turbidity	-0.615	0.364	0.091	-0.672	0.320	0.036*
Predators	-0.350	0.340	0.303	-0.334	0.239	0.163
Substrate	0.354	0.156	0.024*	0.283	0.126	0.025*
Flow rate	1.800	0.474	<0.001*	0.794	0.339	0.019*
Temperature	-0.101	0.095	0.286	-0.152	0.072	0.036*
pH	1.169	0.315	<0.001*	0.988	0.264	<0.001*
***Anopheles maculatus* s.s. larvae only**						
(Intercept)	-8.671	3.878	0.025*	-5.697	4.124	0.167
Perimeter	0.046	0.196	0.815	0.049	0.209	0.815
Vegetation	0.770	0.279	0.006*	0.780	0.336	0.021*
Canopy cover	0.258	0.228	0.256	0.490	0.247	0.048*
Water depth	-0.123	0.207	0.551	0.209	0.216	0.333
Turbidity	-0.171	0.454	0.706	-0.958	0.531	0.071
Predators	0.202	0.418	0.628	0.143	0.396	0.717
Substrate	0.193	0.155	0.212	0.271	0.187	0.146
Flow rate	1.421	0.458	0.002*	0.864	0.547	0.114
Temperature	-0.056	0.119	0.636	-0.267	0.113	0.019*
pH	0.511	0.392	0.193	0.954	0.437	0.029*
***Anopheles dirus* s.s. larvae only**						
(Intercept)	-7.392	4.835	0.126	-6.761	5.238	0.197
Perimeter	0.365	0.230	0.113	0.070	0.210	0.739
Vegetation	-0.083	0.359	0.817	-0.258	0.389	0.508
Canopy cover	0.067	0.259	0.794	-0.372	0.230	0.106
Water depth	-0.930	0.395	0.019*	-0.704	0.347	0.042*
Turbidity	0.030	0.518	0.954	0.302	0.548	0.581
Predators	-0.447	0.467	0.339	-0.932	0.354	0.008*
Substrate	0.141	0.202	0.484	0.139	0.234	0.551
Flow rate	0.237	0.538	0.659	0.312	0.594	0.599
Temperature	-0.153	0.142	0.282	-0.066	0.159	0.677
pH	1.253	0.506	0.013*	1.301	0.529	0.014*

Anopheles species composition and distribution

A total of 242 *Anopheles* larvae were identified to species using molecular techniques (Additional file 3: Figure S5.3). The predominant species was *Anopheles maculatus sensu stricto* (65.3%, *n* = 158), followed by *Anopheles dirus sensu stricto* (21.9%, *n* = 53), *Anopheles scanloni* (7%, *n* = 17), *Anopheles kochi* (5.4%, *n* = 13), and *Anopheles vagus* (0.4%, *n* = 1). Of the 50 pupae identified molecularly, three were *An. maculatus s.s.* (6.0%), while the remaining were Culicine species: *Aedes albopictus* (28.0%, *n* = 14), *Armigeres subalbatus* (12.0%, *n* = 6), and *Culex* spp. (54.0%, *n* = 27) (Additional file 3: Figure S5.3).

Anopheles maculatus s.s. (*n* = 64), *An. dirus s.s.* (*n* = 33), *An. scanloni* (*n* = 13), and *An. kochi* (*n* = 7) were detected across a variety of larval habitats, spanning natural habitats, man-made habitats from natural materials, and man-made habitats from artificial materials (Table 5.4; Figure 5.3). Natural stream margins were the most frequently occupied habitat type for *An. maculatus s.s.* (n = 39), while *An. dirus s.s.* was most commonly found in tyre tracks (n = 14). *An. scanloni* was most frequently detected in stream margins (n = 4), whereas *An. kochi* and *An. vagus* were observed at low frequencies across a limited range of habitats (Table 5.4; Figure 5.3).

Co-occurrence was observed in multiple sites: *An. dirus s.s.*, *An. scanloni* and *An. kochi* coexisted in two tyre tracks, while *An. dirus s.s.*, *An. scanloni* and *An. maculatus s.s.* cohabitated two stream margin sites. Additionally, *An. dirus s.s.* and *An. kochi* coexisted in a natural rock hole and a human dug hole. Four *Anopheles* species (*An. maculatus s.s.*, *An. dirus s.s.*, *An. scanloni* and *An. kochi*) were observed in artificial containers (e.g. plastic buckets and jerrycans (<10L)) in mixed and oil palm plantations (Table 5.4). Tyre tracks were the only habitat type in which all five *Anopheles* species were detected (Table 5.4).

Figure 5.3. Larval habitats with positive *Anopheles* larvae. Natural habitats (left to right: ground pools, rock holes and stream margins); man-made from natural materials (left to right: coconut shells, ditch, fishpond, dug holes and tyre tracks); man-made from artificial materials (small plastic containers < 10 l).

Table 5.4. *Anopheles* species larval habitats in Dusun II and V, Ujung Bandar Village, Salapian Subdistrict, Langkat Regency, North Sumatra, Indonesia

Habitat Class	Habitat subclass	No. of occurrences*	Number larval habitats occupied				
			An. maculatus s.s.	*An. dirus s.s.*	*An. scanloni*	*An. kochi*	*An. vagus*
Natural	Groundwater pools	38	3	4	2	0	0
	Rock holes	15	5	4	0	1	0
	Stream margins	62	39	7	4	0	0
	Total	**115**	**47**	**15**	**6**	**1**	**0**
Man-made from natural materials	Coconut shells	9	1	0	0	0	0
	Ditches	8	3	2	0	0	0
	Fishponds	16	3	0	1	0	0
	Dug holes	22	2	1	0	1	0
	Tyre tracks	69	7	14	3	2	1
	Total	**124**	**16**	**17**	**4**	**3**	**1**
Man-made from artificial materials	Small containers (<10 L)	65	1	1	3	3	0
Overall Total		**304**	**64**	**33**	**13**	**7**	**1**

*"No. of occurrences" refers to the total number of mosquito larval habitats recorded during the survey, regardless of whether they were occupied by *Anopheles* larvae.

Presence of *An. maculatus s.s* and *An. dirus s.s.*

The two most common species were modelled using GLMMS (Tables 5.1 and 5.3). The presence of *Anopheles* larvae was influenced by several environmental characteristics, with clear differences between species. Both species were significantly affected by habitat class, the presence of these species was negatively associated with man-made artificial habitats (*An. maculatus s.s.* GLMM, β = 1.452, P = 0.004; *An. dirus s.s.* GLMM, β = -1.084, P = 0.036; Table 5.1). For *An. maculatus s.s.*, significant positive associations were observed with increasing vegetation (GLMM, β = 0.770, P=0.006) and water flow rate (GLMM, β = 1.421, P=0.002) (Table 5.3). In contrast, *An. dirus s.s.* larvae were more likely to occur in shallow water (GLMM, β = -0.930, P=0.019) and under more alkaline pH conditions (GLMM, β = 1.253, P=0.013) (Table 5.3; Additional files 6 and 7: Figures S5.6 and S5.7).

Density of *An. maculatus s.s* and *An. dirus s.s.*

Habitat class was negatively associated with larval density for both *An. maculatus s.s.*(GLMM, β = -2.168, $P < 0.001$) and *An. dirus s.s.* (GLMM, β = -2.027, P = 0.001) (Table 5.1), reinforcing the reduced productivity of artificial habitats. *Anopheles dirus s.s.* density was also associated with dusun (hamlet) (GLMM, β = 2.180, P = 0.004) (Table 5.1), indicating higher densities in Dusun V, potentially due to spatial variation in environmental or ecological factors.

Anopheles maculatus s.s. larval density was positively associated with aquatic vegetation (GLMM, β = 0.780, P = 0.021), canopy cover (GLMM, β = 0.490, P = 0.048), and pH (GLMM, β = 0.954, P = 0.029), and negatively associated with temperature (GLMM, β = -0.267, P = 0.019) (Table 5.3).

Anopheles. dirus s.s. larvae showed increased density in shallow water (GLMM, β = -0.704, P = 0.042), at higher pH (GLMM, β = 1.301, P = 0.014), and in the absence of aquatic predators (GLMM, β = -0.932, P = 0.008) (Table 5.3). Observed aquatic predators included tadpoles, dragonfly nymphs, water striders, *Toxorhynchites* larvae, and fish.

Spatial distributions of larval habitats by species

Although land use type was not significantly associated with species presence, some observations are made here. *Anopheles maculatus s.s.* were found most frequently in mixed plantations (n = 35), followed by oil palm plantations (n = 26), gardens (n = 2), and residential areas (n = 1) (Figure 5.4). Garden habitats, although limited in size, had the highest larval habitat density for *An. maculatus s.s.* were in gardens (333.33 positive habitats/km^2), followed by mixed plantations (73.38 habitats/km^2), oil palm plantations (32.30 habitats/km^2) and residential areas (16.67 habitats/km^2) (Table 5.5; Additional file 3 and 4: Figures S5.3 and S5.4). In contrast, *An. dirus s.s.* larvae were not found in the residential areas or gardens (Figure 5.4), but were most commonly found in mixed plantations (44.03 positive habitats/km^2), followed by oil palm plantations (14.91 habitats/km^2) (Table 5.5).

Table 5.5. Density of *An. dirus s.s.* and *An. maculatus s.s.* larval habitats across different land use type.

Land use type	Surveyed area (km^2)	*An. dirus s.s.* larval habitat		*An. maculatus s.s.* larval habitat	
		number (n)	density (n/km^2)	number (n)	density (n/km^2)
Gardens	0.006	0	0.00	2	333.33
Residential areas	0.06	0	0.00	1	16.67
Mixed plantations	0.477	21	44.03	35	73.38
Oil palm plantations	0.805	12	14.91	26	32.30

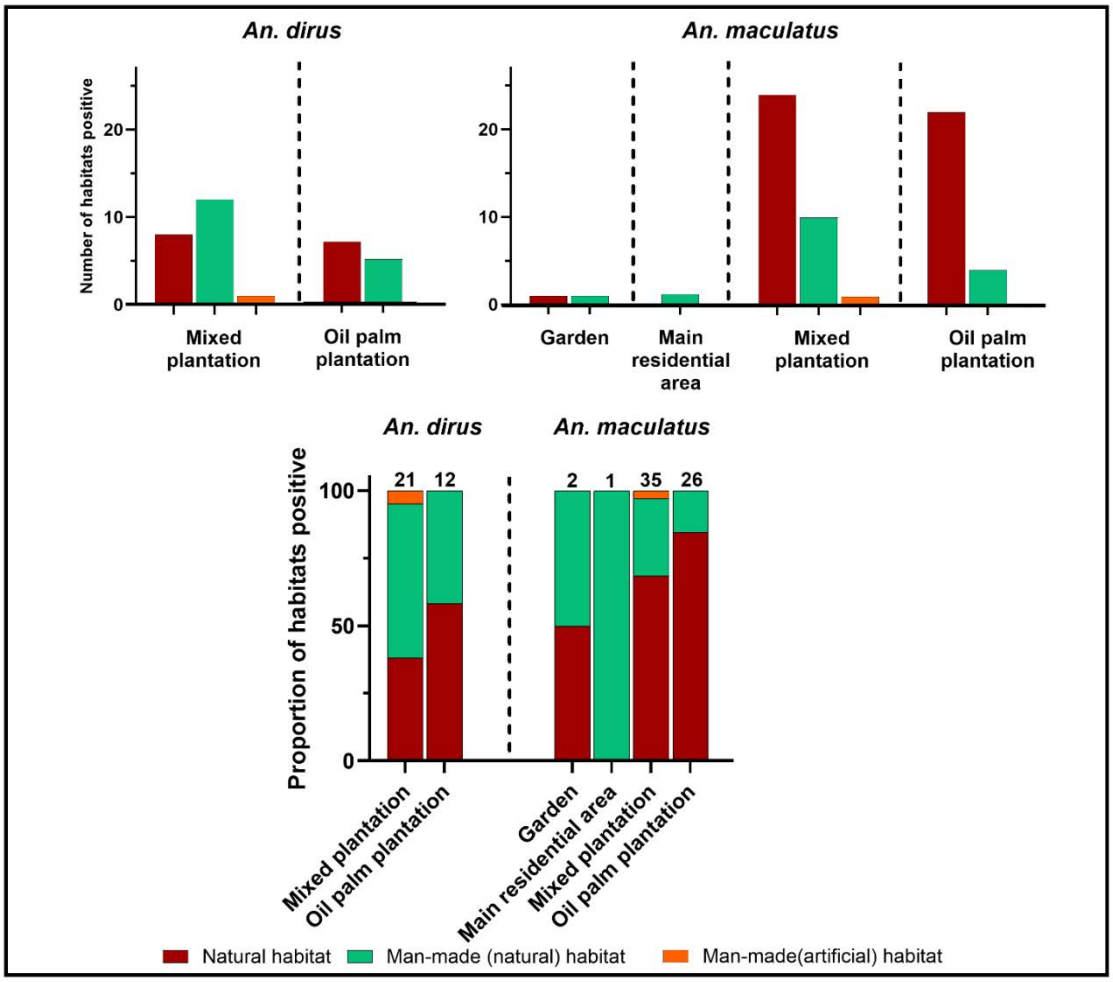

Figure 5.4. The number of positive larval habitats by species in land use type and habitat class

Discussion

This study identified five *Anopheles* species from larval habitats in Ujung Bandar Village, North Sumatra: *An. maculatus s.s.*, *An. dirus s.s.*, *An. scanloni*, *An. kochi* and *An. vagus*. These results were similar to adult mosquito species caught by human landing catch

(HLC) from a concurrent longitudinal study in the same locations [B. v. d. Straat et.al., unpublished]. Four of these species are recognised as malaria vectors in Indonesia [36]. *Anopheles kochi* is a secondary human malaria vector in Central Java, northern Sumatra and Sulawesi [36], with *An. vagus* being a secondary human malaria vector in Banten and East Nusa Tenggara (Sumba) [339, 340]. *Anopheles maculatus s.l.* and *var. manoreh* is a primary human malaria vector in Central Java (Menoreh Hills) [307] and a secondary human malaria vector in western Indonesia [36] and was positive for enzootic and zoonotic malaria DNA (i.e. *Plasmodium inui* and *P. knowlesi*) in North Sumatra (Langkat) (see chapter 4). *Anopheles dirus s.s.* and *An. scanloni* are members of the Dirus Complex within the Leucosphyrus Group. Both species identities were confirmed by PCR (targeted ITS2 gene) for the first time in Indonesia with evidence that *An. dirus s.s.*, is a probable vector of human and zoonotic malarias in Langkat (see chapter 3). While the vector status of *An. scanloni* in Indonesia remains unknown, this species is a secondary malaria vector in Thailand [38, 288].

Spatial patterns in larval habitat use varied by species. *An. maculatus* s.s. exhibited a strong preference for natural aquatic environments, especially stream margins, whereas *An. dirus* s.s. was more adaptable, frequently using man-made habitats formed from natural substrates, such as tyre tracks. Both species were rarely found in artificial habitats, but their presence—even in low numbers—suggests some capacity to exploit novel environments. These findings align with previous research in Malaysia and Indonesia but provide new evidence for habitat use in a region where *P. knowlesi* transmission was detected.

While land use type did not significantly predict the presence or density of *Anopheles* larvae, habitat class—particularly the distinction between natural and artificial environments—was a strong determinant. Natural and semi-natural habitats (created by human modifications of natural substrates) supported higher densities of both *An. maculatus* s.s. and *An. dirus* s.s., likely due to favourable ecological conditions such as shaded vegetation, flowing water, and suitable substrates. These abiotic and biotic factors emerged as significant predictors in the GLMMs, underlining the complex interplay between vector ecology and habitat characteristics.

Consistent patterns were not observed when comparing Anopheles abundance across individual land use categories, possibly due to small sample sizes within categories and overlapping vegetation structures across the study sites. However, a study from Sabah, Malaysia, showed that certain land use types, such as eucalyptus plantations and oil palm plantations, can support significantly higher mosquito numbers. than restored dipterocarp forest [281], suggesting that vegetation type and structure may influence mosquito populations and warrant further investigation in the North Sumatra context.

The detection of *An. dirus* s.s. and *An. scanloni* primarily outside residential areas is consistent with their known associations with forested and peri-domestic environments [253]. However, given the flight range of *Anopheles* mosquitoes (being approximately 542 m [341] and with *An. dirus s.l.* reported to fly distances up to 1.5 km [342, 343]) larval habitats located up to 1-2 km from households could pose a significant risk of human-vector contact [332, 333]. However, in a parallel HLC study, there was an absence of significant biting by *An. dirus s.s.* and *An. maculatus s.s*. in the residential areas [B. v. d. Straat et.al., unpublished].

A second factor that may have limited the presence of positive larval habitats and adults sampled by HLC in the residential areas might be the absence of either wild or pet macaques, the natural blood meal sources of *An. dirus s.s.*, in the main residential areas of the two dusuns surveyed.-Studies have shown that macaques are primarily active in rubber and oil palm plantations and that members of the Leucosphyrus Group tend to maintain their presence near macaques [332, 344].

A key contribution of this study lies in its implications for vector control, particularly the potential application of larval source management (LSM). The success of LSM is generally considered more likely in areas where larval habitats are limited in number, fixed in location, and easily accessible, particularly in settings with seasonal transmission or peri-urban environments [29, 331]. In this context, *An. dirus* s.s. represents a promising target: the species frequently utilised habitats such as tyre tracks, which are often clustered, stable, and amenable to physical modification, drainage, or larviciding. These characteristics make LSM a potentially feasible and cost-effective strategy for reducing *An. dirus* s.s. populations in Langkat. Obsomer et al.

(2007) also found that *An. dirus (s.s.* and *s.l)*, were most commonly found in pools, including those formed in tyre tracks [253].

In contrast, LSM appears less viable for controlling *An. maculatus* s.s., whose larvae were primarily found in natural stream margins—habitats that are flowing, dispersed, and difficult to treat consistently. The complexity and scale of such environments limit the practical application of LSM tools. Similar findings were reported in various sites in Indonesia [36] and Malaysia [128], which found that *An. maculatus s.l.* larvae were predominantly in natural habitats such as stream margins as was found here or along riverbanks.

Environmental management strategies targeting mosquito breeding sites in hilly areas, such as sub-soil drainage, covered channels, and stream-flushing systems, have been documented in various regions [345], including in West Malaysia by Sir Malcolm Watson in the 1910s [346]. These interventions effectively reduced breeding sites of An. maculatus and other vector species in specific hill regions where terrain, hydrology, and infrastructure facilitated sustained implementation [345, 346]. However, the ecological and operational context of the present study area differs considerably. Here, *An. maculatus s.s.* breeding sites are widely scattered along natural stream margins without existing drainage infrastructure, in locations that are difficult to access and subject to seasonal variability. Implementing large-scale engineering solutions similar to those used in other hill regions would present significant logistical, financial, and operational challenges. Therefore, despite historical successes elsewhere, the feasibility of LSM for *An. maculatus s.s.* in this context remains limited.

Nevertheless, the detection of four *Anopheles* species, *An. maculatus* s.s. and *An. dirus* s.s., *An. scanloni* and *An. kochi*, in artificial containers is noteworthy. Though rare, these findings suggest emerging behavioural plasticity and may indicate an early adaptation to anthropogenic environments. Such trends warrant close surveillance, as they could signal a shift in vector ecology that expands breeding sites into urban or peri-urban zones. The presence of *Anopheles* larvae in artificial habitats is most notably seen in the urban malaria vector, *Anopheles stephensi,* that oviposits in a diverse array of artificial

sites in urban environments [347]. Similarly, *Anopheles vagus* larvae were reported in tyres, drums and dinghies in Indonesia [36].

The density and distribution of *An. dirus s.s.* and *An. maculatus s.s*. larval habitats reflect both ecological availability and the detection sensitivity of larval surveillance. Detection rates are influenced by sampling effort—number of dips per site, number of collectors, site accessibility, and whether locations are fixed or opportunistic. While greater effort improves detection, it may limit geographic coverage due to time and staffing constraints. This trade-off is particularly relevant in extensive oil palm and mixed plantations, which in this study supported the highest numbers of *An. dirus s.s.* and *An. maculatus s.s.* habitats. Operational assessments are therefore essential to balance thoroughness and coverage, guiding resource allocation and goal setting.

These considerations reinforce the importance of detailed larval habitat mapping to guide targeted vector control. Where LSM is appropriate, interventions should prioritise human-made habitats from natural substrates that are accessible and relatively stable. More broadly, the results support integrated vector management strategies that combine entomological surveillance, habitat modification, and responsive control measures tailored to species-specific behaviours and environmental contexts.

Conclusion

This study highlights the importance of habitat class in shaping the distribution and density of *Anopheles* larvae in North Sumatra, particularly for *An. dirus s.s.* and *An. maculatus s.s.*. Naturally occurring and man-made habitats from natural materials consistently supported larval populations. The occasional detection of *An. maculatus s.s., An. dirus s.s., An. scanloni,* and *An. kochi* in artificial containers, suggests limited but notable adaptability to human-associated environments. These findings have practical implications for vector control: *An. dirus* s.s., which frequently utilised accessible habitats like tyre tracks, presents a more feasible target for larval source management (LSM), whereas *An. maculatus* s.s. was primarily found in less accessible stream margins. Continued larval surveillance is essential to monitor these patterns,

particularly in landscapes undergoing rapid environmental change, and to guide locally adapted vector control strategies informed by species-specific ecological preferences.

Supplementary information

Additional file 1: Figure S1. The environmental condition of Ujung Bandar Village in 2022 and 2023.

Additional file 2: Figure S2. Analyses of larvae and pupae of *Anopheles* identification.

Additional file 3: Figure S3. Proportion of the area of each surveyed land use class.

Additional file 4: Figure S4. Spatial distribution of the surveyed land use class.

Additional file 5: Figure S5. The gel electrophoresis results from mosquito larvae.

Additional file 6: Table S1. Definition of habitat subclasses.

Declarations

Acknowledgements

We sincerely appreciate the residents of Dusun II and V in Ujung Bandar Village, Salapian Sub-district, Langkat Regency, North Sumatra, for their invaluable support in mosquito sampling and their warm hospitality during our fieldwork. Our gratitude also extends to Hidayatullah Nasution and Panusunan Hasibuan from Balai Laboratorium Kesehatan Masyarakat Medan for their significant contributions to the fieldwork. Additionally, we acknowledge Yessica Sianturi, Meilani Sihite and Ariyani Simbolon from Universitas Sumatra Utara for their assistance during the larval surveys.

Authors contributions

B.F.S., T.R.B., T.L.R. and M.J.G. designed the study. B.F.S., and A.H. conducted larval surveys in the fieldwork. B.F.S. and A.M.H. conducted the laboratory. B.F.S. wrote the original draft of the manuscript. T.R.B. and T.L.R. provided supervision and extensive editing of the manuscript. All authors reviewed the manuscript and approved the final version.

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Competing interest

The authors declare no competing interests

Supplementary Information

Additional file 1

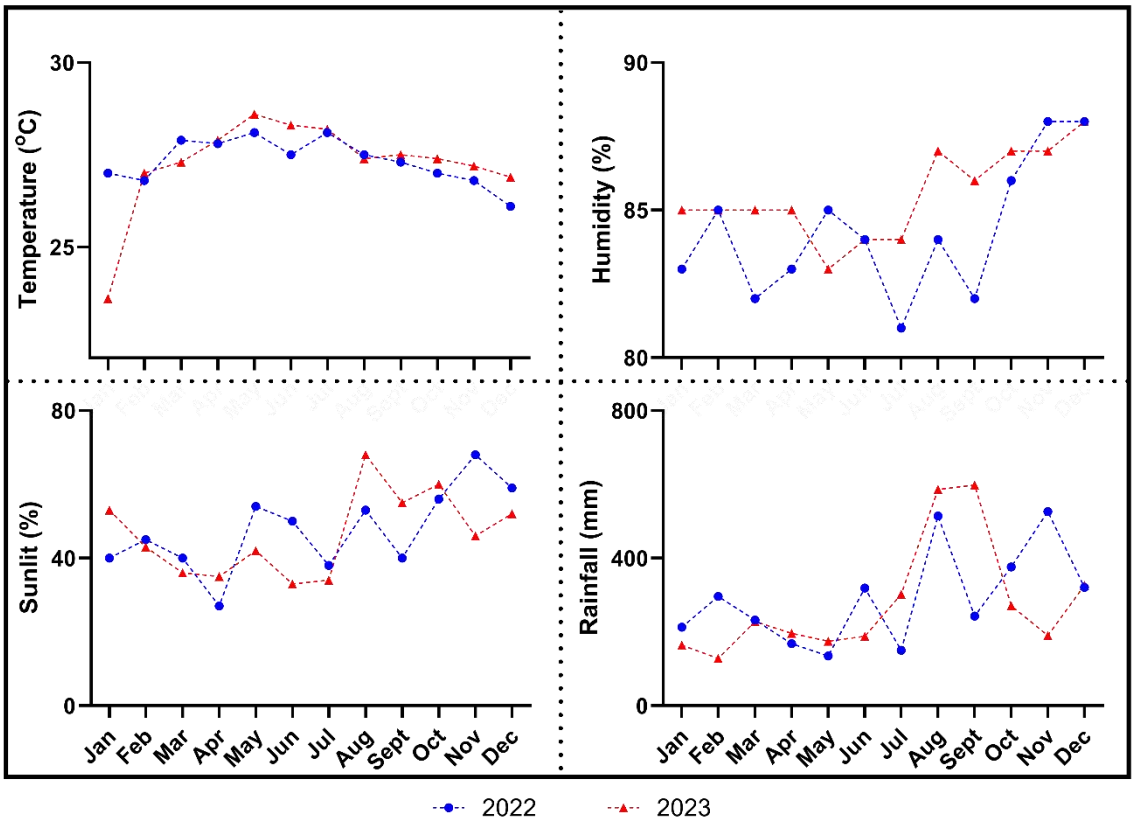

Figure S5.1. Environmental conditions of Ujung Bandar Village in 2022 and 2023 (Temperature, Humidity, sunlight and rainfall).

Additional file 2

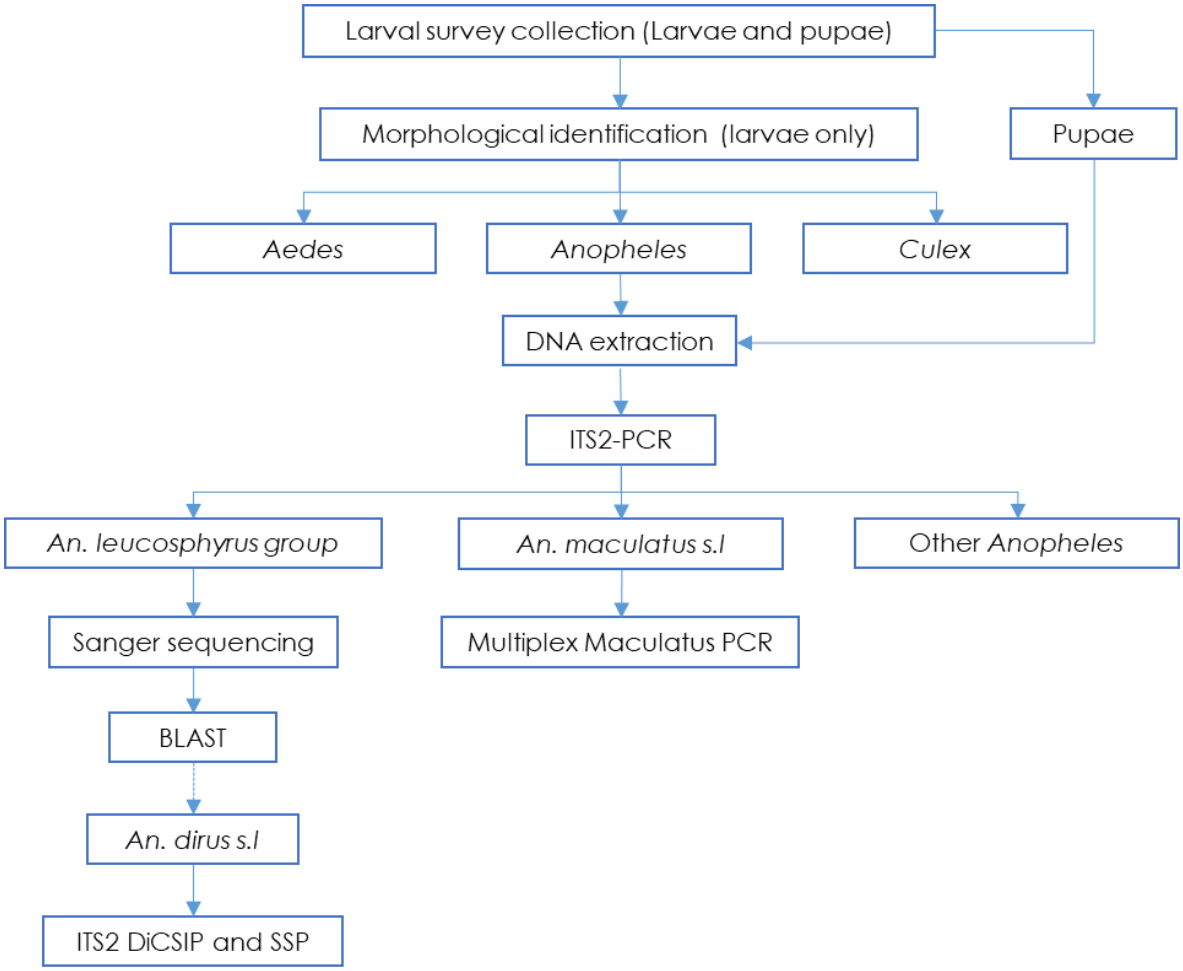

Figure S5.2. Workflow for *Anopheles* identification of larvae and pupae

Additional file 3

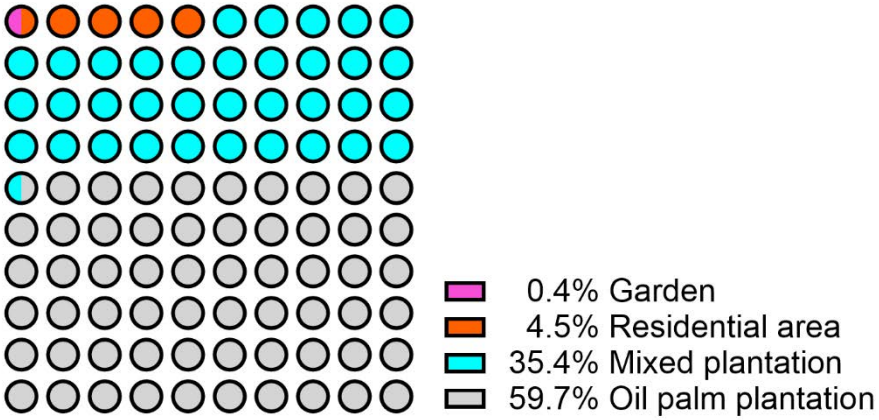

Figure S5.3. Proportion of the area of each surveyed land use type.

Additional file 4

Figure S5.4. Spatial distribution of the surveyed land use types. Some areas cannot be surveyed due to the lack of access or permission from landowners.

Additional file 5

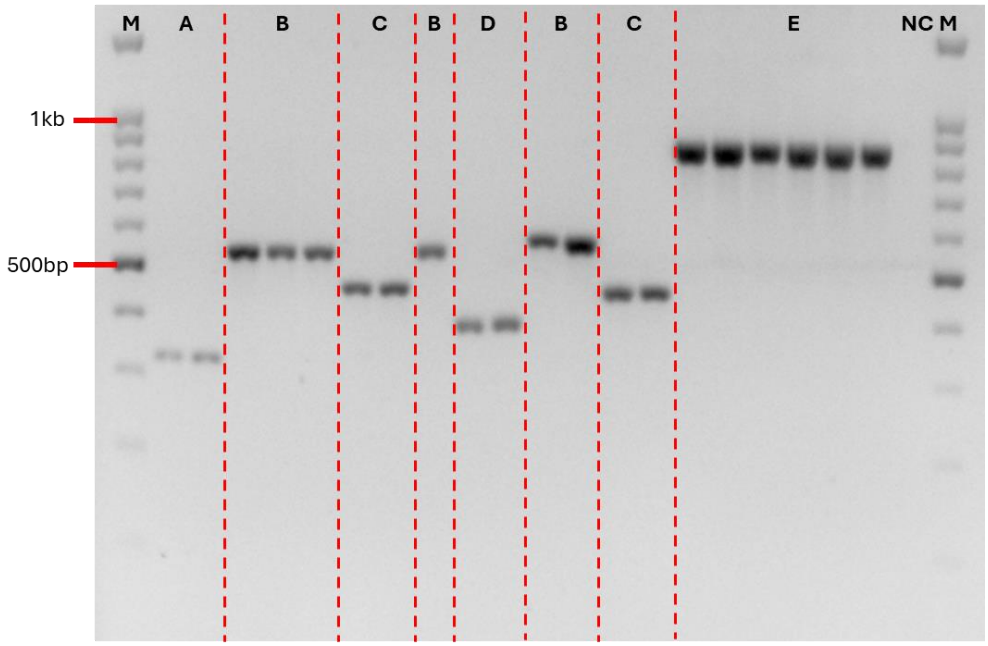

Figure S5.5. The gel electrophoresis results of mosquito larvae. (A) *Ar. subalbatus* ; (B) *Ae. albopictus* : (C) *An. maculatus s.l.* (460bp) ; (D) *An. kochi* (E) *An. leucosphyrus* group (±880bp), NC: negative control, M= 100 bp ladder marker. Gel was running out at 100V for 100 mins.

Additional file 6

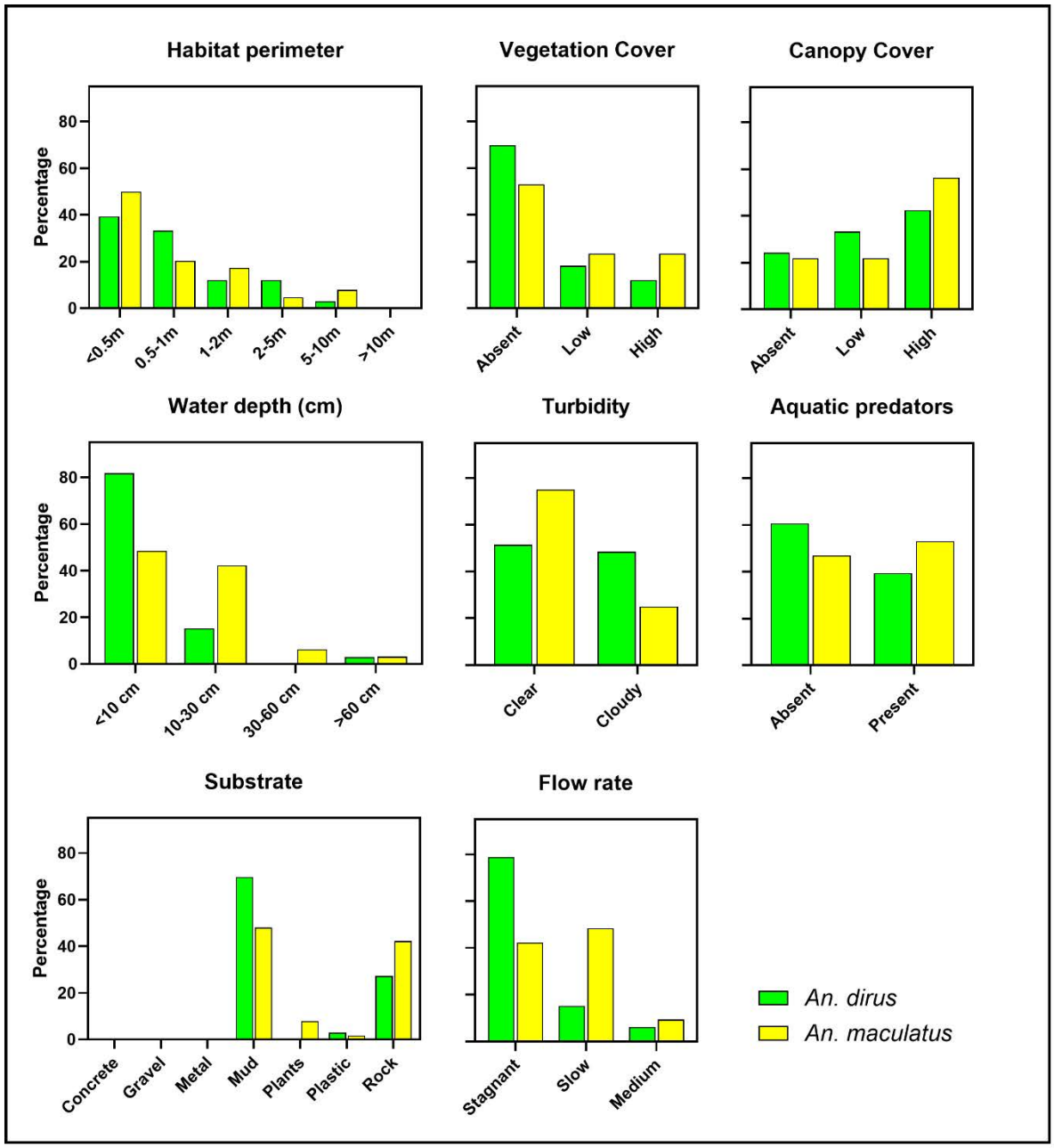

Figure S5.6. Percentage of abiotic and biotic characteristics associated with *Anopheles* larvae habitats

Additional file 7

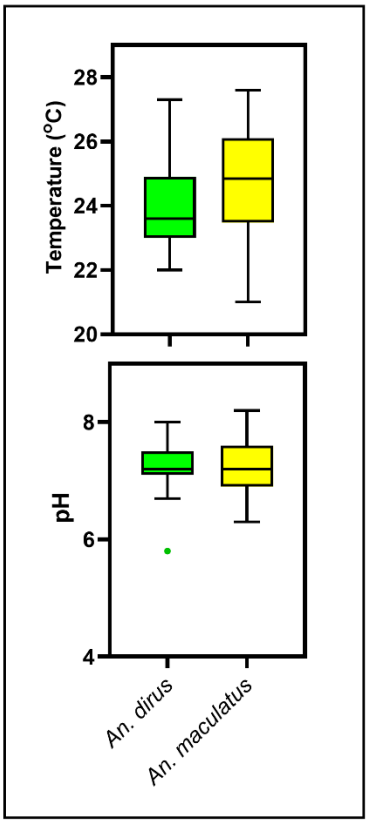

Figure S5.7. Associations between water temperature and pH parameters and the presence of *Anopheles* larvae in aquatic habitats.

Additional file 8

Table S5.1. Definitions of habitat subclass

Subclass	Definition
Class: Natural	
Groundwater pools	Natural water bodies are formed by the accumulation of groundwater or rainwater in surface depressions
Rock holes	Natural depressions in rock surfaces that collect and retain water, often forming due to erosion, weathering or geological processes
Stream margin	Banks of stream where water meets the surrounding land
Tree holes	Natural cavities found in branches of trees
Plant axils	The angles or junctions between a plant's steam and its leaves, where water can accumulate
Class: Man-made from natural materials	
Coconut shells	Man-made openings in coconut shells and water can accumulate
Ditches	Man-made shallow and narrow channels dug into the ground to manage water flow, where the substrate consists of naturally occurring material such as soil, sand or gravel
Fishponds	Man-made water bodies used for fish cultivation, where the substrate consists of naturally occurring material such as soil, sand or gravel
Footprints and hoof prints	Impressions left on the ground by the feet of humans or animals and can collect and retain water
Dug holes	Depressions in the ground created by human activity. In this study, the dug holes were created to accommodate or collect rubber sap before collecting.
Tyre tracks	The impressions left on the ground by the wheels of vehicles that can collect and retain water
Class: Man-made from artificial materials	
Drain	A channel made of concrete or other artificial materials that is designed to remove excess water from an area.
Large containers (>25l)	Container with a capacity greater than 25 liters, made of artificial materials such as plastic, metal, glass or concrete. Example: plastic or metal drums, plastic or concrete water containers.
Small containers (<10L)	Container with a capacity less than 10 liters, made of artificial materials such as plastic, metal, glass or concrete. Example: buckets, jerrycans, plastic sheets, plastic food containers, and pot saucer.
Tyres	Circular, rubber-made structures used on vehicles. On this study, its discarded or improperly stored tyres that can collect and retain rainwater.

Chapter 6

Chapter 5 examined the abundance, spatial distribution and characteristics of anopheline larval habitats (natural as well as man-made from natural or artificial materials) in Ujung Bandar Village, North Sumatra, to assess the potential of LSM to control the vectors of enzootic, zoonotic and human malarias.

This chapter examines sugar feeding as a potential behavioural target for vector control, through ATSBs. Here the sugar feeding habits of *Aedes albopictus* were evaluated to ascertain the potential of ATSBs as a control strategy in the TSI. ATSBs exploit mosquito sugar-feeding habits by delivering an oral toxicant through feeds on a sugar-based bait, a method that effectively killed both male and female mosquitoes in other regions. The success of this intervention depends on the prevalence of sugar feeding in *Ae. albopictus*. Hence, the potential of ATSBs to control *Ae. albopictus* is dependent on its sugar feeding behaviour. Field surveys were conducted on two islands in the TSI to inform the feasibility of ATSBs for controlling *Ae. albopictus* and reducing the risk of its invasion into mainland Australia.

Sugar feeding by *Aedes albopictus* in the Torres Strait, Australia

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Abstract

Background

The establishment of *Aedes albopictus* in the Torres Strait Islands in 2005 is a threat to dengue control in the Islands and on mainland Australia. Attractive targeted sugar baits (ATSBs) have been proposed as a control strategy for outdoor mosquitoes like *Ae. albopictus*. The sugar feeding behaviours of *Ae. albopictus* was studied to ascertain the potential of ATSBs to mitigate the risk of *Ae. albopictus* invading mainland Australia from the Torres Strait Islands.

Methodology/Principal Findings

Aedes albopictus was sampled by sweep net collections in village and bushland habitats across two islands both in the mornings and afternoons. Samples were analysed to determine adult abundance as well as fructose prevalence and content by cold-anthrone test. Sampling stations were characterised by vegetation surveys and included the prevalence of flowers and fruit, and canopy cover. Among the 6,186 captured *Ae. albopictus*, the prevalence of fructose was 31.6% ± 1.4 in males and 30.5% ± 1.2 in females, with fructose averaging 62.9 μg (± 1.4) in fructose-positive *Ae. albopictus*.

Conclusions

Mosquito sex and collection time were associated with the abundance of *Ae. albopictus* as well as fructose prevalence and content in *Ae. albopictus*. Male and female *Ae. albopictus* exhibited sugar abundance and prevalence comparable to studies where ATSBs were effective suggesting that ATSBs could potentially reduce *Ae. albopictus* populations in the Torres Strait Islands.

Author summary

The establishment of *Ae. albopictus* in the Torres Strait Islands poses an ongoing challenge to dengue control in these islands. Additionally, these mosquitoes threaten to spread to mainland Australia. Baseline data on fructose prevalence in *Ae. albopictus* were thereby collected to ascertain the potential of attractive targeted sugar baits (ATSBs) to control *Ae. albopictus* populations and to mitigate both the risk of local dengue transmission and the spread of *Ae. albopictus* to mainland Australia. Using sweep net sampling, 6,186 *Ae. albopictus* mosquitoes were collected on the Hammond and Yorke Islands. Of these, 24-46% of males and 24-32% of females harboured detectable fructose, averaging 62.9 µg fructose per sugar positive mosquito. The abundance, fructose prevalence and fructose content of *Ae. albopictus* differed by mosquito sex and collection times. The fructose prevalence observed were comparable to those in vector populations that were significantly reduced by ATSBs elsewhere.

Introduction

The Torres Strait Islands, between north Queensland, Australia, and Papua New Guinea, were historically inhabited by *Aedes aegypti*. *Aedes albopictus* was first detected in the Torres Straits in 2005 and has since displaced *Ae. aegypti* across the outer islands [348]. Microsatellite loci and mitochondrial COI sequences of *Ae. albopictus* suggest a likely introduction from Indonesia [94].

Aedes albopictus and *Ae. aegypti* are both vectors of dengue, Zika and chikungunya viruses [62, 349]. *Aedes aegypti* is highly anthropophagic, endophagic and endophilic and oviposits in artificial containers [350]. *Aedes albopictus*, on the other hand, is predominantly exophagic, feeds on a wide range of host blood meal sources, is exophilic and oviposits in both natural and artificial containers [21]. Both species are highly invasive, with their migrations facilitated by ovipositing desiccation-resistant eggs in artificial containers that are transported on planes and ships [90]. Vector control is essential to curb transmission of the arboviruses spread by these vectors and to prevent its dispersal [348, 351].

The establishment of *Ae. albopictus* across the Torres Strait Islands directly threatens dengue control on mainland Australia [352]. This invasive species, which arrived naturally in the Torres Strait in 2005 by human-assisted transport [348] was not intentionally introduced as part of any control strategy. Unlike the *Wolbachia*-infected *Ae. aegypti* released in north Queensland to replace the original dengue competent non-*Wolbachia* infected *Ae. aegypti* [353], *Ae. albopictus* represents a natural invasion threat. Its cold tolerance could enable it to expand the geographic range of dengue transmission to include all major capital cities currently free from *Ae. aegypti* [352].

In the Torres Strait Islands, initial efforts to eliminate *Ae. albopictus* using adult and larval control failed [41]. Adult control was based on outdoor fogging which is expensive and requires multiple insecticide applications by a large workforce. Larval control is challenging to implement effectively due to the abundance of artificial and natural habitats, many of which are cryptic and thus difficult to find and treat. Elimination was not achieved and due to high implementation costs and workforces requirements the program was realigned [41]. In 2009, a cordon sanitaire was established on Horn and

Thursday Islands (the major travel hubs in the Torres Strait) with the goal of preventing *Ae. albopictus* dispersing via these travel hubs to become established in Australia [41]. Intensive vector surveillance coupled with adult and larval control established an *Ae. albopictus* free zone in the cordon sanitaire.

In spite of intensive control within the cordon sanitaire, incursions of *Ae. albopictus* continue to be detected [354]. However, implementing control of *Ae. albopictus* in the outer islands might reduce the risk of ongoing incursions into the cordon sanitaire and correspondingly reduce the risk of its spread to mainland Australia. Effective control strategies for *Ae. albopictus* need to be based on the local *Ae. albopictus* biology, and to be simple and easy to deploy in the remote outer Torres Strait Islands where there is minimal infrastructure and capacity [40].

Recently, attractive targeted sugar baits (ATSBs) were proposed to control outdoor mosquitoes, including *Ae. albopictus* [32, 33, 355-357]. Sugar feeding is essential to the survival of both male and female mosquitoes. The ATSB strategy uses a bait with a sugar based lure to attract mosquitoes which are then killed when they ingest a toxicant in the bait. It has been proposed that attractive targeted sugar baits have the potential to be effective where vectors frequently feed on sugar or where natural sugar sources are limited [358]. Trials of ATSBs on *Ae. albopictus* in Israel and the United States demonstrated over 80% reductions in *Ae. albopictus* populations [32, 33, 356, 357].

An initial study on Yorke Island found fructose prevalences of 36% and 28% in *Ae. albopictus* males and females, respectively [359]. The aim of this study was to quantify *Ae. albopictus* abundance and sugar feeding over time on different islands within the Torres Strait. This information is fundamental for understanding the potential of ATSBs to control *Ae. albopictus* across the islands beyond the cordon sanitaire.

Materials and methods

Study site

The study was conducted in 4th – 26th March 2021 and 23rd March – 14th April 2022 in Hammond (10.5586° S, 142.2072° E) and Yorke (9.7510° S, 143.4114° E) Islands in the

Torres Strait, Australia [360]. Hammond Island (traditionally known as Kirriri) is a granite island formed from volcanic rocks located 6 km northwest of Thursday Island. The island is hilly and varies in altitude to 155 m above sea level (asl) [361] with a narrow coastal strip along the east coast where ~250 residents live [362]. The 1,607 ha area is dominated by bushland, including vine forests, coastal habitats, woodlands and paperbark open forests [361].

Yorke Island (traditionally known as Masig) is a coral cay located 160 kms northeast of Thursday Island. The topography of the 479 ha island is flat, between 0-3 m asl [363]. The 251 residents of Yorke Island mainly live on the northeastern coasts [362]. The dominant habitats are beaches, sand flats and isolated fragmented bushland patches of scrub habitat [363]. The annual rainfall from 2019 to 2023 in Hammond and Yorke Islands averaged 1,749.4 mm and 1,036.1 mm, respectively [364].

Field study

The study was conducted during the wet seasons, in March 2021 and April 2022. Mosquitoes were collected from both village and bushland habitats. Village habitats were defined as small residential settlements. The households usually had large yards or gardens near natural habitats or woodlands. The bushland habitats were defined as vast open areas dominated by trees and shrubs, used intermittently by humans. On each island, repeated sampling was conducted at eight fixed stations, with 4 stations in village and 4 stations in bushland habitats. The distance between stations was at least 100 meters. Additionally, stations in bushland habitats were at least 20 meters from roads or open spaces. A minimum 200 meters buffer zone separated village and bushland habitats to minimise mosquito populations mixing between the habitats (S1 Figure.).

The vegetation at each sampling station was surveyed using a 2 m by 20 m belt transect divided into 10 quadrats (2 x 2 m). Surveys of each quadrat was divided into 4 squares (1 x 1 m). In each quadrat, the following parameters were estimated by direct observation: (1) the abundance and composition of plant specimens, (2) the prevalence of flowers, (3) the prevalence of fruit, and (4) canopy cover.

Parameter estimates were made in each square and then totalled for each quadrat. Plant abundance was estimated by direct counts. The prevalence of grasses, small herbs, seedlings, fruits, and flowers was categorised as absent, low, medium or high. Presence was assessed in each square and scored with a value of 0.25 if present or 0 if absent. The highest possible value in a single quadrat (of 4 squares) was 1, with a maximum potential value possible for a belt transect being 10. The values were summed within each transect and categorised as: absent (0), low (1-4), medium (5-7) and high (8-10). Plants were identified to family level and, if possible, to genus or species based on the distinctive morphology of leaves, flowers, and fruits. Photographs and some plant parts were collected to assist with later identifications in the field laboratory. Plants were identified using a range of methods including specimen collection, information from local communities and photographs before confirmation through dichotomous keys, spatial databases (i.e. Atlas of Living Australia) and later consultation with botanists [365, 366]. Canopy cover was estimated by measuring the distance from each belt transect centre to where the canopy cover started and ended in centimeters. The calculation of canopy cover was defined as a percentage of the belt transect and assigned as one of four categories: absent (no canopy cover), low (≤ 50% canopy cover), medium (>50% - ≤ 85%) and high (>85%).

Mosquitoes were collected twice daily by sweep net between 07:00 and 10:00 and again between 15:00 and 18:00 for eight days in each wet season with the order of stations sampled rotated in a balanced design each day. Sweep net sampling was conducted for 10 minutes at each station using a 38-cm-diameter sweep net treated with a 1.0 g/kg esbiothrin, 0.3 g/kg permethrin, 0.2 g/kg imiprothrin (Mortein) [367]. Daily captured mosquitoes from each station were pooled in individual 1 L clear containers labelled with station identifier and collection time.

Laboratory studies

Sample storage

Twice daily, between 07:00 and 10:00 mosquitoes were collected in the morning and again between 15:00 and 18:00 mosquitoes were collected in the afternoon. Immediately following collection, captured mosquitoes were transported to a field

laboratory and identified by morphology to species, sex and abdominal sugar fed status determined by examination using a stereomicroscope at 20x magnification [368]. *Aedes albopictus* were pooled by sex with a maximum of 10 specimens placed in 1.5 ml Eppendorf tubes labelled with collection date and station identifier. Samples were dried in a dry-heat bath at 100˚C for 60 minutes (with the lid open) before storing at room temperature with silica gel [359].

Cold anthrone test

Fructose was quantified using a modified cold anthrone test in 96-well plates (BD Falcon, Germany) [359, 369-371]. Each 96-well plate included field collected specimens, six duplicate standard fructose samples (ranging from 250 to 10 µg in 25% ethanol), a blank of 25% ethanol, two positive controls, and six negative controls. Control mosquitoes were adult *Ae. albopictus* reared from stage IV larvae and pupae collected in the study sites. Negative controls were adults within 24 hr of emergence that were deprived of sugar before heat fixed. Positive controls were emerged adults given access to a 10% fructose solution for 24 hr after emergence and then heat fixed (as above). Up to three *Ae. albopictus* were selected from each station and time point for analysis.

Each mosquito was mixed with 50 µL of 2% sodium sulphate and homogenised by a 3 mm glass bead (Sigma-Aldrich, USA) in Tissue-Lyser II (Qiagen, USA) at 30 Hz for 2 min. Then, 375 µL of chloroform:methanol solution (1:2 ratio) was added, and the mixture was centrifuged for 15 min at 1500 rpm. Next, 50 µL of supernatant from each sample was added to a microplate well. Under a fume hood, the samples evaporated overnight at room temperature. The next day, 200 µl of anthrone solution (containing 28.15% distilled water, 71.7% sulphuric acid and 0.15% anthrone) was added to each well and mixed. The plate was covered with aluminium foil and incubated for 75 min at room temperature. The optical densities (OD) of the samples were then measured using a microplate reader at 630 nm (POLARstar Omega; BMG Labtech, Australia).

Determining presence and amount of fructose in *Ae. albopictus*

Fructose prevalence estimated the proportion of mosquitoes that had recently taken a sugar meal. The amount of fructose in the mosquito's mid-gut indicates the size of sugar

meals. As fructose digestion is rapid, the amount of fructose rapidly changes, and the amount of sugar provides an indication of when sugar feeding occurred. Thus, prevalence is important to facilitate comparisons of sugar feeding rates with other geographic areas. The OD value generated in the cold anthrone test was used to estimate the presence and amount of fructose in *Ae. albopictus* extracts. A mosquito was considered fructose-positive if its absorbance value exceeded the sum of the mean of the absorbance values from the six negative controls on each plate plus three standard deviations (cut-off = x ± 3 SD).

To calculate the amount of fructose (µg) in a positive mosquito, the OD value of the blank was subtracted from the sample absorbance and then divided by the slope of the standard curve [359]. As the extract assayed was 12% of the total extract volume the fructose content of the whole mosquito was calculated by multiplying the µg of fructose in the sample by 8.5.

Statistical analysis

All fieldwork data, including vegetation surveys and mosquito collections were recorded using the Ona platform (http://ona.io). Cold anthrone test results were recorded using MS Excel (Microsoft Crop., USA). All statistical analyses were executed with the R statistical environment (v4.4.1), and all graphs were visualized using GraphPad (v10.2.2). Initial descriptive statistics of the vegetation differences across the two islands were compared with a Chi-squared test for flower and/or fruit presence and a t-test for canopy cover. The rare plant families which were encountered 5 or less times were removed from the dataset. The influence of plant families on the prevalence of flowers and/or fruits was compared with a GLM. To explore the relationship between the proportion of sugar-positive mosquitoes and the presence of flowers and/or fruit by plant family, we performed a correspondence analysis (CA), using the *ca* package in R. The CA method decomposes associations into principal dimensions, allowing visualization of the most influential components in the dataset and was most suitable for the multidimensional binomial dataset.

Principal Component Analysis (PCA) was conducted to explore the multivariate structure of plant composition data in the study. The dataset was centred and scaled to ensure uniformity among variables before PCA was applied to extract principal components explaining the dataset's variance. Principal components were interpreted using loadings to understand relationships between plant families and components, using the *vegan* package in R. The significance of each PC was assessed using eigenvalues and scree plots. Scores of individual observations of significant PCs were used to visualize and interpret patterns of plant composition variation across sampling sites and habitats.

Generalised linear mixed models (GLMMs) were employed to assess the abundance of *Ae*. *albopictus* (negative binomial model), prevalence of fructose (binomial model) and fructose content (µg) per individual mosquito (gaussian model). These analyses were conducted with the *glmmTMB* package in R [338]. Random factors were collection date, sampling stations and study sites, with independent factors being sex, collection time, flower and fruit prevalence, canopy cover and vegetation PC values. A forward regression approach identified the most significant independent variables influencing the dynamics of the dependent variable. Each potential independent variable was evaluated individually to improve model fit, measured by Akaike's Information Criterion (AIC). The comparative strength of evidence for each alternative model was compared using Akaike weights (*w*AIC), with *w*AIC values ranging from 0 (no support) to 1 (complete support), indicating the likelihood of each model being the best predictor of the data [372, 373]. The forward regression process was continued until no additional independent variable significantly decreased the AIC. During the model development process, predictors were examined to minimize potential multicollinearity by ensuring predictors were conceptually distinct and not highly correlated based on exploratory assessments. The use of AIC for model selection provided an objective criterion to balance model fit and parsimony, reducing overfitting and aiding in selecting models that generalize well.

Results

Description of vegetation

Overall, 57 plant families were identified across Hammond (n=43) and Yorke (n=35) Islands (S6.1 Table). The average family richness per station was 12.5 (±1.13, SEM) and 8.5 (±1.18) on Hammond and Yorke Islands. In terms of habitat types, the average family richness was 11.3 (±0.96) in villages and 9.75 (±1.66) in bushland habitats. Fabaceae (or Leguminosae) and Combretaceae were the most abundant plant families on both islands with *Terminalia catappa* the most frequently recorded plant species (JCU Research Data repository).

On Hammond Island, the predominant plant life forms were seedlings (28%), trees (26%) and herbs (24%). Conversely, on Yorke Island, the most common plant life forms were shrubs (26%), followed by seedlings (19%), herbs (16%) and trees (15%). While village habitats were dominated by herbs (38%) and seedlings (12%), bushland habitats exhibited more balance in plant life forms with trees and seedlings commonly found in both habitat types (S6.2 Figure).

Overall, 40% of plant occurrences had some form of flowers (even at a low flowering density) and 19% had fruits, and the presence of any potential sugar source (flowers and/or fruit) was analysed as a single category. During the vegetation survey, the prevalence of either flowers and/or fruits ($\chi^2 = 46.11$, df = 1, $p < 0.001$) was associated with island, with both being more prevalent on Yorke Island (S6.3 Figure). The average canopy coverage was 90% and there was no difference between islands (t = -0.889, df = 14, p-value = 0.3885) (S6.3 Figure). Village habitats had more flowers and/or fruit than bushland habitats ($\chi^2 = 53.99$, df=1, $p < 0.001$).

The presence of flowers and/or fruit varied significantly across plant families ($\chi^2 = 95.97$, df = 13, $p < 0.001$). Certain families had notably higher flower and/or fruit prevalence compared with the reference, being: Fabaceae, Asteraceae, Euphorbiaceae, Phyllanthaceae and Poaceae (S6.4 Figure). This was mostly driven by flower presence.

Aedes albopictus abundance

A total of 6,186 *Ae. albopictus* were captured by sweep net on Hammond and Yorke Islands, with 4,007 (64.8%) being females (Figure 6.1(I)). *Aedes scutellaris* was also collected on both islands (n = 37), but *Ae. aegypti* was not captured. The results from the GLMM showed that *Ae. albopictus* abundance was significantly associated by island, sex, collection time, canopy, PC2 (variation in vegetation gradients), habitat and the presence of flowers and/or fruits (GLMM Table 1; Figure 6.1(I); Figure 6.2(I)). The abundance of male *Ae. albopictus* was consistently less than female abundance across the different islands, habitats, collection times, flower and/or fruit presence, and canopy cover (Figure 6.1(I); Figure 6.2(I)).

Table 6.1. GLMM with forward regression nested model evaluation of best predictors for *Ae. albopictus* abundance, the prevalence of fructose-positive *Ae. albopictus* and fructose quantity (µg). The model comparison was made on the basis of ΔAIC, wAIC, chi-square and *P*-value. The explanatory variables were sex, collection time, island, habitat, flowers, and fruit prevalence.

Model	Variable entered	*df*	AIC	ΔAIC	*w*AIC	*X2*	*P* value
***Aedes albopictus* abundance**							
1	Island	5	5597.3	182.40	0.00	210.22	<0.001
2	Island + Sex	6	5521.1	106.21	0.00	78.21	<0.001
3	Island + Sex + Time	7	5472.9	58.01	0.00	50.22	<0.001
4	Island + Sex + Time + Canopy	8	5447.6	32.77	0.00	27.26	<0.001
5	Island + Sex + Time + Canopy + PC2	9	5422.3	7.45	0.02	27.35	<0.001
6	Island + Sex + Time + Canopy + PC2 + Habitat	10	5417.6	2.80	0.19	6.69	0.009
7	Island + Sex + Time + Canopy + PC2 + Habitat + FlowersFruits	11	5414.7	0.00	0.79	4.83	0.027
Prevalence of fructose							
1	Time	5	1714.0	9.56	0.01	98.31	<0.001
2	Time + Sex	6	1706.7	2.19	0.25	9.39	0.002
3	Time + Sex + PC2	7	1704.4	0.00	0.74	4.22	0.040
Fructose (µg) per *Aedes albopictus*							
1	Time	5	7690.8	32.45	0.00	119.73	<0.001
2	Time + Sex	6	7674.6	16.25	0.00	18.20	<0.001
3	Time + Sex + Island	7	7659.5	1.20	0.35	17.06	<0.001
4	Time + Sex + Island + FlowersFruits	8	7658.3	0.00	0.65	3.21	0.073

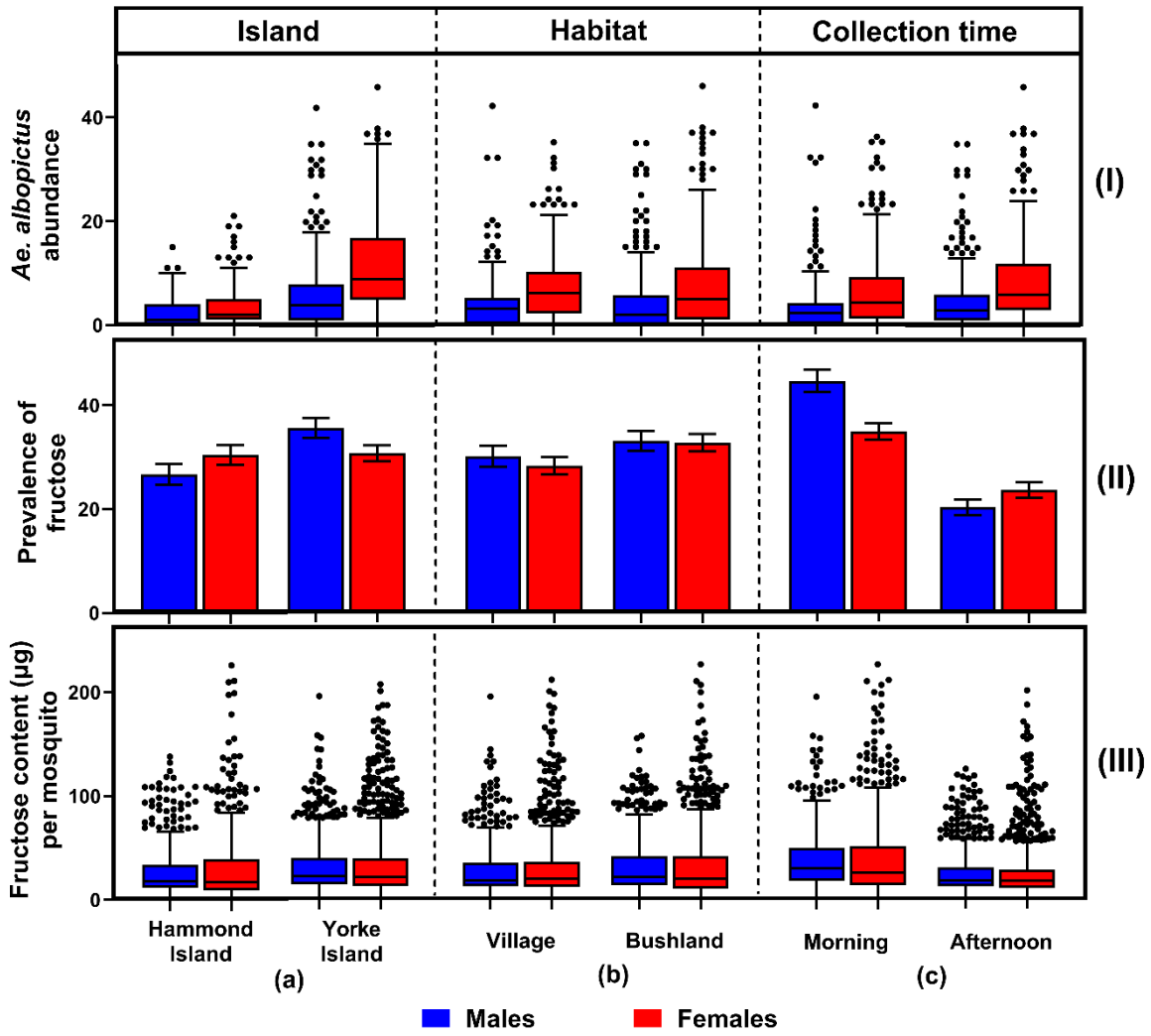

Figure 6.1. (I) *Aedes albopictus* abundance, (II) prevalence of fructose, and (III) fructose content (µg) per *Ae. albopictus* by (a) island, (b) habitat, and (c) collection time.

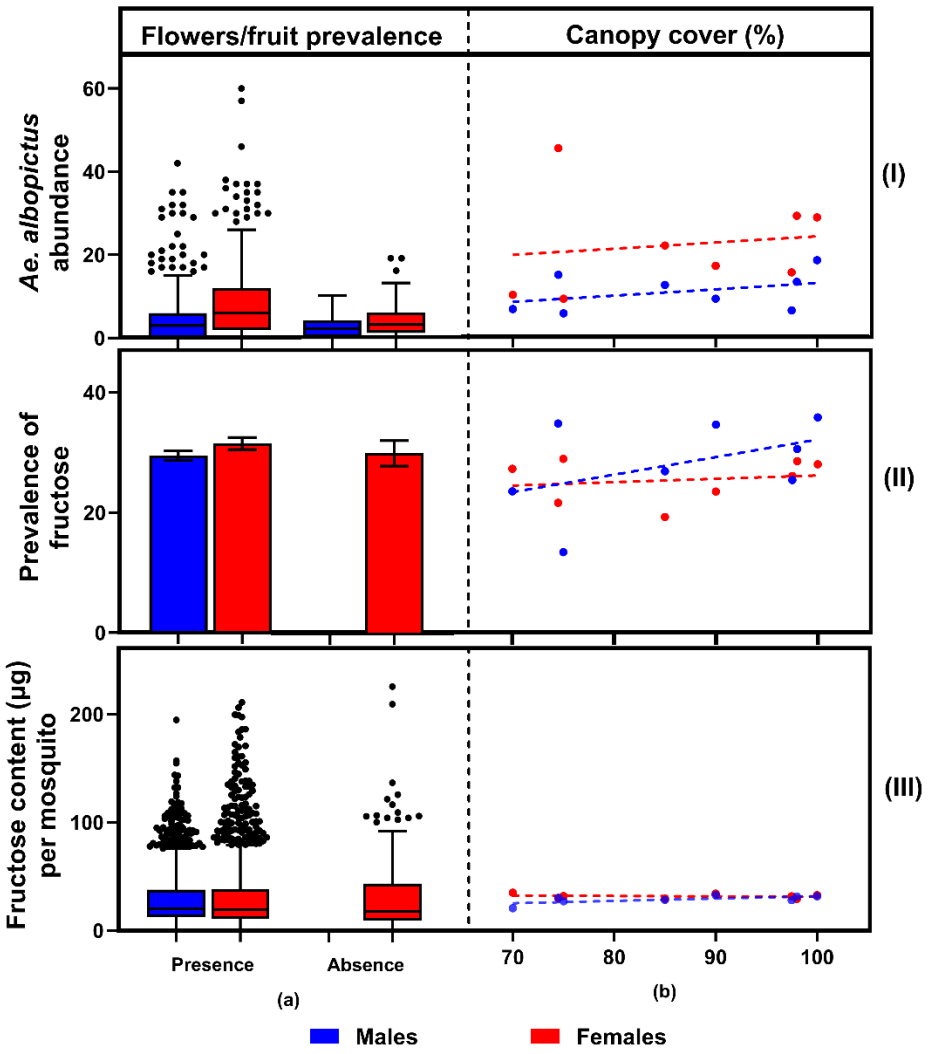

Figure 6.2. (I) *Aedes albopictus* abundance, (II) prevalence of fructose, and (III) fructose content (µg) per *Ae. albopictus* by (a) flowers/fruit prevalence and (b) canopy cover (%).

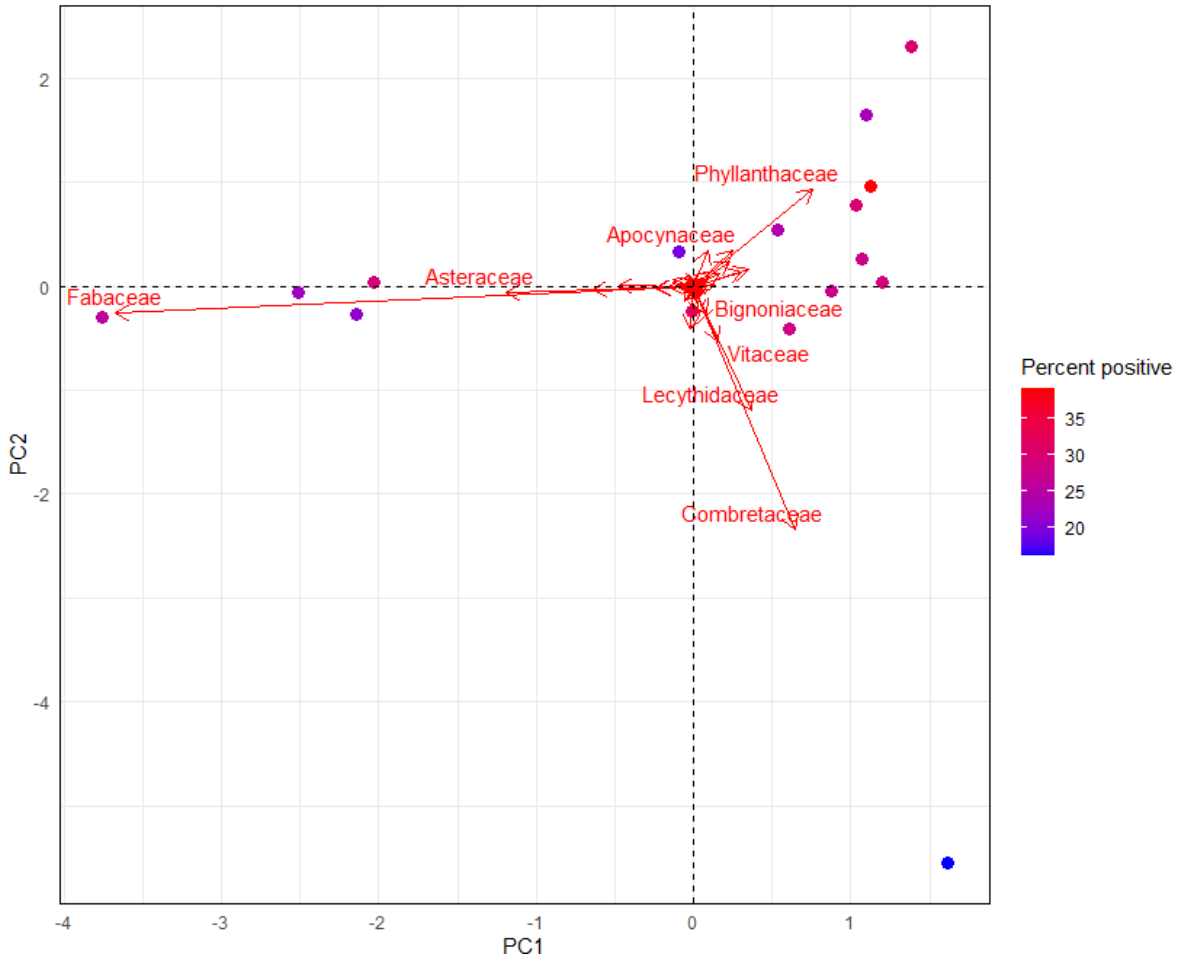

Figure 6.3. Principal component analysis (PCA) of plant family composition variation across sampling sites (islands) and habitats.

Prevalence of fructose

Overall, 2,641 *Ae. albopictus* were tested for fructose. The prevalence of fructose was significantly associated by collection time, sex and PC2 (Table 6.1; Figure 6.1(II)). Fructose positive male *Ae. albopictus* were more prevalent overall than female fructose positives (31.6% ± 1.4 in males and 30.5% ± 1.2 in females; GLMM, *P*=0.002, Figure 6.1(II) and Figure 6.2(II)). Fructose positive *Ae. albopictus* were almost two times more prevalent in the morning than in the afternoon (42.0% ± 1.9 in morning and 22.0% ± 1.9 in afternoon; GLMM, *P* <0.001) (Table 6.1; Figure 6.1 (II)). Principal Component 2, derived from the PCA of plant composition data, exerted a notable influence on fructose prevalence, suggesting that specific combinations of plant species were correlated with variations in fructose positivity. The plant family Phyllanthaceae was strongly associated with PC2, suggesting plant communities dominated by this family were correlated with an increased prevalence of fructose positive *Ae. albopictus* (Figure 6.3, loading PC1: 0.7569, PC2: 0.9312). As noted above, Phyllanthaceae was one of the families that had significantly higher flower and/or fruit presence.

The correspondence analysis identified distinct relationships between the proportion of sugar-positive mosquitoes and the presence of flowers by plant family across the study sites. The first two principal dimensions accounted for a cumulative inertia of 52.5% (28.2% for Dimension 1 and 24.3% for Dimension 2). Dimension 1 likely represents broad patterns of differentiation among plant families, while Dimension 2 captures more specific associations within these broad patterns. Among plant families, significant associations with the principal dimensions were observed. For example, the Phyllanthaceae family exhibited the highest contribution to Dimension 1 ("ctr"[1]: 768), driven by its widespread presence and association with sugar-positive mosquitoes (S6.5 Figure).

[1] In Correspondence Analysis, ctr (contribution) indicates the proportion of a point's influence in defining a given dimension, with higher values reflecting greater importance to that dimension's construction.

Fructose (µg) per *Aedes albopictus*

The average fructose-positive *Ae. albopictus* had 62.9 µg (± 1.4) fructose, with an average cut-off value of 15.9 µg (± 0.3). The amount of fructose per mosquito was significantly associated by sex, collection time, and island; with the addition of flower and/or fruit presence almost significant (Table 6.1; Figure 6.1(II)). Overall, females took larger fructose meals than males (31.2 µg ± 0.9 in females; 29.4 µg ± 0.8 in males; GLMM, P <0.001). The average fructose amount in *Ae. albopictus* collected in the morning was almost 60% more than that found in the afternoon (38.48 µg ± 1.1 in the morning and 24 µg ± 0.7 in the afternoon; GLMM, P <0.001). The amount of fructose found in *Ae. albopictus* on Yorke Island was higher than on Hammond Island (31.5 µg ± 0.8 in Yorke Island; 29.1 µg ± 1.0 in Hammond Island, GLMM, P =0.021).

Discussion

The distribution of *Aedes* in the Torres Strait Islands changed over the past three decades. Following dengue control efforts, including residual spraying in domestic surface containers and the removal of rainwater tanks and wells in 1997, *Ae. aegypti* was displaced by *Ae. scutellaris* [348, 374]. When *Ae. albopictus* was introduced from Indonesia in 2005, the distribution and abundance of both *Ae. scutellaris* and *Ae. aegypti* diminished while *Ae. albopictus* became the predominant *Aedes* species across the Torres Strait Islands [41, 375].

In this study, male *Ae. albopictus* were less frequently collected than females which can be attributed to the collection method as well as behavioural and lifespan differences. The sweep net technique indirectly uses the collector body as bait to attract blood meal seeking female mosquitoes and collaterally captures males seeking females for mating [376]. In general, the survivorship of adult males is shorter than females, which reduces male abundance and therefore lowers the ratio of male to female mosquitoes [377].

Sex and collection time were the strongest factors influencing both the amount and prevalence of fructose in *Ae. albopictus* in the Torres Strait Islands. The prevalence of fructose in both sexes was highest in the morning, indicating that morning may be the

preferred sugar-feeding time for *Ae. albopictus*. This aligns with known mosquito behaviour, as sugar feeding often occurs during periods of reduced activity when energy demands are lower. Female *Ae. albopictus* exhibited a lower prevalence of fructose compared to males; a pattern consistent with other mosquito species [358, 369, 378, 379]. While both sexes sugar feed for energy, females also obtain energy from blood meals, reducing their dependence of sugar feeding [369, 380, 381]. Moreover, a fructose meal is rapidly digested in a mosquito's abdomen, being only detectable for 48 hours after feeding for *Ae. albopictus* and *Ae. aegypti*, depending on factors such as environmental conditions, sugar concentration, and mosquito metabolic rates . As such, the cold anthrone test detects only recent sugar meals. Females, due to their larger body size and the energy requirements for reproduction, consumed larger sugar meals when they sugar fed. Larger sugar meals could result in fructose being detectable for a longer duration in females than in males.

Plant communities that were dominated by species from the Phyllanthaceae family were positively correlated with higher abundance and fructose prevalence of *Aedes albopictus*. During the surveys, the Phyllanthaceae family was one of the families with a higher occurrence of flowers and/or fruit. This plant family was only present on Hammond Island, mostly in bushland areas and comprised three species: *Cleistanthus* sp. (most common), *Glochidion* sp., and *Bridelia* sp. The *Cleistanthus* sp. produces small and often inconspicuous flowers and fruits. While the flowers aren't conspicuous and may not produce abundant nectar, there are some limited reports that the flowers are still attractive to small insects. The positive correlation between fructose prevalence and PC2, as opposed to the overall presence of flowers and/or fruit in the environment indicates that the composition of the vegetation structure does have importance. During the vegetation surveys, fruits were infrequently observed while flowers were present at all but one station, albeit often in low densities. This suggests that the overall measure of flower and/or fruit presence is not nuanced enough to represent the cues that the insect population are likely responding to. The correspondence analysis (ca) highlights meaningful ecological relationships between mosquito sugar-feeding behaviors and local floral resources. These results underscore the need to investigate the role of specific plant families in shaping vector ecology and disease transmission risk.

Experimental trials suggest that ATSBs are a promising approach for controlling outdoor mosquitoes. In a trial conducted in Haifa, Israel, where fructose prevalence prior to ATSB deployment was 68% and 63% in male and female *Ae. albopictus*, *Aedes albopictus* abundance was reduced by 84% in mosquitoes sampled by human landing catches following implementation of ATSBs [356]. Similarly, following deployment of ATSBs in a desert oasis where 72% of *Anopheles sergentii* were positive for sugar, *An. sergentii* populations were reduced by 97.5% [382].

Defining the level of sugar in the environment is complex and authors have used different methods, including estimating the percentage of flowering vegetation or a measure of landscape greenness. Here the percentage of flowering vegetation ranged widely across the sample stations and an overall average of 40% of vegetation occurrences having flowers. An ATSB treatment trial in Bamako, Mali effectively reduced *Ae. aegypti* abundance by approximately 95% in both sugar-rich (50% flowering vegetation) and sugar-poor (<5% flowering vegetation) environments [358], showing that ATSBs were effective in environments within a wide range of natural sugar sources. In Mali, this fructose prevalence was similar to what was found in *Ae albopictus* in this study (24-46% in males and 24-32% in females) and a previous study on Yorke Island (35% in males and 28% in females) [359]. This comparable fructose prevalence seen in Mali and in the Torres Strait Islands suggests that ATSB trials would be warranted to determine their potential to reduce the risk of *Ae. albopictus* mosquitoes being introduced into the cordon sanitaire. This, in turn, would lower the chances of *Ae. albopictus* spreading to mainland Australia via transport hubs on Horn and Thursday Islands.

Conclusion

Understanding the sugar feeding behaviour of *Aedes albopictus* provides fundamental information for assessing the potential of ATSB applications to control this outdoor mosquito. Mosquito sex and collection time were parameters associated with the abundance of *Ae. albopictus*, the fructose prevalence and the amount of fructose in *Ae. albopictus*. Male and female *Ae. albopictus* harboured different sugar amounts and the prevalences of sugar positives by sex varied by time of day. The comparable fructose

prevalence seen in Mali and in the Torres Strait Islands suggests that ATSBs trials would be warranted to determine their potential to both control *Ae. albopictus* and in so doing reduce the risk of *Ae. albopictus* mosquitoes escaping from the cordon sanitaire to the mainland of Australia.

Supporting information

S6.1 Table. List of plant families at each station on Hammond and Yorke Islands. "1" denotes present and "0" denotes absent during the survey.

S6.1 Figure. Distribution of stations on Torres Strait Island. (a) Hammond and (b) Yorke Islands. The basemap was created with Esri World Imagery (WGS84).

S6.2 Figure. Vegetation life-forms. (a) Hammond Island; (b) Yorke Island; (c) Village habitat; (d) Bushland habitat.

S6.3 Figure. Prevalence of flowers and/or fruit, and percentage of canopy cover by islands and habitat types.

S6.4 Figure. Prevalence of flowers and/or fruit by common plant families.

S6.5 Figure. Correspondence Analysis (CA) plot illustrating the relationship between plant families and their presence in flower and fruit occurrence. The analysis identifies patterns of association between locations and plant families based on flower and fruit presence, providing insights into the floristic composition and reproductive traits across the surveyed areas.

Declarations

Acknowledgments

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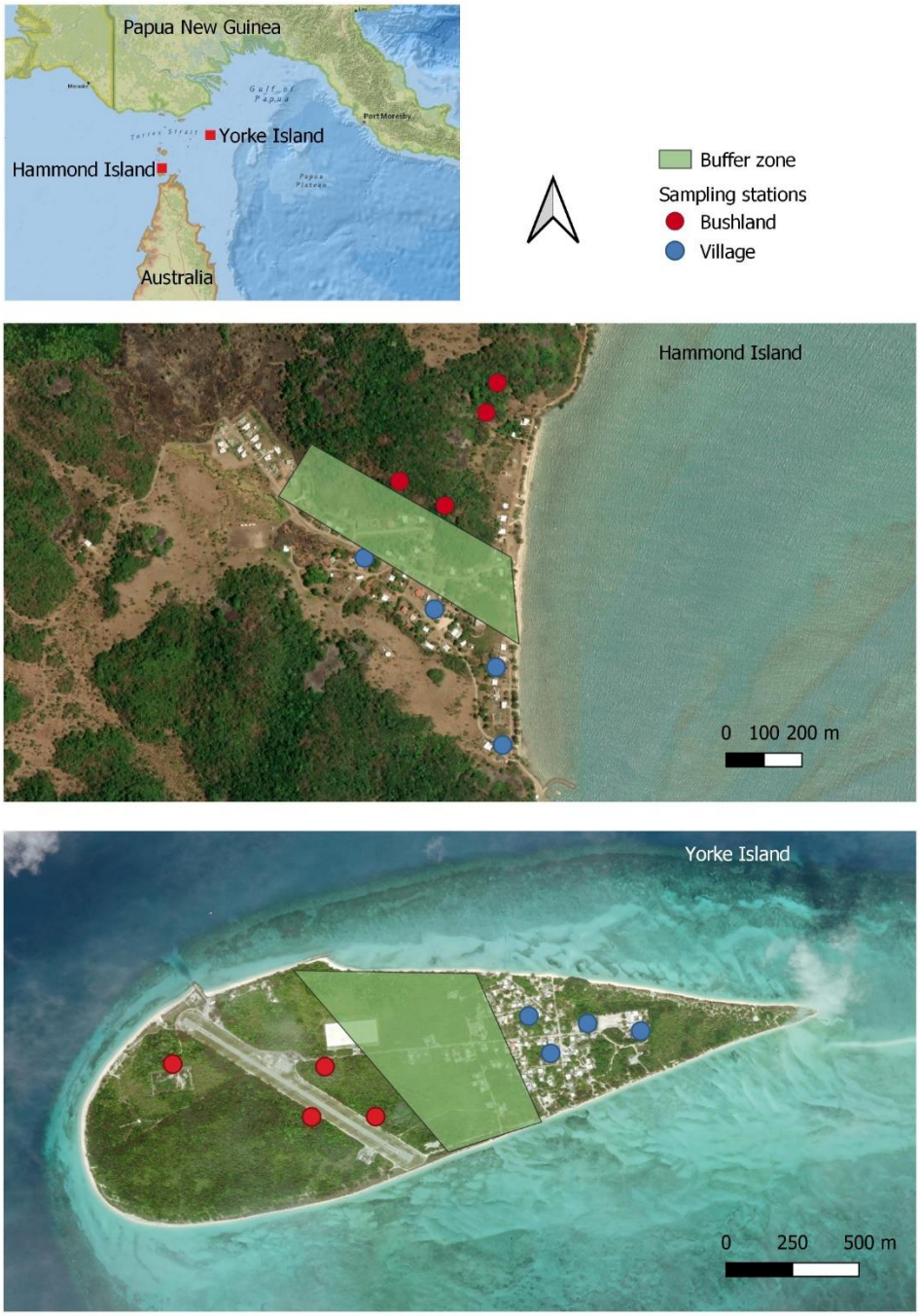
Data Availability Statement:

The dataset supporting the conclusions of this article are available in the JCU Research Data repository (https://doi.org/10.25903/bs5k-f510).

Supporting Information

S6.1 Table. List of plant families at each station on Hammond and Yorke Islands. "1" denotes present and "0" denotes absent during the survey.

No.	Plant family	Hammond	Yorke
1	Amaryllidaceae	1	0
2	Anacardiaceae	1	1
3	Apocynaceae	1	1
4	Araceae	1	1
5	Arecaceae	1	1
6	Aristolochiaceae	0	1
7	Asparagaceae	0	1
8	Asteraceae	1	1
9	Bignoniaceae	1	1
10	Burseraceae	1	0
11	Caesalpiniaceae	1	0
12	Capparaceae	1	0
13	Casuarinaceae	0	1
14	Chrysobalanaceae	1	0
15	Colchicaceae	0	1
16	Combretaceae	1	1
17	Commelinaceae	1	1
18	Convolvulaceae	0	1
19	Crassulaceae	0	1
20	Dilleniaceae	0	1
21	Dioscoreaceae	1	0
22	Elaeocarpaceae	1	0
23	Euphorbiaceae	1	1
24	Fabaceae	1	1
25	Flagellariaceae	1	1
26	Lamiaceae	1	1
27	Lauraceae	1	0
28	Lecythidaceae	1	0
29	Loranthaceae	1	0
30	Malvaceae	1	1
31	Menispermaceae	1	0
32	Mimosaceae	0	1
33	Moraceae	1	0
34	Musaceae	1	0
35	Myristicaceae	1	0
36	Myrtaceae	1	1
37	Oleaceae	1	0
38	Pandanaceae	1	1
39	Passifloraceae	0	1
40	Phyllanthaceae	1	0
41	Pittosporaceae	0	1
42	Poaceae	1	1
43	Polygalaceae	1	0
44	Polypodiaceae	0	1
45	Portulaceae	1	0
46	Putranjivaceae	0	1
47	Rhamnaceae	0	1
48	Rhizophoraceae	1	0
49	Rubiaceae	1	1
50	Rutaceae	1	1
51	Sapindaceae	1	0
52	Sapotaceae	1	1
53	Smilacaceae	1	1
54	Taccaceae	0	1
55	Turneraceae	1	0
56	Verbenaceae	1	1
57	Vitaceae	1	0
Grand Total (df = 1)		**43**	**35**



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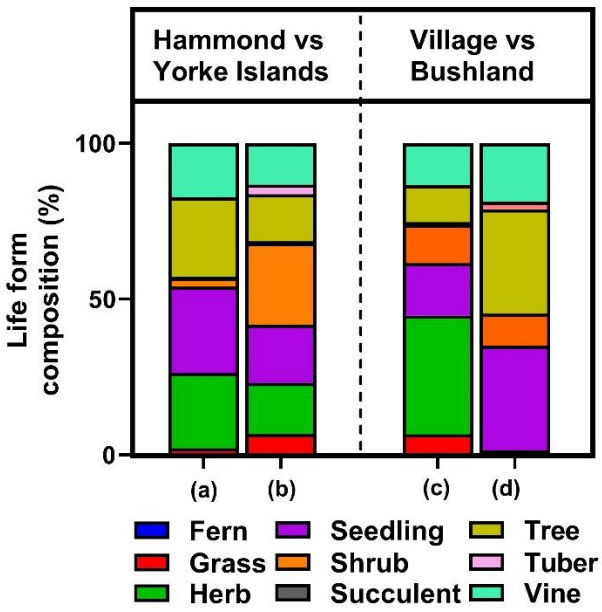

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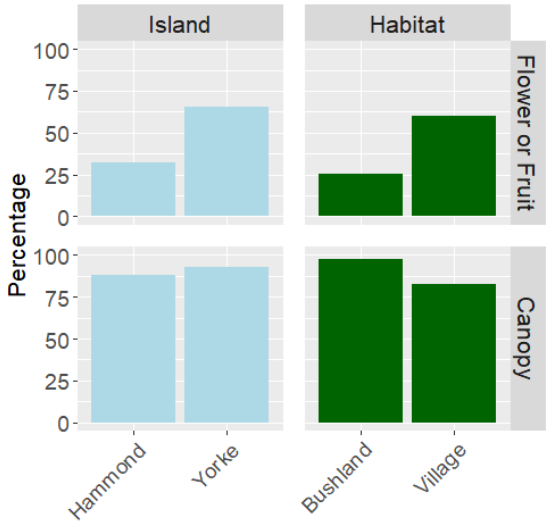

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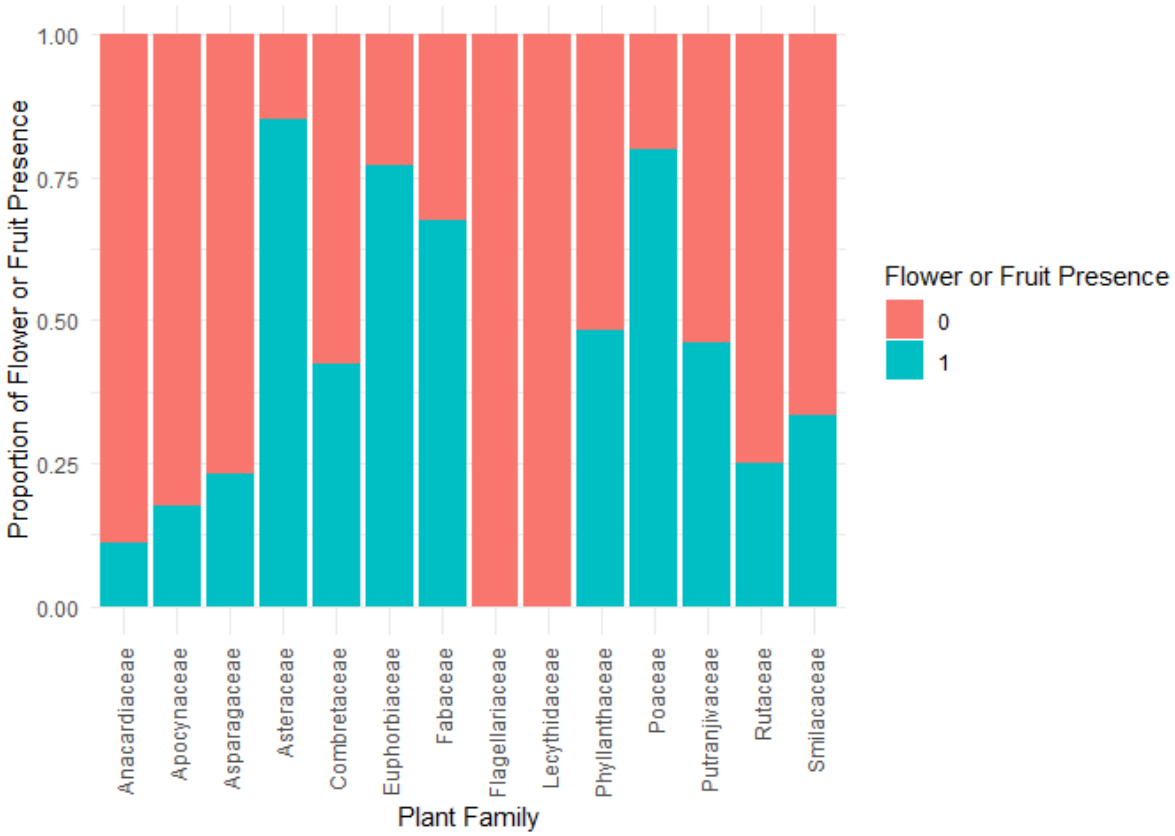
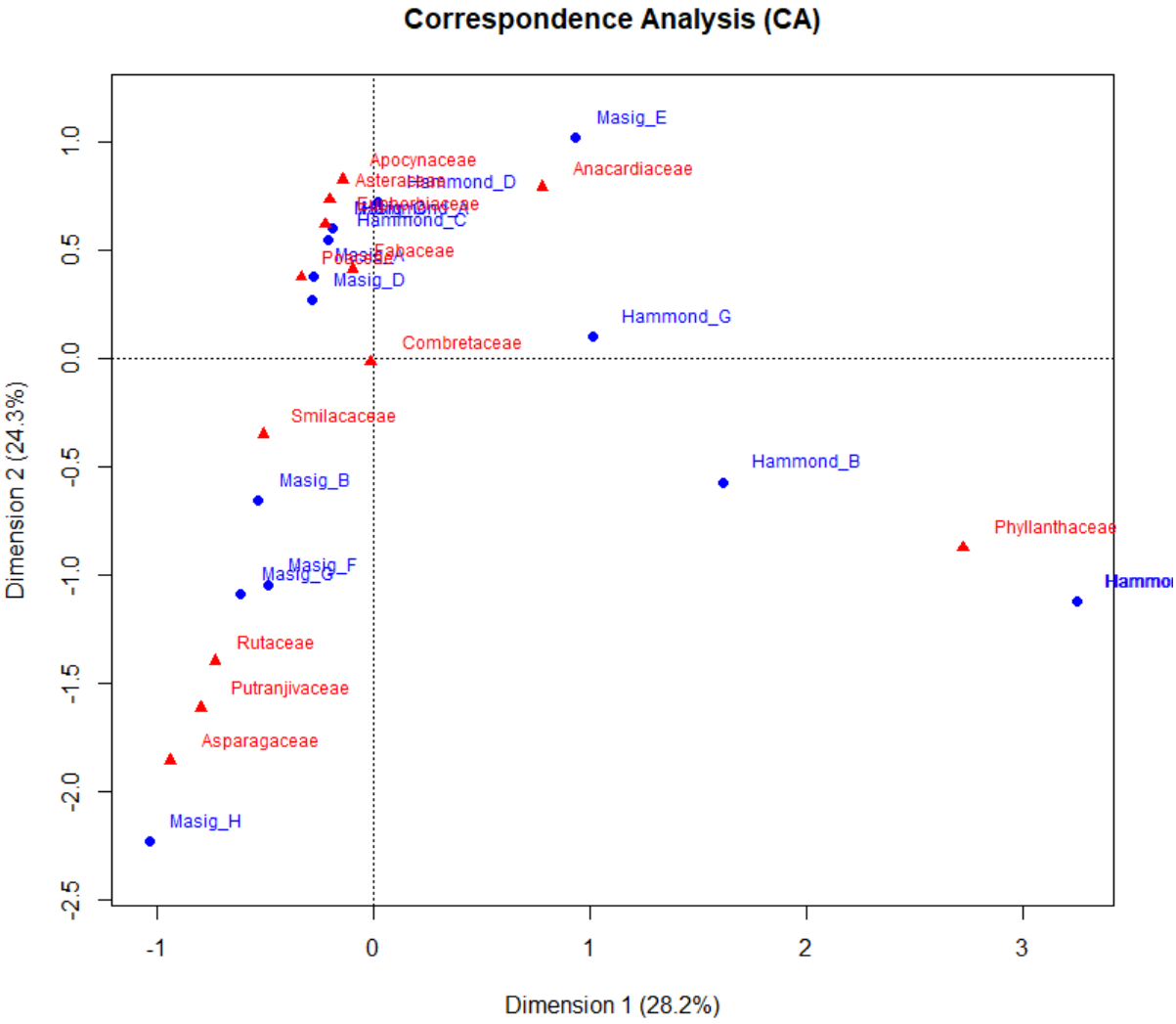
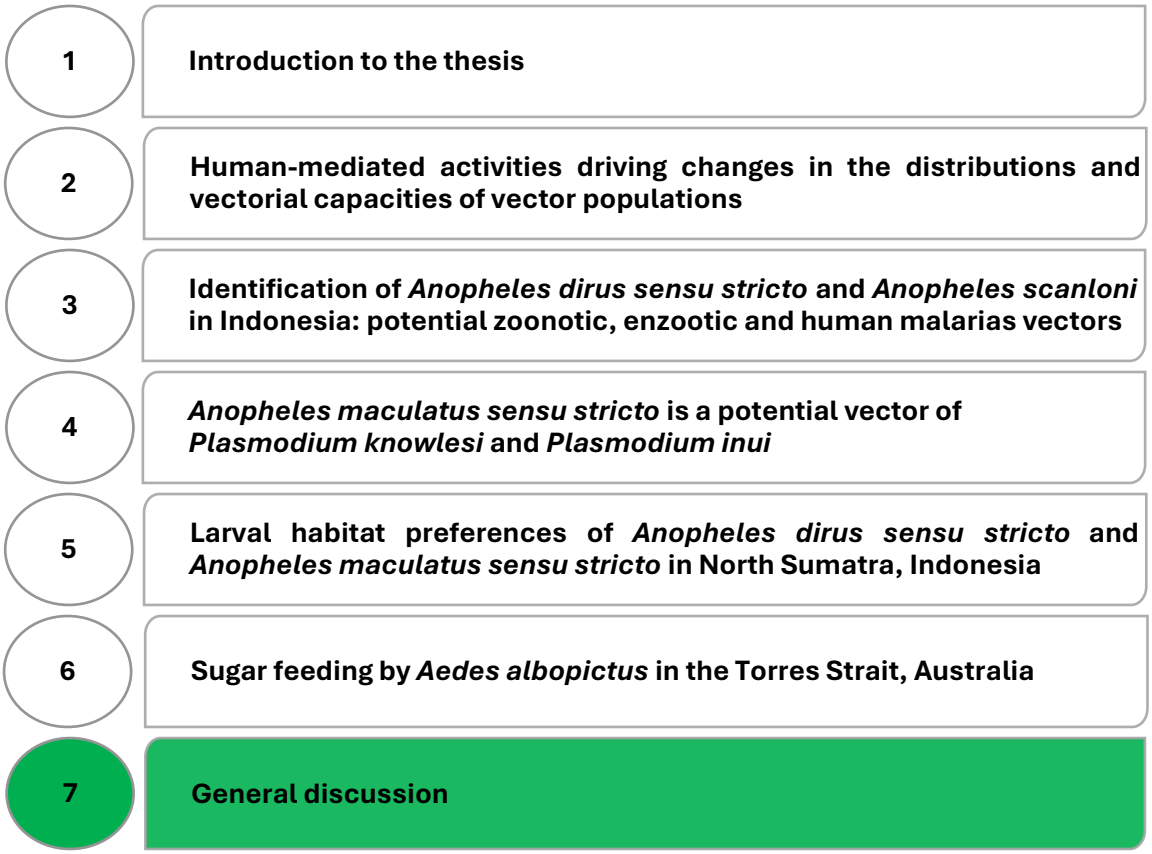


S6.4 Figure. Prevalence of flowers and/or fruit by common plant families.



S6.5 Figure. Correspondence Analysis (CA) plot illustrating the relationship between plant families and their presence in flower and fruit occurrence. The analysis identifies patterns of association between locations and plant families based on flower and fruit presence, providing insights into the floristic composition and reproductive traits across the surveyed areas.

Chapter 7



This thesis addressed two foundational pillars of vector-borne disease control: vector incrimination and the associated behaviours of vectors. These pillars underpin the selection and deployment of context-specific effective control strategies. In this thesis, the vectors of *P. knowlesi* transmission in North Sumatra, Indonesia, and *Ae. albopictus*, as a dengue vector, in the Torres Strait, Australia were detailed in case studies.

This research is situated within the broader context of the Anthropocene, a period marked by the profound impact of human activity on ecosystems and disease dynamics. The literature review presented in this thesis (chapter 2) highlights how anthropogenic forces such as urbanisation, deforestation, agricultural intensification, climate change, and global mobility are reshaping vector ecology. This Anthropocene context frames the complex and evolving relationships between mosquitoes and their environments being shaped by human activities. It also highlights the limitations of conventional, static assumptions about vector ecology and underscores the urgency of developing adaptive,

evidence-driven responses to emerging vector-borne disease risks. Within this shifting context, localised vector incrimination and behavioural profiling are essential to understand and respond to novel transmission ecologies.

Vector incrimination: a critical foundation for control

Establishing the mosquito species responsible for pathogen transmission remains an essential, yet often overlooked, first step in designing control programs. Central to this effort is the precise identification of mosquito species involved in disease transmission. Distinguishing vectors from non-vector species enables targeting vector populations for control [383]. Traditional assumptions in malaria control tend to focus on a narrow set of well-known vectors and rely heavily on historical incrimination data. This approach is increasingly inadequate in regions where zoonotic malaria, ecological change, or anthropogenic disturbance alter transmission dynamics with increased outdoor-biting mosquitoes being responsible for increasing and novel means of transmission.

In North Sumatra, this thesis presents evidence that *P. knowlesi* malaria in humans may be associated with the distributions of *An. dirus s.s.* and *An. maculatus s.s.* (chapters 4 & 5), a finding that contrasts with dominant vector narratives in Indonesia, which have historically focused on human malaria transmission and more recently with transmission of zoonotic malaria by species within the Leucosphyrus Group. These observations demonstrate the empirical value of localised vector surveillance and suggest that knowledge of incriminated vector distributions must be updated regularly. This has significant implications for how emerging zoonotic risks are monitored and managed in Indonesia and similar ecotonal regions.

In the Torres Strait, vector incrimination was initially critical for identifying and responding to the early incursion of *Ae. albopictus*. The species was first detected in 2005 during routine surveillance and rapidly established itself across almost all islands in the Torres Strait [348]. Drawing on published data, early incrimination provided the necessary incentive for implementing first an elimination strategy following by containment and biosecurity efforts. The importance of entomological surveillance

systems capable of detecting invasive species and enabling timely responses is underscored by this case. The incursion of *Ae. albopictus* reinforces the necessity of responsive and ongoing surveillance [40, 354] to prevent its expanding its range to mainland Australia.

Theoretically, these findings challenge the longstanding assumption that vector incrimination is a resolved problem in most regions. Instead, they support an emerging view in vector ecology that incrimination must be continuous and surveillance of vectors responsive to environmental change, species invasions, and zoonotic spillover.

Targeting vector behaviour: designing interventions around vulnerabilities

Understanding mosquito behaviours—particularly biting and resting patterns, host preferences, and larval habitats—is critical for developing targeted interventions. Noting that much of the global malaria vector control strategy continues to rely on insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS), which assume indoor, late-night biting and resting by vectors.

Research from both Indonesia and Australia that the dominant vectors in these settings engage in outdoor and early-evening biting suggests that the traditional WHO recommended control tools have limited effectiveness. In parallel studies in North Sumatra, *Anopheles* spp. were observed biting outdoors and earlier in the evening, raising questions about the suitability of ITNs and IRS in zoonotic malaria zones. In the Torres Strait, *Ae. albopictus* exhibited strong exophilic and exophagic behaviours, thriving in peri-domestic environments and artificial containers. These findings add to a growing literature questioning the one-size-fits-all application of control tools designed for specific species in Africa and Southeast Asia. Highlighting the need for behavioural surveillance as a routine component of vector control planning.

Chapters 5 and 6 of this thesis provide further data on vector behaviours, with particular attention to spatial and temporal patterns on larval habitat use and sugar feeding behaviour. Chapter 5 highlighted the *Anopheles* larval habitats used by *An. dirus s.s.* and

An. maculatus s.s., particularly noting the ability of these species to exploit habitats made by humans from artificial materials and by modifying natural substrates which suggests an ecological plasticity and adaptability to human-modified environments. In chapter 6, improved knowledge on sugar feeding in *Ae. albopictus* lays the foundation for the potential of attractive targeted sugar baits as a vector control tool in the TSI.

Control programmes must now grapple with the specific challenge of outdoor-biting mosquitoes that routinely evade the reach of traditional tools like ITNs and IRS. Behavioural vulnerability must become a central organising principle in control programme design. This requires new tools (e.g. spatial repellents, attractive targeted sugar baits) and new paradigms, including community-based environmental management and responsive urban planning. Such approaches have often been underutilised or underfunded due to the entrenched focus on commodity-based interventions.

This research also highlights the potential of mosquito bionomics as a tool for spatial risk assessment. By understanding the specific ecological preferences and behaviours of vectors—such as biting times, larval habitats, and host-seeking strategies—public health practitioners can identify high-risk areas and periods of transmission more accurately. This allows for better targeting of resources and interventions, particularly in settings where control efforts must contend with outdoor-biting vectors or fragmented ecologies. Integrating bionomic data into spatial decision-support systems can enhance anticipatory governance and enable more efficient, risk-based approaches to vector surveillance and control.

Institutional and governance considerations

While vector biology defines the technical parameters of control, the implementation of those interventions depends on the broader systems in which public health operates.

In North Sumatra, decentralisation and limited vertical integration of health services have contributed to fragmented malaria control responses. However, the entomological findings presented in this thesis, particularly around vector habitat preferences, suggest

a vulnerability for targeted prioritisation of a control strategy with the potential to control outdoor vectors. Local evidence on larval habitats can inform LSM efforts and community-level interventions, even within a constrained system. Strengthening technical capacity and enhancing collaboration between health, forestry, and agriculture sectors could amplify the utility of these findings.

In the Torres Strait, while institutional capacity is comparatively high, human resource implementation costs and the adaptability of *Ae. albopictus* underscores the logistical and ecological challenges inherent to vector control in this remote setting. The islands are difficult and costly to access, complicating timely responses and routine surveillance. Moreover, the exophilic and exophagic behaviours of *Ae. albopictus* reduce the efficacy of traditional indoor-based tools and necessitates approaches such as the use of ATSBs.

These findings align with broader observations in global health about the difficulties of translating evidence into action. They suggest that improving vector control requires attention to the practical challenges that health workers and programme managers face. Better tools, clearer accountability, and stronger local systems are key to making sure evidence leads to action, followed by results.

Cross-case insights: common challenges and divergent realities

Comparing the two case studies reveals both shared challenges and context-specific dynamics. Both regions are characterised by high ecological connectivity—whether through zoonotic interfaces or inter-island transport networks—that complicate vector control efforts. In both cases, the dominant vectors are outdoor biters on which standard interventions will only have a limited impact.

But the policy and health system situations are very different. In Indonesia, human malaria is diminishing, while reports of zoonotic malaria are increasing. However, given the evolving nature of surveillance systems, it remains unclear whether this reflects a true rise in incidence or improved detection. In contrast, the Torres Straits, in Australia,

have a unique *cordon sanitaire* that focuses on preventing the spread of *Ae. albopictus* to the mainland using strategies based on vector behavioural vulnerabilities to achieve a status quo to prevent the further spread of *Ae. albopictus* but unable to push back the threat of geographic expansion of the vector. This region also remains malaria-receptive due to the presence of competent *Anopheles* vectors and its proximity to Papua New Guinea. The deployment of ATSBs in the Torres Strait could offer dual benefits: complementing *Ae. albopictus* control and targeting the sugar-feeding behaviour of exophilic and exophagic *Anopheles* species. Such approaches could strengthen efforts to prevent malaria re-establishment by addressing residual outdoor transmission.

Importantly, both case studies illustrate that vector identification, incrimination and behavioural monitoring can open critical windows for interventions. These findings support a growing recognition that surveillance is not simply a preparatory step, but a strategic intervention in its own right.

Limitations and future directions

While this research offers important insights, it is not without its limitations. The fieldwork was time-bound (including by the COVID-19 pandemic which prohibited establishing field work in Sumatra for several years) and was geographically limited, which constrains generalisability. Entomological data relied on specific collection techniques that may under-represent certain behaviours or species.

Future research should prioritise longitudinal studies that capture changing landscapes and the ecological plasticity of outdoor-biting mosquito species, particularly in response to anthropogenic environmental changes. Each vector species exhibits distinct behavioural traits, and even within a single species, these behaviours may vary across land use types. Understanding variations in behavioural behaviours is essential for designing locally tailored and adaptive control strategies that can address shifting vector dynamics. Further research should also explore the role of ecological connectivity in shaping transmission risk—recognising, for instance, how landscape changes or habitat fragmentation may link vector populations, hosts, and human settlements. For example,

there is a need for further investigation into the potential vectors of *P. knowlesi* on Sumatra Island, given the widespread distribution of its natural hosts, long-tailed and pig-tailed macaques, throughout the region [227].

Additional research is warranted to optimise and evaluate the field application of alternative vector control tools. While this thesis demonstrates the potential of LSM and ATSBs, operational research is needed to assess their efficacy, cost-effectiveness, and community acceptability in diverse ecological and socio-economic settings [155, 324, 384]. Ultimately, interdisciplinary research integrating entomology, molecular biology, ecology, and public health will be essential for developing sustainable and responsive vector control frameworks in the face of ongoing environmental change.

Conclusion

This thesis underscores that effective vector control begins with identifying and incriminating the vectors and understanding how they behave. Without this knowledge, control programmes risk being ineffective, misaligned, or unsustainable. Yet, entomological insights alone are not sufficient. The translation of evidence into practice depends on the strength of governance systems, institutional coordination, and community involvement.

The case studies of North Sumatra and the Torres Strait demonstrate that outdoor-biting vectors pose specific challenges that cannot be addressed with conventional tools. They also highlight the value of early incrimination and behavioural surveillance in shaping timely and context-appropriate responses.

This research contributes to a more realistic, context-aware, and ultimately a more effective approach to managing vectors and vector-borne disease. It supports a renewed commitment to local evidence, institutional resilience, and adaptive management—foundational pillars for sustainable vector control in a world marked by increasing complexity and uncertainty.

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Appendix

Published outputs of the thesis

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Sugar feeding by *Aedes albopictus* in the Torres Strait, Australia

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Abstract

Background

The establishment of *Aedes albopictus* in the Torres Strait Islands in 2005 is a threat to dengue control in the islands and on mainland Australia. Attractive targeted sugar baits (ATSBs) have been proposed as a control strategy for outdoor mosquitoes like *Ae. albopictus*. The sugar feeding behaviours of *Ae. albopictus* was studied to ascertain the potential of ATSBs to mitigate the risk of *Ae. albopictus* invading mainland Australia from the Torres Strait Islands.

Methodology/Principal Findings

Aedes albopictus was sampled by sweep net collections in village and bushland habitats across two islands both in the mornings and afternoons. Samples were analysed to determine adult abundance as well as fructose prevalence and content by cold-anthrone test. Sampling stations were characterised by vegetation surveys and included the prevalence of flowers and fruit, and canopy cover. Among the 6,186 captured *Ae. albopictus*, the prevalence of fructose was 31.6% ± 1.4 in males and 30.5% ± 1.2 in females, with fructose averaging 62.9 μg (± 1.4) in fructose-positive *Ae. albopictus*.

Conclusions

Mosquito sex and collection time were associated with the abundance of *Ae. albopictus* as well as fructose prevalence and content in *Ae. albopictus*. Male and female *Ae. albopictus* exhibited sugar abundance and prevalence comparable to studies where ATSBs were effective suggesting that ATSBs could potentially reduce *Ae. albopictus* populations in the Torres Strait Islands.