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Mycobacterium ulcerans in Mosquitoes and March flies captured from endemic areas of Northern Queensland, Australia

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Abstract:

Mycobacterium ulcerans is the causative agent of Buruli ulcer (BU). This nontuberculous mycobacterial infection has been reported in over 33 countries worldwide. In Australia, the majority of cases of BU have been recorded in coastal Victoria and the Mossman-Daintree areas of north Queensland. Mosquitoes have been postulated as a vector of *M. ulcerans* in Victoria, however the specific mode of transmission of this disease is still far from being well understood. In the current study, we trapped and analysed 16,900 (allocated to 845 pools) mosquitoes and 296 March flies from the endemic areas of north Queensland to examine for the presence of *M. ulcerans* DNA by polymerase chain reaction. Seven of 845 pools of mosquitoes were positive on screening using the IS2404 PCR target but only one pool was positive for presence of *M. ulcerans* after confirmatory testing. None of the March fly samples were positive for the presence of *M. ulcerans*. *M. ulcerans* was detected on proboscises of deliberately exposed mosquitoes.

Author Summary:

The causative agent of Buruli ulcer is Mycobacterium ulcerans. This destructive skin disease is characterized by extensive and painless necrosis of skin and underlying tissues usually on extremities of body due to production of toxin named mycolactone. The disease is prevalent in Africa and coastal Australia. The exact mode of transmission and potential environmental reservoir for the pathogen still remain obscure. Aquatic and biting insects have been identified as important niche in transmission and maintenance of pathogen in the environment. In this study we screened mosquitoes and march flies captured from endemic areas of northern Queensland for the presence of *M. ulcerans.* In addition, we conducted artificial blood feeding experiment to identify the role of mosquitoes in transmission of this pathogen. We found one pool of mosquito out of 845 pools positive for *M. ulcerans* and none of the March fly samples

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were positive. This could indicate a low burden of the bacteria in the environment coinciding with a comparatively low number of human cases of *M. ulcerans* infection seen during the trapping period of the study. Evidence to support mechanical transmission via mosquito proboscises was found.

Introduction:

Buruli ulcer (BU), also known regionally as Daintree ulcer in north Queensland, Australia or Bairnsdale ulcer in Victoria, Australia, is an emerging disease of skin and underlying tissue, with a potential to lead to permanent disability, particularly if treatment is inadequate or delayed. The causative agent of this disease, *M. ulcerans* secretes a polyketide exotoxin, mycolactone, the production of which requires expression of a series of contiguous genes on the large pMUM001 plasmid. This exotoxin is the main virulence determinant of the bacteria (1). The outbreaks of BU have been consistently linked with wetland or coastal regions (2). Environmental samples such as water, aquatic plants, soil at endemic areas has been found PCR-positive for *M. ulcerans* DNA (3, 4). Insects such as mosquitoes and aquatic bugs has been proposed as a vital ecological niche for the maintenance of pathogen in environment (5, 6).The detection of *M. ulcerans* DNA in insects does not prove their ability to transmit *M.*

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67 *ulcerans* but could indicate potential to act as either biological or mechanical vector. A study
68 conducted by Marsollier and his colleagues provided evidence of the presence of *M. ulcerans*
69 in the salivary gland of wild caught Naucoridae (aquatic bug). They successfully isolated the
70 pathogen by culture from the salivary glands of aquatic bugs and suggested aquatic insects as
71 having an important ecological niche in the maintenance of the organism in the environment.
72 They were also able to demonstrate transmission to mice in a laboratory environment (6).
73 Similarly, a study conducted by Wallace *et al*. provided evidence of the ability of mosquitoes
74 to act as a mechanical vector of *M. ulcerans* (7). Studies conducted in endemic areas of Africa
75 suggest that conducting farming activities close to rivers (8) and swimming in rivers located in
76 endemic areas (9) are risk factors for exposure to *M. ulcerans.*

77 In Australia, foci of BU infection have been found in tropical Far North Queensland (10, 11),
78 the Capricorn Coast region of central Queensland (10), the Northern Territory (12) and
79 temperate coastal Victoria (5). Victorian researchers detected the presence of *M. ulcerans* in
80 five different species of mosquito during a BU outbreak in an endemic area of Victoria,
81 Australia. They demonstrated the absence of *M. ulcerans* in a neighbouring area, where BU
82 did not occur (5). Together, the evidence was proposed to support a link with mosquitoes in
83 the ecology of BU in Victoria (5, 13). More recently, there was a report of the presence *M.*
84 *ulcerans* in a single mosquito out of two pools collected in the BU endemic region of north
85 Queensland. The isolated detection of *M. ulcerans* in a tropical endemic region in Australia
86 highlighted a need to examine a larger sample size to gauge the significance of the role of
87 mosquito in ecology of BU in Northern Queensland (14). An additional hypothesis put forward
88 by the local population (including people with a history of BU) was that March flies
89 (Tabanidae) might have a role in transmission (Villager *et al*, unpublished manuscript). We
90 therefore aimed, in this study to capture and screen mosquitoes and March flies for the presence
91 of *M. ulcerans* DNA in the BU endemic area of Northern Queensland. In addition, we

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92 conducted a mosquito artificial blood feeding experiment to demonstrate an in vitro basis for
93 mechanical transmission of *M. ulcerans* by blood fed mosquitoes.

94 ## Material and Methodology:

95 Selection of the study site was based on GIS mapping of human cases of BU in Northern
96 Queensland (15). We divided the endemic area of northern Queensland into three regions:
97 Region-1: extending from Miallo to lower Daintree including Wonga/Wonga Beach area,
98 Region-2: Forest Creek area and Region-3: Upper Daintree area for ease of sampling and
99 analysis (Fig. 1).

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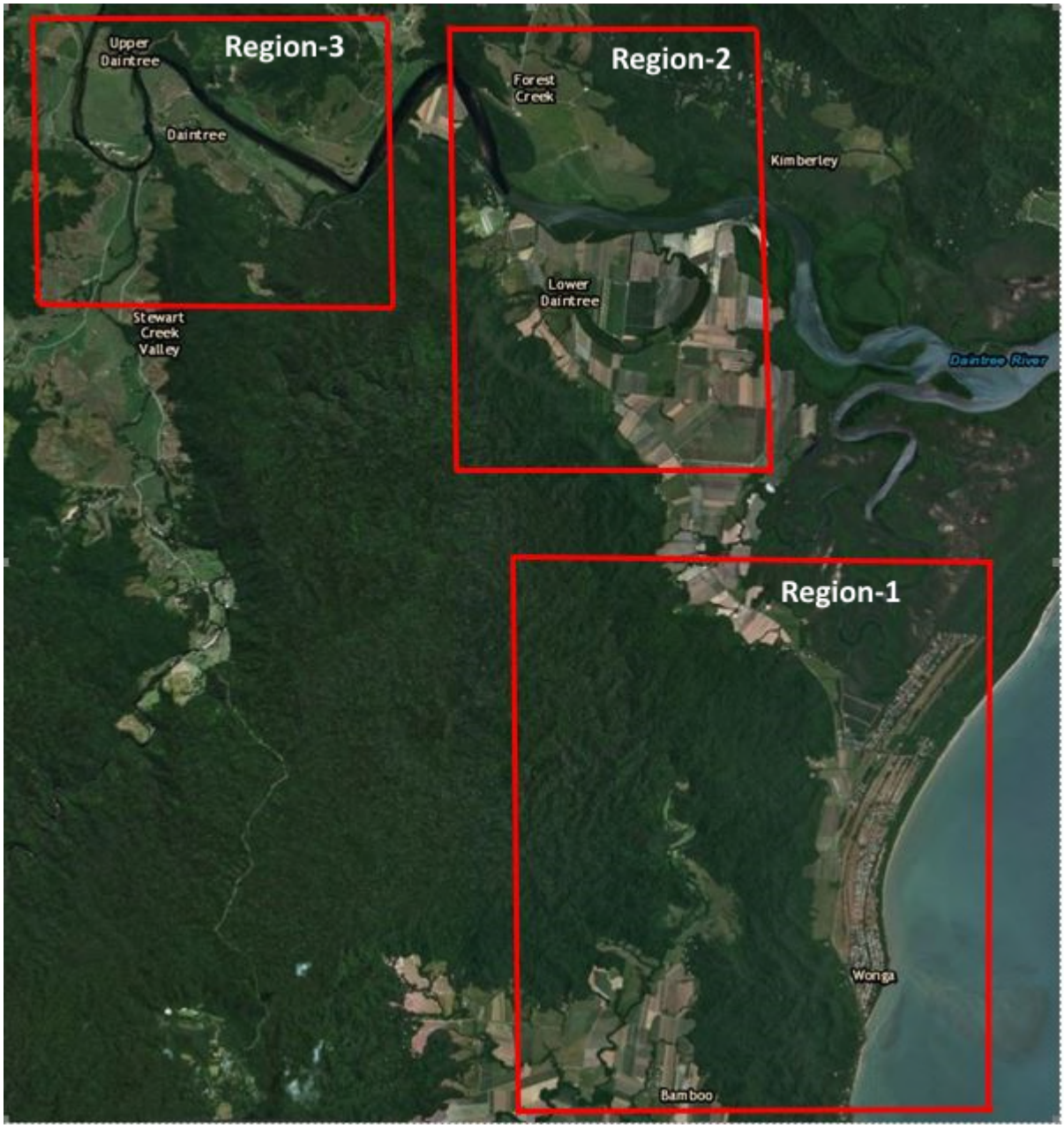

Fig. 1: BU endemic areas of Northern Queensland, Australia and Mosquito trapping regions. This figure was created using base layer obtained from https://landsatlook.usgs.gov/

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Trapping of Mosquitoes:

Mosquitoes were captured using a model 512 "CDC miniature light trap" (John W. Hock Company, Gainesville Florida USA) baited with 1 kg of dry ice as the source of CO_2. This trap is the most reliable, efficient and portable device for trapping mosquitoes and sand flies (16). This trap consists of an electric light and fan just over the collection container and is operated by a 12V battery. A two liter insulated container was used to hold dry ice and a pipe was attached to release CO_2 over the trap to attract mosquitoes (Fig. 2). Thirty overnight trapping sessions were conducted starting from September 2016 through to February 2018, with at least 4 CDC traps placed within a 1 kilometer radius of each-other. Of the 30 trapping sessions, 14 were conducted at eight different sites within region-1, nine at six different sites within region-2 and seven at five different sites of region-3 (Fig. 1). Traps were placed at different sites after obtaining permission to access properties from the owners and selection of sites were based on history of BU cases in humans in nearby households. Geographical Information System (GIS) coordinates of each trap was recorded. On each occasion, traps were set before dusk and checked for mosquitoes after dawn the next morning. After each occasion of trapping, catches were transported to the Mosquito Research Facility, Australian Institute of Tropical Health and Medicine (AITHM), James Cook University, Cairns, Australia where they were counted, sorted and pooled by genus, with each pool containing ≤ 20 mosquitoes of same genus and collected from the same site.

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Fig. 2. CDC miniature light trap baited with dry ice

Trapping of March Flies:

Several attempts were made to trap march flies from endemic areas with an investigator wearing dark clothes to attract them, or with the use of an insect net sprayed with insecticide. These attempts occurred from February 2016 through September 2016. The yield from these attempts were very low. A request was made to residents of region-1 through the local State School to collect march flies. This effort was successful and large numbers of March flies of genus *Tabanus* were collected by the local community. The addresses of properties from which March flies were collected were recorded. Sampling of March flies was restricted to region-1.

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Screening of Mosquitoes and March Flies for MU DNA by PCR:

DNA was extracted from each pools of ≤ 20 mosquitoes of the same genus by using the FastPrep Instrument (MP Biomedicals, Solon, OH, USA) as per manufacturer's instruction with FastDNA Kit (MP Biomedicals). Using the same instrument, DNA from individual March fly was extracted with FastDNA Spin Kit (MP Biomedicals). Extracted DNA was stored at -20 ^{o}C. The extracted DNA samples were screened for the presence of *M. ulcerans* DNA by using a semi-quantitative real-time PCR adapted from a method for the detection of *M. ulcerans* DNA from environmental samples (17). To rule-out the possibility of contamination, three negative controls (double deionized water, MilliQ) and three positive controls (purified *M. ulcerans* DNA obtained from Victorian Infectious Disease Reference Laboratory) were used during RT-PCR assay run. All of the extracted DNA samples were initially screened for the *M. ulcerans* insertion sequence element IS 2404. Samples positive for IS 2404 were re-analyzed by a second real-time PCR for the detection of two additional regions in the genome of *M. ulcerans*: IS 2606 and ketoreductase B domain (KR). This screening process has been validated by Fyfe *et al*. to differentiate *M. ulcerans* from other mycolactone producing mycobacteria (MPM) (17). They suggested that the difference in real-time PCR cycle thresholds (Ct) between IS 2606 and IS 2404 (ΔCt [IS 2606 – IS 2404]) allows for the differentiation of *M. ulcerans* from other MPM that contain IS 2404 but which have fewer copy numbers of IS 2606. The detection of all three targets (IS 2404, IS 2606 and KR) with an expected ΔCt values (< 7) confirms the presence of *M. ulcerans* DNA in the sample. (17, 18).

Mosquito artificial blood feeding experiment:

An isolate of *M. ulcerans* obtained from the Mycobacterium Reference Laboratory at the Royal Brisbane Hospital was used for this experiment. *M. ulcerans* was confirmed via PCR analysis. Our laboratory was not equipped with facilities to safely conduct transmission experiments, so

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158 isolates of *M. ulcerans* were subjected to UV light to kill the pathogen. To confirm the sterilty
159 of the isolates, an aliquot was sub-cultured onto Lowenstein-Jensen (LJ) slants and liquid
160 Middlebrook 7H9 media supplemented with 10% oleic acid-albumin-dextrose enrichment
161 (OADC). Inoculated media was kept at 31°C in 25cm2 tissue culture flasks and observed for 8
162 weeks for growth. No growth was observed confirming the absence of live bacteria. The killed
163 isolates were subjected to RT-PCR targeting IS 2404, IS 2606 and KR as described above. RT-
164 PCR analysis confirmed the presence of *M. ulcerans* DNA.

165 We used an artificial blood feeding method (simple membrane method) described by Finlayson
166 *et al*. with some modification in this study (Finlayson, Saingamsook, & Somboon, 2015). This
167 procedure is a simple and affordable alternative for direct host feeding (DHF). The method
168 involves pouring warmed defibrinated sheep blood (Applied Biological Products Management-
169 Australia) into the indented base on the underside of a plastic container and then covering it
170 with a stretched collagen membrane secured by a rubber band. The container is then turned up,
171 filled with warm water and covered by a lid. The feeder is then placed on the mesh side of the
172 cage, allowing the mosquitoes to pierce the collagen membrane to access the blood.

173 The experiment was conducted using wild type *Aedes aegypti* hatched and reared in the same
174 batch, sorted as pupae into four cages containing 30 female mosquitoes in each. One out of
175 four cages was used as control (Cage-D) where only defibrinated sheep blood was used as feed
176 and in remaining three cages (Cage-A, B and C) defibrinated sheep blood mixed with killed *M.*
177 *ulcerans* isolates were used. All four cages were exposed to blood for 2 hours. Fully blood fed
178 mosquitoes from each cage were aspirated separately and knocked down by freezing. Pool of
179 mosquitoes from cage A, and B were dissected separating the head, abdomen and legs of each
180 insects by sterile fine forceps to avoid contamination during dissections. DNA from the head,
181 abdomen and legs (pooled separately) from the mosquitoes from cage A and B and whole

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mosquitoes from cage C and D were extracted using FastPrep Instrument (MP Biomedicals, Solon, OH, USA) as per manufacturer's instruction with FastDNA Kit (MP Biomedicals). All the extracted DNA were initially screened for IS 2404 and IS 2404-positive samples were re-analyzed for IS 2606 and KR with RT-PCR assay as described above.

Results:

Screening of Mosquitoes:

A total of 16,900 mosquitoes were captured over the course of the study from 30 occasions of trapping at three different regions of northern Queensland. Total mosquitoes captured from region-1, region-2 and region-3 were 7880, 5100, and 3920, respectively. The majority of captured mosquitos belonged to the *Verrallina* genus (specifically *Verrallina lineata*) 82%, followed by *Coquillettida* (9%) and *Mansonia* (3%). The remaining 6% consisting seven other genera that were classified as "other" for screening. See figure 3 below.

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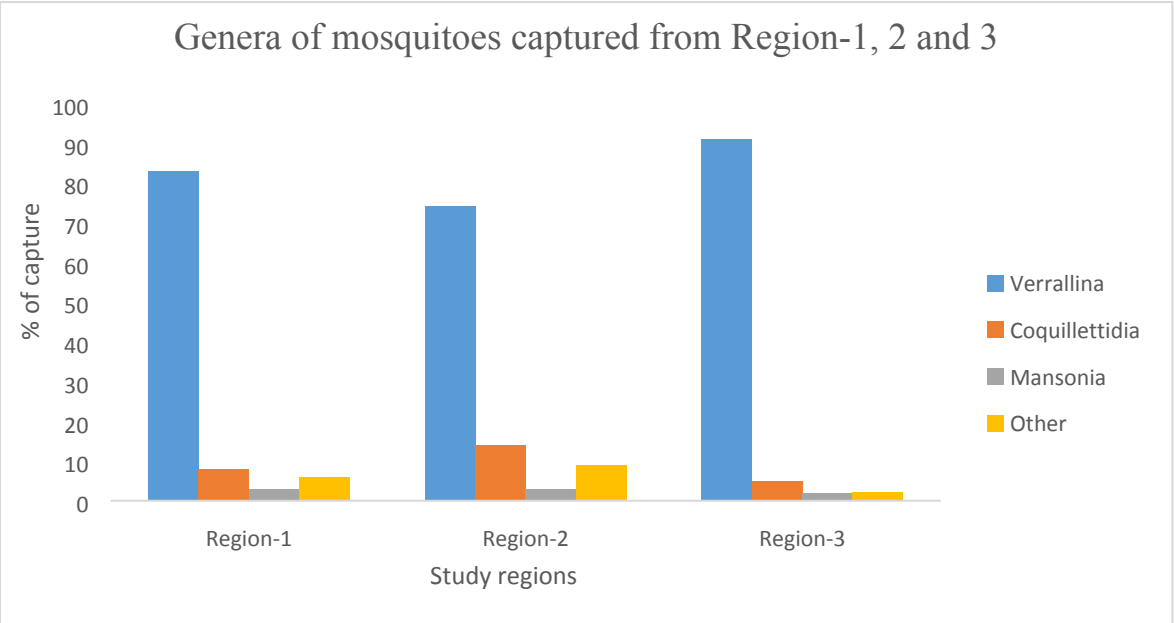

196

197 **Fig. 3. Genera of mosquitoes captured from three different regions: Region-1 comprising**
198 **83% of *Verrallina sp.*, 8% of *Coquillettida sp.*, 3% of *Mansonia sp.* and 6% of**
199 **others; Region-2 comprising 74% of *Verrallina sp.*, 14% of *Coquillettida sp.*, 3%**
200 **of *Mansonia sp.* and 9% of others and Region-3 comprising 91% of *Verrallina***
201 ***sp.*, 5% of *Coquillettida sp.*, 2% of *Mansonia sp.* and 2% of others of total catches.**

202 Of total 16,900 mosquitoes screened (845 pools), seven pools were positive for IS 2404. Three
203 of those seven pools were *Verrallina sp.* from region-1, two pools were *Coquillettida sp.* one
204 each from capture region-1 and 3 and the remaining two pools were *Mansonia sp.* from region-
205 1. Of the seven pools positive for IS 2404, one pool was positive for *M. ulcerans* using the
206 confirmatory PCR assays. This single positive pool of mosquitoes was comprised of genus
207 *Verrallina sp.* and linked to region-1. Thirty pools of mosquitoes which were negative for IS
208 2404 were tested for IS 2606 and KR. None of them were positive for these probes signifying
209 the dependent nature of existence of IS 2606 and KR with IS 2404. Similar findings were
210 reported during the Victorian outbreak (5).

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Screening of March flies:

DNA extracts of 296 March flies were screened for IS 2404. None of the samples were positive for this probe. Twenty-four randomly selected IS 2404 negative samples were tested for IS 2606 and KR and none were positive.

Mosquito artificial blood feeding experiment:

There were a total of seven samples: 2 pools of heads, 2 pools of abdomens, 2 pools of legs (from cage A and B) and 1 pool of whole mosquitoes (Cage C). DNA extracted from pools of heads and abdomens of mosquitoes from cage A and B and the pool of whole mosquitoes from Cage C were positive for IS-2404. Confirmatory assays targeting IS 2606 and KR revealed that three samples: DNA extract from the heads of mosquitoes from cage A and B and the pool of mosquitoes from cage C were positive for *M. ulcerans* DNA. Controls (a pool of mosquitoes from cage D) were negative for all three targets: IS 2404, IS 2606 and KR.

Discussion:

Mosquitos serve as important biological vectors for a variety of pathogens. The movement of pathogens from the gastro-intestinal tract after ingestion to the salivary glands for subsequent transmission is well documented for many diseases. However, this phenomenon has not been demonstrated for *M. ulcerans*. A study conducted by Wallace and colleagues (2010) provided evidence on the maintenance of *M. ulcerans* throughout larval development without further passage of the organisms into pupa or adult mosquitoes (19). They concluded that mosquitoes were an unlikely biological vector of *M. ulcerans*. Wallace *et al* (2017) subsequently provided evidence of mechanical transmission of *M. ulcerans* via anthropogenic skin puncture or mosquito bites (7). Detection of *M. ulcerans* from pools of head of mosquitoes in our mosquito artificial blood feeding experiments indicate a potential for mosquitoes act as an agent for

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234 mechanical transmission of *M. ulcerans*. However, our mosquito artificial blood feeding
235 experiment had some limitations. We were not able to conduct experiments to verify whether
236 *M. ulcerans* positive mosquitoes transmit pathogen to healthy animal or not. Only proboscises
237 of mosquitoes were in direct contact with blood containing *M. ulcerans* DNA. *M. ulcerans*
238 was only detected from the abdomen of mosquitoes using the IS2404 target. This might have
239 been due to insufficient amount of DNA to identify IS 2606 and KR.

240 For mechanical transmission, insect vectors such as mosquitoes must acquire the pathogen
241 either from the environment or an infected host. For this to occur efficiently, the organism must
242 be abundantly present in the environment. A survey in Victoria, Australia has confirmed a
243 strong correlation between mosquitoes found to test positive for carrying *M. ulcerans* and the
244 number of human cases of BU occurring (5). The group found a significantly higher number
245 of mosquitoes screened positive for *M. ulcerans* during an intense outbreak of BU in endemic
246 areas, in comparison to areas with a lower incidence of human cases.

247 The number of human cases of BU has decreased in Northern Queensland, Australia since the
248 largest recorded outbreak in 2011 (> 60 cases). The majority of the cases during the 2011
249 outbreak were from Wonga and the Wonga beach area, referred as region-1 in the study by
250 Steffen and Freeborn (2018) (20). Out of 394 pools collected, only one from region-1 was
251 positive for *M. ulcerans* in this study. Interestingly, mosquitoes of this positive pool were
252 trapped in the backyard of a property in Wonga Beach area (region-1) where two human cases
253 of BU were confirmed in 2017. All other pools of mosquitoes and march flies collected from
254 that properties negative for *M. ulcerans*.

255 In a separate study conducted in Northern Queensland, Australia, one pool of mosquitoes was
256 found positive for *M. ulcerans* out of two pools collected in total (14). However, it must be

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noted that this study was conducted soon after 2011 which raises the possibility that sampling should occur as close as possible in time to when transmission is thought to be occurring.

M. ulcerans is an environmental pathogen and detection of *M. ulcerans* positive mosquitoes may only be an indicator for the presence of the organism in the environment. A significant decrease in human cases of BU in Northern Queensland in recent years could be due to a lower load of bacteria in the environment. This may explain the low detection of *M. ulcerans* positive mosquitoes and March fly populations in the study sites. However, the detection of *M. ulcerans* even in a single pool of mosquitoes from the endemic areas of Northern Queensland is significant, as it corroborates findings in Victoria where five different species of mosquitoes captured from BU-endemic regions during human outbreaks were positive for *M. ulcerans*.

Our detection of *M. ulcerans* in mosquitoes in Northern Queensland does support the earlier report from Victoria in Australia (5). The Victorian study provides evidence for high detection rates of *M. ulcerans* positive mosquitoes if captured during peak times of outbreaks. Our study found that it is less likely to find *M. ulcerans* positive mosquitoes if they are trapped from areas where human incidence of BU is currently low. We hypothesize that mosquitoes and perhaps other biting insects, such as March flies may have a significant role in the ecology and transmission of *M. ulcerans* in endemic areas during outbreaks and that the level of detection of *M. ulcerans* positive mosquitoes in the environment could be an indicator for disease outbreaks.

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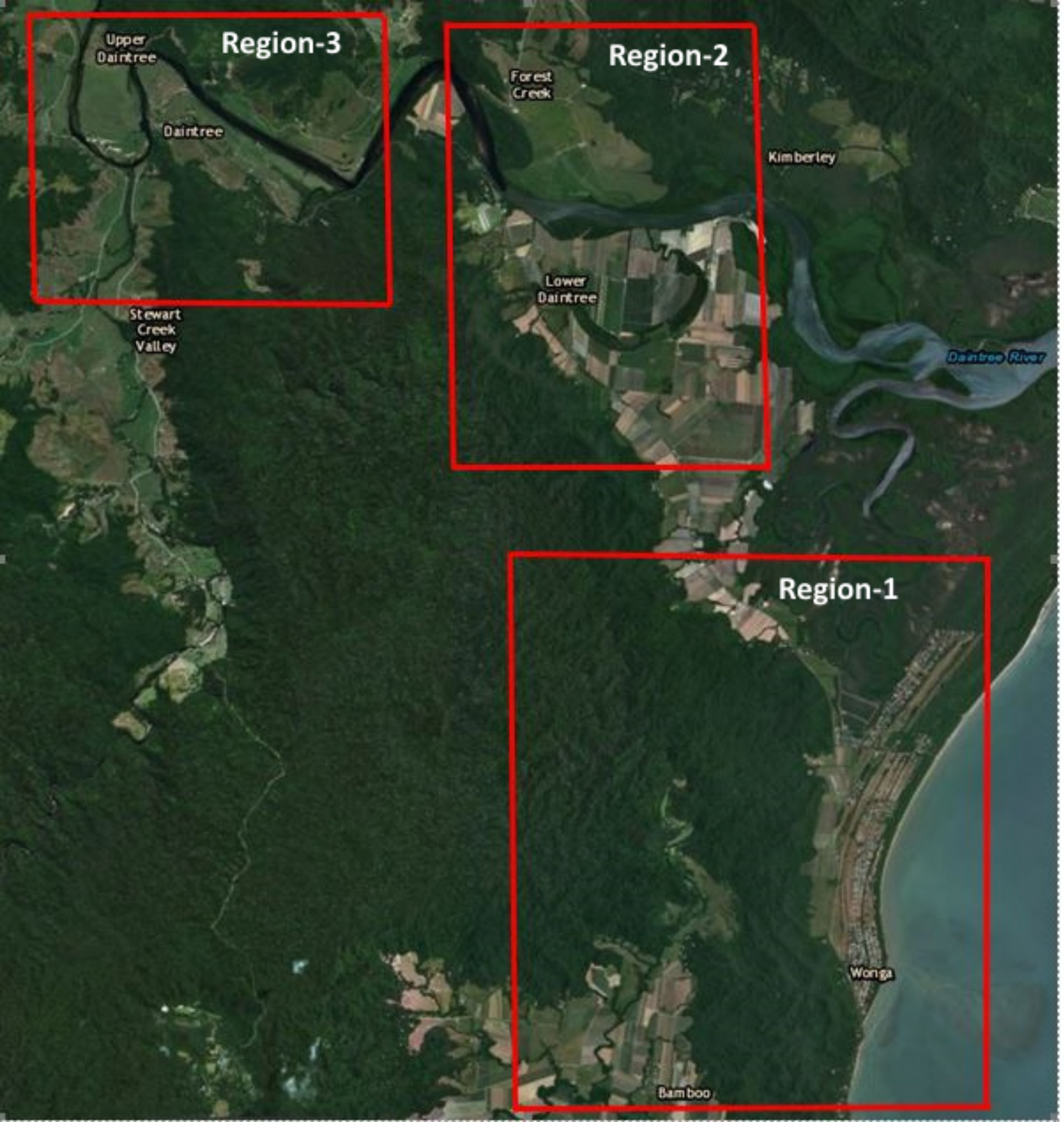
Region-3
Upper Daintree
Daintree
Stewart Creek Valley
Region-2
Forest Creek
Kimberley
Lower Daintree
Daintree River
Region-1
Wonga
Bamboo