A transmission chain linking *Mycobacterium ulcerans* with *Aedes notoscriptus* mosquitoes, possums and human Buruli ulcer cases in southeastern Australia

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Abstract

In temperate southeastern Australia over the past two decades there has been a marked progressive increase in human cases of Buruli ulcer, an infection of subcutaneous tissue caused by *Mycobacterium ulcerans*. Native possums are the major local environmental reservoir of *M. uclerans* as they not only develop Buruli lesions but they also shed *M. ulcerans* in their excreta. However the way humans acquire *M. ulcerans* from possums has not been determined. Previous case-control studies, insect field surveys and vector competence studies have suggested a role for mosquitoes in *M. ulcerans* transmission between possums and humans. To explore these links we conducted an extensive, 4-month structured mosquito field survey and four *ad hoc* field surveys across an area of 350km^2 on the Mornington Peninsula, an area endemic for Buruli ulcer. We then compared spatial and temporal patterns of *M. ulcerans*-positive mosquito occurrence with *M. ulcerans*-positive possums (established by previous possum excreta surveys) and human Buruli ulcer cases across the region. We used metabarcoding to assess mosquito blood-feeding host preference and to reconstruct *M. ulcerans* genomes from positive mosquitoes to test epidemiological inferences. We collected 66,325 mosquitoes spanning 26 different species from 180 repeatedly sampled traps over a 4-month period. *Culex molestus* and *Aedes notoscriptus* were the dominant species (42% and 35% of trapped mosquitoes, respectively). PCR screening 25% of trapped mosquitoes revealed a significant association between *M. ulcerans* and *Ae. notoscriptus* ($p<0.0001$) with a maximum likelihood estimate (MLE) of 5.88 *M. ulcerans* positive mosquitoes per 1,000 tested. Using spatial scanning statistics, we also observed significant overlap between clusters of *M. ulcerans*-positive *Ae. notoscriptus*, *M. ulcerans*-positive possum excreta and human Buruli ulcer cases. Metabarcoding analyses of blood-fed *Ae. notoscriptus* showed individual mosquitoes had fed both on humans and native possums. Enrichment genome sequencing from PCR-positive mosquitoes confirmed identical *M. ulcerans* genome single-nucleotide polymorphism (SNP) profiles between insects, possums and clinical human isolates within the same regions. These findings indicate that certain mosquito species likely transmit *M. ulcerans* in southeastern Australia and highlight mosquito control as a plausible means to control the Buruli ulcer epidemic in our region.

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Introduction

Mycobacterium ulcerans is the causative agent of a neglected tropical skin disease called Buruli ulcer, a necrotising infection of skin and subcutaneous tissue [1]. Buruli ulcer is rarely a fatal condition but can cause severe tissue destruction if not diagnosed and managed effectively [2]. Buruli ulcer has been described in more than 32 countries worldwide [3] and is an ongoing public health issue in west and central Africa [4]. Buruli ulcer has also been unexpectedly surging in temperate southeastern Australia (Figure 1) and encroaching on the major metropolitan centres of Melbourne (population 5.1 million) and Geelong (population 274,000), with >250 cases routinely notified each year since 2017 to the Victorian State Government Department of Health [5].

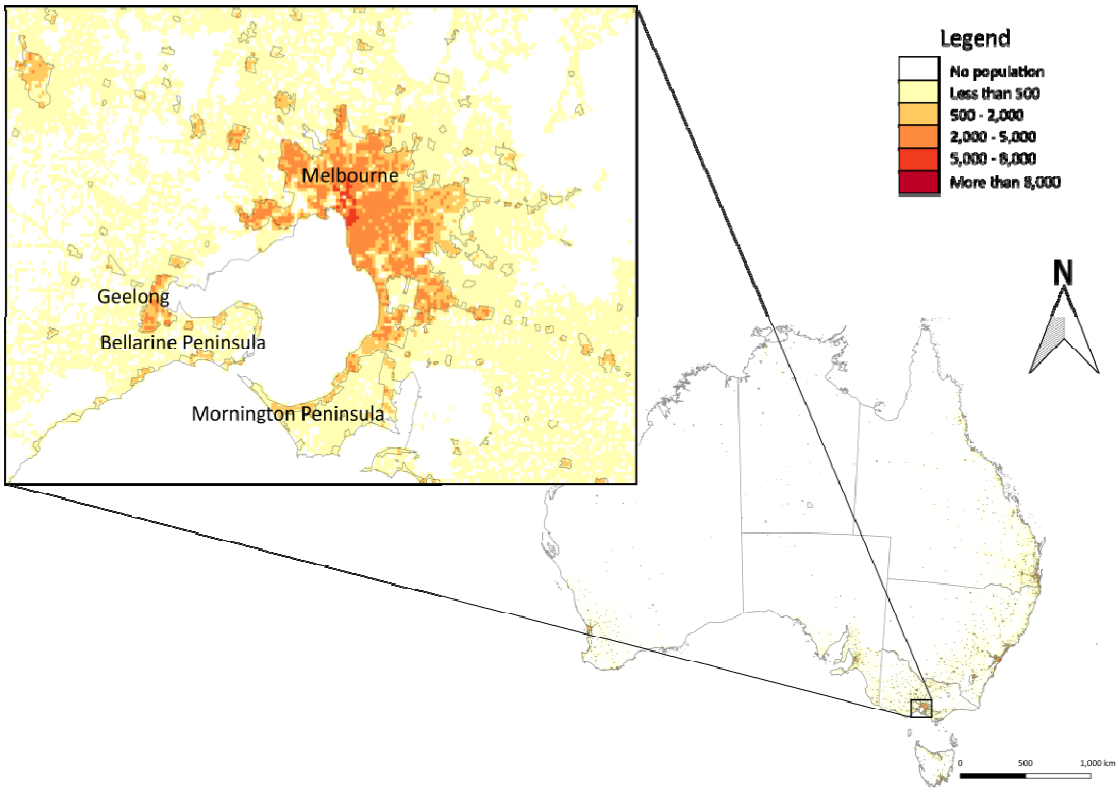

Figure 1. *Location of the Mornington Peninsula and the Bellarine Peninsula of Victoria, Australia. Population density is represented on the map from low to high (white to red) based on the human population per square kilometre.*

How humans contract Buruli ulcer is a central question that has intrigued scientists and confounded public health control efforts since the discovery of *M. ulcerans* from patients in the Bairnsdale region of Australia in the 1930s and across Africa shortly after [1,6,7]. Buruli ulcer epidemiology can be unpredictable, with outbreaks emerging in specific geographical areas and then disappearing over a number of years. *M. ulcerans* has a 4-5 month median incubation period. It is very challenging to isolate the bacterium in pure culture from the environment, presumably due to its very slow growth, although it can be isolated from human skin lesions. These factors combined have made it incredibly challenging to establish how *M. ulcerans* is spread to humans, despite global efforts to investigate this over more than 80 years [8].

The discovery that Buruli ulcer is a zoonosis and that Australian native possums are a major wildlife source of *M. ulcerans* that is intimately linked with disease transmission has addressed one key component of the transmission enigma [9-14]. The role of mosquitoes as vectors spreading *M. ulcerans* from possums to humans (and between possums) is the subject of the research presented in this current study. The first indications that mosquitoes might be vectors of *M. ulcerans* from possums to humans in Australia came from a series of entomological field surveys in the southeast of the country in response to an increase in Buruli ulcer cases in the seaside township of Point Lonsdale, located on the Bellarine Peninsula [15]. Among the 12 species identified from a trapping effort that collected 11,500 mosquitoes, five different species were IS2404 PCR positive for *M. ulcerans,* including *Aedes camptorhynchus*, *Aedes notoscriptus*, *Coquillettidia linealis*, *Culex australicus*, and *Anopheles annulipes* (maximum likelihood estimate (MLE) was 4.11/1000 mosquitoes) [15]. Note that IS2404 is a *M. ulcerans* specific insertion sequence and molecular target for the gold-standard diagnostic PCR for Buruli ulcer [16].

A concurrent case control study performed in the same geographic area identified only two factors associated with the odds of being diagnosed with Buruli ulcer: insect repellent use (OR 0.37, 95% CI 0.19–0.69) and being bitten by mosquitoes on the lower legs (OR 2.60, 95% CI 1.22–5.53). A variety of outdoor activities were also surveyed but were not independently predictive suggesting that mosquito exposure specifically rather than environmental exposure generally might be the main mode of MU transmission to humans [17]. In Africa, two case control studies conducted in Cameroon both found use of bed nets as a

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protective factor against Buruli ulcer, (OR 0.4, 95% CI 0.2-0.9, p=0.04) [18] and (OR 0.1, 95% CI 0.03-0.3, p<0.001) [19].

However, case control studies and entomological surveys alone are insufficient to indict biological agents as vectors of pathogens. There are formal frameworks used in biomedicine, such as the Barnett criteria [3] that build hierarchies of evidence to implicate a candidate disease vector. Here, we build on this aforementioned research to formally address the Barnett criteria, and test the hypothesis that mosquitoes are vectors of *M. ulcerans* to humans [20-22]. A summary of our findings is shown in Table 1, including the new data presented in this study that specifically address criteria 1-3.

Table 1: Summary of evidence to indict mosquitoes as vectors of *M. ulcerans*

Barnett Criterion	Evidence	Reference
1. Acquire from source reservoir	*M. ulcerans* genome sequences from possum excreta matches genomes recovered from mosquitoes in same area	This study
2. Close links with infected hosts	*M. ulcerans* genome sequences from humans matches genome recovered from mosquitoes in same area. Mosquito bloodmeal analysis confirms human-possum link.	This study
3. Repeat detections from candidate vector	*M. ulcerans* detected with mosquitoes in seven separate field surveys	[15,21], this study
4. Experimental demonstration of transmission	Mosquito-*M. ulcerans* mechanical transmission demonstrated using murine contaminated skin model	[22]

In the research presented here, based on a very substantial field survey of >65,000 mosquitoes undertaken over four months in 2019 and 2020, and four smaller *ad hoc* surveys conducted between 2016 and 2021, we employed spatial clustering analyses, bacterial enrichment genome sequencing and mosquito blood meal metabarcoding to continue building a hierarchy of evidence that implicates mosquitoes as vectors of *M. ulcerans* from local wildlife sources to humans.

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Material and methods

Study Site

Insects were collected from the Mornington Peninsula suburbs of Rye (population 8,416), Blairgowrie (population 2,313), Tootgarook (population 2,869) and Capel Sound (population 4,930) [23]. The study area encompasses an area of 350 km^2 and is located 90 km south of Melbourne, the capital city of Victoria [10] (Figure 1). The area was originally covered in low-lying coastal vegetation [10], receives an average annual rainfall of 740 mm, and an elevation of 60 metres above sea-level [24]. The Mornington Peninsula region continues to maintain among the highest incidences of Buruli ulcer in the world [25] with a conservative local incidence estimate of 55 human cases/100,000 population in 2022 [5 26].

Arthropod collection and identification

Mosquito trapping campaigns used Biogent Sentinal (BGS) traps (Biogent) that were baited with dry ice pellets to provide a source of carbon dioxide over an intended 12-hour period. BGS traps were set out at dusk and collected at dawn in shaded locations on the grassed edges of the roadways in the study area. GPS locations for all traps were recorded. Trapped mosquitoes were knocked down with CO_2 by placing the catch-bag in dry ice before being transported back to the laboratory and kept at -20°C until processing. Mosquito species were morphologically identified using a stereo dissecting microscope (Nikon, SMZ800N) and reference to taxonomic keys [27-29].

Other (non-mosquito) arthropods were collected using Yellow Sticky Traps (YST) and Sticky Ovitraps (SO). Two YST and SO were placed in residential properties where householders had previously noted insect activity to the researchers [9]. The SO traps were placed on the ground and had hay grass infusion (3 Jack Rabbit (clover/lucerne) pellets (Laucke Mills, Barossa Valley, SA, Australia) in 500 mL water) added to them, while the YST (Bugs for Bugs, Toowoomba, QLD, Australia) were placed on the ground with a 14 cm plant tag plastic T-support (Garden City Plastics, Dandenong, VIC, Australia). Within three to four days of

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144 being set, residents were asked to pack up the YST and SO by covering the sticky card with a plastic film,
145 and return the sealed traps to the laboratory, where they were stored at -20°C. Non-mosquito arthropods
146 were morphologically identified to family level and, if PCR positive for *M. ulcerans,* were DNA barcoded for
147 species confirmation by targeting the cytochrome c oxidase subunit I (COI) gene [30].

148

149 DNA extraction from mosquitoes

150 Mosquitoes were sorted by species per trap and by sex and then pooled in 2 mL o-ring tubes, with a
151 maximum of 15 individuals in each pool. A subset of *Ae. notoscriptus* mosquitoes were also screened
152 individually. Mosquitoe(s) were homogenised with 10 x 1.0 mm zirconia-silica beads (BioSpec Products),
153 with 597 µL of Buffer RLT and 2.8 µL of carrier RNA. Homogenisation was performed using a TissueLyser II
154 (Qiagen) at 30 oscillations/sec for 100 sec, repeated twice. Tubes were then centrifuged at 16,000 g for 3
155 mins. A 550 µL volume of supernatant was transferred into a 96 well deep well plate, with extraction
156 performed as is the protocol for the BioSprint 96 One-For-All Vet Kit (Qiagen). Every eleventh of twelve
157 wells in a 96-well plate was a blank DNA extraction control (seven in total) and a synthetic IS2404 positive
158 control was spiked into one of these seven wells to act as a positive extraction control. Extraction was
159 performed on a KingFisher™ Flex Magnetic Particle Processors (Thermo Scientific).

160

161 DNA extraction from arthropods

162 Arthropods other than mosquitoes collected on YST or SO were removed from the sticky cards and placed
163 in 1.5 mL microtubes (Eppendorf). Insects were separated by family and trap location, and were pooled
164 with a maximum of 10 individuals from each family per 1.5 mL tube. Samples were extracted non-
165 destructively to allow species confirmation if positive detections occurred. DNA was extracted using the
166 ISOLATE II Genomic DNA Kit (Bioline). Briefly, 25 µL of Proteinase K and 180 µL of Lysis Buffer GL was added
167 to each tube with samples incubated overnight at 56°C. Following incubation, the insects were removed

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168 and stored to allow for further morphological identification if required, with the DNA extraction completed
169 on the incubation solution as per manufacturer instructions.

170

171 Synthetic PCR positive control

172 A synthetic PCR positive control DNA molecule was designed to discriminate false positives due to
173 contamination with positive control DNA versus the authentic IS2404 amplicon. The synthetic positive
174 control was designed to have an amplicon size of 120 bp to easily differentiate from a true IS2404 PCR
175 positive (59 bp) [16]. The synthetic positive was added at the DNA extraction stage on all 96-well plates, as a
176 positive control for this step and for the subsequent qPCR. The additional DNA sequence used to construct
177 the synthetic positive control was randomly selected from a DNA sequence unlikely to be in the laboratory,
178 in this case, Irrawaddy Dolphin (MK032252).

179 The synthetic positive control had the sequence: 5' –
180 TCCTAAAGCACCACGCAGCATCTATCGCGAGCTTAATCACCATGCCGCGTCCAACGCGATCCCCGCTCGGCAGGGATC
181 CCTCTTCTCGCACCGGGCCACAATCCACTGGGGTCGCTATGA – 3' and was synthesised as an ssDNA oligo
182 (Sigma-Aldrich). The synthesised IS2404 synthetic positive was resuspended in nuclease-free water and
183 diluted to 0.001 pM, with 2 µL being used for extraction and positive controls. To confirm the presence of a
184 true positive as opposed to contamination, 5 µL of the qPCR product was added to 1 µL of DNA Gel Loading
185 Dye 6X (Thermo Scientific™) and run on a 2 % agarose gel (TopVision Agarose Tablets, Thermo Scientific™),
186 with 1% SYBR Safe DNA Gel Stain (Invitrogen™). The size of any positive IS2404 detection was assessed
187 against 2 µL of 100 bp DNA Ladder (Promega) and run at 50 V for 1.5 hrs before being visualised with an EZ
188 Gel Documentation System (Bio-Rad). Before the screening of insects commenced, the synthetic positive
189 control for IS2404 was successfully designed and tested. By running the amplified PCR products on an
190 agarose gel with the synthetic positive control and a real positive control, visual differentiation between a
191 synthetic positive occurring at 120 bp and a true positive at 59 bp (Figure S1).

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193 ## Screening insects by qPCR for *M. ulcerans*

194 The qPCR screening was performed using three independent assays IS2404, IS2606 and KR [16]. All samples
195 were first screened with the IS2404 qPCR; if a positive was detected, additional confirmation was
196 attempted with IS2606 and KR qPCR assays. Reactions were performed using 7.5 µL TaqMan™ Fast
197 Universal PCR Master Mix (2X), no AmpErase™ UNG (Applied Biosystems™), 1 µL of the primer-probe mix,
198 2 µL of DNA and 4.5 µL of nuclease-free water. A final primer-probe concentration for the IS2404 assay was
199 as follows: 250:650:450nM for the forward primer, reverse primer and probe and 800:800:220nM for the
200 forward primer, reverse primer and probe for the IS2606 and KR assay. A 2 µL volume of the synthetic
201 positive control was added for the IS2404 reactions, whereas 2 µL of *M. ulcerans* DNA was used for IS2606
202 and KR. All reactions included six no-template extraction controls and were run in a 96 well plate format.
203 Cycling conditions were as follows: denaturation at 95°C for 2 mins followed by 45 cycles at 95°C for 10 sec
204 and 60°C for 30 sec, with qPCR performed on a QuantStudio™ 5 Real-Time PCR System (Applied
205 Biosystems™). Positives were classified as reactions that produced a cycle quantification (Cq) value less
206 than 40. Data were analysed using the QuantStudio™ Design and Analysis Software v1.4.3 with the Delta
207 Rn threshold set at 0.04 for IS2404 and IS2606 and Delta Rn threshold of 0.1 for KR. The maximum
208 likelihood estimate (MLE) per 1,000 mosquitoes tested (bias corrected MLE for point estimation of
209 infection rate and a skew-corrected score confidence intervals) was calculated from the pooled samples as
210 described [31]. Fisher's Exact test for assessing the significance of differences in IS2404 PCR positivity
211 between mosquito species was calculated in R 4.0.2 [32].

212 All qPCR screening was performed blind with mixed-species 96-well plates. The synthetic positive control
213 was added at the DNA extraction phase to one well of each plate and to qPCR plates to check that both
214 extraction and qPCR detections were successful. All no-template controls (extraction and qPCR stage) were
215 checked to ensure they remained negative, and that synthetic positive controls were detected for both the
216 DNA extraction and qPCR stage in each run. Positive samples were run on agarose gels to confirm they
217 were true positives and not contamination from the synthetic positive control.

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218 An IS2404 qPCR standard curve was prepared using 10-fold serial dilutions of *M. ulcerans* genomic DNA,
219 with quadruplicate testing of each dilution. The DNA was extracted from *M. ulcerans* JKD8049 as described,
220 and quantified using fluorimetry (Qubit, dsDNA HS ThermoFisher Scientific) [22]. A limit-of-detection was
221 defined as the lowest dilution that returned a positive signal for all four replicates. Genome equivalents
222 were calculated based on the estimated mass of the *M. ulcerans* genome of 5.7 femtograms [22]. IS*2404* cycle
223 threshold (Ct) values were converted to genome equivalents (GE) to estimate bacterial load within a
224 sample by reference to a standard curve (r^2 = 0.9956, y = [-3.829Ln(x)+37.17]*Z, where y = Ct and x =
225 amount of DNA [fg] and Z = the dilution factor]) (Figure S2). An IS2404 qPCR standard curve was fitted using
226 non-linear regression in GraphPad Prism (v9.5.1).

227

228 *Mycobacterium ulcerans* genome sequencing

229 Whole-genome sequencing was performed directly on DNA extracted from selected PCR positive mosquito
230 samples and possum excreta specimens using a hybridisation capture approach, based on 120 nucleotide
231 RNA baits spanning the 5.8 Mbp chromosome of the the *M. ulcerans* JKD8049 reference genome
232 [Bioproject ID: PRJNA771185] (Datafile S1) (SureSelect Target enrichment system, Agilent, Santa Clara, CA,
233 USA) and the Illumina *Nextera Flex for Enrichment with RNA Probes* protocol [33]. Resulting sequence reads
234 were submitted to NCBI GenBank and are available under Bioproject PRJNA943595 (Table S2).

235

236 *Mycobacterium ulcerans* phylogenetic analysis

237 To compare genomic variations between clinical isolates and sequence capture datasets, we mapped the
238 sequence reads against a fully assembled *M. ulcerans* chromosome reconstructed from a Victorian clinical
239 isolate (JKD8049; GenBank accession: NZ_CP085200.1) using the Snippy pipeline (v4.4.5)
240 (https://github.com/tseemann/snippy). While standard parameters, including a minimum coverage of 10x
241 were used for the clinical isolates and single possum sequence capture dataset, the mosquito sequence

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242 capture datasets had lower coverage, necessitating the adjustment of parameters. Thus, we lowered the
243 minimum coverage threshold to 1x to facilitate SNP calling for the mosquito sequence capture datasets.
244 The resulting SNPs were combined with those from the clinical isolates to generate an alignment of core
245 genome SNPs, which was used to infer a maximum likelihood tree with the GTR model of nucleotide
246 substitution using FastTree (v2.1.10) [34].

247

248 *Aedes notoscriptus* typing and species confirmation sequencing

249 Mosquito genotyping was performed by sequence comparisons of a partial fragment of the cytochrome c
250 oxidase subunit I (COI) gene [30]. DNA was extracted using the above protocols. PCR was performed using 5
251 µL of 5x MyFi Reaction Buffer, 1 µL of MyFi DNA polymerase, 5 µL of DNA, and primer concentrations as
252 described [35], with reaction made up to 25 µL with nuclease-free water. Reaction conditions were as follows
253 for COI, initial denaturation at 95 °C for 1 min, followed by 35 cycles at 95°C for 20 sec, 46°C for 20 sec, and
254 72°C for 60 sec before a final extension at 72°C for 5 mins. A 5µL volume of the amplified PCR product was
255 added to 1 µL of DNA Gel Loading Dye 6X (Thermo Scientific™) and run on a 1 % agarose gel (TopVision
256 Agarose Tablets, Thermo Scientific™), with 1% SYBR Safe DNA Gel Stain (Invitrogen™). 2 µL of 100 bp DNA
257 Ladder (Promega) was added to confirm amplicons size and run at 100 V for 45 mins. PCR products that
258 produced bands of the correct size were purified using the ISOLATE II PCR and Gel Kit (Bioline) as per
259 manufacturer's protocol and submitted for sequencing using an Applied Biosystems 3730xl capillary
260 analyser (Macrogen), with sequencing occurring on both strands.

261 Sequences were analysed in Geneious Prime (v2019.2.1) and trimmed to high-quality bases, aligned using
262 ClustalW v2.1 and trimmed to a consensus region, *Ae. notoscriptus* COI (874 bp) and for species
263 identification COI (816-882 bp). Sequences were analysed using blastn against the NCBI database. COI
264 sequences generated for species identification are available under accession numbers OQ600123-4 and COI
265 sequences for *Ae. notoscriptus* phylogenetics under accession numbers OQ588831-67 (Table S2).

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267 ## Mosquito bloodmeal analysis

268 Ninety blood-fed mosquitoes were identified as having an engorged abdomen and stilling having a red
269 pigment indicating a fresh bloodmeal and were dissected with a sterile scalpel blade. Blood from the
270 dissected abdomen was absorbed onto a 3 x 20 mm piece of a Whatman FTA™ card (Merck) and placed in
271 a 2 mL tube. DNA was extracted from the FTA™ card using an ISOLATE II Genomic DNA Kit (Bioline) with a
272 pre-lysis in 180 µL of Lysis Buffer GL and 25 µL Proteinase K for 2 hours before completing the extraction as
273 per manufacturer's protocol. Extracted DNA was amplified for Cyt *b* using primers previously described
274 (Townzen et al. 2008), with MyTaq™ HS Red Mix (Bioline), thermocycling conditions used were as follows:
275 95°C for 1 min, 30 cycles of 95°C for 15 secs, 50°C for 20 sec, 72°C for 20 sec, with a final extension at 72°C
276 for 2 mins. Negative extraction and negative PCR controls were included with each PCR reaction. 5 µL of the
277 amplified products was run on a 1% agarose gel containing 0.1% SYBR Safe DNA gel stain (Invitrogen) and
278 visualised with a G:BOX Syngene blue light visualisation instrument. If a band was visualised at ~480 bp the
279 PCR product was purified using an ISOLATE II PCR and Gel Kit (Bioline).

280 PCR products were quantified using a Qubit (Invitrogen) Fluorometer with an HS dsDNA kit. Sequencing
281 libraries were prepared using 10 ng of purified PCR product with a NEXTFLEX® Rapid XP DNA-Seq Kit
282 (PerkinElmer) barcoded using NEXTFLEX® UDI Barcodes (PerkinElmer). As a result, 70 bloodmeal libraries
283 were sequenced, as well as 6 PCR negative control and 3 extraction negatives on a NovaSeq 6000 (Illumina),
284 with 2 Gb requested per sample.

285 Sequence data were analysed by identifying poor-quality reads using Rcorrector (Song & Florea, 2015) and
286 removed with TrimGalore v0.6.5 (Krueger 2019). De novo assembly was performed on the remaining
287 sequence reads using Trinity v2.8.6 (Grabherr et al., 2011). The resulting contigs were filtered to be
288 between 400-480bp and analysed using BLASTN (Altschul et al. 1990) against the nucleotide database
289 publicly available on the National Centre for Biotechnology Information (NCBI) website. The resulting hits
290 were filtered to exclude those sequences which had < 97% sequence identity to the database. Contigs
291 identified as a host bloodmeal were confirmed by mapping raw reads using BWA-MEM v0.7.17 (Li &
292 Durbin, 2009). Sequence reads were submitted to Genbank (Table S2).

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Mosquito phylogenetic analysis

Phylogenetic analysis was performed on trimmed consensus regions of *Ae. notoscriptus* COI. The substitution model was selected using jModelTest2 v2.1.10, with the topology taking the best of nearest neighbour interchange, subtree pruning and regrafting [36]. The most appropriate substitution model was selected based on the Akaike information criterion. Maximum-likelihood trees were constructed in PhyML v3.3.2 with 1,000 bootstrap replicates; the gamma distribution parameter was used to estimate rate variation across sites [37]. The Hasegawa-Kishino-Yano (HKY) substitution model was selected for the COI tree.

Geographical data acquisition and spatial cluster analysis

The population map was created in qGIS v2.18.20 [38], using a 1 km^2 population grid [39] with 2011 Victorian mesh block data. Since 2004, Buruli ulcer has been a notifiable condition in Victoria, requiring Health Department reporting by doctors and laboratories. De-identified case notification data of Buruli ulcer patients who had laboratory-confirmed *M. ulcerans* infection and who lived on the Mornington Peninsula during the years 2019-2020 were provided by the Victorian Department of Health. The cases were defined as patients with a clinical lesion that was diagnosed using IS2404 qPCR and culture [16]. To conduct high-resolution spatial analyses, the data were aggregated at the mesh block level, the smallest geographical census units which typically contain 30-60 dwellings. The 2011 Victorian mesh block boundaries and the Victorian mesh block census population counts datasets were obtained from the Australian Bureau of Statistics (ABS) website [40]. The datasets were joined using the unique mesh block IDs using the QGIS v.3.16.7 geographic information system software [41]. The latitude and longitude (projected in GDA94) were derived from the centroids of the mesh block polygon. The dataset was then down-sampled to include only the Mornington Peninsula study area, specifically the Point Nepean and Rosebud-McCrae ABS level 2 Statistical Area.

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SaTScan version 10.1.0 [42] was utilised to identify spatial clusters among trapped mosquitoes positive for *M. ulcerans*, possum excreta positive for *M. ulcerans*, and human Buruli ulcer cases. The software searches for instances where the observed number of spatial incidences exceeds the expected number within a circular window of varying size across a defined study area. A log likelihood ratio statistic is calculated for each window by comparing the number of observed and expected cases inside and outside the circle against the assumption of randomly distributed cases. In addition to the most likely cluster, there are usually secondary clusters with almost as high likelihood that substantially overlap with the primary clusters. These secondary clusters can be indicative of sub-clusters within the primary cluster or potentially distinct clusters that are spatially adjacent to the primary cluster. The Mornington Peninsula surveillance data used in these analyses consisted of trapped mosquitoes (177 traps screened for IS2404 collected: 12/11/19 to 20/03/20), *M. ulcerans* detected in possum excreta collected during the summer (December to February) of 2019 using data from a previous study [43] and notified human Buruli ulcer cases from the study area in the years 2019-2020. The use of possum excreta collected during the summer was appropriate as Buruli ulcer transmission is most likely to occur during that time of year [44,45]. For each of the three data sources (trapped mosquitoes, possum excreta, and human cases), the null hypothesis assumes that *M. ulcerans* detections or Buruli ulcer cases are uniformly distributed across the study area, where the alternative hypothesis suggests that there may be certain locations with higher rates than expected if the risk was evenly distributed. Primary and secondary clusters were accepted only if the secondary clusters did not overlap with previously reported clusters with a higher likelihood. Given that the trapped mosquito and possum excreta IS2404 PCR results were binary (positive or negative), a Bernoulli model was used to scan for spatial clusters, with the maximum cluster size set to 50% of the population size. The human Buruli ulcer case data aggregated at the mesh block level varied in number, with some mesh blocks having zero cases and others having one or more. We applied the Poisson probability model to the notified Buruli ulcer case counts, using a background population at risk that was derived from the 2011 population census. The maximum cluster size was limited to 14,481 individuals, 10% of the total population at risk. To determine the likelihood of a triple-cluster overlap between the three SaTScan analyses occurring by chance, we conducted a

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permutation test (https://github.com/abuultjens/BU-3-way-SatScan). In each of 10,000 iterations, the geographical coordinates for each variable were randomly shuffled. The number of SaTScan clusters with triple overlap was determined using the 'sf' package [46] in the R statistical programming language [47].

Results

Mosquito surveys of the Mornington Peninsula

A primary goal of this research was to test the hypothesis that mosquitoes are associated with *M. ulcerans* transmission, as reflected by IS2404 PCR positivity at a certain frequency in areas of the Mornington Peninsula with human Buruli ulcer cases. A total of 73,580 mosquitoes were collected, consisting of 72,263 females (Table 2) and 1,317 males (Table S2). The majority (90%) of these insects were collected in the large survey of 2019-2020 (Datafile S2). Across all five surveys, 26 different mosquito species were collected covering six genera (Table 2). The most dominant species identified during the 2019-2020 survey was *Cx. molestus* accounting for 42% of mosquitoes, followed by *Ae. notoscriptus* (35%) and *Cx. australicus* (8%). Twenty-three other species comprised the remaining 15% of mosquitos. The distribution of the two dominant species across the survey area is shown in Figure 2. This mapping revealed an asymmetric distribution of each species, with *Ae. notoscriptus* dominant to the eastern end and *Cx. molestus* dominant towards the western end of the Peninsula (Figure 2).

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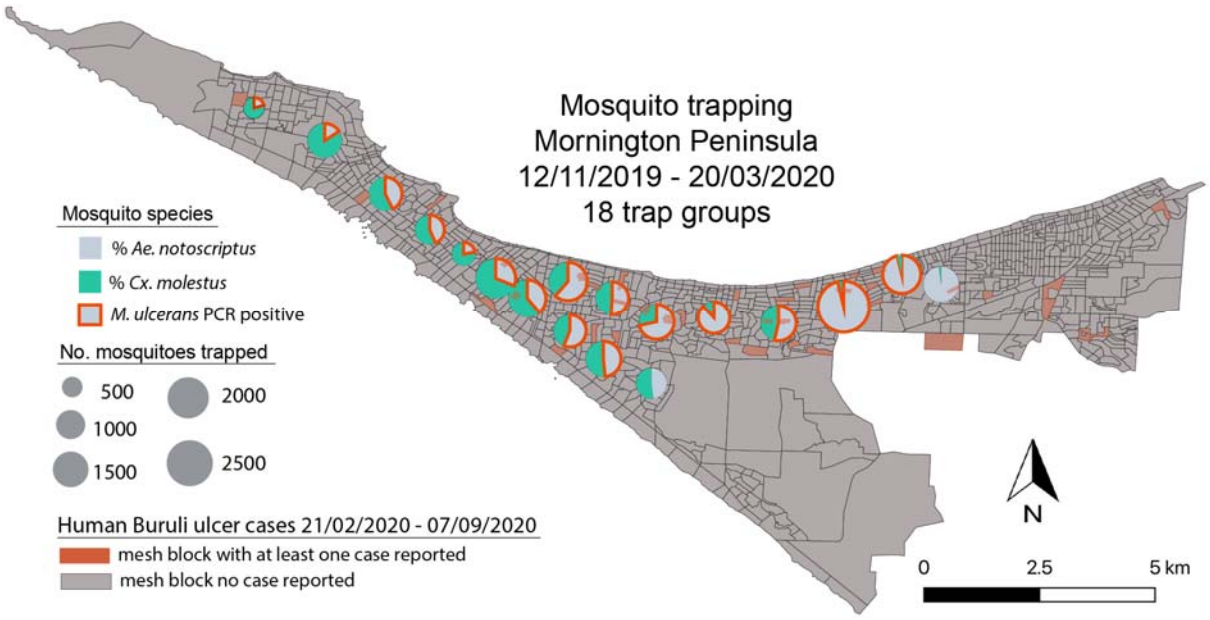

Figure 2. Dominant mosquito species distribution across the Mornington Peninsula. *Map showing the proportional distribution of the two dominant mosquito species trapped during 2019/2020. The pie charts are an aggregation of the 180 different trap sites. Trap groups containing mosquitoes that were PCR positive for M. ulcerans are also indicated. Shown too, are the meshblock statistical areas, with those in red containing at least one human* Buruli ulcer *case diagnosed in 2019-2020.*

M. ulcerans *PCR positive mosquitoes were predominantly* Aedes notoscriptus

The IS2404 qPCR assay was used to infer the presence of *M. ulcerans* in association with mosquitoes. Of the 73,580 mosquitoes trapped across all years, 18,610 (25%) were screened by IS2404 qPCR for the presence of *M. ulcerans* (Table 2). *Mycobacterium ulcerans* qPCR positives were observed in 53 pools or individuals of *Ae. notoscriptus*, with detections occurring in each year across all survey events (Figure 2, Table 2). Only one other mosquito species tested IS*2404* positive, which was a two-insect pool of *Ae. camptorhynchus* (Table 3). The positive association between *M. ulcerans* and *Ae. notoscriptus* compared to other

375 mosquitoes, in particular the other abundant mosquito species, *Culex molestus*, was highly significant (P
376 <0.0001, Fisher's exact test).

377

378 Subsets of *Ae. notoscriptus* were screened individually to better estimate the prevalence of *M. ulcerans*
379 positive mosquitoes in this species. Individually tested mosquitoes included all of the 2017/2018 collection
380 (367/367 individuals with 8 positives), 448/4,330 of the 2019/2020 collection with 3 positives, and all 1,247
381 individuals of the 2021 collection with 7 positives (Table S3). Thus, based on screening individual insects,
382 18/2,062 (0.87%) *Ae. notoscriptus* were IS2404 PCR positive. All other *Ae. notoscriptus* and other mosquito
383 species in this study were screened in pools (up to 15 insects per pool) with 24/747 *Ae. notoscriptus* pools
384 (3%), PCR positive (Table S4).

385

386 **Table 2. Female mosquitoes trapped on the Mornington Peninsula and screened by IS2404 PCR.**

Species	No. of mosquitoes/No. mono-species pools screened for *M.ulcerans* (No. pools positive for *M. ulcerans*, IS*2404**)					
	Dec 2016 - Apr 2017	Nov 2017- May 2018	Dec 2018- May 2019	Nov 2019- Mar 2020	Feb 2021- Mar 2021	Total
Ae. alboannulatus	9/9	4/4	3/3	183/96	21/21	220/133
Ae. australis	0	0	0	1/0	0	1/0
Ae. bancroftianus	0	0	0	3/2	0	3/2
Ae. camptorhynchus	258/258	5/5	0	121/64 **(1)**	8/8	392/335 **(1)**
Ae. clelandi	0	0	0	41/32	0	41/32
Ae. flavifrons	1/1	0	0	31/26	13/13	45/40
Ae. imperfectus	0	0	0	266/226	0	226/226
Ae. notoscriptus	174/174 **(2)**	367/367 **(8)**	1,793/1,779 **(15)**	23,360/4,330 **(14)**	1,247/1,247 **(7)**	26,941/7,897 **(46)**
Ae. rubrithorax	9/9	27/27	230/230	1,402/335	45/45	1,713/646
Ae. sagax	0	0	0	2/2	0	2/2
Ae. theobaldi	0	0	0	1/1	0	1/1
Ae. vigilax	0	0	0	2/0	0	2/0
Ae. vittiger	0	0	0	2/0	0	2/0
An. annulipes	1/1	24/24	4/4	751/376	1/1	781/406
An. atratipes	0	0	0	2/0	0	2/0

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Cq. linealis	89/89	142/142	2/2	1,254/258	11/11	1,498/502
Cu. inconspicua	0	0	0	2/2	3/3	5/5
Cx. annulirostris	0	3/3	0	42/1	0	45/4
Cx. australicus	3/3	198/198	17/17	4,910/534	33/33	5,161/785
Cx. cylindricus	0	0	2/2	160/81	0	162/83
Cx. globocoxitus	0	24/24	0	3,207/854	9/9	3,240/887
Cx. molestus	61/61	29/29	384/377	28,107/3,308	1,465/1,465	30,046/5,240
Cx. orbostiensis	0	0	0	131/69	0	131/69
Cx. quinquefasciatus	3/3	257/257	37/37	1,098/401	163/163	1,558/861
Tp. atripes	0	0	0	18/0	12/12	30/12
Tp. tasmaniensis	0	0	0	15/13	0	15/13
Total	**608/608 (2)**	**1,080/1,080 (8)**	**2,512/2,451 (15)**	**65,112/11,011 (15)**	**3,031/3,031 (7)**	**72,263/18,181 (47)**

* pool size ranged from 1 individual up to 15 individuals

Of the 46 *Ae. notoscriptus* that tested positive for IS2404, 26 (56%) were confirmed positive for IS2606 and 31 (67%) for KR, with 24 (52%) pools positive by all three qPCR assays (Table S3). Of the 46 IS2404 positive *Ae. notoscriptus*, eight (17%) pools were not tested using the IS2606 assay and one (2%) pool using the KR assay due to limited template DNA available. The average Ct value for IS2404 positive *Ae. notoscriptus* was 36.00 (range 29.74-39.65). The average Ct value for IS2606 was 37.73 (range 32.49-45.00), and the average Ct value for KR was 34.19 (range 28.52-39.26). With reference to an IS2404 qPCR standard curve (Figure S2), we estimated the *M. ulcerans* burden per mosquito. The mean bacterial genome equivalents (GE) per insect was 294 GE (range: 11 – 4200) (Table S3). The MLE of estimated infection rate for all *Ae. notoscriptus* was 5.88 based on the IS2404 *M. ulcerans* qPCR-positive mosquitoes per 1,000 tested (95% CI 4.37-7.76) for all *Ae. notoscriptus* tested over the years and 2.96 for *Ae. camptorhynchus* (95% CI 0.17-14.19) (Table 3).

IS2404 PCR positive Ae. notoscriptus and possum excreta yield M. ulcerans genomic SNP profiles that match human patient isolates

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402 Genomic epidemiological studies have shown there are unique SNP signatures associated with *M. ulcerans*
403 clinical isolates from specific endemic areas in southeastern Australia [48]. To test if the *M. ulcerans* genotype
404 present in mosquitoes from our study area matched that found in possums and human Buruli ulcer cases in
405 the same region, we conducted genome sequence enrichment using *M. ulcerans* custom RNA baits
406 (Datafile S1). This method facilitates whole genome sequencing of target pathogen genomes in complex
407 microbial samples or when competing background DNA precludes sequencing the target directly. As
408 reported in other pathogen sequence enrichment studies, we observed decreasing genome sequence
409 recovery with decreasing pathogen load, as indicated by increasing IS2404 Ct values (Figure 3A) [49]. While
410 DNA sequence reads were obtained from three mosquitoes, only DNA from one *Ae. notoscriptus* extract
411 (sample ID: 5675, Ct 32.66) had a high enough concentration of *M. ulcerans* DNA to yield sufficient reads
412 for SNP-calling following read mapping. Nevertheless, the fact that sequence reads mapped across the
413 length of the *M. ulcerans* chromosome for all three insects, despite low coverage for two of them, confirms
414 the presence of the pathogen on (or in) *Ae. notoscriptus* (Figure 3B). We also included sequence capture
415 enrichment results for two IS2404 positive possum excreta specimens, collected as part of a large field
416 survey of *M. ulcerans* in Australian native possums in the region [43]. More than 500,000 reads were obtained
417 from these samples (primary IS2404 Ct values <23). We then inferred a phylogeny, using a 5.6Mbp core-
418 genome alignment that identified 23 SNP positions from mapping the reads from these possum excreta
419 samples, the *Ae. notoscriptus* sample (ID: 5675) and a reference panel of sequence read sets from 36
420 published *M. ulcerans* genomes from southeastern Australia [48]. These 36 genomes were selected because
421 they span the population structure of *M. ulcerans* in this locale (Table S1). The resulting phylogeny (tree
422 tips aligned by sample/isolate geographic origin) showed possum and mosquito *M. ulcerans* genotypes co-
423 clustering with each other and with human *M. ulcerans* isolates from the Mornington Peninsula (north)
424 area, with no SNP differences between them.

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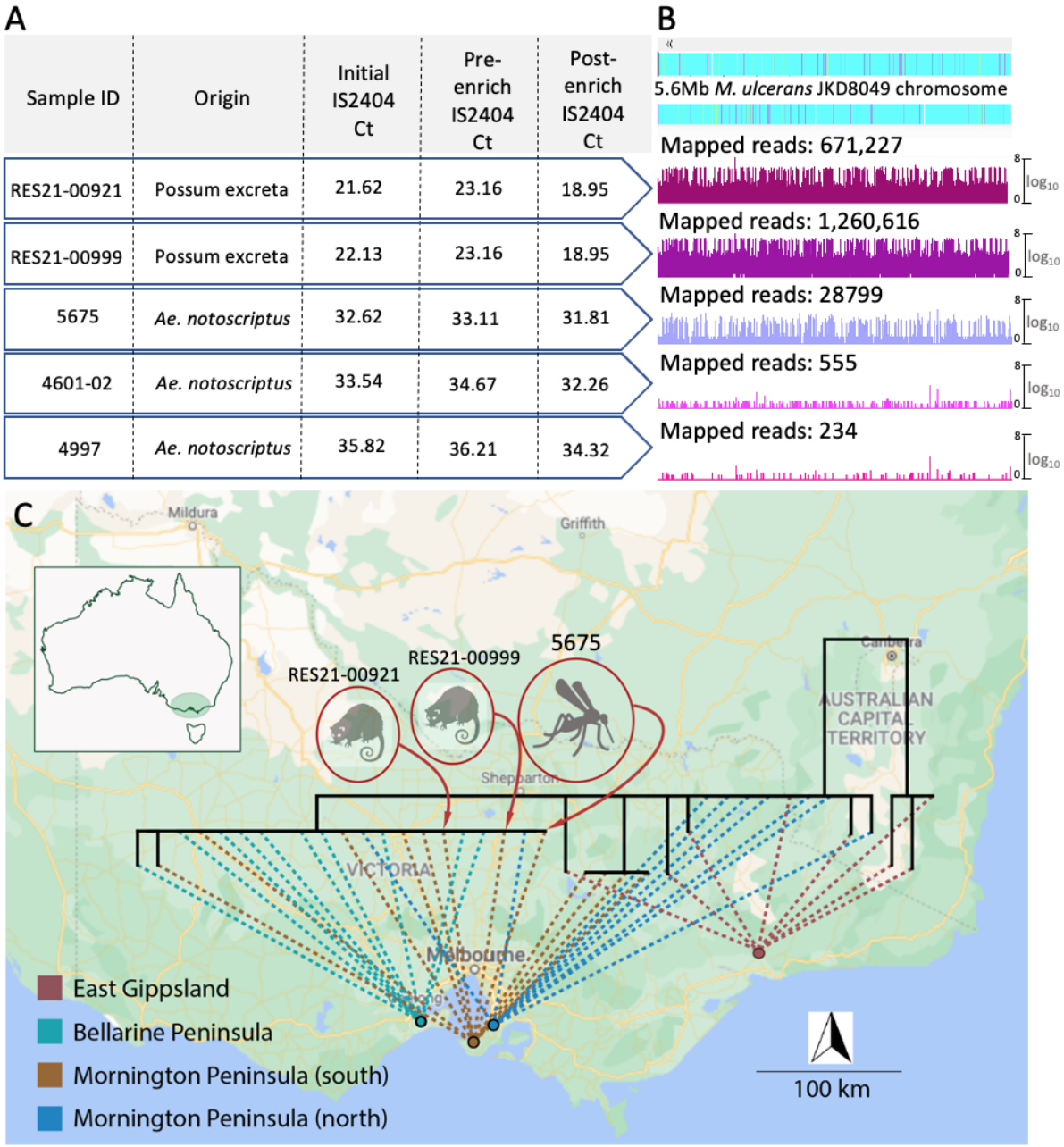

Figure 3. Comparison of M. ulcerans genome from human Buruli ulcer cases compared with sequences recovered from possum excreta and mosquitoes on the Mornington Peninsula. *(A) Summary of IS2404 qPCR screening of primary samples and the sequence capture libraries pre- and post-enrichment. Note that possum excreta were enriched as barcoded sequence library pools so share the same pre- and post-enrichment Ct values; (B) Artemis coverage plots showing sequence capture reads mapped to the M. ulcerans JKD8409 chromosome from possum excreta samples and three qPCR positive mosquitoes; (C) Maximum-likelihood phylogeny inferred from an alignment of 23 core-genome SNPs using reads mapped to*

433 *the JKD8049 reference chromosome, and with tips aligned with environmental sample or patient origin.*
434 *Dataset includes reads from the five environmental samples listed in panel (A) and a reference collection of*
435 *36* M. ulcerans *genomes representing the genomic diversity of the bacterial population in southeastern*
436 *Australia* [48]*. The shortest vertical branch length represents a single SNP difference.*

437

438 *Genetic characterisation of* Ae. notoscriptus *collected on the*
439 *Mornington Peninsula*

440 To assess if *M. ulcerans* presence was associated with a particular clade of *Ae. notoscriptus* on the
441 Mornington Peninsula we compared COI gene sequences for 18 *M. ulcerans* positive (confirmed by all
442 IS2404, IS2606 and KR qPCR assays) *Ae. notoscriptus* and 19 *M. ulcerans* negative *Ae. notoscriptus* (Figure
443 2). Based on the COI phylogenetic tree, *Ae. notoscriptus* from the Mornington Peninsula spanned the three
444 previously identified clades and there was no association between *M. ulcerans* and a particular mosquito
445 lineage (Figure S3) [35].

446

447 **Table 3. The number *of M. ulcerans* positive pools tested by insertion sequence IS2404 qPCR and**
448 **Maximum likelihood estimate (MLE) of *M. ulcerans* per 1,000 mosquitoes trapped in the Mornington**
449 **Peninsula.**

Species	No. mosquitoes	No. positive/No. pools tested	MLE‡	95% Confidence interval
Ae. alboannulatus	133	0/97	0	0-27.71
Ae. bancroftianus	2	0/2	0	0-657.62
Ae. camptorhynchus	335	1/85	2.96	0.17-14.19
Ae. clelandi	32	0/3	0	0-67.97
Ae. flavifrons	40	0/16	0	0-78.10
Ae. imperfectus	226	0/27	0	0-14.96
Ae. notoscriptus	7897	46/2937	5.88	4.37-7.76
Ae. rubithorax	646	0/220	0	0-5.84

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Ae. sagax	2	0/1	0	0-545.52
Ae. theobaldi	1	0/1	0	0-793.45
An. annulipes	406	0/148	0	0-9.16
Cq. linealis	502	0/139	0	0-7.43
Cu. Inconspicua	5	0/4	0	0-408.11
Cx. annulirostris	4	0/3	0	0-450.75
Cx. australicus	785	0/150	0	0-4.76
Cx. cylindricus	83	0/38	0	0-41.60
Cx. globocoxitus	887	0/150	0	0-4.22
Cx. molestus	5240	0/609	0	0-0.73
Cx. orbostiensis	69	0/9	0	0-42.95
Cx. quinquefasciatus	861	0/237	0	0-4.39
Tp. atripes	12	0/9	0	0-230.35
Tp. tasmaniensis	13	0/10	0	0-215.26

450 [‡]MLE bias was corrected when ≥ 1 pool was positive.

451

452 ## *IS2404 PCR screening of arthropods other than mosquitoes*

453 We also investigated the association of *M. ulcerans* with arthropods other than mosquitoes in the region
454 using yellow sticky traps (YST) and sticky ovitraps (SO). A total of 21,000 specimens were collected and
455 sorted from 278 YST and 33 SO traps. We were able to classify 2,696 as insects that may bite or pierce
456 human skin (Table 4). Flies were the largest group collected on the sticky traps. YSTs collected more insects
457 than the SOs, but this was proportional to the number of traps set (Table 4). Of the 2,696 insects screened
458 by PCR, only two flies tested positive for *M. ulcerans* (Table S3). Both flies had high Ct values for IS2404 (Ct
459 35.92 and 37.54) and each sample was only confirmed for either IS2606 or KR assay (Table S3), but not
460 with all three assays. Both insects were blowflies (*Calliphora hilli* Patton) based on morphology and
461 sequencing of the COI region with sequences having >99.86 nt identity to *C. hilli* (Table S1).

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463 **Table 4. Number of arthropods collected on Yellow Sticky Traps (YST) and Sticky Ovitraps (SO) on the**

464 **Mornington Peninsula that might bite or pierce human skin.**

Trap	Ants	Biting midges	Flies	Mosquitoes	Spiders	Ticks	Wasps	Total
YST	28	58	1,562	10	76	21	562	2,317
SO	70	1	144	11	141	4	8	379
Total	**98**	**59**	**1,706**	**21**	**217**	**25**	**570**	**2,696**

465

466

467 ## *Mosquito bloodmeal analysis*

468 Of the mosquitoes collected, a proportion of individuals identified as being engorged (bloodfed) were PCR
469 screened and the resulting amplicon sequenced for Cytochrome B (CytB) to identify host bloodmeal
470 sources. A total of 90 individual engorged mosquitoes were extracted, with 70 DNA preparations producing
471 high quality amplicons that were of sufficient concentration to permit Illumina amplicon sequencing. After
472 quality filtering, 36 individuals were identified as having had a recent bloodmeal: 14 *Cx. molestus*, 13 *Ae.*
473 *notoscriptus*, 2 *Ae. rubrithorax*, 2 *Cx. globocoxitus*, 2 *Cx. quinquefasciatus*, 2 *Cq. linealis* and 1 *Cx.*
474 *australicus*. Of the bloodmeals detected, common ringtail possum was the most commonly identified with
475 20 detections across the 36 samples, followed by 17 blackbirds, 13 humans, 11 red wattle birds, and 5 little
476 wattle birds; a further 16 bloodmeals identified 10 minor host species (Figure 4, Figure S4). Dual
477 bloodmeals were commonly identified, with 55% (20/36) of individuals having more than one bloodmeal
478 identified. Additionally, two mosquitoes had evidence of three different bloodmeal sources within an
479 individual insect. Three individuals (2 x *Ae. notoscriptus* and 1 x *Ae. rubrithorax*) were also identified as
480 having dual bloodmeals from ringtail possum and human origins (Figure 4).

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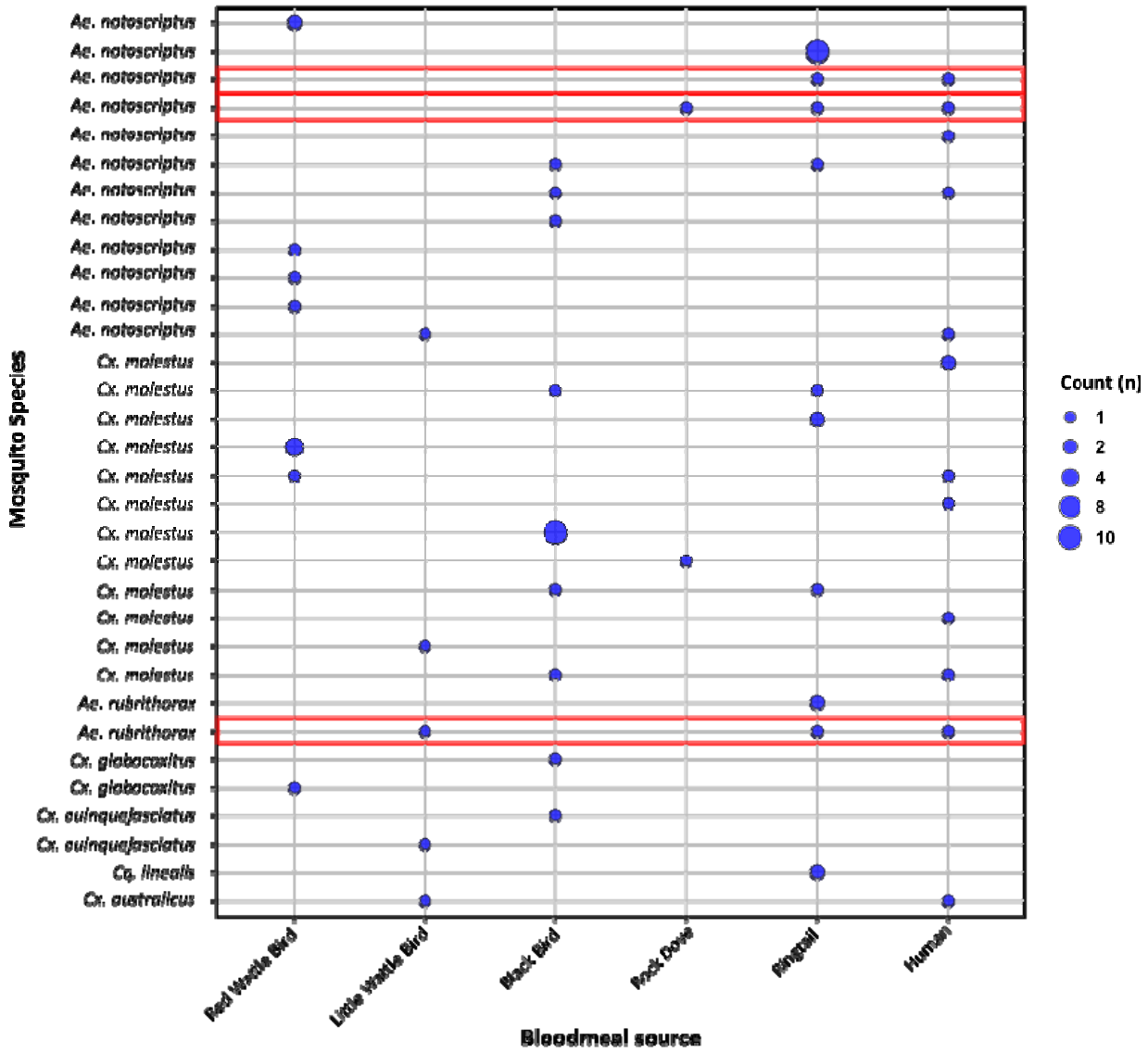

Figure 4. Mosquito blood meal analysis. *Summary of cytochrome B (Cytb) gene sequences from the 36 bloodfed mosquitoes to identify host bloodmeal sources . A blue circle indicates positive for host blood source; the larger the circle, the more individual mosquitoes with an identical bloodmeal profile. Red boxes indicate individual mosquitoes which have dual bloodmeals for both humans and ringtail possum sources.*

Spatial clustering links M. ulcerans *positive mosquitoes with positive possum excreta and human Buruli ulcer cases*

In our previous research, we demonstrated a spatial association between possum excreta containing *M. ulcerans*, as detected by structured surveys, and clusters of human Buruli ulcer cases [50]. To expand on this finding, we investigated whether further spatial clustering associations could be detected by analysing qualitative qPCR (IS2404) data of trapped mosquitoes using SaTScan. Here, three separate analyses were conducted: (i) human Buruli ulcer case data from individuals reported to have acquired the disease in the study area during 2019-2020, (ii) qPCR data from possum excreta collected in a previous investigation (2018-2019), and (iii) qPCR data from trapped mosquitoes (2019-2020). These analyses identified a single mosquito cluster, four possum clusters and six human Buruli ulcer clusters (Figure 4A-B). Notably, one human Buruli ulcer cluster and two possum excreta clusters had higher numbers of observed Buruli ulcer cases or *M. ulcerans* detections, respectively, than expected if uniformly distributed (p-value <0.05) (Figure 4B). Importantly, the analyses revealed an instance of triple cluster overlap (mosquito/possum excreta/human) in the Mornington Peninsula suburb of Rye, where all three SaTScan analyses had an overlapping cluster (Figure 4A).

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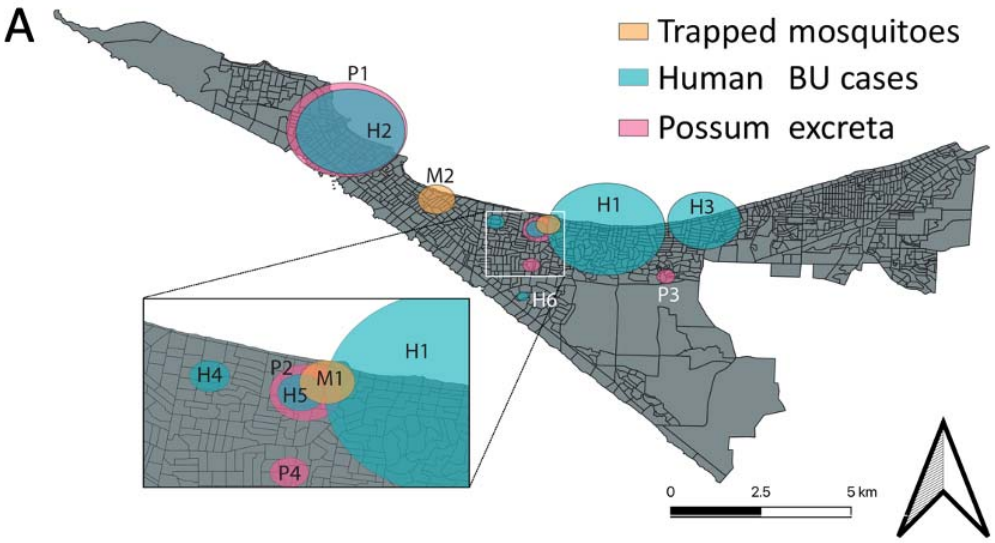

B

	CLUSTER	P_VALUE	OBSERVED	EXPECTED	LLR
	M1	0.915	2	0.26	4.16
	M2	0.472	3	0.52	4.17
	H1	2.06E-05*	31	9.35	17.75
	H2	0.148	13	3.56	7.80
	H3	0.265	15	4.95	7.03
	H4	0.702	4	0.42	5.53
	H5	0.820	4	0.46	5.14
	H6	0.999	2	0.12	3.74
	P1	9.21E-05*	41	17.54	17.05
	P2	0.002*	9	1.350	13.58
	P3	0.804	4	0.562	6.42
	P4	0.997	3	0.450	4.46

Figure 4. *SaTScan analyses of trapped mosquitoes, possum excreta, and human* Buruli ulcer *cases on the Mornington Peninsula, revealing spatial clustering. A) Map illustrating the clusters identified by the three separate SaTScan analyses: (I) trapped mosquitoes (177 traps screened for IS2404 collected: 12.11.19 to 20.03.20), (ii) M. ulcerans detected in possum scats collected during the summer of 2019 (Dec 2018 – Feb 2019) using data from a previous study, and (iii) notified human* Buruli ulcer *cases from the study area in the years 2019-2020. The zoomed insert highlights an instance where all three analyses had overlapping clusters in the suburb of Rye. B) Table summarising the SaTScan results for all identified clusters. Log likelihood ratio is abbreviated as "LLR." Clusters with p-values <0.05 are marked with asterisks.*

The results of our permutation test to explore the probability of these three categories (mosquitoes, possums and humans) overlapping showed that a triple-cluster overlap occurred with randomly rearranged location labels at a low frequency of 8.9%. This analysis adds support to a causal relationship between the presence of *M. ulcerans* in possums and mosquitoes and humans contracting Buruli ulcer in the Mornington Peninsula suburb of Rye.

Discussion

Using insect field surveys augmented with genomics we have addressed *Barnett Criteria* 1 – 3 (Table 1) to add to a hierarchy of evidence indicting mosquitoes as principal vectors in the spread of *M. ulcerans* from the environment to humans [3,15,17,21,22]. Our survey area was the Mornington Peninsula, where human Buruli ulcer cases have increased progressively over the past 20 years [12]. Our key findings were that *M. ulcerans* was almost exclusively associated with one mosquito species in this region, *Aedes notoscriptus*, a mammalian host feeder, at a frequency of 0.87%. The estimated pathogen burden in each insect (Table S3) was consistent with the reported low infectious dose for *M. ulcerans* [22,51]. Pathogen genomics provided confirmation of a linked transmission chain between mosquitoes, native possums and humans in this region. The metabarcoding bloodmeal analysis of trapped mosquitoes revealed dual feeding by individual mosquitoes on both possums and humans, providing an example of a potential transmission pathway between infected possums and humans. Finally, spatial clustering analysis showed a striking overlap between clusters of possums shedding *M. ulcerans*, mosquitoes harbouring *M. ulcerans* and human Buruli ulcer cases, reinforcing the importance of possums and mosquitoes in the spread of *M. ulcerans* to humans.

Indications that invertebrates might be playing a role in the spread of Buruli ulcer first came from field surveys in West Africa in the late 1990s, where surveys in Benin identified water bugs in the genera *Naucoris* and *Diplonychus* as IS2404 PCR positive for *M. ulcerans* [15,16,52]. Subsequent culture isolation of *M.*

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535 *ulcerans* from an aquatic insect in Benin (*Gerris* sp.) and potentially from *Naucoris* sp. in neighbouring Cote
536 d'Ivoire further suggested a role for water bugs in disease transmission, at least in west Africa [53,54]. A role
537 for mosquitoes in the transmission of *M. ulcerans* anywhere was first revealed from entomological surveys
538 in the mid-2000s on the Bellarine Peninsula and then more recently in Far North Queensland [15,21,55]. Other
539 observations that supported a role for mosquitoes in transmission included an analysis of the Buruli ulcer
540 lesion location on the human body, which included more than 600 human Buruli ulcer cases, and showed a
541 preponderance of lesions on ankles, elbows and backs of legs. These areas are frequently exposed and
542 insects may preferentially bloodfeed there [56]. Laboratory studies under one transmission scenario have also
543 shown the competence of *Ae. notoscriptus* as mechanical vectors of *M. ulcerans* (Barnett criteria No. 4,
544 Table 1) [22].

545

546 Of the ~18,600 mosquitoes tested by IS2404 PCR from five different survey periods in the present study,
547 *Ae. notoscriptus* was consistently positively associated with *M. ulcerans*. Where and how *M. ulcerans* is
548 contaminating (or infecting) these mosquitoes remains to be determined. However, we made the
549 somewhat unexpected observation that the most abundant mosquito species trapped on the Mornington
550 Peninsula, *Culex molestus,* which predominately feeds on avian species [57] was consistently IS2404 PCR
551 negative. This observation might be explained by a difference in the ecology of these two mosquito species,
552 such as how (or where) they are encountering *M. ulcerans.* The apparent *Ae. notoscriptus*/*M. ulcerans*
553 tropism might also indicate a specific biological interaction between this insect and the mycobacterium, or
554 alternatively, a *Cx. molestus* antagonism to mycobacterial carriage.

555 MLE is a parameter used in vector ecology to estimate the proportion of infected mosquitoes that are
556 pathogen positive, based on a pooling-based screening assay, where the proportion is the parameter of a
557 binomial distribution [58] . The MLE of *Ae. notoscriptus* over all 5 years of our surveys was 5.88 *M. ulcerans*
558 positive mosquitoes/1,000 tested (95% CI 4.37-7.76). The MLE for the only other positive mosquito species,
559 *Ae. camptorhynchus* (consisting of two insects that tested positive for *M. ulcerans*), was 2.96/1,000 tested
560 (95% CI 0.17-14.19). These estimates are consistent with those previously reported for *Ae. camptorhynchus*

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561 (10,558 mosquitoes tested, MLE 3.98 per 1,000) and *Ae. notoscriptus* (221 mosquitoes tested, MLE of 4.47
562 per 1,000) during the Bellarine Peninsula survey of that Buruli ulcer endemic area [15]. Interestingly, these
563 values are significantly higher than the MLE values from an *M. ulcerans* mosquito survey conducted in
564 tropical far north Queensland, Australia which reported an MLE value of 0.13 /1,000 (95% CI 0.01–0.61) for
565 different species of mosquito [55]. To note though, there were few reported cases of Buruli ulcer in this area
566 of Queensland during the field survey period, consistent with the suggestion that mosquito surveys for *M.*
567 *ulcerans* will be useful for predicting Buruli ulcer risk in humans.

568

569 In tropical far north Queensland, Australia, host-seeking mosquitoes that harboured *M. ulcerans* collected
570 through similar carbon-dioxide light traps to those used in this study were within the genera *Verallina*,
571 *Coquillettidia* and *Mansonia* [55]. These comparisons highlight that the mosquitoes encountering *M. ulcerans*
572 appear to be region/environment specific, and these observations support the idea that mosquitoes are
573 mechanical vectors of *M. ulcerans*. The major mosquito species associated with detection of *M. ulcerans* in
574 Victoria, *Ae. notoscriptus, Ae. camptorhynchus*, *An. annulipes* and *Cq. linealis,* are all diverse opportunistic
575 feeders.

576

577 To explore host sources for mosquitoes trapped in our study, we used metagenomic amplicon sequencing
578 of engorged mosquitoes [59-64]. Of the 90 blood-fed mosquitoes processed, 36 individual insects successfully
579 had their host blood source identified. The 54 bloodmeals that were unidentified were probably due to
580 degradation of the host DNA that occurs approximately 36 hours post-feeding [65]. As the mosquitoes
581 analysed in this study were collected from baited mosquito traps, the exact time post-feeding they were
582 collected is unknown. Of the host blood sources identified, common ringtail possum was the most
583 commonly identified bloodmeal, followed by birds and humans, with dual bloodmeals observed in 55% of
584 mosquitoes (Fig. 4, Fig. S4). Most notably, three insects were determined to have bloodmeals from both
585 common ringtail possums and humans. These insects consisted of two *Ae. notoscriptus* and one *Ae.*
586 *rubrithorax*. Common ringtail possums are major wildlife reservoirs of *M. ulcerans* [11], and in this study we

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587 identified *M. ulcerans* in frequent association with *Ae. notoscriptus*. The identification of these dual
588 bloodmeals provides evidence that *Ae. notoscriptus* mosquitoes are bloodfeeding between possums and
589 humans within a relatively short timeframe. Dual bloodmeals may provide an opportunity for a mechanical
590 transmission event of *M. ulcerans* from a possum with a Buruli ulcer teeming with *M. ulcerans,* from which
591 a mosquito bloodfeeds and then moves to a nearby human. However, the observed positive association
592 specifically between *Ae. notoscriptus* and *M. ulcerans* is not explained by our bloodmeal analysis, which
593 showed several other mosquito species that were not *M. ulcerans* positive, such as *Cx. molestus*, *Ae.*
594 *rubrithorax* and *Cq. Linealis,* also fed on possums. An alternative source of *M. ulcerans* acquisition for *Ae.*
595 *notoscriptus* might be from possum excreta contaminating the *Ae. notoscriptus* breeding sites, as this
596 species breeds in small artificial containers [59]. Previous studies have shown that mosquito larvae can ingest
597 *M. ulcerans* and test positive for *M. ulcerans* during the larval stage, but *M. ulcerans* is not detected in the
598 pupa or adult, which is largely thought to be a result of the larval midgut being purged [66]. However, if
599 possum excreta, which can have a high concentration of *M. ulcerans* [11], falls into these artificial breeding
600 sites, then this may provide a means to contaminate the adult *Ae. notoscriptus* during eclosion and
601 emergence on top of the water's surface.

602

603 In common with other arthropod-borne bacterial pathogens, *M. ulcerans* also has the genomic hallmarks of
604 niche adaptation [67,68]. For instance, the bacterial diseases vectored by blood-feeding arthropods, such as
605 bubonic plague, spread to humans through the bite of fleas harbouring the bacterium *Yersinia pestis* [69], the
606 tick-borne infections such as Lyme disease, Rocky Mountain Spotted Fever, ehrlichiosis (among others)
607 caused by *Borrelia burgdorferi*, *Rickettsia rickettsii* and *Ehrlichia* sp., respectively [70], or tularemia, caused by
608 infection with *Francisella tularensis* spread through the bite of infected ticks (with some subspecies also
609 spread by mosquitoes) [71-73], are all caused by bacteria that have degenerating genomes. That is, like M.
610 ulcerans, their genomes bear the distinctive hallmarks of evolutionary bottlenecks and niche-adaptation
611 (plasmid acquisition, pseudogene accumulation, insertion sequence expansion), a genomic pattern thought
612 indicative of a shared trajectory towards symbiosis with the arthropod host [74]. In addition, the absence of

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recombination and highly clonal population structure of *M. ulcerans* is aligned with the population structure of bacteria in 'closed symbiosis' with their host animal [75].

Mosquitoes were not the only arthropods from which *M. ulcerans* were detected in the Mornington Peninsula. With over 20,500 arthropods collected using sticky traps (YST and SO), this study screened other insects that might broadly act as a mechanical vector, that is, an insect that is involved in the accidental transport of a pathogen [76 22]. *M. ulcerans* was successfully identified on two of ~1,800 flies tested, identified as blowflies (*Calliphora hilli*) . Previous studies have screened other species of flies, such as March flies (Tabanidae), with no positive detections [55]. *C. hilli* is a native species occurring along the Australian east coast and in South Australia [77]. This species is a carrion breeder occurring year-round throughout Victoria, with the female laying her eggs in decaying flesh where the larvae emerge [77]. Possum carcases are readily infested by *C. hilli* [78], which may explain how this fly species is becoming contaminated with *M. ulcerans*, as possums are wildlife reservoirs of *M. ulcerans* [10]. The likelihood of *C. hilli* carrying *M. ulcerans* to humans is relatively low due to this species rarely biting humans [79].

Interestingly, all species that have tested positive for *M. ulcerans* share quite different breeding environments and feeding preferences, although most feed on humans. *Aedes camptorhynchus* and *Verrallina sp.* are saltmarsh mosquitoes [80,81], while *Ae. notoscriptus* is a freshwater container breeder occurring in close association to humans [82]. *Anopheles annulipes* and *Culex australicus* are also freshwater breeding mosquitoes [83], although *Culex australicus* typically feeds on avian species and is not considered to feed on humans [84]. This diversity in mosquito species, habitats, flight range from breeding sites and host-preference feeding raises interesting questions about how these mosquitoes are coming into contact with *M. ulcerans* and how potential vector mosquito species may change based on the study area. Additionally, the ecology of the mosquito sub-species are generally poorly understood, including *Ae. Notoscriptus,* which occurs as a series of genetically differentiated clades within Australia [35].

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639 In this study, we also deployed for the first time targeted enrichment genome capture using the Agilent
640 RNA bait system to demonstrate beyond reasonable doubt the presence of *M. ulcerans* in association with
641 mosquitoes [49]. The IS2404, IS2606 and KR PCR assays have provided robust evidence for the presence of *M.*
642 *ulcerans* in the environment, but detecting pathogen genomic DNA and using those sequences to
643 forensically dissect transmission chains by matching pathogen genome SNP profiles is a powerful
644 technological advance [49]. While the method lost sensitivity for IS2404 qPCR Ct values >32, we generated
645 sufficient genome coverage from one IS2404 PCR positive mosquito and from two possum excreta
646 specimens to show that the *M. ulcerans* genotypes of the captured genomes were identical to those
647 associated with human Buruli ulcer cases in the study area, rather than Buruli ulcer cases linked to other
648 regions (Fig. 3). These findings provide strong support for mosquitoes and possums playing a role in the
649 transmission of *M. ulcerans* in the Mornington Peninsula. These results also add to the growing body of
650 evidence demonstrating the utility of pathogen genomics in specifically identifying the sources and
651 transmission pathways of environmental pathogens and suggest that continued surveillance of *M. ulcerans*
652 genotypes in mosquitoes and possums could be useful in guiding public health interventions that seek to
653 control the spread of Buruli ulcer in the region. We are now exploring the use of genome sequence
654 enrichment to dissect the genomic epidemiology from the perspective of environmental sources, in
655 particular to track the spread of Buruli ulcer in Australian native possums across the region [50].

656

657 The SaTScan clustering analyses provide compelling evidence for the spatial association between the
658 presence of *M. ulcerans* in possums, mosquitoes, and human Buruli ulcer cases in the study area (Figure 4).
659 The triple-cluster overlap observed in our SaTScan analyses suggests that the spatial distribution of these
660 three factors is likely to be closely linked and may be contributing to the persistence of Buruli ulcer in the
661 Mornington Peninsula. Our finding of a low frequency of triple-cluster overlap among 10,000
662 randomisations, with only 8.9% of replicates showing this phenomenon, provides a level of confidence that
663 our observations have not occurred randomly. This further supports the validity of our findings and
664 reinforces the idea that the presence of *M. ulcerans* in possums and mosquitoes may play a crucial role in
665 the transmission of *M. ulcerans* to humans in the study area. Our findings highlight the importance of

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ongoing surveillance of possum and mosquito populations, which may provide critical insights into the epidemiology of Buruli ulcer in the region and inform targeted public health interventions to control the disease.

Our research has some limitations. Although we found a spatial association between the presence of *M. ulcerans* in possums and mosquitoes, and human Buruli ulcer cases, we cannot absolutely conclude causality or directionality. In addition, the very specific set of circumstances that have led to the rise of Buruli ulcer in temperate southeastern Australia restricts the generalisability of our results. One must be cautious to draw parallels with African Buruli ulcer endemic countries for instance, where a highly susceptible mammalian reservoir equivalent to the Australian native possum is yet to be identified. Further investigations are needed to better understand the underlying mechanisms that drive the association between the presence of *M. ulcerans* in these different species and the development of human Buruli ulcer cases.

Despite these caveats, our collective research over more than 15 years makes it very clear that mosquitoes are likely vectors and native possums are major wildlife reservoirs of *M. ulcerans* in southeastern Australia. Mosquito surveillance with *M. ulcerans* screening coupled with mosquito control and public health messaging to avoid mosquito bites are practical interventions that would be expected to reduce the incidence of human Buruli ulcer.

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Ethics

Ethical approval for the use in this study of de-identified human BU case location, aggregated at mesh block level, was obtained from the Victorian Government Department of Health Human Ethics Committee under HREC/54166/DHHS-2019-179235(v3), 'Spatial risk map of Buruli ulcer infection in Victoria.'.

Author Contributions

Conceptualisation PTM, JO, RF, SRC, PDRJ, JRW, AAH, KBG, TPS, SEL; Data curation PTM, KB, JO; Formal Analysis PTM, AHB, TPS; Funding acquisition SRC, PDRJ, KBG, JRW, AH, KG, TPS, SEL; Methodology PTM, AHB, KB, JCC, JRW, TPS, SEL; Writing – original draft PTM, AHB, SEL, TPS; Writing – review & editing all authors.

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