Developing molecular barcoding and egg sampling tools to underpin vector surveillance in Great Britain.

by

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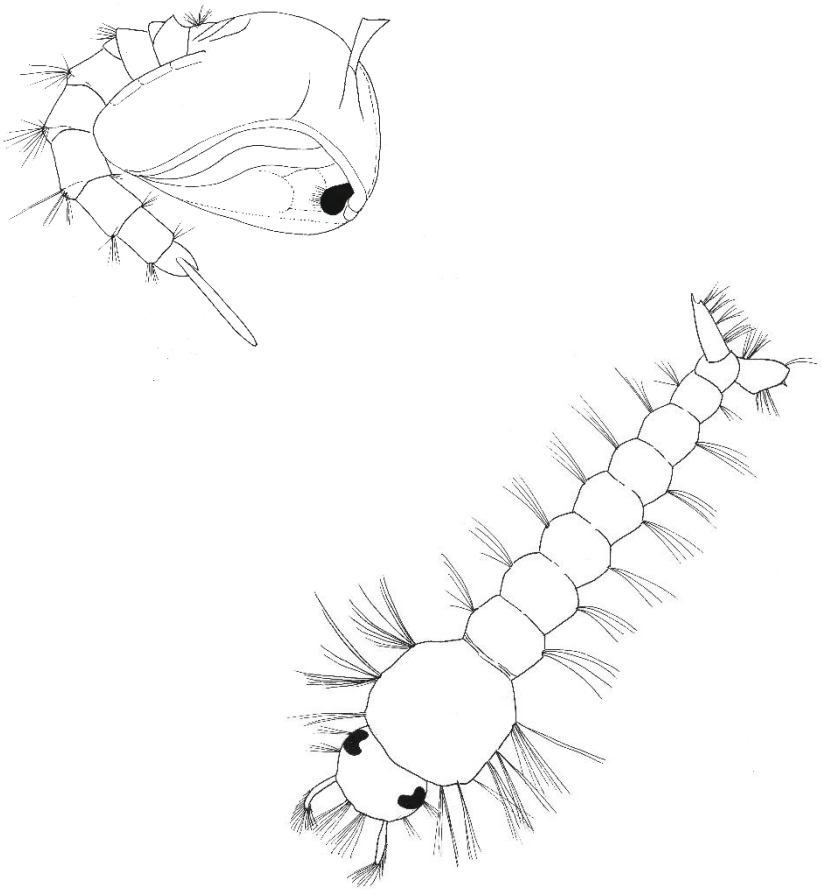
For Pauline….



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ABSTRACT

Coined as ‘a huge global burden’, the surveillance of mosquito species considered high-risk for disease transmission is currently undertaken in almost every country on the planet. The monitoring of the, approximately 36, species native to Great Britain for their potential as disease vectors (of which 14 are considered to have significant vector potential in other countries) should also be included. Similarly, there is now an urgent need for effective surveillance in Britain for the two most rapidly dispersing and globally invasive species, *Aedes* (*Stegomyia*) *albopictus* (Skuse 1895), and *Aedes* (*Stegomyia*) *aegypti* (L. 1762). The former having swept across Europe since its introduction in 1990 bringing autochthonous outbreaks of dengue fever and chikungunya virus. For the surveillance of these species to be effective, however, a solid underpinning of strategies and tools must be available.

Here we attempt to improve the surveillance of native and invasive mosquitoes by undertaking the first large scale effort to collect and characterise all the species found in Britain, by using the mitochondrial barcoding genes, cytochrome c oxidase subunit I (*COI*) and internal transcribed spacer 2 (*ITS2*). A detailed analysis of both genes, as well as descriptions of the folded secondary RNA structures of *ITS2,* are also included. To gauge the taxonomic status of British species against those from across their range, data collected was compared to sequences from other countries. We collected samples of 28 species, those unaccounted for are currently considered rare, or have a questionable presence. Examination of *COI* and *ITS2* suggests they are good predictors of species; however, high levels of intraspecific genetic variation were recorded in some (i.e. *Aedes vexans*), and too low to support others (i.e. *Ochlerotatus annulipes / cantans* and *Oc. daciae / messeae*). The addition of species range data indicated several distinct clusters within species groups over long distances, this could signify a need for taxonomic review and reclassification (i.e. *Ae. vexans, Culex territans, Culiseta alaskaensis, Cs. morsitans*). Most clear separations were between those that occupy Nearctic and Palearctic biogeographical regions.

Alarmingly, both *Ae. albopictus* and *Ae. aegypti* were discovered in England during the period of this study. A description of the post surveillance survey for the *Ae. aegypti* find is also included here. This discovery highlights a need to improve methods of detecting imported *Aedes*. Therefore, a study into the effectiveness of sticky tape as a cheap and tactile medium to test car tyres for mosquito eggs was also undertaken. This study suggests that affordable and rapid methods of surveying for invasives can be achieved, and although microscopic analysis of eggs collected in this manner is not possible, molecular identification is not prohibited. Additionally, the removal of eggs by adhesive tape does not prevent rearing of larvae for further analysis.

Underpinning the surveillance of important disease vectors, such as mosquitoes, by using the methods tested above, could be paramount to the avoidance of autochthonous transmission in Britain. However, their rapid dissemination into practice will be key to their future usefulness.

Keywords: Vector surveillance; Barcoding; *COI*; *ITS2*; RNA structures; Native species; Mosquitoes; *Aedes aegypti*; Eggs; Tyres.

ABBREVIATIONS

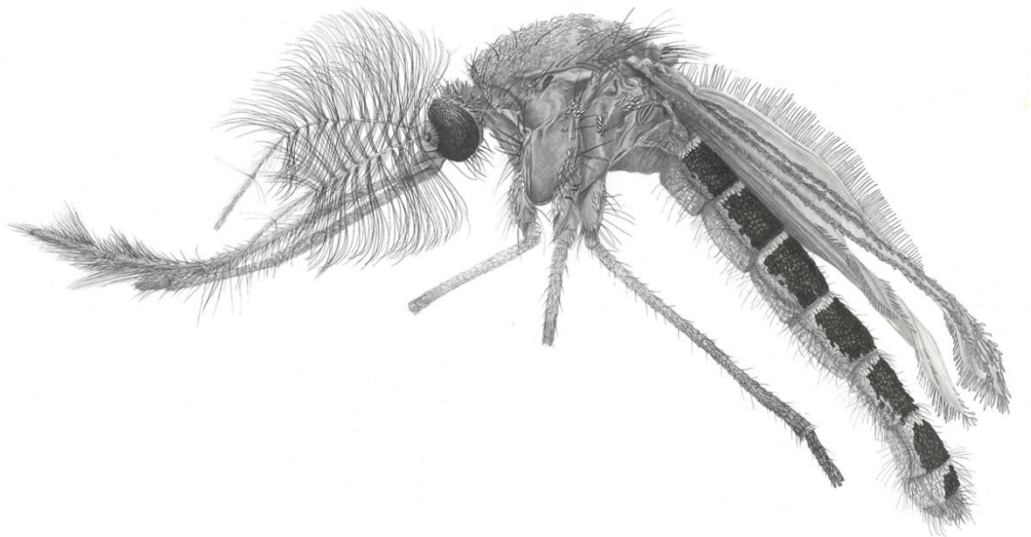
~	Approximately
+G	gamma categories
+I	invariant sites
°C	Centigrade
A	Adenine
ABGD	automated barcode gap discovery
AIMs	*Aedes* invasive mosquitoes
BINS	barcode index number system
BOLD	Barcode of Life Data System
bp	base pair
bPTP	Bayesian Poisson tree processes
BT	body tape
C	Cytosine
CBC	compensatory base changes
CHIKV	chikungunya virus
cm	Centimetres
CO_2	carbon dioxide
COI	Cytochrome c oxidase subunit I (gene)
COII	Cytochrome c oxidase subunit II (gene)
DENV	dengue fever virus
df	degrees of freedom
dH_20	deionised water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DT	duct tape
ECDC	European Centre for Disease Prevention and Control
eDNA	environmental DNA
EHU	Edge Hill University
ESE	East South East
EU	European Union
EXOSAP	exonuclease I/shrimp alkaline phosphatase
f.	Form
Fig.	Figure
FT	double sided floor tape
G	Guanine
g	Grams
GB	Great Britain
GC	guanine + cytosine
GTM	general time reversible model
HF	high-fidelity
INKV	Inkoo virus
GMYC	generalized mixed yule coalescent (model)
iTOL	Interactive Tree of Life
ITS2	Internal transcribed spacer 2 (gene)
IUCN	International Union of the Conservation of Nature
JEV	Japanese encephalitis virus
K2P	Kimura two-parameter
km^2	square kilometres
kV	kilovolt
L	litres
L.	Linnaeus
LNR	local nature reserve
LWM	Liverpool World Museum
m	metres
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization
MBD	Mosquito borne disease
MCMC	Markov chain Monte Carlo
mg	milligrams
mins	minutes
MJN	median joining network (analysis)
ML	maximum likelihood (analysis)
mL	millilitres
mm^2	square millimetres
N	North
n/a	not applicable
USUV	Usutu virus
W	watts
W	West
WD	working distance
WNV	West Nile virus
$\bar{x}$	mean
NBN	National Biodiversity Network
NCBI	National Centre for
YELV	yellow fever virus
ZIKV	Zika virus

	Biotechnology Information
ND2	NADH dehydrogenase 2 (gene)
ND4	NADH dehydrogenase 4 (gene)
ng	Nanogram
NHM	Natural History Museum (London)
no. or *n*	Number
NR	nature reserve
Nr.	Near
NUMTs	nuclear copies of mitochondrial origin
OTU	operational taxonomic unit
PCR	polymerase chain reaction
pers. comm.	personal communication
PHA	Port Health Authorities
PHE	Public Health England
PIMS	Port Invasive Mosquito Surveillance
POD	point of discovery
PT	clear packaging tape
PTP	Poisson tree processes
ref.	Reference
RH	relative humidity
RIFV	Rift Valley fever
RNA	Ribonucleic acid
S	South
s	Seconds
s.l.	sensu lato (in the broad sense)
SC	serosal cuticle
SD	standard deviation
SEM	scanning electron microscopy
SINV	sindbis virus
SNP	single nucleotide polymorphism
sp.	species (unknown)
SR	species range
SW	South West
T	Thymine
TAHV	Tahyna virus
Tm	melting temperature
TS/TV	transitions/transversions
U	Uracil
U	Units
UK	United Kingdom
ųl	Microlitre
ųm	Micromolar
USA	United States of America

CONTENTS

CHAPTER ONE:

Introduction



1. Introduction

Mosquitoes (family Culicidae Meigen) are infamous around the world for their blood feeding behaviour and as a prolific carrier of disease, albeit in a minority of species. They are possibly the most disreputable of all the two-winged flies (order Diptera L.). The Culicidae are a globally diverse family, with 3 554 currently recognised species (Harbach, 2019), from 111 genera, and 137 subgenera (Reinert, 2009). Found across all contents, with the exception of Antarctica, they have adapted to a broad gradient of relative temperature and humidity. This behavioural elasticity also applies to feeding and reproductive preferences, which has resulted in the globalisation of many species, and an ensuing capability for zoonosis for some.

1.1 The risk of invasive *Aedes* mosquitoes (AIMs)

The surveillance of species that inflict significant impacts on human health are a high priority for international public health bodies in countries they are not native to, such as the European Centre for Disease Control (ECDC), and Public Health England (PHE) (ECDC, 2012; Schaffner et al., 2013). The most impacting of these species are arguably the mosquitoes, whose blood feeding behaviour is an optimal transport network for endoparasites such as viruses and bacteria that have evolved to utilise them as primary vectors between hosts. Consequently, vectors, hosts and parasites have implicitly linked behavioural lifecycles (Koella, 1999; Koella et al., 1998; Koella and Agnew, 1997). For hosts, such as humans, these diseases can have a severe impact on health, with often high rates of morbidity. It is estimated that

malaria alone causes 219 million cases of infectious disease every year, from which 435 000 result in the loss of life (WHO, 2018). The severity and distribution of mosquito borne disease (MBD) outbreaks are closely linked to their population abundance (Scott and Morrison, 2003) and vector dispersal capabilities (Harrington et al., 2005; Rezza, 2012), with differing severity of health symptoms depending on the nature of infection, and the causal parasite. Outbreaks of MBD are commonly associated with warmer climates, however, this view is misleading as temperate regions are, and historically have been, at risk from relatively under recorded emerging diseases.

In Great Britain (GB), the surveillance of potential MBD vectors prioritises two invasive container breeding species, *Aedes* (*Stegomyia*) *aegypti* L., native to Africa with the vernacular 'Yellow Fever Mosquito', and *Aedes* (*Stegomyia*) *albopictus* Skuse, the 'Asian Tiger Mosquito'(Fig. 1.1). The latter of the two is of great concern to the European community. It is considered by the International Union for the Conservation of Nature (IUCN) as one of the top 100 invasive species globally (Lowe et al., 2004), and since its first introduction in Italy in 1990 (Bonizzoni et al., 2013; Sebatini et al., 1990), is now recorded in 31 EU countries and established in 24 (Becker et al., 2013; Osório et al., 2018; Roche et al., 2015; Roiz et al., 2011; Schaffner and Mathis, 2014; O. Šebesta et al., 2012) (Fig. 1.1). *Aedes aegypti* is also a significant carrier of MBDs and has also become established on most continents. In Europe, however, it is less well distributed than *Ae. albopictus*, being currently only established in Russia, Georgia, Turkey and Madeira (Fig. 5.1 Chapter 5). The mechanism for the rapid global dispersal of both species is by anthropogenic means, via the transportation of second-hand car tyres and wet-footed plants, such as lucky bamboo (Brown et al., 2011; Demeulemeester et al., 2014; Hofhuis et al., 2009; Jupp

and Kemp, 1992). Recent surveys in Spain also suggests that passive dispersal inside road vehicles could also be a likely mode of movement (Eritja et al., 2017). Both species have distributions restricted by climactic condition with species behavioural and distribution modelling intimating that *Ae. aegypti* is less cold tolerant than *Ae. albopictus*, and is therefore less likely to establish in the cooler temperate climates found in the Northern Palearctic (Kamal et al., 2018; Medlock and Leach, 2015). However, the discovery of a male *Ae. aegypti* found in England (described in Chapter 5) aligned with reports of regular introductions to the Netherlands (Brown et al., 2011; Scholte et al., 2010) and adaptive overwintering observed in Washington DC, USA (Severson et al., 2016), demonstrates a need for vigilance towards both invasive species of *Aedes* (Van De Vossenberg et al., 2015).

The medical importance of these species is not understated as both species are known vectors of similar pathogenic arboviruses, with *Ae. aegypti* as the primary vector. These diseases include: (i) Dengue fever (DENV) (Flaviviridae: *Flavivirus*), a considerable global burden to human health, can cause high fevers, fatal hypertension and haemorrhagic disease. Over two billion people are at risk of exposure globally, and it is the second highest cause of death by MBD after malaria (~20, 000 per year) (Brady et al., 2012; Carabali et al., 2015). (ii) Zika virus (ZIKV) (Flaviviridae: *Flavivirus*), causes mild fever, and myalgia like symptoms and although this disease is unlikely to be fatal, it causes microcephaly in the unborn children of infected pregnant women (Calvet et al., 2016; Mlakar et al., 2016). The vectoral competence of *Ae. aegypti* for ZIKV has been established (Roundy et al., 2017), however, the status of *Ae. albopictus* is still unconfirmed but laboratory testing is suggestive (Di Luca et al., 2016). (iii) Yellow fever virus (YELV) (Flaviviridae: *Flavivirus*), an acute haemorrhagic disease that can lead to liver failure

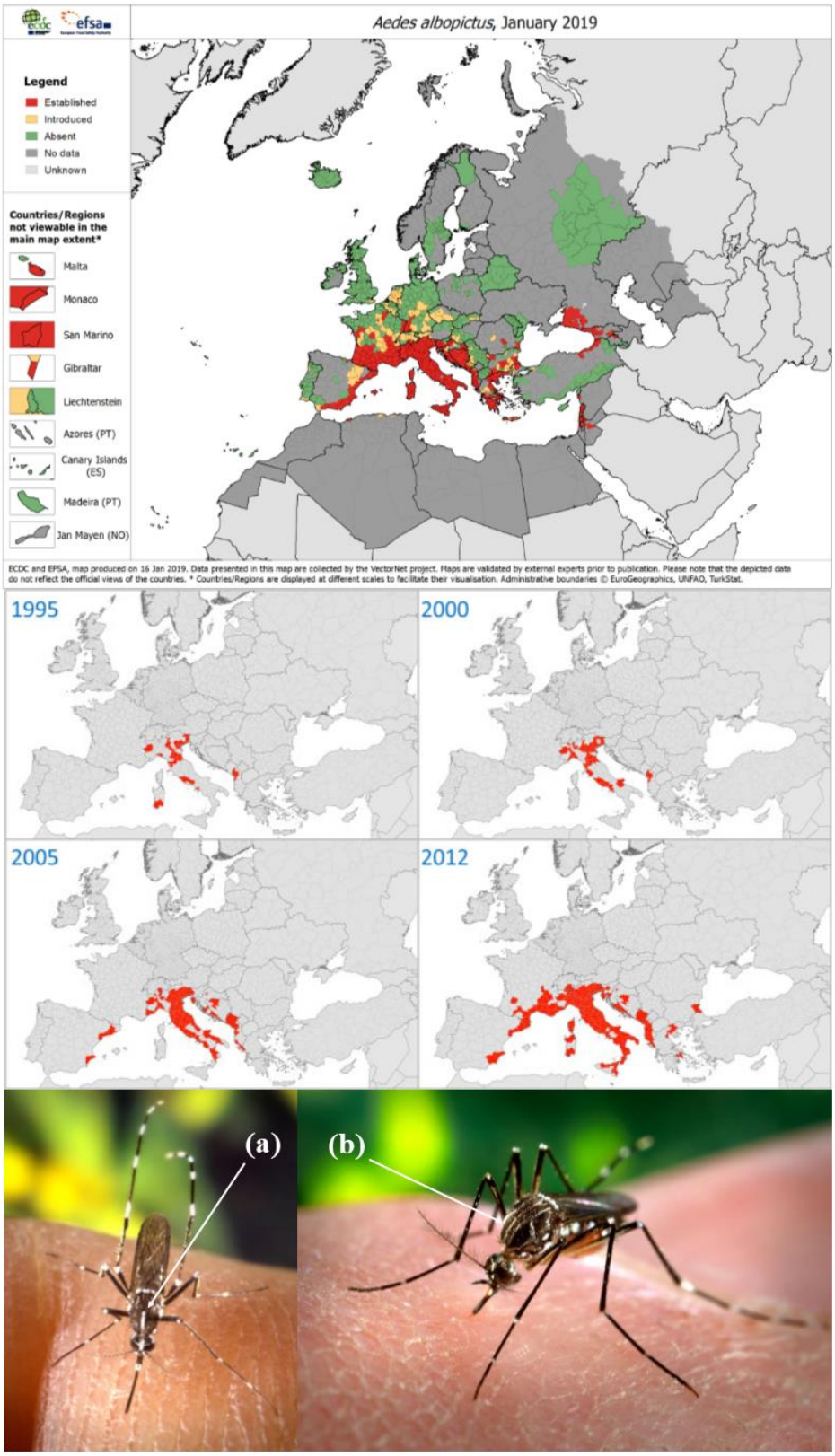


Fig. 1.1 The spread of *Ae. albopictus* across Europe (1995 to 2019), and identifying features alongside *Ae. aegypti*.
Images from the ECDC (available from https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/mosquito-maps) and (Schaffner et al 2013). (a) And (b) show the separation of *Ae.*

albopictus and *Ae. aegypti*, by characteristic of a single central stripe on the scutum of the former, and the shape of a Lyre in the latter. Both *Aedes* images from Wikimedia Commons.

and side effects of jaundice, giving the 'yellow' appearance which gives the disease its name (Christophers, 1960). (iv) Chikungunya virus (CHIKV) (Togaviridae: *Alphavirus*), presents similar symptoms to ZIKV, no reported microcephaly, but can cause long term joint and muscle pain.

Of the above-mentioned viruses, locally transmitted (autochthonous) outbreaks of DENV and CHIKV in France and Italy (Gould et al., 2010; Tomasello and Schlagenhauf, 2013) have occurred in the wake of the recent *Ae. albopictus* dispersal across Europe. The importance of surveillance for this species cannot be underestimated, as an established introduction is likely to lead to localised outbreaks here in Britain.

1.2 Risks posed by the native mosquitoes of Great Britain.

It has been 100 years since the last reported case of autochthonous MBD in Great Britain (GB), where 330 cases of autochthonous malaria (probably *Plasmodium vivax*) were reported from the Isle of Sheppey, Kent (James, 1920; Newman, 1919). Subsequently, a nescient approach towards mosquito surveillance has persisted, and only a handful of researchers and public health officers are studying their movements and ecology, with often limited resources (Vaux et al., 2019). However, a recent incentive to increase surveillance has come from two technical reports issued by the European Centre for Disease Control (ECDC) which act as guidelines for the surveillance of invasive (ECDC, 2012) and native (ECDC, 2014) mosquito species in Europe. Within the latter, native species risk is reflected in a 5-level ranking system (summarised in Table. 1.1) based on the most current understanding of vector competence and potential for transmission at the time of publication. Of the 96

European native species, 36 are currently considered as potential vectors with 26 posing a significant risk (ranked >3). Notable mosquito borne diseases found within European species include: (i) Sindbis virus (SINV) (Togaviridae: *Alphavirus*) found across Europe, most notably reported in Scandinavia (Lundström and Pfeffer, 2010), and can develop high levels of morbidity, appearing in seven year cycles (Sane et al., 2010). SINV is a precursor to Pogosta disease (Finland), Ockelbo virus (Sweden), and Karelian fever (Russia). Symptoms of which include fevers, arthritis, skin rashes and non-pruritic skin lesions (Medlock et al., 2006), 20-50% of SINV cases lead to long term chronic osteoarthritis, fibromyalgia or other forms of joint pain (Laine et al., 2004, 2000; Niklasson et al., 1988). Potential European vectors include; *Ae. cinereus/geminus, Oc. communis, Oc. sticticus, An. hyrcanus, Cx. perexiguus, Cx. pipiens s.l., Cx. theileri*. *Cx. torrentium, Cs. morsitans*. (ii) West Nile virus (WNV) (Flaviviridae: *Flavivirus*), parasitises wild birds, however, vertebrates such as humans and equine species can become dead end hosts (Hubálek and Halouzka, 1999). Cases of WNV have increased in Europe, with the highest (1670 cases) recorded in 2018 (Barrett, 2018). Symptoms are influenza-like, often with fatigue, conjunctivitis, a maculopapular or roseolar rash, diarrhoea, nausea, abdominal pain and associated eating disorder, and respiratory problems. In ~15% of cases WNV can develop into neuroinvasive encephalitis which can be fatal (Wang et al., 2004). Potential European vectors include; *Ae. cinereus/geminus, Ae. vexans, Ochlerotatus caspius, Oc. dorsalis, Oc. excrucians s.l., Oc. sticticus, Anopheles maculipennis s.s., An. plumbeus, Coquillettidia richiardii, Cx. modestus, Cx. perexiguus, Cx. pipiens s.l., Cx. theileri, Cx. tritaeniorhynchus, Cs. morsitans.* (iii) Tahyna virus (TAHV) (Bunyaviridae: *Orthobunyavirus*) also known as 'Valtice fever', is principally hosted by lagomorphs (hares and rabbits), *Erinaceus roumanicus (*hedgehogs*)* and rodentia (rodents), but when in humans (primarily children) manifestations are similar to the

influenza-like symptoms of WNV. It is the main cause of arboviral encephalitis in the USA. There are currently no reported fatalities from TAHV (Hubálek, 2008). Reported cases of TAHV have come from many European countries, including France, Germany, Austria, Sweden, Romania, Serbia and Norway (Medlock et al., 2006). Potential European vectors include; *Oc. caspius*, *Ae. vexans*, (Bardos and Danielova, 1953; Gligic and Adamovic, 1976) and *Cx. pipiens s.l.*(Arcan et al., 1974).

Prior to this study, in 2014, 34 species were listed as present in GB (Table 1.1.), 32 listed by Cranston et al (1987), with the recent additions of *Aedes geminus* Peus (Medlock and Vaux, 2009), and *Anopheles daciae* Linton, in Southern England (Linton et al., 2005). These species fall within eight genera; *Aedes* Meigen (*Ae*.) (3 species), *Anopheles* Meigen (*An*.) (6), *Coquillettidia* Dyar (*Cq*.) (1), *Culex* L. (*Cx*.) (4), *Culiseta* Felt (*Cs*.) (7), *Dahliana* Reinert, Harbach & Kitching (*Da*.) (1), *Ochlerotatus* Lynch Arribálzaga (*Oc*.) (11), and *Orthopodomyia* Theobald (*Or*.) (1).

Despite there being no recent recordings of autochthonous mosquito borne diseases from humans in GB, there are historical records of such incidences. The malaria parasite *Plasmodium vivax* transmitted by *An. atroparvus* (Chin and Welsby, 2004; Hutchinson and Lindsay, 2006), occurred prolifically in the lowland fens of England between the mid-16th to mid-19th centuries, often referred as 'The Ague', or Marsh Fever (MacArthur, 1951), it was suspected of causing high numbers of human fatalities (Dobson, 1980; Hutchinson and Lindsay, 2006; Reiter, 2000, 2000). There are no recent records of *P. vivax* persisting in GB, however, *An. atroparvus* appears to have remained well distributed and abundant in lowland wetland areas (Snow, 1998). Between 1987 and 2013, 50 187 cases of non-autochthonous malaria were

reported in GB from returning travellers, of which 25.4% (*n* = 12 769) were caused by *P. vivax* (Broderick et al., 2015). Current estimates suggest that ~2% of all people travelling from the tropics (West Africa, India and Pakistan in particular) to European and North American are infected with malaria (Gerard, 2002). Incidences where associated airport staff, and members of the public in proximity to airports are afflicted by malaria by proxy are also reported. This pattern of transmission has been coined 'airport malaria' and is mostly transmitted by accidental introductions of infected mosquitoes (Bradley, 1989; Rodger et al., 2008; Whitfield et al., 1984). This effect is not only restricted to airports, or indeed to malaria. In Swansea, 1865, 29 cases of yellow fever (of which 17 were fatal) were well recorded, its origins traced to a barque named 'The Hecla' that had recently returned from Cuba with infected crew, as well as a surviving population of infected vectors (most likely *Ae. aegypti*) (Smith and Gibson, 1986).

Although the different forms of human-affecting malaria are not currently present in GB, other species of *Plasmodium* are still present in avian populations. A study of *Cyanistes caeruleus* (Blue Tit) from Wytham Woods recorded a prevalence of *Plasmodium sp.* in 27.8% (*P. relictum* accounting for 10.2%) of sampled birds (Wood et al., 2007). The importance of such transmissions for bird numbers and as a potential reservoir for zoonosis is not yet understood (Wood and Cosgrove, 2006). However, forecast changes to the annual temperatures across GB as a result of climate change could be favourable for *P. vivax* and *P. falciparum* for three to four months of the year, within the next 50 years (Lindsay et al., 2010; Medlock and Leach, 2015), but would likely be restricted by two factors; the location of the primary vector (*An. atroparvus*) and regions within this distribution where host biting is most prevalent. Modelling approaches by Lindsay et al (2010) suggests that any risk would be restricted to the marshlands of the South East of England.

Family	Sub-family	Tribe	Genus	Subgenus	Species	ECDC Risk
Culicidae Meigen, 1818	Anophelinae Grassi, 1900		*Anopheles* Meigen, 1818	*Anopheles* Meigen, 1818	*algeriensis* Theobald, 1903	3
					atroparvus van Thiel, 1927*	5
					claviger Meigen, 1804	4
					daciae Linton, Nicolescu & Harbach, 2004*	0
					messeae Falleroni, 1926*	3
					plumbeus Stephens, 1828	2
	Culicinae Meigen, 1818	Aedini Neveu-Lemaire, 1902	*Aedes* Meigen, 1818	*Aedes* Meigen, 1818	*cinereus* Wiedemann, 1818	5
					geminus Peus, 1970	0
				Aedimorphus Theobald, 1903	*vexans* Meigen, 1830	4
			Dahliana Reinert, Harbach & Kitching, 2006	-	*geniculata* Olivier, 1791	2
			Ochlerotatus Lynch Arribálzaga, 1891	-	*annulipes* Meigen, 1830	0
					cantans Meigen, 1818	1
					caspius Pallas, 1771	5
					communis de Geer, 1776	5
					detritus Haliday, 1833	1
					dorsalis Meigen, 1830	3
					flavescens Müller, 1764	0
					leucomelas Meigen, 1804	0
					punctor Kirby, 1837	2
					sticticus Meigen, 1838	1
				Rusticoidus Shevchenko & Prudkina, 1973	*rusticus* Rossi, 1790	0
		Culicini Meigen, 1818	*Culex* Linnaeus, 1758	*Barraudius* Edwards, 1921	*modestus* Ficalbi, 1890	5
				Culex Linnaeus, 1758	*pipiens* Linnaeus, 1758	5
					torrentium Martini, 1925	5
				Neoculex Dyar, 1905	*territans* Walker, 1856	0
		Culisetini Belkin, 1962	*Culiseta* Felt, 1904	*Allotheobaldia* Brolemann, 1919	*longiareolata* Macquart, 1838	2
				Culicella Felt, 1904	*fumipennis* Stephens, 1825	0
					litorea Shute, 1928	0
					morsitans Theobald, 1901	5
					alaskaensis Ludlow, 1906	0
				Culiseta Felt, 1904	*annulata* Schrank, 1776	1
					subochrea Edwards, 1921	0
		Mansoniini Belkin, 1962	*Coquillettidia* Dyar, 1905	*Coquillettidia* Dyar, 1905	*richiardii* Ficalbi, 1889	3
		Orthopodomyiini Belkin, Heinemann & Page, 1970	*Orthopodomyia* Theobald, 1904	-	*pulcripalpis* Rondani, 1872	0

Table 1.1 Species of mosquitoes present in Great Britain at the beginning this study (2014), and their vector potential.

* Constituent species of *An. maculipennis s.l.* ECDC RISK: 0 = Species not implicated in disease transmission, or no data; 1 = Species infected in nature only; 2 = Species competent in the laboratory only; 3 = Species infected in nature and competent, for the same pathogen or for different pathogens, or, for malaria, secondary vector only; 4 = Species known as vector outside Europe; 5 = Species known as past/present vector in Europe.

Of the 36 European mosquito species classified as potential vectors, 14 found in GB are ranked as ‘significant’ (ranks 3-5), and eight considered ‘lower’ risk (ranked <3), the remaining are currently unclassified, most likely due to a lack of empirical evidence (Table 1.1). At the time of this study there is no active screening of mosquitoes in GB for diseases, although some host organisms, primarily birds, have been tested for the presence of arboviruses, with SINV, WNV and Usutu virus (USUV) positively identified by serological testing (Buckley et al., 2003; Gould et al., 2006). Additionally, limited laboratory testing for vector competence of some mosquito species as putative vectors for disease has also been undertaken. The saltmarsh mosquito *Oc. detritus* is a prolific nuisance biter of humans in GB and has been shown to be a potentially competent vector of WNV (Blagrove et al., 2016) and Japanese encephalitis virus (JEV) in specimens collected in England (Mackenzie-Impoinvil et al., 2015).

To assign a risk of autochthonous disease to GB mosquitoes, several factors should be considered that include; vector competence of a given species/population, the mosquito’s exposure risk to diseases, localised population of competent vectors, climate, species host preference, proximity to human populations, and localised abundance (Kilpatrick et al., 2005). Given the high vector potential of some GB species, the proximity to sources of infection (i.e. ‘airport malaria’, and seropositive birds), a forecast shift to optimal climactic conditions for parasites such as malaria, and a history of mosquito borne diseases, all the above emphasise the importance of a rigorous surveillance programme for native species.

1.3 The challenges of surveillance in Great Britain.

Several efforts have taken place to pre-empt possible routes of introduction of AIMs via international shipping ports and airports. In 2010, the use of Mosquito Magnets®, BG-Sentinels (CO_2 and pheromone adult attractants traps), and larval surveys, were carried out in 12 locations across GB by PHE (Murphy et al., 2013; Vaux and Medlock, 2015). Resource shortages resulted in only Liverpool Ports continuing surveillance by 2014, with PHE switching efforts into locating AIMs by prioritising possible routes of incursion. This included two major imported used tyre yards and motorway service stations located on thoroughfares from major ports in the south of England. The passive introduction of *Ae. albopictus* into Germany, Austria, the Czech Republic, Spain and Switzerland via public transport networks (Becker et al., 2013; Eritja et al., 2017; Flacio et al., 2016; Kampen et al., 2013a; Scholte and Schaffner, 2007; O Šebesta et al., 2012) and the recorded movement of *Aedes* eggs via car tyres (Craven et al., 1988; Dalla Pozza et al., 1994; Jupp and Kemp, 1992) supported this change in strategy. In 2015, the surveillance of ports was reinitiated by the formation of the Port Invasive Mosquito Surveillance project (PIMS). A collaboration between PHE, Edge Hill University (EHU) and the Port Health Authorities (PHA) to include the surveillance of 39 ports and airports. Due to their high costs, and high maintenance requirements, the use of CO_2 and pheromone attractants (Mosquito Magnets® and BG-Sentinels) was replaced by a combination of oviposition and BG-GAT (Gravid *Aedes* Traps) for detection of eggs laid, or females looking for oviposition sites. These are cheap and easy methods of detecting recently laid eggs/hatched larvae (oviposition traps), or adults (killed by pesticide loaded netting in the BG-GAT traps). The findings of this project are summarised in Vaux et al (2019) a key finding was the discovery of *Ae. albopictus* in Southern

England on four occasions (September 2016, July 2017, July 2018 and September 2019). Confirmation of species identification was via a combination of egg hatching and larval rearing, examination of the egg morphology using scanning electron microscopy (SEM), and comparative BLASTn searches (NCBI database) of the mitochondrial cytochrome oxidase I gene (*COI*), and Internal Transcribed Spacer 2 (*ITS2*) (using the methods outline in chapters 3 & 4). Post-discovery surveillance did not find established populations; however, these are the first records of this medically important species in GB. Despite these discoveries the resources available for the location of AIMs remains low, and therefore a reliance on the efficiency of trapping, and on methods of identification, have become of paramount importance to gain coverage of a large network of possible incursion routes. Current methods are advised and summarised in the ECDC guidelines for invasive mosquitoes (ECDC, 2012). This advisory prioritises the detection of adult female mosquitoes looking for a blood feed, or oviposition sites, often requiring the female to have laid eggs after introduction. Appropriate methods for the detection of passively introduced eggs is not available, which is problematic, as the global transportation of egg loaded car tyres is believed to be one of the most likely causes of introduction across the globe.

Mosquito surveillance in GB requires monitoring from two fronts, those posed by the influx of *Aedes* invasive mosquitoes (AIMs), and the risks associated with native species. The same concern for these species, however, has been rarely addressed. The surveillance of native species in GB is not extensive but has increased since the commencement of this project (Kampen et al., 2015). Passive recording has historically been the most used method of determining species distribution via the National Mosquito Recording Scheme (https://www.brc.ac.uk/scheme/mosquitoes-recording-scheme). This approach to surveillance has had some success over its

duration, with approximately ~10 000 data points, including collated historical records from 1827 to the present. Species identification, however, is questionable using this method as many records are without authentication. In 2005, PHE formed 'Mosquito Watch' as an attempt to promoting the recording of nuisance biting by the public and pest controllers. Over seven years the project only recorded 116 reports, with the majority being of *Cs. annulata* and *Cx. pipiens s.l.*, species found commonly within houses.

Additional research to assess the effects of wetland creation on mosquito populations has led to the discovery of some species that were once considered rare in Britain, such as *Aedes vexans* in Norfolk (Medlock et al., 2017a), and *Cx. modestus* in Kent, England, both species are considered competent for WNV (Balenghien et al., 2007; Tiawsirisup et al., 2008). Research work is currently underway to ascertain if these species are native to GB, or recent introductions (J. Medlock (PHE), pers. comm.). Currently, *Cx. modestus* is the only native species actively under surveillance as populations appear to be spreading along the Thames Estuary and Kent coastlines in the South East of England (Vaux et al., 2015).

The lack of investment for the surveillance of GB native mosquito species is likely to have many causes. Additional government investment in such programmes is not forthcoming, despite several surveillance programmes in other temperate European countries gaining important data on candidate vector distribution (Culverwell, 2018; Krüger and Tannich, 2013; Zeller et al., 2013). A lack of expertise in the identification of mosquito species is also evident across GB, and improvements to current taxonomic keys are required to increase the number of regional recorders. The most recent reference materials (Cranston et al., 1987; Snow, 1990) are now 30

years old, do not include invasive species, and have not been updated alongside taxonomic developments. Two European texts, Becker et al (2010) and Schaffner et al (2001) are also accessible but are expensive (Becker et al = £219.99 at NHBS on the 14.05.2019), or 'out of print' as with the latter.

1.4 Improving surveillance of mosquitoes in Great Britain

During the process of vector surveillance, the accurate confirmation of native or AIM species is vital for any downstream decision-making process; distribution mapping, screening for MBDs and application of control measures.

Morphological and genetic identification is well described for *Ae. aegypti* and *Ae. albopictus* (Becker et al., 2010; Chen et al., 2015; Nene et al., 2007). In native species, however, morphological identification can be ambiguous between species and within complexes, such as *Cx. pipiens s.l.* (Harbach, 2012). Additionally, the application of genetics in mosquito taxonomy has revealed the existence of cryptic species within *Anopheles*, and *Cx. pipiens s.l.* complexes (Alquezar et al., 2010; Dumas et al., 2016; Lehr et al., 2006; Müller et al., 2013; Nicolescu et al., 2004; Paredes-Esquivel et al., 2009; Silva-Do-Nascimento et al., 2006; Wang et al., 2012). These studies bring into question the accuracy of current morphological approaches for accurate species identification, especially in regions where species review by genetic analysis has yet to be applied.

Additional to morphological vagaries, mosquito specimens are often fragile and features of taxonomic importance, such as scales, setae, legs and wings, are regularly damaged or lost during collection. In these instances resolution to species can be impossible for damaged specimens, DNA analysis is the only confirmatory procedure available. The barcoding genes of the mitochondrial cytochrome oxidase c

subunit I (*COI*) and the nuclear internal transcribed spacer II (*ITS2*) are the most used approach, with large repositories of data available for statistical comparisons. In GB, however, not only is little known about the genetic composition of its mosquito populations, but the current list of species is considered to be incomplete (Harbach et al., 2017; Medlock and Vaux, 2010). This is problematic for surveillance as the recording of new species becomes a complex query of 'native' vs 'invasive' that can have serious implications when intraspecific regional differences can reveal varying vector competence (Bennett et al., 2002). Conspecifics considered native, for example, may be of lower risk compared to those from a population introduced from other countries where MBD outbreaks are a regular occurrence. This can lead to difficult decisions as to whether control is appropriate. A dilemma currently faced by the discovery of spreading populations of *Cx. modestus* in Kent, that has unknown origins.

The surveillance of mosquitoes in GB is only as effective as the surveillance tools at the disposal of field and laboratory workers at any given time. It is therefore important to constantly assess their usefulness and update if necessary. Here we develop the use of tested molecular based methods to significantly improve our understanding of species genetic profiles in GB mosquitoes, by constructing and testing a database of *COI* and *ITS2* barcoding genes. Additionally, we present a report on the finding of *Ae. aegypti* in the North West of England during this process, discuss the implications of this discovery, and develop a novel method of screening car tyres for the dormant eggs of AIMs to aid in their detection at ports and tyre yards. Both approaches we hope will significantly contribute to GB mosquito surveillance programmes.

1.5 Research goals (summary)

The surveillance of potential disease vectors in GB is still relatively new and under-resourced. Therefore, the methods that underpin surveillance strategy must be constantly adapted to improve affordability and efficiency of detection. Based on current inadequacies in surveillance methods we have developed the research to address gaps in our knowledge and developed a potential tool for field application. To this end the study has addressed;

a) A need for improvement for the identification of species present in GB by the development of genetic barcoding data on British mosquitoes.
b) A new method to improve the screening of used car tyres for AIMs eggs in diapause.

Within this thesis we attempted to address these using the following methods;

i) Attempt to sample specimens of all species currently considered present in GB by undertaking a survey to collect new specimens by utilising historical records. (Chapter 2)
ii) Develop a genetic database of the *COI* and *ITS2* of GB mosquitoes sampled during the survey phase and test the ability of these genes for effective species partitioning. (Chapters 3 & 4)
iii) Investigate the possibility of taxonomic error, or the presence of new cryptic species, by comparing species sampled from GB to those from across their range. (Chapter 3 & 4)
iv) Record and investigate any invasive mosquitoes collected in the duration of the study. (Chapter 5)
v) Develop a low-cost method of screening car tyres for AIM eggs. (Chapter 6)

CHAPTER TWO:

A survey of native and non-native British mosquito species

Findings from this chapter contributed towards two separate publications. The first describing the discovery of *Ochlerotatus nigrinus* in GB for the first time;

Harbach, R.E., Dallimore, T., Briscoe, A.G., Culverwell, C.L., Vaux, A.G.C., Medlock, J.M., 2017. *Aedes nigrinus* (Eckstein, 1918) (Diptera, Culicidae), a new country record for England, contrasted with *Aedes sticticus* (Meigen, 1838). Zookeys 671, 119–130. https://doi.org/10.3897/zookeys.671.12447

And secondly, for the species confirmation of *Ae.albopictus* in the south of England;

Vaux, A.G.C., Dallimore, T., Cull, B., Schaffner, F., Strode, C., Pflüger, V., Murchie, A., Rea, I., Newham, Z., Mcginley, L., Catton, M., Gillingham, E.L., Medlock, J.M., 2019. The challenge of invasive mosquito vectors in the U.K. during 2016–2018: a summary of the surveillance and control of *Aedes albopictus*. Med. Vet. Entomol. mve.12396, 1–10. https://doi.org/10.1111/mve.12396

2.1 Abstract

Native British mosquito species are disproportionately under-recorded compared to other insect groups considered of high ecological, or medical importance. A lack of awareness towards vector potential, and a need for the improvement of available identification material could be compounding the problem. Recent advice from the European Centre for Disease Control (ECDC) reinforces a need for surveillance of native mosquito species in addition to invasive species. Therefore, a survey of mosquitoes was carried out between 2016 and 2018 with the aim of collecting new specimens of all known mosquito species from across Great Britain (GB), to characterise these species using DNA barcoding methods, and to confirm whether species with few historical records were still present.

A targeted sampling approach utilised current records as well as available habitat suitability information to select 106 locations, in which a variety of sampling methods collected 2989 mosquitoes. The data presented here also includes specimens donated by Public Health England (PHE) from invasive species surveillance programmes, as well as by entomologists from Liverpool World Museum and Edge Hill University. Of the 34 suspected species reported prior to the survey, 25 species were recorded, with the addition of *Ochlerotatus nigrinus* Eckstein to the GB species list, as well as the invasive mosquitoes *Aedes aegypti L.* and *Ae. albopictus* Skuse. The latter being contributed by national mosquito surveillance projects. Of the ten species unaccounted for, three are from morphologically similar species complexes and were found to be absent after genetic profiling (Chapters 3 & 4). Two species, *Oc. dorsalis* and *Or. pulcripalpis* are considered rare, and the status of the remaining five in GB remains questionable.

This survey located 18 native species currently highlighted as a high-risk for vector borne diseases in a European. The vector potential of these species in GB is still unknown, therefore an increase in passive and formal surveillance strategies are suggested.

2.2 Introduction

Disease risk from native mosquito species in Great Britain (GB) is currently considered low (Medlock et al., 2006, 2005; Medlock and Leach, 2015). However, reports compiled by the ECDC highlights gaps within our knowledge of mosquitoes at a European level. In particular, the possibility of under recorded disease potential in native species, as well as risks from future introductions of conspecifics from different geographic areas, and potentially more serious, invasive non-natives such as *Ae. albopictus* and *Ae. aegypti* (ECDC, 2012; Schaffner et al., 2013). Despite being an island separated from the European mainland, Great Britain may not be isolated from these issues. For example, autochthonous outbreaks of Inkoo virus (INKV) (Putkuri et al., 2016), Sindbis virus (SINV), Usutu virus (USUV) and West Nile virus (WNV) are transmitted by European mosquito species that are also native to GB (Brummer-Korvenkontio et al., 2002; Buckley et al., 2003; Kurkela et al., 2008; Lindström and Lilja, 2018). Of the 36 species categorised by the ECDC as having vector potential 25 are currently believed to be native to GB, including several classified as high risks (Fig. 1.1); of these, *Ae. cinereus/geminus*, *Oc. communis*, *Cx. torrentium*, *Cx. pipiens s.l.* and *Cs. morsitans* are primary vectors of SINV, *Cx. modestus* and *Cx. pipiens s.l.* of WNV and *Dirofilaria sp.*, and *An. atroparvus* of the malarial parasite *Plasmodium vivax*. All the above are suspected of causing disease outbreaks in other EU countries, with the latter having historical significance in GB as the vector of malaria, or the 'Ague', until the mid-19th century (MacArthur, 1951). The current risks of major disease from GB mosquito species are summarised by Medlock and Leach (2015) but are cautiously considered low, however, no screening of diseases within wild caught mosquitoes has been undertaken.

The mosquito (Family: Culicidae) fauna of GB are taxonomically composed of two sub-families, Anophelinae Grassi, 1900 and Culicinae Meigan, 1818, within which seven Genera are currently described, those of *Anopheles* Meigan, 1818, *Aedes* Meigan, 1818 (containing the subgenera *Aedes* Meigan, 1818 and *Aedimorphus* Theobald 1903), *Dahliana* Reinert, Harbach and Kitching, 2006, *Ochlerotatus* Lynch Arribálzaga, 1891 (including the subgenus *Rusticoidus*, Shevchenko & Prudkina), 1973, *Culex* L. 1758 (containing the subgenera *Barraudius* Edwards, 1921, *Culex* L., 1758, and *Neoculex* Dyar, 1905), *Culiseta* Felt, 1904 (including the subgenus Culicella Felt, 1904) and *Coquillettidia* Dyar, 1905 and *Orthopodomyia* Theobald, 1904. At the commencement of this study records suggested the presence of 34 species and are listed within their associated genera and subgenera in Table 1.1. This figure, however, is debatable as relatively little is still known about the GB mosquito fauna. The number of biological records for mosquitoes in the GB, prior to 2015, was ~10 000 (Vaux and Medlock, 2015). Of this data, a significant proportion are collated historical records dating back to the mid-19th century, or collected by Public Health England (PHE) from invasive species surveillance from 2010 to 2015, and the UK Mosquito Recording Scheme running from 2009 to the present. A small number of additional records were contributed from Mosquito Watch (~130), a nuisance biting reporting scheme that ran from 2009 to 2015 (Vaux and Medlock, 2015). The success of passive recording schemes is debatable, with concerns over recorder and spatial bias, accuracy of identification, lack of expert confirmation, and complications caused by historical changes in taxonomic nomenclature (Geldmann et al., 2016; Isaac and Pocock, 2015; Sutherland et al., 2015; van der Wal et al., 2015; Ward, 2014). Mosquito recording is not exempt from these problems with most records being unconfirmed leading to questionable specimen identifications. Additionally, there has been much alteration to the classification of species and

genera over time that has resulted in complications caused by synonyms. The historical name changes in GB mosquitoes are well summarised in Harbach et al., (2017).

The number of records for mosquitoes are comparable to other members of the same Superfamily: *Culicoidea*, such as *Chironomidae* (Non-biting midges) = ~21 000 records, *Chaoboridae* (Phantom, or ghost midges) = ~850, *Dixidae* (Meniscus midge) = ~4 000, but are unsurprisingly lower than those of charismatic insect groups considered to have high importance, such as *Apidae* (Bees) = ~180 000, and Lepidoptera (Butterflies and Moths) = ~18 800 000 (records from the NBN Atlas prior to 2015) (https://species.nbnatlas.org/species/NBNSYS0000040182). This imbalance in the number records is reflected in our knowledge of GB mosquitoes and records do very little to increase our knowledge of basic biological traits such as habitat preference, or biting habits, which appears to have only marginally improved since the work of Marshall (1938). Very little is known about seasonal abundance, and our understanding of species distribution is incomplete (Snow et al 1998). For example, floodwater specialists that are abundant across Europe such as *Ae.vexans* and *Oc.sticticus* are currently reported as rare (Cranston 1987), however, it is probable that they have simply been overlooked as their life cycle is dictated by climactic events rather than stable seasonal cycles. It is therefore more difficult to plan surveillance for species with these traits. Likewise, much of this data has been collected as part of public health surveillance that targets sites, and species, that are nuisance biters to humans. Many species considered rare according to Cranston (1987), such as *Cx. territans* of *Or. pulcripalpis* are primarily believed to feed on non-anthropophilic food sources, such as amphibians, or birds. The likely bias of mosquito biological recorders being individuals working in the vector biology sector has likely led to a sampling bias towards human biting mosquitoes (Vaux and

Medlock, 2015).However, current datasets are useful in providing rudimentary information on distribution possibilities for high priority species, a useful tool for developing surveillance strategies against disease vectors (Kampen et al., 2015; Medlock et al., 2015; Vaux and Medlock, 2015).Several previous efforts to survey GB mosquitoes have been undertaken, however, this has mostly been focussed on the presence of species within localised regions (Chapman et al., 2017; Hernández-Triana et al., 2019; Medlock and Vaux, 2015, 2013; Service, 1994; Snow and Medlock, 2008; Vaux et al., 2015). With the increased movement of global trade and tourism, the effects of climate change, the spread of invasive mosquito species across the EU, and a greater understanding of the vector potential of native species, now is an appropriate time to reassess the mosquito species found in GB. Here we attempted to collect specimens of all the species suspected of being present in GB with a mind to characterising those using DNA barcoding methods (Chapters 3 & 4), to confirm their presence, and to add any new species to the current lists. This chapter is a summary of this survey and a report of significant findings.

2.3 Methodology

2.3.1 Sampling

The aim of this survey was to obtain specimens of all GB species, not to collate quantitative data on distribution, seasonal abundance, or habitat preference. Such an approach would be useful for gaining ecological reference material, but likely to miss uncommon species, as was found by similar attempts to characterise mosquito species by country (Engdahl et al., 2014; Hernández-Triana et al., 2019; Versteirt et al., 2015). Therefore, a targeted, ad hoc, qualitative sampling approach was taken using a system for sample site selection using the following criteria: (1) a known

location for target species using records from the Mosquito Recording Scheme, journal articles, and grey literature, (2) locations with habitats that are preferential to target species, and (3) locations not reported in the literature, but are known as having mosquitoes present (pers. comm). Where species were identified as present from multiple locations across the GB, the sampling of these locations was selected using a stratified method that encompassed a selection of the known habitats for any specific species (or suggested by knowledge of sample site), with a geographical distance with a minimum of 50 miles. This method was applied to ensure a reasonable spread of genetic data. Additional fresh samples were also collected and donated from unrelated surveys carried out by entomologists from Liverpool World Museum (LWM), and PHE.

Sample collection was undertaken from 2014 to 2018, from the months of March to November. The date of each sampling visit was selected based on historically recorded seasonal activity for each targeted species using the Mosquito Recording Scheme database, and literature references (Cranston et al., 1987; Snow and Medlock, 2008; Snow et al., 1998). The number of sample visits ranged from 1 to 10. Repeat visits were made when no positive specimens of target species were collected. Sampling was primarily undertaken by sweep netting with a 0.5 m net diameter, 0.7 m depth and a 0.3 m handle length (193325, NHBS, Devon) and larval dipping with a 250 mm diameter net head and 0.3 m bag depth (1 mm mesh size) and a 1.48 m length handle (175601, NHBS, Devon). These methods, although labour intensive and with lower yield, reduce sex-bias found when using attractants, and inflict less overall damage to adults for ease of morphological examination and voucher specimen retention. Additional use of Mosquito Magnets® (Midge Tech, Callander, Scotland) baited with Octenol, BG-Sentinels (NHBS, Devon, UK) loaded with BG-Sweetscent™ lures run on 12v batteries, both methods of attracting

mosquitoes by using CO_2 and pheromone lures, were used during the early stages of sampling in 2014 and 2015, these methods were abandoned due to specimen damage that reduced accurate morphological identification. Mosquito searches were undertaken during the daylight hours. Specimens donated to the project from LWM were mostly collected by sweep net and pinned, samples from PHE were collected by attractant, or oviposition, traps.

A broad habitat description was given to each sample location based on vegetation type, but not quantitively assessed, as well as longitudinal and latitudinal readings to ~ 6 m accuracy using a Garmin eTrex 20x (Kansas, USA). These are summarised in Table 2.1.

The attempt to sample all known British mosquito taxa for DNA analysis was preferred to destructive sampling of museum specimens which produces varying results (Dean and Ballard, 2001). This also gave us the opportunity to investigate the presence/absence of species from historical locations that are considered, rare or absent from GB records for many decades.

All collected adult specimens were placed directly into individual sterile pots immediately after collecting to minimise the potential of DNA cross contamination, and then subsequently euthanised and stored by freezing at -20ºC within 8 hours of collection. Donated specimens were either pinned and air dried, or otherwise frozen at -20ºC and shipped in insulated containers.

Collected larvae and pupae, were reared to adults in 30 cm^3 BugDorms (211283, NHBS), or mini insect breeders (BD7001, NHBS) at EHU under ambient temperature and humidity. Individual adult females were collected on emergence, to aid in identification, males were given a minimum of 24 hours to allow the genitalia to mature (observed when genitalia are fully rotated) before euthanising at -20ºC.

Due to mosquitoes being an under recorded group, the possibility of discovering novel species was high, therefore identification to species was undertaken using both GB (Cranston et al., 1987; Marshall, 1938; Snow, 1990) and European (Becker et al., 2010; Schaffner et al., 2001) taxonomic keys. Due to inconsistencies in the GB keys, males were identified only using features of the hypopygia. Difficult, rare, or novel species were confirmed by Ralph Harbach at the Natural History Museum London.

2.3.2 Species nomenclature

The last two decades have seen multiple revisions in the Culicidae, most notably the elevation of number of sub-genera within the *Aedes* genus as a result of different approaches to morphological analysis (Reinert, 2000; Reinert et al., 2009, 2004). This has led to inconsistencies in current global nomenclature (Savage, 2005; Wilkerson et al., 2015). In the main body of text we follow the terminology used by Becker et al (2010) to ensure that species names can be correlated with the primary source of morphological identification. The only exception is that of the species reported as *Ochlerotatus geniculatus*, which was more recently changed to *Dahliana geniculata*, as described within the Mosquito Taxonomic Inventory (http://mosquito-taxonomic-inventory.info/).

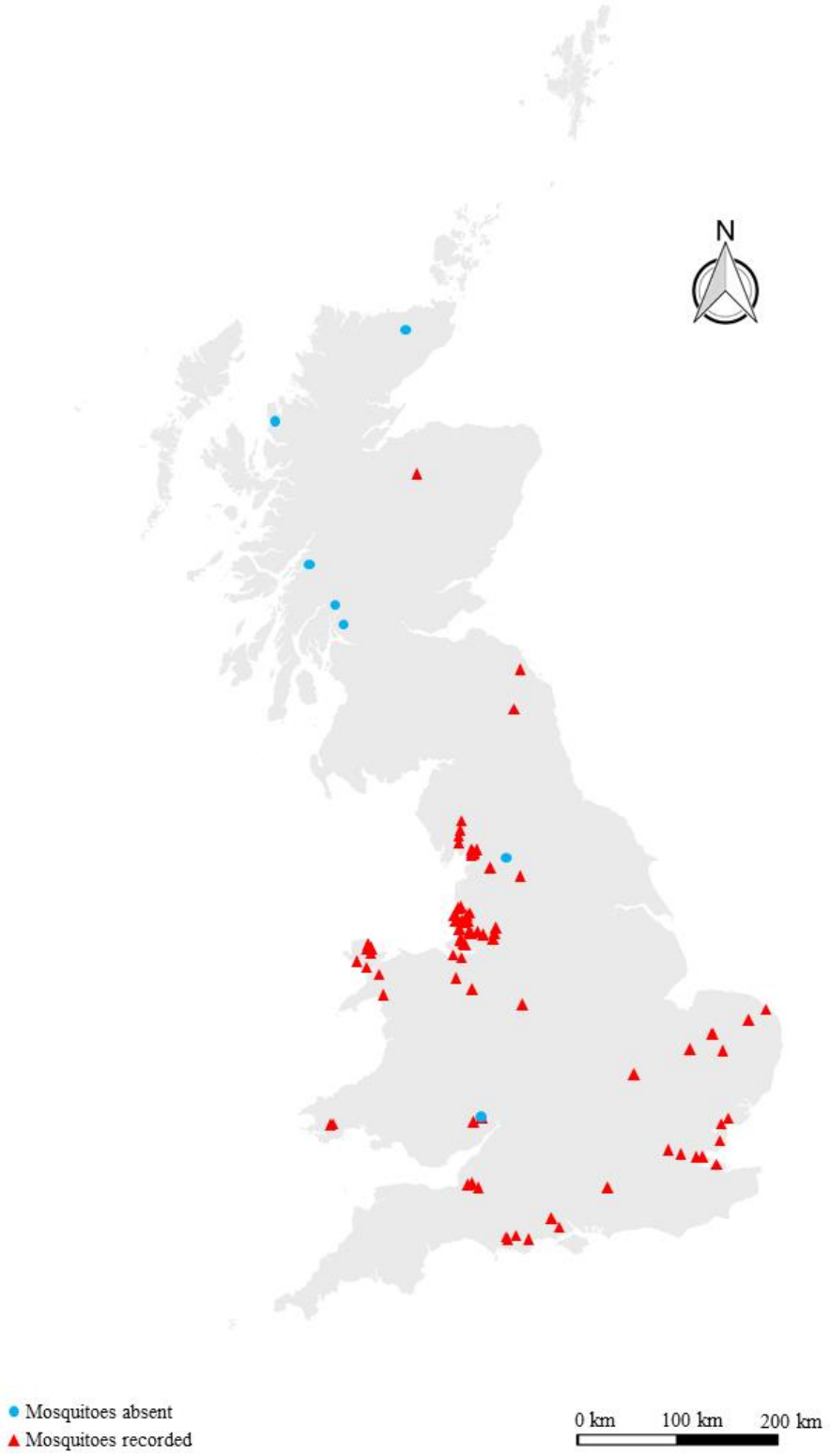


Fig. 2.1 GB mosquito survey sample locations.

Red triangles depict locations where specimens were collected. Locations where mosquitoes were absent are represented by blue circles, and therefore not collected.

Ref.	Location	Long. Lat.	Sampling method	Habitat description	Species
	Scotland				
1	The Trossachs	56.322943, -4.7164478	Sweepnet, Mosquito Magnet	Acid wetland, coniferous woodland, riparian	None
2	Nr. Glencoe	56.689458, -5.0991712	Sweepnet	Mixed woodland	None
3	Nr. Gairloch	57.712953, -5.6874955	Sweepnet, dipping	Scrubbed coastal heathland	None
4	Abernethy, Perth and Kinross	57.252390, -3.7148702	Sweepnet, dipping	Marshy grassland, acid fen, acid pools under coniferous woodland	*An.claviger, Cs.annulata, Cs.morsitans/fumipennis, Cx.territans, Oc.punctor*
5	Forsinard Flows, Forsinard	58.427152, -3.8353014	Sweepnet, dipping	Open acid bog, with coniferous plantations	None
6	Luss, Loch Lomond	56.101532, -4.6416593	Sweepnet, Mosquito Magnet	Rural	*Oc.punctor*
	England **Cumbria**				
7	Roudsea Wood and Mosses National Nature Reserve	54.231795, -3.0258579	Sweepnet, dipping	Mixed broadleaf woodland, wet woodland, open acid raised bog, fen	*Ae.cinereus/geminus, An.plumbeus, Cs.morsitans/fumipennis, Cx.pipiens s.l., Oc.annulipes, Oc.cantans, Oc.detritus, Oc.punctor*
8	Blelham Bog National Nature Reserve	54.396740, -2.9793119	Sweepnet, dipping	Mixed broadleaf woodland, wet woodland, fen, open water margin	*An.claviger, Cs.morsitans/fumipennis, Cx.pipiens s.l./torrentium*
9	Moor Top, Grizedale	54.360307, -3.0139146	Hand collected	Mixed plantation	*An.claviger*
10	Stoney Hazel, Rusland, Lake District	54.302296, -3.0199344	Hand Collected	Mixed broadleaf woodland	*Oc.punctor*
	Cheshire				
11	Holcroft Moss Nature Reserve	53.435300, -2.4757519	Dipping	Lowland raised bog	*Oc.punctor*
12	Rixton Claypits Nature Reserve	53.409813, -2.4750996	Sweepnet, dipping	Mixed broadleaf woodland, open pools, fen	*An.claviger, An.plumbeus, Cs.morsitans/fumipennis, Cs.annulata, Oc.rusticus, Cx.pipiens s.l./torrentium*
13	Chester Zoo	53.226082, -2.8852029	Sweepnet	Parkland	*Oc.rusticus*
14	Ness Botanic Gardens, Neston	53.272174, -3.0453629	Sweepnet	Parkland	*Cs.annulata, Oc.detritus*
15	Risley Moss LNR	53.422628, -2.4994583	Sweepnet, dipping	Post-industrial lowland raised bog, wet woodland, mixed broadleaf woodland, parkland	*Cs.morsitans/fumipennis*

Table 2.1 Sample locations and additional information collected for the GB mosquito survey 2014-2018.

Ref.	Location	Long. Lat.	Sampling method	Habitat description	Species
	Lancashire				
16	St.Helens, Lancashire	53.455615, -2.7338791	Hand collected	Urban garden	*Oc.annulipes*
17	Martin Mere Nature Reserve	53.617240, -2.8656292	Mosquito Magnet	Reedbed, open fen, wet meadow	*An.claviger, Cs.annulata, Oc.caspius, Oc.detritus, Cq.richiardii, Cs.annulata*
18	Burtonwood, Warrington	53.431199, -2.6531467	Dipping	Urban garden	*Cx.pipiens s.l.*
19	Mere Sands Wood Nature Reserve	53.636038, -2.8358545	Sweepnet, Mosquito Magnet, dipping	Mixed woodland, wet woodland, open fen, wet meadows	*An.claviger, An.maculipennis ssp., Cs.annulata, Cx.pipiens s.l./torrentium, Oc.annulipes, Oc.cantans, Oc.detritus, Cq.richiardii*
20	Park Wood, Gisburn Forest	54.004298, -2.4020405	Sweepnet	Mixed plantation, mixed broadleaf woodland	*An.claviger, An.plumbeus*
21	Edge Hill University Campus, Ormskirk	53.562186, -2.8782710	Dipping	Urban garden	*Cs.annulata, Cx.pipiens s.l. Cx.torrentium*
22	Burscough Lancashire	53.589441, -2.8525532	Hand collected, dipping	Urban garden, urban house	*Cx.pipiens s.l.*
23	Long Wood, Silverdale	54.164489, -2.8152080	Sweepnet	Mixed mature woodland	*Oc.annulipes*
24	St.Bedes School, Ormskirk	53.562726, -2.8951271	Hand collected	Urban house	*Cx.pipiens s.l.*
25	Ormskirk	53.566598, -2.8804736	Hand collected	Urban house	*Cx.pipiens s.l.*
26	Astley Moss Nature Reserve	53.471833, -2.4617615	Sweepnet, dipping	Mixed broadleaf woodland, post-industrial lowland raised bog, fen, wet meadow	*Ae.cinereus, Cx.pipiens s.l./torrentium, Cs.morsitans/fumipennis, Cs.annulata, Oc.cantans, Oc.punctor*
27	Gait Barrows National Nature Reserve	54.187056, -2.7920465	Sweepnet, dipping	Mixed broadleaf woodland, limestone pavement	*An.claviger, Cs.morsitans/fumipennis, Oc.annulipes*
28	Eaves Wood, Silverdale	54.179918, -2.8174868	Sweepnet, dipping	Mixed broadleaf woodland	*Aedes sp., An.plumbeus, Cs.annulata, Oc.annulipes*
29	Haskayne Cutting Local Nature Reserve	53.566248, -2.9826593	Sweepnet	Marsh, wet woodland	*An.claviger, Cx.pipiens s.l., Cs.annulata*
30	Haskayne Village	53.566597, -2.9712219	Hand collected	Urban house	*Cx.pipiens s.l., Cs.annulata*
31	Grubbins Wood, Arnside	54.194674, -2.8508062	Sweepnet	Mixed broadleaf woodland	*An.plumbeus*
32	Bottom Wood, Silverdale	54.163479, -2.8227825	Sweepnet	Mixed broadleaf woodland	*Oc.annulipes*
33	Pointer Wood, Silverdale	54.165866, -2.8169117	Sweepnet	Mixed broadleaf woodland	*Oc.annulipes*
34	Scout Wood, Silverdale	54.164977, -2.8213577	Sweepnet	Mixed broadleaf woodland	*Oc.annulipes, Oc.sp.*
35	Clarks Lot, Silverdale	54.164426, -2.8139334	Sweepnet	Mixed broadleaf woodland	*Oc.annulipes*
36	Halsall	53.582234, -2.9576740	Hand collected	Urban house	*Cs.annulata*
37	Scutchers Achre, Lathom	53.588796, -2.8293915	Sweepnet, dipping	Mixed woodland, mixed plantation	*Oc.cantans*

Table 2.1 Continued.

Ref.	Location	Long. Lat.	Sampling method	Habitat description	Species
38	Cuerden Valley Park, Bamber Bridge	53.708042, -2.6558590	Sweepnet	Mixed woodland, parkland	*Cx.pipiens s.l./torrentium*
39	Scaresbrick Hall Woods, Scaresbrick	53.607888, -2.9227774	Sweepnet	Mixed woodland	*Cx.pipiens s.l./torrentium, Cs.annulata*
	Merseyside				
40	Ainsdale National Nature Reserve	53.590905, -3.0638638	Sweepnet	Coastal dunes, plantation woodland, young mixed woodland	*Cs.litorea*
41	Birkdale Village	53.634405, -3.0151484	Hand collected	Urban house	*Oc.detritus*
42	Homer Wood, Knowsley	53.456471, -2.8626637	Sweepnet, dipping	Mixed woodland, shaded ponds	*Cs.annulata, Cx.pipiens s.l./torrentium, Cx.torrentium, Oc.annulipes, Oc.cantans, Oc.sp.*
43	Knowsley Safari Park	53.435441, -2.8120537	Sweepnet	Parkland	*Cx.pipiens s.l.*
44	Knowsley Village	53.460813, -2.8650670	dipping	Urban garden	*Cx.pipiens s.l.*
45	Ribble Estuary National Nature Reserve	53.682602, -2.9546442	Sweepnet	Coastal	*Oc.caspius, Oc.detritus*
46	Aigburth, Liverpool	53.362935, -2.9310279	Sweepnet	Allotments	*Cx.pipiens s.l./torrentium*
47	Marshside	53.678673, -2.9810200	Sweepnet	Coastal, salt marsh	*Oc.caspius*
48	Liverpool City	53.410064, -2.9798012	Sweepnet, dipping	Urban	*Cx.pipiens s.l./torrentium*
49	Liverpool Promenade	53.372874, -2.9526465	Sweepnet	Urban, parkland	*Oc.annulipes/cantans*
50	Liverpool Docks	53.390526, -2.9824619	Mosquito Magnet, Oviposition traps	Urban, coastal, mixed woodland	*An.claviger, Cs.annulata, Cx.pipiens s.l., Da.geniculata, Oc.caspius, Oc.detritus, Oc.rusticus*
51	Formby	53.558958, -3.0506654	Dipped	Urban garden	*Cx.pipiens s.l./torrentium*
52	Jubilee Wood, Lunt	53.511648, -2.9825156	Sweepnet, dipping	Mixed plantation, farm yard	*An.claviger, Cs.morsitans, Cx.pipiens s.l., Cs.annulata, Cx.torrentium*
53	Lunt Meadows, Lunt	53.515894, -2.9863501	Sweepnet, Mosquito Magnet, dipping	Fen, wet meadow, agricultural fields	*An.claviger, Cs.annulata, Cs.morsitans, Oc.caspius*
54	Woolton, Liverpool	53.375608, -2.8680325	Dipped	Urban garden	*Cx.pipiens s.l.*
55	Marine Lake, Crosby	53.473294, -3.0358830	Sweepnet	Coastal, parkland	*Cs.annulata*
56	Kirkby Village	53.477299, -2.8721952	Hand collected	Urban house	*Cx.pipiens s.l./torrentium*
	Shropshire				
57	Fens, Whixall and Bettisfield Mosses National Nature Reserve	52.919326, -2.7595940	Sweepnet, dipped	Post-industrial lowland raised bog, wet woodland, mixed broadleaf woodland, agricultural pasture	*Ae.cinereus, Cq.richiardii, Cx.pipiens s.l., Cx.torrentium, Cs.annulata, Cs.morsitans/fumipennis, Oc.annulipes, Oc.cantans, Oc.punctor, Oc.sp.*

Table 2.1 Continued.

Ref.	Location	Long. Lat.	Sampling method	Habitat description	Species
	Shropshire				
57	Fens, Whixall and Bettisfield Mosses National Nature Reserve	52.919326, -2.7595940	Sweepnet, dipped	Post-industrial lowland raised bog, wet woodland, mixed broadleaf woodland, agricultural pasture	*Ae.cinereus, Cq.richiardii, Cx.pipiens s.l., Cx.torrentium, Cs.annulata, Cs.morsitans/fumipennis, Oc.annulipes, Oc.cantans, Oc.punctor, Oc.sp.*
	Yorkshire and the Humber				
58	Skipton	53.962183, -2.0252051	Hand collected	Urban house	*Cx.pipiens s.l./torrentium*
59	Malham Tarn	54.100295, -2.1722202	Sweepnet, dipping	Blanket bog, open fen, wet woodland	None
	Staffordshire				
60	Brocton Coppice	52.774649, -2.0272865	Hand collected	Mixed broadleaf woodland, plantation	*Da.geniculata*
	Gloucestershire				
61	Highbury Wood National Nature Reserve	51.773735, -2.6696434	Sweepnet	Mixed broadleaf woodland	*Da.geniculata*
	Somerset				
62	Catcott Nature Reserve, Avalon Marshes	51.166040, -2.8509865	Sweepnet, dipping	Wet meadow, mixed broadleaf woodland, reedbed	*Cq.richiardii, Oc.annulipes, Oc.annulipes/cantans*
63	Nr.Glastonbury	51.151724, -2.6855553	Sweepnet	Agricultural field margin	*Cq.richiardii*
64	Westhay Moor National Nature Reserve, Westhay	51.193675, -2.7785969	Sweepnet, Mosquito Magnet, dipping	Lowland raised bog, wet woodland (*Salix*), mixed fen	*An.maculipennis sp., Cs.morsitans/fumipennis, Oc.annulipes, Oc.punctor, Oc.sp.*
	Norfolk				
65	Norwich City	52.626077, 1.3366585	Hand collected	Urban, riparian	*Ae.vexans, Oc.sticticus*
66	Hickling Broads	52.736624, 1.5933952	Mosquito Magnet	Fen, mixed woodland, grazed marsh	*An.algeriensis*
67	Thetford Forest	52.447338, 0.84826217	Hand collected	Broadleaf woodland	*Oc.cantans*
68	Thetford Forest	52.447508, 0.85074306	Hand collected	Conifer plantation	*Oc.cantans*
69	West Hall Wood, Thetford	52.316664, 0.97320843	Hand collected	Mixed woodland (coppice), wet meadow	*Cx.pipiens s.l./torrentium, Da..geniculata, Oc.rusticus*

Table 2.1 Continued.

Ref.	Location	Long. Lat.	Sampling method	Habitat description	Species
	Dorset				
70	Brownsea Island	50.691847, -1.9722691	Sweepnet, dipping	Mixed woodland, mixed fen types, parkland, coastal	*Ae.geminus, An.claviger/plumbeus, Cs.annulata, Cx.pipiens s.l., Cx.pipiens s.l./torrentium, Cs.sp.(Culicella), Oc.detritus, Oc.punctor, Oc.sp.*
71	Nr. Wareham	50.720145, -2.1525908	Sweeping	Mixed woodland, plantation	*Cs.morsitans/fumipennis, Oc.punctor*
72	Moreton	50.704323, -2.3144352	Sweeping	Mixed woodland, arable field margin	*Cx.pipiens s.l., Cx.torrentium*
73	Tadnoll & Winfrith Nature Reserve	50.686089, -2.2953873	Sweeping, dipping	Acid fen, heathland, mixed woodland scrub (Salix)	*Ae.geminus, Cs.annulata, Cx.pipiens s.l., Cq.richiardii, Oc.annulipes, Oc.caspius, Oc.punctor, Cs.sp. (Culicella)*
	Kent				
74	Service stations		Oviposition trap	Service station	*Ae.albopictus*
75	Northward Hill NR	51.457585, 0.55984783	Mosquito Magnet	Mixed woodland scrub, open marsh, wet meadow, coastal	*Ae.vexans*
76	Elmley Marshes National Nature Reserve, Isle of Sheppey	51.383095, 0.78531647	Mosquito Magnet	Coastal, marsh, salt marsh, wet meadow	*Cx.modestus*
77	Cliffe Pools Nature Reserve	51.459205, 0.47364807	Mosquito Magnet	Coastal, marsh, salt marsh, estuarine	*Cx.modestus*
	Cambridgeshire				
78	Chippenham Fen	52.297908, 0.41574669	Mosquito Magnet	Wet woodland, fen	*Oc.sticticus*
	Bedford				
	Fenlake Meadows NR, Bedford	52.128414, -0.44412231	Mosquito Magnet	Wet meadow, riparian	*Ae.vexans, Oc.sticticus*
	Priory Country Park	52.130891, -0.43038082	Mosquito Magnet	Parkland	*Cs.sp.*
	Essex				
79	Rainham Marshes	51.491960, 0.22395802	Mosquito Magnet	Marsh, wet meadow, mixed scrub, estuarine	*Cx.modestus, Oc.flavescens*
80	Wallasea Island	51.606696, 0.85755158	Mosquito Magnet	Coastal, salt marsh, wet meadow, estuarine	*Cx.modestus*
81	Old Hall Marshes NR	51.774537, 0.87149906	Mosquito Magnet	Coastal, salt marsh, wet meadow, estuarine	*Cx.modestus*
82	Fingringhoe Wick NR	51.818813, 0.95189667	Mosquito Magnet	Grassland, scrub heath, salt marsh, estuarine	*Cx.modestus*

Table 2.1 Continued.

Ref.	Location	Long. Lat.	Sampling method	Habitat description	Species
	Surrey				
83	Alice Holt Forest, nr Farnham	51.153051, -0.84093905	Hand collected	Mixed plantation	*Oc.cantans*
	London				
84	Newham	51.512738, 0.048820495	Unknown	Urban, riparian	*Ae.vexans, Oc.sticticus*
	Hampshire				
85	Puckpits Enclosure, New Forest	50.888545, -1.6371086	Sweepnet, dipping	Acid pond, mixed broadleaf woodland	*Ae.cinereus/geminus*
86	New Forest	50.899347, -1.6407909	Sweepnet, dipping	Open water, acid heath	None
87	Beaulieu Heath, New Forest	50.801880, -1.5137469	Sweepnet, dipping	Open wet/dry heath, scrub (gorse), riparian	*Cx.pipiens s.l./torrentium, Oc.nigrinus*
	Northumberland				
88	Ford Moss Nature Reserve	55.630036, -2.0514736	Sweepnet	Acid mixed woodland, lowland raised bog	*Cx.pipiens s.l./torrentium, Cx.torrentium, Cs.annulata, Cs.morsitans/fumipennis, Oc.punctor*
89	River Coquet, Nr. Altwistle	55.344346, -2.1194730	Sweepnet	Scrub woodland, riparian	*Cx.pipiens s.l./torrentium*
	Wales				
90	Nr. Worgreen, Forest of Dean	51.805830, -2.5438821	Sweepnet, dipping	Acid wetland, mixed woodland	*Oc.punctor*
91	Worgreen, Forest of Dean	51.814019, -2.5445849	Sweepnet, dipping	Ponds, mixed plantation	None
92	Cors Erddreiniog National Nature Reserve, Isle of Anglesey	53.308047, -4.2952380	Sweepnet, dipping	Wet woodland, base rich open fen, reedbed, mixed broadleaf woodland, wet meadow, wet heath	*An.claviger, An.plumbeus, Cs.annulata, Cs.litorea, Cs.morsitans/fumipennis, Cx.pipiens s.l.*
93	Cors Bodeilio National Nature Reserve, Isle of Anglesey	53.272030, -4.2421067	Sweepnet, dipping	Base rich open fen, reedbed	*An.claviger, An.plumbeus, Cs.annulata, Cs.sp.(Culicella), Cx.pipiens s.l./torrentium*
94	Cors Gogh National Nature Reserve, Isle of Anglesey	53.308221, -4.2527304	Sweepnet, dipping	Base rich fen, reedbed, wet meadow, scrub woodland, mixed broadleaf woodland	*An.algeriensis, An.maculipennis ssp., An.plumbeus, Cs.sp.(Culicella), Cx.pipiens s.l./torrentium*
95	Newborough Warren National Nature Reserve, Isle of Anglesey	53.158248, -4.3772449	Sweepnet, dipping	Conifer plantation, coastal, dunes, wet meadow	*Cs.annulata, Cs.sp.(Culicella)*
96	Benllech, Isle of Anglesey	53.320646, -4.2399073	Sweepnet	Not recorded	*Cs.sp.(Culicella)*
97	Traeth Glaslyn Nature Reserve	52.925887, -4.1015053	Sweepnet, dipping	Wet meadow, wet woodland, mixed broadleaf woodland, estuarine	*Cs.sp.(Culicella), Oc.sp.*
98	Marian-glas, Isle of Anglesey	53.337820, -4.2573133	Sweepnet	Vegetation next to stream	*Cx.pipiens s.l./torrentium*
99	Llanallgo, Isle of Anglesey	53.349212, -4.2719640	Sweepnet	Disused mine, pine scrub	*Oc.detritus*

Table 2.1 Continued.

Ref.	Location	Long. Lat.	Sampling method	Habitat description	Species
	Wales cont.				
100	Lligwy, Isle of Anglesey	53.348313, -4.2728928	Sweepnet	Disused mine, pine scrub	*Cx.pipiens s.l./torrentium*
101	Plas Glanrafon, Isle of Anglesey	53.318487, -4.2281227	Sweepnet	Not recorded	*Cx.torrentium*
102	Porth Trecastell, Isle of Anglesey	53.207332, -4.5036929	Sweepnet	Flying at burial chamber, coastal grassland	*Cx.pipiens s.l./torrentium*
103	Felin Puleston, Denbighshire	53.036042, -3.0065546	Sweepnet	Country park	*Cx.pipiens s.l./torrentium*
104	Parc Dudley Nature Reserve, Waunfawr	53.103369, -4.2016997	Sweepnet	Mixed woodland	*Cs.morsitans/fumipennis*
105	Long Wood, Minwear Woods, Pembrokeshire	51.786321, -4.8282003	Hand collected	Mixed broadleaf woodland	*An.claviger/plumbeus*
106	Canaston Wood, Pembrokeshire	51.787130, -4.7826582	Hand collected	Mixed plantation	*An.claviger*

Table 2.1 Continued.

2.4 Results

A total of 2989 mosquitoes were collected, female (1833), male (1154) and eggs (2), as part of a targeted sampling strategy between 2014 and 2018. This also includes fresh samples collected ad hoc during other surveys by entomologists from Liverpool World Museum: female (49), and male (31), and PHE: female (178) 25 of the 34 previously known species were found with the addition of three newly recorded species to GB, *Ochlerotatus nigrinus* Eckstein, *Aedes aegypti* L. and *Aedes albopictus* Skuse, 1895. *Ochlerotatus nigrinus* was discovered in temporary pools within the open riparian/heathland at Beaulieu Heath, in the New Forest, Hampshire, these findings are summarised in Harbach et al (2017). *Aedes aegypti* was collected from the surface of herbaceous ground cover in proximity to a farmyard near Maghull in Merseyside and is reported in Chapter 5. The eggs of *Ae. albopictus* were collected by PHE in an oviposition trap from a lorry service station in Kent. The results of post discovery surveillance and controls methods are summarised in (Vaux et al., 2019).

The species not recovered were; *Anopheles atroparvus, An. daciae, Cs. alaskaensis, Cs. longiareolata, Cs. subochrea, Oc. communis, Oc. dorsalis, Oc. leucomelas, and Or. pulcripalpis.* Of those collected, *Ae. vexans, An. algeriensis, Cs. litorea, Cx. territans,* and *Oc. sticticus* were considered rare in GB (Cranston et al., 1987), with *Oc. nigrinus* considered rare across Europe (Becker et al., 2010). A summary of the species collected, the habitats in which they were found, and method of collection are summarised in Table 2.2. Species collected by site, location habitats and longitude and latitudinal information are summarised in Table 2.1.

	Specimen information							Vector potential		
Species	**Nº Eggs**	**Nº Female**	**Nº Male**	**Total**	**Nº Sites**	**Located habitat/aquatic habitat/aquatic cover**	**Collection method**	**ECDC risk***	**Associated diseases**	**Feeding preference**
Genus *Anopheles*										
Anopheles algeriensis	-	48	5	**53**	2	FE/P,F/O	SN	***2***	PLS	HUM
Anopheles atroparvus	-	-	-	-	-	-	-	***5***	PLS[5]	HUM, NHM, AVN
Anopheles claviger	-	75	5	**80**	13	FE,FL,MP, BL/P,C,F/O,PS	MM, SN, LD	***4***	PLS[4], DIR[1]	HUM, NHM
Anopheles daciae	-	-	-	-	-	-	-	***0***	-	-
Anopheles maculipennis s.s.	-	2	1	**3**	3	FE,WW/-/-	SN	***3***	PLS[3], DIR[3], WNV[1]	HUM, NHM, AVN
Anopheles messeae	-	-	-	-	-	-	-	***3***	PLS[3]	NHM, AVN
Anopheles plumbeus	-	13	6	**19**	8	BL/-/-	SN, LD	***2***	WNV[2], DIR[2]	HUM, NHM,
Anopheles claviger/plumbeus	-	39	26	**65**	5	BL,FE,CO/-/-	SN, LD	***4***	-	
Genus *Aedes*										
Aedes spp.	-	1	1	**2**	2	-	SN	***-***	-	
Aedes aegypti	-	-	1	**1**	1	BL,FY/-/-	SN	***5***	YLFV, ZIKV, CHIKV, DENV	
Aedes albopictus	2	-	-	**2**	2	SS/C,F/PS	OT	***5***	DENV, CHIKV	
Aedes cinereus	-	118	62	**180**	2	LRB/P,F/O	SN, LD	***5***	EEEV[1], SINV[5], WNV[1]	HUM, NHM, AVN‡
Aedes geminus	-	116	103	**219**	3	FE,WW/-/-	SN	***0***	-	-
Aedes cinereus/geminus	-	14	6	**20**	5	FE,MBW/-/PS	SN, LD	***5***	-	-
Aedes vexans	-	6	-	**6**	4	UR,WM, RI/-/-	MM	***4***	EEEV[1], RVFV[4], WNV[3], DIR[3]	

Table 2.2 British native and invasive mosquito species collected, associated habitats and vector potential with a European context.

	Specimen information							Vector potential		
Species	**Nº Eggs**	**Nº Female**	**Nº Male**	**Total**	**Nº Sites**	**Located habitat/aquatic habitat/aquatic cover**	**Collection method**	**ECDC risk***	**Associated diseases**	**Feeding preference**
Genus *Dhaliana*										
Dahliana geniculata	-	6	6	**12**	4	MBW, MP, UR/-/-	MM, SN	***2***	WNV	HUM, NHM
Genus *Ochlerotatus*										
Ochlerotatus spp.	-	9	10	**19**	10	MBW,WW/P,F/ PS	SN	***-***	-	
Ochlerotatus annulipes	-	101	10	**111**	16	MBW,UR,WM/P,F/O,PS	MM, SN, LD	***0***	-	HUM, NHM
Ochlerotatus cantans	-	93	5	**98**	10	MBW,MP/P,F/PS	SN, LD	***1***		HUM, NHM, AVN†
Ochlerotatus annulipes/cantans	-	17	-	**17**	8	MBW/-/-	MM, SN	***1***	-	
Ochlerotatus caspius	-	130	98	**228**	5	FE,CO/P,F,B/O	MM, SN, LD	***5***	RVFV[4], WNV[1], DIR[5]	NHM
Ochlerotatus communis	-	-	-	-	-	-	-	***5***	SINV[5]	
Ochlerotatus detritus	-	49	53	**102**	9	MBW,PK, FE, UR, CO/P,F,B/O	MM, SN, LD	***1***	USUV[1]	HUM, NHM, AVN
Ochlerotatus dorsalis	-	-	-	-	-	-	-	***3***	WNV[3]	HUM, NHM
Ochlerotatus flavescens	-	10	-	**10**	1	WM, FE,CO/-/O	MM	***0***	-	HUM, NHM
Ochlerotatus leucomelas	-	-	-	-	-	-	-	***0***	-	
Ochlerotatus nigrinus	-	36	28	**64**	1	RI/T,F/O	SN, LD	***0***	-	
Ochlerotatus punctor	-	202	59	**261**	13	WW,BL,MP,LRB/P,F/PS,O	SN, LD	***2***		HUM, NHM, AVN†
Ochlerotatus sticticus	-	15	-	**15**	4	RI/-/O	MM	***1***	WNV[1]	
Ochlerotatus rusticus	-	8	6	**14**	5	WW,BL/-/-	MM, SN, LD	***0***	-	HUM

Table 2.2 Continued

	Specimen information							Vector potential		
Species	**Nº Eggs**	**Nº Female**	**Nº Male**	**Total**	**Nº Sites**	**Located habitat/aquatic habitat/aquatic cover**	**Collection method**	**ECDC risk***	**Associated diseases**	**Feeding preference**
Genus *Culiseta*										
Culiseta (Culicella) spp.	-	263	231	**494**	25	WW,BL,MP/P,T/PS	SN, LD	***5***	SINV[5], WNV[1], DIR[3]	HUM, NHM, AVN, REP†
Culiseta annulata	-	100	102	**202**	25	BL,WW,MP,FE/UR/P/PS	MM, SN, LD	***1***		HUM, NHM, AVN
Culiseta alaskaensis	-	-	-	-	-	-	-	***0***	-	
Culiseta subochrea	-	-	-	-	-	-	-	***0***	-	
Culiseta longiareolata	-	-	-	-	-	-	-	***2***		
Culiseta spp.	-	1	-	**1**	1	WW/-/-	SN, LD	***-***	-	NHM, AVN
Genus *Culex*										
Culex pipiens s.l.	-	57	68	**125**	19	WW,BL,FE,CO,UR/C,T/PS	MM, SN, LD, GAT	***5***	RVFV[4], SINV[5], USUT[1], WNV[5], DIR[5]	HUM, NHM, AVN†
Culex torrentium	-	20	5	**25**	9	BL,WW,UR/C/O,PS	SN, LD	***5***	SINV[5]	NHM, AVN†
Culex pipiens/torrentium	-	133	191	**324**	31	WW,FE,WM,UR/P,C/O, PS	SN, LD	***5***	-	
Culex modestus	-	97	0	**97**	7	FE/-/-	MM	***5***	WNV[5], DIR[5]	HUM, AVN
Culex territans	-	2	2	**4**	1	WW/P/PS	SN	***0***	-	
Genus *Coquillettidia*										
Coquillettidia richiardii	-	52	64	**116**	7	WW,BL,WM/P/PS	MM, SN	***3***	WNV[1], DIR[3]	HUM, NHM, AVN
Genus *Orthopodomyia*										
Orthopodomyia pulcripalpis	-	-	-	-	-	-	-	***0***	-	
Total	**2**	**1833**	**1154**	**2989**						

Table 2.2 Continued

Table 2.2 Footnotes.

*vector potential score reproduced from the ECDC (2014). 1 = infected in nature only, 2 = competent in the laboratory only, 3 = species infected in nature and competent, 4 = species known as a vector outside of Europe, 5 = past/present vector in Europe, - = not significant, or unknown. PLS = Plasmodium spp., DIR = Dirofilaria spp., WNV = West Nile virus , EEEV = Eastern equine encephalitis virus, SINV = Sindbis virus, RVFV = Rift Valley fever virus, USUV = Usutu virus, YELV = Yellow fever virus, ZIKV = Zika virus, CHIKV = Chikungunya virus, DENV = Dengue virus. Recorded feeding behaviour, HUM = Human blood feeding, NHM = Non-human mammal, AVN = Avian. Individual mosquitoes recorded double feeding on † = NHM + AVN, ‡ = HUM + NHM, §= HUM + AVN. Collection methods, MM=MosquitoMagnet™, SN=sweepnet, LD=larval dipping, OT=oviposition trap, GAT=BG-GAT trap. Associated habitats: FE = fen (including reedbed), FL=flushes, MP = mixed tree plantation, BL = broadleaf woodland, WW = wet woodland, FY = farm yard, SS = service station, LRB = lowland raised bog, UR = urban, WM = wet meadow, RI = riparian, PK = urban parkland CO = Coastal / P = permanent water body, C = container (manmade or natural), T = temporary, F = fresh water, B = brackish / O = open, PS = partial shade

2.5 Discussion

2.5.1 Sampling limitations

As stated above, this survey used a targeted sampling method, with only a small number of surveyors to cover all locations. This method deliberately applied a heavy user bias to any historical collected data in order to target the collection of specimens of each species; therefore, the number of samples and date of collection should not be taken as a proof of seasonal abundance. We have removed this information from summary tables so as not to imply any information to the latter. This information will be submitted to help improve the larger national passive Mosquito Recording Scheme. The results of this survey, however, are worthy of discussion as the discoveries of new and rare species, which includes those classified as AIMs, suggests a need for larger scale studies of GB species to determine fluctuations in seasonal abundance. The data we have collected can be used in providing additional sample locations for future studies with repeated seasonal visits.

2.5.2 Species difficult to separate by morphology

Morphological separation of some species complexes can be difficult and identification only possible by examination of features within the larval stages, or by male genitalia dissection. This is problematic for surveillance as not all species of the same complexes carry the same vector potential (de Buck et al., 1930; Hackett and Missiroli, 1935). Those species within the Subgenus *Culicella* Felt (Genus: *Culiseta*) that include *Cs. morsitans*, *Cs. fumipennis*, *Cs. litorea*, showed overlapping variation across their key morphological identifiers, such as depth/presence/absence of leg banding, presence/absence of inverted ‘v’ on the sternites and proboscis scale

patterns. The overlap between species proved to be too great to accurately identify to species level using females only. Where possible, males were identified using genitalia features where the gonocoxite of *Cs. morsitans* and *Cs. fumipennis* displays greater elongation than that of *Cs. litorea* (Fig. 2.2). We found the separation of *Cs. morsitans* and *Cs. fumipennis* to be unclear in both male and female forms, and so all specimens reported here we considered to be the more common *Cs. morsitans* based on the habitat from which they were collected, typically in shaded woodland. *Culiseta fumipennis* recorded as having a preference for open pools (Cranston et al., 1987). These species were later clarified by molecular methods where it appeared that of the two, it is likely that only those of *Cs. morsitans* was collected (Chapters 3 & 4). The dichotomy of accurate identification of these species is troubling and

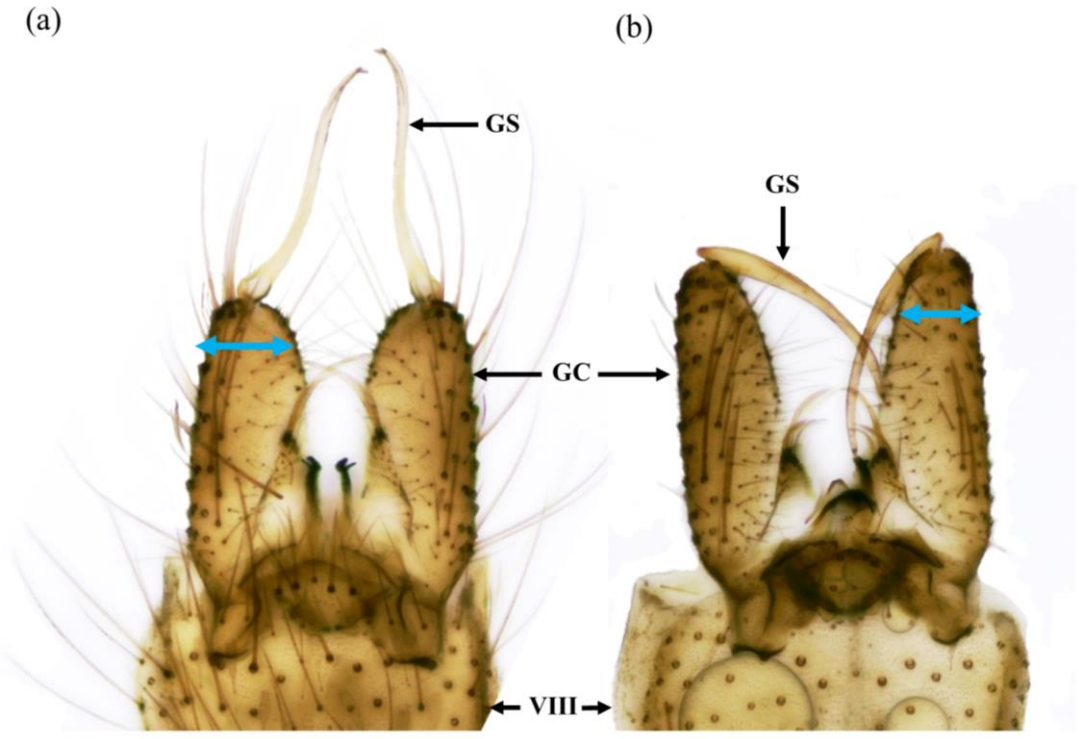


Fig 2.1 Comparison of the distinguishing feature of the male genitalia for (a) *Cs. litorea*, and (b) *Cs. morsitans*.

Broadening of the gonoxocoxite represented by blue arrows. GS = gonostylus, GC = gonocoxite, VIII = eighth tergal segment.

could be significant. *Cs. morsitans* is a reported vector of SINV in Sweden, and of Eastern Equine Encephalomyelitis Virus in the USA (Bergqvist et al., 2015; Morris and Zimmermann, 1981), however, the vector potential of other species within the complex is still unknown. Therefore, the difficulty in separating species of this group through morphological identification alone may have inadvertently affected our understanding of the distribution of potential SINV vectors.

2.5.3 Status of native species not collected

2.5.3.1 *Anopheles* (*Anopheles*) *atroparvus* van Thiel 1927 & *Anopheles* (*Anopheles*) *daciae* Linton, Nicolescu & Harbach 2004

The sampling method employed for this survey biased against the collection of night-time flying Anophelines such as those within the maculipennis group. As a result this group is under-represented in this study. However, the distribution of *An. atroparvus* in GB is reasonably well known and is restricted to lowland wetland sites and coastal marshland (Snow, 1998). *An. daciae* was only recently classified as a unique species in 2004 (Nicolescu et al., 2004), being separated from *An. messeae* by only five single nucleotide polymorphic (SNP) locations on the *ITS2* gene. However, this division is controversial, and the two may only be forms of unique haplotypes rather than distinct species (Bezzhonova and Goryacheva, 2008; Kronefeld et al., 2012). This group is discussed within Chapter 4, where *ITS2* secondary structures are compared between these groups.

2.5.3.2 *Culiseta* (*Culiseta*) *alaskaensis* Ludlow 1906

There are only 21 records for this species in GB and represent a Northern distribution (latitude = 54° N and above). This species is typically recorded from the

boreal and tundra zones of the Holarctic region. Nothing is known about habitat preference in GB, but elsewhere favourable habitats are reported to include swampy tundra and small, permanent, litter filled water pools with low marginal vegetation (Becker et al., 2010). Attempts to locate this species was carried out in closely resembling habitats in Scotland, and several locations in Cumbria. This species has not been recorded since 1983 and was not discovered during this survey. The status of this mosquito is unknown, but if present, may occur in low numbers or at only a few locations. Similar observations have been recorded in Germany where they are seldom encountered due to limited distribution and low population densities (Kampen et al., 2013b). It is possible that this species is still present in GB, but a more intense search for this species in the north is required for confirmation. Due to its low numbers, it is unlikely to present a risk of disease transmission (Medlock et al., 2006).

2.5.3.3 *Culiseta* (*Allotheobaldia*) *longiareolata* Macquart 1838

The presence of *Cs. longiareolata* in GB is questionable, and despite being reported as present in Southern England (Becker et al 2010), is represented by only three records, the most recent from brackish pools on Brownsea Island, Dorset (Service, 1969). A repeat visit to this site, and sampling of similar habitat to those described by Service was negative for this species. However, limited time spent at this location (only two days) could have easily resulted in missing seasonal cues. This species is abundant in the Mediterranean and would be at the limit of their range in GB, where little is known about their behaviour, or ecology, in such extremes. It has been suggested that this species may be periodically introduced but is not established

(Medlock et al., 2005). It is not a prolific biter of humans (Becker et al., 2010), and is therefore not a likely candidate for disease in GB.

2.5.3.4 *Culiseta* (*Culiseta*) *subochrea* Edwards 1921

Considered rare in GB, this species is recorded as having a mostly Southern distribution in England, particularly along the South coast (Snow et al., 1998). It is very similar to *Cs. annulata* in both behaviour and morphology, and its taxonomic status as a distinct species is controversial (Becker et al., 2010). Repeated visits to recorded locations in Dorset (e.g. Brownsea Island, Tadnoll and Winfrith Heath Nature Reserve), were unsuccessful. This species, if genuine, is likely still present in the Britain but with restricted distribution (Snow and Medlock, 2008).

2.5.3.5 *Ochlerotatus communis* De Geer 1776

Although widely distributed across the Holarctic region, *Oc. communis* was last recorded in GB in 1965 in the Isle of Jersey (Cranston et al., 1987). In other temperate zones, this species is associated with strongly acidic, sphagnum dominated, waters in early spring (Becker et al., 2010). It is possible that their distribution and abundance has been reduced by the severe decline in lowland acid wetlands in GB in the latter half of the 20th century. Efforts to locate these species from areas of similarly described habitat at the Forsinard Flows in Northern Scotland was unsuccessful, however, forested acid pools in this area were mostly of dense Scots Pine (*Pinus sylvestris*) plantation, and no natural afforestation now occurs in this region. Similar forested acid habitats, and open sphagnum dominated pools, were also checked at several locations along the West Coast of Scotland (Fig. 2.1)

without any success. It is possible that low numbers, sporadic distribution (in possibly isolated locations) and the monocyclic springtime lifecycle of *Oc. communis*, has resulted in it being under recorded. However, given the length of time since the last report of this species, it is unlikely that this species is still present.

2.5.3.6 *Ochlerotatus dorsalis* Meigen 1830

There has been historical debate as to the taxonomic position of *Oc. dorsalis* in relation to *Oc. caspius* with re-classification from species to subspecies occurring at least nine times (Milankov et al., 2009). However, laboratory breeding experiments from France suggests that these species are reproductively isolated (Lambert et al., 1990). In Britain this species is rarely recorded but demonstrates a wide distribution. The most recent record was from 1993 from salt marsh at Morcha Uchaf SSSI in North Wales. Attempts to locate this species in historically recorded areas in Dorset (Brownsea Island) and Cumbria was unsuccessful. It is believed to present at Wareham Forest, Dorset, (R. Cummings, pers. comm.), however, this information was discovered outside of the timeframe of this study and a follow up visit for confirmation was not possible. It is probable that this is an under recorded species often mis-identified as *Oc. caspius*, which favours coastal and brackish conditions. Illustrations separating these species are incorrectly recorded in Cranston et al (1987), where the two species are inversely labelled. Caution should be taken with all records of *Oc. dorsalis* post 1987 as a result of this error.

2.5.3.7 *Ochlerotatus leucomelas* Meigen 1804

Ochlerotatus leucomelas has only been recorded four times in GB (Vaux and Medlock, 2015). The most recent of which was from a single specimen from Roudsea Woods National Nature Reserve, Cumbria in 2013 by sweepnetting, and two sightings from Anglesey, Wales in 2007. Both locations were treated to 15 and 18 repeated visits, respectively, throughout 2014 to 2018, and included searches for larvae (dipping), and adults (sweepnetting). No specimens were found. Adult females of *Oc. leucomelas* are distinguished by scattered pale scales on the base of the wings, and an absence of scattered pale scales on the abdominal tergites (Becker et al., 2010; Cranston et al., 1987). We have observed, however, that the amount of 'speckling' on the morphologically similar *Oc. dertitus*, particularly on the terga, can vary to extremes. As a result of this variation, it is possible that records of *Oc. leucomelas* are misidentified *Oc. detritus*, however, no specimens of the former from the collections in 2007 and 2013 were available for confirmation.

2.5.3.8 *Orthopodomyia pulcripalpis* Theobald 1904

A dendrolimnocolous species (tree-hole specialist), *Or. pulcripalpis* is rare in GB, being only reported in mature trees from the South-East of England, most recently in 2008 by Snow and Medlock (2008). This mosquito is recorded as staying in the tree canopy where it feeds on birds (Snow and Medlock, 2008). Specimens were not collected due to the lack of canopy access.

2.5.4 <u>*Ochlerotatus nigrinus* Eckstein, a new species to Great Britain</u>

The discovery of a new species to Britain, *Oc. nigrinus*, at Beaulieu Airfield (50°48.53'N; 1°29.79'W and 50°48.11'N; 1°30.83'W), New Forest, Hampshire,

England, in May 2016, during this survey, highlights the possibility of further unrecorded species in GB. This species was misidentified in early attempts to characterise GB mosquitoes by Marshall (Marshall, 1938) who also collected specimens from the New Forest (possibly even the same population), described them as *Aedes sticticus* (Synonym of *Oc. sticticus*), a mistake that has persisted in all similar studies that followed (Cranston et al., 1987; Snow, 1990). The 'true' *Oc. sticticus* was also discovered in GB by Ralph Harbach (Natural History Museum London) and PHE in Hurcott Wood (32°23.92'N; 2°12.73'W), Kidderminster, Worcestershire, England, and re-described along with the specimens collected during this survey (Harbach et al., 2017). Both species are similar in appearance, however, adults can be distinguished by the base of the costal and subcostal wing veins pale being in *Oc. nigrinus* and dark in *Oc. sticticus*, and in the form of the basal dorsomesal lobe in the male genitalia. The two species are also considered to have differing ecology, both are flood water specialists, with *Oc. sticticus* found in flooded woodland, and *Oc. nigrinus* in open situations (Becker et al., 2010). This discovery emphasises the need for a reassessment of the British mosquitoes, and to compare specimens to those from across their species ranges in order to isolate misidentified conspecifics.

2.5.5 Other rare British species

The species *An. algeriensis* and *Cx. territans* were also recorded during this survey. Of these species the former is reported within the ECDC guidelines as having vector potential (level 2) and is a candidate competent vector of *Plasmodium sp*. (ECDC, 2014). Due to it its exophilic tendencies, however, it is classified as a secondary vector. This species shows a potentially restricted distribution in the East coast

(Snow, 1998), and from isolated base rich fens on Anglesey, North Wales from where we collected specimens in July 2015. Likewise, *Cx. territans* (Syn. *Culex apicalis*) has been recorded only 43 times from a handful of sites across GB, and we were only able to confirm this species from a single location in Abernethy, Scotland, where it appeared to be utilising small permanent waterbodies, in partially shaded Caledonian woodland. Samples of *Cx. territans* were collected in June 2016. This species is widely distributed across Europe, feeding primarily on amphibians and reptiles, and is not considered to be a potential risk to humans. The taxonomic status of this species is questionable. A recent analysis of morphological differences between specimens from the European Palearctic and Nearctic, were considered significant enough to separate the two species (Da Cunha Ramos et al., 2003). This observation, however, has still not become widely accepted by mosquito biologists and may only be resolved by additional molecular scrutiny.

2.5.6 Status of invasive mosquitoes in Britain

Parallel to this survey, a Port Invasive Mosquito Surveillance (PIMS) project was developed by EHU in collaboration with PHE and the Port Health Authorities to locate AIMs entering GB via international transport routes. In three consecutive years from 2016, the eggs of *Ae. albopictus* were found by PHE in oviposition traps at service stations in Kent, England (Vaux et al., 2019). Specimens of these eggs were sent to EHU for identification and so have been included within this dataset. Additionally, a male specimen of the 'Yellow Fever mosquito', *Ae. aegypti* was discovered in Merseyside, England and described in Chapter 5. Both species are important vectors of DENV, CHIKV, ZIKV and YELV, and although their establishment has not yet been proven (survival over winter periods), these

discoveries are significant, and therefore samples have been included within this study.

The discovery of these species exemplifies a need to develop additional methods of scrutinising cargo that enters the GB for possible introductions. Therefore, alongside a genetic analysis of these species, we have developed and tested a novel method of sampling imported car tyres for the presence of their eggs (Chapter 6).

2.6 Conclusion

Of the 28 species collected, 18 are considered to have a potential vector role in Europe (ECDC, 2014) (Table 2.2). Human affected arboviruses such as Tahyna virus, SINV, USUV and WNV have been detected in British birds (Buckley et al., 2003Gould et al., 2006; Medlock et al., 2005), and although there have been no recent autochthonous mosquito borne diseases reported in humans in GB, the possibility of existing, or future, transmission should not be taken lightly.

The distribution, ecology and seasonal variance of British mosquitoes was not examined here, however, this survey highlights the need for greater surveillance of native species, to plug gaps in current knowledge. Additionally, a revision of taxonomic guidance for mosquito workers is important, as this study has highlighted several difficulties in current GB literature. Prior to attempting such a feat, the first steps in resolving questions over species delimitations is required, and therefore the need for the exploration of GB mosquitoes by molecular characterisation is fully justified.

CHAPTER THREE:

Species profiling of GB mosquito species using cytochrome oxidase I (*COI*) with an eye to potential vector surveillance

3.1 Abstract

The surveillance of mosquito species with disease vector potential is of paramount importance to public health, however, challenges in species identification can make this process difficult. Many mosquito species form morphologically similar complexes with differing levels of disease potential. It is critical, therefore, that a genetic profile of species exists to provide rapid identification of vector candidates. Additionally, species genotyping can help to classify damaged specimens collected from surveillance traps, or eggs that are difficult to identify. We present here the first effort to profile all species of mosquito reported in Great Britain using *COI* along with the addition of sequences from specimens found across their global range.

The power of *COI* to separate GB mosquitoes was similar when using both 600 bp and 348 bp sequences. *COI* was effective at species delimitation in most GB mosquitoes, except for *Oc. annulipes* and *Oc. cantans* that were not separable using this method. Additionally, potentially cryptic species were found in specimens of both *Ae. vexans* and *Oc. flavescens*. The addition of global sequence data showed high levels of genetic variation within *COI* as well as several cryptic groups. A median-joining network analysis (MJN) suggested geographical distance as the underlying cause of many of these cryptic groups. Particularly between Nearctic and Palearctic regions. MJN also reveals a high number of haplotype and haploclade clusters within species complexes.

DNA barcoding is a useful tool for the surveillance of native and non-native mosquito species in GB as it provides a fast and cheap method of identification for most species and required only short reads of DNA for an accurate match. Most country specific barcoding projects do not include species range data from outside their area of interest. By doing so, we have shown that more cryptic clusters, either

species, or haploclades, may exist than previously believed. *COI* may therefore be a useful tool to determine taxonomic inferences, and to detect introductions of non-native conspecifics.

3.2 Introduction

The global movement of disease vector mosquitoes has become a global phenomenon leading to an increase in the distribution of major diseases (Mayer et al., 2017). Transportation of invasive alien mosquitoes is primarily by anthropogenic means (Grubaugh et al., 2018; Kindhauser et al., 2016), coupled with the effects of climate change expanding areas of habitability (Campbell et al., 2015; Metelmann et al., 2019). Species of the genus *Aedes*, have been particularly efficient at adapting to human influences, and are now one of the most prolific of invasive species (Ramasamy and Surendran, 2016).

This century has seen the spread of the invasive *Ae. albopictus* across Europe, with the first cases of autochthonous transmitted dengue fever virus (DENV) reported in 2010 in France, and chikungunya virus (CHIKV) appearing in Italy from 2007 (Tomasello and Schlagenhauf, 2013; Venturi et al., 2017). Introductions of other invasive *Aedes* mosquitoes (AIMs) are being reported frequently, with sporadic appearances of *Ae. aegypti* (Brown et al., 2011; Dallimore et al., 2017) and the establishment of *Ae. japonicus* in central and northern Europe (Eritja et al., 2019; Schaffner et al., 2009; Seidel et al., 2016; Zielke et al., 2016). Predicted effects of climate change suggest a future increase in the distribution of these vectors and their associated diseases (Cunze et al., 2016; Metelmann et al., 2019). Alongside the movement of invasive mosquitoes, disease transmission from native European species are also a concern with an upsurge in locally acquired transmission of the malaria parasite, *Plasmodium vivax* in Greece (Danis et al., 2011; Vassalou et al., 2017), Sindbis virus (SINV) and Inkoo virus (INKV) in Scandinavia (Bergqvist et al., 2015; Brummer-Korvenkontio et al., 2002; Putkuri et al., 2016), and West Nile

virus (WNV) in Eastern, Central and Southern Europe (Aberle et al., 2018; Calistri et al., 2010; Hubálek and Halouzka, 1999; Napp et al., 2018). Surveillance programmes have increased as a response, along with a revitalised awareness of the resurgent vector potential in native species (Calzolari, 2016; ECDC, 2014; Hubálek, 2008). The collection and identification of mosquito species is the foundation of these programmes. The identification of mosquitoes can be difficult, however, and species level taxonomy is far from resolved (Beebe, 2018). Identification problems arise mostly from morphologically similar complexes (i.e. *Culex pipiens* complex) (Smith and Fonseca, 2004) and species showing overlapping, and variable, morphological features (particularly those of subgenus *Culicella*). Additionally, specimens collected as part of vector surveillance programmes are often damaged or of a life stage (such as eggs, pupae, and some adult females) that makes species identification more difficult (Becker et al., 2010).

The characterisation of mosquitoes using genetic markers has been essential for bypassing these issues and allows for non-experts to carry out identification work in almost any molecular facility. The most frequently utilised method for mosquito genetic characterisation is the cytochrome c oxidase subunit I (*COI*) gene, one of three subunits that are a component of respiratory enzyme production in mitochondrial genomes. Approximately 1500 base-pairs in length this gene is located adjacent to the tRNA regions of Y and L1 and flanked by the protein coding genes of ND2 and COII (Fig. 3.1). Partial fragments of this gene, approximately 600 bp, have been used extensively in the ‘barcoding’ of species due to its slow rate of mutation, lack of indels and is minimally affected by recombination events (Hebert et al., 2003). In mosquitoes, *COI* has been highlighted as an effective gene for the

delimitation of species, it has few reported problems with amplification, and is capable of being used to identify cryptic or closely related species (Beebe, 2018). Large scale efforts in mosquito barcoding have already been undertaken in China (Wang et al., 2012), Belgium (Versteirt et al., 2015), Colombia (Andean region) (Rosera-Garcia et al., 2018), India (Kumar et al., 2007), Turkey (Gunay et al., 2015), Sweden (Engdahl et al., 2014), Canada (Cywinska et al., 2006), Singapore (Chan et al., 2014) and Southern Australia (Batovska et al., 2016). The Mosquito Barcoding Initiative, part of the International Barcode of Life project (iBOL), now holds ~45 000 *COI* sequences. Despite positive first appearances, the efficiency of *COI* in the delimitation of mosquito species has been mixed. Reported instances demonstrate *COI*'s inability to distinguish some species previously separated by morphology, such as specimens of *Oc. portonovoensis* and *Oc. wardi* (Kumar et al., 2007), and more relevant to this study, *Oc. annulipes* and *Oc. cantans* (Khrabrova et al., 2013; Versteirt et al., 2015), with both of the latter species being abundant in GB. Likewise, the ability of *COI* to sort species into monophyletic groups at deeper phylogenies can be poor, as has been reported for *Aedes*, *Ochlerotatus*, *Lutzia* and *Culex* (Chan et al., 2014; Khrabrova et al., 2013). However, the act of species identification for the purpose of surveillance, arguably takes priority over accurate taxonomic positioning of genera.

The comprehensive barcoding of the *COI* gene in GB species has been limited to several small localised projects that have relied heavily on inferences from specimens from other countries (Hernandez-Triana et al., 2017; Hernández-Triana et al., 2019). Here we have undertaken the first attempt to collect specimens of all the GB mosquito fauna (Chapter 2), and the partial sequencing of their *COI* genes (~600

bp). Additionally, we analysed our sequences against a larger data set that covers conspecific sequences from across global ranges. In doing so we hoped to test the GB species definitions for any synonymous confusion, and to investigate diversity of the *COI* gene across geographic distances outside of GB.

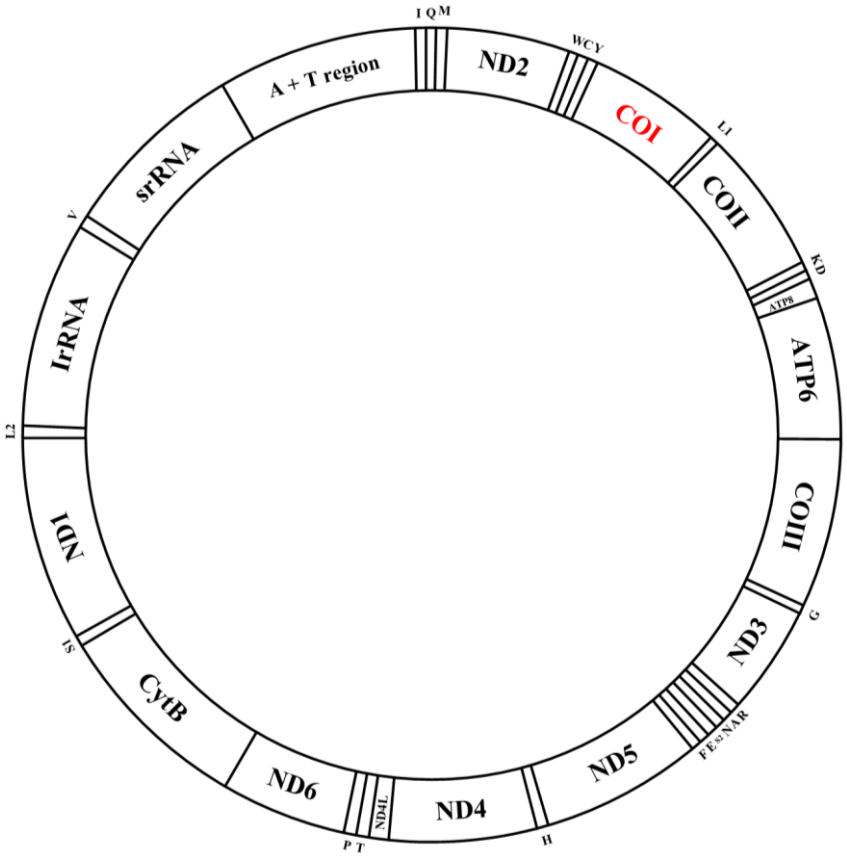


Fig 3.1 Mitochondrial genome structure of an *Anopheles* mosquito. Including the location of the cytochrome oxidase subunit I (*COI*) gene.

Location of *COI* highlighted in red. Whole mitochondrial structure approximately 15 400 bp. Illustration reproduced from Hao et al., (2017).

3.3 Methods

3.3.1 Specimen collection and storage

An attempt was made to collect representative specimens of all GB mosquito species by using a targeted sampling approach. Sample locations were selected using historical species, or suitable habitat, records and options filtered based on likelihood of collecting specimens. Where possible efforts were made to collect a minimum of five specimens of each species, 106 locations were sampled across GB. All samples were collected primarily by sweepnet, or by dipping for larvae (method and results summarised in Chapter 2). All collected samples were stored at -20ºC prior to species confirmation by morphology and tissue extraction. DNA was extracted from a mean of six individuals from each species. In the instances where the total number of specimens collected was below five all individuals were sequenced. A higher number of extractions were performed for species with a broad distribution (>5 sites) (*An. claviger, Cs. annulata, Cq. richiardii, Oc. detritus, Oc. punctor, Oc. rusticus*) or from those within a species complex (*Cx. pipiens s.l./torrentium, Oc. annulipes/cantans*), allowing for additional exploration for cryptic clades. Individuals within a species were selected for sequencing by using a random stratified sampling method to ensure unbiased coverage from across, and within, all samples sites. Any mosquitoes that showed signs of fungus were rejected due to, downstream complications with non-specific amplification. The number of extracted sequences per species is given in Table 3.1.

3.3.2 DNA extraction

Two legs were removed from each adult mosquito and coarsely ground using a motorised, hand-held, homogeniser and disposable pestles (431-0094, VWR, Leicestershire, UK). Samples were homogenised in the same 1.5 µl microcentrifuge tubes used for the lyses phase to avoid cross contamination. DNA extraction was undertaken using DNeasy Blood & Tissue spin columns (69506, Qiagen, Manchester, UK) in accordance to the manufacturer's instructions. Modifications to the protocol included an extended lysis time of 10-15 hours at 56 ºC on an orbital incubator (SI500, Stuart®, Staffordshire, UK) to increase yield. DNA yield was checked, and purity measured using A260/280 and A260/230 absorption with a Thermo Scientific, NanoDrop™ Lite spectrophotometer (Thermofisher, Altrincham, UK). Failed extractions were excluded from PCR amplification.

3.3.3 Polymerase chain reactions

A 735 bp region of the *COI* gene was targeted for amplification by direct (non-cloned) polymerase chain reaction (PCR) using the primer set of Chan et al (2014) and tested against all species. Suspected polymorphic SNP sites within the primer regions resulted in some taxa being unsuccessfully amplified. To gain full species coverage, existing *COI* sequences for non-amplifying species were downloaded from GenBank and aligned using the multiple sequence alignment tool MUSCLE. Novel oligos were designed using conserved regions and tested for secondary structures, primer dimer, Tm, and GC content (%) using the Sigma-Aldrich® (Merck) online OligoEvaluator™ (https://www.sigmaaldrich.com/technical-documents/articles/biology/oligo-evaluator.html). Additional primers designed by Folmer et al (1994) were also used (see Table 3.2 for all primer codes). Primer

AGEMTDf/r was designed to amplify *COI* from both closely related species *Ae.cinereus* and *Ae.geminus,* however, testing of primer efficiency suggests that it only targets those of *Ae.geminus*.

A 25 µl PCR reaction was undertaken for each sample and consisted of 2.5-20 ng of DNA template, 0.5 U of Phusion® High-Fidelity Polymerase (New England Biolabs® Ltd. Herts, UK(NEB)), 1x Phusion HF Buffer (NEB), 200 µM of dNTP mix (NEB), 0.5 µM each primer, and 2% DMSO (NEB).

PCR amplification was performed using a Primer Thermal Cycler (Techne, Staffordshire, UK) programmed with an initial denaturation of 98°C for 30 s, followed by 35 cycles of 98°C for 10 s for denaturation, 20 s of annealing (COIF/R + LCO1490/ HC02198= 54ºC, AMACTDf/r + AALGTD + AGEMTDf/r = 48ºC, CMORTDf/r = 50ºC), and 72°C for 20 s extensions, followed by a final extension of 72°C for 7 mins. A 10 µl sample of each PCR product was resolved by electrophoresis prior to purification. Non-successful amplifications were screened out prior to the purification step. PCR products were purified by EXOSAP reaction where 0.19 µL of Exonuclease I (NEB), 0.56 µL of Shrimp Alkaline Phosphatase (NEB), and 0.375 of dH_20 was added to the remaining PCR product (on ice) and passed through a single cycle of 37ºC for 30 mins and 72ºC for 20 mins followed by a 4ºC hold.

Zoogeographic regions	**Palearctic**								**Oriental**			**Afro-tropical**	**Nearctic**		**Neo-tropical**			**Accession no.†**
Species	GB*	GB (Other)	Europe	Russia	China	Tajikistan	Iran	Afghanistan	East Asia	Pakistan	S.E Asia	Central Africa	Canada	USA	South America	**Total**	
An. algeriensis	1	-	4	-	-	-	-	-	-	-	-	-	-	-	-	**5**	KP942714, KU214675, KT876467, KF754804
An. atroparvus	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**	KU877020
An. claviger	11	-	7	-	-	1	1	-	-	-	-	-	-	-	-	**20**	KP942736, MF095665, AF253042, KU308382, JF966742, KM243938, KF754806, KM457607, JX255721
An. daciae	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	**2**	AY757954, AY757924
An. maculipennis s.l.	1	-	7	1	-	-	1	-	-	-	-	-	-	-	-	**10**	AY258190, AF342722, GU908010, KY196462, DQ118177, KM258236, MF095667, LK054514, KF318189, KM457608
An. messeae	-	1	8	-	-	-	-	-	-	-	-	-	-	-	-	**9**	KU876989, KM280597, KU214663, KP942740, AY258182, AY258172, HE659586, AF342723, KM258223
An. plumbeus	5	-	4	-	-	-	1	-	-	-	-	-	-	-	-	**10**	KM258217, KM258218, KM258219, JF966740, KM280577
Ae. aegypti	1	-	-	-	-	-	-	-	-	-	-	4	-	1	1	**7**	GQ165783, KX446447, GBMIN56180-17, KT881425, KX446464, MF371170
Ae. albopictus	1	-	1	-	1	-	-	-	1	-	1	-	-	1	-	**6**	ACMIP154-07, ENTJR153, GBDCU001-12, GBDCU676-12, GBDP16960-15

Ae. cinereus	-	-	6	1	-	-	-	-	-	-	-	-	4	2	-	**13**	KR965255, GU907871, KR395489, KF761595, JX259553, MG242481, KP942718, JX040511, KY607730, KM258353, AF253027, KT876487, KM457571
Ae. geminus	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**	
Ae. vexans	6	-	6	-	1	-	-	-	-	-	-	-	3	1	-	**17**	KM258257, KY609204, KP942710, KR525482, GU907995, MF822045, MG242525, AY917213, MF179152, AF253041, KT876477, KM457605
Da. geniculata	3	-	8	-	-	-	-	-	-	-	-	-	-	-	-	**11**	KM258315, KM258314, KM258309, KP942768, KP942767, KP942766, KM280584, GMGMA1396-14
Oc. annulipes	12	3	5	-	-	-	-	-	-	-	-	-	-	-	-	**20**	KM258382, KM258380, KM258389, KC602651, KC602652, KC602659, GU289201, JX040507
Oc. cantans	9	4	5	2	-	-	-	-	-	-	-	-	-	-	-	**20**	KY607731, KX064675, KP942755, JX040506, KU876958, KU876946, KC602647, KC602641, KM258370, KM258376, KM258375
Oc. caspius	5	-	12	1	-	-	3	-	-	2	-	-	-	-	-	**23**	KT361856, KT361864, KT361861, KC855625, KT876464, KP942727, KP942758, LC090042, LC090050, KJ768114, KJ768092, KM258362, KM258365, KM258360, HM140418, HM140416, KM452948, KM452934
Oc. communis	-	-	5	1	-	-	-	-	-	-	-	-	5	-	-	**11**	KP942762, JX040509, KM258335, KM258338, KM258339, KC855584, GU907877, ACMIP058-07, ACMIP238-07, JF868932, KR464826
Oc. detritus	9	4	4	-	-	-	-	-	-	-	-	-	-	-	-	**17**	KM258322, KM258321, KM258326, KU876965, KU876977, KU876998, KC602685, KT876476
Oc. dorsalis	-	1	2	1	-	-	-	-	1	-	-	-	4	1	-	**9**	JQ246392, KC855609, KP942728, KP942726, KT358408, JF868954, MF827467, KR689712, KM940705, IUP695-14
Oc. flavescens	5	3	3	-	-	-	-	-	-	-	-	-	4	-	-	**15**	JF868910, JF868922, KR688348, KR689544, KC602636, KC602637, KC602639, KT876484, KP942764, KM457575
Oc. leucomelas	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	**2**	KP942730, KP942729
Oc. nigrinus	5	-	2	-	-	-	-	-	-	-	-	-	-	-	-	**7**	KP942770, KP942769
Oc. punctor	19	3	6	3	-	-	-	-	-	-	-	-	2	2	-	**35**	KM258292, KM258293, KM258285, ASDIP399-15, MOSN090-17, GBDCU1104-14, KC602675, KC602677, KC602667, KC855582, KC855579, KC855577, JX040508, KP942772, KP942771

Oc. sticticus	6	-	8	1	-	-	-	-	-	-	-	-	3	1	-	**19**	KM243959, KX064673, KM243960, KM280585, KM280587, KU875641, KM258268, KM258266, KM258261, JX040512, GU907942, KF535011, KM936953
Oc. rusticus	7	1	4	-	-	-	-	-	-	-	-	-	-	-	-	**12**	KP942776, MSEMV428-15, KM258272, KM258277, KM258269
Cs. fumipennis	-	1	4	-	-	-	-	-	-	-	-	-	-	-	-	**5**	KM258139, KM258140, KM258138, KM457616, KU748471
Cs. litorea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	
Cs. morsitans	2	3	7	-	-	-	-	-	-	-	-	-	4	2	-	**18**	GU908114, MF823656, KR383868, MF827272, JX259982, NEONU376-11, KM258137, KM258134, KM258133, KP942747, KP942748, GBMTM750-15, GBMTM944-15, KU748460, MSEMV309-15, KU748453
Cs. annulata	14	3	5	-	-	-	-	-	-	-	-	-	-	-	-	**22**	KM258146, KM258145, KM258156, KT876473, KM280593, KU748481, KU748423, KU748438
Cs. alaskaensis	-	-	1	-	-	-	-	-	-	-	-	-	2	2	-	**5**	ACMIP205-07, ACMIP215-07, KP942744, KU874741, KU874740
Cs. subochrea	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	**1**	KP942749
Cs. longiareolata	-	-	3	-	-	-	-	1	-	-	-	-	-	-	-	**4**	KJ124849, KJ124850, JQ388785, HG931139
Cx. pipiens s.l.	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**15**	GU908083, JQ728284, JQ958370, GQ255648, JN592736, KP293424, KP728870, KP728861, MONSW042-17, KU495007, MOAV036-15, KC407759, KC407758, LC054466, JQ253834, JQ253835, JQ253836, KU175256, GU289209, GU289211, GU289221, GU289218, KP728874, KP728856, KU175252
Cx. torrentium	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**5**	JQ253809, JQ253810, JQ253812, JQ253815, JQ253816, JQ253818, JQ253819, JQ253822, JQ253823, JQ253827, JQ253831, JQ253833, JQ253832, KU175265, KM439054, KM439055, KM258159, GU300725, GU300731, GU300729, GU300749, FN395198, FN395194
Cx.pipiens/torrentium	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**7**	
Cx. modestus	4	2	9	1	3	-	-	-	-	-	-	-	-	-	-	**19**	MF537266, MF537267, KJ012102, KU877022, KM452947, JN592731, JN592748, JQ728375, JQ728112, JQ728108, FM177758, HF562837,

																	KT876488, KU214653, KM280578
Cx. territans	1	-	4	-	-	-	-	-	-	-	-	-	5	2	-	**12**	KM258164, KM258162, KM258165, KM280581, GU908103, ACMIP208-07, BBGCO942-15, KR425704, KR389985, JX259926, JX259921
Cq. richiardii	15	3	6	-	-	-	-	-	-	-	-	-	-	-	-	**24**	KM258213, KM258202, KM258205, JX040513, KT876478, KT876472, KU876993, KU876980, KU876982
Or. pulcripalpis	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	**1**	KY608735
Total	**170**	**32**	**142**	**10**	**5**	**1**	**6**	**1**	**1**	**2**	**1**	**4**	**27**	**14**	**1**	**440**	

Table 3.1 Geographic locations of *COI* sequences acquired for analysis.

*Samples collected during this study. †Includes accession numbers from both GenBank® and BOLD databases.

3.3.4 Sequencing reactions

Sequencing reactions were undertaken using the BigDye™ Terminator v.3.1 Cycle Sequencing Kit (4337455, Applied Biosystems® (AB), Paisley, UK) using the following modified protocol for 10 µL reactions. 1 µL of template DNA (PCR product) modified depending on yield requirement specified by the manufacturer, 0.32 µL of primer (100 µM), 0.5 µL of BigDye™ v. 3.1 (undiluted from manufacturers specifications), 1.75 µL of BigDye™ buffer (undiluted from manufacturers specifications), made up to 10 µL (total reaction volume) with dH_20. Sequencing reaction clean-up was by EDTA-ethanol precipitation with final resuspension in 10 µL of Hi-Di™ Formamide (4311320, AB). Sequencing was carried out on an AB3500 genetic analyser, 50 cm capillary array (AB) using a z-type dye matrix (4404312, AB).

3.3.5 Raw sequence treatment

Contigs were constructed using forward and reverse sequences for each specimen, and aligned and merged using MEGA7 (Kumar et al., 2016), ambiguous flanking regions were removed. Any unscored nucleotides were visually checked on the chromatograph and where appropriate corrected. All *COI* reads were checked for congruent sequences in the NCBI database, GenBank (https://www.ncbi.nlm.nih.gov/genbank/), using the BLASTn search function retaining default settings.

Ref.	loci	Primer (5'→ 3')	Target species	Source
COIF	*COI*	GGATTTGGAAATTGATTAGTTCCTT	*An. claviger, An. plumbeus, Ae. vexans, Cs. annulata, Cq. richiardii, Cx. modestus, Cx. pipiens, Cx. territans, Cx. torrentium, Da. geniculata, Oc. annulipes, Oc. cantans, Oc. caspius, Oc. detritus, Oc. flavescens, Oc. nigrinus, Oc. punctor, Oc. rusticus, Oc. sticticus*	Chan *et al.*, 2014
COIR	*COI*	AAAAATTTTAATTCCAGTTGGAACAGC		
LCO1490	*COI*	GGTCAACAAATCATAAAGATATTGG	*Ae. aegypti, Ae. albopictus, Da. Geniculata*	Folmer *et al.*, 1994
HC02198	*COI*	TAAACTTCAGGGTGACCAAAAAATCA		
CMORTDf	*COI*	GGATTTGGTAATTGATTAGTTCCTT	*Cs. morsitans, Cs. litorea*	Novel
CMORTDr	*COI*	AAAAATTTTAATTCCTGTAGGTACTGC		
AGEMTDf	*COI*	GGATTTGGTAATTGACTTGTTCCTT	*Ae. cinereus, Ae. geminus*	Novel
AGEMTDr	*COI*	AAAAATTTTAATTCCAGTTGGAACAGC		
AALGCOIf*	*COI*	GGATTTGGTAATTGATTAGTACCAT	*An. algeriensis*	Novel
AMACCOIr	*COI*	GGATTTGGAAACTGATTAGTTCCTT	*An. messeae*	Novel
AMACCOIr	*COI*	AAAAATTTTAATTCCTGTTGGTACAGC		

Table 3.2 Primers used for *COI* gene amplification in GB mosquitoes

*Primer was used in combination with COIR for amplification of *An. algeriensis COI*.

3.3.6 Additional sequence data mining

To gain a genetic overview of each of the species found in the GB, barcoding genes were compared to available sequence data from across their range. A search of additional *COI* DNA data was carried out using the BOLD© database (http://www.boldsystems.org/index.php/databases), and GenBank®. Specimens that could add value to the dataset were selected based on sampling location. To gain broad coverage a maximum of two sequences were selected from each sample location using a random number generator. Any additional GB specimens were also mined and added to the dataset. The number of additional sequences per species is given in Table 3.1.

3.3.7 Alignments

COI is a protein-coding gene that lacks indels such as those found in the non-coding *ITS2* (Internal Transcribed Spacer 2, investigated in Chapter 4) and, as a result, alignments were generally efficient. Any ambiguous nucleotides were removed or modified from within regions that show no polymorphisms across closely related species. Alignments were carried out using a nucleotide approach in MUSCLE with default settings, followed by a visual inspection for errors in MEGA7. When additional library sequences were added to the GB dataset the variability in *COI* primer usage across datasets was limited by overlapping read length to 348 bp. To determine whether the reduced sequence length data affected analyses, two datasets were created using the same GB sequences with (i) 600 bp coverage, and (ii) edited to 348 bp, representing total coverage from overlapping sequences.

3.3.8 Methods of analysis

3.3.8.1 Phylogenetic approach

A maximum likelihood (ML) approach was used for phylogenetic analysis for all datasets. Selection of suitable nucleotide substitution models was undertaken to account for transition/transversion bias and invariant sites by testing both datasets against 28 candidates in Findmodel (HFV/Ebola Database, https://hfv.lanl.gov/content/sequence/findmodel/findmodel.html), a web-based implementation of Modeltest (Posada and Crandall, 2001, 1998), and confirmed using the Find Best DNA/Protein Models (ML) function in MEGA7. A General Time Reversible model with two discrete gamma categories and compensating for invariant sites (GTM+G+I) was deemed most appropriate for both alignments. The robustness of the phylogeny was tested by applying 1000 bootstrap replications. Phylogenetic trees were rooted using two species of Family: Chaobridae as an outgroup for datasets including global data, and two species of Family: Dixidae for testing sequence length efficiency in GB sequences. Visualisation of the trees was produced using the Interactive Tree of Life web-based interface v.4.3.2 (iTOL) (https://itol.embl.de/).

3.3.8.2 Interspecific and intraspecific divergence

Interspecific and intraspecific distances were calculated by pairwise distances using the Kimura-2-parameter model with 1000 permutations (substitutions included transitions + transversions with uniform rates amongst sites), using MEGA7.

3.3.8.3 Median joining network reconstruction

A median joining network (MJN) analysis was also applied to the *COI* sequence data for each genus. This method provides additional perspective on genetic clustering by visualising cycles of evolutionary possibilities compared to phylogenetic trees that only display a single possible evolutionary outcome. This is particularly useful when high levels of homoplasy struggle to be accounted for by traditional phylogenetic distance models (Bandelt et al., 1999; Zecca et al., 2012). MJN analysis was undertaken using the programme NETWORK version 5.0.1.1 (http://www.fluxus-engineering.com). Reticulations were broken using rules described by Pfenninger and Posada (Pfenninger and Posada, 2002).

3.3.8.4 ABGD (barcode gap) analysis

Automated Barcode Gap Discovery (ABGD) uses alignment data to best assign hypothetical species using the distribution of pairwise distances, also known as 'barcoding gaps' (Puillandre et al., 2012). Analysis on whole family data as well as by genus was undertaken using the graphic web version (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) were Pmin = 0.01, Pmax = 0.1 and 10 steps, X = 1.5 Nb bins = 20, a Kimura (K80) TS/TV approach to distance was applied.

3.3.8.5 Poison tree processes (PTP and bPTP) of species delimitation

A poisson tree process (PTP) model was applied to the phylogenetic tree outputs to infer putative species boundaries using branching events by number of substitutions, as well as adding Bayesian support values (bPTP). PTP outputs are calculated as part of the bPTP implementation process and settings for a Bayesian approach are reliant on meeting a strict set of assumptions for convergence of Markov chain Monte Carlo (MCMC) chains. If convergence is not achieved, then Bayesian support values are meaningless. This was checked visually using the bPTP web server trace plot

function (Zhang et al., 2013). Where convergence was not met the number of MCMC was incrementally increased from 100 000 to a maximum of 500 000, and 'seed' function increased across the range from 123 to 2 000 and Burn-in 0.1 to 0.5. Where this had no effect on improving convergence, the bPTP model was considered unsuitable for the data and a PTP (maximum likelihood) approach was utilised solely based on default settings. A generalized mixed yule coalescent model (GMYC) analysis was not used, as tree inputs requires time calibration to conduct the analysis.

3.4 Results

A total of 170 specimens from the GB survey (Chapter 2) from 28 species identified using morphological characteristics were sequenced. An additional 32 sequences from specimens collected in the GB was mined from repositories and added to the dataset giving a total of 202 GB mosquito sequences representing 29 species. Preliminary cryptic diversity across species ranges, plus additional missing species, was investigated using an additional 238 sequences. This final dataset represented all the 34 species of mosquito reported to be found in the GB, plus the newly discovered *Oc. nigrinus* and both the reported AIMs, *Ae. aegypti* and *Ae. albopictus*.

3.4.1 Recoverability (Sequence success)

Overall sequencing success of the *COI* region was high ($\bar{x}$ = 80%) with a mean sequence length of 626 bp achieved across all samples. However, the ratio of sequencing success against failure was species dependant (where sequencing failure was considered as <500 bp continual reads) with some more problematic than others, particularly those of *An. algeriensis* (1(success):7(failure)), *Ae. geminus* (0:4), *Oc. punctor* (9:17) and *Cs. morsitans* (2:24). All sequencing failures were despite

positive amplification of DNA from PCR reactions confirmed by gel electrophoresis. These issues did not appear to be caused by poly regions, but possibly due to NUMTs (nuclear copies of mitochondrial origin) which are found to be common in some species of mosquitoes (Black and Bernhardt 2009). Further research would be required to confirm this cause. A summary of sequence success rates is found in Table 3.3.

3.4.2 Nucleotide characteristics

The frequency of guanine (G) and cytosine (C) was low (A = 28.1%, C = 15.4%, G = 15.6%, T = 38%), but comparable to those found in other mosquito *COI* studies (Batovska et al., 2016, Cywinska et al., 2006; Torres-Gutierrez et al., 2016; Wang et al., 2012), as well as for other animal groups such as Insecta: Hymenoptera (Li et al 2008), Crustacea: Malacostraca (da Silva et al., 2011), and Acari: Tetranychidae (Ros and Breeuwer, 2007). Across the different genera; *Anopheles* (GC% = 33.1), *Aedes* (Inc.*Stegomyia*) (34%), *Dahliana* (33.6%), *Ochlerotatus* (33.6%), *Culiseta* (34.2%), *Culex* (33.7%), *Coquillettidia* (38.2%) and *Orthopodomyia* (31.3%). GC frequency for individual species is reported in Table 3.4.

3.4.3 Distance analysis

The mean pairwise nucleotide sequence divergence, using a Kimura 2-parameter model, across all GB sequences was 15.5%, and 15.6% across global ranges. Similar to scores reported by Khabrova et al (2013) (14.3%) and Cywinska et al (2006) (16%). Congeneric K2P divergence ranged from 5.7% to 11.6% (not including genera represented by a single species). Conspecific sequence divergence increased with the addition of sequences from across the global range, with the mean K2P

distance across the datasets increasing from 0.9% to 1.5%. Most notably, maximum observed K2P distances scores within *An. claviger* 4.8% (1.5% mean across species), *Ae. vexans* 5.7% (2.9%), *Oc. caspius* 3.5% (1.4%), Cs. alaskaensis 5.7% (2.5%), *Cx. modestus* 4.8% (1.8%), *Cx. territans* 6% (3.2%), *Oc. flavescens* 7.9% (0.02%) and *Oc. punctor* 8.2% (2.5%) were higher when global range data was analysed. Conversely, several species showed little variation across all four datasets, *Oc. rusticus* 0.3 to 0.7% max (0 to 0.2% mean), *Cq. richiardii* 0.2 to 1.8% (0 to 0.3%), *Da. geniculata* 0.3 to 0.9% (0.2 to 0.4%), *An. plumbeus* 0 to 0.6% (0 to 0.1%). A summary of genetic distance measurements can be found in Table 3.5.

	Anoph.	***Aedes***	***Dahl.***	***Ochl.***	***Culiseta***	***Culex***	***Coquil.***	***Ortho.***	**All**
COI									
n of species barcoded	4/6	4/5	1/1	9/12	2/5	4/4	1/1	0/1	**25/35**
Total no. successful sequences *	15	8	5	67	15	28	13	-	**151**
Sequencing success rate (%)	72	69.2	87.5	88.5	46.1	97	100	-	**80**
Partially recovered sequences (%) †	12	7.7	25	2.6	7.7	12.1	20	-	**12.3**
Mean seq. length (bp)	613.4	654.9	558.3	680.8	598.9	655.7	620.9	-	**626.1**
Mean (SD) no. of specimens barcoded per species	4.5 (4.5)	1.5 (2.2)	8 (0)	7.6 (2.9)	9 (7.1)	6.4 (5.3)	16 (0)	-	**6.5 (4.5)**
GC richness (% mean)	34.1	33.4	33.6	33.9	34.5	33.5	38.2	-	**34.5**
Ts/Tv									**1.147**

Table 3.3 Sequence recoverability and additional information of *COI* gene from samples collected in the GB (by genus).

* Total sequence read > 500 bp. † Total sequence read <500 bp. Genera: Anoph.= *Anopheles*, Dahl. = *Dahliana*, Ochl. = *Ochlerotatus*, Coquil. = *Coquillettidia*, Ortho. = *Orthopodomyia*.

Genus/species	UK only			Across species range			GC (%)
	n	**Within species mean distance (K2P)**	**Within species max. observed distance (K2P)**	***n***	**Within species mean distance (K2P)**	**Within species max. observed distance (K2P)**	
Anopheles							**33.1**
An.algeriensis	1	-	-	4	0.016	0.027	33.6
An.claviger	10	0.003	0.009	17	0.015	0.048	34.6
An.maculipennis s.l.	3	0.03	0.045	23	0.024	0.054	31.5
An.plumbeus	5	0	0	10	0.001	0.006	34.2
***Aedes* (inc. *Stegomyia*)**							**34**
Ae.aegypti	1	-	-	5	0.011	0.021	34.4
Ae.albopictus	1	-	-	6	0.001	0.003	35.7
Ae.cinereus/geminus	1	-	-	10	0.03	0.06	34.8
Ae.vexans	6	0.026	0.048	14	0.029	0.057	32.6
Dahliana							**33.6**
Da.geniculata	3	0.002	0.003	8	0.004	0.009	33.6
Ochlerotatus							**33.6**
Oc.annulipes	15	0.005	0.017	19	0.006	0.018	34.1
Oc.cantans	13	0.011	0.02	20	0.01	0.02	34
Oc.caspius	6	0.007	0.012	21	0.014	0.035	31.4
Oc.communis	0	-	-	9	0.001	0.006	33
Oc.detritus	12	0.002	0.006	16	0.005	0.018	33.9
Oc.dorsalis	1	-	-	6	0.016	0.026	31.7
Oc.flavescens	7	0.025	0.076	14	0.02	0.079	35.1
Oc.leucomelas	0	-	-	2	0.003	0.003	35.2
Oc.nigrinus	5	0.009	0.015	7	0.009	0.023	33
Oc.punctor	12	0.008	0.015	21	0.025	0.82	35.7
Oc.sticticus	6	0	0	17	0.026	0.135	33
Oc.rusticus	8	0	0	12	0	0.003	32.5
Culiseta							**34.2**
Cs.alaskaensis	0	-	-	5	0.025	0.057	32.9
Cs.annulata/subochrea	16	0.001	0.006	22	0.001	0.006	34.4
Cs.fumipennis	1	-	-	5	0.062	0.102	34.4
Cs.longiareolata	0	-	-	4	0	0	32.5
Cs.morsitans	4	0.046	0.086	12	0.068	0.122	34.5
Culex							**33.7**
Cx.modestus	6	0.007	0.015	18	0.018	0.048	33.9
Cx.pipiens s.l.	23	0.002	0.018	37	0.004	0.029	33.1
Cx.territans	1	-	-	9	0.032	0.06	34.9
Cx.torrentium	19	0.001	0.006	23	0.001	0.006	33.9
Coquillettidia							**38.2**
Cq.richiardii	17	0.003	0.018	23	0.003	0.018	38.2
Orthopodomyia					-		**31.3**
Or.pulcripalpis	0	-	-	1	-	-	31.3
Across all genera	**203**	**0.0094**		**420**	**0.015**		**31**

Table 3.4 Intraspecific distances (K2P), and GC content for GB mosquitoes and from across species ranges.

Numbers in bold refer to the mean GC% score across a genus.

3.4.4 Phylogenetics

Phylogenies at the generic level or below were weakly supported by bootstrap values (bootstrap = <75). This includes sub-genera that tended to cluster well, but still scored poorly. This problem was observed in both GB and species range phylogenies. To test whether the efficiency of deep phylogeny reconstruction was affected by sequence length, two datasets were created using only GB sequences with 600bp and 348bp alignments and analysed using the same phylogenetic approach. Longer sequence reads did marginally improve bootstrap values, however, neither approach was able to add enough power to support monophyletic grouping (where bootstrap scores should minimally be > 75) (Figure 3.2).

Species level determination performed much more consistently, with clustering strongly supported by bootstrap values (>75). However, *Oc. annulipes/cantans* were not strongly supported in phylogenies of GB specimens, or after the inclusion of sequences from across the species ranges. The addition of species range data was also unable to split *Oc. caspius/dorsalis* by bootstrap values alone. The analysis of GB sequences showed possible cryptic species grouping in *Ae. vexans* and *Oc. flavescens,* with both supported by bootstrap scores of 100.

The introduction of additional data from across species ranges increased the number of potential cryptic species by phylogenetic analysis with strongly supported monophyletic groups in *Ae. vexans* (4 groups*), Cs. alaskaensis* (2), *Cs. morsitans* (3), *Cx. modestus* (2), *Cx. pipiens* (2), *Cx. territans* (3), *Oc. flavescens* (2), *Oc. punctor* (5), *Oc. sticticus* (2).

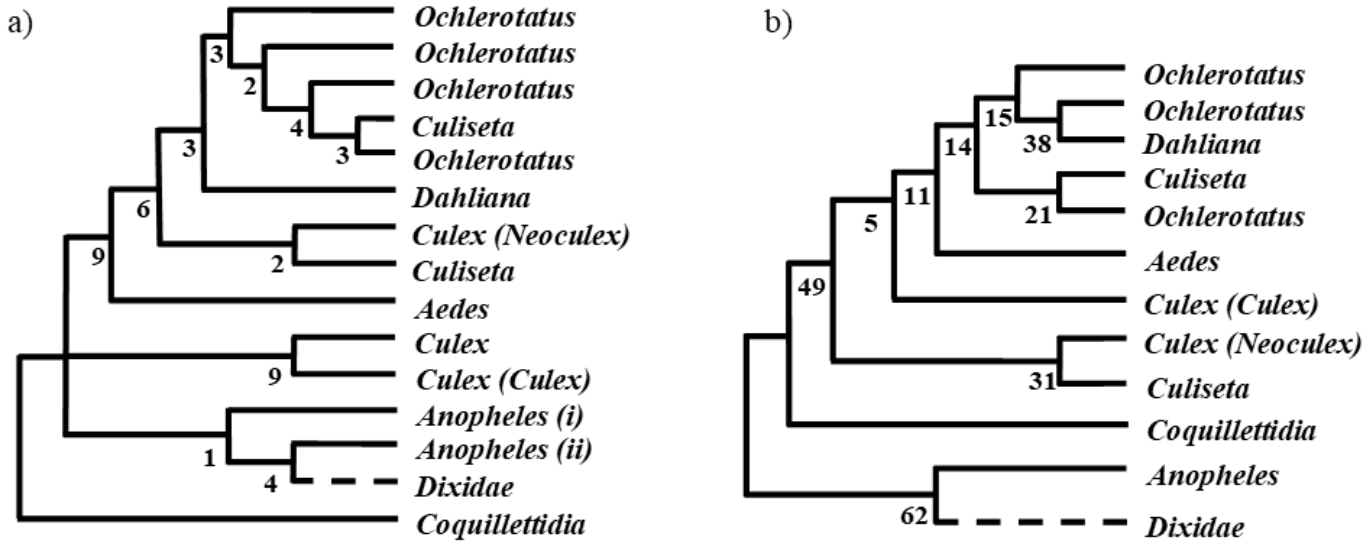


Fig. 3.2 Cladograms displaying deeper phylogenetic bootstrap scores using only GB specimens of a) 348 bp sequence lengths, and b) 600 bp.

Cladograms constructed using bootstrap values from maximum likelihood phylogenetic analysis, using a GTM model (+*G*(2), +I). Branch lengths are not representative of evolutionary distance

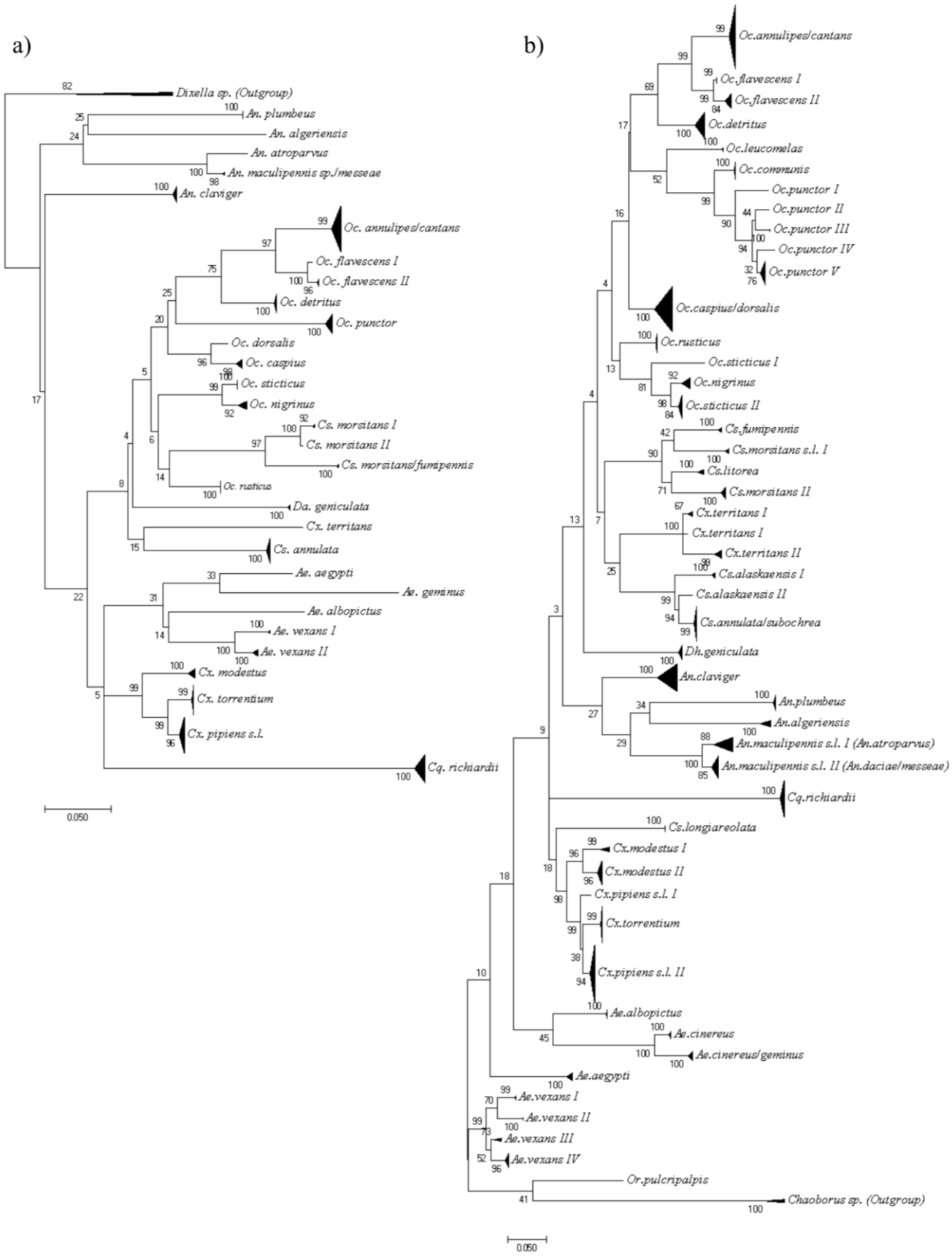


Fig. 3.3 Phylogenetic analysis of *COI* gene sequences where a) shows GB mosquito data only, and b) includes sequences from across species ranges.

a) and b) were inferred using ML trees by GTM. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution (+*G*) was used to model evolutionary rate differences among sites (2 categories). The rate variation model allowed for some sites to be evolutionarily invariable (+*I*). Trees are drawn to scale, with branch lengths measured in the number of substitutions per site. a) Involved 205 nucleotide sequences b) 423, both had a total of 348 positions. Nodes at lower taxonomic resolution have been collapsed where BS values are <75, higher phylogenetic inferences have been retained to visualise deeper phylogeny issues within *COI*.

3.4.5 Species delimitation

Phylogenetic analysis should not be solely relied upon for the delimitation of unique species groups (Knowles and Carstens, 2007). Here we applied additional methods to assign hypothetical species to the global range dataset using ABGD and PTP analyses. ABGD uses barcoding gaps where intraspecific divergence is delimited from the interspecific diversity based on pairwise distance data. This method automatically, recursively, applies a model-based, one-sided, confidence limit for intraspecific divergence. This means that hypothetical delimitation can be applied to groups at even the population level and are presented based on several probable partitions, without the need for strict monophyletic groupings (Puillandre et al., 2012). The results from the initial analysis suggested 7 different partition possibilities, ranging from 34 (P = 0.021544) to 143 taxonomic groups (P = 0.001000). The 6^{th} recursive partition suggests 40 delimited groups (P= 0.012915) and is the closest to the hypothetical number of species based on K2P and phylogenetic observations alone. These taxonomic assignments can be seen in Table 5. Distances were bimodal for the whole dataset, this was also consistent when the data was viewed by genus, although there is a noticeable overlap in the maximal interspecific and minimal intraspecific distance values (max-inter = 0.01 to 0.09, min-inter = 0.02 to 0.12) (Fig. 3.4).

Due to ABGD's ability to push hypothetical species concepts to represent taxonomic subdivision by population, data should be interpreted with an additional statistical perspective (Puillandre et al., 2012). PTP analysis is a good comparison to ABGD as it uses an operational taxonomic unit picking method (OTU-picking), by applying a Poisson tree model to infer putative species boundaries. A different approach to that of methods of barcode gap analysis.

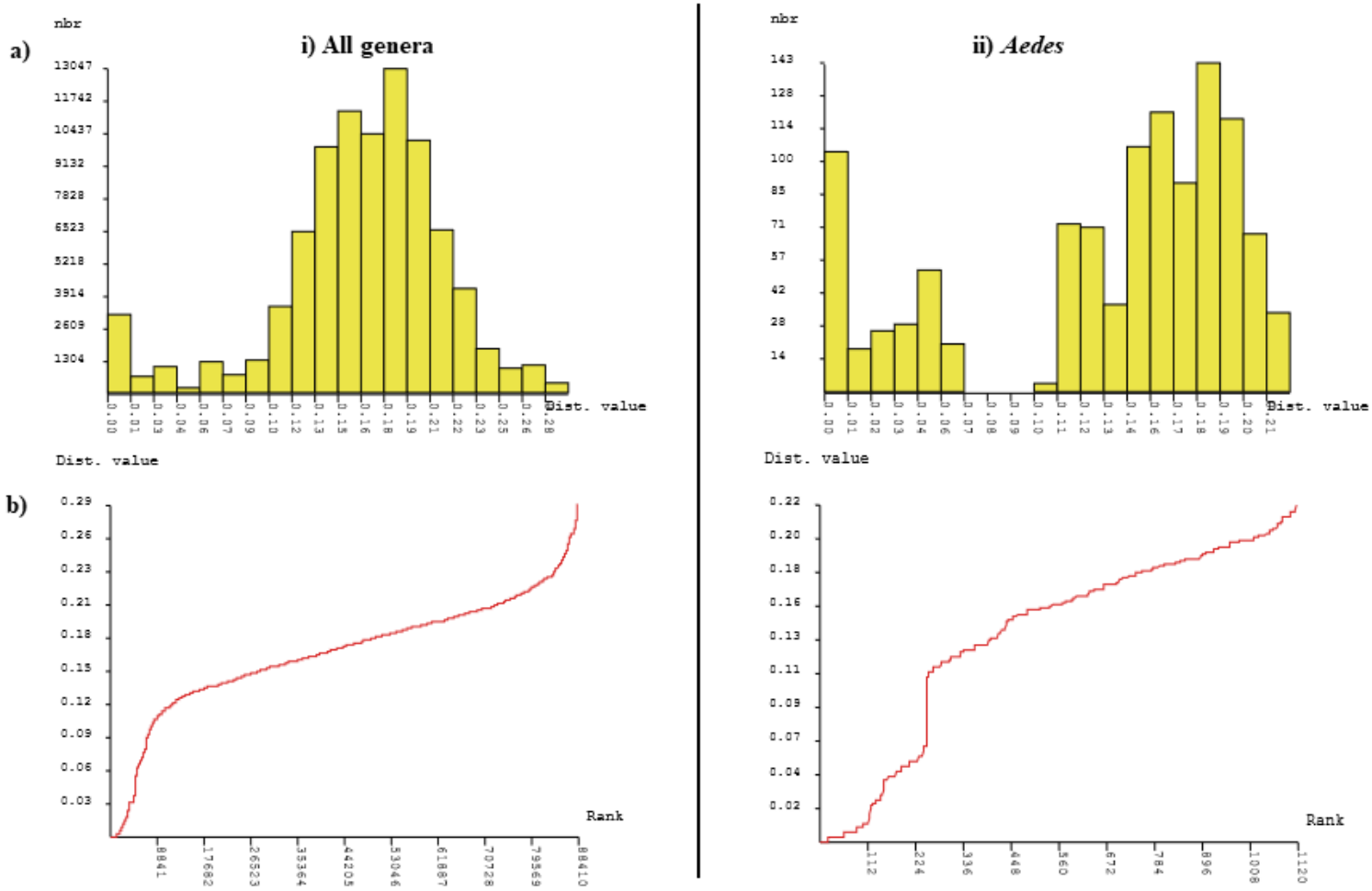


Fig. 3.4 ABGD 'barcode gap' histograms a) and ranked distance b) for species range *COI* sequence data by genus and family.

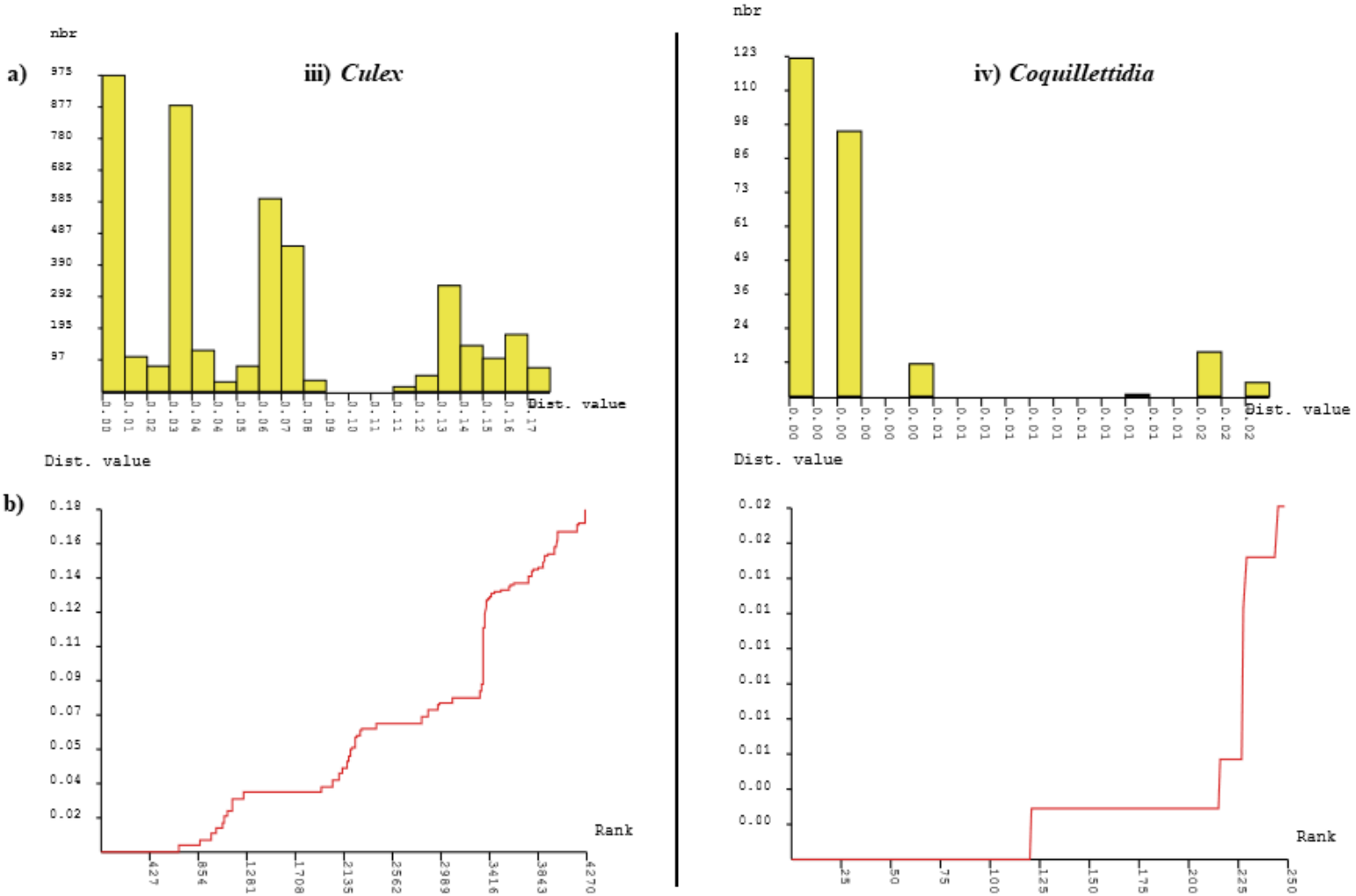


Fig. 3.4 Continued.

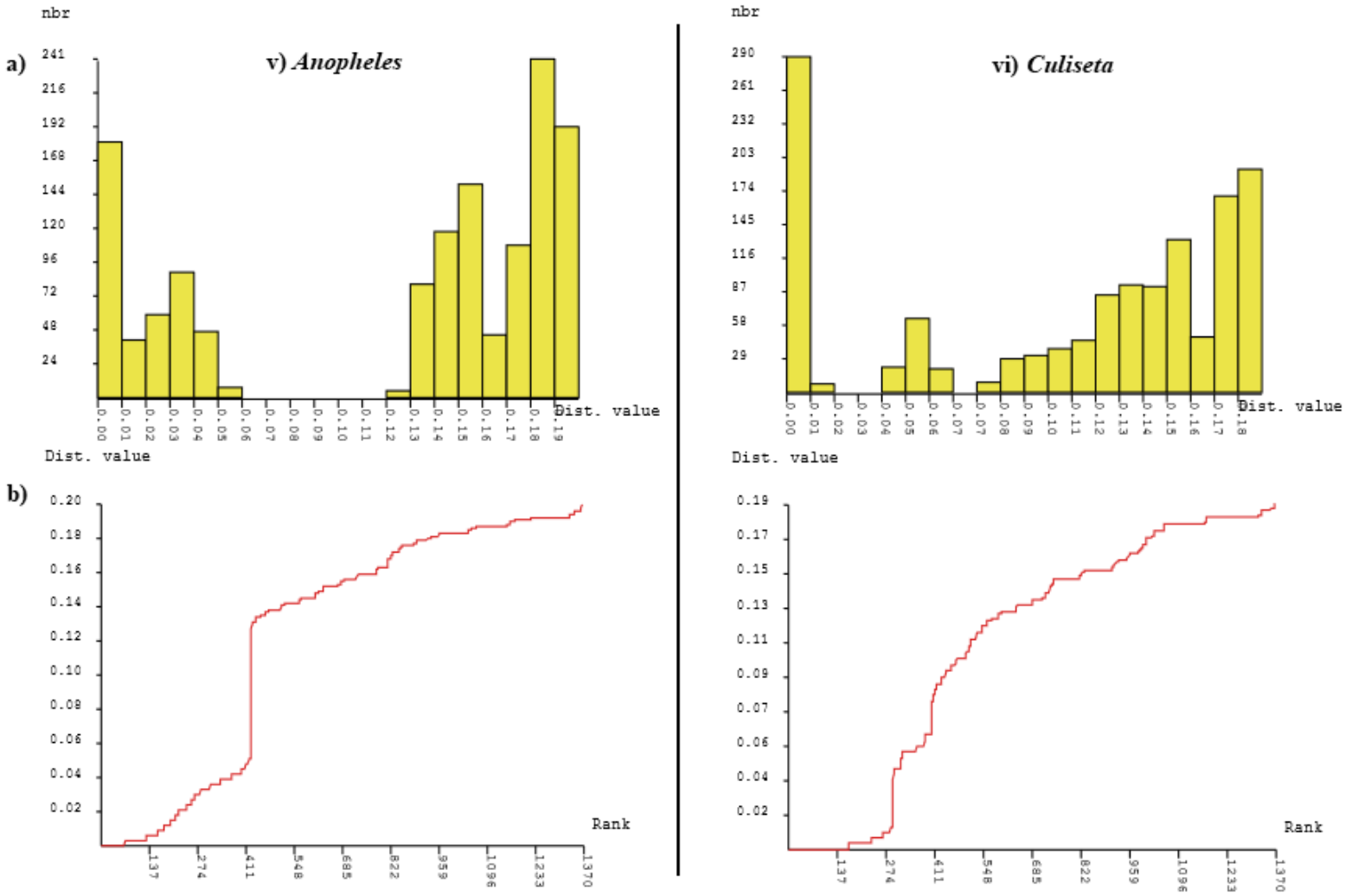


Fig. 3.4 Continued.

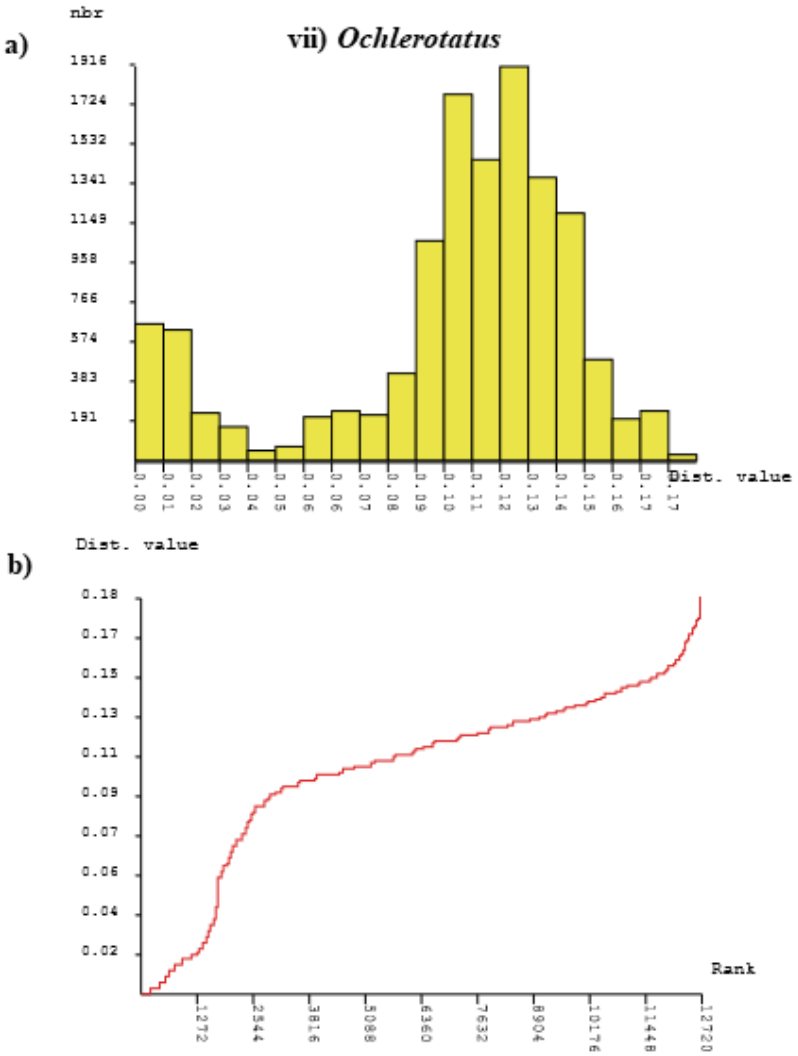


Fig. 3.4 Continued.

PTP analysis supported a 55 species partition using a maximum likelihood solution. Support for the division of *Ae. vexans* in the GB was upheld with an additional partition for Nearctic samples. Likewise, partitions for Nearctic vs. Palearctic species splitting was also suggested for *Cx. territans* and *Cs. alaskaensis*. Genetic diversity within *Oc. punctor* was also highlighted by PTP but grouped with ABGD. Other geographical groupings were also suggested by PTP but not by ABGD, a potentially new *Cx. modestus group* from the Sino-Japanese region, and divisions of *An. claviger* from the Middle-East and Saharo-Arabian from the Palearctic is also accentuated. The *Cq. richiardii* splits inferred by ABGD is not supported by PTP which groups all samples into one hypothetical species. *An. macculipennis* s.l. groups are split into three by PTP which may reflect the current view of three species identified within a European context i.e. *An. atroparvus,* and a split between *An. messeae* and *An. daciae* (Nicolescu et al., 2004). PTP also supported the subdivision of the subgenus *Culicella* (which currently includes, *Cs. morsitans*, *Cs. litorea* and *Cs. fumipennis*) into four groupings.

3.4.6 Network analysis

Network analysis uses MJN to produce a haplotype network with median vectors, points that represent hypothetical ancestors, or missing specimens connected via maximum parsimony and branch length is subdivided by mutated positions. The interpretation of these locations, and the number of mutations can aid in determining haplogroups and haploclades, as well as haplotypes. A network analysis for the GB plus species range dataset revealed 211 haplotypes, from the 423 taxa, with the total number of mutations disregarding the torso = 694, and shortest tree within the torso = 702 mutations. The data set was then broken down into genera for dissemination (Fig. 3.5 to 3.7). Haplotype scores were as follows; *Aedes* = 26 haplotypes from 35

taxa (176 mutations for the shortest tree). *Dahliana* = 6 haplotypes from 8 taxa (6). *Anopheles* = 36 haplotypes from 54 taxa (221). *Coquillettidia* = 6 haplotypes from 23 taxa (8). *Culiseta* = 23 haplotypes from 48 taxa (191). *Culex* = 33 haplotypes from 87 taxa (134). *Ochlerotatus* = 78 haplotypes from 165 taxa (383).

The MJN reveals eight haploclades within the *Aedes* genus and appears to support four distinct clades in the *Ae. vexans* group, two found to be present in GB samples, as well an individual group represented by a single oriental specimen (possibly the subspecies considered to be *Ae. vexans nipponii* Theobald 1907. Both clades present in the GB samples appear to be from separate lineages, the first (vexHC1) is more closely related to a North American haploclade (vexHC2), the second (vexHC3) to the oriental clade (vexHC4). Both sets of British samples are most closely related to other Palearctic groups, suggesting that the *Ae. vexans* group may have historically colonised this region at least twice. The observation on two distinct clades of *Ae. vexans* in the palearctic region has also been reported by Lilja et al (2018). This analysis also supports the recent separation of *Ae. cinereus* into two species, *Ae. cinereus* and *Ae. geminus* (Medlock and Vaux, 2009). The two distinct haploclades are likely to reflect this as incorrect species assignments in the repository descriptors would be likely, given their recent separation. However, this group also appears to have a North American/Palearctic geographical split. A larger dataset would be required to resolve any additional genetic diversity within this group.

The genus *Culex* was represented by 6 haploclades, of which *Cx. territans* has a clear split into two distinct clades by Nearctic and Palearctic separation. Revisions of this species was suggested by da Cunha Ramos et al (2003), whom suggested a new European species (*Cx. europaeus*) separate from *Cx. territans* found in the Nearctic.

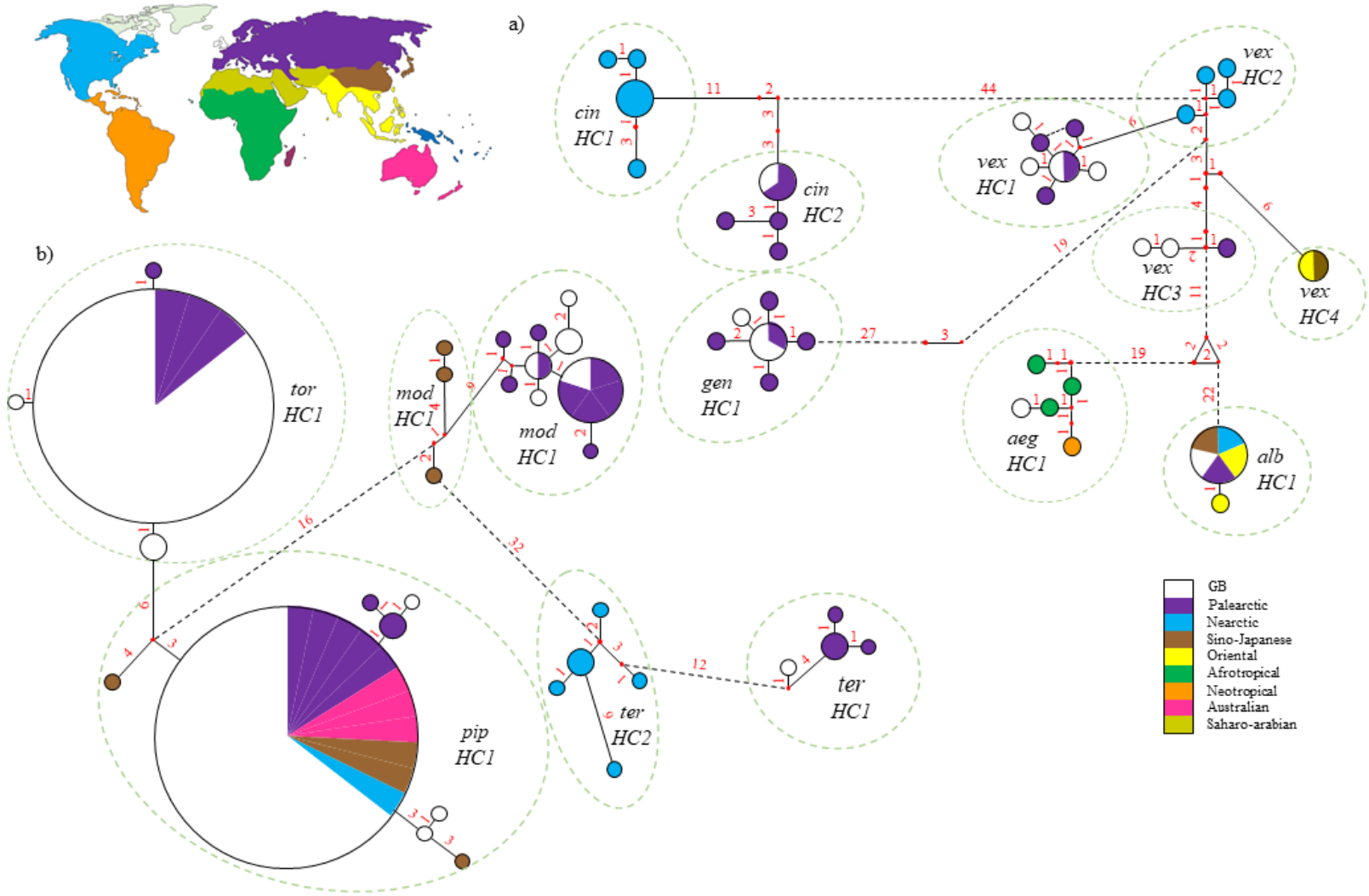


Fig. 3.5 Minimum joining network analysis of the genus a) *Aedes* (including *Dahliana*), and b) *Culex*

The size of frequency charts is relative to the number of individuals and coloured according to zoogeographic location. Solid lines are proportional to the number of point mutations and dashed lines refer to non-proportional. The number of point mutations are given in red and assigned haploclades within green chequered areas.

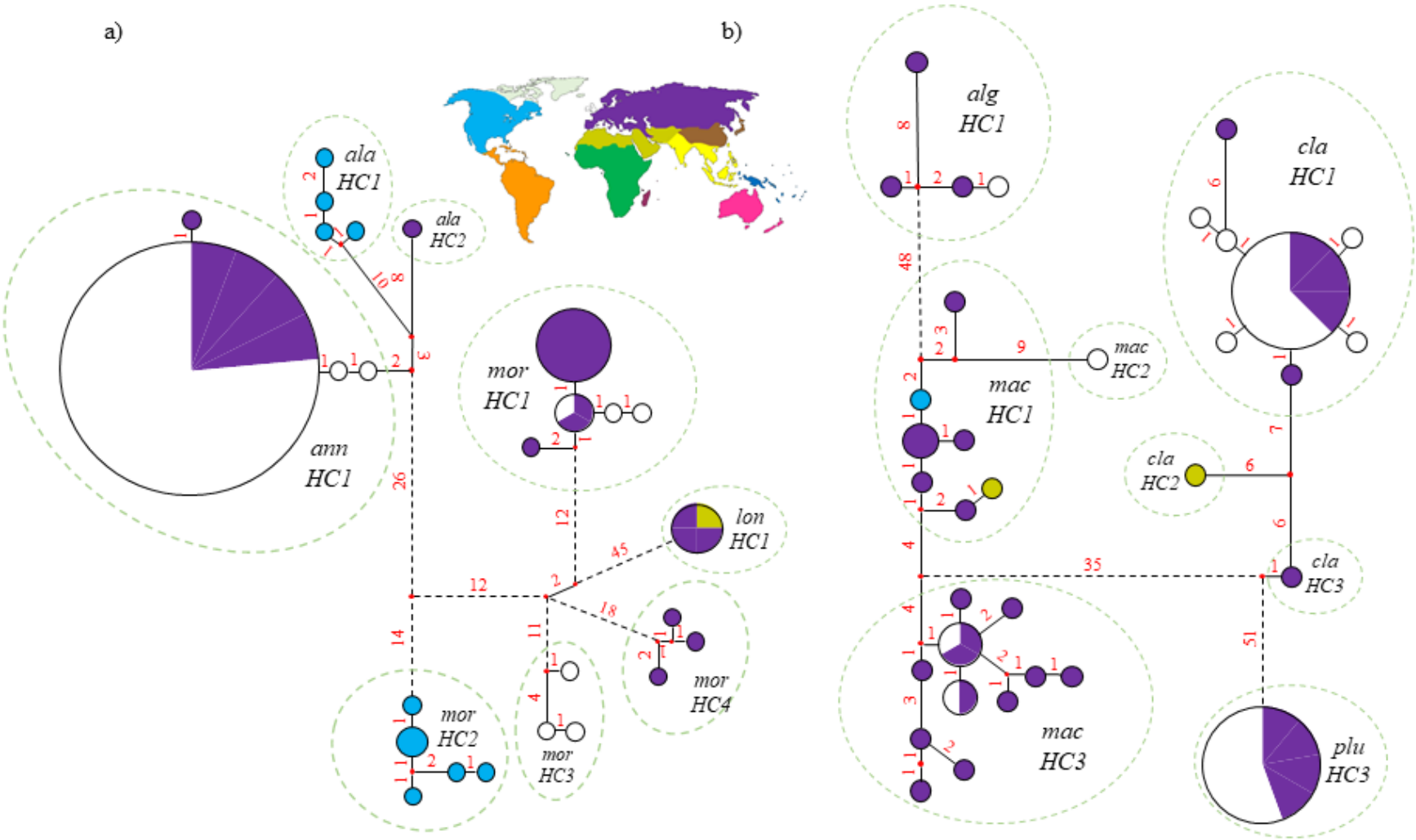


Fig. 3.6 Minimum joining network analysis of the genus a) *Culiseta*, and b) *Anopheles*

The size of frequency charts is relative to the number of individuals and coloured according to zoogeographic location. Solid lines are proportional to the number of point mutations and dashed lines refer to non-proportional. The number of point mutations are given in red and assigned haploclades within green chequered area.

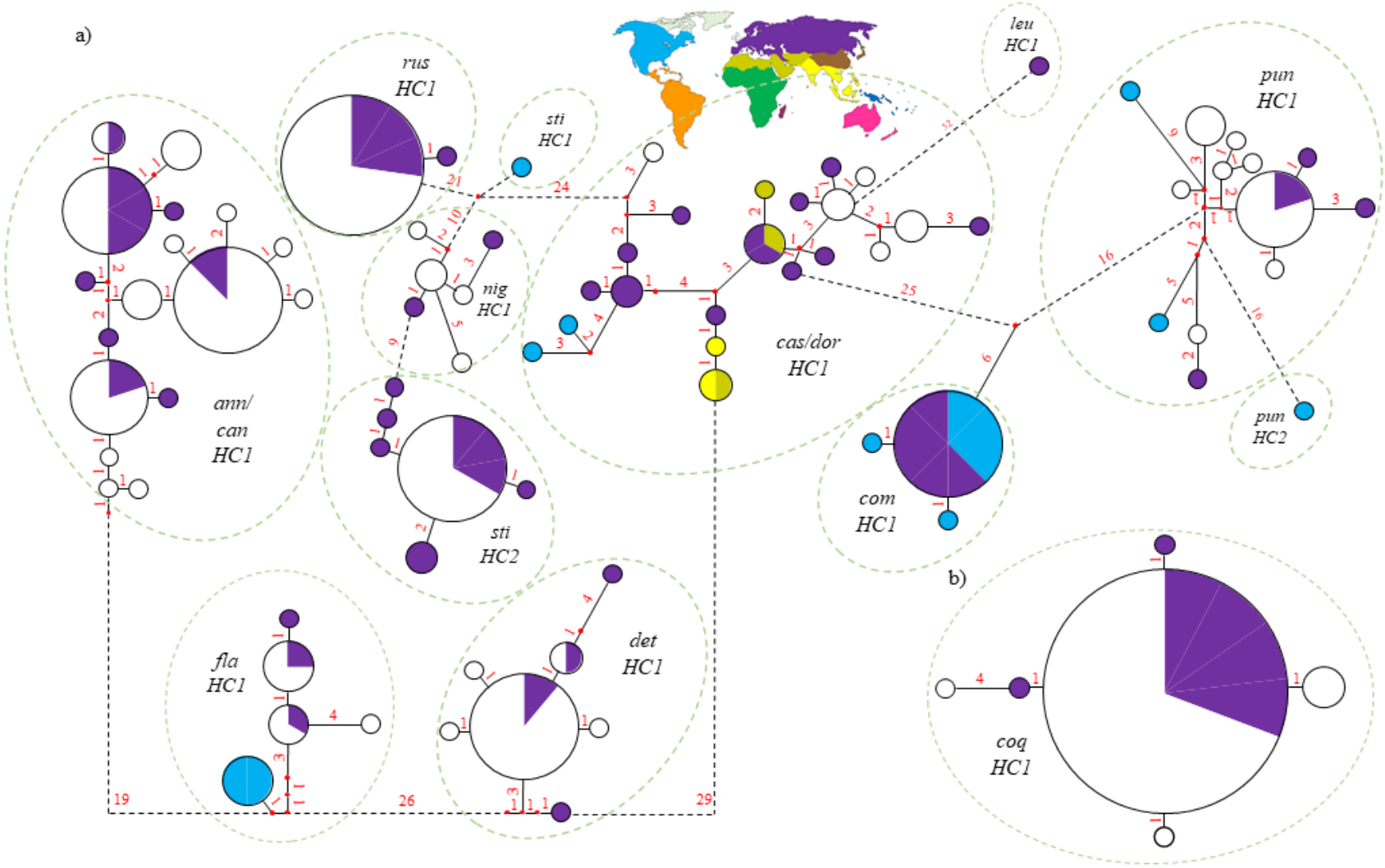


Fig. 3.7 Minimum joining network analysis of the genus a) *Ochlerotatus*, and b) *Coquillettidia*

The size of frequency charts is relative to the number of individuals and coloured according to zoogeographic location. Solid lines are proportional to the number of point mutations and dashed lines refer to non-proportional. The number of point mutations are given in red and assigned haploclades within green chequered area.

Gunay et al (2015) also suggested that *Cx. territans* in Turkey could be divided into several other species, and *Cx. territans* was dropped from the countries species list. An observed K2P distance of ≥ 4.8% was also observed between Belgian and Canadian *Cx. territans* by Versteirt et al (2015). Of this genus, *Cx. modestus* also showed high levels of genetic variation, including a haplotype that represents *Cx. modestus* in the Sino-Japanese region.

The genus *Ochlerotatus* was represented by 12 haploclades, and showed high levels of genetic variation, particularly for *Oc. punctor*, *Oc. caspius*, *Oc. dorsalis*, *Oc. cantans/annulipes* and *Oc. flavescens*. The latter displaying Nearctic/Palearctic separation. *Ochlerotatus cantans/annulipes* showed high levels of genetic variation but did not clearly separate into defined clades. Similarly, *Oc. dorsalis* and *Oc. caspius* were defined but not clearly. Outliers of *Oc. sticticus* and *Oc. punctor* from Nearctic regions have also added some confusion and need refinement by additional sample gathering and further analysis.

Coquillettidia and *Dahliana* were each represented by a single haploclade.

3.5 Discussion

The effective surveillance of disease vectors, such as mosquitoes, relies heavily on the identification of species. It is, therefore, important that such tools are readily available to allow for rapid confirmation of damaged, or difficult species. Here we have shown that the *COI* gene is a useable tool in the identification of GB mosquito species (with some exceptions discussed here), and laboratory processing of sequencing data can be turned over rapidly. It is likely that the application of *COI* analysis in vector surveillance will remain in frequent use in the foreseeable future. It

is, however, important in this context to discuss the pros and cons of such practices. It is regularly the case that practical problems go unreported in journals and this information can be vital, as the difference between sequencing problems by user error, or genuine biochemical technical issues could significantly slow down laboratory processes.

3.5.1 *COI* considerations

Hebert et al (2003) suggests that the use of a 600 bp region towards the 5' prime end of the *COI* gene is sufficient to determine species separation. However, several primer regions from across the gene are typically used for mosquito *COI* amplification (Chan et al., 2014; Folmer et al., 1994). As a result, overlapping sequencing regions are often unavoidable when utilising repositories, limiting the length of comparable sequence data. We found that a shorter sequence alignment of only 348 bp was sufficient for species delimitation and provided enough information to highlight groups with cryptic species potential, as well as picking out haplotypes by geographical location. The use of shorter reads for effective mosquito identification was also described from Sweden (Engdahl et al., 2014).

The ability of *COI* to resolve deeper phylogenies is poor (Remigio and Hebert, 2003), and the mosquito sequences analysed here showed no exception. The length of sequences used did not improve bootstrap values enough to provide meaningful phylogenetic reconstruction, and therefore fails to resolve disputes surrounding the classification of mosquito genera (Reinert et al., 2009; Wilkerson et al., 2015). However, for surveillance, where species identification is necessary for responsive vector control in the face of a disease outbreak, or increased risk of an outbreak, accurate phylogenetic assignment of monophyletic genera is less important.

The practicalities of using these genes is paramount, and sequencing failure rates often go unreported and unrecorded, which is problematic for two reasons. Firstly, sequencing failures, short runs, or outlier sequences can be caused by genuine genome traits such as pseudogenes and nuclear mitochondrial DNA segments (NUMTs) that need to be checked prior to analysis. Secondly, it is important that practitioners in vector surveillance, particularly in gene sequencing, can easily understand difficulties in data interpretation so that results can be obtained without wasting time in failed optimisation tests.

Overall sequencing success for most species of GB mosquito was high. However, most failures came from specific species rather than from sample quality. Specimens of the *Cs. fumipennis/litorea/morsitans* group (Sub-genus: *Culicella*) had an amplification success of only 8.3%. These failures were tested against possible contamination, laboratory errors and primer region polymorphism. However, none of these explained the lack of amplification in this species group. Likewise, attempts to amplify other mitochondrial genes, such as *COII*, *ND4* and cytochrome B was also unsuccessful, despite all groups exhibiting strong gel electrophoretic bands after PCR amplification. Nuclear gene amplification of *ITS2* from the same individuals was more successful (53% full recovery, 66% including partial recovery) (Chapter 4). Due to the limitations of this study we were unable to resolve these amplification anomalies, however, we suspect an unusually high level of NUMTs could be the underlying cause. NUMTs are widely recorded in the Culicidae genome due to breakage and non-homologous recombination (Black IV and Bernhardt, 2009). The insertion of mitochondrial super-contigs could have an amplified affect in an island population if the insertion remained through a species bottleneck, or as part of a founder effect. Additional research for verification is required for future *COI* work on these groups when using GB specimens.

3.5.2 Species identification for surveillance using *COI* genes

The *COI* gene has been described as being both conservative (Engdahl et al., 2014; Versteirt et al., 2015), as well as overestimating species diversity in mosquitoes (Hemmerter et al., 2009). Genetic diversity of species was not consistent using the *COI* gene which was unable to separate *Oc. cantans/annulipes*, *Oc. caspius/dorsalis* and *Oc. nigrinus/sticticus* into monophyletic groups effectively, and neither OTU-picking, nor ABGD were effective at species partitioning. The inability to decipher these species does not appear to significantly affect our understanding of vector status, however, *Oc. caspius* and *Oc. dorsalis* are both considered as potential vectors of WNV (ECDC, 2014; Higgs et al., 2004), but whether this is as a result of genetic relatedness has not yet been studied. Conversely, some species, such as *Oc. punctor*, *Cq. richiardii*, *An. claviger* and *Cx. modestus* displayed inconsistent taxon delimitation by PTP, or ABGD. These species are likely to be at greatest risk of ambiguous splitting/lumping. The use of additional genes, or even whole genome data may be necessary to resolve these groups.

There was clear evidence from median joining network analysis that geographical separation occurs in some species groups. Those of *Cx. territans, Ae. cinereus,* and *Cs. alaskaensis* were genetically differentiated by Palearctic and Nearctic regions (Fig. 3.5 & 3.6). Other groups also showed high levels of variance at the phylogeographic level and require further discussion.

3.5.2.1 *Aedes vexans*

Aedes vexans is a known competent vector of over 30 viruses (Lilja et al., 2018), including laboratory competence in ZIKV (Gendernalik et al., 2017), and Rift Valley

fever (RIFV) (Miller et al., 2002). It is currently recognised as having three sub-species *Ae. vexans arabiensis* Patton (from Mauritania, Sénégal, the Gambia, Ghana, Nigeria, Sudan, Ethiopia, Somalia, and South Africa) (White 1975), *Ae. vexans nipponii* Theobald (from China, Korea and Japan) and *Ae. vexans vexans* Meigen (from the rest of the world) (Reinert, 1973; White, 1975). Network analysis, maximum likelihood and PTP approaches all suggest at least four distinct haploclades from only a small dataset used here. Nearctic, Oriental/Sino-Japanese, and two Palearctic divisions, both of which were found in the GB, where *Ae. vexans* was previously only considered to be one species/subspecies (Medlock et al., 2017a). This confirms results of recent investigations into the *COI* and *ITS2* genes of *Ae. vexans* in Sweden which also highlighted at least two distinct Palearctic haploclades (Lilja et al., 2018). A global population level genetics study of this group is yet to be undertaken, and the small number of *COI* sequences used here suggests further study of this species would be beneficial. The number of haploclades that appear to be present may also display properties of a ring-species effect, however, this theory is tenuous and would need to be confirmed by a larger species specific dataset (Irwin et al., 2001; Monahan et al., 2012). Currently, no research into genetically distinct groups and vector competency has been reported in this species. However, RIFV has only been recorded and isolated from the *Ae.vexans arabiensis* form (Miller et al., 2002), and the competency of European/GB haploclades is still unknown.

3.5.2.2 *Subgenus Culicella*

Mosquitoes of the subgenus *Culicella* (Genus Culiseta) are currently represented by three species in GB, *Cs. fumipennis, Cs. litorea* and *Cs. morsitans.* All three species have variable overlapping morphology making identification difficult (Becker et al.,

2010, Marshall, 1938). Of the 17 *COI* sequences analysed from across the species range (including five from GB) four distinct haploclade were constructed using MJN analysis (all GB specimens fall into two haploclades), supported by ABGD, PTP and phylogenetic analysis. Sequences identified as *Cs. fumipennis* (according to GenBank referencing), were found in two of these clades and are likely a result of misidentification. Haploclade 1 (*morHC1*) is most likely to represent *Cs. morsitans* and haploclade 4 (*morHC4*) as *Cs. fumipennis*. Haploclade 3 (*morHC3*) are samples collected exclusively from littoral habitats in the Sheppey Island, Kent, England (Fernández de Marco et al., 2016), and are therefore most likely to represent the coastal specialist *Cs. litorea*. Haploclade 2 (*morHC2*) represented a Nearctic separation to all Palearctic species and may represent a previously undescribed cryptic species group. The separation of the three European species of *Culicella* is important as *Cs. morsitans* is likely responsible for outbreaks of SINV in Sweden (Bergqvist et al., 2015), and eastern equine encephalitis virus (EEEV) in the USA (Molaei et al., 2006). However, these populations are genetically distinct, and the identification of *Cs. morsitans* from *Cs. fumipennis* and *Cs. litorea* in Europe is poor. Therefore, the true vector potential of these species is still unknown. *Cs. morsitans* has been classified as having a level five vector risk, the highest vector potential risk, by the ECDC for SINV in Europe, and level three for *Dirofilaria* species (ECDC, 2014). Whereas the capacity for SINV and *Dirofilaria* transmission is still unknown for *Cs. litorea* and *Cs. fumipennis.*

3.5.2.3 *Culex modestus*

Phylogenetic approaches, PTP and Network analysis support a possible spilt in *Cx. modestus* where GB and other Palearctic specimens are separate from those found in

the Sino-Japanese zoogeographic region, sequences of which were recorded from the Shanxi and Nei Meng Gu provinces in China (Wang et al., 2012).

3.5.2.4 *Anopheles maculipennis s.l.*

The *An. maculipennis* complex are morphologically very similar and consists of the malaria vector *An. atroparvus,* and *An. messeae,* the latter being recently separated into two distinct species by Nicolescu et al (2004) to include *An. daciae,* by genetic (*ITS2*) and morphological evidence. Sequences of *An. daciae* collected from this study were included within the dataset (AY757954, AY757924) alongside sequences of *An. messeae* from several different Palearctic countries. Although three distinct haploclades were identified by MJN, and ABGD, there was no separation of *An. messeae* and *An. daciae*, therefore, the separation of these species is not clearly supported as a result of this analysis. Delimitation into three groups shows two main partitions representing one from those of *An. messeae/daciae,* a second by specimens only classified as *An. maculipennis s.l.*, and third outlier represented by a single specimen described as *An. atroparvus* (KU877020) collected in GB. Despite, a lack of statistical evidence to suggest delimitation of *An. messeae/daciae,* valid additional variation may exist within this group. A global review of this complex would be needed for clearer resolution.

3.5.3 *COI* barcoding and surveillance

The development of sequencing technology is rapid with the associated costs constantly reducing and the volume of sequence data exponentially increasing (Muir et al., 2016). The development of next generation sequencing (NGS) over the last ten years has revolutionised the quantity of genetic data that can be obtained from a single read, and this is reflected in the development of ambitious projects such as the Earth BioGenome project (Lewin et al., 2018), and the Sanger Institute's Darwin

Tree of Life Project (GB only) (https://www.sanger.ac.uk/science/programmes/tree-of-life) which aim to sequence the genomes of all species. However, there is still a basic need for the use of traditional Sanger sequencing methods as they are fast and affordable in comparison to NGS. The protocol described for the sequencing of the *COI* gene above can be adapted to process mosquito samples in as little as eight hours when required, by reducing lysis time, and providing they are free of contaminants.

How well a species is defined is an important question for vector surveillance. Data collected from GB suggests that accurate identification of most species can be easily obtained using only 348 bp reads, and therefore identification of species for surveillance of 'native' species can be easily achieved using this method. Samples from across the species range, however, show high numbers of haplotypes (and haploclades) in many species clusters and could provide additional data regarding species geographic distribution. The BOLD Barcoding Index Number System (BINS), uses OTU picking methods to cluster large *COI* sequence data repositories into hypothetical species (Kartavtsev, 2018; Ratnasingham and Hebert, 2013), however, this method does not cluster by haplotype (or haploclade), and although this method is useful for detection of synonyms, does not provide enough information to ascertain place of origin.

Species identification using the *COI* barcoding gene tends to rely heavily on matching sequences with online repositories such as GenBank (NCBI) and BOLD. From our searches for species specific sequence data, we have found that there are many misidentified species within these datasets, and geographical location information is often missing.

There are two layers of information from sequencing genes that can be harvested for the benefit of surveillance. The first is the rapid identification of specimens to improve enhanced, and targeted, control efforts should the need arise e.g. disease outbreak or increased risk of disease transmission. For this to happen, a rapid method of species identification is required. The second, is the analysis of these data in a geographical context, to search for cryptic species, or haplotypes that have the potential for different characteristics, such as insecticide resistance profiles and vector carrying capacity. Likewise, the additional analysis of global haplotype distributions can help to detect the movement of non-native conspecifics that could introduce MBDs or alter the susceptibility of a given population through introgression (Hernández-Triana et al., 2018; Roundy et al., 2017; Turell, 2012). Conversely, where *COI* is unable to separate closely related species, the addition of more markers must be tested to determine whether this is a deficit in the gene of choice, or whether this is a genuine taxonomic assignment (Wiemers and Fiedler, 2007). The ability to resolve species identification with the use of multiple markers could be important, as a lack of genetic resolution could also provide a screen to vector potential.

3.6 Conclusion

The misidentification of a mosquito to species is not uncommon even by experts, however, it still remains the foundation of an effective surveillance programme for native or AIMs (ECDC, 2014). Positive identification prevents misconceptions of species vector potential and is required to inform surveillance and potential control options, as well as the accurate determination of behavioural ecology, i.e. response to environmental stimulus, such as climate change and habitat change. The

development of databases can only be as effective as the data that is included. Therefore, regionally specific databases are important to provide both a local and international understanding of species composition. Here we show most of the GB species can be identified using *COI* markers with the addition of possible cryptic species clusters within *Ae. vexans* and *Oc. flavescens*, but conversely shows poor species determination between *Oc. annulipes* and *Oc. cantans*. A further expansion of this dataset to include sequences from across conspecific global ranges shows high levels of genetic variation of the *COI* gene. The analysis of these data using various partitioning and phylogenetic approaches shows geographically separated haploclades, and possible cryptic species across species ranges. Given these results, the process of elucidation by the examination of additional genetic markers is required for validation. Additionally, further studies to analyse species-specific datasets are required to fully resolve the suggestive results highlighted within this study. This research does, however, strengthen the argument for a more holistic phylogeographic based approach to using genetics in vector surveillance programmes.

CHAPTER FOUR:

Going nuclear? The usefulness of the internal transcribed spacer 2 (*ITS2*) gene in the identification of British mosquito species

4.1 Abstract

The use of the nuclear internal transcribed spacer 2 (*ITS2*) gene is commonplace in phylogenetic and phylogeographic studies and has been used to decipher the evolutionary relationships of many groups of species, including some Anopheline mosquitoes. Use of *ITS2* alongside additional genes, such as mitochondrial *COI*, can add increased robustness to species identification as well as resolving complex taxonomic relationships. Currently there have been no efforts to describe the *ITS2* genes of all Great British (GB) mosquito species and its usefulness in the surveillance of native and invasive species has not yet been tested.

Here we investigated the potential use of *ITS2* in GB mosquito surveillance by using a range of analytical methods (PTP, ABGD, *p*-distance and median joining network analysis) to examine the ability of this gene to delineate mosquito species. We also attempted to describe the secondary RNA structures for each species and investigate compensatory base changes (CBC's) as a potential indicator of separation by sexual isolation.

The size of the *ITS2* region varied between 182 to 414 bp with a mean interspecific *p*-distance of 0.313, and intraspecific distance of $p = 0.027$. *ITS2* appears to be a robust method of identifying GB mosquitoes to species level. However, as with *COI*, *ITS2* showed high levels of genetic variation when comparing GB sequences with those from across species ranges, particularly in *Ae. vexans* and *An. claviger*. Conversely, we did not find evidence to support species separation between *An. daciae* and *An. messeae*, or *Oc. annulipes* and *Oc. cantans*. We advise that *ITS2* be used only as a complementary gene to *COI* in GB mosquito surveillance as sequence recovery from direct PCR was low (51.8%) due to intraindividual variation caused

by nucleotide repeats. Additionally, larger datasets of *ITS2* are required to fully gauge the haplotype diversity found in GB, and from across their species ranges.

4.2 Introduction

The ability to accurately identify species is a keystone for mosquito surveillance, without which an understanding of species distribution, and discovery of invasive species would be almost impossible. Barcoding genes provide such an option for rapid identification. The use of *ITS2* has been widespread across all eukaryotes and is the barcoding gene of choice for many mycological studies. This also applies to mosquitoes, where *ITS2* has been utilised in more barcoding publications than those of the arguably more favourable cytochrome c oxidase subunit 1 (*COI)* gene. According to a review by Beebe (Beebe, 2018), ~220 papers published through PubMed contain the use of *ITS2* on mosquitoes, vs. ~150 that utilise *COI* (as of 2018). However, these frequencies are misleading as ~ 90% of *ITS2* publications are applied solely to malaria related *Anopheles*. This demonstrates an almost monocentric approach to gene selection for barcoding, and by proxy, species delimitation of mosquito species (across all genera) in favour of *COI*. Similarly, in GB the use of *ITS2* has been limited to the *An. maculipennis* group (Danabalan et al., 2014), as well as a single specimen of *Orthopodomyia pulcripalpis* Rondani, used in the global phylogenetic analysis of the genus (Byrd et al., 2012).

Over the last 25 years *COI* has become the barcoding gene of choice in distinguishing animal taxa (Folmer et al., 1994). It is a highly conserved region of the mitochondria across groups of plants and animals, it evolves rapidly, and allows for relatively straight forward comparison between species. However, this widely used gene displays varying rates of evolution amongst taxa, and therefore is prone to

over, and under, estimating genetic distances (Pentinsaari et al., 2016). In mosquitoes, the use of *COI* has been shown to be insufficient to separate species of *Oc. annulipes* and *Oc. cantans* (Versteirt et al., 2015) (Chapter 3: Section 3.4), conversely, it suggests additional levels of variation in *Ae. vexans* (Lilja et al., 2018) and *Cx. territans* (Chapter 3). Similar *COI* over/under-estimations of cryptic species groups has also been reported from Australian *Culex* (Hemmerter et al., 2009). As such a reliance on a single genetic marker for species identification is unlikely to be enough to differentiate some closely related species, or may overestimate divergence in others (Beebe, 2018; Dupuis et al., 2012; Gemmellaro et al., 2019). The use of additional nuclear markers will help to elucidate these findings and provide an additional reference for the validation of species collected during surveillance (Beebe, 2018; Gemmellaro et al., 2019).

The internal transcribed spacer 2 (*ITS2*) is a cistron located between *5.8S* and *28S*, a part of the ribosomal (rRNA) family of genes that repeats multiple times across the genomes of Eukaryotes (Han et al., 2013; Yao et al., 2010) (Fig 4.1). They exhibit a concerted evolution driven by non-Mendelian processes that, as a result of multiple recombination mechanisms, change rapidly over species/population distances, but yet remain relatively homogenised within the genome (Bower et al., 2008). The result is relatively low intraspecific versus high interspecific variation, making this gene useful in reconstructing evolutionary lineages between closely related species (Coleman, 2003; Yao et al., 2010). These regions are, however, prone to insertion and deletion, and often contain high numbers of rapidly evolving tandem repeats (Banerjee et al., 2007b; Cornel et al., 1996). This variability can cause difficulties in forming accurate alignments as the more distantly related the taxa, the greater the lack of sequence similarity. This leads to ambiguity in resolving deeper phylogenies and complicates decisions as to the most appropriate analytic method for dealing

with high numbers of indels (Liu et al., 2012). Early efforts to circumvent these issues led to new analytical approaches that compare the secondary RNA structures of *ITS2*. The logic follows that the function of these structures must remain conserved across taxa despite the extensive nucleotide rearrangements and may reveal additional taxonomic information that is not obvious by comparison of the highly variable sequences alone.

To analyse secondary structures an appropriate folding method is required. Producing accurate folds is less than straightforward, however, as a trade-off between thermodynamic laws for minimal energy and the laws that regulate biological function can be hazy. Folding can therefore result in the production of different structural options from which the correct outcome must be selected. A unified approach to deal with this problem has been developed over the last 10 years, after early attempts to use secondary structures showed inconsistencies across studies, and a lack of functional conservation (Coleman, 2003; Mai and Coleman, 1997; Sallum et al., 2009; Wesson et al., 1992). A biological model for secondary *ITS2* folding outlined by Coleman (2007) compared sequence similarities across multiple taxa (including several Aedine mosquitoes) highlighting several conserved features that can now be used for screening, they are as follows: (a) A molecule that displays between three and five helices orientated around a central loop, (b) a conserved secondary helix that includes a non-pairing pyrimidine bulge, (c) a conserved region of sequence that lies on the 5' side of the third helices. In an effort to standardise secondary RNA structure folding following the Coleman model the ITS2 database was developed (Selig et al., 2008), along with step by step guidelines for sequence to structure analysis (Schultz and Wolf, 2009). The use of secondary structures has also provided additional resolution for improved phylogenetic

reconstruction (Gomez-Zurita et al., 2000; Grajales et al., 2007; Marinho et al., 2011; Wiemers et al., 2009).

Compensatory base changes (CBCs) are mutations that occur in paired nucleotides located within helices of the secondary RNA transcript of *ITS2* that result in the pairing being maintained (e.g. A-U to G-C). The rate at which CBC's occur has been correlated to isolation by sexual incompatibility. Generalised testing approximated 93% of closely related species are separated by their presence (Müller et al., 2007). This method has been used successfully in delimitating cryptic species, and the construction of more efficient phylogenies; particularly in other insect groups, such as beetles of the genus *Altica* (Ruhl et al., 2010), and across other major groups of organisms; in Abalone (*Haliotis*) (Coleman and Vacquier, 2002), flukes (*Opisthorchis*) (Sahu et al., 2016), diatoms (Amato et al., 2007; Lim et al., 2018; MacGillivary and Kaczmarska, 2012; Poulíčková et al., 2010), and fungi (*Rhizoctonia*) (Ahvenniemi et al., 2009). The use of CBC's has become an important part of the analysis of secondary *ITS2* structures, as their presence can also be used in checking fold accuracy (Coleman, 2007). This method has not been successfully tested on mosquitoes and may provide additional insight into the closely related species complexes. The absence of CBCs between species, however, is not considered a guarantee of sexual panmixia (Coleman, 2009), and some studies, from where this application has not been successful, caution against using this approach without additional support (Caisová et al., 2011).

Here we set out to obtain the full sequences of as many GB species as possible, validate their complete *ITS2* regions, and accurately fold their secondary structures for comparison and compare samples for CBC locations. We also investigated the ability of *ITS2* to discriminate species by phylogenetic reconstruction, ABGD

(barcode gaps), PTP (OTU picking), and median joining network (MJN) analysis. Additionally, as was included with COI analysis (Chapter 3), the sequence data from other specimens from across the species ranges was included to investigate any undetected variance at the species level.

The high numbers of *ITS2* copies found across the genome, and the presence of heterozygous positions inevitably lead to intraindividual variants that can cause differences in length variation between the alleles. These issues have been observed in mosquitoes (Beebe, 2018; Beebe et al., 1999; Wesson et al., 1992) but the sequential causes are poorly described, and the effect on amplification success rates usually go unreported. Surveillance of any vector species requires fast and responsive identification of species, and therefore reporting these problems can minimise time in troubleshooting, and unnecessary repeated sequencing efforts. To gain an understanding of these problems, we have characterised and discussed problems in the amplification of these genes.

The *ITS2* gene is subject to indels, the insertion and deletion of nucleotides over evolutionary time. The management of gaps that occur in alignment sequences as a result of indels can directly affect monophyletic groupings inferred by phylogenetic approaches (Liu et al., 2012). The removal of these gaps prior to analysis is often the most desirable option as different regions of a gene can evolve at different rates, however, they can also provide additional levels of information to support analysis (Dwivedi and Gadagkar, 2009; Simmons et al., 2007; Simmons and Ochoterena, 2000). The presence of indels has been recorded in mosquitoes (Bargues et al., 2006; Beebe et al., 1999), however, the primary approach to managing subsequent gaps in alignments has been by their complete deletion (Beebe et al., 1999; Byrd et al.,

2012). Here we determine an appropriate method to dealing with gaps in mosquito *ITS2* regions.

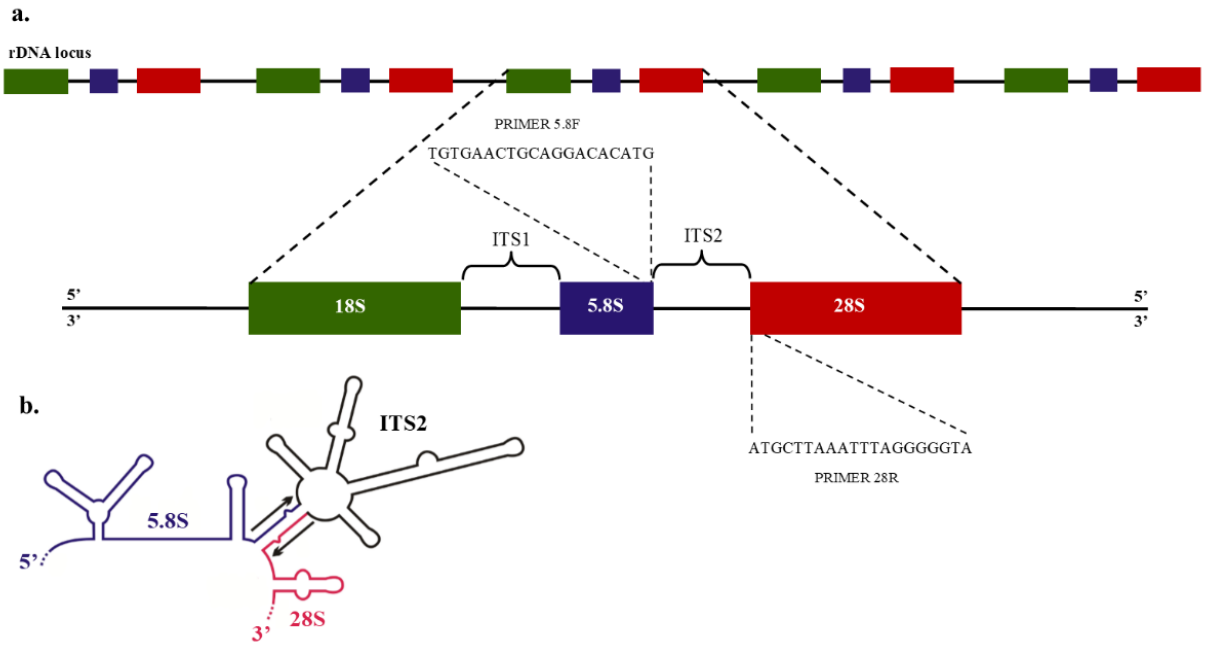


Fig. 4.1 The location of the nuclear internal transcribed spacer 2 (*ITS2*) gene within the ribosomal DNA (rDNA) locus.

(a) Illustration of the repeated nature of the rDNA locus where 5.8F and 28R represent the forward and reverse primer for *ITS2* gene amplification. (b) Schematic folded rRNA structure of *ITS2* with the flanking genes *5.8S* and *28S*. Note the hybridisation of the proximal stem that is diagnostic for the presence of pseudogenes. Reproduced from (Eddy, 1998).

4.3 Methodology

4.3.1 Sample collection and selection

Samples were collected using methods described in Chapter 2. Specimens that were selected for *COI* sequencing were also used for amplification attempts on *ITS2*, and therefore, DNA extractions methods were the same as described in Chapter 3. If *COI* amplification by PCR or sequencing steps were not successful, attempts were still made to amplify *ITS2*. In doing so, negative results could be cross confirmed, and

samples with absence of bands after visual inspection of PCR product using gel electrophoresis after both attempts, were discarded from downstream applications.

After low sequencing success rates from *COI* in mosquitoes from the subgenus *Culicella* (genus *Culiseta*) (2 successful amplifications from 24 specimens), an attempted to sequence an additional 48 specimens (72 total) was undertaken, with up to five specimens from all locations sampled, to ensure a good sample coverage for this species.

4.3.2 PCR amplification and sequencing

ITS2 was amplified by PCR and sequenced directly from the product following the protocol outlined in Chapter 3. PCR amplification was performed using the generic *ITS2* primers from the conserved flanking regions *5.8S* and *28S* (Fig.4.1), of Beebe et al (1999) (ITS2A = 5'-TGT GAA CTG CAG GAC ACA T-3') and (ITS2B = 5'-T ATG CTT AAA TTC AGG GGG T-3'), or the later slightly modified primers of Walton et al (2007) (5.8f = 5′- TGT GAA CTG CAG GAC ACA TG-3′) and (28r = 5′- ATG CTT AAA TTT AGG GGG TA-3′). Conditions for both primer sets were as follows: Initial denaturation of 98°C for 30 s, followed by 35 cycles of 98°C for 10 s for denaturation, 20 s of annealing at 54ºC, 72°C for 20 s extensions, followed by a final extension of 72°C for 7 mins.

PCR reaction clean up and all sequencing steps were as described within the Chapter 3 methodology.

4.3.3 Treatment of sequence data

Sequence quality can be affected by direct PCR, as multiple intraindividual variants are not separated during the sequencing process. Sequence quality was recorded based on the ability to unambiguously sequence the whole *ITS2* gene. Intraindividual variation can result in sequences with multiple peaks within chromatographs, or a drop off in the quality of the trace (Fig. 4.2). Where a single polymorphic event causes double trace peaks a manual attempt was made to separate them resulting in a set of two (occasionally 3) different sequences per specimen. These subsets are identified by an alphabetic code after each species reference (e.g. *Oc. punctor* BM114(a) + BM114(b)). In most traces where sequence signal dropped into three or more peaks, splitting was not possible.

Sequence assembly was done using MEGA7 where forward and reverse contigs for each specimen were aligned and reduced to a single consensus sequence. The *5.8S* and *28S* flanking regions were identified for each sequence using the annotation function on the ITS2 database II website (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) by selecting the Diptera (42/26) model function. This tool also allows for a visual inspection of stem hybridisation of the *5.8S* and *28S* flanking motifs. Any uncharacteristic folding of this region can be indicative of a pseudogene sequence (Harpke and Peterson, 2008) and so was removed from downstream data analysis.

Sequences with successful annotation were then trimmed of extraneous sequence data leaving the flanking regions attached to aid in the construction of alignments. All sequences were compared to the NCBI database using the BLASTn search function to check for conspecific, or closely related, sequences.

4.3.4 Cloning of difficult sequences

To gain an additional overview of intraindividual variation from within problematic species, eight specimens from the genus *Culiseta* (subgenus: *Culicella*) were cloned (*Cs. morsitans* (*n* = 6), *Cs. litorea* (*n* = 2)), and eight colonies picked for sequencing. As above data subsets from each specimen are followed by alphabetic coding.

Cloning was carried out using pGEM®-T Easy Vector System kit (Promega, Southampton) following manufacturers guidelines. PCR products were ligated into pGEM®-T Easy using T4 DNA ligase (Promega) and transformed into chemically competent JM109 *Escherichia coli* (Promega). The transformation was plated on LB nutrient agar with carbenicillin (100 µg/mL), IPTG (1 mM/mL), and X-GAL (200 mg/mL). Once dried the plates were incubated at 37 °C overnight and 8 x white colonies were then selected for sequencing from each sample.

4.3.5 Additional sequences data mining

To gain a genetic overview of each of the species found in GB, barcoding genes were compared to available sequence data from across their range. A search of additional *ITS2* data was carried out using GenBank®, and the ITS2 database II. Specimens that could add additional value to the dataset were selected based on sample location. To gain broad coverage a maximum of two sequences were selected from each sample location using a random number generator. Additional subset of sample data used for the separation of *An. maculipennis s.l.* by Danabalan et al (2014) were also selected. Sequences without *5.8S* and *28S* flanks were rejected, as these regions appeared to be most useful in the detection of pseudogenes. The

number of sequences per species and the geographical distribution is given in Table 4.1.

4.3.6 Alignments and treatment of indels

Sequence alignments were carried using MUSCLE in the programme MEGA7 and discrepancies manually adjusted by eye.

The influence of indels in species delimitation was tested by comparing bootstrap success across several gap treatments (Liu et al., 2012). (1) partial-deletion (PD), where an arbitrary 70% site coverage cut-off was assigned, (2) pairwise-deletion (PWD), were indels are removed during analysis if they do not provide additional information, and (3) complete-deletion (CD) of all indels. All three treatments were replicated using two different evolutionary models; a standard *p*-distance where homoplasy is not considered over time, or for substitution rate biases, and Kimura's 2 parameter distance model; including compensations for Gamma distance (+G). The latter was selected as most appropriate approach for the *ITS2* dataset using the Find Best DNA Model function in MEGA7. Phylogenetic reconstruction was undertaken for each treatment using Neighbour-Joining trees with 1000 bootstrap replications. All the above tests were carried out using MEGA7.

4.3.7 Construction of *ITS2* secondary structures

Initial prediction of *ITS2* secondary structure folding was undertaken using the ITS2 Database II (Selig et al., 2008) following the instructions outlined in Schultz and Wolf (Schultz and Wolf, 2009). This method utilises hidden Markov models (HMMs) against pre-folded sequences stored within the database as a template to

increase the folding efficiency of target sequences. However, due to a lack of existing RNA folds for mosquitoes, some samples did not match anything on existing databases, and homology modelling was rejected when helix transfer scores were less than 70%. For non-matching sequences, folding was undertaken manually, and a consensus taken from mfold (Zuker, 2003), RNAstructure v.5.8.1 (Reuter and Mathews, 2010) and RNAfold (RNAfold WebServer, 2018) using default settings. An accurate folding of the *ITS2* region does not always conform to the lowest energy state but must conform to a functional biological model. All manually folded secondary structures were checked for biological accuracy using the criteria outlined by Coleman (2007).

Additional checks were also made against the presence of pseudogenes by utilising the ITS2 Database II annotation tool. This method confirms hybridisation efficiency of *5.8S* and *28S* which flank the *ITS2* region (Harpke and Peterson, 2008). Their hybridisation forms a proximal stem from which the *ITS2* secondary structure is attached (Fig.4.1.). Pseudogenes in *ITS2* tend not to conform to the biologically restricted fold making them characteristically unstable, therefore, secondary RNA structure folding can itself be a method of detecting the presence of pseudogenes. Low GC content can also be characteristic (Álvarez and Wendel, 2003), therefore, all sequences were checked for nucleotide ratios. Any sequences categorised as a pseudogene candidate using the afore mentioned criteria were removed from any further analysis.

4.3.8 Consensus modelling and compensatory base change (CBC) detection

CBC detection and visualisation of all secondary structures, as well as consensus modelling, was undertaken by aligning dot-bracket string notation for nested RNA

structures (Vienna format) and the ‘structure viewer function’ in 4SALE 1.7.1 (Seibel et al., 2008, 2006).

4.3.9 Network analysis

A median joining network (MJN) analysis was also applied to the *ITS2* sequence data. A lack of conserved similarity across the whole family resulted in analysis failure, and so analysis by genus was most appropriate. This method provides additional perspective on genetic clustering by visualising cycles of evolutionary possibilities compared to phylogenetic trees that only display a single possible evolutionary outcome. This is particularly useful when high levels of homoplasy struggle to be accounted for by traditional phylogenetic distance models (Bandelt et al., 1999; Zecca et al., 2012). MJN analysis was undertaken using the programme NETWORK version 5.0.1.1 (http://www.fluxus-engineering.com). Reticulations were broken using rules described by Pfenninger and Posada (Pfenninger and Posada, 2002).

4.3.10 ABGD (barcode gap) analysis

Automated Barcode Gap Discovery (ABGD) uses alignment data to best assign hypothetical species using the distribution of pairwise distances, also known as ‘barcoding gaps’ (Puillandre et al., 2012). As with MJN, high levels of variation across alignments meant that ABGD struggled to accurately formulate hypothetical species groups using whole family data, and so a by genus approach was undertaken using the graphic web version (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) where: Pmin = 0.01,

Pmax=0.1 and 10 steps, X = 1.5 Nb bins = 20, a Kimura (K80) TS/TV approach to distance was applied.

4.3.11 Poisson tree processes (PTP and bPTP) of species delimitation

A Poisson Tree Processes (PTP) model was applied to the phylogenetic tree outputs to infer putative species boundaries using branching events by number of substitutions, as well as attempting to add Bayesian support values (bPTP). PTP outputs are calculated as part of the bPTP implementation process and settings for a Bayesian approach are reliant on meeting a strict set of assumptions for convergence of MCMC chains. If convergence is not achieved, then Bayesian support values are meaningless. This was checked visually using the bPTP web server trace plot function (Zhang et al., 2013). Where convergence was not met, the number of MCMC was incrementally increased from 100,000 to a maximum of 500,000, and 'seed' function increase across the range from 123 to 2,000 and Burn-in 0.1 to 0.5. Where this had no effect on improving convergence, the bPTP model was considered unsuitable for the data and a PTP (maximum likelihood) approach was utilised solely based on default settings. GYMC analysis was not used, as tree inputs requires time calibration to conduct the analysis.

4.4 Results

4.4.1 *ITS2* sequencing success rates

Direct sequencing of 226 sequences was undertaken. Full recovery of *ITS2* was low (51.8%), however, partial gene recovery was possible for some sequences (18.6%). Loss of sequencing signal was common across all genera, with some species more

problematic than others (Table 4.2). Causes of sequence loss was species specific but general heterogeneity was as a result of intraindividual polymorphisms within *ITS2* manifested primarily from mono-, di-, tri-, tetra-, penta- and hexanucleotidepolymorphisms, and to some extent as indels caused by insertions and non-repeated poly regions (i.e. position 140 in *Oc. flavescens*, CGTCGAGGT to GCGAGA---). A summary of *ITS2* sequencing drop off for each species are summarised in Table 4.2. A confirmation of intraspecific causes was also confirmed by cloning of seven specimens of *Cs. morsitans* and one of *Cs. litorea* eight times per individual, all other samples that presented two overlapping sequences were manually separated and variants added to the downstream analysis (*n* reported in Table 4.2). Those of *Oc. detritus*, *Oc. rusticus*, and *Oc. sticticus* were the only species that showed no intra-individual sequencing difficulties.

The presence of pseudogenes was also a possible cause of a small number of sequencing failures and was found to be persist in only 0.9% of all sequences sampled from GB. A total of thirteen sequences removed from the overall dataset (4.53%) after being characterised as pseudogenes due to hybridisation failure of the *5.8S* and *28S* flanking regions in most instances (Harpke and Peterson, 2008). A single exception of a specimen of *Cs. annulata* (UK956) was considered a possible pseudogene due to a lack of any sequence similarity with any Genbank enquiry (across all organisms) despite the presence of *5.8S* and *28S* respectively. Percentage of AT within *ITS2* was also taken into consideration as an indicator of pseudogenes, however, all GC content ranged from 48.95 to 58.41% across all species and is concordant with findings in other studies of mosquito *ITS2* (Byrd et al., 2012; Zomuanpuii et al., 2013) (summary of GC/AT ratios found in Table 4.7). Examination of *ITS2* flanking regions has proven to be a more useful indicator of pseudogenes in the mosquito species described herein.

4.4.2 *ITS2* gene information

Sequence lengths varied from 182 bp (*Or. pulcripalpis*) to 414 bp (*An. algeriensis)* between species, and within genera: *Anopheles* (mean difference between *ITS2* lengths, *n* = 128 bp), *Aedes* (207 bp), *Ochlerotatus* (27 bp), *Culiseta* (125 bp), *Culex* (146 bp). Species separation by amplicon size may be possible for some genera, such as *Anopheles* and *Culiseta* where size of the *ITS2* is clearly differentiated. However, this method is not efficient for identification of many closely related species due to overlap in sequence length variation. Refer to Table 4.7 for *ITS2* size ranges found in each species.

Despite sequence length heterogeneity, all folded secondary structures resulted in either three or four helices. Successful folds conformed to the paradigm set out by Coleman (2009) with all species demonstrating a characteristic pyrimidine bulge on helix II typically found in functional *ITS2* structures (Fig. 4.6 to 4.11). Short conserved regions were also reported from helix III and summarised in Table 4.7. These regions recorded within the genus *Aedes* (GATAGTCAGRCR) are comparable to those highlighted by Coleman (2007). Additionally, the same conserved sequence was unchanged across closely related genera, *Dahliana* and *Ochlerotatus*. Conserved third helix regions were found to be preserved at the intra-genus level only in: *Anopheles* (ACRCCTCACCRMC), *Culex* (CCCACACWCCARCCTGGCTTGG) and *Culiseta* (TTGATGAATACATCCCAT). Length and complexity varied most in helices I and III, with formation of helix IV as a result of a highly polymorphic regions at the 3' prime end of the *ITS2*. The appearance of a fourth helix was not restricted to species, with some (*An. claviger, Ae. aegypti, Oc. caspius, Oc. flavescens, Cs. litorea* and *Cs. morsitans*) displaying both states intraspecifically. The characteristics of the

Zoogeographic regions	**Palearctic**					**Saharo-arabian**			**Sino-Japanese**	**Oriental**			**Australian**	**Nearctic**		**Neotropical**			
Species	GB*	GB (Other)	Europe	Russia	Tajikistan	Iran	Saudi	North Africa	China	Sri Lanka	India	Vietnam	Australia	Canada	USA	South America	**Total**	**Accession no.†**
An.algeriensis	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**6**	n/a
An.atroparvus	-	1	2	1	-	-	-	-	-	-	-	-	-	-	-	-	**4**	AF504243, AY634518, AM409779, AM076979
An.claviger	2	-	3	-	1	2	-	1	-	-	-	-	-	-	-	-	**9**	AY129232, KP749464, KP749463, HM347501, DQ229314, KF483836, AJ555157
An.daciae	-	2	2	1	-	-	-	-	-	-	-	-	-	-	-	-	**5**	AY634472, JX416349, AY822587, MG727769, EF090200
An.maculipennis s.l.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	-
An.messeae	1	2	3	-	-	-	-	-	-	-	-	-	-	-	-	-	**6**	AF504212, AF504229, AY648983, AF342711, KY695114
An.plumbeus	5	-	1	1	-	2	-	-	-	-	-	-	-	-	-	-	**9**	KC294444, JQ928897, AM076978, AJ555483
Ae.aegypti	1	-	-	1	-	-	1	1	-	1	1	-	-	-	-	1	**7**	GU980956, KJ862124, MF142278, KY382418, MF072936, AY512665
Ae.albopictus	-	-	2	-	-	-	-	-	1	1	-	1	-	-	-	-	**5**	KX495943, DQ168420, AY741377, KF471598, KY382421
Ae.cinereus	3	-	3	-	-	-	-	-	-	-	-	-	-	-	1	-	**7**	MG232614, AM397835, AM397836, AM397837
Ae.geminus	2	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	**5**	AM397838, AM397839, AM397840
Ae.vexans	2	-	7	-	-	1	-	-	1	-	-	-	-	-	2	-	**13**	M95132, AF298626, KY614727, MG232641, KY614783, KY614782, KY614779, KY614777, KY614770, EF539857, AM084684
Da.geniculata	4	-	3	-	-	1	-	-	-	-	-	-	-	-	1	-	**9**	KF471610, KF471605, MG232621, KF471603, KF483833
Oc.annulipes	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**7**	n/a
Oc.cantans	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**2**	n/a
Oc.caspius	4	-	4	1	-	1	-	-	1	-	-	-	-	-	1	-	**12**	HM140420, MG232612, KP642721, KP642705, HM140424, KU880625, KF483843, AM084685
Oc.communis	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	**2**	KF535022, KF535019

Oc.detritus	6	-	2	-	-	-	-	1	-	-	-	-	-	-	1	-	**10**	MG232616, KJ661028, KJ661029, KJ661031
Oc.dorsalis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	**1**	MG232618
Oc.flavescens	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**2**	n/a
Oc.leucomelas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	-
Oc.nigrinus	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**3**	n/a
Oc.punctor	2	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	**4**	KF535076, KF535068
Oc.sticticus	6	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	**8**	KF535083, KF535080
Oc.rusticus	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**5**	n/a
Cs.fumipennis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	-
Cs.litorea	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**4**	n/a
Cs.morsitans	37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**37**	n/a
Cs.annulata	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**	n/a
Cs.alaskaensis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	-
Cs.subochrea	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**	MG954356
Cs. longiareolata	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	**1**	KF483837
Cx.pipiens s.l.	4	-	2	2	-	1	-	1	-	-	-	-	2	-	1	-	**13**	LC120317, LC114272, JQ958369, U22131, AJ850085, AJ850086, KU175324, KX866004, KU495644
Cx.torrentium	1	-	-	1	-	-	-	-	-	-	-	-	-	-	1	-	**3**	AJ850083, U33040
Cx.modestus	2	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	**6**	KU880622, KU880623, KU880649, KU880650
Cx.territans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	**2**	U33035, U33036
Cq.richiardii	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**5**	-
Or.pulcripalpis	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**	FJ867653
Total	**117**	**6**	**38**	**8**	**1**	**9**	**1**	**4**	**7**	**2**	**1**	**1**	**2**	**6**	**11**	**1**	**215**	

Table 4.1 Geographic locations of mosquito *ITS2* sequences acquired for analysis.

GB sequences by number of individuals and does not include intra-individual multiple extracted sequences. * Specimens collected and sequenced from GB. † All *ITS2* additional sequences acquired from GenBank (NCBI).

secondary structures are consistent within genera but not at family level. The *ITS2* secondary structure in *Anopheles, Aedes, Culex, Dahliana* and *Orthopodomyia* has a more elaborate morphology in helix III, in *Ochlerotatus* this appears in helix I. Specimens of *Culiseta* and *Coquillettidia* show approximately equal length and complexity in both helix I and III (Fig. 4.8). Successful folding of ITS2 structures for *Cs.annulata* and *Cs.subochrea* was not possible due to none of the resulting folds meeting *ITS2* structure criteria, therefore, these species were removed from CBC analysis.

4.4.3 Phylogenetic analysis and estimated evolutionary distances

Phylogenetic reconstruction of *ITS2* was hampered by multiple indels and a lack of conserved regions, only a 7 bp (ATATTT) region at the 5' prime end of *ITS2* is consistent across all species. This resulted in difficulty in using complex evolutionary models for accurate phylogenetic reconstruction. To counter this a simplified approach using Kimura-2 and *p*-distance were tested for their ability to handle the alignments with significant indel problems. Complete deletion of indels proved to have the lowest power in predicting species level monophyletic groups (43.3% (with Kimura-2) and 56.6% (*p*-dist.)). The highest bootstrap scores came from the use of *p*-distance models with both partial and pairwise deletions (83% resolution). The number of positions included in the distance calculations was only 75 for complete deletions, 210 for partial deletions and 791 for pairwise deletions (Table 4.3). Therefore, a pairwise deletion of indels and a standard *p*-distance was considered most suitable for building mosquito *ITS2* phylogenies despite its tendency to rapidly saturate.

(a)

(b)

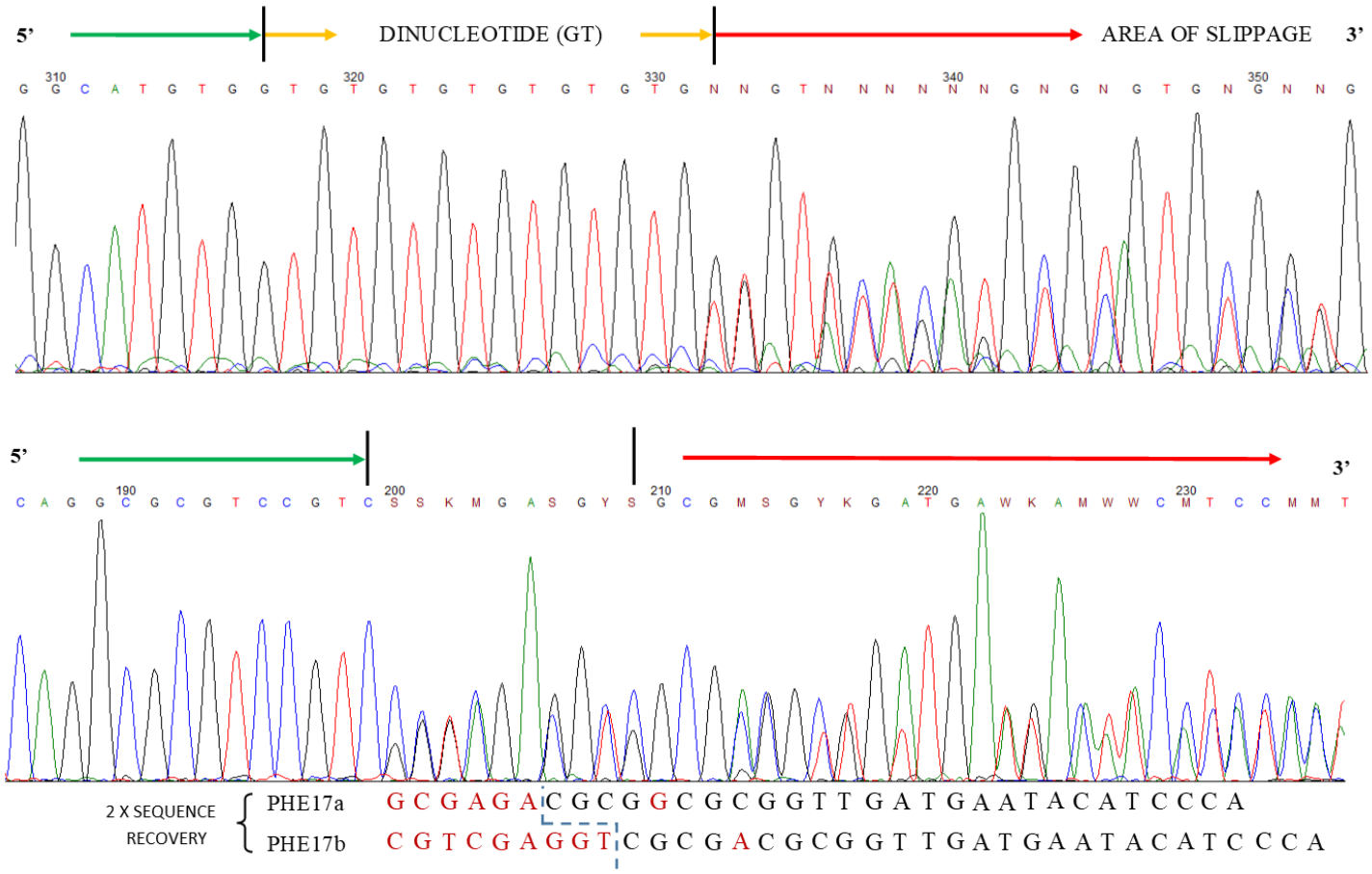


Fig. 4.2 Identification of chromatograph collapse in direct sequencing of *ITS2*. **(a)** *Culiseta morsitans* (BM20) - chromatograph problems as a result of dinucleotide repeat. **(b)** *Ochlerotatus flavescens* (PHE17) - chromatograph problems as a result of polymorphic region (CGTCGAGGT/GCGAGA---).

Phylogenetic reconstruction using the approach above produced strong monophyletic groupings for most morphologically separated species (bootstrap values >75%) using only specimens from GB. However, separation of *Oc. annulipes* and *Oc. cantans* was not strongly supported (bootstrap = 34%). Additional clades were also detected for *Oc. sticticus* (2 clades), *Cx. modestus* (2) and *Cs. morsitans* (2) in GB specimens. Analysis including sequence data from across geographical ranges suggests additional levels of *ITS2* complexity within *Ae. cinereus* and *Ae. geminus*, *Ae. vexans* (x5 clades), *Oc. detritus* (x2) and *An. claviger* (x4) (Fig. 4.4).

Across all samples, a mean inter-specific *p*-distance of 0.313 was recorded. The smallest observed distances were between those of *An. daciae* and *An. messeae* (0.007), and *Oc. annulipes* and *Oc. cantans* (0.017). It is worth noting that the synonymisation of species with similar distances has been suggested for other species of *Anopheles* within the Hyrcanus group (Hwang, 2007). Mean intra-specific distances of 0.012 were observed for GB specimens, and 0.027 with the addition of sequence data from across species ranges suggesting an increase in genetic variation across geographical distances. A summary of all evolutionary distance estimations can be found in Tables 4.4 and 4.5.

4.4.4 Species partitioning using ABGD and PTP analysis

ABGD and PTP analysis present two different approaches to species delimitation. ABGD analysis was able to produce suitable gaps required for the calculation of hypothetical species groups at the genus level but did not produce bimodal distribution across the whole family (Fig. 4.3). PTP estimated between 58 and 114

Species	*n*	Full sequence recovery	% Full coverage	Partial sequence recovery	Multiple sequence recovery	Seq failure	% Seq failure	Types of indels resulting in sequence failures, or multiple sequence overlap
An.algeriensis	7	6	85.7	0	0	1	14.3	n/a
An.atroparvus	0	-	-	-	-	-	-	-
An.claviger	13	2	15.4	9	0	2	15.4	PCR failure, pos[139] DIN(CA), pos[158] TRI(TTG)
An.daciae	0	-	-	-	-	-	-	-
An.maculipennis s.l.	3	0	0.0	1	0	2	66.7	Pseudogenes, PCR failures
An.messeae	1	1	100.0	0	0	0	0.0	n/a
An.plumbeus	6	5	83.3	0	0	1	16.7	n.d
Ae.aegypti	1	1	100.0	0	0	0	0.0	n/a
Ae.albopictus	2	0	0.0	2	0	0	0.0	pos[67] POLY(GTGTGCGCGCA/--GAGCGTACGC), pos[332] INV(GCA/ACG)
Ae.cinereus	4	3	75.0	0	2	1	25.0	pos[22] PEN(CGCGT), pos[178] DIN(CG)
Ae.geminus	4	2	50.0	2	1	0	0.0	pos[22] DIN(CG), pos[124] DIN(GC), pos[204] MON(A), pos[218] MON(A)
Ae.vexans	6	2	33.3	3	0	1	16.7	pos[31] TRI/DIN(GCT&CT), pos[52] MON(A), pol[63] HEX(CGTATG), pos[178] DIN(GC), pos[242] MON(A), pos[262] MON(A)
Da.geniculata	7	4	57.1	1	2	2	28.6	pos[71] TRI(CGG), pos[251] INS(GA)
Oc.annulipes	8	7	87.5	0	0	1	12.5	n.d
Oc.cantans	5	2	40.0	3	1	0	0.0	pos[210] INS(CAAGACACC), pos[224] DIN (AC), pos[232] MON(A)
Oc.caspius	5	4	80.0	1	2	0	0.0	pos[187] DIN(CG), pos[213] INS(CAT), pos[234] INS(CCAGT)
Oc.communis	-	-	-	-	-	-	-	-
Oc.detritus	6	6	100.0	0	0	0	0.0	n/a
Oc.dorsalis	-	-	-	-	-	-	-	-
Oc.flavescens	6	2	33.3	1	1	3	50.0	pos[140] POLY (CGTCGAGGT/GCGAGA---), pos[215] POLY(AC*), pos[226] PEN(ATAGC)
Oc.leucomelas	-	-	-	-	-	-	-	-

Oc.nigrinus	5	3	60.0	1	0	1	20.0	pos[236] DIN(CA)
Oc.punctor	6	2	33.3	3	1	1	16.7	pos[146] DIN(CG), pos[189] DIN(CG), pos[228] TET(TCAA)
Oc.sticticus	6	6	100.0	0	0	0	0.0	n/a
Oc.rusticus	5	5	100.0	0	0	0	0.0	n/a
Cs.fumipennis	-	-	-	-	-	-	-	-
Cs.litorea	5	4	80.0	1	8	0	0.0	pos[230] MON(C), pos[347] TRI(AAG)
Cs.morsitans	72	37	51.4	9	41	26	36.1	pos[30] TRI(GTG), pos[235] DIN(CA), pos[257] DIN(GT), pos[330] POLY (ACAACCCAAACAACAG/TCTCAACAGTC-----)
Cs.annulata	5	1	20.0	0	0	4	80.0	Pseudogene, pos[52] DIN(CG), pos[82] DIN(CG), pos[107] MON(C)
Cs.alaskaensis	-	-	-	-	-	-	-	-
Cs.subochrea	-	-	-	-	-	-	-	-
Cs.longiareolata	-	-	-	-	-	-	-	-
Cx.pipiens s.l.	6	4	66.7	1	1	1	16.7	pos[28] POLY(GT/-C), pos[109] MON(G), pos[224] TRI(CGT), pos[235] DIN(CA), pos[354] MON(C)
Cx.torrentium	9	1	11.1	1	1	7	77.8	PCR failures, pos[256] DIN(TA), pos[267] DIN(GA)
Cx.modestus	5	2	40.0	2	1	1	20.0	pos[46] DIN(GT), pos[241] DIN(TC), pos[252] POLY(A)
Cx.territans	1	0	0.0	0	0	1	100.0	n.d
Cq.richiardii	17	5	29.4	1	0	11	64.7	PCR failures, pos[26] DIN(GC), pos[296] TRI(CAA)
Or.pulcripalpis	-	-	-	-	-	-	-	-
Total	**226**	**117**	**51.8**	**42**	**62**	**67**	**29.6**	

Table 4.2 Sequencing success rates and the causes of sequence failures in *ITS2* from GB mosquitoes.

*multiple combinations in this region. pos[] = nucleotide position within the *ITS2* gene. MON = mononucelotide repeat, DIN = dinucleotide repeat, TRI = trinucleotide repeat, TET = tetranucleotide repeat, PEN = pentanucleotide repeat, HEX = hexanuxleotide repeat, INV = inversion, INS = insertion, POLY = polymorphic loci.

Evolutionary model	Indel treatment	*An.algeriensis*	*An.atroparvus*	*An.claviger*	*An.daciae*	*An.messeae*	*An.plumbeus*	*Ae.aegypti*	*Ae.albopictus*	*Ae.cinereus*	*Ae.geminus*	*Ae.vexans*	*Cx.modestus*	*Cx.pipiens*	*Cx.territans*	*Cx.torrentium*	*Cs.litorea*	*Cs.morsitans*	*Cs.annulata/subochrea*	*Da.geniculata*	*Oc.annulipes*	*Oc.cantans*	*Oc.caspius*	*Oc.communis*	*Oc.detritus*	*Oc.flavescens*	*Oc.nigrinus*	*Oc.punctor*	*Oc.rusticus*	*Oc.sticticus*	*Cq.richiardii*	*Or.pulcripalpis*	Resolution (%)	Reconstrcution (%)	Positions (*n*)
K2+G (+1.3)	CD	94	87	91	n.d	n.d	98	n.d	84	n.d	n.d	53	94	n.d	99	n.d	n.d	56	78	55	n.d	n.d	n.d	n.d	52	n.d	n.d	n.d	n.d	n.d	100	/	43.3	80.9	75
K2+G (+0.7)	PWD	99	88	97	n.d	n.d	78	n.d	97	n.d	n.d	79	95	87	97	94	99	69	90	98	n.d	n.d	n.d	99	66	n.d	76	60	68	68	100	/	70	62.7	791
K2+*G* (+0.7)	PD	96	78	95	n.d	n.d	79	65	96	n.d	n.d	92	95	61	98	89	93	61	93	99	n.d	n.d	92	95	61	n.d	75	80	97	92	100	/	76.6	81.4	210
p -distance	CD	100	97	99	n.d	n.d	100	72	98	n.d	n.d	73	99	n.d	100	n.d	59	49	81	57	n.d	n.d	96	n.d	64	n.d	n.d	n.d	83	n.d	100	/	56.6	100	75
p -distance	PWD	100	100	100	85	76	100	100	100	n.d	n.d	100	100	100	100	100	100	100	99	100	n.d	n.d	99	100	98	n.d	99	93	100	98	100	/	83.3	100	791
p -distance	PD	100	99	100	83	90	100	99	100	n.d	n.d	99	100	99	100	100	100	97	99	100	n.d	n.d	100	100	94	n.d	97	99	100	98	100	/	83.3	100	210

Table 4.3 Bootstrap values (%) for species level determination by *ITS2* using different indel treatments.

CD = complete deletion, PWD = pairwise distance, PD = partial deletion, 70% site coverage cut-off, Resolution = % of monophylogenies confirmed by bootstrap scores >50%. Reconstructions = the number of successful tree constructions from 1000 bootstrap repetitions.

Ae.aegypti	**aeg.**																														
Ae.albopictus	0.285	**alb.**																													
Ae.cinereus	0.235	0.358	**cin.**																												
Ae.geminus	0.251	0.361	0.087	**gem.**																											
Ae.vexans	0.212	0.315	0.310	0.315	**vex.**																										
An.algeriensis	0.445	0.556	0.488	0.489	0.483	**alg.**																									
An.messeae	0.529	0.509	0.521	0.522	0.518	0.576	**mes.**																								
An.atroparvus	0.514	0.558	0.515	0.516	0.545	0.563	0.142	**atr.**																							
An.daciae	0.532	0.517	0.524	0.523	0.518	0.590	0.007	0.143	**dac.**																						
An.claviger	0.410	0.560	0.462	0.461	0.432	0.564	0.568	0.612	0.579	**cla.**																					
An.plumbeus	0.405	0.489	0.450	0.461	0.448	0.482	0.546	0.554	0.550	0.374	**plu.**																				
Cq.richiardii	0.336	0.394	0.377	0.385	0.366	0.536	0.594	0.608	0.602	0.530	0.493	**ric.**																			
Cs.annulata	0.326	0.416	0.364	0.363	0.392	0.478	0.536	0.538	0.525	0.448	0.491	0.386	**ann.**																		
Cs.litorea	0.256	0.369	0.337	0.339	0.325	0.504	0.550	0.537	0.552	0.486	0.426	0.412	0.303	**lit.**																	
Cs.morsitans	0.267	0.366	0.285	0.297	0.292	0.521	0.538	0.552	0.544	0.485	0.432	0.383	0.285	0.170	**mor.**																
Cs.subochrea	0.280	0.359	0.330	0.333	0.331	0.468	0.528	0.518	0.503	0.463	0.469	0.411	0.211	0.300	0.279	**sub.**															
Cx.modestus	0.378	0.489	0.443	0.450	0.404	0.528	0.519	0.584	0.513	0.535	0.509	0.422	0.448	0.456	0.411	0.433	**mod.**														
Cx.pipiens	0.327	0.473	0.417	0.423	0.416	0.529	0.525	0.546	0.518	0.531	0.530	0.432	0.405	0.394	0.371	0.415	0.309	**pip.**													
Cx.torrentium	0.342	0.465	0.415	0.417	0.445	0.573	0.518	0.566	0.511	0.561	0.505	0.405	0.441	0.414	0.384	0.434	0.374	0.232	**tor.**												
Cx.territans	0.329	0.424	0.451	0.454	0.433	0.541	0.544	0.584	0.560	0.472	0.468	0.371	0.389	0.397	0.372	0.425	0.317	0.288	0.274	**ter.**											
Da.geniculatus	0.276	0.393	0.341	0.349	0.328	0.504	0.519	0.535	0.514	0.447	0.455	0.422	0.335	0.331	0.315	0.357	0.436	0.440	0.454	0.448	**gen.**										
Oc.annulipes	0.215	0.290	0.261	0.270	0.296	0.478	0.479	0.490	0.468	0.427	0.405	0.319	0.269	0.251	0.220	0.300	0.310	0.281	0.316	0.363	0.240	**ann.**									
Oc.cantans	0.241	0.285	0.290	0.294	0.320	0.490	0.473	0.485	0.463	0.441	0.414	0.327	0.298	0.259	0.251	0.314	0.348	0.311	0.328	0.351	0.279	0.017	**can.**								
Oc.caspius	0.224	0.322	0.297	0.306	0.314	0.493	0.507	0.514	0.490	0.416	0.415	0.378	0.293	0.304	0.278	0.323	0.389	0.366	0.373	0.404	0.269	0.164	0.162	**cas.**							
Oc.communis	0.203	0.279	0.281	0.286	0.290	0.479	0.479	0.485	0.465	0.431	0.421	0.358	0.275	0.277	0.233	0.306	0.343	0.323	0.334	0.380	0.252	0.104	0.126	0.171	**com.**						
Oc.detritus	0.233	0.296	0.285	0.295	0.280	0.477	0.474	0.485	0.461	0.398	0.415	0.360	0.283	0.255	0.248	0.305	0.361	0.344	0.354	0.383	0.258	0.119	0.144	0.167	0.107	**det.**					
Oc.flavescens	0.229	0.289	0.285	0.290	0.309	0.489	0.475	0.489	0.464	0.423	0.417	0.331	0.288	0.254	0.238	0.313	0.342	0.306	0.317	0.351	0.258	0.029	0.051	0.171	0.120	0.134	**fla.**				
Oc.nigrinus	0.229	0.318	0.278	0.284	0.288	0.479	0.483	0.474	0.478	0.416	0.410	0.350	0.326	0.285	0.272	0.286	0.384	0.356	0.355	0.384	0.242	0.183	0.214	0.178	0.204	0.213	0.191	**nig.**			
Oc.punctor	0.258	0.313	0.293	0.301	0.321	0.477	0.461	0.468	0.445	0.429	0.429	0.360	0.322	0.280	0.258	0.326	0.340	0.342	0.348	0.374	0.292	0.100	0.132	0.174	0.107	0.115	0.122	0.192	**pun.**		
Oc.rusticus	0.212	0.286	0.252	0.267	0.272	0.460	0.502	0.512	0.500	0.412	0.398	0.400	0.286	0.304	0.293	0.257	0.328	0.278	0.291	0.336	0.232	0.160	0.169	0.173	0.160	0.159	0.180	0.174	0.197	**rus.**	
Oc.sticticus	0.218	0.289	0.266	0.268	0.272	0.469	0.476	0.478	0.471	0.416	0.412	0.346	0.303	0.280	0.248	0.289	0.360	0.353	0.361	0.385	0.233	0.183	0.195	0.184	0.197	0.201	0.185	0.043	0.194	0.190	**sti.**
Or.pulcripalpis	0.348	0.356	0.353	0.350	0.306	0.414	0.470	0.474	0.463	0.433	0.431	0.358	0.413	0.335	0.312	0.369	0.382	0.365	0.431	0.427	0.335	0.293	0.304	0.308	0.303	0.274	0.295	0.259	0.275	0.308	0.272

Table 4.4 Evolutionary distance between mosquito species found in the GB, calculated using *p*-distance with pairwise substitutions

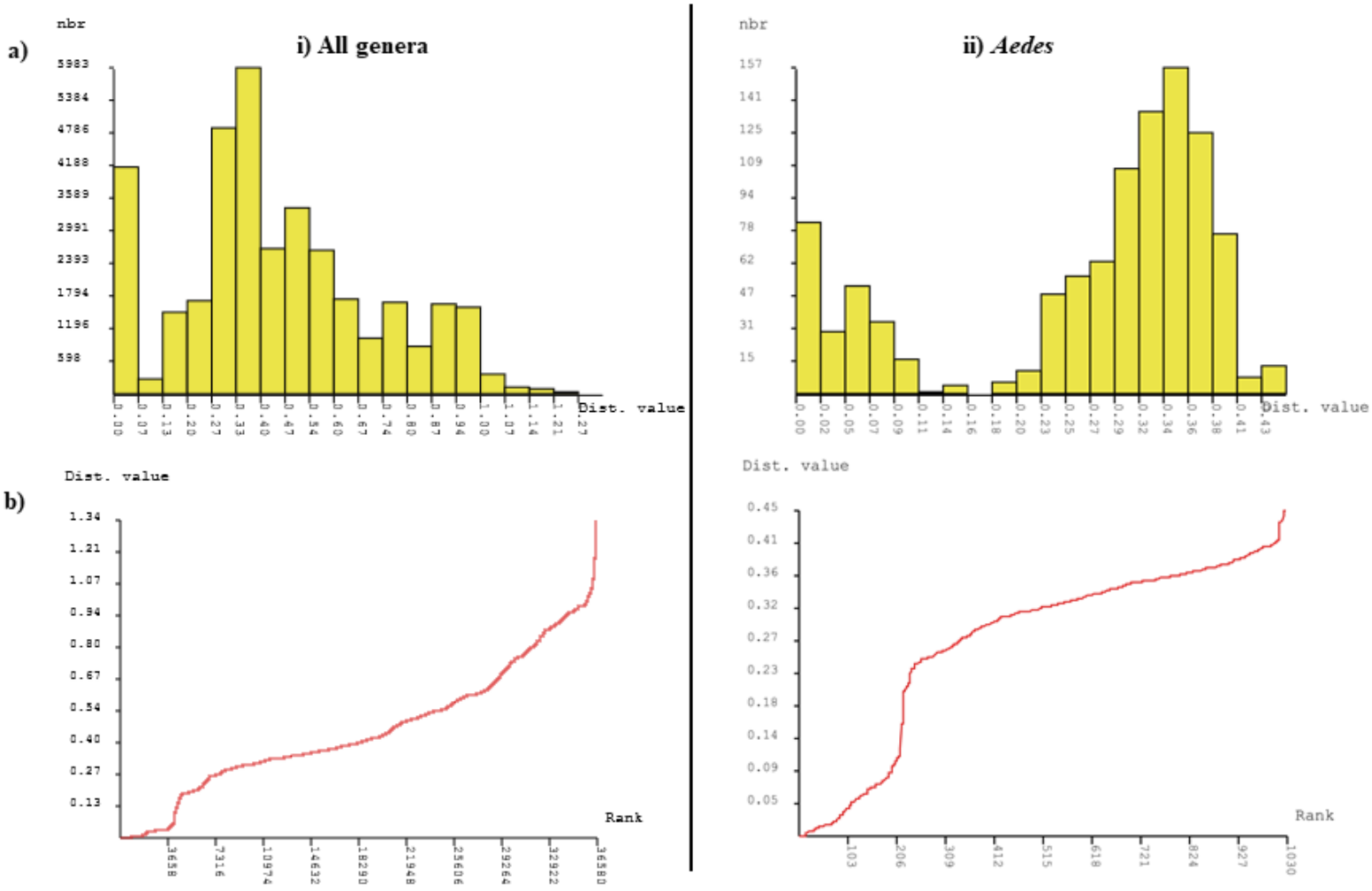


Fig. 4.3 ABGD 'barcode gap' histograms a) and ranked distance b) for species range *ITS2* sequence data by genus and family

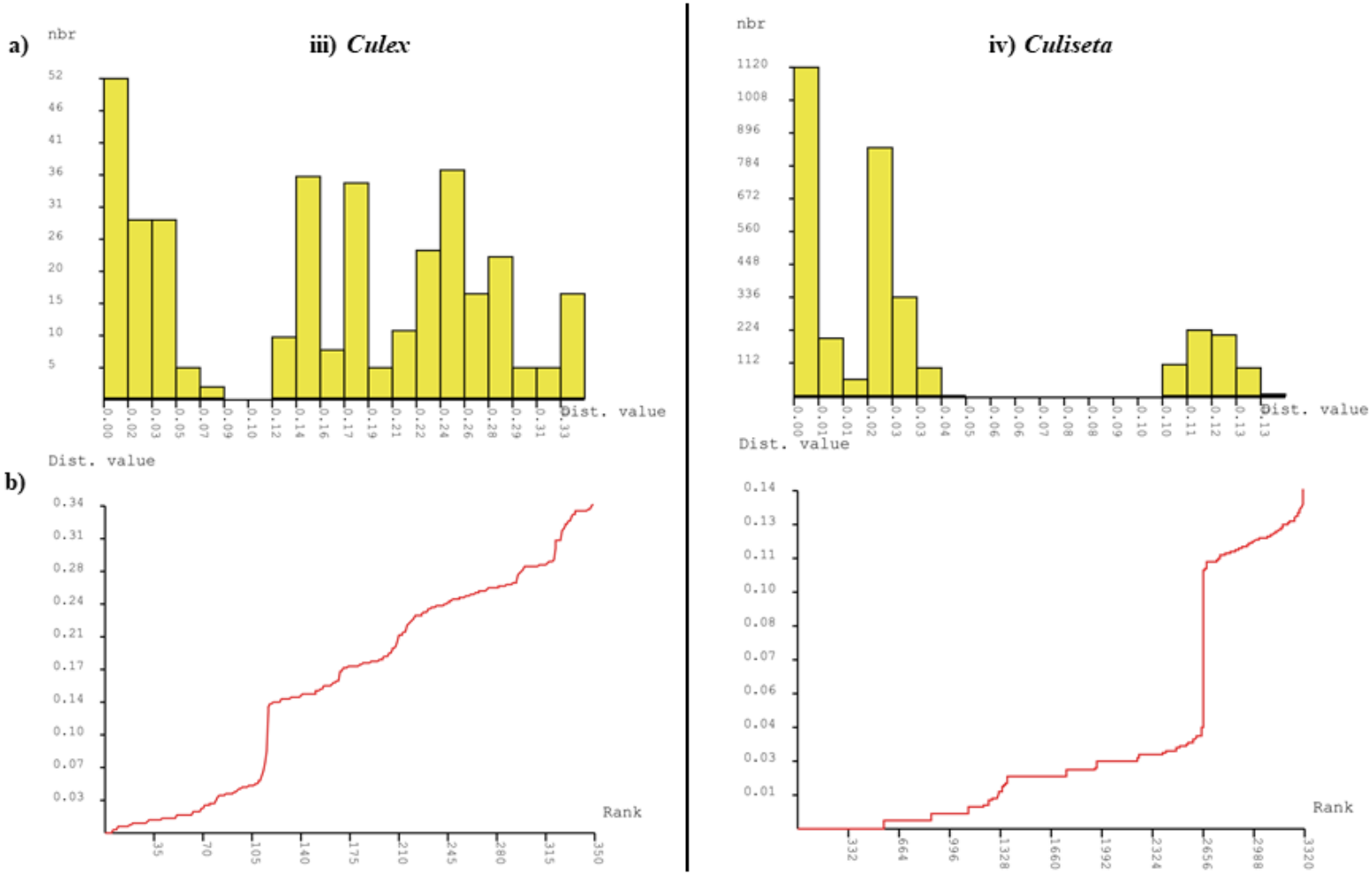


Fig. 4.3 Continued.

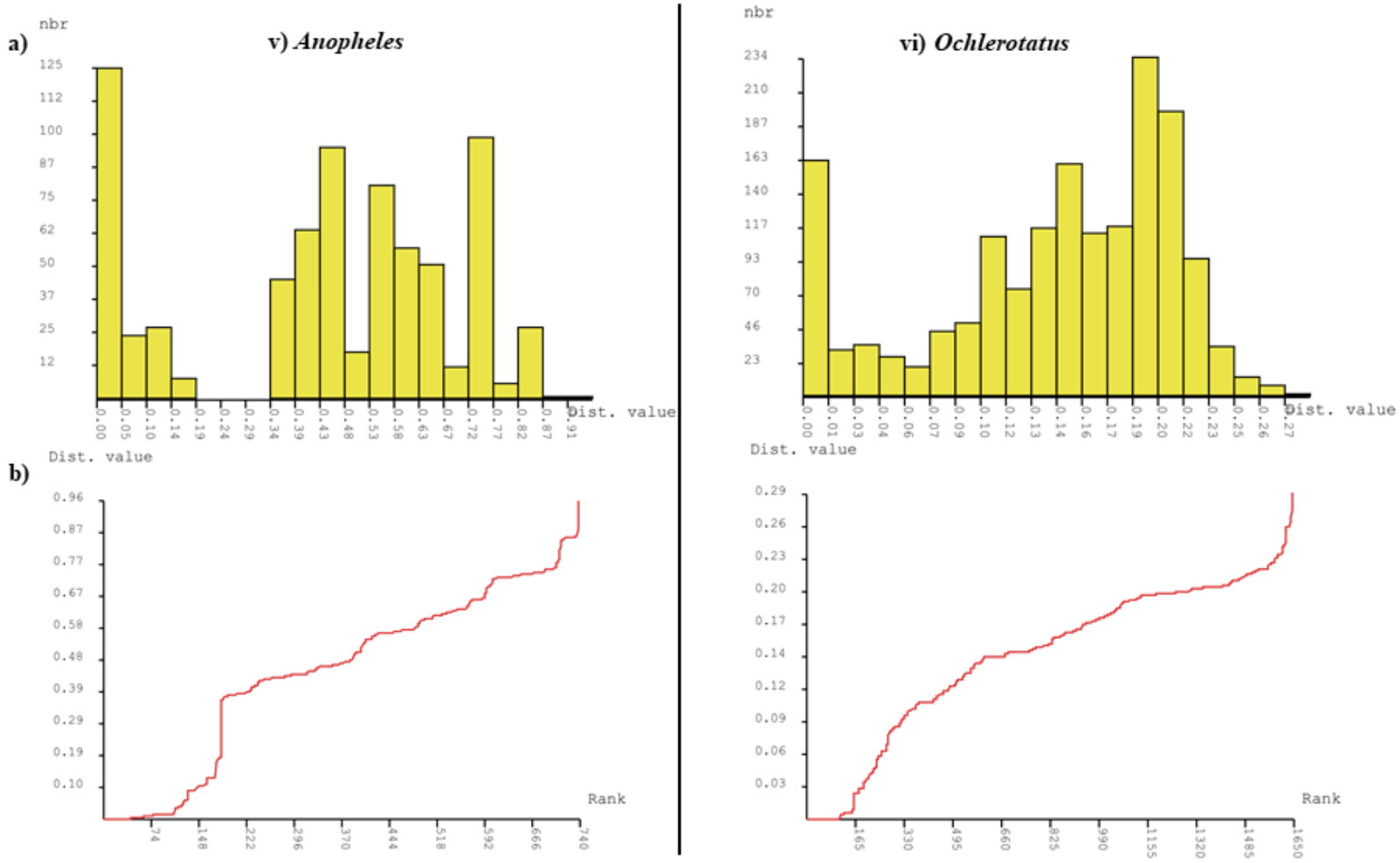


Fig. 4.3 Continued.

species (Acceptance rate = 0.3907) across the family. Both methods showed inconsistency between species numbers delimited in GB samples and those collected across the species range. ABGD analysis of the genus *Anopheles* partitioned samples into 6 groups from GB and 7 from across species ranges (SR) (prior maximal distance P = 0.0215), *Culex* = 3 GB and 4 SR (P = 0.0129), *Culiseta* = 3 GB and 4 SR (P = 0.0077), *Ochlerotatus* = 12 GB and 15 SR, *Aedes* (including *Dahliana*) = 4 GB and 5 SR (P = 0.0077). PTP partitioning of *Anopheles* = 7 GB and 10 SR, *Culex* = 5 GB and 8 SR, *Culiseta* = 4 GB and 5 SR, *Ochlerotatus* = 14 GB and 20 SR, *Aedes* and *Dahliana* = 7 GB and 20 SR. All *Coquillettidia ITS2* sequences were homogenous. A summary of the number of hypothetical species groupings by both ABGD and PTP are summarised in Table 7.1 (Chapter 7). Notably, these approaches did not separate *Oc. annulipes* and *Oc. cantans*, nor those of *An. daciae* and *An. messeae* within the *An. maculipennis* complex. Conversely, *An. claviger*, *Oc. caspius*, and *Oc. punctor* showed additional species groupings in GB and SR compared to those expected by morphological methods of species identification.

4.4.5 Compensatory base change (CBC) locations

An initial analysis of CBC locations using a dataset of all sequences was unsuccessful in reflecting species delimitation consistent with morphological, phylogenetic, ABGD and PTP approaches (results not presented here). However, breaking down data by genus proved to be more consistent with partitioning approaches mentioned above, except for species within the Genus *Culex*, where only a single CBC was recognised within *Cx. torrentium*/*pipiens* against all other species

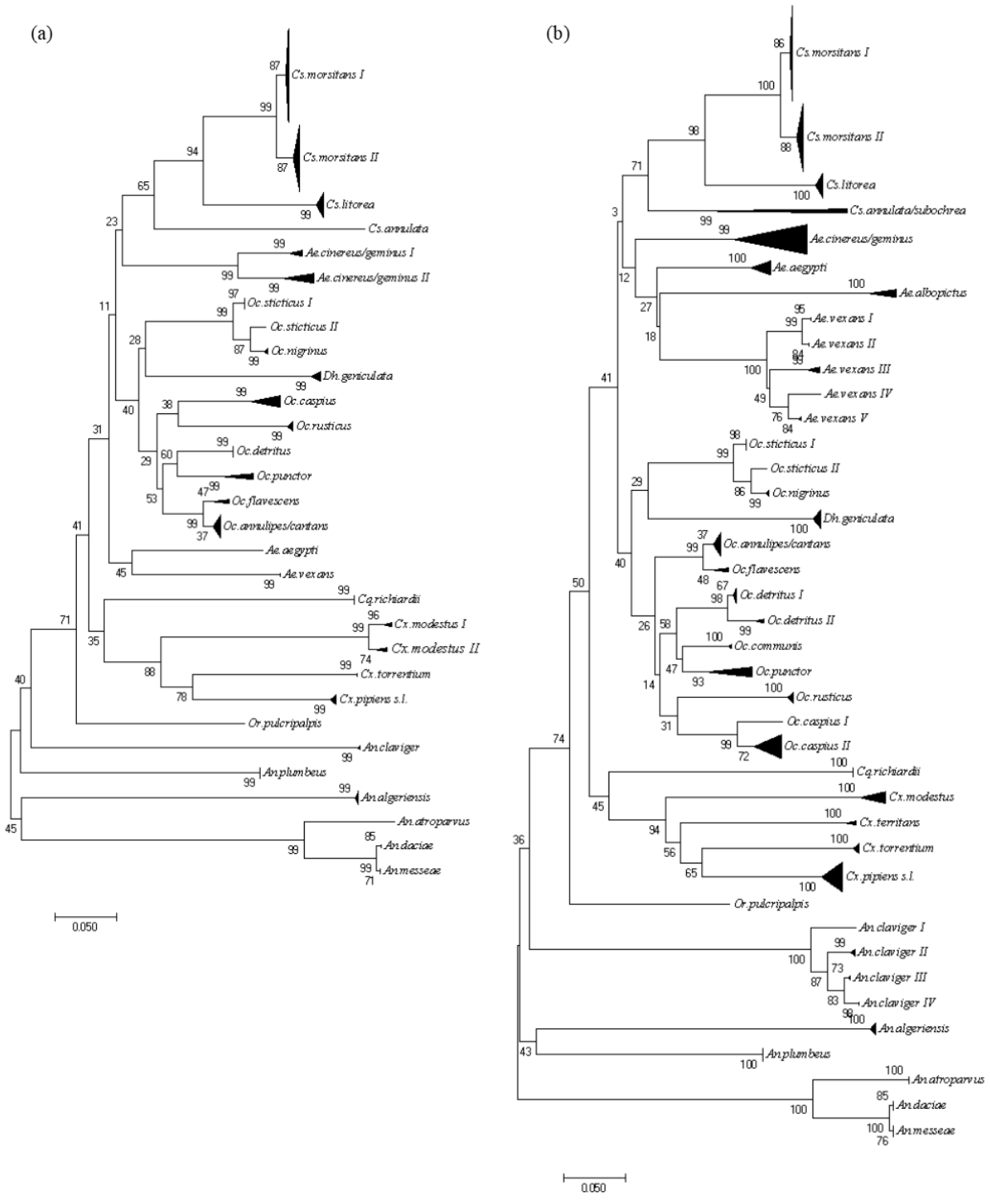


Fig. 4.4 Phylogenetic analysis of the *ITS2* gene from the mosquito species found in (a) GB (b) plus sequences from across their species range.

Phylogenetic tree of (a) and (b) were constructed by neighbour-joining methods using 1000 bootstrap replicates. Evolutionary distance was calculated using the *p*-distance method. The analysis include (a) 193, and (b) 271, sequences with a total of 791 positions. Indels were treated by pairwise deletion. Analysis carried out using MEGA 7.

Genus/Species	GB only			Across species range		
	n	**Within species mean distance (K2P)**	**Within species max. observed distance (K2P)**	***n***	**Within species mean distance (K2P)**	**Within species max. observed distance (K2P)**
Anopheles	**20**	**0.001**	**0.01**	**36**	**0.0385**	**0.076**
An.algeriensis	6	0.003	0.01	6	0.003	0.01
An.claviger	2	0.003	0.003	6	0.045	0.076
An.atroparvus	1	n/c	n/c	5	0	0
An.messeae	3	0	0	6	0	0
An.daciae	2	0	0	4	0	0
An.plumbeus	6	0	0	9	0.183	0.047
***Aedes* (inc. *Stegomyia*)**	**11**	**0.032**	**0.098**	**36**	**0.053**	**0.179**
Ae.aegypti	1	n/c	n/c	7	0.023	0.042
Ae.albopictus	0	-	-	4	0.034	0.054
Ae.cinereus	4	0.054	0.098	8	0.056	0.116
Ae.geminus	4	0.041	0.066	7	0.099	0.179
Ae.vexans	2	0	0	10	0.055	0.109
Dahliana	**5**	**0.01**	**0.016**	**9**	**0.01**	**0.019**
Da.geniculatus	5	0.01	0.016	9	0.01	0.019
Ochlerotatus	**43**	**0.012**	**0.057**	**58**	**0.015**	**0.088**
Oc.annulipes	7	0	0	7	0	0
Oc.cantans	3	0.004	0.056	3	0.004	0.056
Oc.caspius	6	0.023	0.057	13	0.025	0.088
Oc.communis	0	-	-	2	0.004	0.004
Oc.detritus	6	0	0	10	0.017	0.067
Oc.dorsalis	0	-	-	0	-	-
Oc.flavescens	3	0.031	0.057	3	0.031	0.057
Oc.leucomelas	0	-	-	0	-	-
Oc.nigrinus	3	0.005	0.008	3	0.005	0.005
Oc.punctor	3	0.038	0.057	5	0.05	0.079
Oc.sticticus	7	0.008	0.03	7	0.008	0.03
Oc.rusticus	5	0.003	0.008	5	0.003	0.008
Culiseta	**97**	**0.012**	**0.056**	**98**	**0.012**	**0.056**
Cs.alaskaensis	0	-	-	0	-	-
Cs.annulata	1	n/c	n/c	1	n/c	n/c
Cs.subochrea	0	-	-	1	n/c	n/c
Cs.litorea	12	0.007	0.017	12	0.007	0.017
Cs.longiareolata	0	-	-	0	-	-
Cs.morsitans	84	0.017	0.056	84	0.017	0.056
Culex	**11**	**0.011**	**0.041**	**28**	**0.02275**	**0.259**
Cx.modestus	4	0.027	0.041	6	0.028	0.049
Cx.pipiens s.l.	5	0.007	0.015	16	0.044	0.259
Cx.territans	0	-	-	2	0.014	0.014
Cx.torrentium	2	0	0	4	0.005	0.011
Coquillettidia	**5**	**0**	**0**	**5**	**0**	**0**
Cq.richiardii	5	0	0	5	0	0
Orthopodomyia	**1**	**n/c**	**n/c**	**1**	**n/c**	**n/c**
Or.pulcripalpis	1	n/c	n/c	1	n/c	n/c
Across all genera	**193**	**0.012**	**0.098**	**271**	**0.027**	**0.259**

Table 4.5 Intraspecific evolutionary distances using *p*-distance with pairwise deletions.

n = number of sequences including intraindividual variants. n/c = not calculable

(Fig. 4.9; Table 4.6(e)). A single CBC location within species of the *An. maculipennis* group was identified between *An. atroparvus* and *An. daciae/messeae*, but not between *An. daciae* and *An. messeae*, providing additional evidence against the splitting of these species (Fig. 4.6, Table 4.6(a)). Other species of *Anopheles* show higher numbers of CBCs, with *An. claviger* showing up to seven CBC locations against all other *Anopheles* with varying rates depending on geographical locations (Fig. 4.7, Table 4.6(a)). Folding of *Cs. subochrea* and *Cs. annulata* was unsuccessful using the methods here, and the only recoverable sequence of *Cs. longiareolata* was removed as a suspected pseudogene. Therefore, *Culiseta* CBC analysis included only those of the subgenus *Culicella* with either one or two CBC locations between species (Fig. 4.8, Table 4.6(d)). Division of *Ae.* vexans into additional species groupings was supported with a GC-CG CBC on helix III (Fig. 4.11, Table 4.6(b)). *Ae. cinereus* and *Ae. geminus*, also revealed two CBC locations to support the split, and presence of both species in GB (Fig.4.10, Table 4.6(b)). *Ae. albopictus* scored a single CBC location difference from *Ae. cinereus* and *Ae. geminus* but not from other species of *Aedes*, or *Dahliana.* Likewise, three groups of *Ochlerotatus* lacked CBC location separations such as (1) *Oc. flavescens/ Oc. punctor/ Oc. communis* and *Oc. detritus*, (2) *Oc. sticticus/ Oc. nigrinus and* (3) *Oc. annulipes/ Oc. cantans*. No intraindividual CBC's were recorded.

4.4.6 Median joining network analysis (MJN) and biogeographic patterns

Of the 268 individuals (which includes 62 from intragenomic variants) including sequences from across species ranges, MJN analysis calculated 156 novel haplotypes (58.2%), broken down by genus this resulted in; *Aedes* = 38 haplotypes from 43 sequences, *Anopheles* = 15 from 36, *Culex* = 24 from 27, *Culiseta* = 47 from 98,

(a) Genus: *Anopheles*

An.atroparvus	**atr.**							
An.daciae	1	**dac.**						
An.messeae	1	0	**mes.**					
An.plumbeus	3	3	3	**plu.**				
***An.claviger* (i)**	4	4	4	6	**cla.(i)**			
An.algeriensis	3	3	3	1	2	**alg.**		
***An.claviger* (ii)**	4	4	4	5	3	0	**cla.(ii)**	
***An.claviger* (iii)**	4	4	4	6	2	0	0	**cla.(iii)**
***An.claviger* (iv)**	5	5	5	7	3	0	0	0

(b) Genus: *Aedes* (inc. *Dahliana*)

***Ae.vexans* (i)**	**vex.(i)**							
***Ae.vexans* (ii)**	1	**vex.(ii)**						
***Ae.geminus* (i)**	1	1	**gem.(i)**					
***Ae.geminus* (ii)**	2	2	2	**gem.(ii)**				
Ae.cinereus	2	2	2	0	**cin.**			
Ae.albopictus	0	0	1	1	1	**alb.**		
***Ae.aegypti* (i)**	3	3	1	0	0	0	**aeg.(i)**	
***Ae.aegypti* (ii)**	2	2	2	1	1	0	0	**aeg.(ii)**
Da.geniculatus	4	4	2	2	2	0	3	3

(c) Genus: *Ochlerotatus* (inc. *Dahliana*)

***Oc.flavescens* (i)**	**fla.(i)**													
***Oc.flavescens* (ii)**	0	**fla.(ii)**												
Oc.nigrinus	2	2	**nig.**											
Oc.punctor	0	0	2	**pun.**										
Oc.annuilpes	1	0	2	1	**ann.**									
***Oc.cantans* (i)**	1	0	2	1	0	**can.(i)**								
***Oc.cantans* (ii)**	1	0	2	0	0	0	**can.(ii)**							
Oc.rusticus	1	1	0	1	2	2	2	**rus.**						
***Da.geniculatus* (i)**	1	1	0	1	1	0	1	0	**gen.(i)**					
***Da.geniculata* (ii)**	2	2	1	2	2	1	2	1	0	**gen.(ii)**				
Oc.sticticus	2	2	0	2	2	2	2	0	0	1	**sti.**			
***Oc.caspius* (i)**	2	2	3	2	2	2	2	1	0	1	3	**cas.(i)**		
***Oc.caspius* (ii)**	2	2	3	2	2	2	2	1	0	0	3	0	**cas.(ii)**	
Oc.communis	0	0	3	0	1	1	0	1	1	2	3	3	3	**com.**
Oc.detritus	0	0	3	0	1	1	0	1	1	1	3	3	3	0

(d) Genus: *Culiseta*

***Cs.litorea* (I)**	**lit.(i)**		
***Cs.litorea* (II)**	0	**lit.(ii)**	
***Cs.morsitans* (I)**	2	1	**mor.(i)**
***Cs.morsitans* (II)**	2	1	0

(e) Genus: *Culex*

Cx.modestus	**mod.**			
***Cx.pipiens s.l.* (I)**	0	**pip.(i)**		
Cx.pipiens s.l.* (II)	0	1	**pip.(ii)**	
Cx.torrentium	1	1	1	**tor**
Cx.territans	0	0	0	0

Table. 4.6 (a) - (e) CBC matrix for the *ITS2* gene from species of the mosquitoes found in GB, and from across their species range.

* Represented by a single specimen (Acc.U22131 from the USA (Crabtree et al 1995).

Ochlerotatus = 31 from 58, *Coquillettidia* = 1 from 6. This result is not unexpected due to high levels of *ITS2* polymorphisms at the intraindividual (intragenomic) level, as well as intraspecific, reported in other studies (Bezzhonova and Goryacheva, 2008; Li and Wilkerson, 2006; Onyabe and Conn, 1999). To bypass the additional resolution expected from *ITS2*, we partitioned the data into arbitrary haploclades (Figs. 4.13 to 4.15). MJN results for *ITS2* did not produce the same resolution in biogeographical separation as seen from *COI* (Chapter 3 Figs. 3.5 to 3.7) due to only 34% of GB species being found to have more than 10 sequence hits for *ITS2* available from repositories, compared to 78.4% represented in *COI*. As a result, biogeographical distributions were poorly visualised due to low sample resolution. However, haploclades for *Ae. vexans* partition into two/three separate clusters from GB and Palearctic, with some closer to Nearctic and others to Saharo-arabian and Sino-japanese regions (Fig. 13). This multi-origin haplotype distribution was also found in analysis of *COI*. The delimitation of *Oc. annulipes* and *Oc. cantans* was not supported by MJN, with cluster separation by a single mutation. Likewise, of the *An. maculipennis s.l.* group, *An. daciae* and *An. messeae* was not disassociated by this method, whereas *An. atroparvus* formed a clearly isolated cluster by 41 mutations. The *Ae. cinereus/ geminus* split was supported by MJN as two distinct clusters were formed by 337 mutations. However, both groupings showed high levels of variation, with the formation of several haploclades and haplotypes. MJN results for each species are presented in Table 7.1 (Chapter 7).

Species	*n seq*	ITS2 amplicon size	GC%	*n* Pseudo	*n* helices	Δ*G**	Conserved region helices III	Folding method
An.algeriensis	6	414	57.93	0	4	-163.7	ACACCTCACCAAC	RNAstructure
An.atroparvus	4	307	53.75	0	4	-95.93	ACACCTCACCACC	MFold
An.claviger	10	331-346	55.8	2	3-4	-78.6	ACACCTCACCGAC	RNAFold, ITS2database
An.daciae	5	305	52.13	0	4	NR	ACACCTCACCACC	ITS2database
An.messeae	6	305	52.79	0	4	NR	ACACCTCACCACC	ITS2database
An.plumbeus	7	276-277	54.35	1	3	-93.64	ACGCCTCACCAAC	MFold
Ae.aegypti	7	196-206	48.95	0	3-4	-58.8(-66)	GATAGTCAGACG	RNAstructure
Ae.albopictus	4	378-403	56.44	0	3	-157.7	GATAGTCAGACG	RNAstructure
Ae.cinereus	3	225-229	50.66	0	4	-81.14	GATAGTCAGACR	RNAstructure
Ae.geminus	2	226-228	50.44	0	4	-81.14	GATAGTCAGACG	RNAstructure
Ae.cinereus/geminus	7	223-228	50.66	0	4	-81.14	GATAGTCAGACG	RNAstructure
Ae.vexans	13	244-261	51.9	3	4	-97.2(-87.4)	GATAGTCAGACG	RNAstructure
Dh.geniculatus	9	324-331	58.41	2	3	-134.7	GATAGTCAGGCG	RNAstructure
Oc.annulipes	7	228-229	49.81	0	4	-83.2	GATAGTCAGGCG	RNAstructure
Oc.cantans	3	236-238	50	0	4	-85.2	GATAGTCAGGCG	RNAstructure
Oc.caspius	15	229-236	50.34	1	3-4	-73.2(-71.8)	GATAGTCAGGCG	RNAstructure
Oc.communis	2	240	48.96	0	3	-72.3	GATAGTCAGGCG	RNAstructure
Oc.detritus	10	220-247	50.41	0	3	-63.3	GATAGTCAGGCG	RNAstructure
Oc.dorsalis	-	-	-	-	-	-	-	-
Oc.flavescens	3	230-239	50.5	0	3-4	-81.8(-80.7)	GATAGTCAGGCG	RNAstructure
Oc.leucomelas	-	-	-	-	-	-	-	-
Oc.nigrinus	3	243	55.14	0	3	-79.9	GATAGTCAGGCG	RNAstructure
Oc.punctor	5	228-235	50.43	0	3	-75.7	GATAGTCAGGCG	RNAstructure
Oc.sticticus	9	237-238	54.88	2	3	-79.1	GATAGTCAGGCG	RNAstructure
Oc.rusticus	5	244-245	53.19	0	3	-96.9	GATAGTCAGGCG	RNAstructure
Cs.fumipennis	-	-	-	-	-	-	-	-
Cs.litorea	4	359-363	58.21	0	3-4	-154.2	TTGATGAATACATCCCAT	RNAstructure
Cs.morsitans	39	331-347	56.52	0	3-4	-141.6(-135.7)	TTGATGAATACATCCCAT	RNAstructure
Cs.annulata	2	242	57.44	1	UF	UF	TTGATGAATACATCCCAT	UF
Cs.alaskaensis	-	-	-	-	-	-	-	-
Cs.subochrea	1	238	55.46	0	UF	UF	TTGATGAATACATCCCAT	UF
Cs.longiareolata	1	-	-	1	-	-	-	-
Cx.pipiens s.l.	15	330-356	56.95	0	4	NR	CCCACACACCAACCTGGCTTGG	ITS2database
Cx.torrentium	4	277-281	55.92	0	4	NR	CCCACACACCAACCTGGCTTGG	ITS2database
Cx.modestus	6	344-346	54.66	0	4	NR	CCCACACACCAACCTGGCTTGG	ITS2database
Cx.territans	2	210-211	51.54	0	4	-72.20	CCCACACTCCAGCCTGGCTTGG	MFold
Cq.richiardii	6	315	55.56	0	3	-117.85	nd	MFold
Or.pulcripalpis	1	182	50	0	3	-62	nd	MFold
Total	**226**							

Table 4.7 *ITS2* gene and secondary RNA structure folding information for species of mosquitoes found in GB.

*Free energy values predicted for ITS2 secondary structures without 5.8S and 28S flanking regions. UF = Unsuccessful folding (conserved helices III region estimated from sequence alignment), NR=data not available, nd = no data obtainable.

4.4.7 Hybridisation

It is worth noting several sequence variants found within individual specimens were also found within closely related taxa. One of eight clones from a sample of *Cs. litorea* from Anglesey, Wales, amplified a match to sequences found from *Cs. morsitans* (UK1482). Similarly, two sequence variants extracted from *Ae. cinereus* in Shropshire, England (UK390) clustered to both *Ae. cinereus* and *Ae. geminus*. A specimen of *Oc. cantans* (BM93) from Cheshire, England revealed two sequence variants that included a match to *Oc. flavescens*. Although we have not been able to elucidate the origin of these phantom *ITS2* sequences, no other contamination was recorded except for these examples from only closely related taxa. More research is required to clarify the cause of these phenomena, but they also highlight the possibility of sibling species introgression which is reported in some mosquitoes (Bates, 1939; Choochote et al., 2014; Hanemaaijer et al., 2018; Onyabe and Conn, 1999; Urbanelli et al., 2014).

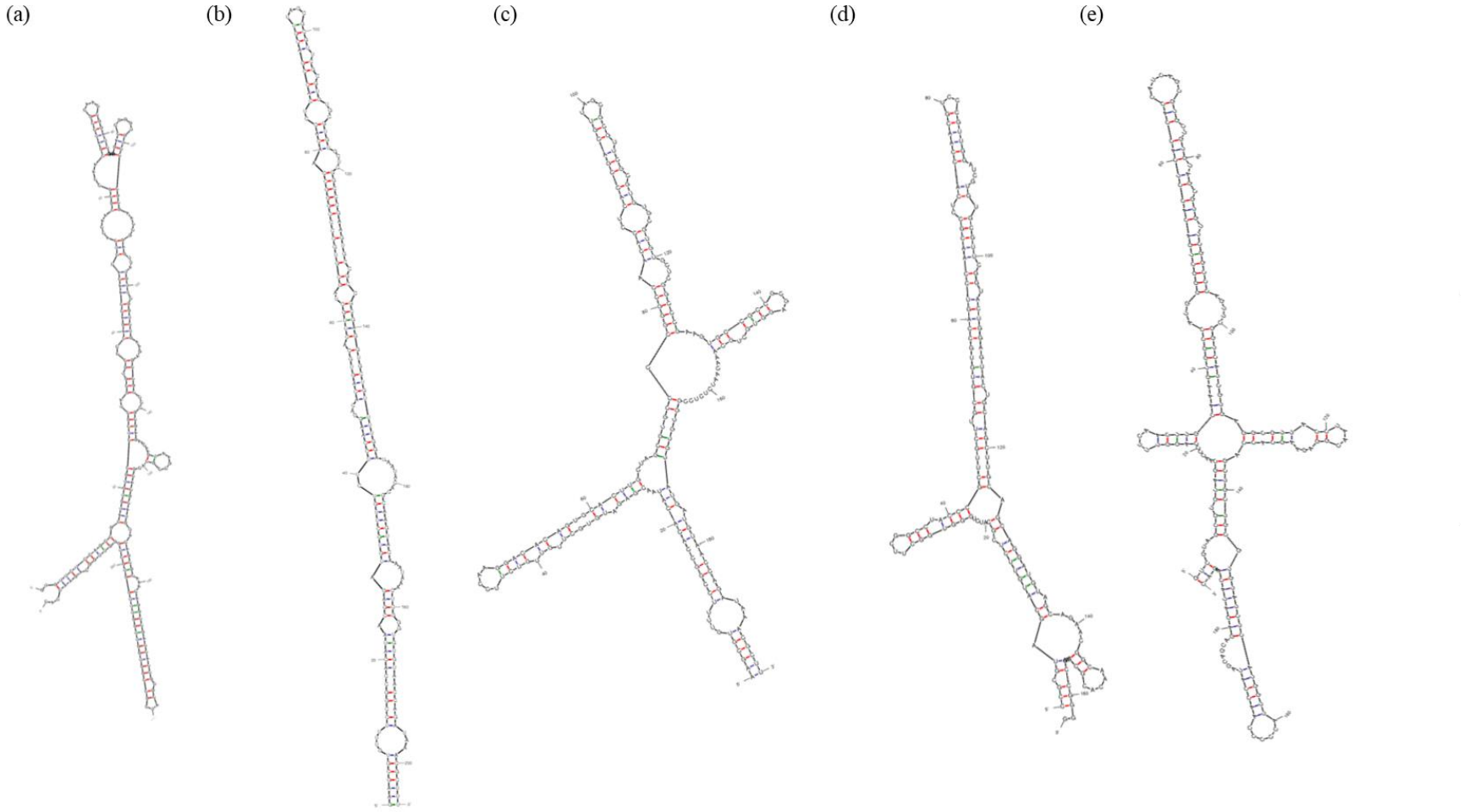


Fig 4.5 Variation found within helix III from the genus: *Anopheles* (a) *An. algeriensis*, (b) *An. claviger* haplotype I, (c) *An. claviger* haplotype II, (d) *An. plumbeus* and (e) *An. atroparvus*.

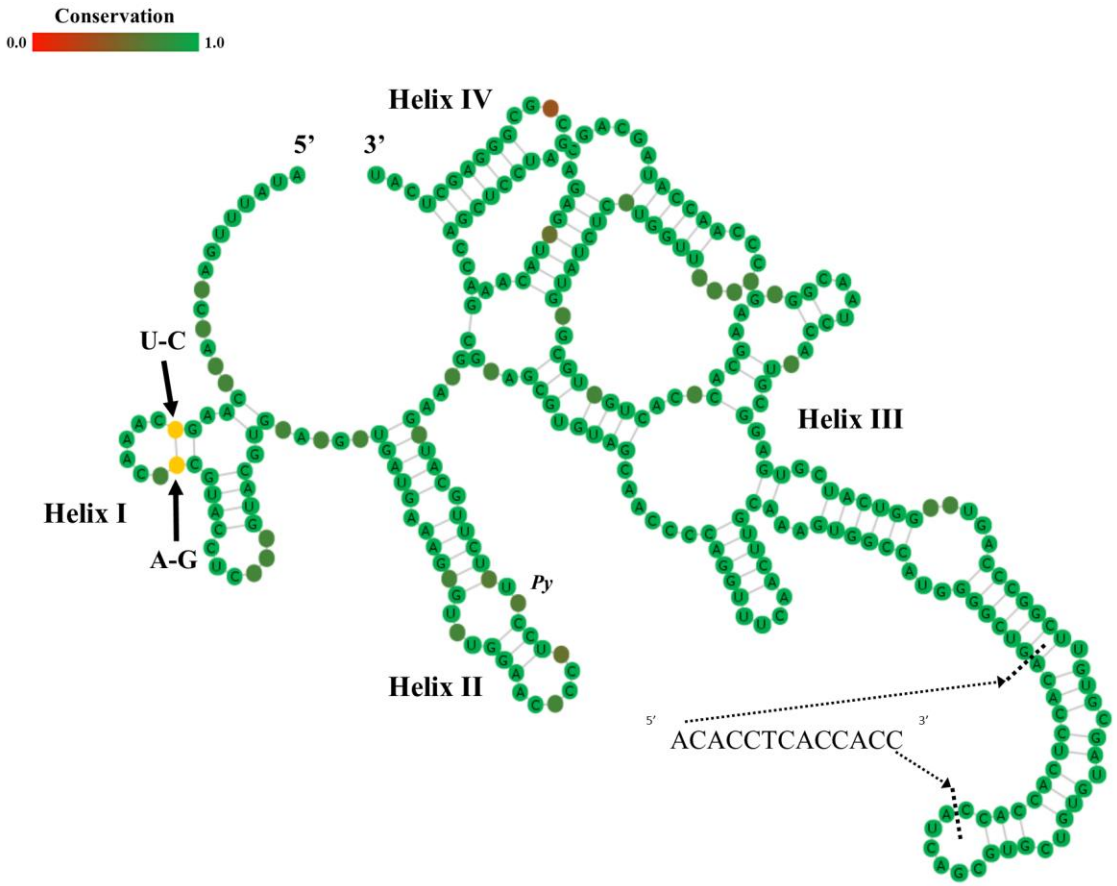


Fig. 4.6 Consensus secondary RNA structure of *ITS2* within the *An.maculipennis* complex.

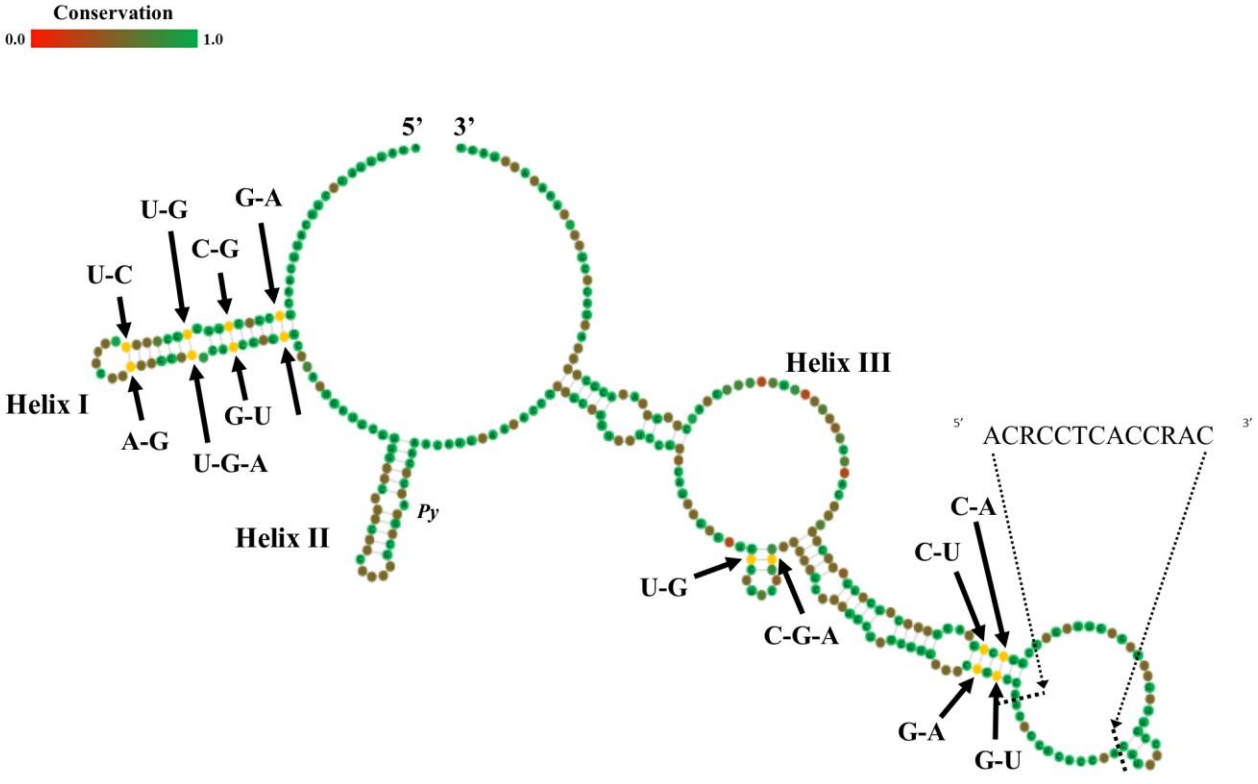


Fig. 4.7 Consensus secondary RNA structure of *An.claviger* and *An.plumbeus.*

CBC's locations are represented in yellow. *Py* = Pyrimidine bulge, typical of *ITS2* secondary structures. Sequence highlighted by dashed lines represent conserved region across genera.

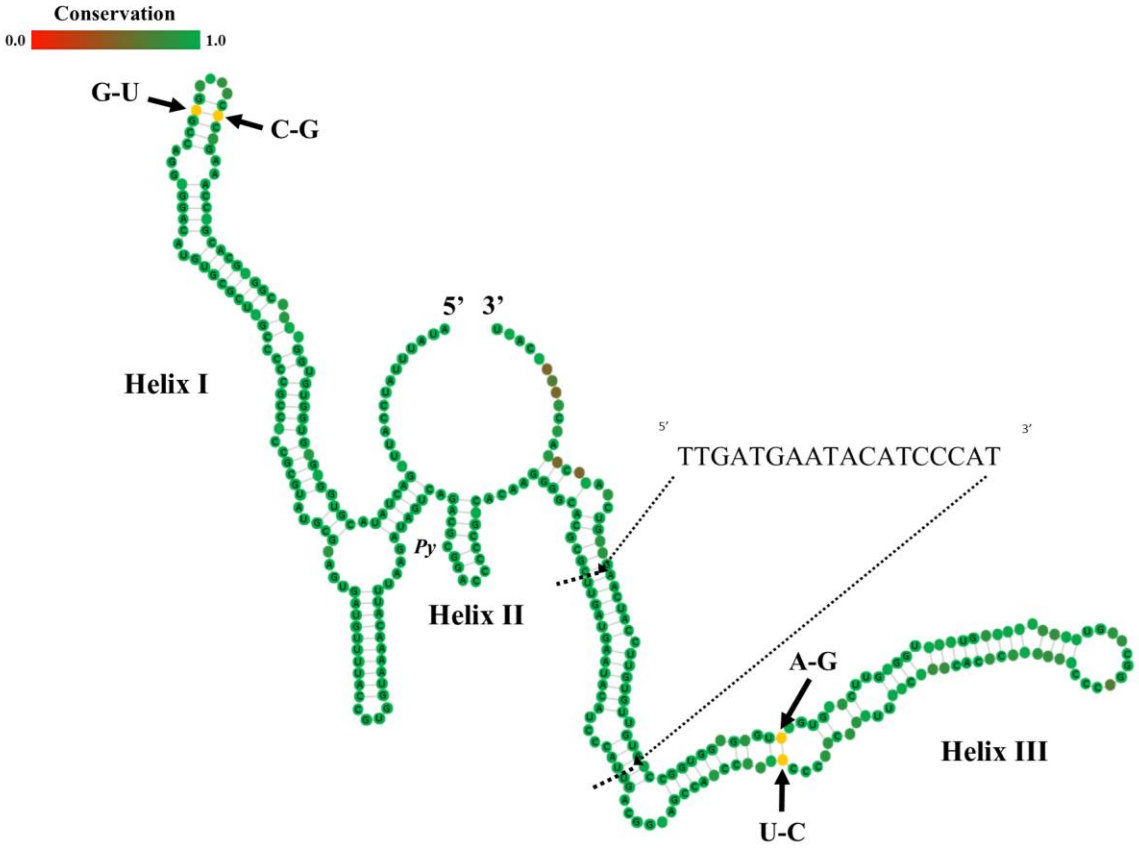


Fig. 4.8 Consensus secondary RNA structure of *ITS2* of *Cs.morsitans* and *Cs.litorea,* including CBC locations.

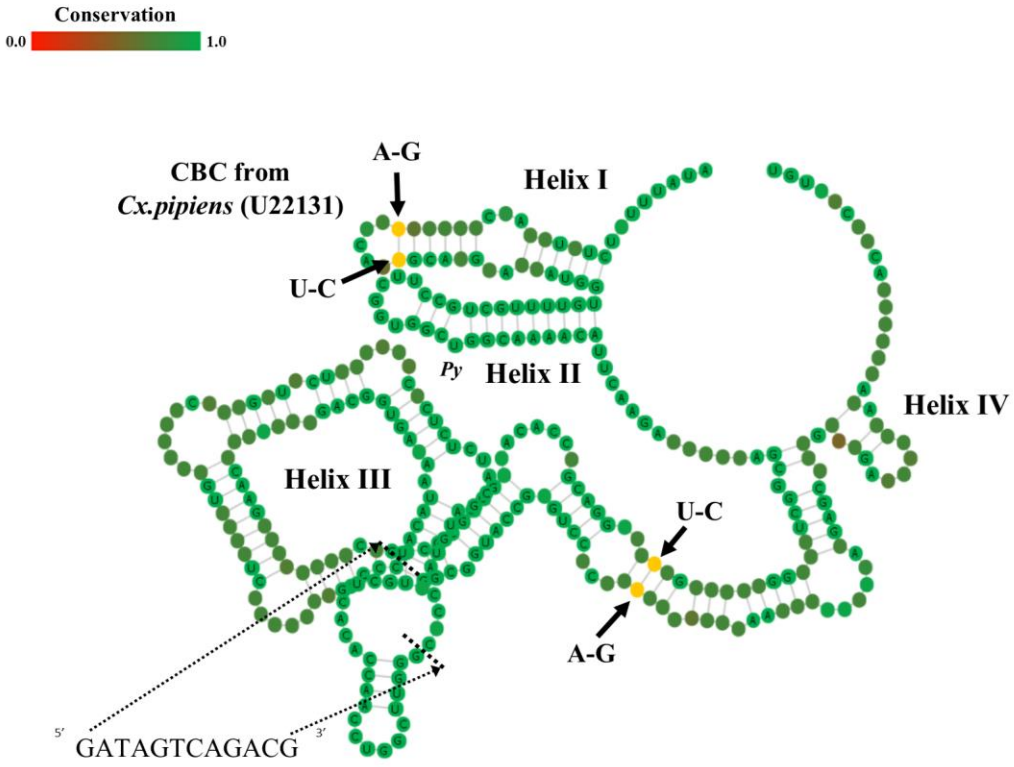


Fig. 4.9 Consensus secondary RNA structure of *ITS2* of *Cx.pipiens* and *Cx.torrentium,* including CBC locations.

CBC's locations are represented in yellow. *Py* = Pyrimidine bulge, typical of *ITS2* secondary structures. Sequence highlighted by dashed lines represent conserved region across genera.

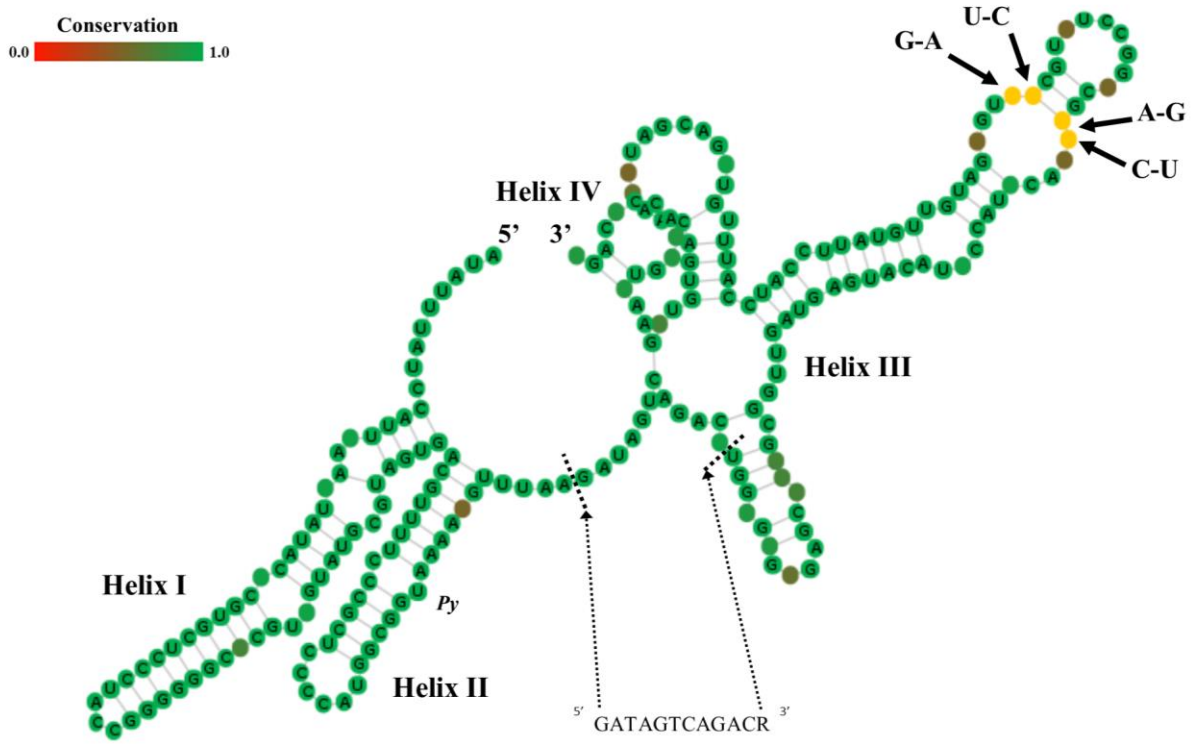


Fig 4.10 Consensus secondary RNA structure of *ITS2* of *Ae.cinereus* and *Ae.geminus,* including CBC locations.

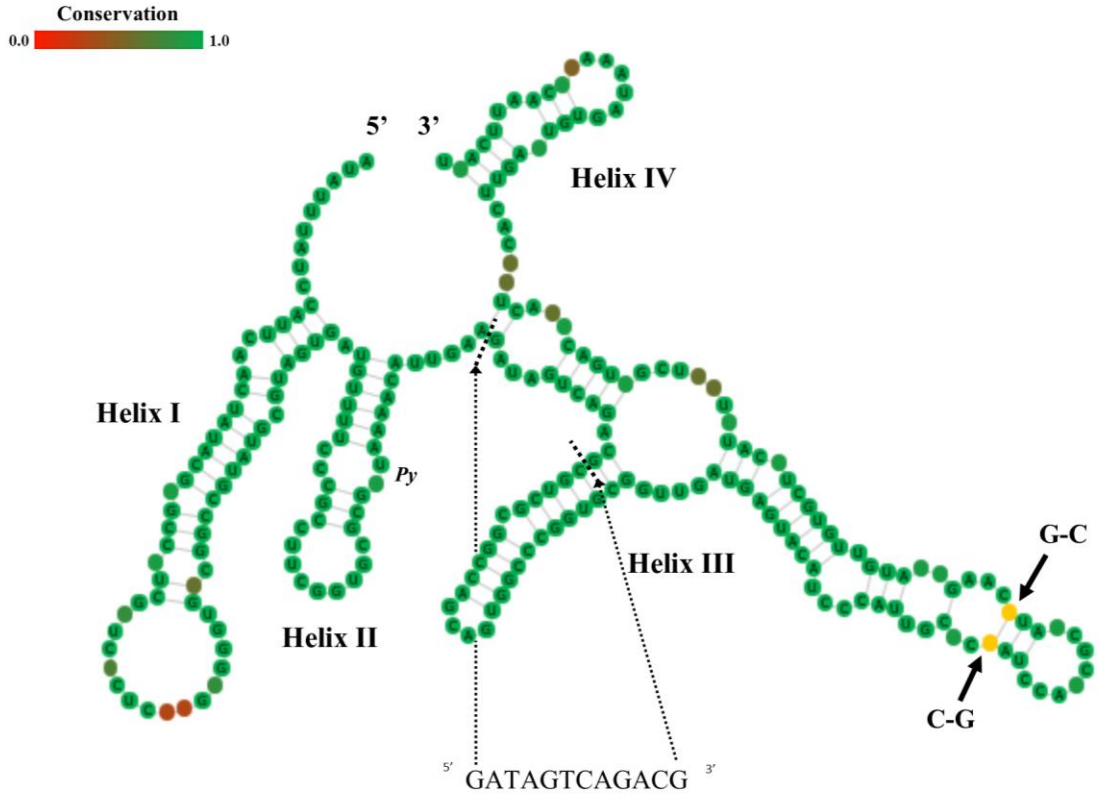


Fig 4.11 Consensus secondary RNA structure of *ITS2* for two distinct *Ae.vexans* genotypes found in Sweden (i) KY614782 (ii) KY614777, this genotype is also found in the within GB.

CBC's locations are represented in yellow. *Py* = Pyrimidine bulge, typical of *ITS2* secondary structures. Sequence highlighted by dashed lines represent conserved region across genera.

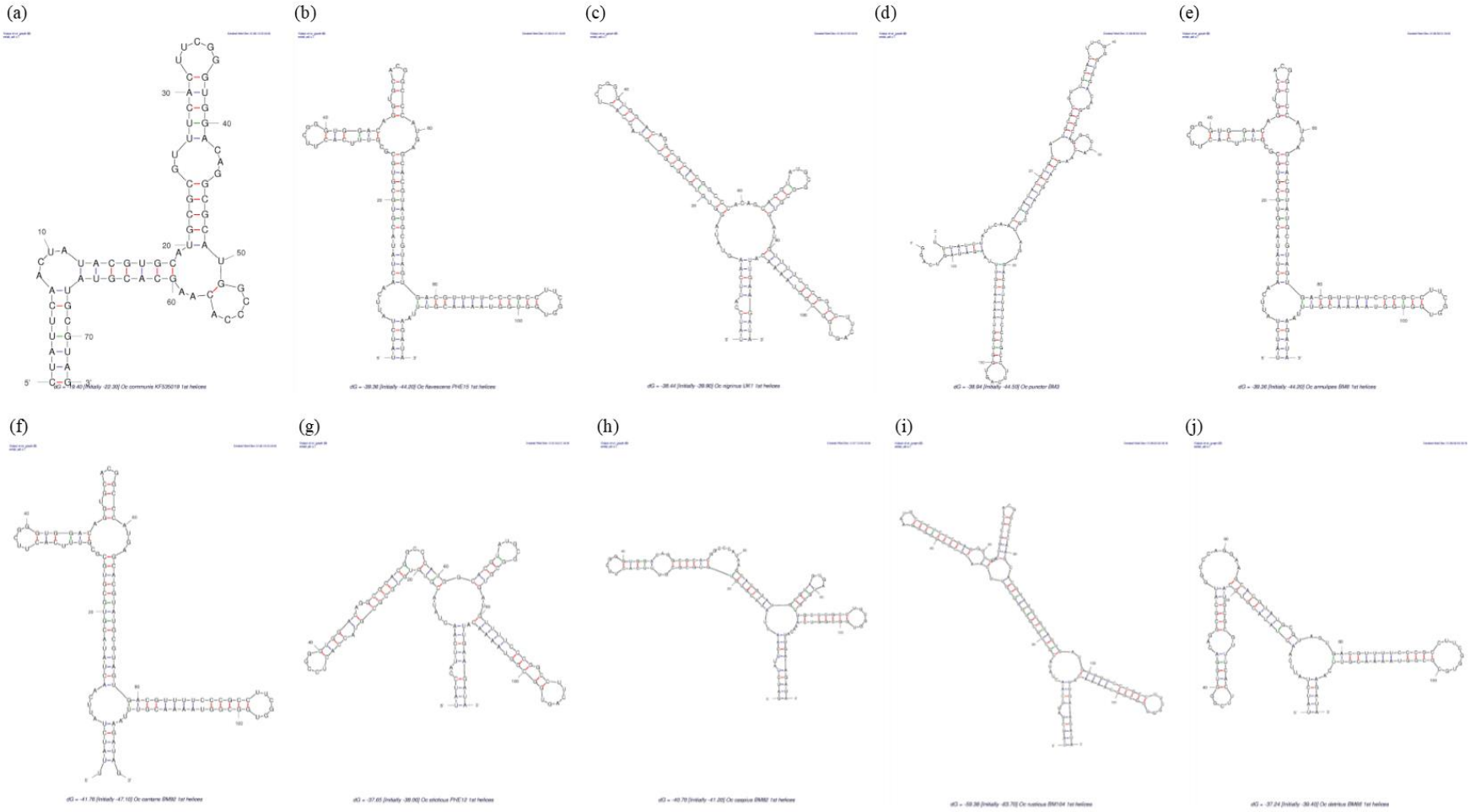


Fig. 4.12 Variation found within helix I of *ITS2* secondary RNA structures for species within the Genera: *Ochlerotatus* a) *Oc. communis* b) *Oc. flavescens* c) *Oc. nigrinus* d) *Oc. punctor* e) *Oc. annulipes* f) *Oc. cantans* g) *Oc. sticticus* h) *Oc. caspius* i) *Oc. rusticus* j) *Oc. detritus.*

4.5 Discussion

The use of a single gene in species identification can lead to ambiguous data, over/underestimated lineages and misclassification (Beebe, 2018). Here we have examined the use of the *ITS2* gene as a complement to *COI* in the identification of GB mosquitoes, along with the inclusion of additional sequences from across their global range. In the context of its application *ITS2* is a 'marmite' gene, loved for its ability to resolve taxonomic problems at the species level, and loathed due to difficulty dealing with indels, homoplasy, amplification issues and intragenomic variation. This study has found that it has practical uses as well as hindrances when assessed using GB mosquito species. The amplification of the *ITS2* gene by direct PCR had relatively low success rates compared to *COI*, problems with microsatellite polymorphism being the main cause of chromatograph problems, along with additional issues with pseudogenes and other intragenomic indel variants. It is important that these issues can be recognised by laboratory users, and those undertaking mosquito surveillance, to minimise wasted time dealing with sequencing errors and quickly picking-out problems caused by genuine genetic artefacts rather than human error. Instances of microsatellite polymorphism can be recognised by observations in the sequencing chromatograph, where multiple peaks, and or, sequence failure occurs after a run of tandem repeats (Fig. 4.2). Intragenomic variation can often manifest itself in a similar form by either a single double peak at the SNP locus, or by the presence of indels, usually without tandem repeating regions (Fig. 4.2). Both problems can only be resolved by cloning of the PCR product, and in some cases by a manual separation of the sequence code if poly-regions cause only two overlapping sequences. A method of identifying specimens

that requires cloning has been developed using a heteroduplex analysis of the *ITS2* PCR product via a native acrylamide gel (Beebe et al., 2001).

The presence of pseudogenes was uncommon across all mosquito species, but still present, and therefore all users of *ITS2* should be checking sequencing results for this phenomenon, prior to analysis. Within this study, 92.3% of all identified pseudogenes were characterised by the lack of hybridisation in the *5.8S* and *28S* flanking regions, and this can be easily checked by using the annotation tool found on the ITS2 database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/).

4.5.1 Indels, do they stay, or do they go now?

The treatment of indels in *ITS2* sequences has been much debated as they can cause problems with alignments and subsequent analysis, however, they can also reveal additional resolution, and their use must therefore be carefully considered prior to analysis (Liu et al., 2012). The presence of indels did cause complications when selecting an appropriate form of analysis, with phylogenetic methods such as maximum likelihood unable to deal with the number of gaps in the dataset, however, alignment was straightforward. Likewise, ABGD analysis and NETWORK analysis were unable to handle excessive amounts of indels. To bypass these problems, a pairwise deletion approach appeared to produce the strongest results when applied to neighbour-joining and *p*-distance methods and was able to utilise 791bp positions, against only 75 bp after complete indel deletion. Problems with species delimitation could easily be overcome dealing with data on a by genus basis where specific conserved regions were found on the 5' of helix III (Table 4.7), and lower overall variance allows for more successful partitioning.

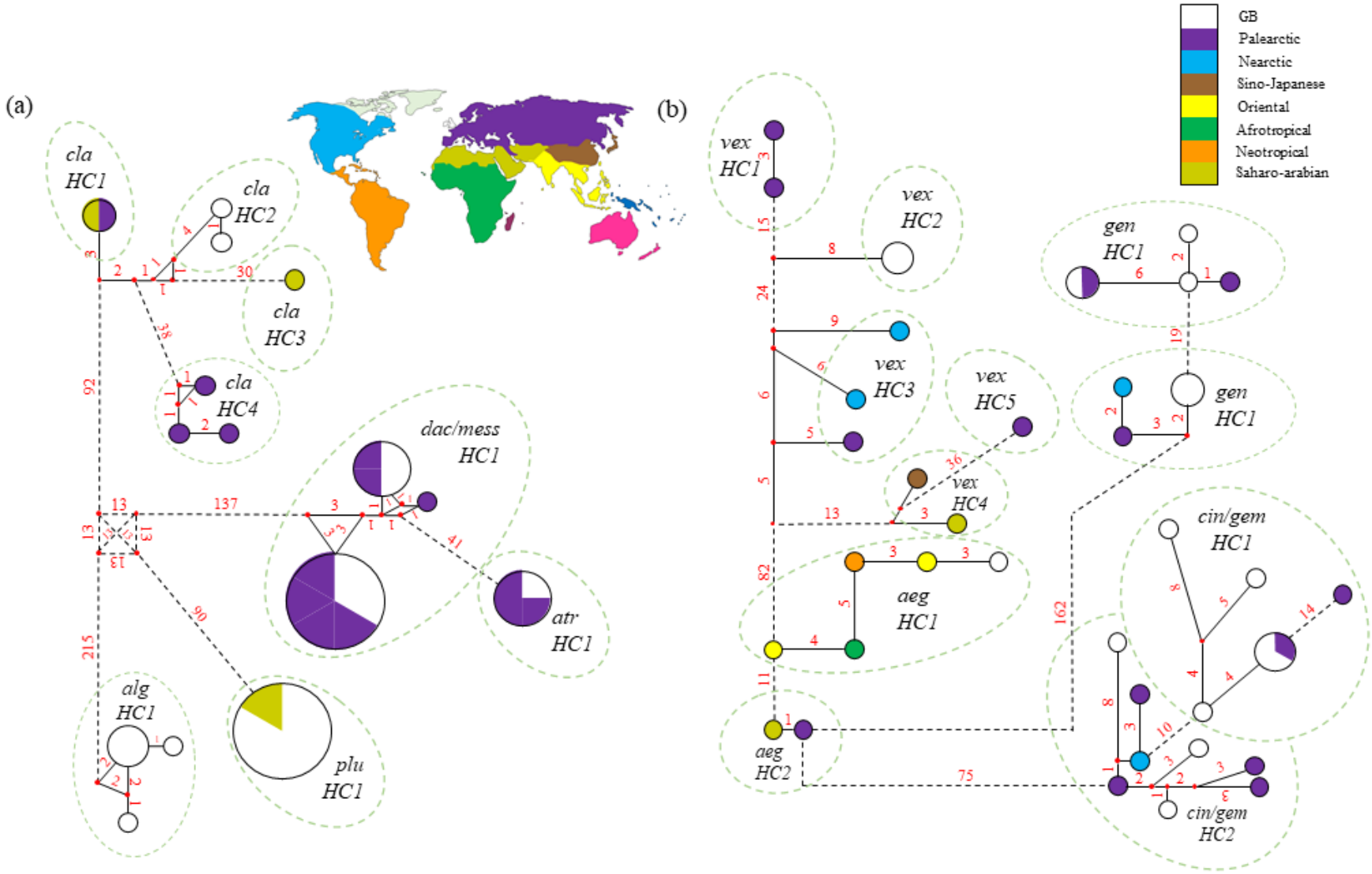


Fig. 4.13 Median joining network analysis of *ITS2* for the genus (a) *Anopheles* and (b) *Aedes and Dhaliana*.

The size of frequency charts is relative to the number of individuals and coloured according to zoogeographic location. Solid lines are proportional to the number of point mutations and dashed lines refer to non-proportional. The number of point mutations are given in red and assigned haploclades within green chequered areas.

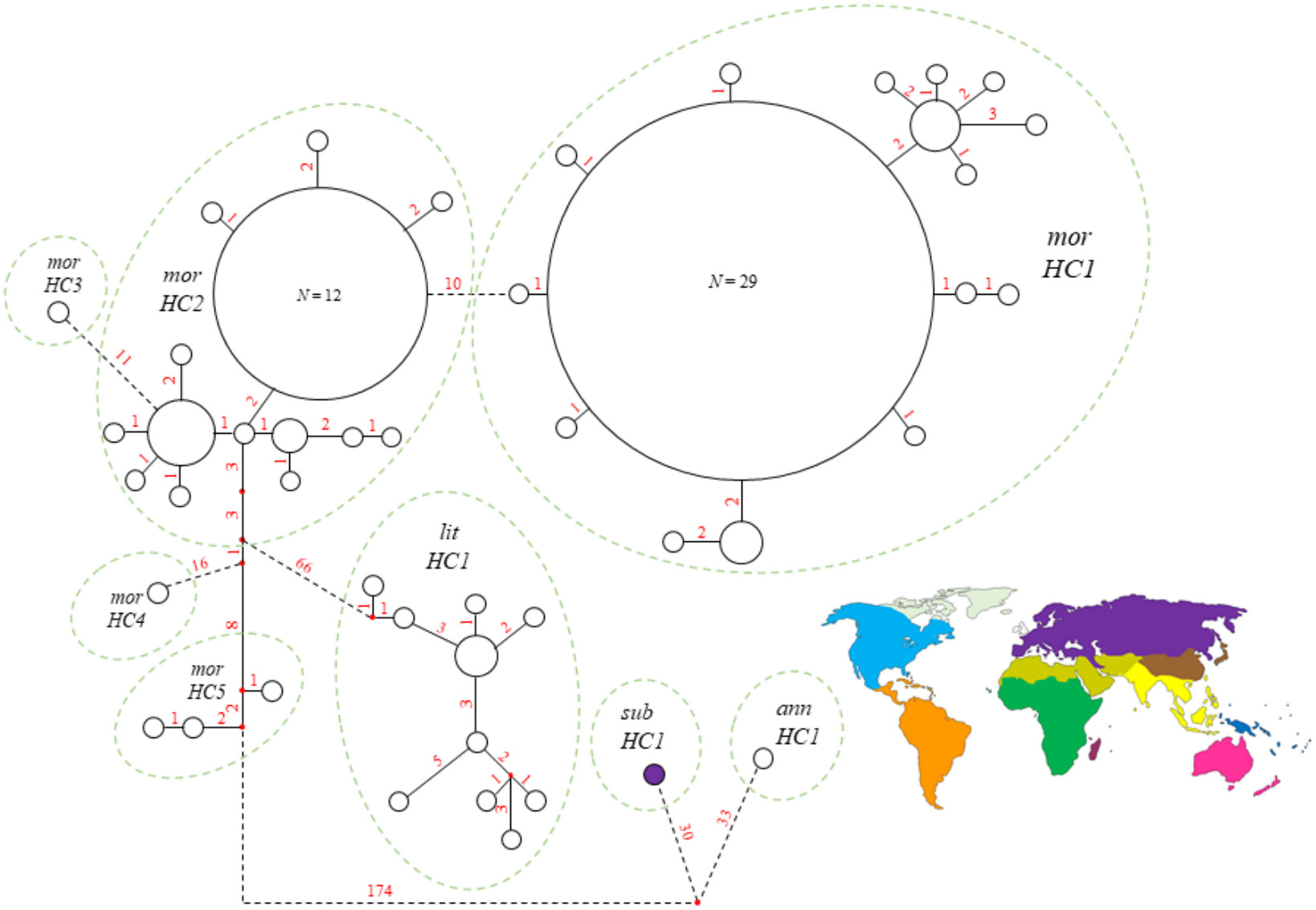


Fig. 4.14 Median joining network analysis of *ITS2* for the genus *Culiseta.*

The size of frequency charts is relative to the number of individuals and coloured according to zoogeographic location. Solid lines are proportional to the number of point mutations and dashed lines refer to non-proportional. The number of point mutations are given in red and assigned haploclades within green chequered areas.

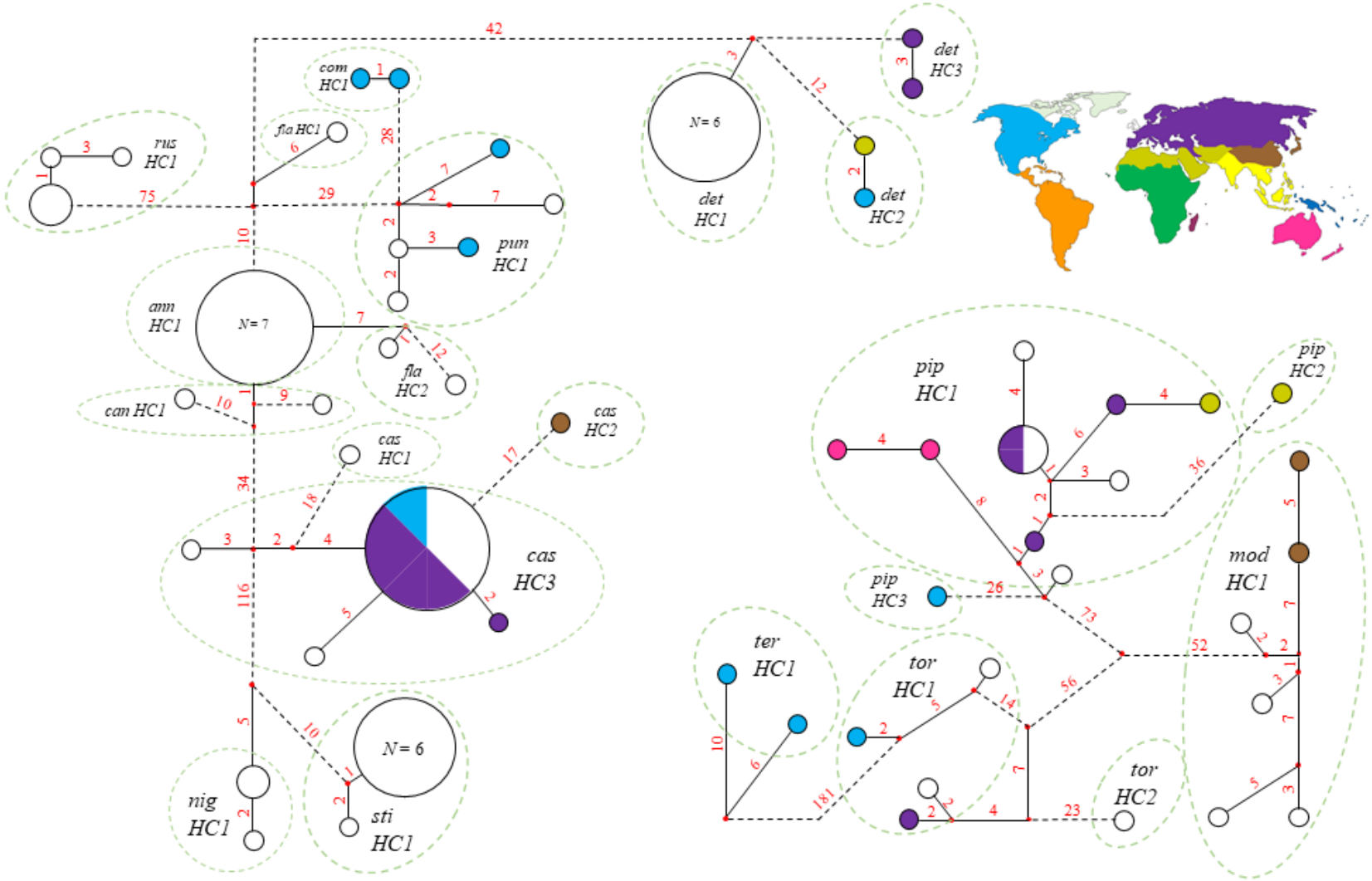


Fig. 4.15 Median joining network analysis of *ITS2* for the genus (a) *Ochlerotatus* and (b) *Culex.*

The size of frequency charts is relative to the number of individuals and coloured according to zoogeographic location. Solid lines are proportional to the number of point mutations and dashed lines refer to non-proportional. The number of point mutations are given in red and assigned haploclades within green chequered areas.

4.5.2 *ITS2* folding and CBC's as useful species indicators

The folding of *ITS2* sequences into their complimentary RNA structures allows for an additional approach to understanding where useful information is situated within the gene, and for indicators of possible sexual isolation via CBC locations (Müller et al., 2007). The folding of *ITS2* secondary structures has been attempted before for different groups of mosquitoes, in *Orthopodomyia* (Byrd et al., 2012), *Culex* (Severini et al., 1996), early (and incorrect) attempts on *Aedes* (Wesson et al., 1992), and most frequently for *Anopheles* (Banerjee et al., 2007a; Beebe et al., 1999; Dassanayake et al., 2008; Zomuanpuii et al., 2013). Of these, only Wesson et al (1992) have attempted to test the usefulness of CBC locations. At first glance this method appears to be effective, however, the folding of secondary structures during this study was prior to the Coleman model, and as such not comparable to the dataset we have produced here. Indeed, many of the early attempts to describe these structures (pre 2007) do not meet the assumption required for functional *ITS2* molecules (Coleman, 2007). Here we have attempted to do both and have found that variation in the structure of the secondary RNA can vary interspecifically, intraspecifically, as a well as intraindividually, by length and complexity of the first and third helices (Fig. 4.5 & 4.12), and by the presence/absence of a fourth proto-helix. However, these differences are likely explained by rapidly evolving indels and nucleotide repeats. The presence of CBCs at the species level, when compared to other methods of species delimitation, appeared to be indicative in some groups, and less so in others such as those found in *Culex*, some closely related *Ochlerotatus*, and *Ae. albopictus.* Previous studies of CBCs suggest the absence of CBCs cannot be used to indicate a lack of sexual isolation between species, but their presence is indicative of isolation with ~93% accuracy (Müller et al., 2007). However, some studies have found discrepancies between *ITS2* evolution and the CBC clade concept

(Caisová et al., 2011). Therefore, the application of CBCs as a tool for species delimitation should be taken cautiously when applied on its own, but in the context of other analytical tools and additional reference genes, could provide insightful information.

4.5.3 Gene length separation

Some species complexes that are difficult to separate morphologically appear to be separable using *ITS2* fragment length alone, most notably, *Cs. morsitans* (330-347bp) and *Cs. litorea* (358-363bp), *Cx. pipiens* (330-356bp) and *Cx. torrentium* (277-281bp). The separation of the AIM's *Ae. aegypti* (196-206) and *Ae. albopictus* (378-403) from the native container breeding species *Da. geniculata* (324-331) is also possible. Therefore, preliminary separations of these groups can be made by visualisation of the PCR product using gel electrophoresis, resulting in early confirmation within 3 to 4 hours. However, due to the highly polymorphic nature of these genes, confirmation by sequencing of barcoding genes would be recommended. Amplicon size information for each species are summarised in Table

4.5.4 The practicalities of using *ITS2* in surveillance

Given that amplification success was low compared to the use *COI*'s (see Appendix I for comparisons) it is reasonable to question whether the efficiency of this marker is appropriate for mosquito surveillance where accurate identification could be required for very low numbers of collected individuals. Such an example is summarised within Chapter 5, where only a single specimen of the invasive species *Ae.aegypti* was discovered. In these instances, the use of *ITS2* on its own would

likely be insufficiently reliable for surveillance as sequencing was only successful half of the time. The identification to species, however, has proven to be efficient and therefore testing against a second barcoding gene may render this marker as a useful tool to reassure results of more reliable markers, such as *COI* .

4.5.5 *ITS2* in GB mosquito species

4.5.5.1 *Anopheles*

The analysis of *ITS2* from specimens of the *An. maculipennis* group was undertaken by Nicolescu et al (2004) leading to a controversial taxonomic revision (Bezzhonova and Goryacheva, 2008) and the addition of the species *An. daciae* from *An. messeae*, based on genetic differentiation alone. Here we included some of the sequences defined as each species using the genetic differences described by Nicolescu (5 x *An. daciae*, and 5 x *An. messeae* (Genbank references included in Table 4.1)) alongside to assess whether species delimitation can support the various species partitioning approaches. Five fixed variable sites accounting for 1.03% difference in *ITS2* were the determinant factor for species separation, however, we find no secondary RNA structural alterations that support the addition of *An. daciae* as a unique taxonomic unit. No CBC locations were found between the *An. daciae* and *An. messeae*. Only *An. atroparvus* within the *An. maculipennis s.l.* group was separated by the presence of a single (UA-CG) CBC mutation on helices I. Species delimitation was also not supported by PTP or ABGD analysis, and *p*-distance values between these species (0.007) was below the range expected for intragenomic variation (0.027 mean across all Culicidae; 0.0385 mean across all *Anopheles* (Table 4.5)). Phylogenetic reconstruction did form bootstrap supported (bs = 99 – 100) monophylogenies, however, this was not backed up by MJN which separated these species by only a

single mutation. These findings therefore support the additional *ITS2* analysis carried out by Bezzhonova and Goryacheva (2008) as resolving *An. daciae* as a likley variant within the *An. messeae* species, and not as a unique toxonomic unit.

Two/three distinct haplotype groups were found to exist within *An. claviger*, which demostrated very different helix III structures and one haplotype exhibiting a fourth proto-helix. However, despite these different structures, no CBC differences were observed within the species, but the delimitations were supported by ABGD and PTP anlaysis (Fig 7.1). *An. claviger* has been considered part of a complex along with a sibling species *An. petragnani* (Schaffner et al., 2000). Isozyme analysis of *An. claviger s.s.* has also recorded two distinct haplotypes relating to Western, and a second in Eastern and Northern Europe (Schaffner et al., 2003). Analysis of *ITS2*, supported by *COI*, suggest the possibility of more distinct haplotype groups within this complex. However, more samples with greater species coverage are required to confirm this.

4.5.5.2 *Culex*

Specimens of *Cx. torrentium* and those of the *Cx. pipiens s.l.* complex can be difficult to separate morphologically, requiring genitalia dissection, and or, intact larvae, for species confirmation. Both are vectors of SINV in Europe, although the level of competence is variable with *Cx. torrentium* considered the most efficient (Lundström et al., 1990). Additionally, *Cx. pipiens s.l.* may have significantly contributed to outbreaks of WNV in the USA (Hamer et al., 2008), and possibly Europe (Rizzoli et al., 2015). Recent susceptibility tests suggest that *Cx. torrentium* may be more permissive for the disease than *Cx. pipiens s.l.* (Leggewie et al., 2016). Both *Cx. pipiens s.l.* and *Cx. torrentium* can be sympatric (Smith and Fonseca,

2004), and it is likely that difficulties in identification of females, which are sampled more readily than males, has resulted in unreliable species distribution data. To combat this problem, several molecular methods have been devised to split them (Danabalan et al., 2012; Weitzel et al., 2011; Zittra et al., 2016). Here we also found the division between these species groups to be robust. Phylogenetic analysis, ABGD, and PTP gave distinct species separation between the two, however, MJN analysis (and to an extent PTP) showed higher levels of genetic variation within the *ITS2* of these species. *Cx. pipiens s.l.* has been broken down into seven often indistinguishable species, based on behavioural and feeding preference differences. In Europe these include, the two forms *Cx. pipiens f. pipiens* and *Cx. pipiens f. molestus*, and *Cx. pipiens f. quinquefasciatus* (Becker et al., 2012). Genetic variation found here could account for these groupings but were not confirmed by species definitions in repository data. We have not attempted to make any judgement on the position of those within *Cx. pipiens s.l.*, as stated above, this group has been well studied with more robust datasets than used here. The data presented here does not contribute anything additional to this discussion.

Secondary RNA structures for the Genus *Culex* showed some structural variation, with *Cx. pipiens s.l.* and *Cx. torrentium* presenting four helices, whereas *Cx. modestus* and *Cx. territans* presented three. However, CBC analyses were inconclusive between *Cx. pipiens*, *Cx. modestus* and *Cx. territans*, and only a single CBC between *Cx. torrentium* against all others was present on helices I and III. As all other methods of species delimitation support the separation of these species, it is reasonable to presume that CBC's are not an appropriate gauge of species differentiation within this genus.

4.5.5.3 *Orthopodomyia/ Coquillettidia*

Only a single reference gene was available for *Or. pulcripalpis* from GB (FJ867653). Secondary structure folding did not match that of Byrd et al (2012), and only produced three helices, and not the four as previously described. The folding of *ITS2* secondary structure we present here using MFOLD, conforms to the biological folding paradigms, whilst still conforming to the universal model with the conserved GARTACATCC region on the 5' of helix III (as described by Bryd et al (2012)) and the helix II pyrimidine bulge. However, we suggest that a greater number of *Or. pulcripalpis* samples from across the species distribution are required for a taxonomic review of this group, and to validate the secondary RNA structure.

All specimens of *Cq. richiardii* analysed were collected from GB and showed no genetic variation of the *ITS2* gene across all specimens. Three helices were recorded for this species with complex structures in both the first and third.

4.5.5.4 *Culiseta*

Out of all mosquito complexes, those found in the subgenus Culicella are probably the least known and currently includes three species, *Cs. morsitans*, *Cs. litorea*, and *Cs. fumipennis.* Of these mosquitoes *Cs. moristans* is a reported vector of SINV in Northern Europe (Bergqvist et al., 2015) and classified as a risk level 5 by the ECDC (Table 1.1, Chapter 1), and recorded as feeding on multiple species groups, particularly birds and some mammals including humans (Service, 1994). Females of this complex can be difficult to identify due to overlapping phenotypic variation. For this reason and because of difficulties in sequencing *COI* from this group (Chapter 3), an attempt was made to sequence more specimens for this group than any other (*n*

= 77) Morphological identification using the keys of Becker et al (2010) and Cranston et al (1987) using male genitalia, suggested that only *Cs. litorea* and *Cs. morsitans* had been collected. *Culiseta morsitans* was by far the most common but was also found in sympatry with *Cs. litorea* at Cors Eddreiniog National Nature Reserve, Anglesey (Wales). The division of these two species was backed by ABGD analysis, however, PTP and phylogenetic analysis predicted two hypothetical species groups within *Cs. morsitans.* An MJN approach revealed a complex series of haplotype networks, some of which are likely to be caused by intragenomic variation detected in the sequence data. One to two CBC locations were detected between these two species groups, but none were found within *Cs. morsitans* identified specimens. The dataset analysed using these methods also included multiple sequences from individuals that represent intraindividual variation. Many of these appear across identified MJN haploclades and so it is likely that the high levels of variation seen in *Cs. morsitans* can be explained by intraindividual differences within *ITS2*. The only exception here is that of from specimen UK1482, collected from coastal wetlands on Anglesey, Wales (confirmed by genitalia identification as *Cs. litorea*), was found to contain the *ITS2* sequences from both *Cs. litorea* and *Cs. morsitans* within its genome. It is possible that this phenomenon could be a product of past introgression, however, more evidence would be needed to verify this observation. Given the vector potential of this species group, and the haplotype numbers observed in *ITS2* from GB specimens, it is important that more is done with this group to fully understand the causes of the observed genetic variation using additional genes, or NGS.

4.5.5.5 *Aedes / Dahliana / Ochlerotatus*

Mosquitoes from these genera are arguably the most important for GB surveillance. In particularly the globally distributed disease vectors *Ae. albopictus* and *Ae. aegypti*. CBCs appear to support species delimitation within the genus, with notable CBC locations supporting the *Ae. cinereus/geminus* separation suggested by Peus (1972). Two distinct *Ae. vexans* partitions based on CBC locations (KY614782 from Sweden, distinct from all others) was also observed. PTP analysis also supported delimitation within these species groups, however, barcode gap analysis did not conform to these results.

Data from the *Ochlerotatus* genus did not show any intraspecific CBC locations, however, results for interspecific differences was mixed. Although these locations appear to be useful for the reinforcement of species delimitation, the lack of CBCs between some suggests a cautious approach when applied to this genus. Despite a bimodal distribution of intra vs. intergenomic variation across specimens from the genus *Ochlerotatus*, some overlap occurs within the dataset, a feature also noted within the *COI* gene (Fig. 3.4, Chapter 3). ABGD analysis does not differentiate *Oc. annulipes, Oc. cantans* and *Oc. flavescens*, however, the latter is differentiated by PTP and MJN analysis. ABGD also clusters *Oc. communis* from *Oc.punctor* but suggests additional distinct groups within *Oc. caspius* and *Oc. sticticus*. *Oc. punctor* and *Oc. communis* are differentiated by PTP and MJN, with both methods also indicating possible haplotype groupings within *Oc. caspius* and *Oc. sticticus* (Table 7.1).

Observation from phylogenetic analysis suggests that two intraindividual sequence reads (spec. PHE17a + b) display types from both *Oc. flavescens* and from *Oc. annulipes/cantans*. No contamination was detected during *COI* analysis of this specimen suggesting that this is a genuine genetic artefact. Interestingly, CBCs exist

between some specimens of these species and none between others suggesting that the possibility of hybridisation events between these species should not be ruled out.

As with *COI* analysis, it appears that *ITS2* is not sufficient to separate the *Oc. annulipes/cantans* group as supported by ABGD and maximum likelihood bootstrap values (bs = 55). More research is needed to determine whether these are genuine distinct species, or subspecies within the same complex, as data presented here gives no molecular evidence to support a split, and no further information was located on their reproductive isolation.

Despite support from high bootstrap values (bs = 98) and ABGD/PTP partitioning, *Oc. sticticus/nigrinus* show no CBC locations. A similar effect was also found between *ITS2* sequences of *Oc. communis* and *Oc. detritus*. However, it is likely that CBCs are not an appropriate indicator for the division of these species.

4.6 Conclusion

A single gene approach to barcoding and resolving species has become frequent, and this has often been the case for regional efforts to characterise mosquitoes, where there has been a preference for the sole use of mitochondrial *COI* (Chan et al., 2014; Engdahl et al., 2014; Hernández-Triana et al., 2019; Versteirt et al., 2015). Despite its common use, genes such as the nuclear *ITS2* are becoming less popular within these large-scale barcoding projects but remain in use for the development of rapid PCR based identification systems (Das et al., 2012; Higa et al., 2010). Historical use of *ITS2* in mosquitoes has been mostly applied to those of the *Anopheles* genus, and is underused, and therefore undertested, in other species groups. The use of *ITS2* is a double-edged sword, with a highly polymorphic nature that is both a help and a

hindrance, particularly when using direct PCR sequencing methods. In the surveillance of potential disease vectors, rapid methods of species identification are paramount, and therefore direct PCR should be preferred over traditional cloning methods as a complete sequence can be obtained in only several hours. An essential quality when fast response is an essential requirement, for example when an AIM is suspected, rapid confirmation is required to allow for effective control. This makes *ITS2* additionally problematic, as compared to *COI* which has 80% sequencing success in GB (including IMS) mosquitoes, *ITS2* chromatograph collapse occurs 48.2% of the time. Although not without difficulties in practical application, the positives of this gene are not to be ignored. *ITS2* evolves at a rate close to neutral and is efficient in delimitation of species. Additionally, current methods available for the folding of the secondary RNA structures allow for an additional level of analysis to aid in species delimitation by way of CBC locations. A feature that is not available in *COI* analysis. The inclusion of nuclear markers can also aid in the identification of possible introgression between species, the prevalence and implications of which is not well studied in the natural environment. The results of this study suggest that *ITS2* has a place in GB surveillance strategies and could provide significant insight into species taxonomy. Due to a relatively low amplification success rate, we suggest that *ITS2* sequence data (as standard barcoding) be used as a compliment in the identification of species, alongside that of *COI*. The use of CBC locations as a tool for the identification of species could be further expanded, however, vagaries about its efficiency in the *Culex* and *Ochlerotatus* genera suggest that its use would ultimately be restrictive. As relatively little is known about introgression in wildtype mosquitoes, CBCs may have more use in introgression based studies, which may be better placed to confirm the informative power of this feature.

Additional findings from the study of *ITS2* reflect those from *COI* in GB mosquitoes. Our preliminary characterisation of species by analysis of barcoding genes, complemented by data from across their species ranges, suggests that a closer investigation with a broader subset of samples from many species groups is required to accurately disseminate taxa from across broader geographical ranges, as well as in the GB.

CHAPTER FIVE:

Discovery of a single male *Aedes aegypti* (L.) in Merseyside, England and post discovery surveillance

Published in Parasites and Vectors:

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5.1 Abstract

The mosquito *Aedes aegypti* (L.) is found in tropical and sub-tropical regions where it is the major vector of dengue fever, yellow fever, chikungunya and more recently Zika virus. Given its importance as a vector of arboviruses and its propensity to be transported to new regions, the European Centre for Disease Prevention and Control (ECDC) has placed *Ae. aegypti* on a list of potentially invasive mosquito species. It was previously reported in Great Britain (GB) in 1865 and 1919 but did not establish on either occasion. It is now beginning to reappear in European countries and has been recorded in the Netherlands (not established) and Madeira (Portugal), as well as southern Russia, Georgia and Turkey.

During summer 2014, a single male *Ae. aegypti* was captured during mosquito collections in north-western England using a sweep net. Morphological identification complemented by sequencing of the *ITS2* rDNA, and *COI* mtDNA regions, confirmed the species. Following confirmation, a programme of targeted surveillance was implemented around the collection site by first identifying potential larval habitats in greenhouses, a cemetery, a farm and industrial units. Despite intensive surveillance around the location, no other *Ae. aegypti* specimens were collected using a combination of sweep netting, larval dipping, Mosquito Magnets®, BG-sentinel traps and ovitraps. All species collected were native to GB.

The finding of the single male *Ae. aegypti*, while significant, presents no apparent disease risk to public health, and the follow-up survey suggests that there was no established population. However, this report does highlight the need for vigilance

and robust surveillance, and the requirement for procedures to be in place to investigate such findings.

5.2 Background

The mosquito *Aedes* (*Stegomyia*) *aegypti* (L.) is found in tropical and subtropical regions where it is the primary vector of arboviruses, such as dengue (DENV), chikungunya (CHIKV) and yellow fever (YELV). This species is also a vector of Zika virus (ZIKV) and is currently responsible for widespread cases throughout the Americas (Galindo-Fraga et al., 2015). The immature stages of the ancestral form *Ae. aegypti formosus* develop in natural containers (e.g. tree holes, bamboo internodes and leaf axils) but the internationally occurring form *Ae. aegypti aegypti* has adapted its habitat preferences to exploit human-made containers such as water storage tanks, discarded tyres and jars, and water-filled pots. Consequently, it is found near human dwellings making it a particularly effective vector of human diseases. This adaptation to artificial containers, coupled with the ability of *Aedes* eggs to withstand prolonged periods of desiccation, has led to its invasion of new territories globally.

Increasing urbanisation and globalisation, including international trade, have been implicated in the passive dispersal of *Aedes* invasive mosquitoes (AIMs) such as *Ae. aegypti,* and to a greater extent *Ae. albopictus*. The international trade in used tyres, lucky bamboo and wet-footed plants have all been implicated in the movement of AIMs between countries and continents. *Aedes aegypti* introductions into the Netherlands, for instance, was via the importation of used tyres from Miami, Florida (Brown et al., 2011).

The European Centre for Disease Prevention and Control (ECDC) considers AIMs a serious public health threat to Europe and has produced guidelines for the

surveillance of such species (ECDC, 2012). While *Ae. albopictus* remains the most prolific AIM in Europe, having greatly expanded its range across 28 countries, the geographical extent of *Ae. aegypti* in Europe is much more limited. Historically, *Ae. aegypti* occurred widely throughout the Mediterranean but largely died out in the post-WW2 period (Schaffner and Mathis, 2014). However, it has begun to re-colonise parts of southern and south-eastern Europe with populations found in Madeira (Portugal) and the Black Sea coast of Russia, Georgia and more recently Turkey (Kraemer et al., 2015; Medlock et al., 2015) (Fig. 5.1). Unlike *Ae. albopictus*, which has adapted to cooler climates by entering winter diapause, *Ae. aegypti* has not become established in northern Europe. It has never been recorded as established further north than 44°30′N latitude and its distribution is limited to areas with a January isotherm of 10 °C and mean annual temperatures of 15 °C, making northern Europe including the GB, inimical for their survival (Schaffner and Mathis, 2014). The species was responsible for an outbreak of YELV in Swansea, Wales, in 1865 where *Ae. aegypti*, introduced via shipping, were reported to transmit the virus from infected sailors to the local population. The mosquitoes were not recorded as having survived the winter (Surtees et al., 1971).

Within GB, AIMs surveillance includes both passive and active operations (Vaux and Medlock, 2015). Passive surveillance has involved the collection of existing and historical data on mosquito distribution, as well as an identification service for mosquitoes collected by entomologists, academics, environmental health officers and members of the public (e.g. the Mosquito Recording Scheme and Mosquito Watch) (Kampen et al., 2015; Medlock et al., 2012; Vaux and Medlock, 2015). Active surveillance includes deploying traps and performing larval sampling at strategic sites such as seaports and airports, used tyre import yards, and motorway service

stations close to southern ferry ports and the Eurotunnel. Prior to this finding, there had been no reports of AIMs *via* either passive or active surveillance.

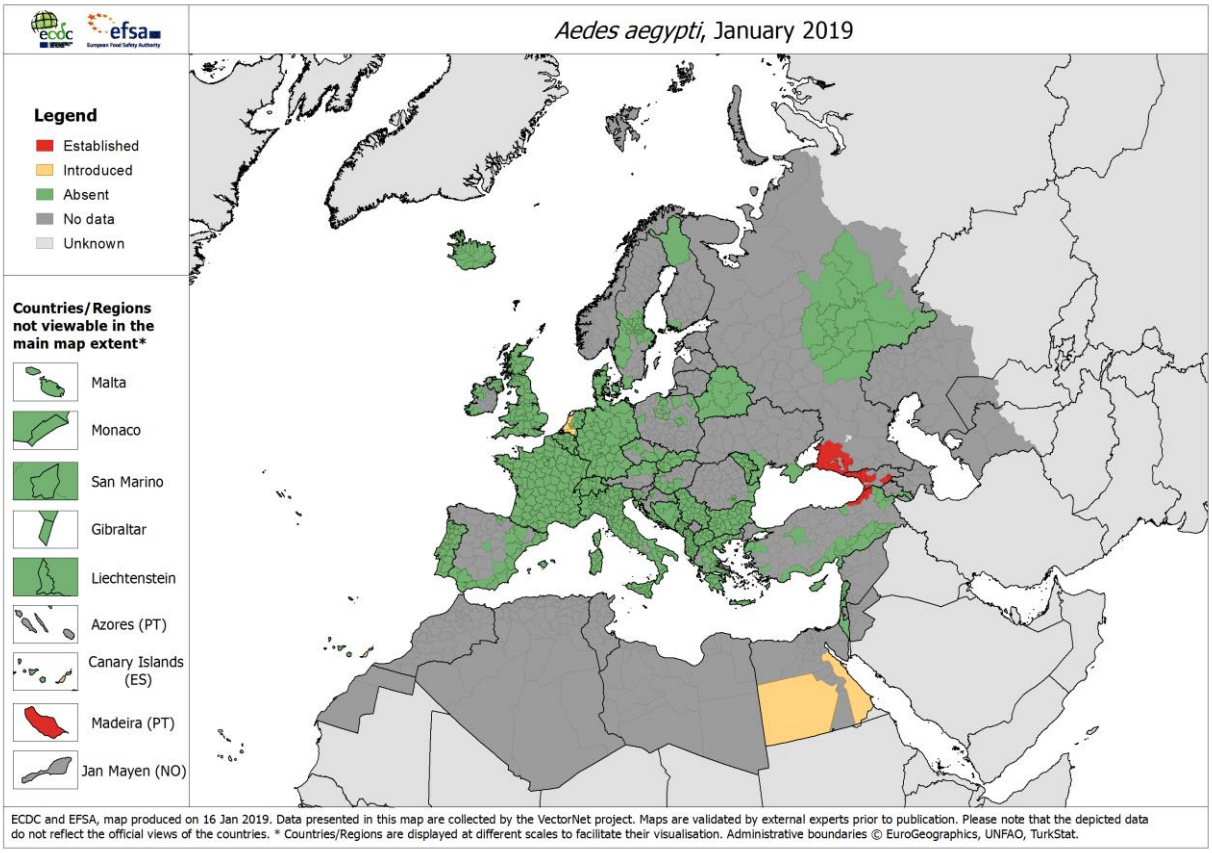

Fig. 5.1 Current known European distribution of *Aedes aegypti*, January 2019.

(Source: ECDC-EFSA/VECTORNET.)

5.3 Methods

5.3.1 Mosquito collections

A single male mosquito was collected on 13.07.2014 during sweep netting of ferns and other low vegetation, in and around a young mixed broadleaf plantation (< 10 years old) 6 km to the north of Liverpool, England (53°30'42.13"N, 2°59'01.74"W) (Fig. 5.2). The location was ~100 m from an active arable farm yard, and ~ 250 m from a recently established wetland nature reserve, of wet grassland, fen, reed bed and open water (77 km^2).

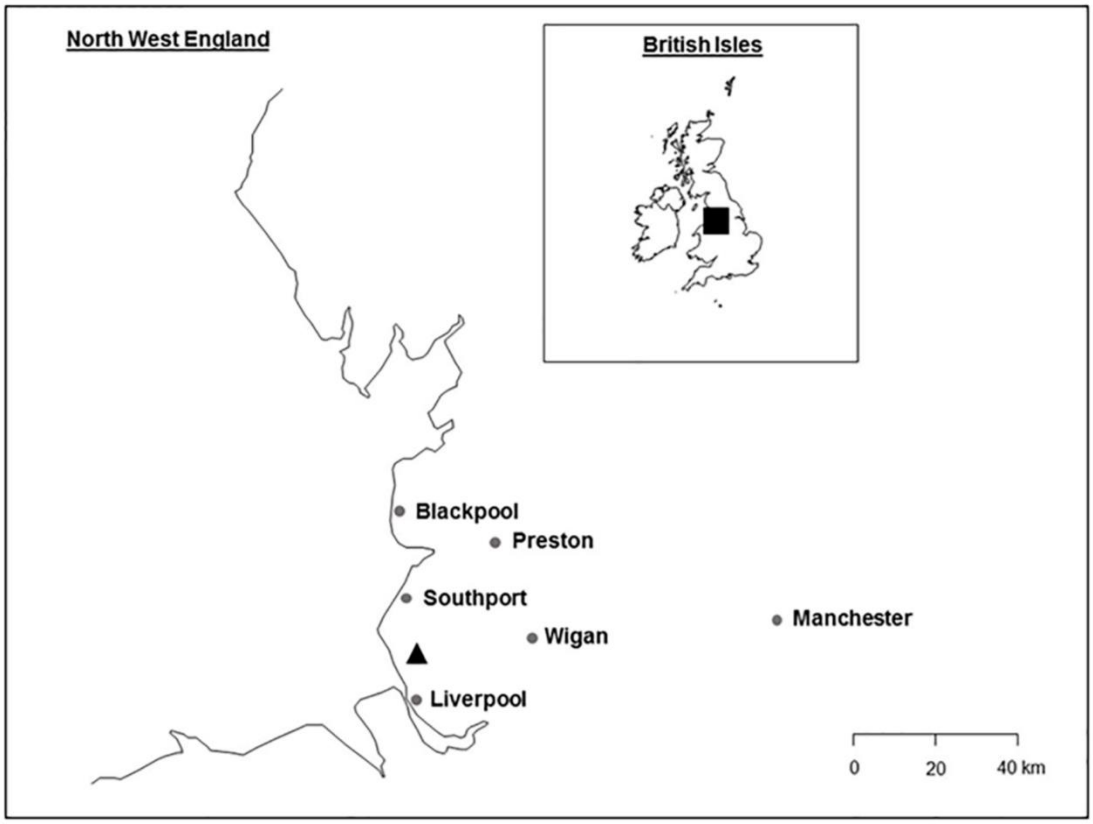

Fig. 5.2 Location of *Aedes aegypti* specimen collection site.

Black triangle denotes location point.

5.3.2 Morphological identification

Specimen identification was undertaken using the keys of Becker et al. (2010) and Schaffner et al. (2001), and identification was confirmed at the Natural History Museum (NHM), London, by further examination and genitalia dissection.

5.3.3 Genetic identification

To further confirm the identification, DNA was extracted from a single leg using a Qiagen DNeasy® Blood and Tissue kit (Manchester, UK) according to the manufacturer's instructions and amplified by PCR. Target amplification was carried out using Phusion® high-fidelity polymerase (New England Biolabs. Hitchin, UK) on the internal transcribed spacer 2 (*ITS2*) of nuclear ribosomal DNA (rDNA), and the mitochondrial cytochrome c oxidase subunit I (*COI)* region using the following 5′-TGT GAA CTG CAG GAC ACA TG-3′ (*ITS2* forward) and 5'-ATG CTT AAA TTT AGG GGG TA-3' (*ITS2* reverse) primers (Walton et al., 2007), and 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' (*COI* forward) and 5'-TAA TAT GGC AGA TTA GTG CAT TGGA-3' (*COI* reverse). The PCR products were purified using the ThermoFisher Scientific GeneJET Purification Kit (Paisley, UK), and amplification success was confirmed by gel electrophoresis. The products were subsequently sequenced using an Applied Biosystems 3730 DNA Analyser with BigDye v. 3.1 (University of Sheffield, Core Genomic Facility). The sequences were then blasted in GenBank for sequence similarity matches (http://www.ncbi.nlm.nih.gov/genbank/). DNA sequencing was replicated five times for *ITS2* and three times for *COI* regions to remove discourse through PCR error.

5.3.4 Targeted surveillance

As part of the ongoing sampling of mosquitoes, and prior to the confirmation of the *Aedes* specimen, sweep netting, and larval dipping close to the point of discovery (POD) was conducted by Edge Hill University (EHU) and the Liverpool World Museum (LWM) in August 2015. Additionally, three Mosquito Magnet® Independence traps were deployed within the adjacent wetland nature reserve ~ 200 m, ~ 450 m and ~ 1,000 m from the POD: the first was positioned adjacent to a vegetated drainage ditch, the second close to a blocked water filled ditch and an open water pond. The third was placed in an area of wet grassland.

Once the specimen was confirmed as *Aedes aegypti* a programme of targeted surveillance was implemented by entomologists at EHU and Public Health England (PHE). Species dispersal was considered as *Ae. aegypti* has been reported to have poor dispersal capabilities, with approximately a 50–363 m mean life-time dispersal distance (Harrington et al., 2005; Maciel-de-freitas et al., 2007; Trpis and Hausermann, 1986). Merseyside and West Lancashire comprises flat open expanses that are often subject to high winds so normal dispersal distance may be exaggerated in these conditions. As a precaution, a 2 km² search area was designated around the POD to search for established populations. Potential larval habitats within the search area were identified using a combination of local knowledge, on the ground investigations and Google Maps (https://www.google.co.uk/maps). The strategy was developed utilising the ECDC guidelines (ECDC, 2012).

The landscape surrounding the POD is predominately arable farmland and residential housing. Four locations found within this area were considered as potential *Ae.*

aegypti habitat: an active farm yard with used tyres (~ 1 km^2), a disused garden centre with extensive greenhouses (~ 0.6 km^2), a large cemetery with numerous flower vases (~ 9 km^2) and an industrial estate (~ 9 km^2), with distances from the POD of ~ 100 m (S), 1,300 m (SW), 1,600 m (SW) and 2,000 m (ESE), respectively. Surveillance was concentrated within these areas using BG-Sentinel (Biogents) adult traps and ovitraps, with larval dipping, also carried out where appropriate (Table 5.1). The BG-Sentinels were deployed with CO_2 and BG-Sweetscent™ lures and run on 12V car batteries that were re-charged weekly. The traps were activated on all sites from 9–11th September 2015 until the 27th October 2015. A total of 38 ovitraps where deployed concurrently with the BG-Sentinels at three of the target sites (with only the BG-Sentinel deployed at the industrial estate). Traps were checked once per week.

The working farm was the closest site to the POD with suitable larval habitats including water-filled containers, blood sources and shelter in farm buildings. The industrial estate was selected due to the presence of tyres, which on inspection were newly manufactured and not stored outside for long enough periods of time that would allow water to accumulate, this was, therefore, an unlikely source of introduction. Transportation of horticultural goods has proven to be an active method of AIM movement (Deblauwe et al., 2015; Demeulemeester et al., 2014) and the disused garden centre provided potential larval habitat for *Ae. aegypti* with water-filled containers. Several large greenhouses were also present providing shelter and higher temperatures. This could have potentially permitted over-wintering. This site has been demolished since this investigation. Cemeteries have proven to be ideal sites for container inhabitants such as *Ae. aegypti*, with an abundance of water-filled flower vases, sugar source from flowers, blood sources from cemetery workers,

visitors, birds and animals, as well as providing shelter around grave stones and surrounding trees and vegetation (Vezzani, 2007). A local cemetery was identified as a priority for surveillance, and at the request of the cemetery owners, sampling was limited to methods that were inconspicuous (e.g. ovitraps and BG-Sentinels) to respect the sensitivity of the location. Therefore, larval dipping of vases at grave sites was not undertaken.

Method of mosquito surveillance	Surveillance site					
	Forestry plantation	**Wetland nature reserve**	**Active farm yard**	**Disused garden centre**	**Cemetery**	**Industrial estate**
Sweep net	Yes	No	Yes	No	No	No
Larval dipping	Yes	Yes	Yes	Yes	No	Yes
Mosquito Magnet®	No	Yes	No	No	No	No
BG-Sentinel trap	No	No	Yes	Yes	Yes	Yes
Ovitrap	No	No	Yes	Yes	Yes	No

Table 5.1 Types of mosquito traps deployed at potential *Ae.aegypti* larval habitats.

5.3.5 Literature search

A comprehensive search of historical records for *Ae. aegypti* was undertaken to determine if the species had previously established itself in GB. This included museum records, historical journal articles and grey literature sources. Data from the NBN Gateway (https://data.nbn.org.uk/), Merseyside BioBank (http://www.merseysidebiobank.org.uk/) and Mosquito Recording Scheme/Mosquito Watch (https://www.brc.ac.uk/scheme/mosquitorecording-Scheme) biological recording centres were also searched.

5.4 Results

5.4.1 Morphological identification

On discovery, the specimen was in a reasonably good condition except for missing scutal scaling, a foreleg and tarsomere five from one of the hindlegs. The validity of the identification using the key by Schaffner et al. (2001) was questioned, as the length of tarsomere 4 was observably shorter than tarsomere 5. This feature is used as a generic characteristic of *Orthopodomyia* and resulted in an initial misidentification. Defacement of scales on the scutum also made clear determination difficult as the diagnostic lateral lyre-shaped white lines were not clearly visible (ECDC, 2012). As a result, additional confirmation was sought from the NHM. Further careful examination and dissection of the genitalia were required to make and confirm identification, respectively, of the specimen as *Ae. aegypti*. The specimen (Fig. 5.3) is deposited in the NHM collection (Specimen barcode no. 010630631).

5.4.2 Genetic identification

Blasted *COI* and *ITS2* regions were shown to match several *Ae. aegypti* sequences in the GenBank database. The closest matches to the *COI* was 100% identity to KY022527 and for *ITS2* 100% identity to KF471584. Sequence data from this study was deposited within the GenBank database (accession numbers; BM9ITS2, MF043260 and BM9COI, MF043259).

Fig.5.2 Images of the *Aedes aegypti* specimens collected in Merseyside, England.
A.Foreleg tarsomeres 3 to 5. B. Dorsal view of the abdomen and wings. C. Lateral view.

5.4.3 Targeted surveillance

A total of 366 mosquitoes (161 adults, two pupae and 203 larvae) were collected across all the surveillance sites, with six species identified (Table 5.2). Species recorded in the order of greatest abundance were *Culex pipiens s.l.* L., *Anopheles claviger* Meigen, *Culiseta annulata* Schrank, *Cs. morsitans* Theobald, *Cx. torrentium* Martini and *Ae. caspius* Pallas. No specimens of *Ae. aegypti* were found.

Searching by dipping and sweep netting was by far the most productive method of sampling, followed by the Mosquito Magnets®, ovitraps and the BG-Sentinels, respectively. Both the BG-Sentinels and the ovitraps captured very few specimens. These traps are designed for AIMs and do not regularly capture *Cx. pipiens s.l.*

Method of mosquito surveillance	**Surveillance site**					
	Forestry plantation	**Wetland nature reserve**	**Active farm yard**	**Disused garden centre**	**Cemetery**	**Industrial estate**
Sweep net	*An.claviger* *Cs.annulata* *Cs.morsitans* *Cx.pipiens s.l.*	n/a	*Cx.pipiens s.l.*	n/a	n/a	n/a
Larval dipping	None	*An.claviger*	*An.claviger* *Cs.annulata* *Cx.pipiens s.l.* *Cx.torrentium*	*Cx.pipiens s.l.* *Cx.torrentium*	n/a	None
Mosquito magnet	n/a	*An.claviger* *Cs.annulata* *Cs.morsitans* *Oc.caspius*	n/a	n/a	n/a	n/a
BG sentinel trap	n/a	n/a	None	*Cx.pipiens s.l.*	None	None
Ovitrap	n/a	n/a	*An.claviger*	None	None	n/a

Table 5.2 Mosquito species found at the various surveillance sites based on collection method.

Most specimens were found at the active farmyard, and the wetland nature reserve, with no specimens recorded at the cemetery or the industrial estate. However, dipping and netting were limited in both locations due to restrictions on site activity,

therefore trapping methods were limited to BG-Sentinels and ovitraps. As the primary aim of the surveillance was to find *Ae. aegypti*, which is known to be effectively surveyed by both methods, the lack of specimens found is indicative of species absence (Carrieri et al., 2011; Harwood et al., 2015).

5.5 Discussion

Given that only a single male specimen was found in 2014 and no other individuals were collected during surveys in 2015, it can be assumed that *Ae. aegypti* was not locally established. Furthermore, any population would be unlikely to reach its biotic potential. There are several well-recorded factors that can affect the fecundity of *Ae. aegypti*, namely food availability (Arrivillaga and Barrera, 2004; Canyon et al., 1999), suitability of the physical environment (Jansen and Beebe, 2010), humidity (Canyon et al., 1999) and particularly temperature (Chang et al., 2007; Farnesi et al., 2009; Mohammed and Chadee, 2011; Rueda et al., 1990; Yang et al., 2009). However, attempts to determine the survival ability of *Ae. aegypti* at different temperatures has been heavily weighted towards laboratory-based experiments rather than studies in the field (Brady et al., 2013). Additionally, little research has been done to establish the adaptability of *Ae. aegypti* at the extremes of its temperature range. Despite this gap in the available literature, current estimates for *Ae. aegypti* survival range from 10–35 °C for adults (Brady et al., 2013) and 10–30 °C for larvae (Yang et al., 2009), although the successful development of larvae, and the metabolising of food, is difficult at the extremes.

Northerly latitudes have previously been considered unsuitable for the establishment of *Ae. aegypti*. Our current knowledge of the life history of this species suggests that

it is unable to survive winters at these extremes. The temperature thresholds for the persistence of *Ae. aegypti* populations are thought to be the January isotherm of 10 °C or the annual mean temperature of 15 °C (Schaffner and Mathis, 2014). To put this in context, January isotherms for Scotland are 4-5 °C, and in England mostly 5-6 °C with 7 °C in SW Cornwall. According to the UK Met Office (officially the Meteorological Office until 2000) in January 2016 mean temperatures were 5.4 °C in Wales, 5.2 °C in England, 5.0 °C in Northern Ireland and 3.0 °C in Scotland. Records for January 2015 were colder. In some years (2001 - 2016) some parts of London and the south coast experienced mean January isotherms above 6 °C, with > 8 °C reported in a few localities. Annual mean temperatures across GB (1981 - 2010) vary between 4–11 °C, with > 11 °C in parts of London and the south coast of England (http://www.metoffice.gov.uk/). It is unlikely, therefore, that *Ae*. *aegypti* would establish in GB. This is supported by the discovery and subsequent monitoring of *Ae*. *aegypti* in the Netherlands (Brown et al., 2011). However, to accurately predict the extension of its range, *Ae*. *aegypti* behavioural studies are needed to determine if urban refugia, such as heated houses, are a potential resource for assisted overwintering.

For AIMs to establish in a new territory and overwinter, their population size must be large enough not to suffer from a lack of genetic variation (Deblauwe et al., 2015). Regions in southern Germany, for example, have suffered repeated re-introductions of *Ae. albopictus via* ground transport (Becker et al., 2013). Great Britain benefits from being a small island compared to the large landmass of continental Europe, so re-introductions may not be as common.

In this instance, we were unable to determine the point of entry for the specimen. The working farm was the closest site to the POD that contained suitable breeding habitats including water-filled containers, blood sources and shelter in farm buildings. From a site survey conducted at the time of the surveillance, there were no obviously introduced/planted material in the mixed broad-leaf plantation which would otherwise be a risk for the introduction of AIMs. The site was planted 11 years ago with native species with minimal subsequent intervention. The industrial estate was selected due to the presence of tyres which on inspection during active surveillance were in fact newly manufactured and not stored outside for periods of time long enough that would allow water to accumulate. We still believed it was prudent to continue with monitoring at this site.

Transport of horticultural goods has been demonstrated as a method of AIMs movement, as such the garden centre had been disused for several years and presented an ideal breeding site for *Ae. aegypti*, as there was plenty of water filled containers and the greenhouses, presented ideal shelter and warmth for adult mosquitoes. Surveillance time at the garden centre was limited due to its scheduled demolition for a building development. Despite the time restriction, no additional AIMs were found.

The initial identification by EHU using the morphological keys of Cranston et al. (1987), for mosquitoes in the GB, and Schaffner et al. 2001, for mosquitoes in Europe, was not straight forward. It proved that morphological features alone could make identification difficult if the specimen is missing key features and, particularly in this case if it belongs to a non-endemic species not included in regional specific keys. This situation has highlighted the need for supplementing morphological

identification with genetic methods to circumvent these issues, which are important to AIM surveillance projects.

The recent introduction of *Ae*. *aegypti* into the Netherlands (Scholte et al., 2010) and the rapid response to eliminate this population, along with the specimen reported here, highlights the continued need for passive and active surveillance methods for mosquito reporting, as highlighted by Vaux and Medlock (2015). We encourage individuals collecting mosquitoes in GB, either through entomological work and environmental health investigations of nuisance reporting, to submit specimens to entomologists at PHE, NHM or EHU for identification. This record of *Ae*. *aegypti* remains enigmatic and based upon the evidence presents no public health concern.

5.6 Conclusions

The discovery of a single *Ae*. *aegypti* male mosquito in the North-West region of GB led to targeted surveillance of the local area. As no other specimens were found, there is no risk to public health. Despite this, this study demonstrates the need to for surveillance and vigilance in countries believed to be climatically unsuitable for *Ae*. *aegypti* and other invasive mosquito species that pose a health risk. It is equally important that procedures are in plan to deal with situations such as the one encountered in this study.

CHAPTER SIX:

A potential global surveillance tool for effective, low cost, sampling of invasive *Aedes* mosquito eggs from tyres using adhesive tape

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6.1 Abstract

The international movement of used tyres is a major factor responsible for global introductions of *Aedes* invasive mosquitoes (AIMs) (Diptera: Culicidae) that are known disease vectors (e.g. dengue, Zika, chikungunya and yellow fever). Surveillance methods are restricted by expense, availability and efficiency to detect all life stages. Currently, no tested method exists to screen imported used tyres for eggs in diapause, the life stage most at risk from accidental introduction. Here we test the efficiency of adhesive tape as an affordable and readily available material to screen tyres for eggs, testing its effect on hatch rate, larval development, DNA amplification, and structural damage on the egg surface.

We demonstrate that the properties of adhesive tape can influence pick up of dormant eggs attached to dry surfaces. Tapes with high levels of adhesion, such as duct tape, removed eggs with high levels of efficiency (97% ± 3.14). Egg numbers collected from cleaned used tyres were found to explain larval hatch rate success well, particularly in subsequent larval to adult emergence experiments. The strength of this relationship decreased when we tested dirty tyres. Damage to the exochorion was observed following scanning electron microscopy (SEM), possibly resulting in the high variance in the observed model. We found that five days was the optimal time for eggs to remain on all tested tapes for maximum return on hatch rate success. Tape type did not inhibit amplification of DNA of eggs from three, five or ten days of exposure. Using this DNA, genotyping of AIMs was possible using species-specific markers.

We demonstrated for the first time that adhesive tapes are effective at removing AIM eggs from tyres. We propose that this method could be a standardised tool for surveillance to provide public health authorities and researchers with an additional method to screen tyre cargo. We provide a screening protocol for this purpose. This method has a global applicability and in turn can lead to increased predictability of introductions and improve screening methods at high risk entry points.

6.2 Introduction

The spread of RNA based flaviviruses and alphaviruses such as dengue (DENV), yellow fever, Zika (ZIKV) and chikungunya viruses (CHIKV) have become a major global concern. Annual infection rates of DENV (family *Flavivirdae*) have been predicted at 284- 528 million (Bhatt et al., 2013), resulting in ~20,000 reported deaths per year (Carabali et al., 2015). It is a multi-form disease with varying side effects and severity, symptoms range from flu-like fevers and a characteristic skin rash, to severe haemorrhagic bleeding and potentially fatal hypertension. Some 30 - 54.7% (2.05 – 3.74 billion) of the world's population is now believed to be at risk from infection across 128 countries (Brady et al., 2012) with little substantial progress being made in reducing the spread of both the vectors and the disease.

ZIKV (family *Flaviviridae*) has also become well reported with 440 000 – 1 300 000 cases from the 2015 Brazil epidemic alone (ECDC, 2015) and can be asymptomatic or present as mild flu like symptoms concurrent with some DENV manifestations. ZIKV can also spread via intrauterine transmission leading to congenital microcephaly in unborn children (Calvet et al., 2016; ECDC, 2015; Mlakar et al., 2016), as well as Guillain–Barré syndrome (Musso et al., 2014). Phylogenetic

analysis of whole ZIKV genomes suggests the disease originated in East Africa in the 1920s (Gatherer and Kohl, 2016), and until recent pandemics across the Pacific and Americas, had a slow dispersal (Roth et al., 2014). Reasons for accelerated ZIKV are yet to be confirmed but are likely to be multi causal with lack of localised immunity, increased mobility of competent vectors, delayed detection and expanding levels of globalisation the likely candidates (Grubaugh et al., 2018; Kindhauser et al., 2016).

The primary vectors of these arboviruses are *Aedes (Stegomyia) aegypti* (L.) (Yellow Fever Mosquito) and *Aedes (Stegomyia) albopictus* (Skuse) (Asian Tiger Mosquito). Both species have immature aquatic stages that require natural water filled tree holes, bamboo nodes and leaf axils for egg and larval development but have become successfully adapted to living in proximity to humans by utilising human made water filled containers and subterranean drainage systems as a viable alternative (Ngoagouni et al., 2015; Russell et al., 2001; Severson et al., 2016).

The global spread of these species is associated with the transportation of human goods such as the international trade in tyres and wet footed plants, such as lucky bamboo (Brown et al., 2011; Demeulemeester et al., 2014; Hofhuis et al., 2009; Jupp and Kemp, 1992). *Aedes aegypti* and *Ae. albopictus* have biological characteristics that, to differing extents, favour invasiveness. Both have developed anthropophilic adaptive tendencies, resulting in their proximity to humans for all aspects of their life cycle, and feed primarily on human blood (Lounibos, 2002; Lounibos and Kramer, 2016).

Aedes aegypti deposits 85 - 125 eggs (Christophers, 1960) on the margins of small temporary pools that form within these containers where they utilise a process of diapause, an adaptation to allow maximum return for larval development in small water bodies that can quickly evaporate (Beckel, 1958; Clements, 1992; Rezende et al., 2008). Oviposition technique is an integral part of this trait, with eggs being placed directly adjacent to the meniscus where a secretion on the exochorion adheres the egg ventrally to the material margin of the pool (Bosworth et al., 1998; Padmaja and Sundara Rajulu, 1981). This prevents eggs from falling into the water body and allows repeated submergence over several flooding cycles resulting in staggered larval emergence, also referred to as instalment hatching. After egg laying, a drying period of 11-13 hours is required for the development of the serosal cuticle (SC), an inner membrane of the egg shell that allows the embryo to survive desiccation (Rezende et al., 2008). In captive bred populations of *Ae. aegypti*, eggs have been reported as surviving such periods for 6-12 months depending on environmental conditions (J. Longbottom, pers. comm.). These adaptations allow Aedine eggs to survive long journeys attached to the surface of vessels whilst remaining in a state of diapause. This key physiological feature has allowed for the colonisation of new territories from their ancestral origin of sub-Saharan Africa (Powell and Tabachnick, 2013).

Global modelling of Aedine species distribution suggest that the range of both *Ae. aegypti* and *Ae. albopictus* is still expanding, this has been particularly well recorded across European and American continents (Kraemer et al., 2015). In Europe, the movement of *Ae. albopictus* has led to the first documented cases of autochthonous transmission of CHIKV and DENV (Tomasello and Schlagenhauf, 2013) and reports of *Ae. aegypti* well beyond its expected range (Dallimore et al., 2017), aligned with

recently recorded adaptive behaviour of AIMs (Goubert et al., 2017; Severson et al., 2016) this presents an argument for greater surveillance at the current limits of their geographical distribution (Brown et al., 2011).

The movement of AIMs appears to be multi-causal, but primarily through human transport networks. Active dispersal of these species is limited with a reported life-time mean mobility of approximately 50 – 363 m (Harrington et al., 2005; Maciel-De-Freitas et al., 2007; Trpis and Hausermann, 1986). Recent evidence suggests that adults can be passively dispersed by cars, the detection of *Ae. albopictus* in motorway service stations in Kent, England (Medlock et al., 2017b) and Bavaria and Baden-Wuerttemberg, Germany (Becker et al., 2013), supports this theory. However, the global movement of car tyres has been highlighted as a primary method of distributing *Ae. albopictus* and *Ae. aegypti* (Reiter, 1998; Reiter and Sprenger, 1987). It was by this method that *Ae. aegypti* was introduced into the Netherlands from Florida, USA, in 2010 (Brown et al., 2011). To combat the further spread of AIMs, international efforts have been made to increase surveillance at major ports and airports, tyre yards and service stations, as well as areas of suitable habitation (Doosti et al., 2016; ECDC, 2012; Kumar et al., 2014; Porse et al., 2015; Schaffner et al., 2001, 2013; Vaux and Medlock, 2015).

Traditional surveillance techniques for AIMs mostly utilise oviposition-based traps, larval dipping, and attractants such as CO_2, pheromones, light and human bait (ECDC, 2012). The efficiency of such traps is well documented (Silver, 2008). However, the deployment of different sampling methods between surveillance programmes is highly variable at an international and national scale, possibly a reflection of resource availability, as well as the varying inclination of local and

national authorities to promote active surveillance (Vazquez-Prokopec et al., 2010). These techniques have been fruitful in locating AIMs. However, these methods target larvae, pupae, adults and *in situ* egg deposition. Dormant eggs attached to the surface of car tyres, or dry containers are overlooked (Brown et al., 2011; Scholte et al., 2010). In surveillance systems where port authority screening is relied upon as the first line of defence against AIMs, any cargo containing eggs passes freely without discovery. Additionally, current surveillance requires the presence of active females in various reproductive states. Oviposition trapping techniques rely on recently introduced females to blood feed, or to have been imported in a gravid state. Likewise, CO_2 and pheromone attractants require mosquitoes to be actively seeking a blood source. Such surveillance methods target adult mosquitoes that are attempting, or even succeeded, in reproducing. These methods can reduce response times for post-discovery control, and often bypass populations of *Aedes* eggs being transported in a desiccated state that later emerge, when wetted, into a new territory. In this instance, early detection may only occur in countries with robust surveillance, or by chance reports of nuisance biting.

Although there is no singularly effective method of surveillance for AIMs it is essential that a range of sampling tools are available to determine their presence, to quantify the extent of any infestation and to provide accurate species identification and rapid initiation of control measures.

To develop a low cost and easy to use tool for mosquito workers to standardise screening of car tyres for *Aedes* eggs, we tested the efficacy of four distinct types of sticky tape for their ability to remove the mosquito eggs from the tyre surface. Additionally, we investigated the post-removal impact of this technique on the ability to 1) rear the sampled eggs to larvae and adulthood for identification, and

whether time exposed to the tape had any negative effects, 2) identify the eggs by morphology and for possible damage, using SEM, and to 3) investigate whether the tape types had any notable effect on DNA integrity by genotyping extracted eggs using species specific markers.

6.3 Methods

6.3.1 Maintenance of colonies

Aedes aegypti (New Orleans strain) laboratory colonies were reared within the Edge Hill University Vector Biology Research Group insectaries at 27°C and 70% RH, on an 11 h day/night cycle with a simulated 60 min dawn/dusk period, using a lighting system of 4 x Osram Dulux 26 W 840 lights positioned approximately 2 m from the rearing cages. Several hundred eggs (~1 000 to 1 500) deposited on filter paper were stimulated to hatch by submerging filter papers in a broth of 0.1 g brewer's yeast (Holland & Barrett, Ormskirk, UK) and 0.5 g of nutrient broth (Sigma Aldrich, Dorset, UK) dissolved in 1.4 L of dH_20 (Zheng et al., 2015). Of those successfully hatched (~500 to 1 000), first and second instar larvae were separated into four or five separate trays to avoid overcrowding and fed using ground fish flakes (Aquarian Tropical Fish Food, UK), ~ 0.08 mg per larvae after hatching with volumes doubled for each day there after until pupation. All fourth instar larvae and pupae were then transferred into 30 x 30 x 30 cm insect rearing cages (#211261, BugDorm-1. NHBS, Totnes, UK) and emergent adults fed for the first three days on 10% sugar solution soaked into cotton wool. Emergent male and female mosquitoes where allowed to mix for a minimum of three days to ensure that most females had the opportunity to copulate. After three days all females (~400 to 500 mosquitoes) were removed and placed into rearing cages and starved for 24 hours prior to blood feeding. All females

were engorged on defibrinated horse blood (Fisher Scientific, UK) using the Hemotek® membrane feeding system (Hemotek Ltd, UK) until fully distended and then left for 24 hours with a supplement of 10% sugar solution until gravid. Any gravid females were then separated into two equal batches; the first batch was used within experimental treatments (~200), and the second batch for rearing the next generation (~200). Specimens used during experimentation did not exceed more than three generations to reduce possible effects of inbreeding depression on egg viability (Ross et al., 2018).

6.3.2 Egg laying on car tyres

Two used car tyres (175/70 R13, Michelin, UK) were obtained from an outdoor store at a farmyard in Burscough, Lancashire, and each divided into eight equal sections. A first batch of eight sections were scrubbed with a detergent, sterilised with bleach and thoroughly rinsed using dH_2O to ensure no residual contaminants were present (hereafter referred to as 'clean'). A further eight were left in the condition they were found in (hereafter referred to as 'dirty'). Each section was ~ 21 (L) x 17 (W) x 13 (H) cm in size, just large enough to move in and out of bugdorms without dislodging or disturbing *in situ* eggs. Four tyre sections were used per round of egg laying with a total of 16 replicates/tape type/treatment (treatment = no. days exposed to the tape). For each replicate, a section of car tyre was added to each rearing cage along with 20 gravid females, and 75 mL of dH_2O deposited into the centre of each tyre section to create a small pool in the central depression. The aim here was to encourage female oviposition and egg adherence around the margin (Fig. 6.1). Females were left for 72 hours to lay eggs and then removed by aspiration. The remaining dH_2O was drawn from each tyre by pipetting to prevent dislodging the eggs and ensure that they were

not stimulated to hatch. Tyre sections were then removed from the bugdorms and allowed to dry at room temperature for 24 hours.

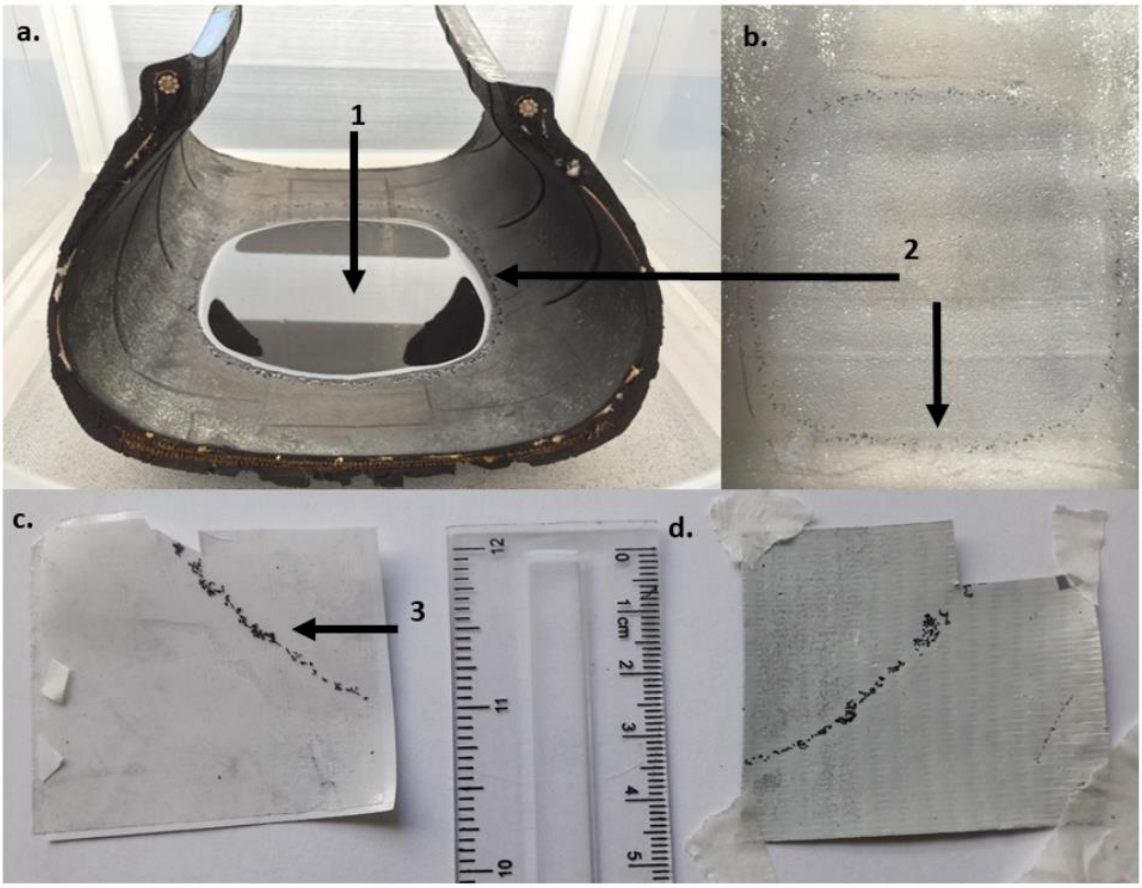


Fig. 6.1 Visibility of *Ae. aegypti* egg deposition on tyre and tape sections.

(a) Section of tyre placed within a bugdorm. (b) Dorsal view of the tyre after drying. (c) After sampling, eggs attached to adhesive surface of double-sided carpet tape. (d) After sampling, eggs attached to adhesive surface of duct tape. (1) 75 mL of dH_20 added to the tyre to encourage oviposition. (2) The deposited eggs can be seen by the naked eye as a dark speckled ring around the margin of the reservoir. (3) Increased visibility of eggs on adhesive tape after sampling.

6.3.3 Egg sampling/larval rearing with sticky tape

Four tape types were selected based on different properties (summarised in Table 6.1). Each tape type was cut into a 50 mm² sections and applied to the tyre along the line of deposited eggs, using a stratified random sampling method, and adhered with gentle pressure from the tip of the index finger. Tape application was carried out by

the same two individuals to reduce possible user bias. The outline of each piece of tape was marked using white chalk. Each tyre section accommodated 8 x 50 mm² pieces of tape allowing for two replicates of each tape type per tyre section. The tape was removed using fine tipped entomological forceps and placed into a dry 250 mL glass beaker and eggs counted under a stereomicroscope (Leica M80, GT vision, Suffolk, UK) at x40 magnification, along with any remaining eggs within the marked areas on the tyre.

Tape type	Code	Properties	Cost
Body tape	BT	Medium levels of adhesion. Less commercially available but contains fewer chemicals that potentially have less deleterious effects on egg viability.	£4.90 for 27 strips (Eylure Body Tape Pre-Cut Adhesive Strips, Boots UK, Ormskirk, England)
Clear packing tape	PT	Low-medium levels of adhesion, wide commercial availability, low cost, may be less damaging to eggs during transfer.	£3.28 for (L) 100 M (W) 50 mm (Diall Clear Packing Tape, B&Q, Aintree, England)
Double-sided flooring tape	CT	Medium-high levels of adhesion. Widely available in DIY stores, double-sidedness may prove advantageous for adhering samples for transportation.	£8.90 for (L)25 M (W)50 mm (Diall White Double-sided Tape, B&Q, Aintree, England)
Duct tape	DT	High levels of adhesion, wide commercial availability, a white background to improve visibility, easily torn without the need for cutting apparatus.	£ 2.70 for (L) 5 M (W) 50 mm (Duck Tape®, B&Q, Aintree, England)

Table 6.1 Properties of adhesive tape used during experimentation.

Three egg exposure treatments of three-, five- and ten-day intervals were undertaken to determine if the tapes have any effect on hatch rate success. After the allotted exposure time, the egg-laden tapes were submerged in 100 mL of hatching broth in a 250 mL beaker. Emergent larvae were counted after 24 hours and transferred into beakers with 100 mL of dH_20 and reared as described above. A laboratory control was also established whereby the same number of gravid females were encouraged to lay on filter paper and put through the same treatments as applied to the different tape types.

After the first 24 hours of submergence, tapes were removed and dried at room temperature for 48 hours and re-submerged for a second time. Larvae were once again removed and counted after 24 hours. All larvae were left to develop into adults and numbers recorded to determine if tape exposure affected later development.

6.3.4 Scanning electron microscopy (SEM)

A descriptive approach was used to observe potential damage to the egg morphology caused by the removal using tape. Observations were made through scanning electron microscopy (6010LV, JEOL(UK) Ltd., Herts, UK). Sections (10 mm^2) of egg loaded tape of each type and treatment, plus controls, were cut away and attached to 12.5 mm SEM stubs using carbon tabs (Agar Scientific Ltd., Essex, UK). Samples were then coated in gold for four minutes (~ 4 nm) using sputter coater (Q150R ES, Quorum Technologies Ltd., East Sussex, UK). Eggs were left *in situ* throughout this process. Samples were viewed in high vacuum mode where accelerated voltage = 10 kV, WD = 12 and spot size = 50.

6.3.5 PCR based identification – molecular methods

Additional checks were made to determine if amplification of egg DNA was still possible after three, five and ten days attached to the different tape types. A subsample (10 mm^2 squares) from each tape type, exposure and treatment were removed from egg-loaded tapes and placed into individual 1.5 mL tubes and stored at -20ºC until extraction. The number of eggs in each subsample ranged from 2 to 16. Each sample was homogenised (including the tape) for 30 s whilst dry using an electronic pestle and mortar (431-0094, VWR, Leicestershire, UK), 180 µL of buffer ATL and 20 µL of proteinase k (Qiagen, Manchester, UK) was added and homogenised for several seconds. Samples were thoroughly vortexed and left to lyse on an orbital shaker at 56 °C for 15 hours after which when the remaining pieces of tape were removed from each sample and discarded. DNA extraction was completed using the DNeasy® Blood and Tissue spin column kit (Part no. 69506, Qiagen, Manchester, UK) following the provided protocol, with 50 µl elution buffer held in the columns for 5 mins and passed through the column twice to increase DNA yield. To test amplification, a polymerase chain reaction (PCR) based analyses was carried out using species specific primers developed by Das et al. (2012) and Higa et al. (2010) (Table 2). Each 25 µL PCR reaction for the Das primers consisted of 2.5-20 ng of DNA template, 0.5 U of Phusion® High-Fidelity Polymerase (New England Biolabs® Ltd. Herts, UK), 1x Phusion HF Buffer (NEB), 200 µM of dNTP mix (NEB), 0.5 µM of primers AUF and AUR, and 0.7 µM of AEG, and 2% DMSO (NEB). PCR amplification was performed using a Primer Thermal Cycler (Techne, Staffordshire, UK) programmed with an initial denaturation of 98 °C for 30 s, followed by 35 cycles of 98°C for 10 s (denaturation), 59°C for 20 s (annealing), 72°C for 20 s (extension), followed by a final extension of 72°C for 7 mins. Each 25 µL PCR reaction for the Higa primers consisted of 2.5-20 ng of template, 0.5 U of Phusion® HF Polymerase, 1 x Phusion HF Buffer, 200 µm of dNTP mix, 0.5 µM of

primers 18SFHIN and CP16 and 0.7 µM of aeg.r1. Thermal cycler setting as outlined above, but with an annealing temperature of 70°C. PCR products were resolved on a 2% agarose/ethidium bromide gel with HyperLadder™ 100 bp (Bioline Reagents Ltd, London).

Species	**Primer codes**	**Sequence (5'-3')**	**Ref.**
Universal forward primer	AUF	TCA AAA TTA AGG GTA GTG GT	Das et al 2012
Universal reverse primer	AUR	GAC TTC AAC TGG CTT GAA CT	Das et al 2012
Ae.aegypti	AEG	GAC ACC GAG GCG CCC ATT GC	Das et al 2012
Universal forward primer	18FHIN	GTA AGC TTC CTT TGT ACA CAC CGC CCG	Higa et al 2010
Universal reverse primer	CP16	GCG GGT ACC ATG CTT AAA TTT AGG GGG TA	Higa et al 2010
Ae.aegypti	aeg.r1	TAA CGG ACA CCG TTC TAG GCC CT	Higa et al 2010

Table 6.2 Species-specific PCR primers for the identification of *Ae. aegypti.*

6.3.6 Analysis

A comparison of the egg pick-up efficiency of the different tape types was carried out using a Kruskal-Wallis test, after a Shapiro-Wilks test showed that the data was non-parametric. Multiple comparisons between tape types for the egg pick-up efficiency was investigated using a post-hoc Nemenyi Test.

To test how the number of larvae and adults were affected by the number of eggs and larvae respectively, and regarding different tape types and egg exposure time to the

tape (i.e. 3, 5 and 10 days), mixed effect models were used. The best fit model was selected using Akaike Information Criterion (AIC) and from there total explained variance was calculated for each fixed and random effect term. In addition, linear models were used to investigate individual tape treatments and to test the correlations between number of eggs picked up and larval hatch rate, and number of larvae and emergent adults. A Shapiro-Wilks test showed that the hatch rate and adult emergence data were non-parametric, henceforth data was square root transformed before running the linear models. To account for differences in the starting number of eggs and larvae for each sample, transformed data was weighted proportionately by the total number of adults in the population and accounted for differences of larval success from the previous treatments as follows:

$$weight = \left(\left(\frac{A_1}{\sum A_{Total}} \right) A_{1+2} - L_{1+2} \right) - 1$$

Where A_1 is the number of adults in a sample following the first emergence, A_{Total} is the total number of adults in the population, A_{1+2} is the number of adults in a sample following first and second emergence and L_{1+2} is the number of larvae in a sample following first and second hatching. All analysis was carried out using RStudio v.3.4.1.

6.4 Results

Within the parameters of the experiment 41 337 eggs were laid in total, and across all tyre replicates (eggs, *n* = 29 170) and filter paper controls (eggs, *n* = 12 167). A sum of 25 670 (88%) eggs were picked up by all tape type replicates, 11 056 (43.07%) of tape treatments and 7771 (63.87%) of controls developed into 1st instar larvae during first submergence. The second submergence produced 1469 (5.04%)

larvae from tape treatments, and 61 (0.55%) from controls, 10 808 (51.06%) first submergence eggs successfully hatched from clean tyres replicates, 1 033 (4.88%) from second submergence. A considerably lower hatch-rate was recorded for samples collected from dirty tyres; 248 (5.51%) of first submergence eggs were hatched and 436 (9.68%) from the second submergence (Table 6.3).

Analysis of the egg-pick up efficiency using a Kruskal-Wallais test showed significant differences between the efficiency of tape types (Chi Sq = 114.52, df = 3, P = 2.2E – 6). Further exploration of the data using a post-hoc Nemenyi Test demonstrated that the difference in pick-up efficiency was between the PT and all other tapes (Table 6.4). There were no statistically significant differences between all other treatments (Table 6.4). Notably, the egg pick-up efficiency of DT, BT and FT showed high levels of egg pick up efficiency (>96%) and relatively low levels of variation (s = 3.14 – 1.39) compared to that of PT (55.18%, s = 22.19).

6.4.1 The effect of tape type on hatch-rate success and adult emergence

To test if the number of eggs picked up can be used to explain the number of larvae hatching, and whether the number of larvae hatching can explain the number of emergent adults between the different tape types and days of exposure, two approaches were used. In the case of hatch rate success (i.e. the transition from eggs picked up to hatched larvae) the best fit mixed effect model (R^2 = 0.77) included number of larvae and time of exposure as a fixed factor, and tape type as a random factor (Table 6.5; Table 6.6). Similarly, in the case of adult emergence (i.e. the transition of hatched larvae to adult) the best fit mixed effect model (R^2 = 0.99) included number of larvae and time of exposure as a fixed, and tape type as a

Tape type	Exposure	Treatment	Results														
			Egg pick-up			Larval hatch rate (1st sub.)			Larval hatch rate (2nd sub.)			Adult emergence (1st sub.)			Adult emergence (2nd sub.)		
			n	***$\bar{x}$* (%)**	***SD (n-1)***	***n***	***$\bar{x}$* (%)**	***SD (n-1)***	***n***	***$\bar{x}$* (%)**	***SD (n-1)***	***n***	***$\bar{x}$* (%)**	***SD (n-1)***	***n***	***$\bar{x}$* (%)**	***SD (n-1)***
BT	3	Clean	1517	95.22	4.23	428	27.65	22.11	134	15.87	15.18	341	83.54	17.14	97	69.89	25.83
PT	3	Clean	651	38.78	19.4	83	13.92	14.8	60	6.08	7.13	68	94.25	24.18	34	74.55	23.74
CT	3	Clean	1943	92.13	5.86	414	23.84	21.1	281	22.27	16.81	342	81.75	12.65	205	81.75	16.56
DT	3	Clean	1897	95.36	4.52	397	20.94	25.1	304	24.19	15.02	292	70.45	32.44	117	44.02	28.1
n/a	3	Control	n/a	n/a	n/a	2713	64.14	8.71	24	3.15	3.79	2131	81.52	15.31	21	36.11	45.26
BT	5	Clean	1807	94.97	6.22	1294	67.74	19.71	66	6.32	19.91	1112	84.95	10.97	62	89.66	22.18
PT	5	Clean	1130	56.42	13.8	800	69.45	7.99	17	4.08	13.02	692	85.14	8.17	16	100	0
CT	5	Clean	2098	97.43	1.72	1392	64.56	26.52	158	8.67	21.63	1209	87.94	6.32	152	98.84	2.1
DT	5	Clean	2324	97.78	2.39	1818	76.75	6.66	7	2.13	4.6	1453	81.25	11.52	6	100	0
n/a	5	Control	n/a	n/a	n/a	2592	69.35	9.77	24	4.49	5.38	2119	83.22	11.24	15	55	44.44
BT	10	Clean	2059	97.47	2.39	1082	49.24	23.15	1	0.12	0.48	964	87.9	10.16	0	0	0
PT	10	Clean	1220	52.64	15.8	577	50.58	26.56	2	0.08	0.33	535	89.7	11.82	0	0	0
CT	10	Clean	2135	98.01	1.53	1206	55.33	25.09	0	0	0	1038	86.2	4.27	n/a	n/a	n/a
DT	10	Clean	2387	98.18	1.22	1317	54.52	26.28	3	0.18	0.74	1163	86.12	11.35	3	100	0
n/a	10	Control	n/a	n/a	n/a	2466	59.42	7.93	13	4.75	5.85	2089	86.5	7.22	13	100	0
BT	5	Dirty	1918	98.77	1.06	82	4.35	4.51	153	6.94	4.76	30	72.08	47.7	64	43.25	22.63
PT	5	Dirty	499	90.57	7.62	24	3.79	3.04	71	12.15	9.12	23	85.71	37.8	50	69.94	26.17
CT	5	Dirty	1212	98.97	2.37	73	6.44	4.81	109	8.95	6.06	66	92.6	7.83	55	45.96	19.9
DT	5	Dirty	873	99.49	1.44	69	7.92	8.14	103	11.82	6.37	24	66.23	43.11	31	37.38	36.57

Table 6.3 Synopsis of data testing tape pick up efficiency, larval hatch rate and adult emergence.

n = total egg pick-up across all samples, $\bar{x}$ = mean percentage, SD = standard deviation from the percentage mean. Exposure = no. of days that eggs were exposed to the tape. n/a (within egg pick-up) = where eggs were hatched directly from filter paper controls. n/a (within adult submergence) = where no adults could submerge due to zero hatch-rate scores.

	egg pick-up (%)				
Tape type	$\bar{x}$	**BT**	**PT**	**FT**	**DT**
BT	96.30 ± 4.39	-	-	-	-
PT	55.18 ± 22.19	5.20E-14	-	-	-
CT	96.3 ± 4.34	1	4.90E-14	-	-
DT	97.45 ± 3.14	0.54	3.60E-14	0.59	-

Table 6.4 Egg pick up efficiency in different sticky tape treatments. Post-hoc Nemenyi results from a Kruskal Wallis Test.

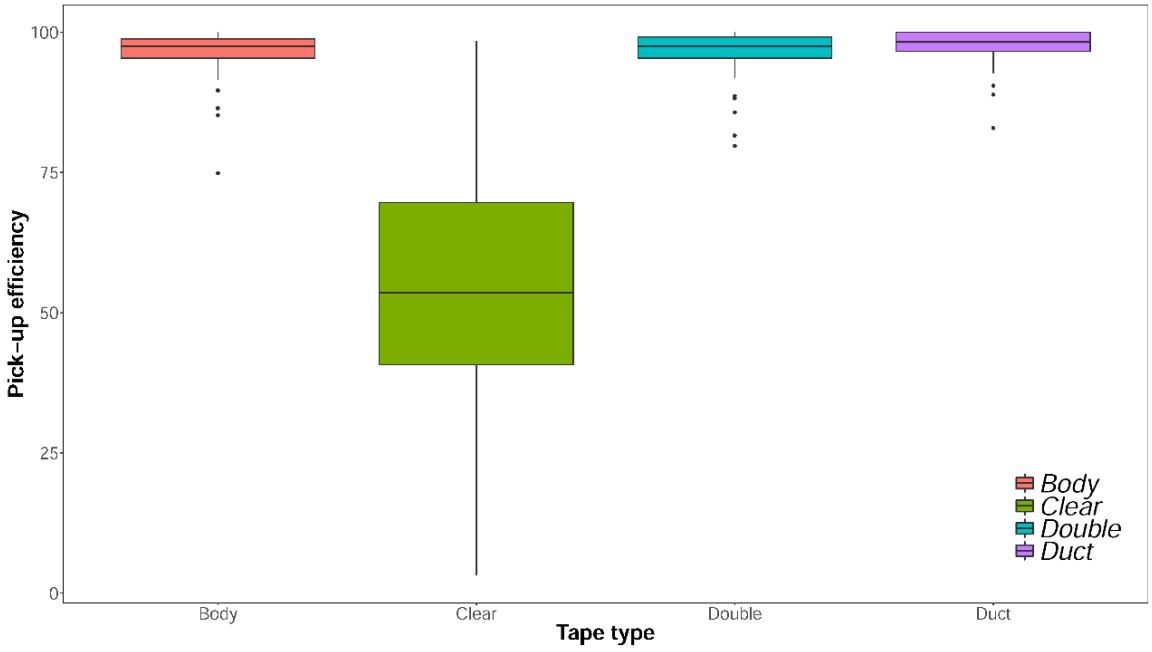


Fig. 6.2 Box plots with error bars of tape types vs. percentage egg pick up from the surface of clean tyres.

random factor (Table 5; Table 6). In both models most of the variance was explained by the number of eggs (R^2 = 0.64) or the number of larvae (R^2 = 0.97; Table 6.6). This was followed by tape type and exposure (Table 6.6).

To investigate the effect of individual tape types a linear model was fitted for each treatment combination (Fig. 6.3; Tables 6.6 & 6.7). The results indicated that the number of larvae that hatch is a good indicator of the number of adults that will emerge (Table 6.5). All treatments fell close to the 1:1 line, except for BT and DT from the dirty tyres. Therefore, if eggs hatch, it is likely that they will make it to adulthood, however, the number of eggs less efficiently explained the number of successfully hatched larvae. Generally, the five-day treatment resulted in stronger correlations compared to the three- and ten-day treatments (Tables 6.6 & 6.7; Fig. 6.3 & 6.4). Regardless of the length of exposure to the tapes, the dirty tyre tape always resulted in poorer explanatory power of either emerged larvae number from egg number, or adult emergence number from larvae number.

6.4.2 Cuticular condition and species identification by SEM

Descriptive observations were made of the condition of the eggs *in situ* upon both the control and tape treatments using SEM, revealing that most of the eggs removed from car tyres showed varying levels of damage to the exochorion (Fig. 6.5), but less so to the endochorionic and serosal cuticle (Farnesi et al., 2015). Additionally, SEM micrographs demonstrate that identification of the mosquito eggs via morphology alone is unlikely to be possible using this sampling technique, as only a dorsal perspective of the egg is visible after pick-up.

	Model	Form	*DF*	*AIC*	*BIC*	*P-value*	
Egg to larvae emergence	M1	No. Larvae ~ No. eggs + Exposure + (1|Tape type)	7	2635.1	2660.3	2.00E-16	***
	M2	No. Larvae ~ No. eggs + Exposure	6	2647.4	2669.0	2.00E-16	***
	M3	No. Larvae ~ No. eggs + (1|Tape type)	4	2757.9	2772.3	1	
	M4	No. Larvae ~ Exposure + (1|Tape type)	6	2882.8	2904.4	1	
	M5	No. Larvae ~ Tape type	6	2908.8	2930.5	1	
	M6	No. Larvae ~ Exposure	5	3016.0	3034.1	1	
Larvae to adult emergence	M7	No. Adults ~ No. larvae + Exposure + (1|Tape type)	7	684.3	709.5	2.00E-16	***
	M8	No. Adults ~ No. larvae + Exposure	6	747.4	769.1	2.00E-16	***
	M9	No. Adults ~ No. larvae + (1|Tape type)	4	706.4	720.9	1	
	M10	No. Adults ~ Exposure + (1|Tape type)	6	2861.1	2882.7	1	
	M11	No. Adults ~ Tape type	5	2998.1	3016.1	1	
	M12	No. Adults ~ Exposure	5	2998.1	3016.1	1	

Table 6.5 Mixed effect model results of number of eggs hatching and the numbers of larvae to adult emergence for the different tape types and exposure treatments.

The model with the lowest AIC (Akaike information criterion) and BIC (Bayesian information criterion) was retained (i.e. the best-fit model) and Chi-squared test was used to test for significance. *P<0.05, ** P<0.01, ***P<0.001

	Variables	Effect	Explained variance (Adj-R^2)	Unexplained variance (Adj-R^2)
Egg to larvae emergence	Total variance		0.77	0.23
	No. eggs	Fixed	0.64	0.36
	Tape type	Random	0.42	0.58
	Exposure	Fixed	0.14	0.86
Larvae to adult emergence	Total variance		0.99	0.01
	No. larvae	Fixed	0.97	0.03
	Tape type	Random	0.43	0.57
	Exposure	Fixed	0.14	0.86

Table 6.6. Summary of the variance from the models that best explained eggs hatching and the numbers of larvae to adult emergence.

For each contributing variable (i.e. tape type and exposure) the explained and the unexplained are presented.

6.4.3 DNA amplified from mosquito eggs

DNA isolation from mosquito eggs following the use of tape, produced DNA yields suitable for successful PCR amplification. Isolation from a single egg extracted from clear tape should produce enough yield to amplify species-specific markers that could be easily visualised with EtBr gel electrophoresis. All treatments including controls successfully amplified using the species-specific markers, indicating that tape treatment does not inhibit amplification of DNA after a period of up to 10 days of exposure to tape types followed by storage at -20°C.

		Results					
Tape type	**Exposure (days)**	*R^2*	*F*	*DF*	*Residual std. error*	*P -value*	
Control	3	0.8926	125.6	14	1.233	2.24E-08	***
BT	3	0.5537	16.61	14	2.514	5.73E-04	***
PT	3	0.6197	25.44	14	1.683	1.79E-04	***
CT	3	0.416	11.69	14	2.604	4.16E-03	**
DT	3	0.3552	9.265	14	3.021	8.76E-03	***
Control	5	0.7588	48.2	14	1.015	6.84E-06	***
BT	5	0.9509	291.2	14	0.6495	9.14E-11	***
PT	5	0.9565	331.1	14	0.4084	3.86E-11	***
CT	5	0.9156	163.7	14	0.6466	4.09E-09	***
DT	5	0.9793	711.8	14	0.4631	2.10E-13	***
Control	10	0.8934	126.7	14	1.001	2.11E-08	***
BT	10	0.5921	22.78	14	1.662	2.98E-04	***
PT	10	0.4102	11.43	14	2.508	4.49E-03	**
CT	10	0.3118	7.795	14	2.062	1.44E-02	*
DT	10	0.3967	10.86	14	2.047	5.30E-03	***
BT (Dirty)	5	0.5239	8.703	6	2.112	2.56E-02	*
PT (Dirty)	5	0.7902	27.37	6	1.063	1.95E-03	**
CT (Dirty)	5	0.416	5.987	6	2.728	5.00E-02	
DT (Dirty)	5	0.387	5.42	6	2.227	5.88E-02	

*P< -0.05, ** P<0.01, ***P<0.001.

Table 6.7 Linear model results of tape type and exposure vs. hatch rate success of eggs from egg pick-up.

		Results					
Tape type	**Exposure (days)**	*R^2*	*F*	*DF*	*Residual std. error*	*P -value*	
Control	3	0.7052	36.88	14	1.528	2.87E-05	***
BT	3	0.9493	282	14	0.5956	1.13E-10	***
PT	3	0.9436	252.1	14	0.417	2.39E-10	***
CT	3	0.9549	318.6	14	0.4294	4.99E-11	***
DT	3	0.7707	51.41	14	1.389	4.78E-06	***
Control	5	0.7629	49.26	14	0.9915	6.04E-06	***
BT	5	0.9765	624.9	14	0.4265	5.13E-13	***
PT	5	0.9764	622.1	14	0.2704	5.29E-13	***
CT	5	0.9734	550.1	14	0.3548	1.23E-12	***
DT	5	0.9436	251.8	14	0.6989	2.41E-10	***
Control	10	0.9564	330.3	14	0.5021	3.92E-11	***
BT	10	0.9802	744.4	14	0.4131	1.54E-13	***
PT	10	0.9939	2432	14	0.2093	2.00E-16	***
CT	10	0.9941	2518	14	0.2319	2.00E-16	***
DT	10	0.9823	831.2	14	0.4154	7.22E-14	***
BT (Dirty)	5	0.2725	3.622	6	2.005	1.06E-01	
PT (Dirty)	5	0.9797	338.9	6	0.2538	1.66E-06	***
CT (Dirty)	5	0.9671	206.6	6	0.3911	7.10E-06	***
DT (Dirty)	5	0.4051	5.768	6	1.433	5.32E-02	

*P< -0.05, ** P<0.01, ***P<0.001.

Table 6.8 Linear model results of tape type and exposure vs. adult emergence success from hatched.

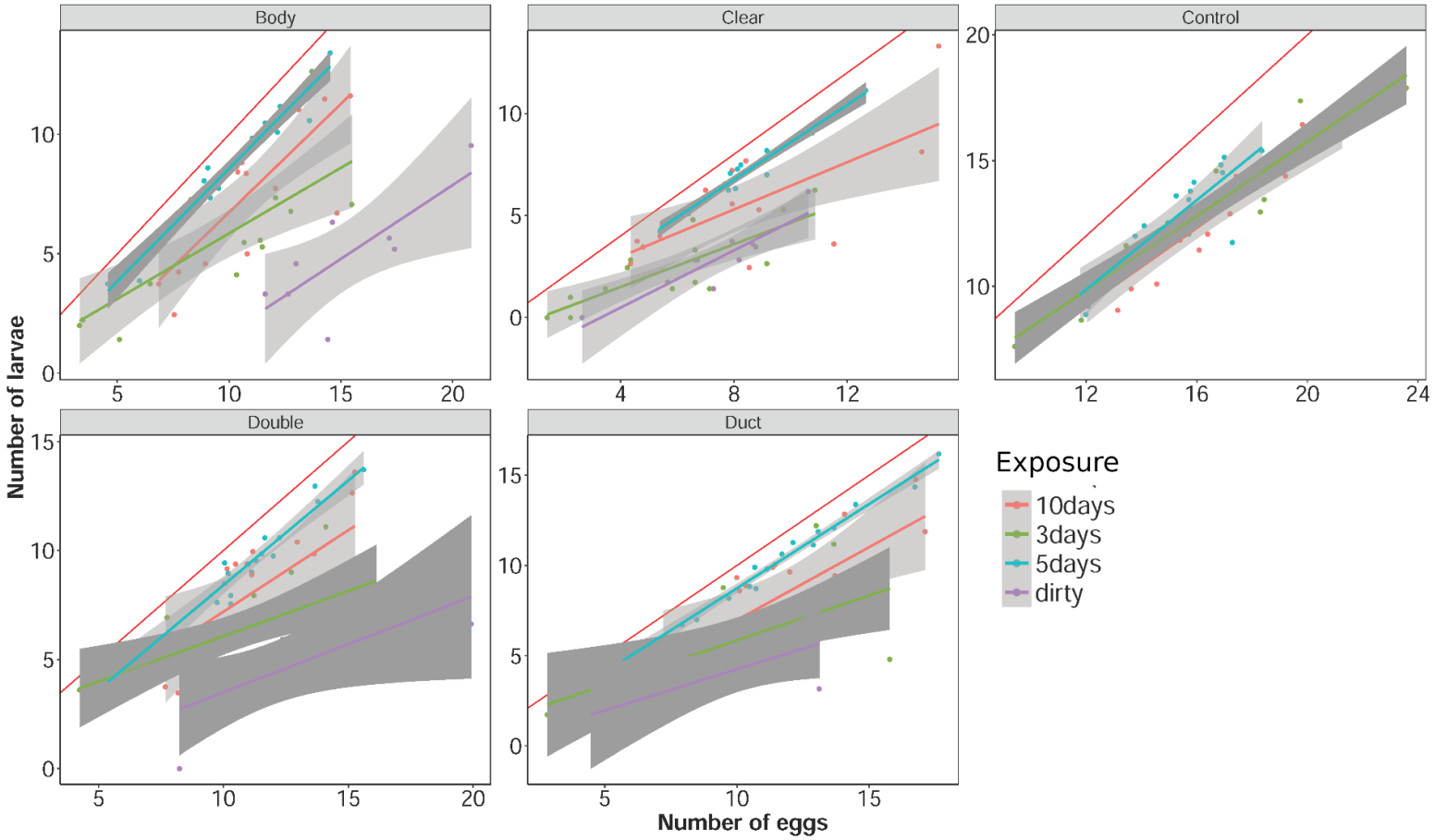


Fig. 6.3 Visualisation of linear model R^2 data from hatch rate success of eggs picked up by different adhesive tape treatments.

Body = Hypoallergenic body tape, Clear = Clear packaging tape, Double = Double-sided flooring tape, Duct = Duct tape, Control = Eggs hatched from standard laboratory reared egg papers.

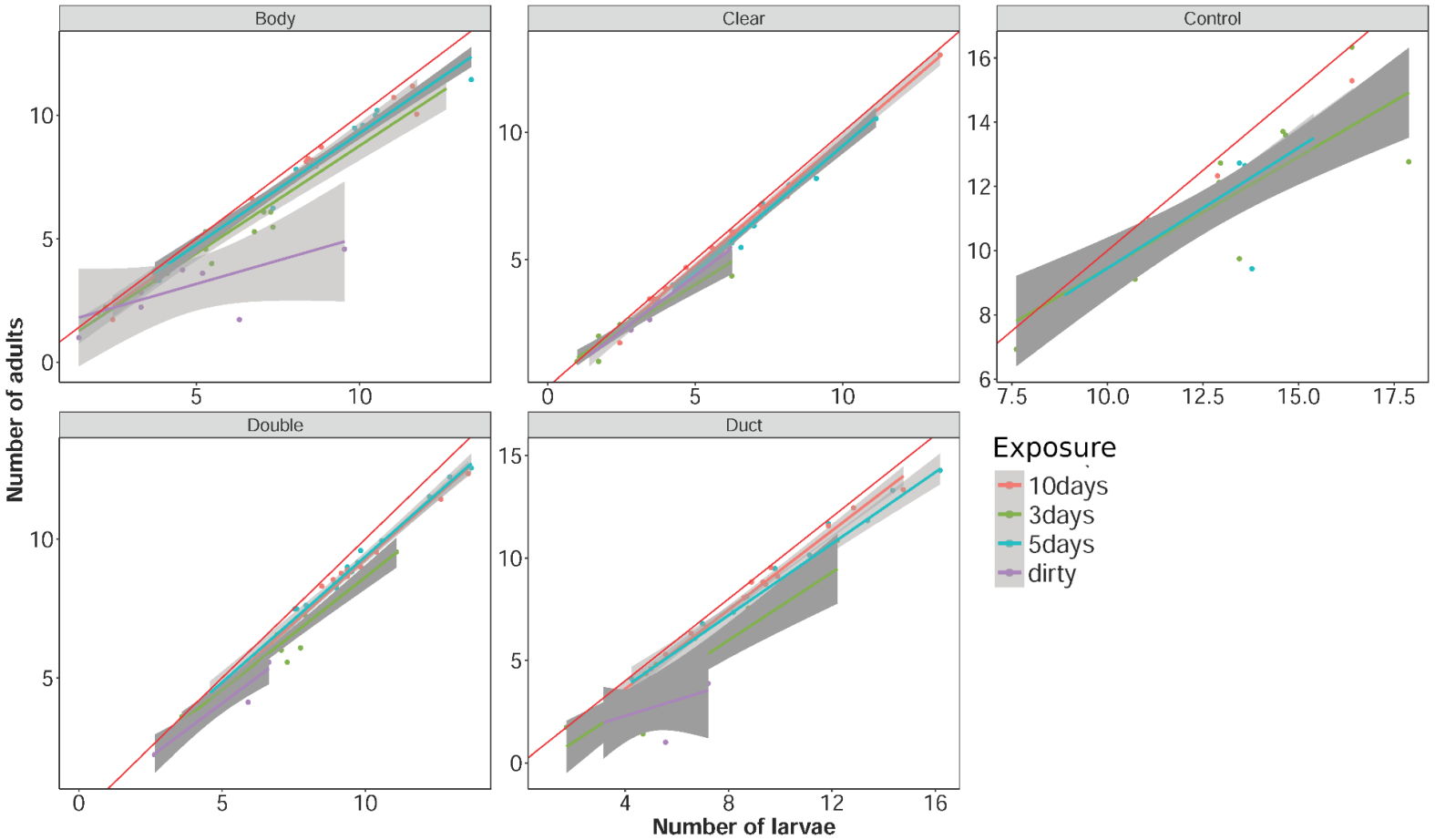


Fig. 6.4 Visualised linear model of R^2 data of adult emergence from successfully hatched eggs.

Body = Hypoallergenic body tape, Clear = Clear packaging tape, Double = Double-sided flooring tape, Duct = Duct tape, Control = Eggs hatched from standard laboratory reared egg papers.

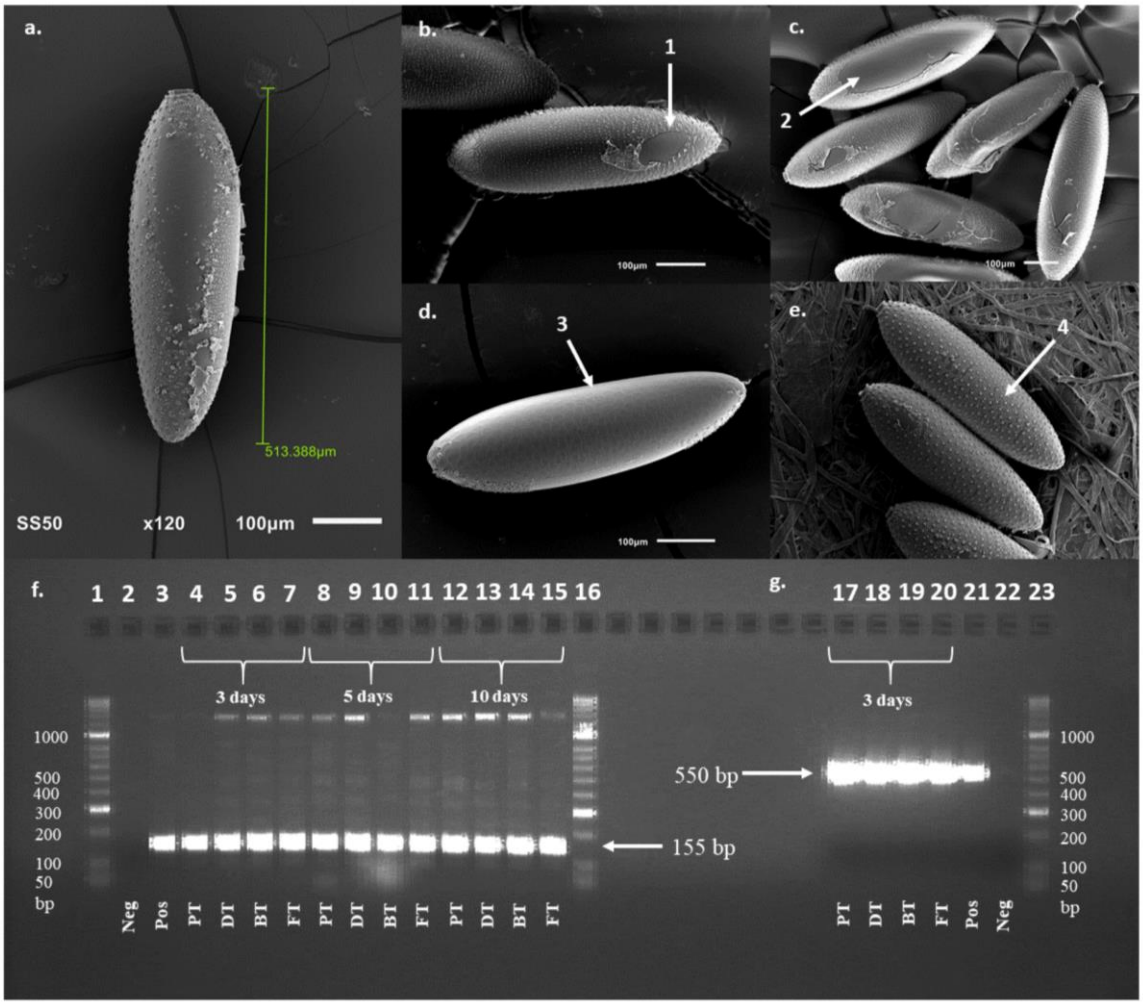


Fig. 6.5 PCR amplification of *Ae. aegypti* using species-specific markers and visualisation of tape damage to the egg surface under SEM.

a – e) SEM micrographs of *Ae. aegypti* mosquito eggs. a) Example of egg size, dorsal view, lifted from filter paper. b) Dorsal view on BT (1 = damage to chorion). c) Dorsal view on DT (2 = damage to chorion). d) Dorsal view on FT (3 = total removal of chorionic layer). e) Ventral view, controls on filter paper (4 = chorion intact). f) Gel electrophoresis results of PCR of eggs from car tyres using primers of Higa et al 2010 and g) of Das et al 2012. 1200 bp bands in (f) represent the positive control for partial *ITS1* and *ITS2* genes from which the species specific markers were designed.

6.5 Discussion

6.5.1 Identification of Aedine species from eggs

Adhesive tape has previously been used in the trapping of other adult insects (Carrol et al., 2011; Noireau et al., 2002), and the application of adhesive tape to locate mosquito eggs is not unknown between entomologists working in the field, although it is only rarely reported in grey literature (ECDC, 2012). We find no previous validation of tape in surveillance or ecological studies of mosquito eggs. It is probable that the development of adhesive tapes for this purpose has been overlooked, as the identification of mosquito eggs to species was historically by morphology, often difficult and requiring an elevated level of expertise with often ambiguous results. However, the recent increase in accessibility, doubled with reduced costing, for technologies such as Matrix Assisted Laser Desorption/Ionization (MALDI-TOF) (Schaffner et al., 2014), Scanning Electron Microscopy (SEM) (Suman et al., 2011), and genetic techniques such as species-specific markers (Beebe et al., 2007; Das et al., 2012; Higa et al., 2010), DNA barcoding (Werblow et al., 2016; Zamora-Delgado et al., 2015) and eDNA analysis (Schneider et al., 2016), has eased the burden of egg identification. Rearing techniques for *Aedes* has also improved over the last 50 years with many publications providing a myriad of workable methodologies (Morlan et al., 1963; Munstermann, 1997; Munstermann and Wasmuth, 1986; Zheng et al., 2015).

The rapid development of such new species identification systems must be matched with a progressive approach to field sampling techniques. Surveillance for eggs is advantageous as it allows for a more timely response to introductions.

6.5.2 Adhesive tape in *Aedes* surveillance

Currently 50% of the world's population is at risk from DENV due to the presence of AIMs such as *Ae. aegypti* and *Ae. albopictus*. Aligned with this, the threat of autochthonous transmission of arbovirus diseases (e.g. DENV, CHIKV and ZIKV) in areas where such diseases are not endemic is becoming a serious public health issue (Gould et al., 2010; Huang et al., 2013; Kutsuna et al., 2015; Rey, 2014; Tomasello and Schlagenhauf, 2013; Venturi et al., 2017). The international movement of AIMs are a serious threat to human health, and as such it is vital that the scientific community, and public bodies, develop novel methods of surveillance to increase efficiency. Here we have demonstrated the value of adhesive tape as a method of improving surveillance by assisting in the identification of tyres that carry AIM eggs in diapause. Adhesive tape has a global availability and can be acquired inexpensively, making it a readily available material for use in tyre screening at any location around the world. However, the application of this method must be carefully considered before use in the field. Locating mosquito eggs via the application of adhesive tape is not a cause to assume that AIMs, or those with vector potential have been located. Eggs could be in fact from container breeding, non-vector, species of mosquitoes. Therefore, understanding the viability of eggs after taping on downstream processing, such as rearing and identification, is essential. Additionally, the use of such tapes to sample dirty tyres in the field could result in unpredictable variance in hatch rate success when using a rearing approach for species identification. Therefore, we do not discourage but suggest caution when using this approach, until field validation of this method is undertaken.

6.5.3 Adhesive quality effect on egg pick-up

From the data, we can infer that all tape types were able to pick up mosquito eggs from the surface of tyres with differing levels of efficiency. The tapes with greater adhesive strength (BT, DT, CT) were able to remove most of the eggs from any given area consistently, whereas clear packaging tape (PT), with the lowest adhesion of all those tested, proved to be significantly more variable. Suggesting that adhesive quality of tape is important if a surveillance strategy requires accurate population census information, but less important if only ascertaining presence/absence.

6.5.4 Rearing from collected eggs

The data collected shows that if a larva hatches from an egg collected by adhesive tape then it is likely to make it to adulthood. The success of hatching from eggs is much more variable and has a lower explanatory power than the controls. This suggests a possible underlying effect caused by egg removal using tape and could be explained by damage to the egg cuticle observed using SEM. However, mean hatch rate success over five and ten days of exposure was above 50% for the first submergence, therefore this method is still useful for surveillance despite the negative impact caused by sampling methods. If only a small number of eggs are recovered during sampling, then we would advise a cautioned use of the rearing method.

This experiment also included a test on a set of dirty tyres with five days of exposure. Results showed that dirty tyres resulted in a lower explanatory power of number of larvae from the number of eggs when using different tapes. It is likely that

this is the result of (a) contamination of the tested tyres by an unknown introduced pathogen at source, or (b) damage to the egg structure resulting in a vulnerability to infection from the unclean surface, or a loss in the ability to retain internal humidity. Although these hypotheses are plausible explanations for our results, further work using soiled tyres would be required to assess this.

We suggest that if the only facilities available for egg identification is through the rearing of larvae to adult, a tape with lower adhesive qualities is preferable, but will likely come at a cost of the total percentage of eggs picked up in a given area. As previously mentioned, caution should be taken when sampling dirty tyres in the field, as our result suggest it could affect both pick up and hatch rate success.

In the instance where larval rearing is the preferred approach, our model showed that using egg number to explain larvae number, or larvae number to explain adult number, is highest (highest R^2) at five days. Therefore, larval rearing at five days from the date of sampling would be optimal. The experiments tested here are from a lab-based study only, during field application, eggs are likely to have been attached to the tyre surface for an unknown period producing an additional unpredictability factor when estimating hatch rate success in wild sampled populations. It would be interesting to test egg viability in combination with our methods based on actual shipping conditions (e.g. duration of the journey, predicted climate condition inside the containers etc.) in order to develop a predictability model on the likelihood of emergent risk of AIMs.

6.5.5 PCR based identification

Where larval/adult rearing is not practical, or a faster method of species identification is required, species genotyping is a viable alternative. We tested this method using egg loaded tape sections from each of the treatments to observe whether amplification was possible after exposure to the tape surface. A targeted species-specific approach was chosen as a preferable method to eliminate amplification from potential sources of contamination from the surface of the tyres. We would recommend that similar tests are undertaken during any field trials that expand this sampling method. However, there have been only several targeted species-specific assays produced for regions with a unique assemblages of problematic species (Beebe et al., 2007; Cook et al., 2005; Das et al., 2012; Higa et al., 2010; Hill et al., 2008; Van De Vossenberg et al., 2015). Alternative methods of species identification using eDNA, or the application of metabarcoding could prove to be a valuable alternative to species specific assays but will require further development.

6.6 Conclusion

This study has shown that a method of screening used tyres for mosquito eggs with sticky tape could prove to be a useful tool in the surveillance of AIMs that pose a serious threat to human health. Despite the global threat there is currently no surveillance technique that screens for the presence of AIM eggs as a primary introduction route. Identification at this point is important, as AIMs have proven to be biologically adaptive to new conditions, and a successful invasive in many areas around the world (Lounibos, 2002; Lounibos and Kramer, 2016). This study has demonstrated that low-cost adhesive tape can be used to detect the eggs of *Ae. aegypti*, from tyres and could also be used for other species notably *Ae. albopictus*.

The benefits of this are three-fold. Firstly, and most importantly, suspected eggs on the tape can be visualized with a hand-held lens which means fast screening of cargo can be achieved. This could result in a shipment being held or tracked to the onward destination where targeted control methods could be deployed if eggs are confirmed to be from AIMs. We have provided a flow chart to describe how the procedural process for such screening processes could take place (Fig. 6.6). Additionally, this method could be used to survey any location where tyre sampling is required (i.e. tyre yards, waste piles). Secondly, the ability to hatch and rear eggs through larvae to adults is possible although further field studies will be required to understand how environmental variables could affect factors such as egg mortality. Larval and adult rearing allows for morphological identification and additional downstream investigations (e.g. screening for insecticide susceptibility, and transovarial transmission of diseases) (Da Costa et al., 2017). Insecticide resistance screening is important given the worldwide spread of insecticide resistance and because AIMs can be imported from any country where they are present, irrespective of disease presence or absence. Lastly, the tapes tested showed no inhibition of PCR amplification, therefore, additional information can be gained from the DNA of any samples collected (e.g. population genetics).

Due to the low cost, and potentially high levels of efficiency, further development of this method could allow it to be deployed internationally, acting as an early warning system for new introductions. However, additional validation of this technique in the field would be advantageous to quantify the effects of sampling soiled tyres. We are currently devising a convenient method of applying tape to the tyre surface to produce a standardised methodology.

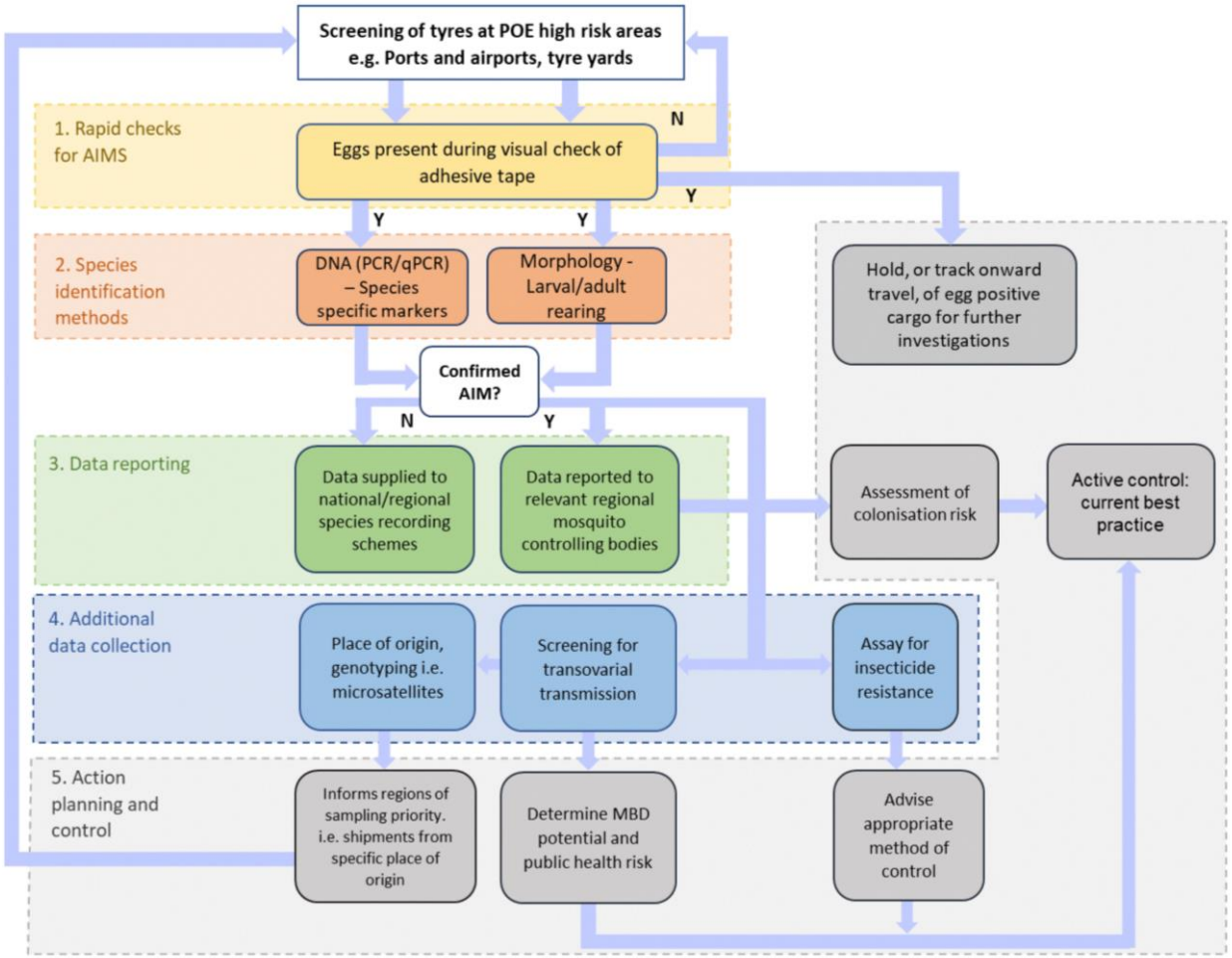
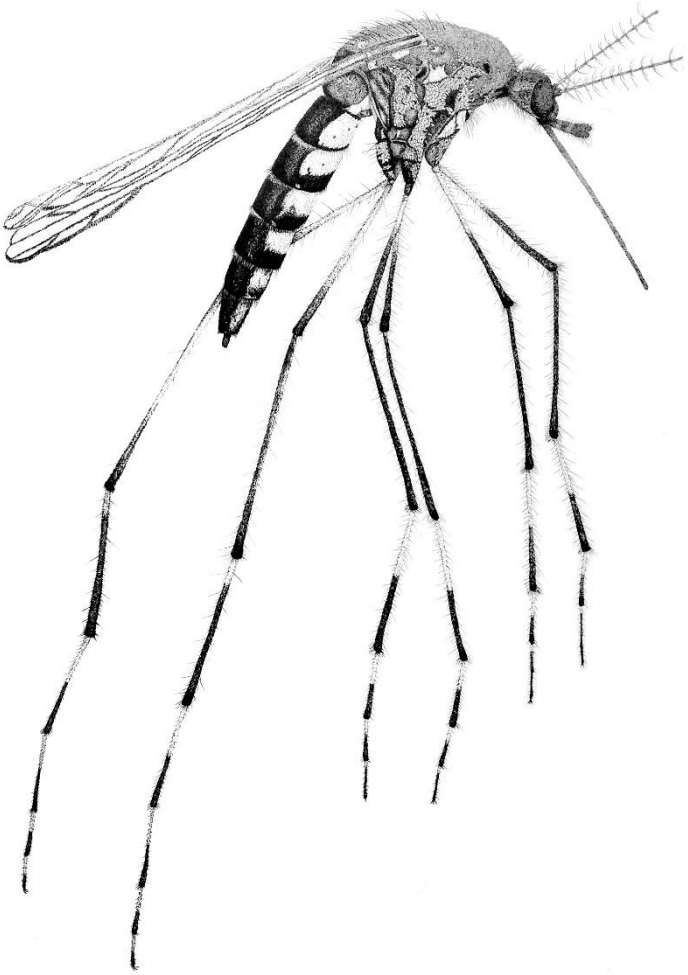


Fig. 6.6 Flow diagram of a recommended procedural process for screening tyres with adhesive tape.

POE = point of entry, AIM = Aedes invasive mosquito, MBD = mosquito borne disease.

CHAPTER SEVEN:

Conclusion



7.1 Conclusion

Globalisation and climate change have aided the movement and establishment of AIMs to new territories (Bhatt et al., 2013), as witnessed by the influx of *Ae. albopictus* into southern and central Europe. Repeated incursions from the importation of egg loaded goods, and the passive dispersal of adults by public transport systems has resulted in the established of uncontrollable AIM populations with associated autochthonous outbreaks of DENV, CHIKV and ZIKV (ECDC, 2016; Tomasello and Schlagenhauf, 2013).

Given the temperate climate of GB and its island status, the threat of AIMs was believed to be lower than for other European countries. The survey carried out as part of this study (Chapter 5), and the associated PHE led surveillance projects (Vaux et al., 2019) have demonstrated with the discovery of *Ae. aegypti* and *Ae. albopictus* in England, that we can no longer be complacent about the threat of AIMs. It is likely, however, that GB is only experiencing the beginning of the influx of these species, but as a cooperative of nations there is still opportunity to curtail the risks they pose. Fundamental to this is the development of tools that not only enhance the efficiency of surveillance strategies by improving detection of AIMs, but also improve response times to potential incursions.

The effective monitoring of AIMs is of highest priority to vector control in GB, however, the risk from native species should not be taken lightly (Schaffner et al., 2013). Of the 34 of mosquito species considered native to GB at the commencement of this study, 22 were reported as competent vectors in other countries, or by laboratory testing for diseases such as SINV, WNV and malaria (Chapter 1). The latter of which is responsible for 435 000 related deaths worldwide (during 2017) (WHO). The knowledge of current distribution for native high-risk species is

relatively poor, most likely due to a lack of trained and well distributed, passive, recorders. Unhelpfully, mosquitoes can often be difficult to identify, especially when damaged, and current British identification keys are now, as a result of scientific developments, inaccurate, or taxonomically invalid. Likewise, the screening of mosquitoes for disease is diffuse, and so our understanding of the disease potential of GB mosquitoes is limited to our knowledge of conspecifics from other geographical regions. The need for improved species identification by DNA barcoding has therefore been of paramount importance in understanding the genetic composition of GB taxa, and to improve accuracy in the surveillance of native species.

7.1.2 Using molecular barcoding as an aid to species surveillance

The use of barcoding genes for the identification of species is now common place in ecological studies and has proven to be effective at informing wider research. The applications of barcoding databases can also aid in the development of downstream applications such as metabarcoding, and eDNA (Lim et al., 2014; Lucas et al., 2018; Ronca et al., 2012). To develop postliminary uses, however, requires a firm foundation of data supported by coverage of the genetic variation found within and between species for any given gene of interest, in this instance those of *COI* and *ITS2*. Our understanding of variation is limited by the geographic area, and the number of analysed specimens per species that is representative of the region of interest. Additionally, for use in vector surveillance, an understanding of how these relate across species ranges can also provide additional elucidation (Lilja et al., 2018), especially given that vector competency can vary within species based on regionality, as well between closely related species (Bennett et al., 2002). We

attempted to collect individuals of each species from across a specific region (the British Isles) and compared them to a subset of sequences from across their geographical species ranges (SR). This study, as with similar attempts in other countries (Engdahl et al., 2014; Versteirt et al., 2015), was limited by our current understanding of species distribution based on historical records, including questionable sightings of species unrecorded for several decades, additionally, the ability to sample was limited by available resources and available sequences from genetic repositories. Consequently, we note a variance in the number of specimens sequenced for each species in our dataset. We have, however, been able to analyse the available sequence information to gain a greater understanding of how effective barcoding genes are at species identification, and highlighted groups that have shown potential for cryptic species. Additionally, we have produced a database that utilises both *COI* and *ITS2* genetic markers to aid in the elucidation of these groups. Likewise, we have analysed both sets of data independently using multiple approaches to species delimitation (traditional phylogenetics, ABGD, MJN, OTU picking etc.), to investigate the possibility of cryptic species, or haplotype grouping. We have summarised the outputs for these methods for each species, and each gene in Table 7.1.

We found that both *COI* and *ITS2* were mostly successful at identifying GB species (Chapters 3 & 4). However, there were several exceptions, the separation of *Oc. annulipes* and *Oc. cantans* failed with the use of both genes, likewise, separation of *An. daciae* and *An. messeae* could not be confirmed. The current status of these two groups requires careful reconsideration and closer analysis with whole genome sequences, or a greater number of samples, may be required to provide more robust partitioning.

Identification of novel cryptic species clusters such as those found across GB specimens of *Ae. vexans* could be important. It raises questions of niche partitioning, and how these species are biologically different. As discussed, the vector competence of mosquito species can be variable depending on population and regional differences. Therefore, the ability to easily identify population genotypes prior to vector competence screening could be insightful as to potential disease epidemiology.

Given the size of the dataset compiled within this study, and the high number of haplotypes partitioned by MJN analysis (Table 7.1), there would be a distinct benefit to carrying out species specific studies to including additional sequences. This would help to clarify the variation for any given species that currently exists in GB, and across species ranges. This study should, therefore, be considered as the foundation from which to build such projects. Species of interest from our results include; *Cx. modestus, An. claviger, Cs. morsitans/litorea, Ae. vexans, Oc. punctor, Ae. cinereus/geminus,* all of which show high numbers of intraspecific haplotypes.

The inclusion of genetic data from specimens collected across species ranges proved to be important in this study, but these are often missed during similar barcoding projects country (Engdahl et al., 2014; Hernández-Triana et al., 2019; Versteirt et al., 2015). Clear cryptic species divergence was found within species from different global regions. For example, the European species *Cx. territans* is genetically distinct from its North American conspecifics (Fig. 3.5). A trend was also found in *Ae. cinereus, Cs. morsitans,* and *Cs. alaskaensis*. High levels of genetic variation were also found in *An. claviger, Oc. detritus*, *Oc. caspius* and *Ae. vexans* within European populations. The confirmation of high levels of intraspecific genetic variation in these species likely follows the suspicions that cryptic clusters exist

within these groups, and many are afforded the title of species complexes, according to Becker et al (2010). Of these 'complexes', *Ae. vexans* is a competent vector of WNV and is a primary vector of Rift Valley fever outside of Europe (Schaffner et al., 2013), *Cs. morsitans* is responsible for outbreaks of SINV in Europe and eastern equine encephalitis in America (Bergqvist et al., 2015; Molaei et al., 2006; Morris and Zimmermann, 1981), *An. claviger* is named as a potential malaria vector (ECDC, 2014), and *Cx. territans* is a suspected vector of several parasitic species of *Trypanosoma,* parasitic protozoa that infect amphibians (Barlett-Healy et al., 2009). These results emphasise the need for population genetic approaches to vector potential studies.

7.1.3 Practical application of DNA barcoding in surveillance

The turn-over time from sample to sequence using our method was relatively quick, and samples could be processed within 24 hours by an experienced laboratory biologist (Chapters 3 and 4). The 'fast' option of direct PCR was used preferential within this study, a method that bypasses the cloning of DNA into *E. coli,* which can take several days. This method, however, can be problematic as the process cannot differentiate contaminants, or intraindividual gene variants. Therefore, the trade-off between time of processing and sequencing success is important. Despite issues with both sets of barcoding genes, we found that using both genes simultaneously provided good coverage. Additionally, the use of whole genes is not required for accurate species assignments. We found that a region of only 348 bp was required for the delimitation of GB species using *COI*. The information from this sequence data could also provide additional, and potentially vital, information regarding

haplotype distributions. Information that is otherwise lost in restriction-PCR based assays (Das et al., 2012).

7.1.4 Integrating new tools into current surveillance practice

The development of new tools for surveillance could become an academic exercise if the application and integration of these ideas is not carefully considered. This study has presented different, but not unrelated, approaches to surveillance; the use of DNA barcoding for species identification, aligned with a novel method of screening used tyres for AIMs. Both methods require experienced operatives in the field and laboratory for them to be effective. There is, therefore, a need to emphasise the importance of collaborative work between academic (or research institutions) and public health authorities, as well as clear route for feedback of information regarding the success of these methods.

The cost of vector surveillance must also be accounted for and may influence the choice of tools available for use. The methods we have trialled here, are both relatively inexpensive, with genotyping by sequencing costing approximately £2 to £3 per sample, and the screening of tyres only the cost of a roll of tape (£1 to £5).

For new techniques to become useful, it is important for them to be applied and trialled in the field. Therefore, we have already begun a process of testing the application of tape for screening of eggs and is currently being trialled by PHA in selected ports across GB. Likewise, the barcoding methods tested here has already proven to be effective, resulting in the confirmation of both *Ae. aegypti* and *Ae. albopictus*, as well as the newly recorded species *Oc. nigrinus* (not considered to be a major vector) to GB (Harbach et al., 2017; Vaux et al., 2019). Furthermore,

preferential processing of candidate AIM eggs from oviposition traps in the South of England, are now being confirmed using barcoding methods.

Both methods tested within this study were designed to complement the tools that are already available for the surveillance of native and AIM's. Prior to this study no method existed for detecting eggs in tyres. The flow chart presented below (Fig. 7.1) suggests a choice making paradigm to help integrate these methods into current surveillance strategies.

7.1.5 Future direction

Three consecutive years (2016 to 2019) of *Ae. albopictus* discovery in the South East of England (Vaux et al., 2019), and the individual specimen of *Ae. aegypti* found in the North West of England (2014), emphasise the need for increased vector surveillance in GB. The reactive nature of this sector demands rapid and continual improvements for methods to integrate field and laboratory techniques, but not at a compromise to their overall effectiveness. To roll out new sampling methodologies promptly is paramount, where multiple years of academic testing could result in their application being too late. The methods that we have tested and developed here are still only in their early stages, with further research to be carried out to optimise them for practical use. It is important, however, that these steps are now followed through with front line field testing, including efficacy on dirty tyres. We, therefore, have several questions that if answered could aid in their continual development:

	Predicted species (*n*)															
	COI								*ITS2*							
	ABGD		PTP		MJN		ML		ABGD		PTP		MJN		NJ	
Species (morphological classification)	**GB**	**+SR**	**GB**	**+SR**	**GB**	**+SR**	**GB**	**+SR**	**GB**	**+SR**	**GB**	**+SR**	**GB**	**+SR**	**GB**	**+SR**
Aedes (Aedes) cinereus	-	1	-	2	1(1)	2(8)	*	1	*	*	2	5	*(7)	*(13)	*	*
Aedes (Aedes) geminus	1	1	1	1	1(2)	1(6)	*	1	*	*	3	6	*(7)	*(13)	*	*
Aedes (Aedomorphus) vexans	2	3	2	4	2(6)	4(15)	2	4	1	1	1	4	1(1)	5(9)	1	5
Aedes (Stegomyia) aegypti	1	1	1	1	1(1)	1(5)	1	1	1	1	1	1	1(1)	2(7)	1	1
Aedes (Stegomyia) albopictus	1	1	1	1	1(1)	1(2)	1	1	-	1	-	4	-	-	-	1
Anopheles (Anopheles) algeriensis	1	1	1	1	1(1)	1(4)	1	1	1	1	1	1	1(3)	1(3)	1	1
Anopheles (Anopheles) atroparvus	1	2	ŧ	ŧ	1(1)	2(8)	1	1	1	1	1	1	1(1)	1(1)	1	1
Anopheles (Anopheles) claviger	1	1	1	1	1(6)	3(10)	1	1	1	1	1	3	1(2)	4(7)	1	4
Anopheles (Anopheles) daciae	§	§	ŧ	ŧ	§(2)	§(11)	§	§	§	§	§	§	§(2)	§(3)	1	1
Anopheles (Anopheles) maculipennis	2	3	1	1	2(3)	3(19)	2	2	1	1	2	2	2(3)	2(4)	3	3
Anopheles (Anopheles) messeae	§	§	ŧ	ŧ	§(2)	§(11)	§	§	§	§	§	§	§(2)	§(3)	1	1
Anopheles (Anopheles) plumbeus	1	1	4	7	1(1)	1(1)	1	1	1	1	3	4	1(1)	1(1)	1	1
Coquillettidia (Coquillettidia) richiardii	2	2	4	7	1(4)	1(6)	1	1	1	1	4	4	1(1)	1(1)	1	1
Culex (Barraudius) modestus	1	1	1	4	1(5)	2(12)	1	2	1	1	3	3	1(4)	1(6)	2	1
Culex (Culex) pipiens s.l.	1	2	1	2	1(4)	1(8)	1	2	1	1	1	3	1(4)	3(11)	1	1
Culex (Culex) torrentium	1	1	1	1	1(3)	1(4)	1	1	1	1	1	1	2(3)	2(5)	1	1
Culex (Neoculex) territans	1	2	1	8	1(1)	2(9)	1	3	-	1	-	1	-	1(2)	-	1
Culiseta (Allotheobaldia) longiareolata	-	1	-	1	-	1(1)	-	1	-	-	-	-	-	-	-	-
Culiseta (Culisella) fumipennis	-	1	-	1	-	1(3)	-	1	-	-	-	-	-	-	-	-
Culiseta (Culisella) litorea	1	1	1	1	1(3)	1(3)	1	1	1	1	1	1	1(10)	1(10)	1	1
Culiseta (Culisella) morsitans	1	2	1	2	1(3)	2(13)	1	2	1	1	2	2	5(35)	5(35)	2	2
Culiseta (Culiseta) alaskaensis	-	2	-	2	-	2(5)	-	2	-	-	-	-	-	-	-	-
Culiseta (Culiseta) annulata	1	1	1	1	1(3)	1(4)	1	1	1	1	1	1	1(1)	1(1)	1	¥
Culiseta (Culiseta) subochrea	-	-	-	-	-	-	-	-	-	1	-	1	-	1(1)	-	¥
Dahliana (Dahliana) geniculatus	1	1	1	1	1(2)	1(6)	1	1	1	1	1	1	2(4)	2(7)	1	1
Ochlerotatus (Ochlerotatus) annulipes	†	†	†	†	†(13)	†(17)	†	†	ǂ	ǂ	†	†	1(1)	1(1)	ǂ	ǂ
Ochlerotatus (Ochlerotatus) cantans	†	†	†	†	†(13)	†(17)	†	†	ǂ	ǂ	†	†	1(2)	1(2)	ǂ	ǂ
Ochlerotatus (Ochlerotatus) caspius	1	‡	1	‡	‡(5)	‡(21)	1	‡	3	5	3	5	2(4)	3(6)	1	2
Ochlerotatus (Ochlerotatus) communis	-	1	-	1	-	1(3)	-	1	-	#	-	1	-	1(2)	-	1
Ochlerotatus (Ochlerotatus) detritus	1	1	1	1	1(5)	1(7)	1	1	1	1	2	3	1(1)	3(5)	1	2
Ochlerotatus (Ochlerotatus) dorsalis	-	‡	-	‡	‡(5)	‡(21)	1	‡	-	-	-	-	-	-	-	-
Ochlerotatus (Ochlerotatus) flavescens	1	1	1	1	1(3)	1(5)	2	2	ǂ	ǂ	2	2	2(3)	2(3)	ǂ	ǂ
Ochlerotatus (Ochlerotatus) leucomelas	-	1	-	1	-	1(1)	-	-	-	-	-	-	-	-	-	-
Ochlerotatus (Ochlerotatus) nigrinus	Δ	Δ	2	2	1(4)	1(6)	1	1	1	1	1	1	1(2)	1(2)	1	1
Ochlerotatus (Ochlerotatus) punctor	1	2	1	3	1(8)	2(14)	1	5	1	#	3	5	1(3)	1(6)	2	2
Ochlerotatus (Ochlerotatus) sticticus	Δ	Δ	1	2	1(1)	1(6)	1	2	2	2	2	2	1(2)	1(2)	2	2
Ochlerotatus (Rusticoidus) rusticus	1	1	1	1	1(1)	1(2)	1	1	1	1	1	1	1(3)	1(3)	1	1
Orthopodomyia pulcripalpis	-	1	-	1	-	1(1)	-	-	1	1	1	1	1(1)	1(1)	1	1
Total (*n*)	**27**	**41**	**33**	**65**	**29(90)**	**45(227)**	**29**	**46**	**26**	**31**	**44**	**70**	**34(109)**	**50(156)**	**30**	**40**

Table 7.1 Status of species before and after *COI* and *ITS2* analysis.

ABGD = Automatic Barcode Gap Discovery analysis. PTP = Poisson Tree Processes analysis. MJN = Median Joining Neighbour analysis, *n* haploclades (*n* haplotypes). ML = Maximum likelihood phylogenetic analysis. UK = data from GB only. +SR = GB and additional species range data. * Data insufficient to differentiate *Ae. cinereus/geminus.* † unable to differentiate *Oc. annulipes/cantans.* ‡ unable to differentiate *Oc. caspius/dorsalis.* § unable to differentiate *An. daciae/messeae.* ŧ unable to differentiate those within the *An. maculipennis* group. Δ unable to differentiate *Oc. caspius/nigrinus.* ǂ unable to differentiate *Oc. annulipes/cantans/flavescens.* # unable to differentiate *Oc. communis/punctor.* ¥ unable to differentiate *Cs. annulata/subochrea.*

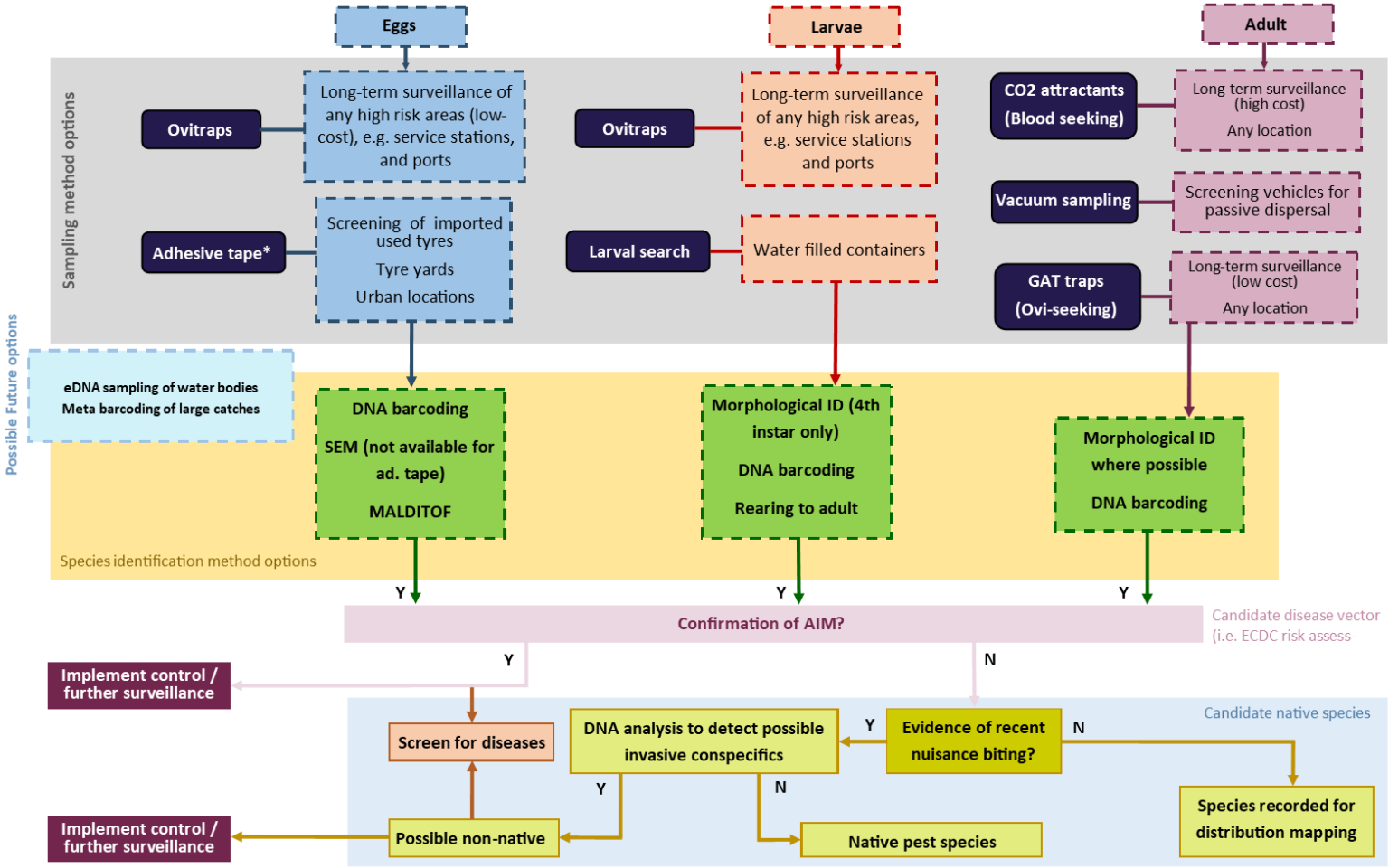


Fig 7.1 Flow chart to aid in decision making for best approach to surveillance. Integrating approaches tested within this study.

*Developed within this study (Chapter 6).

i) There are instances where egg rearing is not possible (e.g. where there are no accessible insectaries, but warmer climate will suffice for rearing). What effect does this have on hatch rate success?

ii) The efficiency of tape to pick-up or damage eggs may be influenced by user bias, and therefore the development of a tool to aid in the application of tape, may be beneficial and make the sampling process easier (an adhesive roller could be useful).

Molecular tools in biodiversity monitoring have already developed beyond the methods used within this study, and new techniques are likely to continue to become available. Presented here, however, is a foundation to the development of such techniques as metabarcoding, and the use of eDNA, that could be applied to mosquito surveillance. Methods such as these are already in development for AIMs (Schneider et al., 2016), however, a similar approach to native vector candidates may streamline the assessment of species assemblages (and therefore an assigned risk-factor) by analysis of freshwater samples alone (Thomsen et al., 2012; Valentini et al., 2016). Both metabarcoding and eDNA are most efficient when tested against a robust dataset of short barcode sequences such as those we have produced here, making this a likely next step in the development of GB mosquito genetics. In the short term, the development of PCR based assays to separate target species is also recommended, as they can provide species confirmation in only a matter of hours. Such tests exist to separate GB species of *An. maculipennis* (Danabalan et al., 2014), however, caution should be taken with PCR based assignment of *An. daciae* and *An. messeae* within this group, due to a questionable species separation (Chapters 3 & 4).

A phylogeographic assessment of genetic variation across species ranges also needs investigating further, and it would have benefits for surveillance strategies to do so. The accurate identification of species is a primary objective for using DNA

barcoding, however, the ability to undertake population differentiation may also be possible for some species groups. As MJN analysis of *COI* and *ITS2* data suggested, phylogeographic partitioning as well as species separations are present and supported by alternative analytical approaches (PTP etc). This could be particularly useful for the detection of conspecifics of native species that may appear morphological identical but carry differing vector potential. The importation of such individuals may occur passively but would be unlikely to be detected using morphological identification alone.

This study has advanced our understanding of mosquitoes of GB, highlighted deficiencies of current methods for surveillance, discovered *Ae. aegypti* and *Ae. albopictus,* and developed a new screening tool for detection of AIMs eggs at the point of entry. What has also become clear during this work is that resources are urgently needed for the surveillance of native and invasive mosquito species as the threat of MBDs to GB is getting closer.

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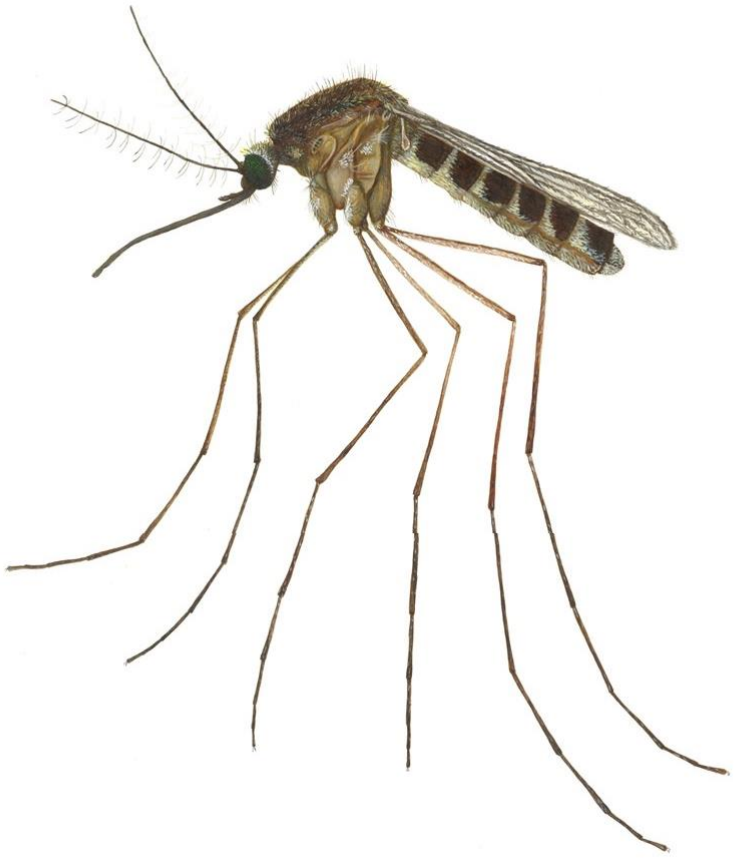

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Appendices

Appendix I – Comparison of *COI* and *ITS2* PCR and sequencing efficiency

		PCR amplification		Sanger sequencing	
Species	**Sample ref.**	***COI***	***ITS2***	***COI***	***ITS2***
Ae. aegypti	BM9	✓	✓	✓	✓
Ae. albopictus	ASH2.1	✓	✓	x	P
Ae. albopictus	PHE8	✓	✓	✓	P
Ae. cinereus	BM100	x	✓	x	x
Ae. cinereus	BM60	x	✓	x	✓
Ae. cinereus	UK285	x	✓	x	✓
Ae. cinereus	UK390	x	✓	x	✓
Ae. cinereus/geminus	BM101	x	✓	x	x
Ae. cinereus/geminus	BM117	x	-	x	-
Ae. cinereus/geminus	BM118	x	-	x	-
Ae. geminus	UK855	✓	✓	x	✓
Ae. geminus	UK873	✓	✓	P	P
Ae. geminus	UK874	✓	✓	x	✓
Ae. geminus	UK954	✓	✓	x	P
Ae. vexans	PHE1	-	✓	✓	✓
Ae. vexans	PHE2	-	✓	✓	✓
Ae. vexans	PHE19	-	✓	✓	P
Ae. vexans	PHE20	-	✓	✓	P
Ae. vexans	PHE22	-	✓	✓	x
Ae. vexans	PHE23	-	✓	✓	P
Aedes sp. (eggs)	PHE25	x	-	x	-
Aedes sp. (eggs)	PHE26	x	-	x	-
Aedes sp. (eggs)	PHE27	x	-	x	-
An. algeriensis	BM10	✓	✓	x	✓
An. algeriensis	BM11	✓	✓	x	✓
An. algeriensis	BM12	✓	✓	x	x
An. algeriensis	BM13	✓	✓	✓	✓
An. algeriensis	BM14	✓	✓	x	✓
An. algeriensis	PHE3	✓	✓	x	✓
An. algeriensis	PHE4	✓	✓	x	✓
An. claviger	BM28	✓	✓	✓	P
An. claviger	BM29	✓	✓	✓	P
An. claviger	BM30	✓	✓	✓	P
An. claviger	BM31	✓	✓	✓	P
An. claviger	BM35	✓	✓	✓	P
An. claviger	BM55	✓	✓	✓	P
An. claviger	BM71	✓	✓	✓	P
An. claviger	UK142	✓	✓	✓	✓
An. claviger	UK144	✓	✓	x	✓

		PCR amplification		Sanger sequencing	
Species	**Sample ref.**	***COI***	***ITS2***	***COI***	***ITS2***
An. claviger	UK1455	✓	✓	✓	P
An. claviger	UK1467	x	x	x	x
An. claviger	UK149	✓	✓	✓	P
An. claviger	UK154	✓	✓	x	x
An. claviger	UK960	✓	✓	✓	✓
An. maculipennis	BM27	✓	✓	x	P*
An. maculipennis	UK134	✓	x	x	x
An. maculipennis	UK2455	✓	x	P	x
An. messeae	UK2430	-	✓	-	✓
An. plumbeus	BM32	✓	✓	✓	✓
An. plumbeus	BM33	✓	✓	✓	✓
An. plumbeus	BM34	✓	✓	✓	✓
An. plumbeus	UK1006	✓	✓	✓	✓
An. plumbeus	UK1011	✓	✓	x	x
Cq. richiardii	BM123	✓	-	✓	-
Cq. richiardii	BM36	✓	x	P	x
Cq. richiardii	BM37	✓	-	P	-
Cq. richiardii	BM38	✓	x	x	x
Cq. richiardii	BM39	✓	x	x	x
Cq. richiardii	BM40	✓	x	x	x
Cq. richiardii	BM46	✓	✓	✓	✓
Cq. richiardii	BM47	✓	✓	✓	x
Cq. richiardii	BM48	✓	✓	✓	x
Cq. richiardii	BM49	✓	✓	✓	✓
Cq. richiardii	BM50	✓	✓	✓	P
Cq. richiardii	BM51	✓	✓	✓	✓
Cq. richiardii	BM52	✓	✓	✓	✓
Cq. richiardii	BM53	✓	✓	✓	P
Cq. richiardii	UK160	✓	✓	✓	✓
Cq. richiardii	UK209	✓	-	✓	-
Cq. richiardii	UK400	✓	-	✓	-
Cq. richiardii	UK877	✓	✓	✓	✓
Cq. richiardii	UK902	✓	✓	✓	x
Cs. annulata	BM54	✓	✓	✓	x
Cs. annulata	BM56	✓	✓	✓	x
Cs. annulata	BM57	✓	✓	✓	✓
Cs. annulata	BM58	✓	✓	✓	x
Cs. annulata	BM59	✓	✓	P	x
Cs. annulata	UK1246	✓	-	✓	-
Cs. annulata	UK1286	✓	-	✓	-
Cs. annulata	UK13	✓	-	✓	-
Cs. annulata	UK1424	x	-	x	-
Cs. annulata	UK1438	✓	-	✓	-
Cs. annulata	UK2527	✓	-	✓	-

		PCR amplification		Sanger sequencing	
Species	**Sample ref.**	***COI***	***ITS2***	***COI***	***ITS2***
Cs. annulata	UK731	✓	-	✓	-
Cs. annulata	UK786	✓	-	✓	-
Cs. annulata	UK833	✓	-	✓	-
Cs. annulata	UK956	✓	✓	✓	x
Cs. litorea	BM112	✓	✓	x	✓
Cs. litorea	BM18	-	✓	-	✓
Cs. litorea	UK1482	✓	✓	x	✓
Cs. litorea	UK1488	-	✓	-	✓
Cs. morsitans	BM107	✓	✓	x	x
Cs. morsitans	BM108	✓	✓	x	P
Cs. morsitans	BM109	-	✓	-	✓
Cs. morsitans	BM110	-	✓	-	P
Cs. morsitans	BM111	-	✓	-	P
Cs. morsitans	BM116	-	✓	-	x
Cs. morsitans	BM19	-	x	-	x
Cs. morsitans	BM20	-	✓	-	✓
Cs. morsitans	BM21	-	x	-	x
Cs. morsitans	BM22	-	x	-	x
Cs. morsitans	BM23	-	✓	-	P
Cs. morsitans	BM24	-	✓	-	x
Cs. morsitans	BM25	-	✓	-	x
Cs. morsitans	BM26	-	✓	-	x
Cs. morsitans	PHE24	-	✓	-	✓
Cs. morsitans	UK1072	-	✓	-	✓
Cs. morsitans	UK1073	-	✓	-	x
Cs. morsitans	UK1074	✓	✓	x	✓
Cs. morsitans	UK1076	-	✓	-	✓
Cs. morsitans	UK1078	✓	-	x	-
Cs. morsitans	UK1079	-	✓	-	P
Cs. morsitans	UK1084	-	✓	-	✓
Cs. morsitans	UK1093	✓	-	x	-
Cs. morsitans	UK1095	✓	✓	x	✓
Cs. morsitans	UK1097	✓	✓	x	P
Cs. morsitans	UK1102	✓	✓	x	x
Cs. morsitans	UK1104	-	✓	-	✓
Cs. morsitans	UK1105	✓	-	✓	-
Cs. morsitans	UK1106	-	✓	-	✓
Cs. morsitans	UK1115	-	✓	-	✓
Cs. morsitans	UK1121	✓	✓	x	x
Cs. morsitans	UK1126	-	✓	-	✓
Cs. morsitans	UK1128	-	✓	-	✓
Cs. morsitans	UK1130	-	✓	-	✓
Cs. morsitans	UK1136	-	✓	-	✓
Cs. morsitans	UK1145	-	✓	-	x

		PCR amplification		Sanger sequencing	
Species	**Sample ref.**	***COI***	***ITS2***	***COI***	***ITS2***
Cs. morsitans	UK1153	✓	✓	✓	✓
Cs. morsitans	UK1158	✓	-	x	-
Cs. morsitans	UK1159	-	x	-	x
Cs. morsitans	UK1162	✓	-	x	-
Cs. morsitans	UK1163	-	x	-	x
Cs. morsitans	UK1178	-	✓	-	✓
Cs. morsitans	UK1179	-	x	-	x
Cs. morsitans	UK1180	-	x	-	x
Cs. morsitans	UK1183	-	x	-	x
Cs. morsitans	UK1184	-	✓	-	P
Cs. morsitans	UK1185	✓		x	
Cs. morsitans	UK1187	-	✓	-	x
Cs. morsitans	UK1188	-	x	-	x
Cs. morsitans	UK1189	-	x	-	x
Cs. morsitans	UK1196	-	x	-	x
Cs. morsitans	UK1198	-	✓	-	x
Cs. morsitans	UK1203	-	✓	-	x
Cs. morsitans	UK1218	-	✓	-	x
Cs. morsitans	UK1237	-	✓	-	✓
Cs. morsitans	UK1253	-	✓	-	x
Cs. morsitans	UK1259	-	x	-	x
Cs. morsitans	UK1333	-	✓	-	✓
Cs. morsitans	UK1367	-	✓	-	x
Cs. morsitans	UK1427	-	✓	-	x
Cs. morsitans	UK1434	-	✓	-	✓
Cs. morsitans	UK1460	-	✓	-	✓
Cs. morsitans	UK1475	-	✓	-	x
Cs. morsitans	UK1505	✓	-	x	-
Cs. morsitans	UK1507	-	✓	-	✓
Cs. morsitans	UK1513	-	✓	-	✓
Cs. morsitans	UK1514	-	✓	-	x
Cs. morsitans	UK2094	✓	-	x	-
Cs. morsitans	UK2419	-	✓	-	P
Cs. morsitans	UK2428	-	✓	-	x
Cs. morsitans	UK2429	-	✓	-	P
Cs. morsitans	UK408	-	x	-	x
Cs. morsitans	UK414	-	✓	-	✓
Cs. morsitans	UK426	-	✓	-	P
Cs. morsitans	UK438	-	✓	-	✓
Cs. morsitans	UK439	-	✓	-	x
Cs. morsitans	UK445	✓	✓	x	✓
Cs. morsitans	UK446	-	✓	-	x
Cs. morsitans	UK461	-	✓	-	✓
Cs. morsitans	UK465	-	✓	-	✓

		PCR amplification		Sanger sequencing	
Species	**Sample ref.**	***COI***	***ITS2***	***COI***	***ITS2***
Cs. morsitans	UK467	-	✓	-	✓
Cs. morsitans	UK469	-	✓	-	✓
Cs. morsitans	UK471	-	✓	-	✓
Cs. morsitans	UK473	-	✓	-	✓
Cs. morsitans	UK476	-	✓	-	x
Cs. morsitans	UK478	-	✓	-	x
Cs. morsitans	UK480	-	✓	-	✓
Cs. morsitans	UK481	-	x	-	x
Cs. morsitans	UK486	-	✓	-	✓
Cs. morsitans	UK487	✓	✓	x	✓
Cs. morsitans	UK492	-	✓	-	✓
Cs. morsitans	UK493	-	✓	-	✓
Cs. morsitans	UK496	-	✓	-	✓
Cs. morsitans	UK497	-	✓	-	x
Cs. morsitans	UK498	-	✓	-	x
Cs. morsitans	UK889	✓	✓	x	✓
Cs. morsitans	UK900	-	x	-	x
Cs. morsitans	UK928	-	x	-	x
Cs. morsitans	UK937	✓	✓	x	x
Cs. morsitans	UK957	✓	x	x	x
Cx. modestus	BM61	✓	✓	✓	✓
Cx. modestus	BM62	✓	✓	✓	P
Cx. modestus	BM63	x	✓	x	x
Cx. modestus	BM64	✓	✓	P	✓
Cx. modestus	BM65	✓	✓	✓	P
Cx. pipiens form molestus	BM124	x	-	x	-
Cx. pipiens form molestus	BM125	x	-	x	-
Cx. pipiens form molestus	BM126	x	-	x	-
Cx. pipiens form molestus	BM127	x	-	x	-
Cx. pipiens form molestus	BM128	x	-	x	-
Cx. pipiens form molestus	BM129	x	-	x	-
Cx. pipiens s.l.	BM113	✓	✓	✓	✓
Cx. pipiens s.l.	BM72	✓	✓	P	✓
Cx. pipiens s.l.	BM73	x	x	x	x
Cx. pipiens s.l.	BM74	✓	✓	✓	✓
Cx. pipiens s.l.	BM75	✓	✓	P	P
Cx. pipiens s.l.	BM76	✓	✓	✓	✓
Cx. pipiens s.l.	PHE5	✓	-	✓	-
Cx. pipiens s.l.	PHE6	✓	-	✓	-
Cx. pipiens s.l.	PHE7	✓	-	✓	-
Cx. pipiens s.l.	UK638	✓	-	✓	-
Cx. pipiens s.l.	UK642	✓	-	✓	-
Cx. pipiens s.l.	UK658	✓	-	✓	-
Cx. pipiens s.l.	UK666	✓	-	✓	-

		PCR amplification		Sanger sequencing	
Species	**Sample ref.**	***COI***	***ITS2***	***COI***	***ITS2***
Cx. pipiens s.l.	UK681	✓	-	✓	-
Cx. pipiens s.l.	UK702	✓	-	P	-
Cx. pipiens s.l.	UK717	✓	-	✓	-
Cx. pippiens/torrentium	UK1019	✓	-	✓	-
Cx. pippiens/torrentium	UK1157	✓	-	✓	-
Cx. pippiens/torrentium	UK1168	✓	-	✓	-
Cx. pippiens/torrentium	UK1193	✓	-	✓	-
Cx. pippiens/torrentium	UK1269	✓	-	✓	-
Cx. pippiens/torrentium	UK1872	✓	-	✓	-
Cx. pippiens/torrentium	UK1879	✓	-	✓	-
Cx. territans	UK683	✓	✓	✓	x
Cx. torrentium	BM41	-	x	-	x
Cx. torrentium	BM42	-	x	-	x
Cx. torrentium	BM43	-	x	-	x
Cx. torrentium	BM44	-	x	-	x
Cx. torrentium	BM45	-	x	-	x
Cx. torrentium	BM77	-	x	-	x
Cx. torrentium	BM78	-	x	-	x
Cx. torrentium	BM79	-	x	-	x
Cx. torrentium	BM80	✓	✓	✓	P
Cx. torrentium	BM81	✓	✓	✓	✓
Cx. torrentium	UK466	✓	-	✓	-
Cx. torrentium	UK686	✓	-	✓	-
Cx. torrentium	UK716	✓	-	✓	-
Da. geniculata	BM119	-	x	-	x
Da. geniculata	BM97	✓	✓	P	✓
Da. geniculata	BM98	✓	✓	✓	✓
Da. geniculata	BM99	✓	✓	✓	✓
Da. geniculata (eggs)	CL5.1	✓	✓	x	✓
Da. geniculata (eggs)	CL7.1a	✓	✓	✓	x
Da. geniculata (eggs)	CL7.1b	✓	✓	x	x
Da. geniculata (eggs)	CL7.1c	✓	✓	x	x
Da. geniculata (eggs)	CL7.2	✓	✓	x	x
Da. geniculatus (eggs)	PHE28	✓	✓	x	x
Da. geniculatus (eggs)	PHE29	✓	✓	x	✓
Oc. annulipes	BM6	✓	✓	✓	✓
Oc. annulipes	BM7	✓	✓	✓	✓
Oc. annulipes	BM8	✓	✓	✓	✓
Oc. annulipes	BM87	x	✓	x	x
Oc. annulipes	BM88	✓	✓	✓	✓
Oc. annulipes	BM89	✓	✓	✓	✓
Oc. annulipes	BM90	✓	✓	✓	✓
Oc. annulipes	BM91	✓	✓	✓	✓
Oc. annulipes	UK541	✓	-	✓	-

		PCR amplification		Sanger sequencing	
Species	**Sample ref.**	***COI***	***ITS2***	***COI***	***ITS2***
Oc. annulipes	UK544	✓	-	✓	-
Oc. annulipes	UK552	✓	-	✓	-
Oc. annulipes	UK555	✓	-	✓	-
Oc. annulipes	UK557	✓	-	✓	-
Oc. annulipes/cantans	UK938	✓	✓	✓	x
Oc. cantans	BM92	✓	✓	✓	✓
Oc. cantans	BM93	✓	✓	✓	✓
Oc. cantans	BM94	✓	✓	✓	P
Oc. cantans	BM95	✓	✓	✓	P
Oc. cantans	BM96	✓	✓	✓	P
Oc. cantans	UK1045	✓	-	✓	-
Oc. cantans	UK1055	✓	-	✓	-
Oc. cantans	UK1133	✓	-	✓	-
Oc. cantans	UK560	✓	-	✓	-
Oc. caspius	BM82	✓	✓	✓	✓
Oc. caspius	BM83	✓	✓	✓	x
Oc. caspius	BM84	✓	✓	✓	✓
Oc. caspius	BM85	✓	✓	✓	✓
Oc. caspius	BM86	✓	✓	✓	✓
Oc. caspius	UK835	✓	-	✓	-
Oc. detritus	BM115	✓	✓	✓	✓
Oc. detritus	BM66	✓	✓	✓	✓
Oc. detritus	BM67	✓	✓	✓	✓
Oc. detritus	BM68	✓	✓	✓	✓
Oc. detritus	BM69	✓	✓	✓	✓
Oc. detritus	BM70	✓	✓	✓	✓
Oc. detritus	UK1537	✓	-	✓	-
Oc. detritus	UK1762	✓	-	✓	-
Oc. detritus	UK966	✓	-	✓	-
Oc. flavescens	PHE15	✓	✓	✓	✓
Oc. flavescens	PHE16	✓	✓	✓	P
Oc. flavescens	PHE17	✓	✓	✓	✓
Oc. flavescens	UK936	✓	✓	x	x
Oc. nigrinus	UK1	✓	✓	✓	✓
Oc. nigrinus	UK16	✓	✓	✓	x
Oc. nigrinus	UK1869	✓	✓	✓	✓
Oc. nigrinus	UK19	✓	✓	✓	✓
Oc. nigrinus	UK569	✓	-	✓	-
Oc. punctor	BM1	✓	✓	x	P
Oc. punctor	BM114	✓	✓	x	✓
Oc. punctor	BM120	-	x	-	x
Oc. punctor	BM121	-	x	-	x
Oc. punctor	BM122	-	x	-	x
Oc. punctor	BM2a	✓	✓	x	x

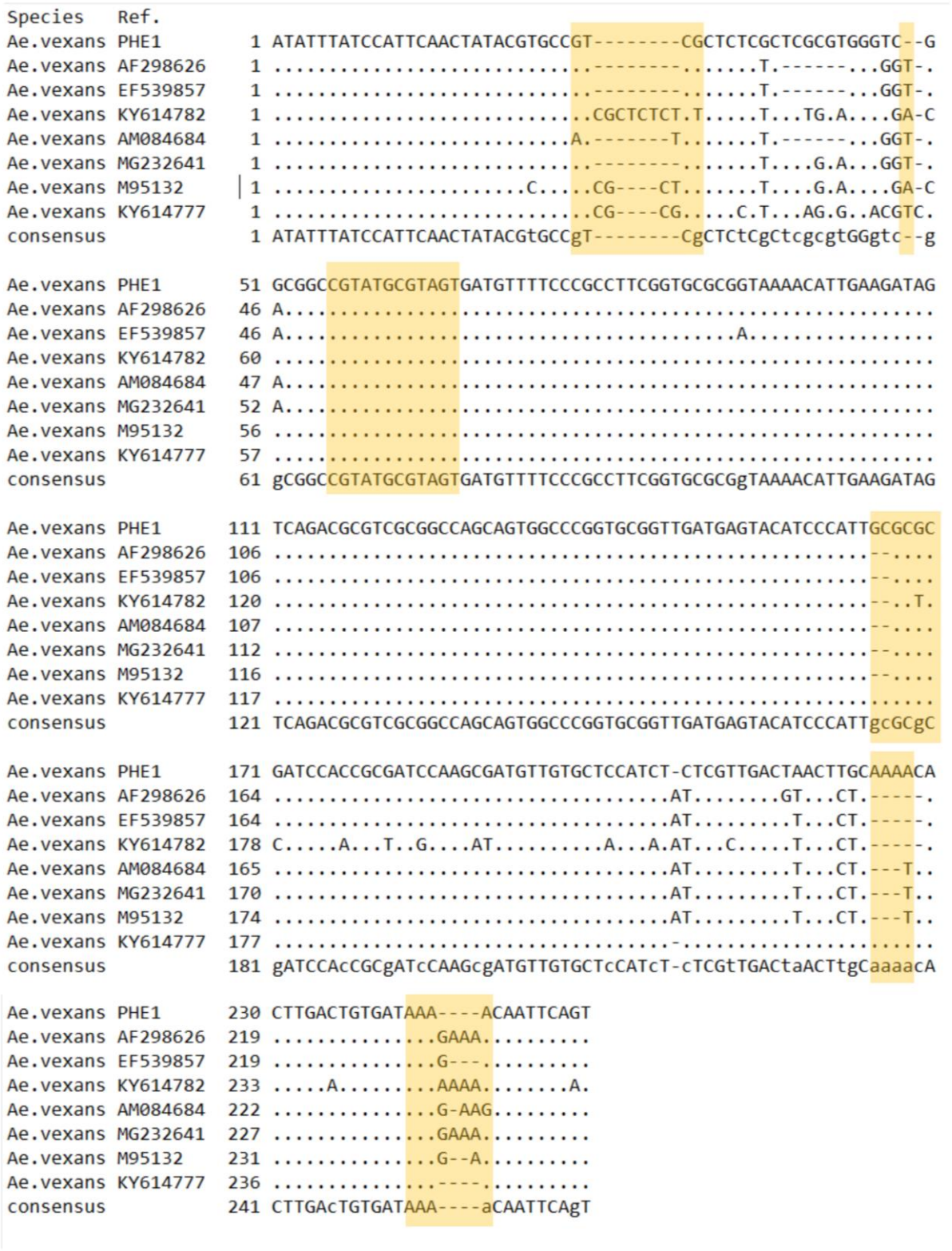
		PCR amplification		Sanger sequencing	
Species	**Sample ref.**	***COI***	***ITS2***	***COI***	***ITS2***
Oc. punctor	BM3a	✓	✓	x	✓
Oc. punctor	BM4a	✓	✓	x	P
Oc. punctor	BM5a	✓	✓	P	P
Oc. punctor	UK1088	✓	-	✓	-
Oc. punctor	UK1092	✓	-	✓	-
Oc. punctor	UK1509	✓	-	x	-
Oc. punctor	UK1523	✓	-	x	-
Oc. punctor	UK2316	✓	-	✓	-
Oc. punctor	UK237	✓	-	✓	-
Oc. punctor	UK241	✓	-	✓	-
Oc. punctor	UK244	✓	-	✓	-
Oc. punctor	UK705	✓	-	✓	-
Oc. punctor	UK858	✓	-	✓	-
Oc. punctor	UK998	✓	-	✓	-
Oc. rusticus	BM102	✓	✓	✓	✓
Oc. rusticus	BM103	✓	✓	✓	✓
Oc. rusticus	BM104	✓	✓	✓	✓
Oc. rusticus	BM105	✓	✓	✓	✓
Oc. rusticus	BM106	✓	✓	✓	✓
Oc. rusticus	UK1003	✓	-	✓	-
Oc. rusticus	UK1685	✓	x	✓	x
Oc. sticticus	UK923	✓	✓	P	x
Oc. sticticus	PHE11	✓	✓	✓	✓
Oc. sticticus	PHE12	✓	✓	✓	✓
Oc. sticticus	PHE13	✓	✓	✓	✓
Oc. sticticus	PHE14	✓	✓	✓	✓
Oc. sticticus	PHE18	✓	✓	✓	✓
Oc. sticticus	PHE21	✓	✓	✓	✓
Or. pulcripalpis	BM130	-	x	-	x

Appendices I. Table outlines the PCR and sequencing success of individual samples used within the study.

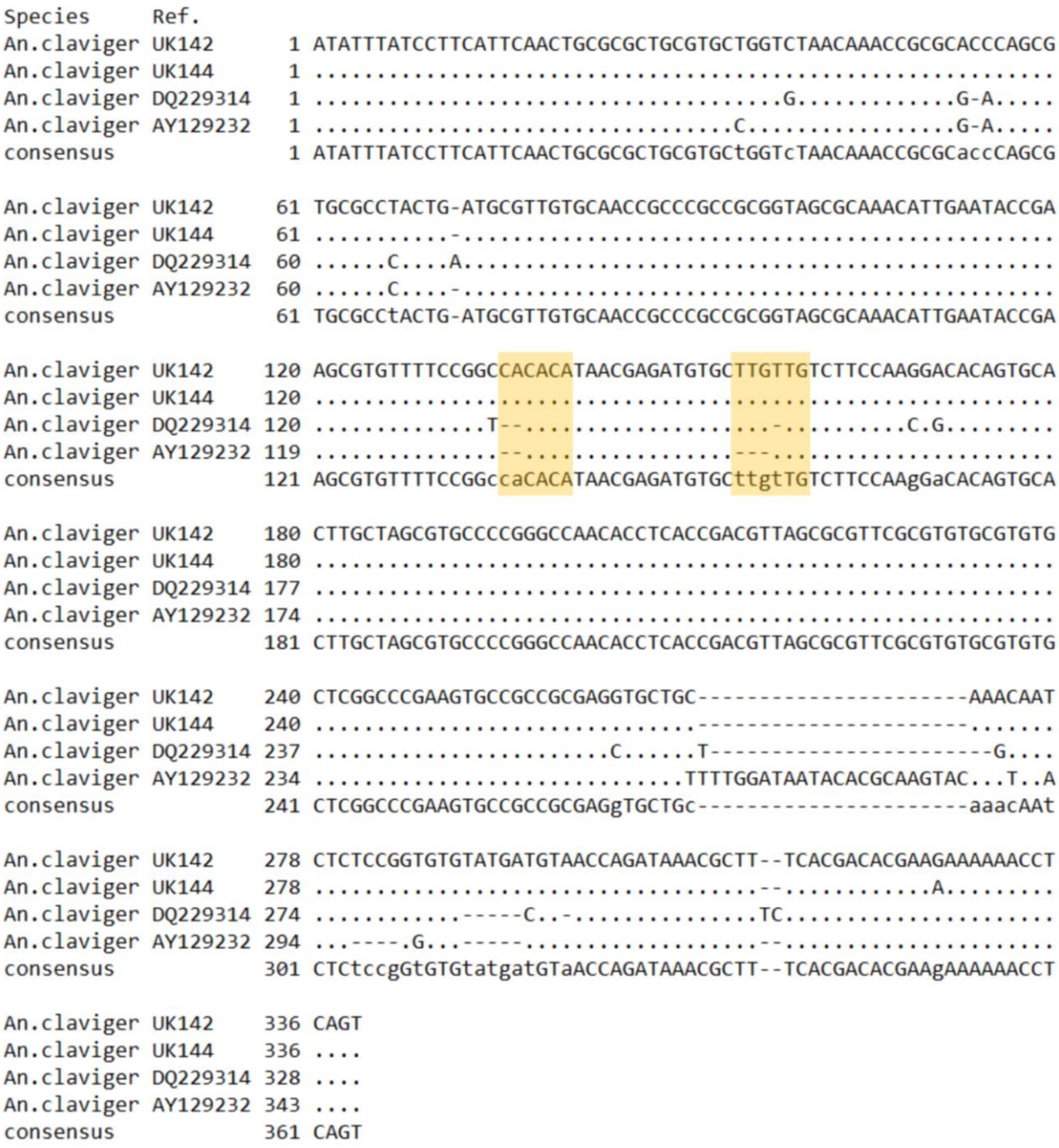
*sample possesses no similarity to other sequences on GenBank, probable pseudogene. ✓=Positive result, x = negative results, P = partial sequence data recovered (in COI this equates to sequence length of <500bp.

Appendix II – *ITS2* alignments of difficult sequence regions within GB mosquitoes.

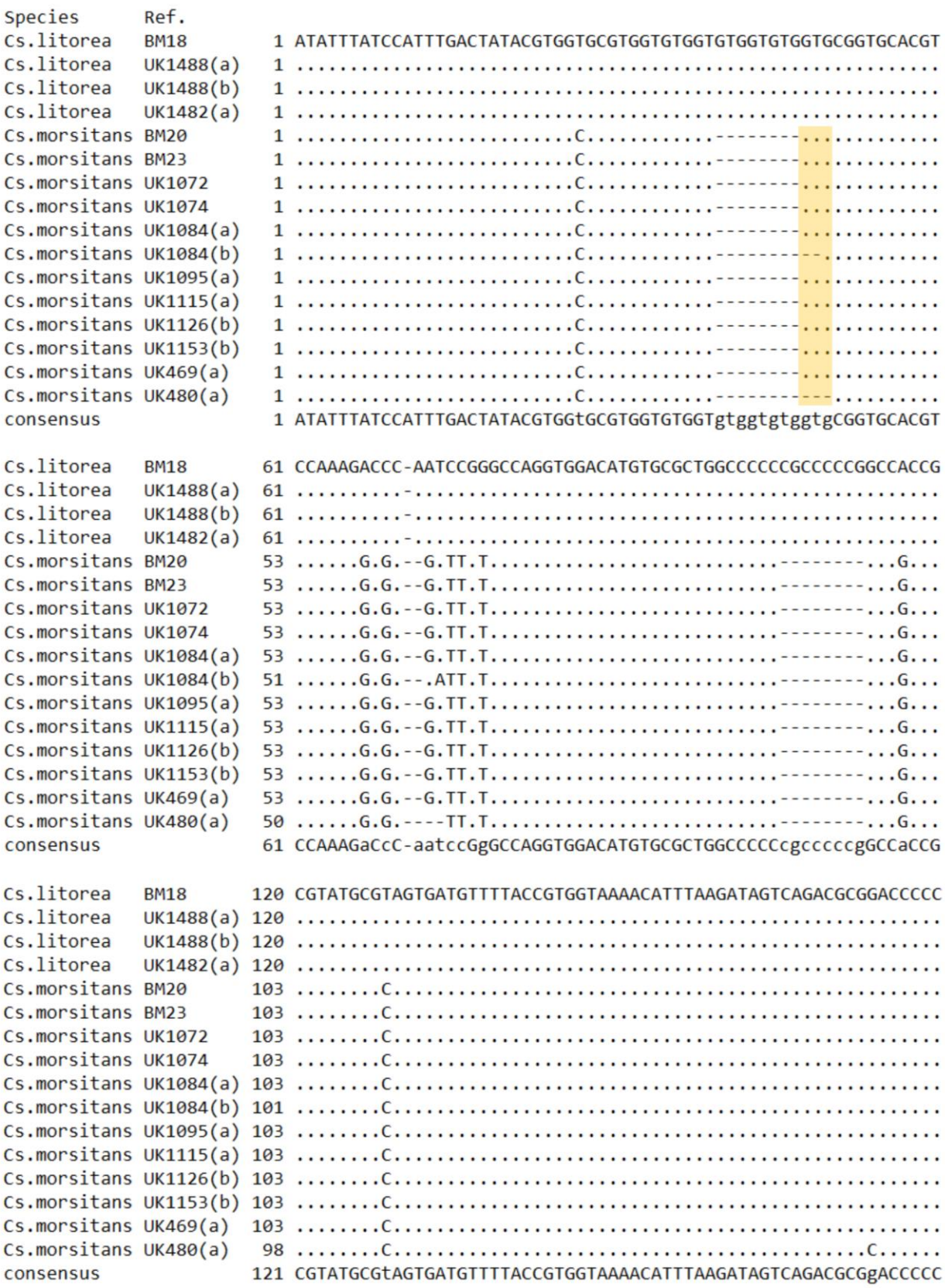
Appendix IIa. Alignment showing differences between the *ITS2* of closely related species *Ae. cinereus* and *Ae. geminus*. Highlights indicate areas that posed sequencing difficulties (as described in Table 4.2). Dots indicate homologous nucleotides, dashes represent gaps. Alignment constructed using BOXSHADE.

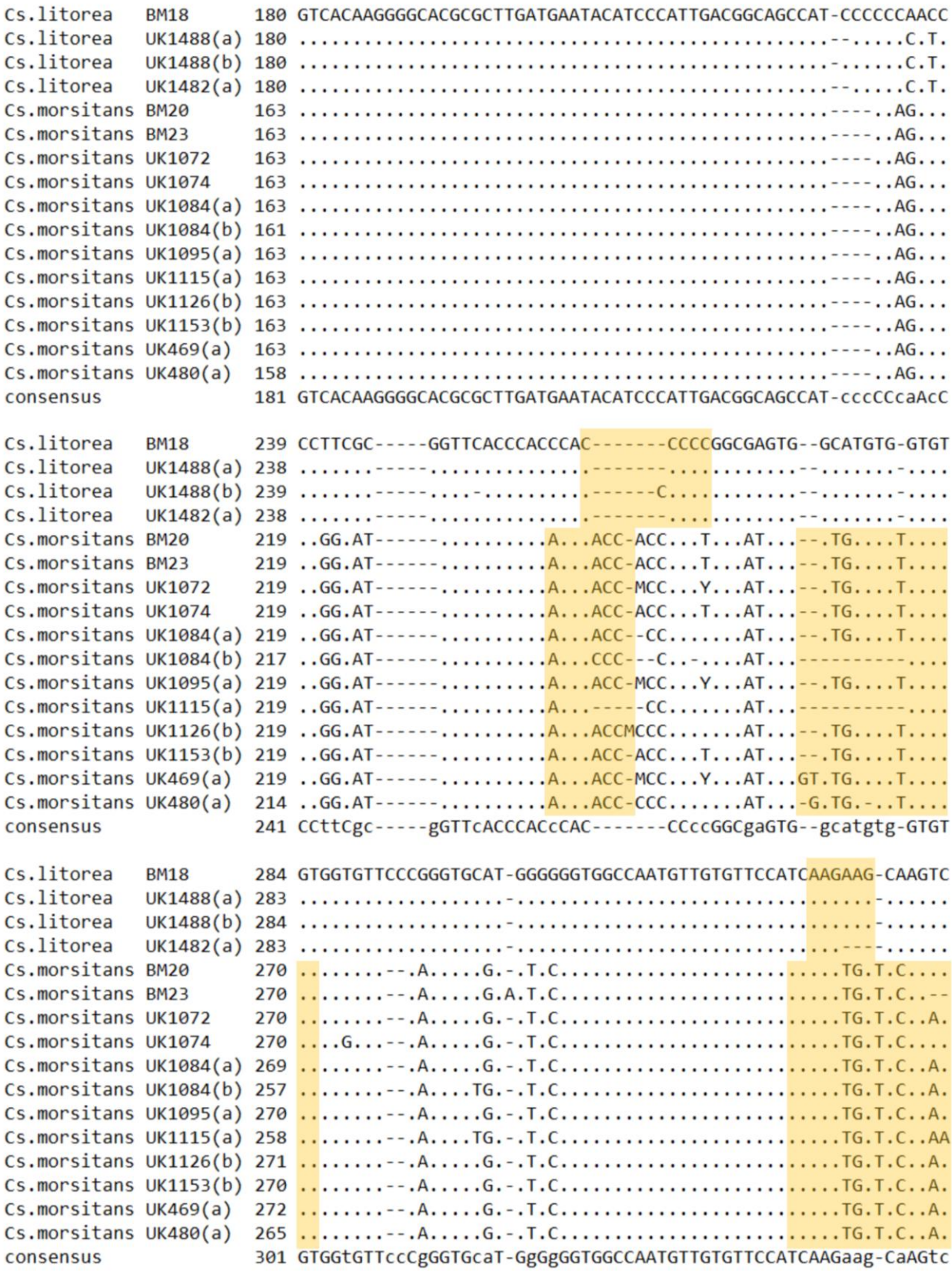


Appendix IIb. Alignment showing intraspecific differences between the *ITS2* of *Ae. vexans*. Highlights indicate areas that posed sequencing difficulties (as described in Table 4.2). Dots indicate homologous nucleotides, dashes represent gaps. Alignment constructed using BOXSHADE.



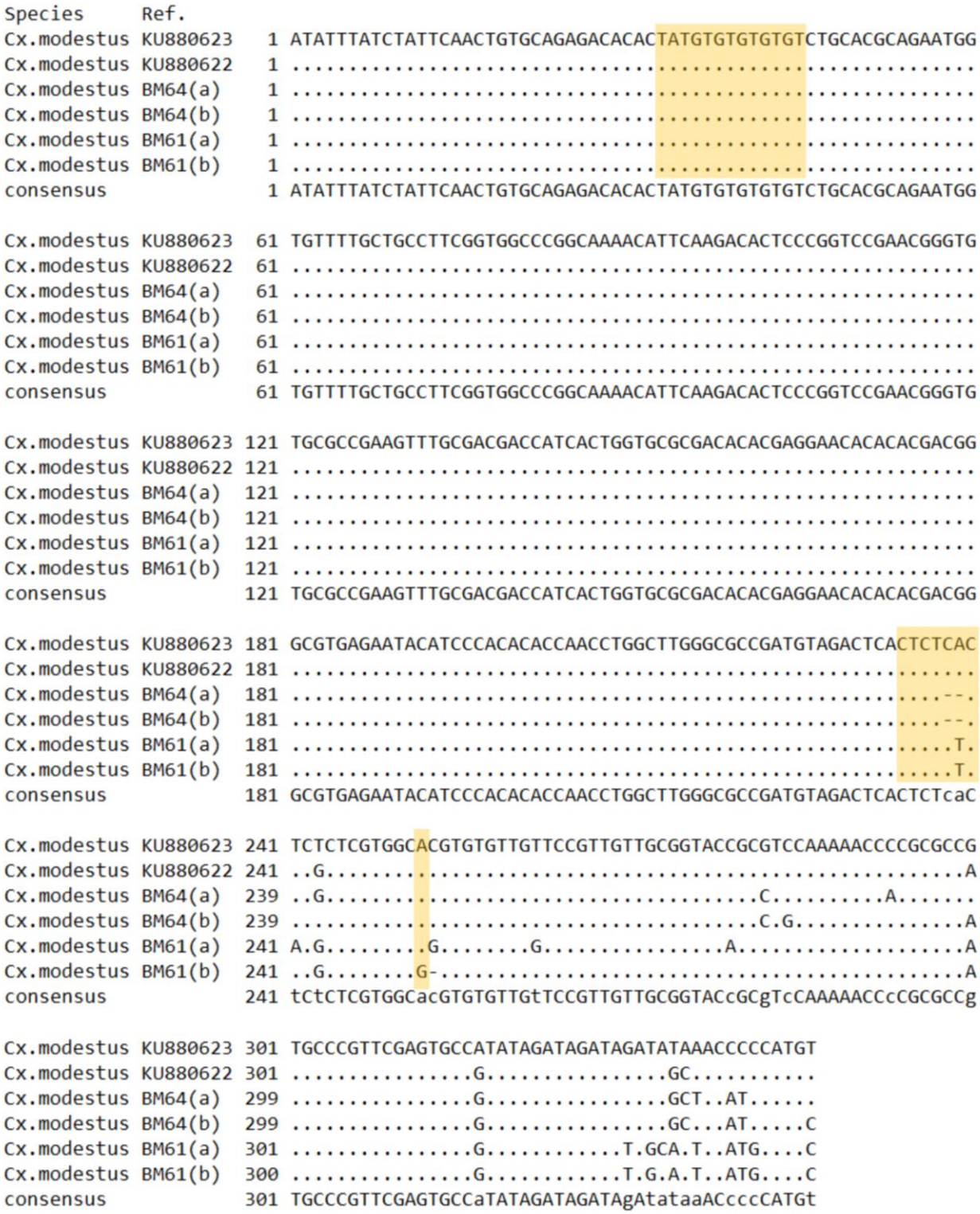
Appendix IIc. Alignment showing intraspecific differences between the *ITS2* of *An. claviger*. Highlights indicate areas that posed sequencing difficulties (as described in Table 4.2). Dots indicate homologous nucleotides, dashes represent gaps. Alignment constructed using BOXSHADE.


Species Ref.
Cs.litorea BM18 1 ATATTTATCCATTTGACTATACGTGGTGCGTGGTGTGGTGTGGTGTGGTGCGGTGCACGT
Cs.litorea UK1488(a) 1 .....
Cs.litorea UK1488(b) 1 .....
Cs.litorea UK1482(a) 1 .....
Cs.morsitans BM20 1 .....C.....-----...
Cs.morsitans BM23 1 .....C.....-----...
Cs.morsitans UK1072 1 .....C.....-----...
Cs.morsitans UK1074 1 .....C.....-----...
Cs.morsitans UK1084(a) 1 .....C.....-----...
Cs.morsitans UK1084(b) 1 .....C.....-----...
Cs.morsitans UK1095(a) 1 .....C.....-----...
Cs.morsitans UK1115(a) 1 .....C.....-----...
Cs.morsitans UK1126(b) 1 .....C.....-----...
Cs.morsitans UK1153(b) 1 .....C.....-----...
Cs.morsitans UK469(a) 1 .....C.....-----...
Cs.morsitans UK480(a) 1 .....C.....-----...
consensus 1 ATATTTATCCATTTGACTATACGTGGtGCGTGGTGTGGTgtggtgtggtgCGGTGCACGT
Cs.litorea BM18 61 CCAAAGACCC-AATCCGGGCCAGGTGGACATGTGCGCTGGCCCCCCGCCCCCGGCCACCG
Cs.litorea UK1488(a) 61 .....-.....
Cs.litorea UK1488(b) 61 .....-.....
Cs.litorea UK1482(a) 61 .....-.....
Cs.morsitans BM20 53 .....G.G.--G.TT.T.....---------...G...
Cs.morsitans BM23 53 .....G.G.--G.TT.T.....---------...G...
Cs.morsitans UK1072 53 .....G.G.--G.TT.T.....---------...G...
Cs.morsitans UK1074 53 .....G.G.--G.TT.T.....---------...G...
Cs.morsitans UK1084(a) 53 .....G.G.--G.TT.T.....---------...G...
Cs.morsitans UK1084(b) 51 .....G.G.--.ATT.T.....---------...G...
Cs.morsitans UK1095(a) 53 .....G.G.--G.TT.T.....---------...G...
Cs.morsitans UK1115(a) 53 .....G.G.--G.TT.T.....---------...G...
Cs.morsitans UK1126(b) 53 .....G.G.--G.TT.T.....---------...G...
Cs.morsitans UK1153(b) 53 .....G.G.--G.TT.T.....---------...G...
Cs.morsitans UK469(a) 53 .....G.G.--G.TT.T.....---------...G...
Cs.morsitans UK480(a) 50 .....G.G.----TT.T.....---------...G...
consensus 61 CCAAAGaCcC-aatccGgGCCAGGTGGACATGTGCGCTGGCCCCCcgccccgGCCaCCG
Cs.litorea BM18 120 CGTATGCGTAGTGATGTTTTACCGTGGTAAAACATTTAAGATAGTCAGACGCGGACCCCC
Cs.litorea UK1488(a) 120 .....
Cs.litorea UK1488(b) 120 .....
Cs.litorea UK1482(a) 120 .....
Cs.morsitans BM20 103 .....C.....
Cs.morsitans BM23 103 .....C.....
Cs.morsitans UK1072 103 .....C.....
Cs.morsitans UK1074 103 .....C.....
Cs.morsitans UK1084(a) 103 .....C.....
Cs.morsitans UK1084(b) 101 .....C.....
Cs.morsitans UK1095(a) 103 .....C.....
Cs.morsitans UK1115(a) 103 .....C.....
Cs.morsitans UK1126(b) 103 .....C.....
Cs.morsitans UK1153(b) 103 .....C.....
Cs.morsitans UK469(a) 103 .....C.....
Cs.morsitans UK480(a) 98 .....C.....C.....
consensus 121 CGTATGCGtAGTGATGTTTTACCGTGGTAAAACATTTAAGATAGTCAGACGCGgACCCCC

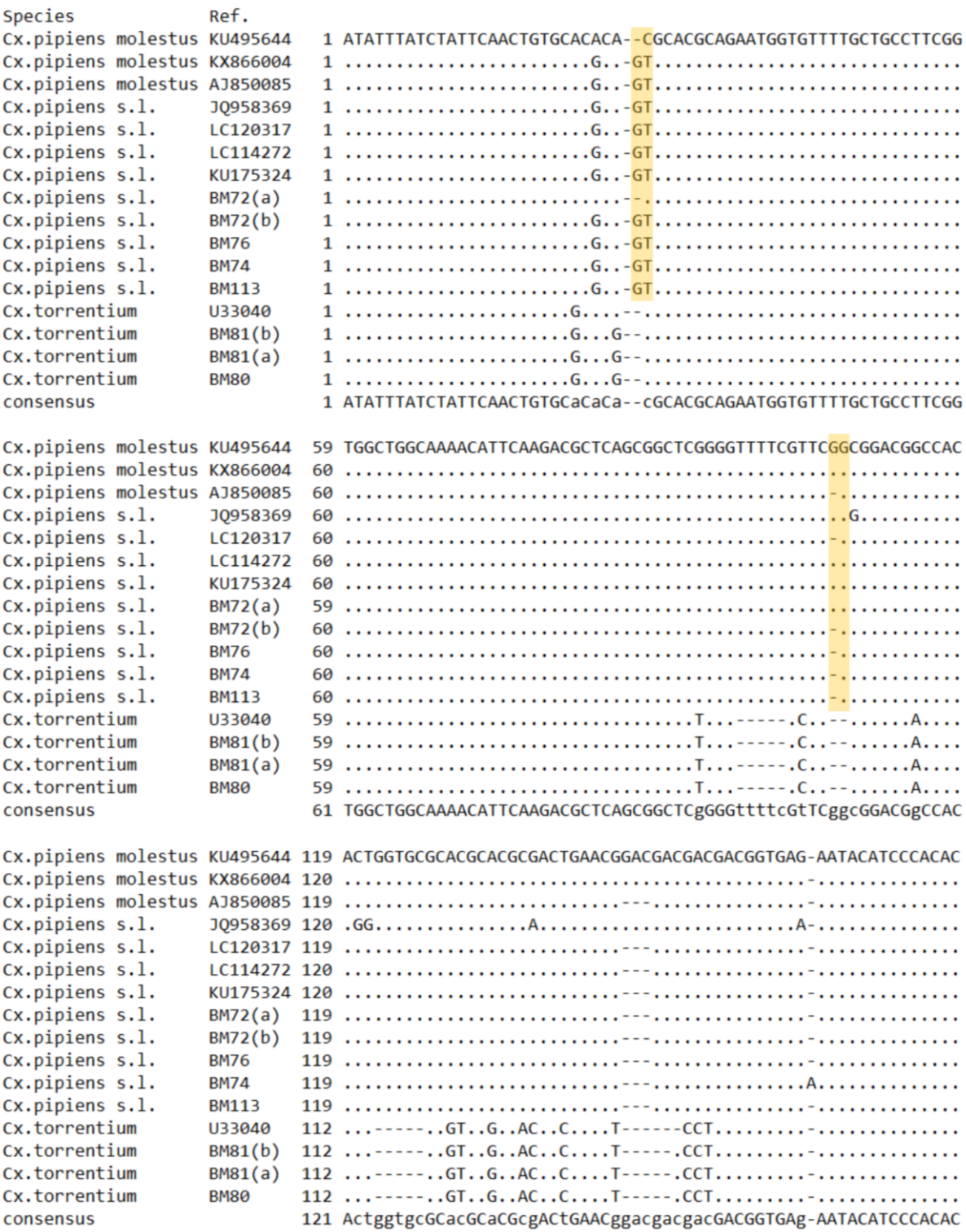

Cs.litorea BM18 180 GTCACAAGGGGCACGCGCTTGATGAATACATCCCATTGACGGCAGCCAT-CCCCCCAACC
Cs.litorea UK1488(a) 180 .....--.....C.T.
Cs.litorea UK1488(b) 180 .....-.....C.T.
Cs.litorea UK1482(a) 180 .....--.....C.T.
Cs.morsitans BM20 163 .....----..AG...
Cs.morsitans BM23 163 .....----..AG...
Cs.morsitans UK1072 163 .....----..AG...
Cs.morsitans UK1074 163 .....----..AG...
Cs.morsitans UK1084(a) 163 .....----..AG...
Cs.morsitans UK1084(b) 161 .....----..AG...
Cs.morsitans UK1095(a) 163 .....----..AG...
Cs.morsitans UK1115(a) 163 .....----..AG...
Cs.morsitans UK1126(b) 163 .....----..AG...
Cs.morsitans UK1153(b) 163 .....----..AG...
Cs.morsitans UK469(a) 163 .....----..AG...
Cs.morsitans UK480(a) 158 .....----..AG...
consensus 181 GTCACAAGGGGCACGCGCTTGATGAATACATCCCATTGACGGCAGCCAT-cccCCcaAcC
Cs.litorea BM18 239 CCTTCGC-----GGTTCACCCACCCAC-------CCCCGGCGAGTG--GCATGTG-GTGT
Cs.litorea UK1488(a) 238 .....-----.....-------.....--.....-....
Cs.litorea UK1488(b) 239 .....-----....-.....------C.....--.....-....
Cs.litorea UK1482(a) 238 .....-----.....-------.....--.....-....
Cs.morsitans BM20 219 ..GG.AT------.....A...ACC-ACC...T...AT...--.TG....T....
Cs.morsitans BM23 219 ..GG.AT------.....A...ACC-ACC...T...AT...--.TG....T....
Cs.morsitans UK1072 219 ..GG.AT------.....A...ACC-MCC...Y...AT...--.TG....T....
Cs.morsitans UK1074 219 ..GG.AT------.....A...ACC-ACC...T...AT...--.TG....T....
Cs.morsitans UK1084(a) 219 ..GG.AT------.....A...ACC--CC.....AT...--.TG....T....
Cs.morsitans UK1084(b) 217 ..GG.AT------.....A...CCC---C..-....AT...----------....
Cs.morsitans UK1095(a) 219 ..GG.AT------.....A...ACC-MCC...Y...AT...--.TG....T....
Cs.morsitans UK1115(a) 219 ..GG.AT------.....A.....-----CC.....AT...----------....
Cs.morsitans UK1126(b) 219 ..GG.AT------.....A...ACCMCCC.....AT...--.TG....T....
Cs.morsitans UK1153(b) 219 ..GG.AT------.....A...ACC-ACC...T...AT...--.TG....T....
Cs.morsitans UK469(a) 219 ..GG.AT------.....A...ACC-MCC...Y...AT...GT.TG....T....
Cs.morsitans UK480(a) 214 ..GG.AT------.....A...ACC-CCC.....AT...-G.TG.-..T....
consensus 241 CCttCgc-----gGTTcACCCACcCAC-------CCccGGCgaGTG--gcatgtg-GTGT
Cs.litorea BM18 284 GTGGTGTTCCCGGGTGCAT-GGGGGGTGGCCAATGTTGTGTTCCATCAAGAAG-CAAGTC
Cs.litorea UK1488(a) 283 .....-.....-......
Cs.litorea UK1488(b) 284 .....-.....-......
Cs.litorea UK1482(a) 283 .....-.....---.....
Cs.morsitans BM20 270 ..........--.A.....G.-.T.C.....TG.T.C....
Cs.morsitans BM23 270 ..........--.A.....G.A.T.C.....TG.T.C..---
Cs.morsitans UK1072 270 ..........--.A.....G.-.T.C.....TG.T.C..A.
Cs.morsitans UK1074 270 ....G.....--.A.....G.-.T.C.....TG.T.C....
Cs.morsitans UK1084(a) 269 ..........--.A.....G.-.T.C.....TG.T.C..A.
Cs.morsitans UK1084(b) 257 ..........--.A....TG.-.T.C.....TG.T.C..A.
Cs.morsitans UK1095(a) 270 ..........--.A.....G.-.T.C.....TG.T.C..A.
Cs.morsitans UK1115(a) 258 ..........--.A....TG.-.T.C.....TG.T.C..AA
Cs.morsitans UK1126(b) 271 ..........--.A.....G.-.T.C.....TG.T.C..A.
Cs.morsitans UK1153(b) 270 ..........--.A.....G.-.T.C.....TG.T.C..A.
Cs.morsitans UK469(a) 272 ..........--.A.....G.-.T.C.....TG.T.C..A.
Cs.morsitans UK480(a) 265 ..........--.A.....G.-.T.C.....TG.T.C..A.
consensus 301 GTGGtGTTccCgGGTGcaT-GgGgGGTGGCCAATGTTGTGTTCCATCAAGaag-CaAGtc

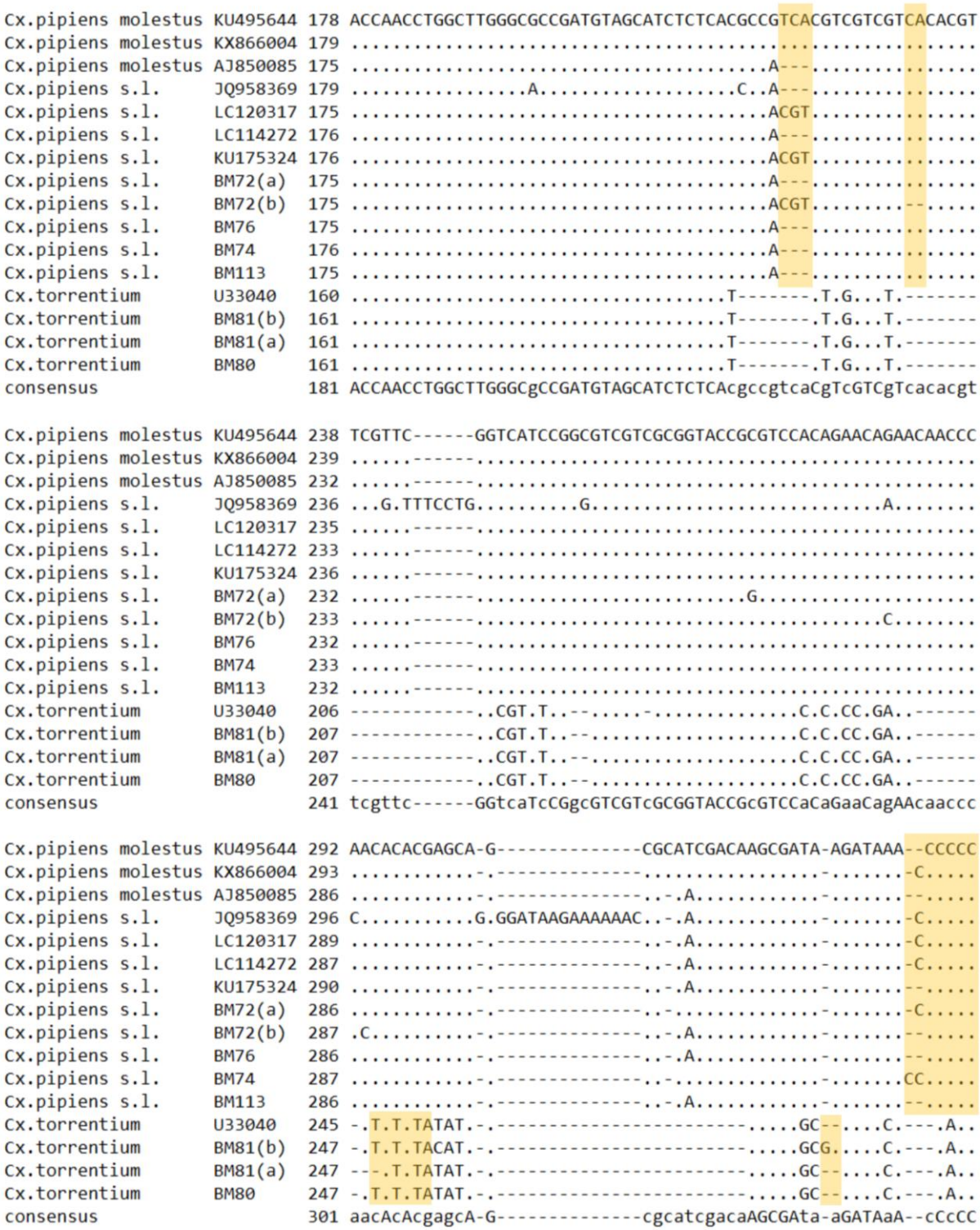
```
Cs.litorea   BM18      342 AAGTCATAGTGCGCATATCAGT
Cs.litorea   UK1488(a) 341 ...C.G..............
Cs.litorea   UK1488(b) 342 ...C.G..............
Cs.litorea   UK1482(b) 338 ...C.G..............
Cs.morsitans BM20      327 TCAA..GT---.-----.....
Cs.morsitans BM23          ----------------------
Cs.morsitans UK1072    327 ..CC..A.---.AACAG.....
Cs.morsitans UK1074    327 TCAA..GT---.-----.....
Cs.morsitans UK1084(a) 326 ..CC..A.---.AACAG.....
Cs.morsitans UK1084(b) 314 ..CC..A.---.AACAG.....
Cs.morsitans UK1095(a) 327 ..CC..A.---.AACAG.....
Cs.morsitans UK1115(a) 315 ..CC..A.---.AACAG.....
Cs.morsitans UK1126(b) 328 ..CC..A.---.AACAG.....
Cs.morsitans UK1153(b) 327 ..CC..A.---.AACAG.....
Cs.morsitans UK469(a)  329 ..CC..A.---.AACAG.....
Cs.morsitans UK480(a)  322 ..CC..A.---.AACAG.....
consensus              361 aagtcatagtgcgcatatcagt
```

Appendix IId. Alignment showing intra and interspecific differences between the *ITS2* of closely related species *Cs. litorea* and *Cs. morsitans*. Highlights indicate areas that posed sequencing difficulties (as described in Table 4.2). Dots indicate homologous nucleotides, dashes represent gaps. Alignment constructed using BOXSHADE.



Appendix IIe. Alignment showing intraspecific differences between the *ITS2* of *Cx. modestus*. Highlights indicate areas that posed sequencing difficulties (as described in Table 4.2). Dots indicate homologous nucleotides, dashes represent gaps. Alignment constructed using BOXSHADE.


Species Ref.
Cx.pipiens molestus KU495644 1 ATATTTATCTATTCAACTGTGCACACA--CGCACGCAGAATGGTGTTTTGCTGCCTTCGG
Cx.pipiens molestus KX866004 1 .....G..-GT.....
Cx.pipiens molestus AJ850085 1 .....G..-GT.....
Cx.pipiens s.l. JQ958369 1 .....G..-GT.....
Cx.pipiens s.l. LC120317 1 .....G..-GT.....
Cx.pipiens s.l. LC114272 1 .....G..-GT.....
Cx.pipiens s.l. KU175324 1 .....G..-GT.....
Cx.pipiens s.l. BM72(a) 1 .....--.....
Cx.pipiens s.l. BM72(b) 1 .....G..-GT.....
Cx.pipiens s.l. BM76 1 .....G..-GT.....
Cx.pipiens s.l. BM74 1 .....G..-GT.....
Cx.pipiens s.l. BM113 1 .....G..-GT.....
Cx.torrentium U33040 1 .....G....--.....
Cx.torrentium BM81(b) 1 .....G...G--.....
Cx.torrentium BM81(a) 1 .....G...G--.....
Cx.torrentium BM80 1 .....G...G--.....
consensus 1 ATATTTATCTATTCAACTGTGCaCaCa--cGCACGCAGAATGGTGTTTTGCTGCCTTCGG
Cx.pipiens molestus KU495644 59 TGGCTGGCAAAACATTCAAGACGCTCAGCGGCTCGGGGTTTTCGTTCGGCGGACGGCCAC
Cx.pipiens molestus KX866004 60 .....
Cx.pipiens molestus AJ850085 60 .....-.....
Cx.pipiens s.l. JQ958369 60 .....G.....
Cx.pipiens s.l. LC120317 60 .....-.....
Cx.pipiens s.l. LC114272 60 .....
Cx.pipiens s.l. KU175324 60 .....
Cx.pipiens s.l. BM72(a) 59 .....
Cx.pipiens s.l. BM72(b) 60 .....-.....
Cx.pipiens s.l. BM76 60 .....-.....
Cx.pipiens s.l. BM74 60 .....-.....
Cx.pipiens s.l. BM113 60 .....-.....
Cx.torrentium U33040 59 .....T..------.C..--.....A....
Cx.torrentium BM81(b) 59 .....T..------.C..--.....A....
Cx.torrentium BM81(a) 59 .....T..------.C..--.....A....
Cx.torrentium BM80 59 .....T..------.C..--.....A....
consensus 61 TGGCTGGCAAAACATTCAAGACGCTCAGCGGCTCgGGGttttcGtTCggcGGACGgCCAC
Cx.pipiens molestus KU495644 119 ACTGGTGCGCACGCACGCGACTGAACGGACGACGACGACGGTGAG-AATACATCCCACAC
Cx.pipiens molestus KX866004 120 .....-.....
Cx.pipiens molestus AJ850085 119 .....---.....-.....
Cx.pipiens s.l. JQ958369 120 .GG.....A.....A-.....
Cx.pipiens s.l. LC120317 119 .....---.....-.....
Cx.pipiens s.l. LC114272 120 .....---.....-.....
Cx.pipiens s.l. KU175324 120 .....---.....-.....
Cx.pipiens s.l. BM72(a) 119 .....---.....-.....
Cx.pipiens s.l. BM72(b) 119 .....---.....-.....
Cx.pipiens s.l. BM76 119 .....---.....-.....
Cx.pipiens s.l. BM74 119 .....---.....A.....
Cx.pipiens s.l. BM113 119 .....---.....-.....
Cx.torrentium U33040 112 ...------..GT..G..AC..C....T------CCT.....-.....
Cx.torrentium BM81(b) 112 ...------..GT..G..AC..C....T-----.CCT.....-.....
Cx.torrentium BM81(a) 112 ...------..GT..G..AC..C....T-----.CCT.....-.....
Cx.torrentium BM80 112 ...------..GT..G..AC..C....T-----.CCT.....-.....
consensus 121 ActggtgcGCacGCaCGcgACtGAACggacgacgacGACGGTGAg-AATACATCCCACAC


Cx.pipiens molestus KU495644 178 ACCAACCTGGCTTGGGCGCCGATGTAGCATCTCTCACGCCGTCACGTCGTCGTCACACGT
Cx.pipiens molestus KX866004 179 .....
Cx.pipiens molestus AJ850085 175 .....A---.....
Cx.pipiens s.l. JQ958369 179 .....A.....C..A---.....
Cx.pipiens s.l. LC120317 175 .....ACGT.....
Cx.pipiens s.l. LC114272 176 .....A---.....
Cx.pipiens s.l. KU175324 176 .....ACGT.....
Cx.pipiens s.l. BM72(a) 175 .....A---.....
Cx.pipiens s.l. BM72(b) 175 .....ACGT.....--.....
Cx.pipiens s.l. BM76 175 .....A---.....
Cx.pipiens s.l. BM74 176 .....A---.....
Cx.pipiens s.l. BM113 175 .....A---.....
Cx.torrentium U33040 160 .....T-------.T.G...T.-------
Cx.torrentium BM81(b) 161 .....T-------.T.G...T.-------
Cx.torrentium BM81(a) 161 .....T-------.T.G...T.-------
Cx.torrentium BM80 161 .....T-------.T.G...T.-------
consensus 181 ACCAACCTGGCTTGGGCgCCGATGTAGCATCTCTCAcgccgtcaCgTcGTCgTcacacgt
Cx.pipiens molestus KU495644 238 TCGTTC------GGTCATCCGGCGTCGTCGCGGTACCGCGTCCACAGAACAGAACAACCC
Cx.pipiens molestus KX866004 239 .....------.....
Cx.pipiens molestus AJ850085 232 .....------.....
Cx.pipiens s.l. JQ958369 236 ...G.TTTCCTG.....G.....A.....
Cx.pipiens s.l. LC120317 235 .....------.....
Cx.pipiens s.l. LC114272 233 .....------.....
Cx.pipiens s.l. KU175324 236 .....------.....
Cx.pipiens s.l. BM72(a) 232 .....------.....G.....
Cx.pipiens s.l. BM72(b) 233 .....------.....C.....
Cx.pipiens s.l. BM76 232 .....------.....
Cx.pipiens s.l. BM74 233 .....------.....
Cx.pipiens s.l. BM113 232 .....------.....
Cx.torrentium U33040 206 ------------..CGT.T..--.....-.....C.C.CC.GA..------
Cx.torrentium BM81(b) 207 ------------..CGT.T..--.....C.C.CC.GA..------
Cx.torrentium BM81(a) 207 ------------..CGT.T..--.....C.C.CC.GA..------
Cx.torrentium BM80 207 ------------..CGT.T..--.....C.C.CC.GA..------
consensus 241 tcgttc------GGtcaTcCGgcGTCGTcGCGGTACCGcGTCCaCaGaaCagAAcaaccc
Cx.pipiens molestus KU495644 292 AACACACGAGCA-G--------------CGCATCGACAAGCGATA-AGATAAA--CCCCC
Cx.pipiens molestus KX866004 293 .....-.....-.....-.....-.....-C.....
Cx.pipiens molestus AJ850085 286 .....-.....-..-.A.....-.....--.....
Cx.pipiens s.l. JQ958369 296 C.....G.GGATAAGAAAAAAC..-.A.....-.....-C.....
Cx.pipiens s.l. LC120317 289 .....-.....-..-.A.....-.....-C.....
Cx.pipiens s.l. LC114272 287 .....-.....-..-.A.....-.....-C.....
Cx.pipiens s.l. KU175324 290 .....-.....-..-.A.....-.....--.....
Cx.pipiens s.l. BM72(a) 286 .....-.....-..-.....-.....-C.....
Cx.pipiens s.l. BM72(b) 287 .C.....-.....-..-.A.....-.....--.....
Cx.pipiens s.l. BM76 286 .....-.....-..-.A.....-.....--.....
Cx.pipiens s.l. BM74 287 .....-.....-..-.....-.....CC.....
Cx.pipiens s.l. BM113 286 .....-.....-..-.A.....-.....--.....
Cx.torrentium U33040 245 -.T.T.TATAT.-.-----.....GC--....C.---.A..
Cx.torrentium BM81(b) 247 -.T.T.TACAT.-.-----.....GCG.....C.---.A..
Cx.torrentium BM81(a) 247 ---.T.TATAT.-.-----.....GC--....C.---.A..
Cx.torrentium BM80 247 -.T.T.TATAT.-.-----.....GC--....C.---.A..
consensus 301 aacAcAcgagcA-G--------------cgcatcgacaAGCGAta-aGATAaA--cCcCC

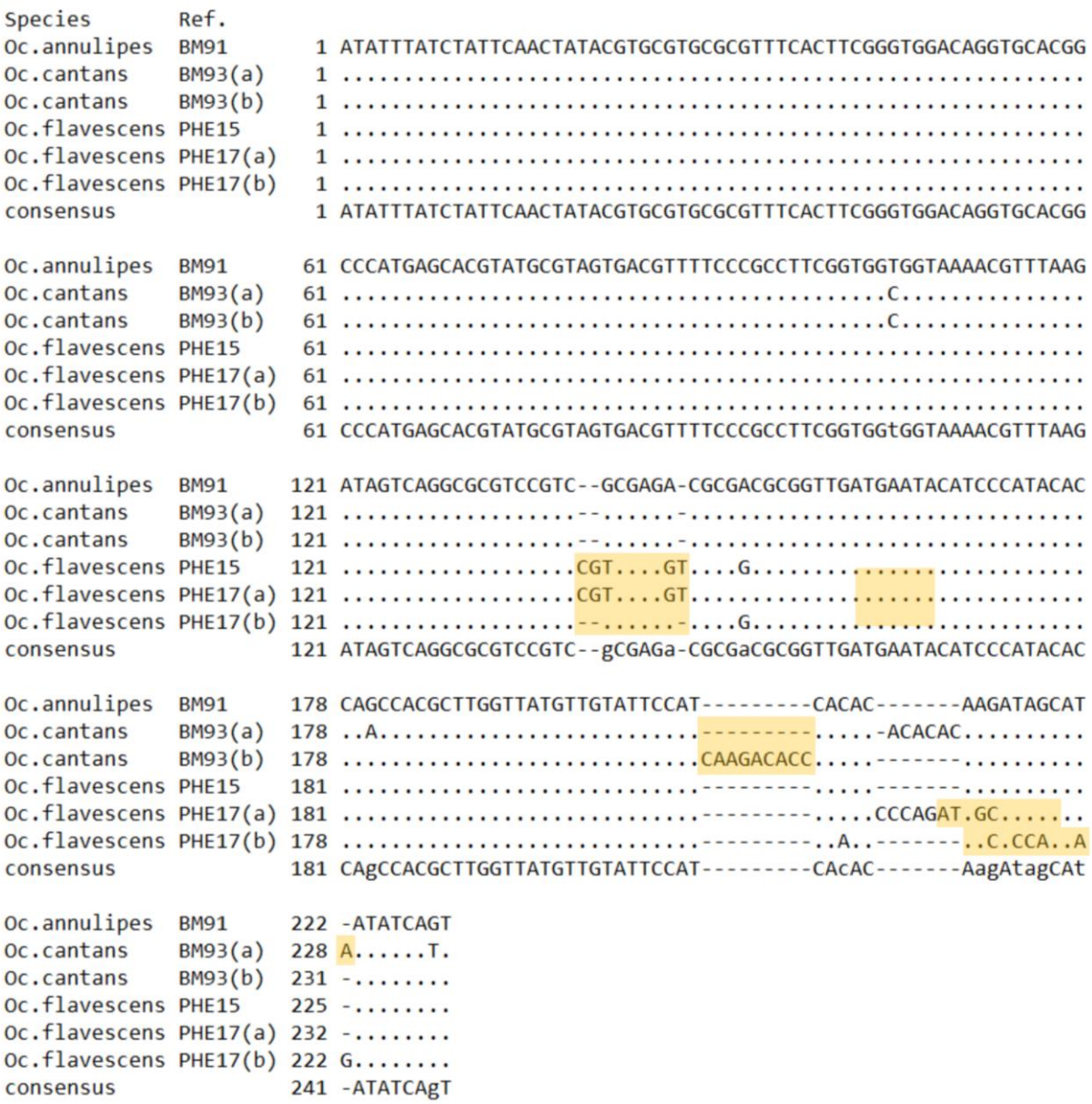
```
Cx.pipiens molestus KU495644 334 ATGT
Cx.pipiens molestus KX866004 336 ....
Cx.pipiens molestus AJ850085 327 ....
Cx.pipiens s.l.     JQ958369 353 ....
Cx.pipiens s.l.     LC120317 331 ....
Cx.pipiens s.l.     LC114272 329 ....
Cx.pipiens s.l.     KU175324 331 ....
Cx.pipiens s.l.     BM72(a)  328 ....
Cx.pipiens s.l.     BM72(b)  328 ....
Cx.pipiens s.l.     BM76     327 ....
Cx.pipiens s.l.     BM74     330 ....
Cx.pipiens s.l.     BM113    327 ....
Cx.torrentium       U33040   274 ....
Cx.torrentium       BM81(b)  278 ....
Cx.torrentium       BM81(a)  274 ....
Cx.torrentium       BM80     276 ....
consensus                    361 ATGT
```

Appendix IIf. Alignment showing intra and interspecific differences between the *ITS2* of *Cx. pipiens s.l. and Cx. torrentium*. Highlights indicate areas that posed sequencing difficulties (as described in Table 4.2). Dots indicate homologous nucleotides, dashes represent gaps. Alignment constructed using BOXSHADE.


Species Ref.
Da.geniculata BM98(a) 1 ATATTTATCCATTCAACTATACGTGTGCGTGCGCGTCCCACCCCGGGTGGACAGGCGCAC
Da.geniculata BM98(b) 1 .....
Da.geniculata PHE29(a) 1 .....
Da.geniculata PHE29(b) 1 .....
Da.geniculata CL5.1 1 .....
Da.geniculata KF471603 1 .....T.....
Da.geniculata KF471605 1 .....
Da.geniculata MG232621 1 .....
Da.geniculata KF471610 1 .....
consensus 1 ATATTTATCcATTCAACTATACGTGTGCGTGCGCGTCCCACCCCGGGTGGACAGGCGCAC
Da.geniculata BM98(a) 61 GGGCCGAACC---CGGCGGCAACCGACCAACCAAGAGTTGCTTGCTTGCCGGGTGCACGT
Da.geniculata BM98(b) 61 .....---.....
Da.geniculata PHE29(a) 61 .....CGG.....--A.....
Da.geniculata PHE29(b) 61 .....---.....G.....
Da.geniculata CL5.1 61 .....---.....
Da.geniculata KF471603 61 .....---.....
Da.geniculata KF471605 61 .....CGG.....--.....
Da.geniculata MG232621 61 .....CGG.....--.....
Da.geniculata KF471610 61 .....---.....
consensus 61 GGGCCGAACC---CGGCGGCAACCGACCAACCaagaGTTGCTTGCTTGCCGGGTGCACGT
Da.geniculata BM98(a) 118 ATGCGTAGTGATGTTTTCCCAGTTGGCCCCGTTCGCGCGGGGTTGGAAAAACATTGAAGA
Da.geniculata BM98(b) 118 .....
Da.geniculata PHE29(a) 119 .....
Da.geniculata PHE29(b) 118 .....
Da.geniculata CL5.1 118 .....
Da.geniculata KF471603 118 .....
Da.geniculata KF471605 119 .....
Da.geniculata MG232621 119 .....
Da.geniculata KF471610 118 .....
consensus 121 ATGCGTAGTGATGTTTTCCCAGTTGGCCCCGTTCGCGCGGGGTTGGAAAAACATTGAAGA
Da.geniculata BM98(a) 178 TAGTCAGGCGGGCATCGTCTCCCATGGCGGCGGCATGCCCGGTTGGTGAATACATCCCAT
Da.geniculata BM98(b) 178 .....
Da.geniculata PHE29(a) 179 .....
Da.geniculata PHE29(b) 178 .....
Da.geniculata CL5.1 178 .....
Da.geniculata KF471603 178 .....
Da.geniculata KF471605 179 .....
Da.geniculata MG232621 179 .....
Da.geniculata KF471610 178 .....
consensus 181 TAGTCAGGCGGGCATCGTCTCCCATGGCGGCGGCATGCCCGGTTGGTGAATACATCCCAT
Da.geniculata BM98(a) 238 AGACCCCCGC--TCTCCTAGCCGAGAGTGG-GTTCCTAGTTGTATTCCATCAAACGCACC
Da.geniculata BM98(b) 238 .....--.....-.....
Da.geniculata PHE29(a) 239 .....GAC.....C..-.....
Da.geniculata PHE29(b) 238 .....--.....-.....
Da.geniculata CL5.1 238 .....GAC.....C..-.....
Da.geniculata KF471603 238 .....--.....-.....
Da.geniculata KF471605 239 .....--.....-.....
Da.geniculata MG232621 239 .....--.....TA.....
Da.geniculata KF471610 238 .....GAC.....C..-.....
consensus 241 AGACCCCCGC--tCTCCTAGCCGAGAGtGG-gTTCCTAGTTGTATTCCATCAAACGCACC

```
Da.geniculata BM98(a)  295 TACCGTGCACTCACTCACCC------GCAATCAGT
Da.geniculata BM98(b)  295 ..................AACGCAA.G......
Da.geniculata PHE29(a) 298 ..................AACGCAA.G......
Da.geniculata PHE29(b) 295 ..................AACGCAA.G....T.
Da.geniculata CL5.1    297 ..................AACGCAA.G......
Da.geniculata KF471603 295 ..................AACGCAA.G......
Da.geniculata KF471605 296 ....................------.........
Da.geniculata MG232621 297 ....................------.........
Da.geniculata KF471610 297 ..................AACGCAA.G......
consensus              301 TACCGTGCACTCACTCACCC------gCaATCAgT
```

Appendix IIg. Alignment showing intraspecific differences between the *ITS2* of *Da. geniculata*. Highlights indicate areas that posed sequencing difficulties (as described in Table 4.2). Dots indicate homologous nucleotides, dashes represent gaps. Alignment constructed using BOXSHADE.



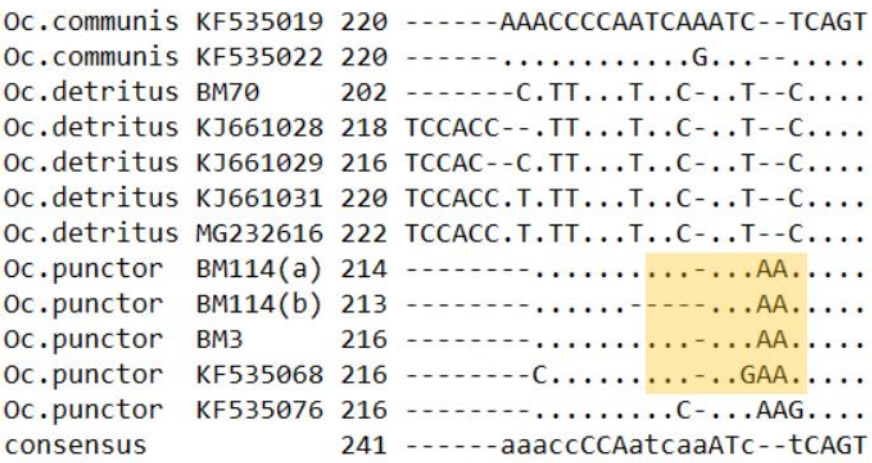
Appendix IIh. Alignment showing intra and interspecific differences between the *ITS2* of *Oc. annulipes, Oc. cantans and Oc. flavescens*. Highlights indicate areas that posed sequencing difficulties (as described in Table 4.2). Dots indicate homologous nucleotides, dashes represent gaps. Alignment constructed using BOXSHADE.

```
Species     Ref.
Oc.communis KF535019   1 ATATTTATCTATTCAACTATACGTGCATGCGC-GTTTCACTTCGGGTGGACAGGCGCATG
Oc.communis KF535022   1 ................................-.............................
Oc.detritus BM70       1 ................................-.............................
Oc.detritus KJ661028   1 ................................-.............................
Oc.detritus KJ661029   1 ................................-.............................
Oc.detritus KJ661031   1 ................................G.............................
Oc.detritus MG232616   1 ................................G.............................
Oc.punctor  BM114(a)   1 ................................-.............................
Oc.punctor  BM114(b)   1 ................................-.............................
Oc.punctor  BM3        1 ................................-.............................
Oc.punctor  KF535068   1 ................................-.............................
Oc.punctor  KF535076   1 ................................-.............................
consensus              1 ATATTTATCTATTCAACTATACGTGCATGCGC-GTTTCACTTCGGGTGGACAGGCGCATG

Oc.communis KF535019  60 GCCCA-CAAGCACGTATGCGTAGTGACGTTTTCCCGCCTTCAGTG-GTGGTAAAACGTTT
Oc.communis KF535022  60 .....-.........................................-............
Oc.detritus BM70      60 .....GG.....................................G...C.C..........C
Oc.detritus KJ661028  60 .....GG.....................................G...C.C..........C
Oc.detritus KJ661029  60 .....GG.....................................G...C.C..........C
Oc.detritus KJ661031  61 .....GG.....................................G...C.C..........C
Oc.detritus MG232616  61 .....GG.....................................G...C.C..........C
Oc.punctor  BM114(a)  60 .....-.........................................-............
Oc.punctor  BM114(b)  60 .....-.........................................-............
Oc.punctor  BM3       60 .....-.........................................-............
Oc.punctor  KF535068  60 .....-T........................................-............
Oc.punctor  KF535076  60 .....-T........................................-............
consensus             61 GCCCA-cAAGCACGTATGCGTAGTGACGTTTTCCCGCCTTCaGTG-GtGGTAAAACGTTt

Oc.communis KF535019 118 AAGATAGTCAGGCGCGTCCGAGGGTGGTAACACATCCGACGACGTGGTTGATGAATACAT
Oc.communis KF535022 118 ............................................................
Oc.detritus BM70     120 ..................----------------C.......................
Oc.detritus KJ661028 120 ..................----------------C.......................
Oc.detritus KJ661029 120 ..................----------------C.......................
Oc.detritus KJ661031 121 ..................----------------C.......................
Oc.detritus MG232616 121 ..................----------------C.......................
Oc.punctor  BM114(a) 118 ....................-----.C..CT.G.GG.GAG.....................
Oc.punctor  BM114(b) 118 ....................-----.C..CT.G.GG.GAG.....................
Oc.punctor  BM3      118 ....................-----.C..CT.G.GG.GAG.....................
Oc.punctor  KF535068 118 ....................-----.C..CT.G.GG.GAG.....................
Oc.punctor  KF535076 118 ....................-----.C..CT.G.GGAGAG.......T.............
consensus            121 AAGATAGTCAGGCGCGTCCgagggtggtaacacatccgaCGACGTGgTTGATGAATACAT

Oc.communis KF535019 178 CCCATACACCAGCCC-CGTT--GGTTATGTTGTATTCCATCACAC---------------
Oc.communis KF535022 178 .............-.....--.....................---------------
Oc.detritus BM70     162 ..........CA.A.-.AGA--.....................----------------
Oc.detritus KJ661028 162 ..........CA.A.-.AGAGG.....................CGGATCA---CCGGA
Oc.detritus KJ661029 162 ..........CA.A.-.AGA--.....................CGGATCA---CCGGA
Oc.detritus KJ661031 163 ..........CA.A.-.AGA--.....................CGGATCACTTCCGGA
Oc.detritus MG232616 163 ..........CA.A.-.AGAGG.....................CGGATCACTTCCGGA
Oc.punctor  BM114(a) 173 ............A.G...---..C.....................----------------
Oc.punctor  BM114(b) 173 ............T.-...---..C.....................----------------
Oc.punctor  BM3      173 ............A.G...---..C.....................----------------
Oc.punctor  KF535068 173 ............A.G...---..C.....................----------------
Oc.punctor  KF535076 173 ..............G...---..C.................C.C.---------------
consensus            181 CCCATACACCagCcC-Cgtt--GGtTATGTTGTATTCCATCaCac---------------
```



Appendix IIi. Alignment showing intra and interspecific differences between the *ITS2* of *Oc. communis, Oc. detritus and Oc. punctor*. Highlights indicate areas that posed sequencing difficulties (as described in Table 4.2). Dots indicate homologous nucleotides, dashes represent gaps. Alignment constructed using BOXSHADE.

```
Species      Ref.
Oc.sticticus PHE13(a)   1 ATATTTATCCATTCAACTATACGTGTGTGCGCGTACCACTCCGGGTGGACAGGCGCACGG
Oc.sticticus PHE13(b)   1 ..........................................................
Oc.nigrinus  UK19       1 ..........................................................
Oc.nigrinus  UK1        1 ................G....G....................................
consensus               1 ATATTTATCCATTCAAcTATAcGTGTGTGCGCGTACCACTCCGGGTGGACAGGCGCACGG

Oc.sticticus PHE13(a)  61 CCCATGGCACGTATGCGGCGTGATGTTTTCCCGGCCTTCAGTGGCGGTAAAACATTGAAG
Oc.sticticus PHE13(b)  61 ..........................................................
Oc.nigrinus  UK19      61 ....CA......................................................
Oc.nigrinus  UK1       61 ....CA......................................................
consensus              61 CCCAtgGCACGTATGCGGCGTGATGTTTTCCCGGCCTTCAGTGGCGGTAAAACATTGAAG

Oc.sticticus PHE13(a) 121 ATAGTCAGGCGTGTCGTACCCGCTCGCGCGGCGATCGGCGCGGTTGATGAATACATCCCA
Oc.sticticus PHE13(b) 121 ..........................................................
Oc.nigrinus  UK19     121 ..................................C.......................
Oc.nigrinus  UK1      121 ..................................C.......................
consensus             121 ATAGTCAGGCGTGTCGTACCCGCTCGCGCGGCGaTCGGCGCGGTTGATGAATACATCCCA

Oc.sticticus PHE13(a) 181 TAGGCCACAGCTCGCTTGGCTATGTTGTATTCCATCTCCAAACAC------CCCAAACTC
Oc.sticticus PHE13(b) 181 ..................................................-----A.......C.
Oc.nigrinus  UK19     181 .........................................C...CAAACA.A..C....
Oc.nigrinus  UK1      181 .........................................C...CAAACA.A..C....
consensus             181 TAGGCCACAGCTCGCTTGGCTATGTTGTATTCCATCTCCAAaCAC------CcCAaACtC

Oc.sticticus PHE13(a) 235 AGT
Oc.sticticus PHE13(b) 236 ...
Oc.nigrinus  UK19     241 ...
Oc.nigrinus  UK1      241 ...
consensus             241 AGT
```

Appendix IIj. Alignment showing intra and interspecific differences between the *ITS2* of *Oc. sticticus and Oc. nigrinus.* Highlights indicate areas that posed sequencing difficulties (as described in Table 4.2). Dots indicate homologous nucleotides, dashes represent gaps. Alignment constructed using BOXSHADE.