

Unveiling mosquito cryptic species and their reproductive isolation

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Abstract

Mosquitoes are major vectors of many infectious pathogens or parasites. Understanding cryptic species and the speciation of disease vectors has important implications for vector management, evolution and host-pathogen and/or host–parasite interactions. Currently, mosquito cryptic species have been reported in many studies, most of which focus on the reproductive isolation of cryptic species and mainly on *Anopheles gambiae* sensu lato complex. Emerging species within the primary malaria vector *Anopheles gambiae* show different ecological preferences and significant prezygotic reproductive isolation, while *Aedes mariae* and *Aedes zammitii* show postmating reproductive isolation. However, data reporting the reproductive isolation in *Culex* and *Aedes albopictus* mosquito cryptic species is absent. The lack of systematic studies leaves many questions open, such as whether cryptic species are more common in particular habitats, latitudes or taxonomic groups; what mosquito cryptic species evolutionary processes bring about reproductive isolation in the absence of morphological differentiation? How does *Wolbachia* infection affect in mosquitoes' reproductive isolation? In this review, we provide a summary of recent advances in the discovery and identification of sibling or cryptic species within mosquito genera.

Keywords: mosquito, evolution, cryptic species, identification, reproduction isolation, *Wolbachia*.

Introduction

Mosquitoes belong to the family of Culicidae, order of Diptera in the class Insecta. There are over 3500 different

species of mosquitoes belonging to 41 genera in the Culicidae worldwide (Wang *et al.*, 2012; Karthika *et al.*, 2018). A species is a basic unit of biological classification and a taxonomic rank is variously defined under different species concepts (Singh, 2016). Mayr (1969) described only three concepts of species: Typological, nominalistic and biological (Mayr 1969, 1970; Singh, 2016). Of these species concepts, only one is the most widely accepted species concept, ie, the biological species concept (BSC), which is the most developed, although it has certain difficulties in its applications (Singh, 2016). Mayr and Ashlock have used a number of terms pertaining to species that have been defined adequately: Subspecies, biological races, superspecies, semispecies, allospecies, sibling species, cryptic species, incipient species, monotypic species and polytypic species (Mayr, and Ashlock, 1991; Singh, 2016). Mayr defined sibling species as 'sympatric forms which are morphologically similar or indistinguishable which possess specific biological characteristics and are reproductively isolated'. Biological races were identified, but were later called sibling species (Singh, 2016). What are cryptic species? The literature is brimming with definitions of cryptic or hidden species. Most authors regard cryptic species to be synonymous with 'sibling species' (Saez and Lozano, 2005; Bickford *et al.*, 2006) whereas others specify that 'sibling' connotes more recent common ancestry than does 'cryptic', implying a sister-species relationship (Knowlton, 1986; Bickford *et al.*, 2006). Although not yet universally adopted, we feel that differentiating the term 'sibling species' in this way gives the phrase enhanced utility. Some advocate that sibling species graduate to the category of pseudo-sibling species once diagnosable characters are found (Saez and Lozano, 2005; Knowlton, 1993; Bickford *et al.*, 2006). Adding confusion, some authors refer to camouflaged or secretive species as being 'cryptic species' (Bickford *et al.*, 2006). Cryptic species are genetically distinct taxa without obvious variation in morphology that are discovered using molecular or sequence datasets of populations previously thought to be a single species (Collins & Paskewitz, 1996). The taxonomic challenge constituted by cryptic species (two or more distinct species classified as a single species) has been

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recognized for long time; however, the advent of relatively inexpensive and rapid DNA sequencing has given biologists a new tool to identify and differentiate morphologically similar species (Bickford *et al.*, 2006).

Mosquitoes can act as vectors of several important pathogens causing diseases, such as dengue fever, filariasis, malaria, yellow fever, encephalitis (in human and livestock) and chikungunya fever. These diseases are a worldwide threat to public health. The discovery of cryptic species in the Culicidae also has serious public health implications because it directly impacts the design of vector control and management programs (Bickford *et al.*, 2006; Chan-Chable *et al.*, 2019). Effective vector control and early diagnosis, along with continuous serosurveillance in animals and humans, are crucial to fight this arboviral diseases. However, the success of vector control operations is linked with the fast and reliable identification of targeted species, and knowledge about their biology and ecology. The correct identification of a species is essential for the development of programs for the control and prevention of diseases transmitted by mosquitoes, as it allows us to focus only on the control of those species that transmit certain diseases (Erlank *et al.*, 2018; Chan-Chable *et al.*, 2019; Karthika *et al.*, 2018).

In this review, the current advances in discovery and identification of sibling or cryptic species within the genera of mosquitoes are summarized and discussed.

Major cryptic species in mosquitoes

Table 1 shows a list of sibling species and groups, and cryptic species for different species groups of mosquitoes. To date, mosquito sibling species or cryptic species have been identified in the following species groups: *Anopheles (An.) gambiae* (Aboagye-Antwi *et al.*, 2015; Fontaine *et al.*, 2015; Barrón *et al.*, 2019), *An. annularis* (Alam *et al.*, 2007), *An. culicifacies* (Wilkerson *et al.*, 2004), *An. cruzii* (Rona *et al.*, 2009), *An. longirostris* (Alquezar *et al.*, 2010), *An. takasagoensis* (Takano *et al.*, 2010), *An. minimus* (Taai *et al.*, 2017), *An. maculipennis* (Novikov and Vaulin 2014), *An. farauti* (Duffield *et al.*, 2019), *An. stephensi* (Firoziyan *et al.*, 2018), *Culex pipiens* (Dumas *et al.*, 2016), *Cx. vishnui* (Karthika *et al.*, 2018), *Aedes aegypti* (L) (Dickson *et al.*, 2016), *Aedes albopictus* (Guo *et al.*, 2018; Minard *et al.*, 2017), *Aedes notoscriptus* (Endersby *et al.*, 2013), *Aedes mariaae* and *Aedes zammitii* (Mastrantonio *et al.*, 2016).

Anopheles species

Anopheles gambiae was actually a complex of several closely related and morphologically indistinguishable sibling species known as the *An. gambiae* complex (Fontaine *et al.*, 2015). The *Anopheles gambiae* sensu lato complex: *Anopheles (kerteszia) cruzii*, *Anopheles*

gambiae, *An. (cellia) annularis*, *An. fontenillei* sp.n., *An. bwambae*, *An. quadriannulatus*, *Anopheles amharicus*, *An. melas*, *An. merus*. Barrón *et al.* reported that *Anopheles fontenillei* sp.n., a new species in the *Anopheles gambiae* complex. In Africa, three of the six major malaria vectors belong to the *Anopheles gambiae* complex: *Anopheles gambiae*, *Anopheles coluzzii*, and *Anopheles arabiensis* (Barrón *et al.*, 2019). This complex includes eight cryptic species that differ in many ecological aspects, particularly in host feeding preference, breeding sites, feeding behaviour and role in malaria transmission (Barrón *et al.*, 2019). In the *An. gambiae* complex, species range from the non-vectors *Anopheles quadriannulatus* and *Anopheles amharicus* to minor vectors *Anopheles melas*, *Anopheles merus* and *Anopheles bwambae*, to the major vectors *An. gambiae*, *An. coluzzii* and *An. arabiensis* (Erlank *et al.*, 2018). Two of the principal malaria vector mosquitoes in tropical Africa belong to the *Anopheles gambiae* sibling species complex. Formerly designated as *An. gambiae* M and S molecular forms (della Torre *et al.*, 2001), and were recently renamed as *An. coluzzii* and *An. gambiae* (Aboagye-Antwi *et al.*, 2015; Coetzee *et al.*, 2013).

Anopheles culicifacies sensu lato is an important vector of malaria in Southeast Asia, contributing almost 70% of malaria cases in India (Sunil *et al.*, 2013; Tyagi *et al.*, 2016). This species occurs as a complex of five sibling species, A, B, C, D and E, among which A, C, D and E act as vectors of *Plasmodium vivax* and *P. falciparum* in several states of India (Green and Miles, 1980; Subbarao *et al.*, 1983, 1988a, 1988b; Vasanthan *et al.*, 1991; Subbarao *et al.*, 1992; Sunil *et al.*, 2013; Tyagi *et al.*, 2016). These sibling species have differential distributions, seasonality, host preferences and susceptibility to parasites and insecticides (Raghavendra *et al.*, 1991, 1992; Subbarao *et al.*, 1988b; Adak *et al.*, 1999; Kaur *et al.*, 2000; Sunil *et al.*, 2013; Tyagi *et al.*, 2016). The five species of the complex can be differentiated by examining the polytene chromosomes and male mitotic chromosomes (Manonmani *et al.*, 2007). The authors reported that using a ribosomal DNA (rDNA)-internal transcribed spacer 2 (ITS2)-polymerase chain reaction (PCR) test could group the five species of the *An. culicifacies* complex into the two categories, species A and D forming one category, and species B, C and E forming another. The major European malaria vector, *An. maculipennis*, was not a single species as had been initially assumed, but was actually a complex of several species with overlapping morphological characteristics. *An. maculipennis*, and its cryptic species *An. messeae* A and B (Collins & Paskewitz, 1996; Novikov and Vaulin 2014). *Anopheles stephensi* is one of the approximately 60 *Anopheles* species considered in malaria transmission as Asian malaria vector. This species is geographically distributed in South Asia and the Arab

TABLE 1. List of sibling species pairs/groups or cryptic species in the genus mosquitoes

Vector species	No. of species	Species found in area	Reference
The <i>Anopheles gambiae</i> Sensu lato complex groups	Sibling species <i>Anopheles gambiae</i> s.s <i>Anopheles (kerteszia) cruzii</i> <i>Anopheles amharicus</i> <i>An. (cellia) annularis</i> <i>An. fontenillei</i> sp.n. <i>An. bwambae</i> <i>An. quadriannulatus</i> <i>An. melas</i> <i>An. merus</i>	Sub Saharans Africa	Aboagye-Antwi <i>et al.</i> (2015) Barrón <i>et al.</i> (2019)
<i>Anopheles (cellia) annularis</i> Van der walp	Species A and B	India	Alam M.T <i>et al.</i> (2007)
<i>Anopheles crucians</i>	A.B.C.E.D	India	Wilkerson <i>et al.</i> , 2004
<i>Anopheles culicifacies</i> Complex	Five cryptic species A.B.C.D.E	India	Manonmani <i>et al.</i> (2007)
<i>Anopheles barbirostris</i> <i>An. stephensi</i>	<i>An. barbirostris</i> A.B.C.	China South Asia and the Arab Peninsula	Wang <i>et al.</i> (2014) Firoozian <i>et al.</i> (2018)
<i>Anopheles farauti</i> Complex	<i>Anopheles farauti</i> (s.s.) and <i>An. hinesorum</i>	Indonesia, Papua New Guinea (PNG), the Solomon Islands, Vanuatu and Australia	Duffield <i>et al.</i> (2019)
<i>An. maculipennis</i>	Cryptic species <i>An. messeae</i> A.B	European	Collins and Paskewitz (1996) Novikov and Vaulin (2014)
<i>Anopheles (kerteszia) cruzii</i> <i>Anopheles longirostris</i> <i>Anopheles takasagoensis</i>	Two sibling species Nine cryptic species A cryptic species <i>Anopheles</i> <i>ff Takasagoensis</i>	Brazil Papua New Guinea Vietnam	Rona <i>et al.</i> (2009) Alquezar <i>et al.</i> (2010) Takano <i>et al.</i> (2010)
<i>Anopheles minimus</i> complex <i>Anopheles albitarsis</i> s.l.	<i>Anopheles minimus</i> Nine cryptic species <i>An. albitarsis</i> s.s. Lynch-Arribálzaga (<i>An. albitarsis</i> A) <i>Anopheles oryzalimnetes</i> Wilkerson & Motoki or <i>An. albitarsis</i> B <i>Anopheles marajoara</i> Galvão & Damascenoor <i>An. albitarsis</i> C <i>Anopheles deaneorum</i> Rosa-Freitas or <i>An. albitarsis</i> D <i>Anopheles janconnae</i> Wilkerson & Sallum or <i>An. albitarsis</i> E <i>An. albitarsis</i> F <i>An. albitarsis</i> G <i>An. albitarsis</i> I	Thailand Paraguay, Brazil Paraguay, Brazil Paraguay, Brazil Argentina, Brazil Northern Brazil, Venezuela and Trinidad Colombia and Venezuela Brazil Colombia and Venezuela	Taai <i>et al.</i> (2017) Fontoura <i>et al.</i> (2014)
<i>Culex pipiens</i> complex <i>Culex</i> <i>Aedes albopictus</i> <i>Aedes albopictus</i> <i>Aedes aegypti</i>	A cryptic species <i>C. vishnui</i> and <i>C. pseudovishnui</i> 11 Cryptic species A cryptic species <i>Aedes aegypti</i> (Aaa) <i>Aedes aegypti</i> formosus (Aaf)	South Africa China Vietnam Global tropical and Sub-Saharan Africa subtropical area Australia	Dumas <i>et al.</i> (2016) Karthika <i>et al.</i> (2018) Guo <i>et al.</i> (2018) Minard <i>et al.</i> (2017) Dickson <i>et al.</i> (2016)
<i>Aedes notoscriptus</i> (Skuse)	A cryptic species	Australia	Endersby <i>et al.</i> (2013)

Peninsula. *An. stephensi* could be considered as complex species including *An. stephensi* sibling A, *An. stephensi* sibling B and *An. stephensi* sibling C (Firoozian *et al.*, 2018). Mosquitoes of the *Anopheles farauti* complex (*An. punctulatus* group) are major vectors of human malaria in Indonesia, Papua New Guinea (PNG), the Solomon Islands, Vanuatu and Australia. *An. farauti* complex. *Anopheles farauti* (s.s.) and *An. hinesorum* (Duffield *et al.*, 2019).

Culex species

C. pipiens is common in temperate regions and is subdivided into two subspecies, *Culex pipiens pipiens* (Europe and North and South Africa) and *Culex pipiens pallens*

(East Asia) (Dumas *et al.*, 2016). In addition, two recognized forms, 'pipiens' and 'molestus', also appeared in *C. pipiens pipiens* in the Northern Hemisphere (Dumas *et al.*, 2016). A second species, *C. quinquefasciatus*, is found across the tropics and the lower latitudes of temperate regions (Dumas *et al.*, 2016). Rasgon *et al.* reported that a cryptic species is present within the *C. pipiens* complex in South Africa (Rasgon and Scott, 2003; Rasgon *et al.* 2006; Dumas *et al.*, 2016). The authors observed the presence of Wolbachia-uninfected *C. pipiens* specimens in several breeding sites in Europe and North Africa. Using a multilocus typing scheme, they further confirmed that these uninfected specimens unambiguously belonged to the *C. pipiens* complex. On the basis of ace-2 DNA sequences, they were included within the *C. pipiens*

pipiens clade (Dumas *et al.*, 2016). Remarkably, novel mitochondrial(mt)DNA haplotypes were found in samples from Europe and North Africa that were related, but different to the mtDNA haplotypes found in Wolbachia-infected *C. pipiens* complex members (Dumas *et al.*, 2016). This genetic pattern demonstrated that uninfected specimens are not a result of imperfect maternal transmission from Wolbachia-infected specimens, but rather belong to a specific lineage (Dumas *et al.*, 2016). Compelling evidence suggested that specimens of the cryptic species do not readily hybridize with Wolbachia-infected *C. pipiens* and *C. quinquefasciatus* specimens. However, Dumas *et al.* suggest that a Wolbachia-uninfected population of *C. pipiens* was present in South Africa and was recently proposed as a cryptic species (Dumas *et al.*, 2016).

Key vectors of Japanese encephalitis are *Cx. tritaeniorhynchus*, *Cx. vishnui*, *Cx. pseudovishnui*, *Cx. gelidus*, *Cx. fuscocephala*, *Cx. quinquefasciatus*, *Culex pipiens pallescens* (Coquillett), *Culex bitaeniorhynchus* Giles and *Culex annulirostris* Skuse. *Cx. tritaeniorhynchus* and *Cx. vishnui* were considered under the *Cx. vishnui* subgroup. Karthika *et al.* using DNA barcoding analysis of *Culex fuscocephala*, *Culex gelidus*, *Culex tritaeniorhynchus*, *Culex pseudovishnui* and *Culex vishnui*, showed that *C. tritaeniorhynchus* exhibited the highest variation in all the ranges. *C. tritaeniorhynchus* exhibited high numbers of polymorphic sites and mutations, suggesting high nucleotide diversity. By contrast, the sister species *C. vishnui* and *C. pseudovishnui* showed a moderate rise. The results suggested that one or more new cryptic subspecies may exist in *Culex* mosquitoes (Karthika *et al.*, 2018).

Aedes species

We reported that cryptic species of *Aedes albopictus* were found in China, including nine individual samples of cryptic species of *Aedes albopictus* from Wuzhou, Guangxi; one individual sample cryptic species of *Aedes albopictus* from Baisha, Hainan; and one individual sample cryptic species of *Aedes albopictus* in Baoting, Hainan. The genetic distances of the 11 *Aedes albopictus* cryptic species were significantly different from those of *Aedes albopictus* in different regions of China. We speculate that these 11 individual samples of cryptic species may belong to more than three populations. In our study, among 14 study populations collected across the tropical, subtropical and temperate climate zones of China, we found three populations in Southern China in which cryptic species of *Ae. albopictus* coexisted in sympatry (Guo *et al.*, 2018). Based on the morphological structure of the cibarial armature, Minard *et al.* identified a cryptic species in the forest park at Bù Gia Mập in the Southeastern region of Vietnam (Minard *et al.*, 2017).

Aedes aegypti (Linne', 1762) is a major vector of arboviruses, such as those causing Yellow Fever, Dengue and Chikungunya. In Africa, where the species exhibits major variations in morphology, ecology, behaviour and vector competence, two subspecies have been described: A light form, named *Ae. aegypti aegypti* (Aaa) with highly domestic and anthropophilic habits and a cosmopolitan distribution; and a dark form, referred to as *Ae. aegypti formosus* (Aaf), which is endemic to Africa and thrives in sylvan environments (Brown *et al.*, 2011). In East Africa, both forms were described to occur in sympatry, whereas only *Aaf* was reported from Central/West Africa. An obvious question that remains is how Senegalese *Aae* are related to the *Aaf* and *Aaa* in East Africa. An earlier study of hybridization and mating behaviour between *Aaa* and *Aaf* in East Africa concluded that these two forms are part of a single, albeit highly polytypic species (Brown *et al.*, 2014). The suggestion that one or more new cryptic subspecies exist in Senegal in no way contradicts the phylogenetic patterns among worldwide *Ae. aegypti* s.l. populations that have been derived over the last 35 years using allozyme markers, microsatellites, nuclear SNP loci, mitochondrial DNA and most recently using a SNP-Chip (Brown *et al.*, 2014).

Aedes notoscriptus (Skuse) is a mosquito from the southwest Pacific region including Australia. Endersby *et al.* assessed the genetic variation and phylogenetic relationships among *Ae. notoscriptus* from the east coast of Australia, Western Australia and New Zealand. Phylogenetic analyses of DNA sequence data from mitochondrial markers indicated that *Ae. notoscriptus* is a complex of divergent genetic lineages, some of which appear to be geographically restricted, while others are widespread in eastern Australia. Samples from New Zealand and Western Australia were related to populations from one southern Australian lineage (Endersby *et al.*, 2013). The morphological character of wing centroid size variation is also associated with genetic lineage (Endersby *et al.*, 2013). These findings point to the possibility that *Ae. notoscriptus* is a complex of species, and suggests that *Ae. notoscriptus* contain two or three cryptic species in the literature (Endersby *et al.*, 2013).

Reproductive isolation in mosquitoes

Revealing the genetic basis of reproductive isolation plays a key role in understanding how biodiversity is generated (Pombi *et al.*, 2017). Reproductive isolation has always been considered as the most critical step in the formation of a species and is the basis for species to maintain their integrity and independence. A fundamental question in biology is what causes a single interbreeding species to diverge into two reproductively isolated species? An essential component of speciation is the formation of

reproductive barriers that hamper and eliminate gene flow. These barriers can be classified as prezygotic (eg, spatial, temporal, and behavioural isolation) (Stump *et al.*, 2005; Weetman *et al.*, 2014; Aboagye-Antwi *et al.*, 2015) or post-zygotic (eg, hybrid nonviability and hybrid sterility) (Stump *et al.*, 2005; Weetman *et al.*, 2014; Aboagye-Antwi *et al.*, 2015). Emerging species within the primary malaria vector *Anopheles gambiae* show different ecological preferences and significant prezygotic reproductive isolation. Stump *et al.* explored the pattern and extent of differentiation between *A. gambiae* incipient species and a sibling species, *A. arabiensis*, from Burkina Faso, West Africa, at 17 microsatellite loci spanning the X chromosome (Stump *et al.*, 2005). Interspecific differentiation was large and significant across the entire X chromosome (Stump *et al.*, 2005). Among *A. gambiae* incipient species, they found some of the highest levels of differentiation recorded in a large region including eight independent loci near the centromere of the X chromosome. Outside of this region, no significant differentiation was detected. The pattern of high differentiation proximally, but little or no differentiation distally, on the X chromosome in M and S is difficult to explain through the action of demographic forces alone. If selection and/or suppressed recombination are responsible for heightened differentiation, then a footprint of linkage disequilibrium and reduced allelic diversity would be expected (Stump *et al.*, 2005). Divergence among species, incipient species or populations in response to demographic factors results from random processes that act on all loci within a taxon. Even limited amounts of gene flow between taxa can preclude divergence at loci that lack fitness effects. Divergence associated with habitat specialization or premating isolation is likely the result of selection acting at a locus or combinations of interacting loci. This pattern suggests that selection is playing a role in the emergence of *A. gambiae* incipient species (Stump *et al.*, 2005).

Reproductive isolation is an intrinsic aspect of species formation. For that reason, the identification of the precise isolating traits, and the rates at which they evolve, is crucial to understand how species originate and persist. In areas where the morphologically indistinguishable malaria mosquitoes *Anopheles gambiae* Giles and *An. arabiensis* Patton are sympatric, hybrids are detected occasionally via species-diagnostic molecular assays. *An. gambiae* and *An. arabiensis* exhibit both pre- and post-reproductive mating barriers, with swarms largely species-specific and male F1 (first-generation) hybrids sterile (Weetman *et al.*, 2014). Weetman *et al.* used data from over 350 polymorphic autosomal SNPs to investigate post F1 gene flow via patterns of genomic admixture between *An. gambiae* and *An. arabiensis* from eastern Uganda. Simulations were used to investigate the statistical power to detect hybrids with different levels of crossing and to identify the hybrid category

significantly admixed genotypes could represent. The result shows that a range of admixture proportions were detected for 11 field-collected hybrids identified via single-locus species-diagnostic PCRs. Comparison of admixture data with simulations indicated that at least seven of these hybrids were advanced generation crosses, with backcrosses to each species identified. In addition, of 36 individuals typing as *An. gambiae* or *An. arabiensis* that exhibited outlying admixture proportions, 10 were identified as significantly mixed backcrosses, and at least four of these were second or third generation crosses (Weetman *et al.*, 2014).

In addition, molecular forms (M and S) have been identified that appear to be reproductively isolated (della Torre *et al.*, 2001). The S form is distributed widely throughout the *An. gambiae* species range, whereas the M form is common, but restricted to western parts of Africa. Hybridization between them is rare in most areas of sympatry. Initial scanning of large numbers of polymorphic markers indicated that the molecular forms were very highly differentiated at only a small number of discrete regions within the two autosomes and the pericentromeric region of the X chromosome, which contains the form-specific markers in the ribosomal (r)RNA genes (della Torre *et al.*, 2005). However, a finding that transposable element insertion sites showed marked differentiation between the molecular forms indicated that reproductive isolation might affect a larger portion of the genome (Esnault *et al.*, 2008). A high-density array to type 400 000 single nucleotide polymorphisms (SNPs) has recently enabled a more thorough genome-wide analyses, confirming that the molecular forms are differentiated at many loci throughout the genome, in addition to those previously identified (Lawniczak *et al.*, 2010; Neafsey *et al.*, 2010; Reidenbach *et al.*, 2012). Lee *et al.* reported that significant exchange of genes between M and S forms in *An. gambiae* occurs, and this gene exchange process is spatially and temporally dynamic (Lee *et al.*, 2013a, 2013b, 2014). The first genome-wide comparison of the M and S forms by Turner *et al.* was consistent with earlier observations of low overall divergence; however, there are small, discrete regions of divergence representing about 3% of the genome (Turner *et al.*, 2005). Genomic island of divergence (speciation): a region of the genome of any size, but usually considered to be relatively small and isolated from other such regions, whose divergence exceeds neutral background expectations in the absence of divergent selection (Feder *et al.*, 2012). These highly divergent genomic regions have been termed 'genomic islands of speciation', implying that they contain a set of linked loci involved in the reproductive isolation process and are under divergent selection in the two species. The functional demonstration of a close association between speciation genes and the X-island of differentiation provides unprecedented support to the island-of-speciation models of sympatric speciation,

facilitated by pericentric recombination suppression (Turner *et al.*, 2005; White *et al.*, 2010). Several ground breaking studies have shown that sympatric speciation in these two incipient species probably involves the divergence of a few 'islands of divergence' that possibly contain clusters of speciation genes and are located in areas of low recombination (White *et al.*, 2010; Aboagye-Antwi *et al.*, 2015). These putative islands of speciation include three pericentromeric islands of divergence located on the X, 2L and 3L chromosomes, as well as smaller islands located in the vicinity of inversion breakpoints (White *et al.*, 2010; Aboagye-Antwi *et al.*, 2015). Perfect linkage disequilibrium between the X, 2L and 3L islands was observed in samples from sympatric populations of *An. coluzzii* and *An. gambiae* s.s. from central West Africa (White *et al.*, 2010). *An. gambiae* and *An. coluzzii* have a geographical range spanning sub-Saharan Africa and encompassing a variety of ecological settings (Foslog *et al.*, 2015). Within pericentromeric regions of chromosomes X, 3 and arm 2R, mosquitoes segregated into two highly distinct clades, largely corresponding to the two species as determined by conventional molecular diagnostics, consistent with previous studies finding that genome regions of reduced recombination are associated with stronger differentiation between closely related species (Cruickshank and Hahn 2014). There were also some genome regions where Miles *et al.* found unusually short genetic distances between individuals from different populations and species, indicating the influence of recent selective sweeps and adaptive gene flow (Miles *et al.*, 2018). Taken together, the comparative genomics data support a model of genomic divergence in which pericentromeric divergence islands play a major role in speciation in the face of varying levels of gene flow (Weetman *et al.*, 2012; Lee *et al.*, 2013a, 2013b). Aboagye-Antwi *et al.* reported the selective introgression of the island of divergence located in the pericentric region of the X chromosome of *An. gambiae* s.s. into its sister taxon *An. coluzzii* through five generations of backcrossing, followed by two generations of crosses within the introgressed strains (Aboagye-Antwi *et al.*, 2015). This resulted in *An. coluzzii*-like recombinant strains fixed for the M and S marker in the X chromosome island (Aboagye-Antwi *et al.*, 2015). The mating preference of the recombinant strains was then tested by giving virgin recombinant individuals a choice of mates with X-islands matching or not matching their own island type (Aboagye-Antwi *et al.*, 2015). Through genetic analyses of transferred sperm, the study showed that recombinant females consistently mated with matching island-type males, thereby associating assortative mating genes with the X-island of divergence. Furthermore, full-genome sequencing confirmed that protein-coding differences between the recombinant strains were limited to the experimentally swapped pericentromeric region.

Finally, targeted-genome comparisons showed that a number of these unique differences were conserved in sympatric field populations, thereby revealing candidate speciation genes (Aboagye-Antwi *et al.*, 2015). These findings suggested an important role of divergence islands for the genetic and behavioural processes associated with speciation (Aboagye-Antwi *et al.*, 2015). Lima reported that heteromorphism of sex chromosomes was associated with strong reproductive isolation (Lima, 2014). Pervasive autosomal introgression between these malaria vectors means that only a small fraction of the genome, mainly on the X chromosome, has not crossed species boundaries (Lima, 2014). Lima results suggest that traits enhancing vectorial capacity may be gained through interspecific gene flow, including between nonsister species (Lima, 2014). Clarkson *et al.* reported that adaptive introgression between *Anopheles* sibling species eliminates a major genomic island but not reproductive isolation (Clarkson *et al.*, 2014). Adaptive introgression can provide novel genetic variation to fuel rapid evolutionary responses, although it may be counterbalanced by potential for detrimental disruption of the recipient genomic background (Clarkson *et al.*, 2014). Clarkson *et al.* examine the extent and impact of recent introgression of a strongly selected insecticide-resistance mutation (Vgsc-1014F) located within one of two exceptionally large genomic islands of divergence separating the *Anopheles gambiae* species pair (Clarkson *et al.*, 2014). Clarkson *et al.* show that transfer of the Vgsc mutation results in homogenization of the entire genomic island region (B1.5% of the genome) between species. Despite this massive disruption, introgression is clearly adaptive with a dramatic rise in frequency of Vgsc-1014F and no discernable impact on subsequent reproductive isolation between species (Clarkson *et al.*, 2014). Introgressive hybridization is now recognized as a widespread phenomenon, but its role in evolution remains contested (Fontaine *et al.*, 2015). Fontaine *et al.* use newly available reference genome assemblies to investigate phylogenetic relationships and introgression in *An. gambiae* complex (*An. gambiae*, *An. coluzzii*, *An. arabiensis*, *An. quadriannulatus*, *An. merus* and *An. melas*). Fontaine *et al.* have identified the correct species branching order to resolve a contentious phylogeny and show that lineages leading to the principal vectors of human malaria were among the first to split (Fontaine *et al.*, 2015).

Intrinsic postzygotic reproductive isolation is famously characterized by "two rules of speciation"—Haldane's rule and the large X-effect. Haldane's rule refers to the preferential sterility or inviability of hybrids of the heterogametic (XY) sex relative to their homogametic (XX) siblings. The large X-effect refers to the disproportionately large role of the X chromosome in reducing hybrid fitness (Presgraves, 2018; Bracewell *et al.*, 2011). The two 'rules

of speciation', Haldane's rule and the large X-effect, describe the genetic basis of postzygotic isolation, and have led to the realization that sex chromosomes play an important role in this process. However, a range of sex determination mechanisms exists in nature, not always involving sex chromosomes. Based on these 'rules of speciation', Lima tested the hypothesis that the presence of sex chromosomes would contribute to a faster evolution of intrinsic postzygotic isolation. The authors showed that taxa that do not have sex chromosomes evolve lower levels of postzygotic isolation than taxa with sex chromosomes, with a similar amount of genetic divergence (Lima, 2014). Taxa with young homomorphic sex chromosomes show an intermediate pattern compared with taxa with heteromorphic sex chromosomes and taxa without sex chromosomes. These results were consistent with predictions from the two 'rules of speciation', and emphasize the importance of sex chromosomes in the evolution of intrinsic postzygotic isolation (Lima, 2014). Crawford *et al.* reported that reticulate speciation and barriers to introgression in the *An. gambiae* species complex. They sequenced 20 genomes from wild *A. gambiae* s.s., *An. coluzzii* and *An. arabiensis*, and compared these with 12 genomes from the "GOUNDRY" subgroup of *An. gambiae* s.l. (sensu lato) against a backdrop of strong reproductive isolation (Crawford *et al.*, 2015). The authors found strong evidence for a speciation continuum with introgression of autosomal chromosomal regions among species and subgroups (Crawford *et al.*, 2015). The X chromosome, however, was strongly differentiated among all taxa, pointing to a disproportionately large effect of X chromosome genes in driving speciation among anophelines (Crawford *et al.*, 2015). Strikingly, the authors found that autosomal introgression had occurred from contemporary hybridization between *An. gambiae* and *An. arabiensis*, despite strong divergence (approximately five times higher than autosomal divergence) and isolation on the X chromosome (Crawford *et al.*, 2015).

Dickson *et al.* reported that reproductive incompatibility involving Senegalese *Aedes aegypti* (L) is associated with chromosome rearrangements (Dickson *et al.*, 2016). An earlier laboratory study did not indicate any pre- or postzygotic barriers to gene flow between *Aedes aegypti* Aaa and *Aedes aegypti* Aaf in East Africa (Dickson *et al.*, 2016). However, similar attempts to construct F1 intercross families between *Aedes aegypti* Aaa laboratory strains and Senegal *Ae. aegypti* (SenAae) failed because of poor F1 oviposition and low F2 egg-to-adult survival. Insemination and assortative mating experiments failed to identify prezygotic mating barriers. Backcrosses were performed to test for postzygotic isolation patterns consistent with Haldane's rule modified for species, like *Aedes*, which have an autosomal sex determining locus (SDL). Egg-pupal survival was predicted to be low in females mated to hybrid F1

males but average when a male mated with a hybrid F1 female. Survival was in fact significantly reduced when females mated to hybrid males; however, egg-pupal survival increased significantly when males were mated to hybrid F1 females. These observations are therefore inconclusive with regards to Haldane's rule. Basic cytogenetic analyses and fluorescent hybridization (FISH) experiments were performed to compare *SenAae* strains with the IB12 strain of Aaa that was used for genome sequencing and physical mapping. Some *SenAae* strains had longer chromosomes than IB12 and significantly different centromeric indices on chromosomes 1 and 3 (Dickson *et al.*, 2016).

The *Anopheles albitarsis* complex comprises at least nine cryptic species, some of which are implicated as vectors of human malaria. Hybridization experiments between *Anopheles albitarsis* s.s. and *Anopheles marajoara*, as well as backcrosses between hybrids and *Anopheles albitarsis* s.s., were performed using the induced mating technique. The result showed that male hybrids had very low insemination rates and nearly complete sterility, apparently because of abnormalities in their reproductive organs. Evidence of partial sterility among the hybrid females was also observed. The data indicated that *Anopheles albitarsis* s.s. and *Anopheles marajoara* show a high level of postzygotic isolation with strong hybrid male sterility. This result is consistent with the Haldane's rule, which states that in interspecific crosses, the heterogametic sex is the first to be affected. However, the fact that the females are not completely sterile raises the possibility of introgression between these two sibling species (Fontoura *et al.*, 2014).

The *Anopheles minimus* complex is known to comprise at least two sibling species (A and C) in Thailand and Vietnam. Somboon *et al.* reported a study which investigated the specific status of *An. minimus* collected in the southern part of Taiwan by crossing experiments with species A from Thailand and species E from Japan. Crosses between Taiwan *An. minimus* and species A revealed genetic compatibilities. Post-zygotic isolation was observed in crosses between Taiwan *An. minimus* and species E (Somboon *et al.*, 2005).

Urbanelli *et al.* investigated two sibling mosquito species, *Aedes mariaae* and *Aedes zammitii*, that which show postmating reproductive isolation (F1 males sterile) and partial premating isolation (different height of mating swarms) that could be reinforced by natural selection against hybridization (Urbanelli *et al.*, 2014). In 1986, they created an artificial sympatric area between the two species and sampled about 20 000 individuals over the following 25 years (Urbanelli *et al.*, 2014). Between 1986 and 2011, the composition of mating swarms and the hybridization rate between the two species were investigated across time in the sympatric area (Urbanelli *et al.*, 2014). The results showed that *A. mariaae* and *A. zammitii* have not

completed reproductive isolation since their first contact in the artificial sympatric area (Urbanelli *et al.*, 2014).

How does Wolbachia infection affect in mosquitoes' reproductive isolation?

Wolbachia is a cytoplasmically inherited bacterium that causes a number of reproductive alterations in insects, including cytoplasmic incompatibility (CI), an incompatibility between sperm and egg that results in loss of sperm chromosomes following fertilization (Bordenstein *et al.*, 2001). The inability of sperm to fertilize eggs creates a reproductive barrier that could subdivide populations into species. How does species-specific fertilization evolve and when does this evolution occur—does it happen at the early stages of species divergence, or do the changes accumulate only after speciation? The study of the mechanisms of speciation is one of the central areas of interest in evolutionary biology. However, the nature of the mechanism responsible for reproductive isolation remains to be determined. Wolbachia might partially contribute to this isolation, because via unidirectional CI, crosses between infected males and uninfected females should be infertile (Gebiola *et al.*, 2016). However, in this case, the other direction of the cross remains fertile, suggesting that the reproductive isolation of the uninfected cryptic species may be driven by other mechanisms, such as behavioural isolation or hybrid nonviability. The main biological traits of the cryptic species are also almost entirely unknown—except for the absence of Wolbachia; however, they probably show distinctive features (Dumas *et al.*, 2016). We reported that Wolbachia infections were detected in all 14 of the *Ae. albopictus* populations. Infection rates ranged from 50 to 100% (in eight populations, with an average of ~90%) suggesting that Wolbachia is highly prevalent in *Ae. albopictus* in China. However, Wolbachia infection in the cryptic *Aedes* species was absent or occurred at a low frequency (Guo *et al.*, 2018). Dumas *et al.* reported that a widespread cryptic species within the *C. pipiens* species complex is absent of Wolbachia infection (Dumas *et al.*, 2013). Wolbachia infection in *Ae. notoscriptus* cryptic species was rare in the sample assessed (1.4%, $n = 591$) (Endersby *et al.*, 2013). Wolbachia is estimated to infect 58–63% of all insect species and is also common in arachnids, isopods and nematodes (Werren and Windsor, 2000; Guo *et al.*, 2018). Therefore, Wolbachia-induced cytoplasmic incompatibility could be an important factor promoting rapid speciation in invertebrates, although this contention is controversial (Bordenstein *et al.*, 2001). Sequence variation in mtDNA can be strongly influenced by Wolbachia infection. Fixation of infection in *Plutella australiana* suggested that Wolbachia manipulates the reproductive biology of this species. Studies found no evidence of sex-ratio distortion, which has been associated with a

Wolbachia strain plutWB1, in *P. xylostella* (Delgado and Cook, 2009; Perry *et al.*, 2018). High infection rates can be driven by CI (Perry *et al.*, 2018; Shoemaker *et al.*, 2004). The high frequency (87%) of a single mtDNA haplotype among *P. australiana* individuals implied that the spread of Wolbachia infection has driven a selective sweep of coinherited mtDNA through the population, causing a loss of mtDNA diversity (Jiggins, 2017; Perry *et al.*, 2018). Does Wolbachia infection induce a reproductive barrier? The contrast in infection status creates the potential for CI between species (Hurst and Jiggins, 2005; Perry *et al.*, 2018). Interspecific crosses showed a pattern of asymmetric isolation consistent with the expected effects of unidirectional CI, where 21% of crosses involving infected *P. australiana* females produced viable offspring, while the reciprocal CI-cross direction (uninfected *P. xylostella* females crossed with infected *P. australiana* males) was nearly all sterile. However, this pattern was not continued in the F1 generation: Infected hybrid males (derived from the *P. australiana* maternal line) produced offspring at comparable rates when back-crossed to either uninfected *P. xylostella* or infected *P. australiana* female parents. The role of Wolbachia-induced postzygotic isolation between the two *Plutella* species requires further study, although the results suggested it could be more important in the F0 generation. Wolbachia could contribute to post-zygotic genetic isolation after speciation by complementing hybrid incompatibilities (Gebiola *et al.*, 2016; Jaenike *et al.*, 2006; Perry *et al.*, 2018). Symbiont infections could also influence mating behaviour and contribute to pre-mating isolation (Shropshire and Bordenstein, 2016). However, the role of Wolbachia-induced reproductive isolation in mosquitoes is not clear.

Summary

Molecular techniques (primarily DNA sequencing) have transformed the ability of scientists to describe and define biological diversity. Although they are not a panacea for species delimitation, molecular data are important and useful when combined with other types of information (Bickford *et al.*, 2006). These newly discovered cryptic species provide opportunities to study important mechanisms of speciation, mate recognition, which natural selection acts upon, and to study non-morphological characters that can be used to differentiate species. Mosquitoes spread many diseases, and unresolved questions still remain with regard to these cryptic species, including: What evolutionary processes in mosquito cryptic species bring about genetic divergence and reproductive isolation in the absence of morphological differentiation? Are mosquito cryptic species more common in the tropics, if so, why? How do different species concepts influence the assessment of mosquito cryptic speciation? How does Wolbachia

infections effect in mosquito reproductive isolation? What kinds of genetic evidence are necessary to demonstrate and formally describe mosquito cryptic species? *Aedes*, *Culex*, and *Anopheles* mosquitoes represent the major group of disease vectors, and more research on cryptic species in different sites is urgently needed, the study of the evolution of these newly discovered cryptic species will be interesting.

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Data Availability Statement

The data sets supporting the results are included within the article.

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