

Short Communication

Detection of *Plasmodium falciparum* Infection in *Anopheles squamosus* (Diptera: Culicidae) in an Area Targeted for Malaria Elimination, Southern Zambia

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Received 12 February 2016; Accepted 11 May 2016

Abstract

Southern Zambia is the focus of strategies to create malaria-free zones. Interventions being rolled out include test and treat strategies and distribution of insecticide-treated bed nets that target vectors that host-seek indoors and late at night. In Macha, Choma District, collections of mosquitoes were made outdoors using barrier screens within homesteads or UV bulb light traps set next to goats, cattle, or chickens during the rainy season of 2015. Anopheline mosquitoes were identified to species using molecular methods and *Plasmodium falciparum* infectivity was determined by ELISA and real-time qPCR methods. More than 40% of specimens caught were identified as *Anopheles squamosus* Theobald, 1901 of which six were found harboring malaria parasites. A single sample, morphologically identified as *Anopheles coustani* Laveran, 1900, was also found to be infectious. All seven specimens were caught outdoors next to goat pens. Parasite-positive specimens as well as a subset of *An. squamosus* specimens from either the same study or archive collections from the same area underwent sequencing of the mitochondrial cytochrome oxidase subunit I gene. Maximum parsimony trees constructed from the aligned sequences indicated presence of at least two clades of *An. squamosus* with infectious specimens falling in each clade. The single infectious specimen identified morphologically as *An. coustani* could not be matched to reference sequences. This is the first report from Zambia of infections in *An. squamosus*, a species which is described in literature to display exophagic traits. The bionomic characteristics of this species needs to be studied further to fully evaluate the implications for indoor-targeted vector control.

Key words: malaria, vector competence, mosquito-borne disease, vector ecology

In Macha, Southern Province, Zambia, malaria cases have dropped by over 90% in the past decade (Moss et al. 2011, 2012; Mharakurwa et al. 2012). Current community surveys indicate the prevalence by rapid diagnostic test to be <1% (W.J. Moss unpublished data) and elimination strategies are now being rolled out. These methods are primarily based on reactive case detection; cases reporting at health facilities are followed up and household members and neighbors are screened and treated with antimalarials if infected. Vector control relies on the routine distribution of long-lasting insecticidal nets (LLINs) to pregnant women and children under 5 yr of age at health facilities and mass distribution of LLINs every 2–3 yr (NMCC 2011). LLINs are currently the most effective tool in preventing exposure to indoor foraging malaria vectors that predominantly feed when people are asleep (Lengeler 2004).

However, this assumption of late night endophagy (Gillies and De Meillon 1968, Pates and Curtis 2005, Killeen et al. 2006) is being challenged in some areas; studies in other parts of Sub-Saharan Africa have shown replacement of vector populations with species that can evade indoor control (Gillies and Smith 1960, Lindblade et al. 2006, Bayoh et al. 2010), or have demonstrated changes in the foraging behavior of existing primary vectors, resulting in biting time shifts, and outdoor feeding and resting (Reddy et al. 2011, Russell et al. 2011, Moiroux et al. 2012, Yohannes and Boelee 2012, Sougoufara et al. 2014, Cooke et al. 2015). Recent studies have focused on determining the extent of exposure to vectors that may not be controlled by indoor targeted methods (Geissbuhler et al. 2007, Govella et al. 2010, Seyoum et al. 2012, Killeen 2014, Cooke et al. 2015). Currently the only vector control deployed in

southern Zambia is use of LLINs, which do not combat exposure to malaria mosquito vectors either outdoors or indoors at times prior or after bed net use.

The majority of programmatic entomological surveillance relies on morphological identification of samples using standard, albeit dated, keys (Gillies and Coetzee 1987). In sub-Saharan Africa, discrimination of specimens focuses on separating and quantifying collections of the main malaria vector complexes, *Anopheles gambiae* Giles, 1902 and *Anopheles funestus* Giles, 1900. Due to limitations of infrastructure in country, samples are rarely identified to sibling species within these complexes by standard PCR-based tools (Scott et al. 1993, Koekemoer et al. 2002), and little attention is paid to other species which are often discarded. However, studies have demonstrated presence of *Plasmodium falciparum* in secondary and unrecognized vectors (Gillies 1964, Nigatu et al. 1994, Wilkes et al. 1996, Antonio-Nkondjio et al. 2006, Stevenson et al. 2012, Degefa et al. 2015, Nepomichene et al. 2015, St. Laurent et al. 2016). Early studies relied on dissection of salivary glands to detect sporozoites, but these labor-intensive methods have generally been superseded by the use of circumsporozoite (CSP) ELISAs of homogenates of mosquito head and thoraces (Burkot et al. 1984). The use of CSP ELISA for zoophagic species has been reported to result in false positives (Durnez et al. 2011) and so some studies have confirmed infectivity by detection of parasite DNA by PCR in the mosquito. In a recent study in central Madagascar, infections of both *P. vivax* and *P. falciparum* detected by CSP ELISA were confirmed by PCR in *An. coustani* Laveran, 1900 caught both indoors and outdoors (Nepomichene et al. 2015). Worryingly, infectivity rates and entomological inoculation rates (the number of infectious bites received per person per annum, EIRs) were comparable with that of *An. funestus*, the recognized vector in the area. Indoor application of insecticides, the mainstay of vector control in Madagascar, as in most other African countries, is unlikely to prevent exposure to *An. coustani* that displays both endophagic and exophagic (indoor and outdoor feeding) behaviors (Gillies and De Meillon 1968, Fornadel et al. 2011, Mwangangi et al. 2013, Degefa et al. 2015, Nepomichene et al. 2015). PCR methods also confirmed presence of *P. falciparum*-positive mosquitoes in the highlands of western Kenya that did not belong to the *An. gambiae* or *An. funestus* species complexes (Stevenson et al. 2012, St. Laurent et al. 2016). Genetic sequencing of these samples to identify the infectious vector species resulted in no match to mosquito species that have been previously sequenced. Many of the infectious specimens were trapped outdoors where they may avoid current control measures. These findings highlight the importance of expansion of entomological surveillance to include potential secondary vectors, especially in low transmission areas where recognized primary vector populations may be marginalized, and programs start to focus on elimination strategies.

As part of the International Centers of Excellence in Malaria Research (ICEMR) in Southern Africa (Conn et al. 2015), outdoor collection methods for anopheline malaria vectors were evaluated in Macha, Choma district, Southern Zambia. Using two 4x4 Latin Square designs, miniature CDC UV light traps, updraft UV light traps (John W Hock Co., Gainesville, FL) and barrier screens (Burkot et al. 2013) were rotated through eight consenting households for a period of 48 nights in the rainy season between February and April 2015. UV light traps were set next to cow, chicken, or goat enclosures, whilst barrier screens were erected within the homestead between houses and breeding site. At the laboratories in Macha, mosquitoes were identified to species level using morphological keys (Gillies and De Meillon 1968, Gillies and Coetzee 1987) and identities confirmed using standard diagnostic PCRs for

Table 1. Details of anopheline specimens sequenced and referenced, GPS locations where specimens were trapped, and results of CSP ELISA and qPCR for *P. falciparum*

Specimen and infection status	GenBank	Longitude	Latitude
<i>Anopheles</i> sp. 475 +*	KU524734	26.9004	-16.2537
<i>Anopheles squamosus</i> 526	KU524735	27.0154	-16.3737
<i>Anopheles squamosus</i> 540	KU524736	27.0154	-16.3737
<i>Anopheles squamosus</i> 564+*	KU524737	27.0154	-16.3737
<i>Anopheles squamosus</i> 609+	KU524738	26.9168	-16.2500
<i>Anopheles squamosus</i> 610	KU524739	26.9168	-16.2500
<i>Anopheles squamosus</i> 671+	KU524740	26.9168	-16.2500
<i>Anopheles squamosus</i> 539	KU524741	27.0154	-16.3737
<i>Anopheles squamosus</i> 541	KU524742	27.0154	-16.3737
<i>Anopheles squamosus</i> 613	KU524743	26.9168	-16.2500
<i>Anopheles squamosus</i> 614	KU524744	26.9168	-16.2500
<i>Anopheles squamosus</i> 440	KU524745	26.8784	-16.2937
<i>Anopheles squamosus</i> 508	KU524746	26.9168	-16.2500
<i>Anopheles squamosus</i> 510	KU524747	26.9168	-16.2500
<i>Anopheles squamosus</i> 611	KU524748	26.9168	-16.2500
<i>Anopheles squamosus</i> 620+*	KU524749	26.9168	-16.2500
<i>Anopheles squamosus</i> 649+	KU524750	26.9168	-16.2500
<i>Anopheles squamosus</i> 706B+*	KU524751	26.9168	-16.2500
<i>Anopheles coluzzi</i>	KU524752	Keele Strain	
<i>Anopheles gambiae</i>	KU524753	28.8072	-9.2569
<i>Anopheles squamosus</i> SQ5	KU524754	26.7906	-16.3929
<i>Anopheles squamosus</i> SQ3	KU524755	26.7906	-16.3929
<i>Anopheles squamosus</i> SQ15	KU524756	26.7906	-16.3929
<i>Anopheles squamosus</i> SQ16	KU524757	26.7906	-16.3929
<i>Anopheles squamosus</i> 392	KU524758	26.9532	-16.3786
<i>Anopheles squamosus</i> 433	KU524759	26.8412	-16.4425
<i>Anopheles squamosus</i> 327	KU524760	26.9004	-16.2537
<i>Anopheles squamosus</i>	JN994170.1	Norris and Norris 2015	
<i>Anopheles pharoensis</i>	JN994163.1		
<i>Anopheles coustani</i>	JN994154.1		
<i>Anopheles rufipes</i>	JN994169.1		
<i>Anopheles pretoriensis</i>	JN994164.1		
<i>Anopheles funestus</i>	JN994155.1		
<i>Anopheles parensis</i>	JN994162.1		
<i>Anopheles vaneedeni</i>	JN994172.1		
<i>Anopheles leesoni</i>	JN994158.1		
<i>Anopheles rivulorum</i>	JN994168.1		
<i>Anopheles theileri</i>	JN994171.1		
<i>Anopheles arabiensis</i>	JN994153.1		
<i>Anopheles quadriannulatus</i>	JN994165.1		

+—CSP-ELISA positive, *—*P. falciparum* qPCR positive.

An. gambiae and *An. funestus* species complexes, the latter which targets a polymorphic region of the ribosomal intergenic spacer 2 (ITS2) region of DNA (Scott et al. 1993, Koekemoer et al. 2002, Kent et al. 2006). Addition of primers to this PCR designed to amplify the ITS2 region of other African anophelines, allowed for detection of species not of the *An. funestus* and *An. gambiae* complexes (Das et al. 2016). All samples underwent CSP ELISA and positive samples were determined by OD readings 2-fold greater than the negative controls (Burkot et al. 1984). Real-time qPCR was performed on DNA extracts from the head and thorax to detect *P. falciparum*.

All parasite-positive samples were sent to Johns Hopkins Bloomberg School of Public Health, Baltimore, for mosquito species confirmation by amplification and alignment of a 698 bp fragment from the mitochondrial cytochrome oxidase subunit I gene (COI) (Norris and Norris 2015). Consensus sequences were aligned and Maximum Parsimony (MP) trees constructed (1,000 bootstraps) with *An. coustani* as an outgroup, using MEGA 6.0 (Tamura et al.

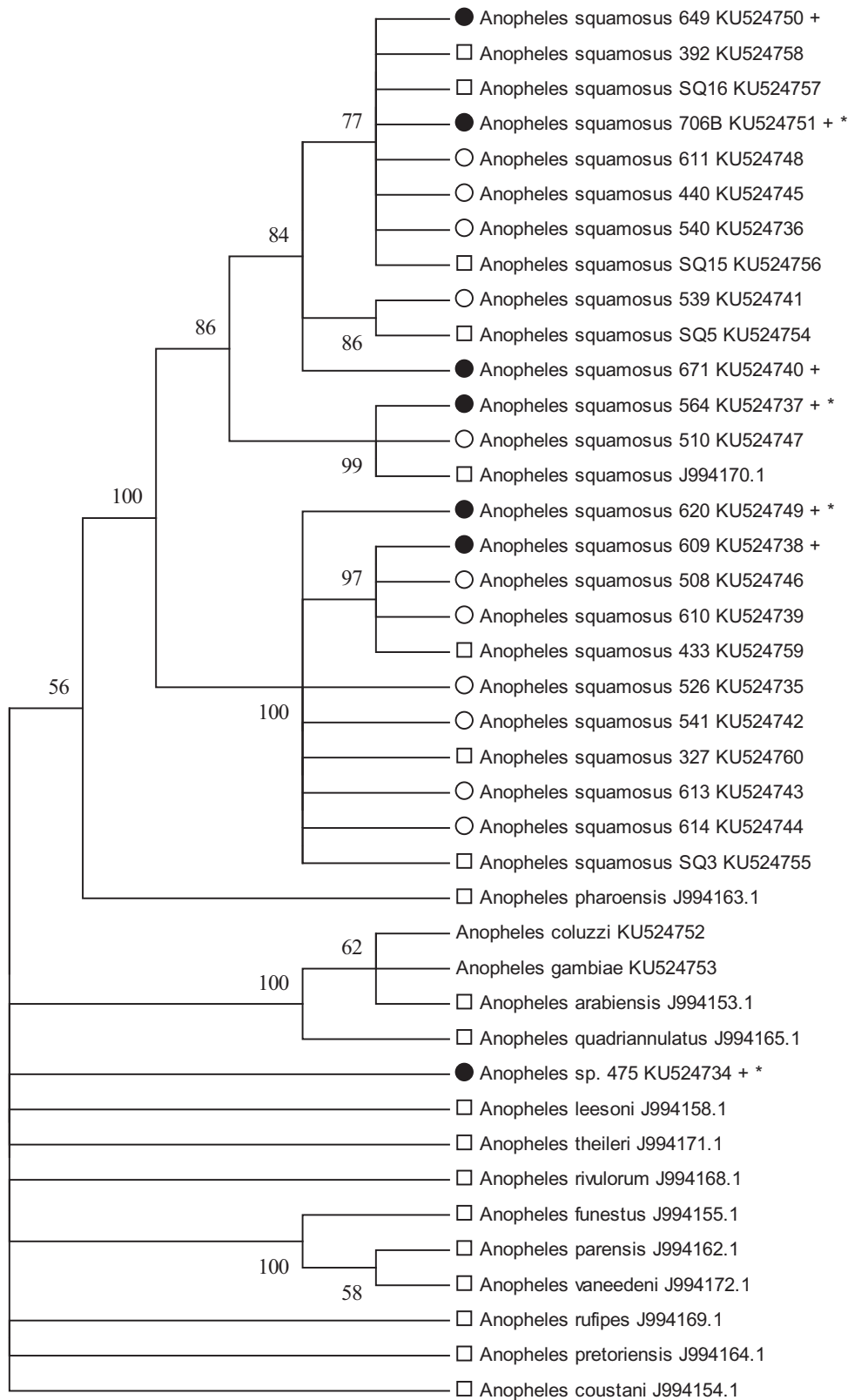


Fig. 1. Cytochrome oxidase subunit I (COI) Maximum Parsimony tree, one of the two most parsimonious trees that did not differ in any arrangements after collapse of all branches with <50% bootstrap support (1,000 replicates). Circle—UV trap outdoors, Square—CDC standard light trap indoors, Filled Circle—CSP-ELISA positive, *An. gambiae* from barrier collection in northern Zambia, *An. coluzzi* from insectary at Johns Hopkins Bloomberg School of Public Health, +—CSP-ELISA positive, *—*P. falciparum* qPCR positive.

2013) as previously described by Norris and Norris (Norris and Norris 2015). The blood feeding host preference of all samples identified as *Anopheles squamosus* Theobald, 1901 were analyzed by PCR (Kent and Norris 2005).

A total of 834 female anophelines were caught during the study from the outdoor light traps and barrier screens. Morphological and molecular identifications were successfully conducted on 812 specimens and revealed domination of catches by *An. squamosus* (40.3%) and *An. arabiensis* (24.8%). Other species identified were *An. coustani* (6.0%), *An. rufipes* (5.5%), *An. quadriannulatus* (4.8%), *An. parensis* (3.7%), *An. lesoni* (1.2%), *An. longipalpis* (1.0%), *An. pretoriensis* (1.0%), *An. rivulorum* (0.1%), and *An. rivulorum-like* (0.1%). Of the 812 samples, 51 were blood-fed with 27 of these identified as *An. squamosus*. These 27 bloodmeals were identified as nonhuman, with 78% of bloodmeals taken from goats. Following standard CSP ELISA, seven of the 812 samples had OD values 2-fold greater than the negative controls. These samples were morphologically identified as *An. squamosus* ($n = 6$) and *An. coustani* ($n = 1$). All samples were analyzed by qPCR of which four gave positive signals for *P. falciparum* (Table 1).

A 698 bp fragment from the mosquito mitochondrial COI was amplified and sequenced from all seven ELISA/qPCR-positive specimens (Norris and Norris 2015). Included with these samples were 11 randomly selected *Plasmodium*-negative specimens caught during the same week, from light traps and identified morphologically as *An. squamosus*, as this morphological taxon dominated the collection. Also included in the phylogenetic analysis were seven mosquitoes morphologically identified as *An. squamosus* from other studies in the Macha area, and specimens of *An. gambiae* s.s. from Nchelenge district, Luapula Province, northern Zambia and *An. coluzzii* specimens from colonies kept at Johns Hopkins Bloomberg School of Public Health (Table 1). These samples were included to address consistency of morphological identifications over time and across studies. The MP analysis and resulting trees revealed that all specimens morphologically identified as *An. squamosus* cluster together with 100% support (Fig. 1). The existence of at least two molecular COI clades is also apparent and strongly supported within *An. squamosus* and warrant further investigation. The molecular identity of specimen 475 remains ambiguous, despite morphological identification as *An. coustani*. This sequence does not cluster with any significance to any other available anopheline COI sequence, most notably any recognized vector species. This lone *P. falciparum*-positive specimen may suggest the existence of yet another potentially important unreported malaria vector species.

Studies are increasingly reporting the potential importance of secondary vectors (Awono-Ambene et al. 2004, Okorie et al. 2011, Stevenson et al. 2012, Animut et al. 2013, Mwangangi et al. 2013, Nepomichene et al. 2015, St. Laurent et al. 2016). Whilst there is the possibility of false positives resulting from ELISAs conducted on zoophagic species (Durmez et al. 2011, Charlwood et al. 2015), our current study in Zambia used both antigen and DNA-based detection methods to determine infection rates and confirm the presence of *P. falciparum* sporozoites and DNA in *An. squamosus* mosquitoes. These anophelines have not been associated with malaria transmission in this area, although historic reports have implicated *An. squamosus* in malaria transmission by sporozoite visualization in nearby Tanzania and Zimbabwe (Gillies 1964, Gillies and De Meillon 1968). The fact that there are no animal reservoirs of *P. falciparum* apart from humans, supports the potential role of *An. squamosus* in malaria transmission in Africa. The COI data generated in this study demonstrated presence of two strongly supported molecular clades in mosquitoes identified as *An. squamosus*, and three

specimens which were positive by both ELISA and qPCR fell into each of the two clades. These mosquitoes were caught outdoors near goat pens and although the extent of their outdoor foraging behavior requires further investigation, such behaviors could undermine current elimination efforts that rely on vector control targeting indoor human sleeping structures. This study did not reveal presence of human blood in any of the *An. squamosus* specimens caught, but previous studies from the area have demonstrated significant anthropophily of *An. squamosus* (Fornadel et al. 2011).

Residual transmission may explain the continued presence of cases in the Macha area. Our findings highlight the utility of molecular tools for both determination of infectivity and accurate identification of anopheline species, and stress the importance of rigorous entomological studies that are not limited to known malaria vectors but also incorporate sympatric anophelines active both indoors and outdoors. Such studies are essential to fully evaluate the epidemiological importance of secondary vectors and to develop appropriate vector control tools. To our knowledge, this is the first molecular confirmation of *An. squamosus* harboring *P. falciparum* sporozoites. Evidence of the existence of *Plasmodium*-infectious exophagic *An. squamosus* and other unidentified taxa indicates that species other than well-recognized malaria vectors could play a role in malaria transmission in Southern Africa, which may jeopardize current malaria elimination efforts, where vector control is solely indoor based. Larval source management which targets both indoor and outdoor resting mosquitoes, is the only recommended programmatic intervention by the World Health Organization (Tusting et al. 2013, WHO 2013), but its use is generally limited to areas where breeding sites are identifiable and limited. It requires a large investment in terms of capacity for sustained program management and entomological monitoring and surveillance. If exophagic and zoophilic species such as *An. squamosus* are found to play an important role in transmission, there are a number of interventions that can reduce exposure to mosquitoes outdoors, such as the use of topical and spatial repellents (Achee et al. 2012, Debboun and Strickman 2013, Wilson et al. 2014), application of topical and systemic insecticides to animals (Hewitt and Rowland 1999, Rowland et al. 2001, Habtewold et al. 2004, Chaccour et al. 2013, Franco et al. 2014, Poche et al. 2015), and deployment of odor-baited traps (Okumu et al. 2010). However, most of these interventions have not been demonstrated to have a marked impact on malaria incidence across multiple sites and so have not received endorsement by the WHO at present. There is urgent need for these technologies to be fully evaluated.

Acknowledgments

We gratefully acknowledge the Southern Africa ICEMR field teams in Macha for their logistical support and participation in field collections and laboratory analysis. We are also very grateful to the communities in Zambia in whose households collections were made. This study was carried out following ethical approval from the Tropical Disease Research Centre IRB, Ndola (TDRC/ERC/2010/4/11) and Johns Hopkins Bloomberg School of Public Health IRB (00003467). This work was supported in part, through funding from the Southern Africa ICEMR (U19AI089680-01) to D.E.N. C.M.J. was supported by a NIH T32 Grant (2T32AI007417-16) and a Johns Hopkins Malaria Research Institute Fellowship. J.C.P. was supported in part by the Johns Hopkins Malaria Research Institute.

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