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DNA barcoding of mosquitoes collected through a nationwide survey in 2011 and 2012 in Malawi, Southeast Africa

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ABSTRACT

We conducted a nationwide survey of mosquito distribution in Malawi from November 2011 to April 2012, and from July to September 2012. Using dried specimens of mosquito adults collected during the survey, we analyzed their cytochrome *c* oxidase subunit I (COI) gene sequences, prepared specimens, and registered the genetic information (658 bp) of 144 individuals belonging to 51 species of 10 genera in GenBank. Using the obtained genetic information, we analyzed the degree of intraspecific variation and investigated the various species from morphological and genetic perspectives. Moreover, we conducted phylogenetic analysis of the medically important species distributed from Africa to Asia and explored their geographical differentiation. Results showed that individuals morphologically classified as *Culex univittatus* complex included a individual of *Cx. perexiguus* which, to date, have not been reported in southern Africa. Furthermore, *Mansonia uniformis,* distributed in Africa and Asia, was revealed to belong to genetically distinct populations, with observed morphological differences of the samples suggesting that they are separate species. The results of genetic analysis further suggested that *Cx. ethiopicus* is not a synonym of *Cx. bitaeniorhynchus*, but that it is an independent species; although, in this study, the only definite morphological difference observed was in the shape of the wing scales. Further morphological and genetic investigation of individuals of these species, including larvae, is highly recommended.

1. Introduction

The Republic of Malawi is situated in the southeastern part of Africa. Like many other African countries, it is plagued with the threat of malaria putting people's lives at risk. The main mosquito-borne diseases reported within Malawi are malaria and filariasis (Merelo-Lobo et al., 2003; Kazembe et al., 2006; Ngwira et al., 2007). Although there are historic reports of Chikungunya fever, O'nyong-nyong fever, Rift Valley fever, and other mosquito-borne viral infections (Lutwama et al., 1999; Ikegami & Makino, 2004; Powers and Logue, 2007), no relevant reports have been filed in recent years. Meanwhile, countries surrounding Malawi have seen frequent epidemics of mosquito-borne viral infections, including Rift Valley fever, dengue fever, West Nile fever, and Chikungunya fever (Amarasinghe et al., 2011; Sumaye et al., 2013; Himeidan et al., 2014; Braack et al., 2018; Matiko et al., 2018). Given that these pathogens have crossed borders and entered neighboring countries, they are highly likely to be spread into Malawi by migrating people, livestock, and wild animals. The risk of epidemics of these infectious diseases depends on mosquitoes as vectors. However, very little is known about the distribution and diversity of mosquitoes inhabiting Malawi, with no recent data available on their ecological details, such as species composition, geographical distribution, and seasonal

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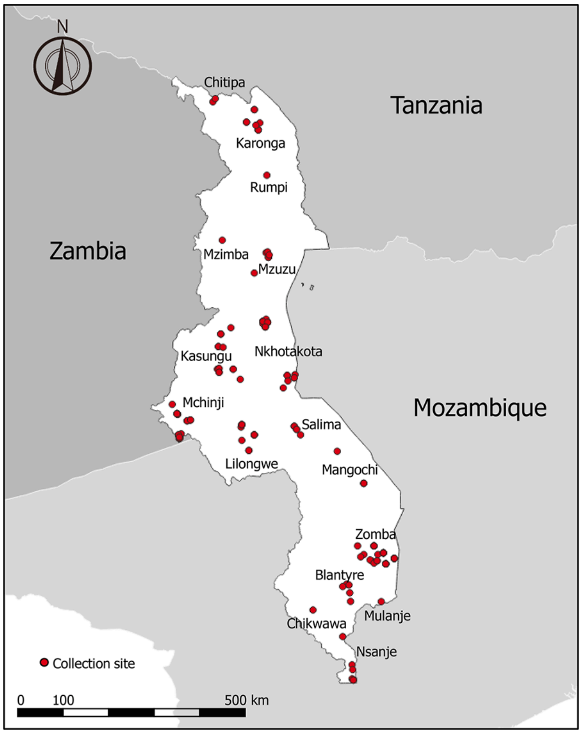

Figure 1. A map showing the collection sites (red circle) and name of localities in Malawi.

prevalence. Highly specialized knowledge, technological expertise, and mobility are required for collecting and surveying mosquitoes, but there are no specialists deeply acquainted with the whole field of mosquito ecology and disease transmission within Malawi. In addition, the underdeveloped infrastructure, in particular the road networks, has precluded attempts to conduct major surveys. If a pathogen were to be carried into Malawi from surrounding countries, Malawian institutions would need to clarify the transmission cycle of the disease domestically. However, without reliable ecological data on mosquito species, including vector species, it would be almost impossible to predict and control epidemics without increasing the risk of subsequent public health problems. Although mosquito species are generally identified based on their external morphological characteristics, many specimens collected in the field tend to be damaged and missing important identification characteristics (such as bristles, scales, parts of legs, and wings), either by aging or from the use of trap fans and sweep nets. It is necessary to identify the mosquito species of the severely damaged specimens if the purpose is to understand the infection cycle of mosquito-borne diseases. Therefore, a method to accurately identify partial mosquito specimens is needed to conduct entomological mosquito surveillance.

In recent years, as a substitute for morphological species identification, a molecular technique has been widely used to identify species. The base sequence of the cytochrome *c* oxidase subunit I (COI) gene domain of an unidentified species is determined and compared to the gene sequences of identified species (i.e., DNA barcoding) (Folmer et al., 1994; Hebert et al., 2003). This method has been reported to be applicable to the identification of mosquito species and is also useful for identification of sibling species and subspecies, and specimens that are too severely damaged to identify morphologically (Cywinska et al., 2006; Kumar et al., 2007). Species identification by DNA barcoding is highly versatile and has many advantages because it can identify related and unknown species. However, this method of species identification is impossible without genetic information for collation (Maekawa et al., 2016). To date, African mosquito COI gene sequences have been registered from Uganda, Kenya, Tanzania, Zambia, South Africa, Benin, and Mayotte (Cook et al., 2009; Le Goff et al., 2013; Lobo et al., 2015; Bennett et al., 2015; Bennett et al., 2016; Ajamma et al., 2016; Mixão et al., 2016). Given the increasing availability of molecular species identification technology in Africa, genetic information needs to be prepared not only for medically important species but also more generally for species indigenous to Africa.

In this study, we analyzed a COI gene sequence (658 bp) using dried specimens of adult mosquitoes collected during a nationwide study to gather genetic information of mosquitoes in Malawi. Additionally, we used the DNA sequences to analyze the degree of intraspecific variation and conduct comparative morphological investigations. Finally, by referencing medically important species distributed across the world—i.e., *Culex quinquefasciatus* Say, *Mansonia uniformis* (Theobald), and *Cx. bitaeniorhynchus* Giles—we compared genetic distances between populations based on the obtained DNA sequences and GenBank-registered sequences to investigate geographical differentiation.

2. Materials and methods

2.1. Sample collection, specimen preparation, and DNA barcoding

We conducted a nationwide survey of mosquito distribution in Malawi from November 2011 to April 2012 (rainy season) and from July to September 2012 (dry season). To collect mosquitoes, we used 20 CDC Miniature Light Traps (John W. Hock Company). Ten houses were selected at each collection site (Fig. 1). In each house, one CDC light trap was hung in the bedroom (for indoor collection) and at the entrance (for outdoor collection) about 1.5m high from the ground. Mosquitoes were collected overnight from 16:00 to 07:00. To identify the species of collected individuals, morphological keys of Edwards (1941), Gillies and De Meillon (1968), Service (1990), and Jupp (1996) were used. Of the classified adult samples, those in good condition, those of rare species, and those requiring reconfirmation were preserved as dried pin specimens. They were placed in specimen boxes for future morphological observation and stored at the Department of Biological Sciences, Chancellor College, University of Malawi, and the Department of Medical Entomology, National Institute of Infectious Diseases (NIID), Japan. DNA analysis was carried out at the laboratory of Medical Entomology, NIID. For DNA extraction, we used the adult pin specimens that were in good condition and morphologically identifiable to the species, which were stored at the NIID. As a gene sample, a middle leg was collected from each dried pin specimen, placed in a 0.2 ml tube, and stored at −20°C. For species with clear characteristics in the middle leg joint, as well as for individuals lacking middle legs, either a fore or hind leg was collected. For specimens morphologically identified as *Anopheles gambiae* complex, a polymerase chain reaction (PCR) was performed to confirm the species, following the method of Scott et al. (1993). The *Cx. univittatus* complex includes three African species that exhibit morphological similarities in all life stages (Mixão et al., 2016). Therefore, the COI sequences of specimens that were morphologically identified as belonging to the *Cx. univittatus* complex were compared against GenBank-registered COI gene sequences to confirm their species identity.

COI gene analysis was conducted following the method used by Maekawa et al. (2016). To extract DNA from the samples, the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich) was used. To amplify DNA, we used LCO1490 and HCO2198 primers (Folmer et al., 1994) and TaKaRa *Ex Taq* Hot Start Version (TaKaRa). The PCR reactions were carried out in a 10 μL volume containing 1.00 μL of 10x PCR buffer, 0.80 μL of 2.5 μM dNTP mixture, 0.05 μL of 5 U/μL *Ex Taq* HS, 0.50 μL of each 2.5 μM primer, 6.15 μL of DDW, and 1.00 μL of DNA template. The temperature settings were based on the PCR conditions given by Kumar et al. (2007), as follows: an initial denaturation at 95°C for 5 min followed by 5 cycles at 94°C for 40 s (denaturation), 45°C for 1 min (annealing), 72°C for 1 min (extension), 35 cycles at 94°C for 40 s (denaturation), 51°C for 1 min (annealing), 72°C for 1 min (extension),

Table 1
. The mosquito specimens used in the study, with the details of their collection sites, specimen code, and GenBank accession number.

Serial no.	Species	Collection details of specimens Region	Locality	Site	GPS coordinates	Date	Method	in/out	Specimen code	GenBank accession no.	R.M.S
1	*Anopheles coustani*	Central	Lilongwe	Lumbadzi	S 14.0244, E 33.8441	February 2012	LT	out	M269	LC473584	
2	*An. coustani*	Central	Mchinji	Mkanda	S 13.5680, E 32.9580	February 2012	LT	out	M282	LC473585	
3	*An. coustani*	Northern	Mzuzu	Chiwanja	S 11.6266, E 34.1588	March 2012	LT	out	M288	LC473586	
4	*An. demeilloni*	Southern	Zomba	Zilindo	S 15.5636, E 35.5005	January 2012	LT	out	M263	LC473587	
5	*An. demeilloni*	Southern	Zomba	Zilindo	S 15.5505, E 35.4805	January 2012	LT	out	M264	LC473588	
6	*An. demeilloni*	Northern	Rumphi	Livingstone	S 10.6338, E 34.1619	March 2012	LT	out	M293	LC473589	
7	*An. demeilloni*	Central	Lilongwe	Lumbadzi	S 13.9541, E 34.0055	February 2012	LT	out	M276	LC473594	
8	*An. demeilloni*	Southern	Zomba	Zilindo	S 15.5933, E 35.5372	September 2012	LT	out	M313	LC473595	
9	*An. arabiensis*	Southern	Zomba	Kachulu	S 15.5386, E 35.7961	January 2012	LT	out	M266	LC473596	
10	*An. arabiensis*	Central	Kasungu	Khamenya	S 12.5844, E 33.7075	February 2012	LT	in	M285	LC473597	
11	*An. arabiensis*	Central	Mchinji	Chidambo	S 13.9808, E 33.0408	February 2012	LT	in	M286	LC473598	
12	*An. maculipalpis*	Central	Lilongwe	Lumbadzi	S 13.9541, E 34.0055	February 2012	LT	out	M272	LC473599	
13	*An. maculipalpis*	Central	Kasungu	Khamenya	S 12.6655, E 33.5761	February 2012	LT	out	M283	LC473600	
14	*An. maculipalpis*	Northern	Chitipa	Kafora	S 9.6963, E 33.4730	March 2012	LT	out	M292	LC473601	
15	*An. pretoriensis*	Central	Lilongwe	Lumbadzi	S 13.9541, E 34.0055	February 2012	LT	out	M270	LC473602	
16	*An. pretoriensis*	Northern	Chitipa	Kafora	S 9.6580, E 33.5088	March 2012	LT	out	M290	LC473603	
17	*An. rufipes*	Southern	Zomba	Kachulu	S 15.5386, E 35.7961	January 2012	LT	out	M267	LC473604	
18	*An. rufipes*	Central	Lilongwe	Lumbadzi	S 13.9541, E 34.0055	February 2012	LT	out	M271	LC473605	
19	*An. rufipes*	Central	Mchinji	Chidambo	S 13.9522, E 33.0338	February 2012	LT	out	M281	LC473606	
20	*An. squamosus*	Southern	Zomba	Kachulu	S 15.6033, E 35.6830	January 2012	LT	out	M265	LC473607	
21	*An. squamosus*	Central	Mchinji	Mkanda	S 13.6861, E 33.0125	February 2012	LT	in	M280	LC473608	
22	*Culex rubinotus*	Central	Mchinji	Chidambo	S 14.0305, E 32.9944	February 2012	LT	in	M190	LC473609	
23	*Cx. rubinotus*	Central	Mchinji	Chidambo	S 14.0305, E 32.9944	February 2012	LT	out	M197	LC473610	
24	*Cx. rubinotus*	Central	Mchinji	Chidambo	S 14.0305, E 32.9944	February 2012	LT	out	M199	LC473611	
25	*Cx. rubinotus*	Central	Kasungu	Chitete	S 13.1172, E 33.5341	February 2012	LT	out	M206	LC473612	
26	*Cx. rubinotus*	Northern	Mzuzu	Chiwanja	S 11.6841, E 34.1875	March 2012	LT	out	M231	LC473613	
27	*Cx. rima*	Central	Mchinji	Mkanda	S 13.6861, E 33.0125	February 2012	LT	out	M144	LC473614	
28	*Cx. rima*	Northern	Mzuzu	Chiwanja	S 11.6841, E 34.1875	March 2012	LT	in	M35	LC473615	
29	*Cx. cinereus*	Southern	Zomba	Matawale	S 15.4838, E 35.4069	January 2012	LT	out	M153	LC473616	
30	*Cx. cinereus*	Central	Mchinji	Mkanda	S 13.7627, E 33.1825	February 2012	LT	in	M196	LC473617	
31	*Cx. poicilipes*	Southern	Zomba	Kachulu	S 15.4694, E 35.6588	January 2012	LT	out	M160	LC473618	
32	*Cx. poicilipes*	Southern	Zomba	Kachulu	S 15.4694, E 35.6588	January 2012	LT	out	M161	LC473619	
33	*Cx. ethiopicus*	Central	Nkhotakota	Chia	S 13.1972, E 34.4227	February 2012	LT	out	M220	LC473620	
34	*Cx. ethiopicus*	Northern	Karonga	Kaporo	S 9.9672, E 34.0744	March 2012	LT	out	M235	LC473621	
35	*Cx. aurantapex*	Southern	Zomba	Kachulu	S 15.4822, E 35.5886	January 2012	LT	in	M159	LC473622	
36	*Cx. aurantapex*	Southern	Zomba	Kachulu	S 15.4694, E 35.6588	January 2012	LT	out	M165	LC473623	

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Table 1 (*continued*)

Serial no.	Species	Region	Collection details of specimens Locality	Site	GPS coordinates	Date	Method	in/ out	Specimen code	GenBank accession no.	R.M.S
37	*Cx. aurantapex*	Central	Nkhotakota	Chia	S 13.1972, E 34.4227	February 2012	LT	in	M219	LC473624	
38	*Cx. annulioris*	Southern	Blantyre	Chigumula	S 15.9716, E 35.2219	January 2012	LT	out	M170	LC473625	
39	*Cx. annulioris*	Central	Lilongwe	Lumbadzi	S 13.8577, E 33.8347	February 2012	LT	out	M184	LC473626	
40	*Cx. annulioris*	Central	Kasungu	Mtunthama	S 13.2450, E 33.8269	February 2012	LT	out	M214	LC473627	
41	*Cx. annulioris*	Northern	Karonga	Kaporo	S 10.0558, E 34.0597	July 2012	LT	in	M246	LC473628	
42	*Cx. duttoni*	Northern	Mzuzu	Chiwanja	S 11.6180, E 34.1766	March 2012	LT	out	M25	LC473629	
43	*Cx. duttoni*	Central	Kasungu	Chitete	S 13.1172, E 33.5341	February 2012	LT	out	M28	LC473630	
44	*Cx. argenteopunctatus*	Central	Kasungu	Khamenya	S 12.6655, E 33.5761	February 2012	LT	out	M201	LC473631	
45	*Cx. argenteopunctatus*	Northern	Karonga	Kaporo	S 10.0558, E 34.0597	March 2012	LT	out	M237	LC473632	
46	*Cx. argenteopunctatus*	Northern	Karonga	Kaporo	S 10.0558, E 34.0597	March 2012	LT	out	M238	LC473633	
47	*Cx. univittatus* complex	Central	Lilongwe	Lumbadzi	S 13.8261, E 33.8452	February 2012	LT	out	M177	LC473634	*Cx. perexiguus*
48	*Cx. univittatus* complex	Central	Kasungu	Khamenya	S 12.6655, E 33.5761	February 2012	LT	out	M203	LC473635	*Cx. neavei* like
49	*Cx. univittatus* complex	Central	Nkhotakota	Illovo	S 12.5080, E 34.1197	February 2012	LT	out	M215	LC473636	*Cx. neavei* like
50	*Cx. univittatus* complex	Central	Nkhotakota	Illovo	S 12.5080, E 34.1197	February 2012	LT	out	M216	LC473637	*Cx. neavei* like
51	*Cx. univittatus* complex	Central	Kasungu	Mtunthama	S 13.1127, E 33.7372	July 2012	LT	out	M242	LC473638	*Cx. univittatus*
52	*Cx. striatipes*	Central	Lilongwe	Lumbadzi	S 13.8261, E 33.8452	February 2012	LT	out	M179	LC473639	
53	*Cx. mirificus*	Central	Lilongwe	Lumbadzi	S 13.8261, E 33.8452	February 2012	LT	out	M176	LC473640	
54	*Cx. mirificus*	Central	Nkhotakota	Illovo	S 12.5080, E 34.1197	February 2012	LT	out	M211	LC473641	
55	*Cx. mirificus*	Central	Nkhotakota	Illovo	S 12.5080, E 34.1197	February 2012	LT	out	M217	LC473642	
56	*Cx. mirificus*	Central	Nkhotakota	Illovo	S 12.5777, E 34.1416	February 2012	LT	in	M218	LC473643	
57	*Cx. terzii*	Northern	Mzimba	Chikangawa	S 11.8813, E 34.0094	March 2012	LT	out	M224	LC473644	
58	*Cx. quinquefasciatus*	Southern	Zomba	Kachulu	S 15.4694, E 35.6588	January 2012	LT	out	M128	LC473645	
59	*Cx. quinquefasciatus*	Central	Lilongwe	Biwi	S 14.1502, E 33.9358	February 2012	BG	in	M133	LC473646	
60	*Cx. quinquefasciatus*	Southern	Zomba	Kachulu	S 15.4694, E 35.6588	January 2012	LT	in	M154	LC473647	
61	*Cx. quinquefasciatus*	Southern	Zomba	Kachulu	S 15.6033, E 35.6830	January 2012	LT	in	M155	LC473648	
62	*Cx. quinquefasciatus*	Southern	Blantyre	Ndirande	S 15.8677, E 35.1830	January 2012	LT	in	M166	LC473649	
63	*Cx. quinquefasciatus*	Southern	Blantyre	Ndirande	S 15.8722, E 35.2136	January 2012	LT	out	M169	LC473650	
64	*Cx. quinquefasciatus*	Central	Lilongwe	Biwi	S 14.1502, E 33.9358	February 2012	LT	in	M171	LC473651	
65	*Cx. quinquefasciatus*	Central	Mchinji	Mkanda	S 13.7627, E 33.1825	February 2012	LT	in	M195	LC473652	
66	*Cx. quinquefasciatus*	Central	Kasungu	Khamenya	S 12.8211, E 33.5447	February 2012	LT	in	M204	LC473653	
67	*Cx. quinquefasciatus*	Central	Kasungu	Khamenya	S 12.8211, E 33.5447	February 2012	LT	in	M205	LC473654	
68	*Cx. quinquefasciatus*	Southern	Mangochi	Chilombo	S 14.1600, E 35.0683	February 2012	LT	in	M222	LC473655	
69	*Cx. quinquefasciatus*	Northern	Mzuzu	Chiwanja	S 11.6550, E 34.1977	March 2012	LT	in	M225	LC473656	
70	*Cx. quinquefasciatus*	Southern	Zomba	Chikanda	S 15.5675, E 35.5713	September 2012	LT	out	M254	LC473657	
71	*Cx. quinquefasciatus*	Southern	Chikwawa	Tomali	S 16.1954, E 34.7501	September 2012	LT	in	M262	LC473658	
72	*Cx. antennatus*	Southern	Zomba	Chilore	S 15.3755, E 35.5314	December 2012	LT	out	M152	LC473659	
73	*Cx. antennatus*	Central	Salima	Chinyamunyamu	S 13.8483, E 34.5186	February 2012	LT	out	M221	LC473660	

(*continued on next page*)

Table 1 (*continued*)

Serial no.	Species	Region	Collection details of specimens Locality	Site	GPS coordinates	Date	Method	in/ out	Specimen code	GenBank accession no.	R.M.S
74	*Cx. perfuscus*	Central	Lilongwe	Lumbadzi	S 13.8261, E 33.8452	February 2012	LT	out	M181	LC473661	
75	*Cx. perfuscus*	Central	Mchinji	Mkanda	S 13.6861, E 33.0125	February 2012	LT	in	M192	LC473662	
76	*Cx. perfuscus*	Central	Mchinji	Mkanda	S 13.6861, E 33.0125	February 2012	LT	in	M193	LC473663	
77	*Aedes scatophagoides*	Southern	Zomba	Kachulu	S 15.5386, E 35.7961	January 2012	LT	in	M15	LC473664	
78	*Ae. aegypti*	Southern	Zomba	Chikanda	S 15.5675, E 35.5713	January 2012	LT	out	M46	LC473665	
79	*Ae. aegypti*	Southern	Mulanje	Mabuka	S 16.1816, E 35.6544	April 2012	LT	out	M48	LC473666	
80	*Ae. luteocephalus*	Northern	Karonga	Kaporo	S 9.7902, E 34.0072	March 2012	LT	in	M106	LC473667	
81	*Ae. luteocephalus*	Central	Nkhotakota	Chia	S 13.1861, E 34.5244	February 2012	LT	out	M94	LC473668	
82	*Ae. simpsoni*	Central	Mchinji	Mkanda	S 13.6861, E 33.0125	February 2012	LT	in	M49	LC473669	
83	*Ae. argenteopunctatus*	Central	Mchinji	Chidambo	S 13.9522, E 33.0338	Feburuary 2012	LT	out	M50	LC473670	
84	*Ae. argenteopunctatus*	Southern	Zomba	Kachulu	S 15.6033, E 35.6830	January 2012	LT	out	M98	LC473671	
85	*Ae. alboventralis*	Central	Kasungu	Khamenya	S 12.8388, E 33.6016	Feburuary 2012	LT	out	M110	LC473672	
86	*Ae. ochraceus*	Northern	Karonga	Kaporo	S 9.9547, E 33.9019	March 2012	LT	out	M76	LC473673	
87	*Ae. ochraceus*	Northern	Karonga	Kaporo	S 9.9547, E 33.9019	March 2012	LT	out	M78	LC473674	
88	*Ae. quasiunivittatus*	Malawi	No data	No data	No data	Jan.- Mar. 2012	LT	-	M256	LC473675	
89	*Ae. dalzieli*	Central	Mchinji	Mkanda	S 13.6861, E 33.0125	February 2012	LT	in	M194	LC473676	
90	*Ae. dalzieli*	Central	Salima	Chinyamunyamu	S 13.8483, E 34.5186	February 2012	LT	out	M66	LC473677	
91	*Ae. dalzieli*	Central	Salima	Chikuni	S 13.9591, E 34.5933	February 2012	LT	out	M84	LC473678	
92	*Ae. dalzieli*	Southern	Zomba	Kachulu	S 15.5386, E 35.7961	January 2012	LT	in	M86	LC473679	
93	*Ae. dalzieli*	Central	Kasungu	Chitete	S 13.1586, E 33.5525	February 2012	LT	out	M95	LC473680	
94	*Ae. hirsutus*	Central	Mchinji	Mkanda	S 13.6913, E 33.0294	February 2012	LT	in	M109	LC473681	
95	*Ae. hirsutus*	Central	Nkhotakota	Illovo	S 12.5777, E 34.1416	February 2012	LT	out	M74	LC473682	
96	*Ae. hirsutus*	Central	Mchinji	Chidambo	S 13.9808, E 33.0408	February 2012	LT	in	M81	LC473683	
97	*Ae. hirsutus*	Central	Mchinji	Mkanda	S 13.7758, E 33.1494	February 2012	LT	in	M83	LC473684	
98	*Ae. hirsutus*	Central	Kasungu	Chitete	S 13.1072, E 33.5597	February 2012	LT	in	M91	LC473685	
99	*Ae. hirsutus*	Central	Salima	Chinyamunyamu	S 13.8836, E 34.5452	February 2012	LT	out	M93	LC473686	
100	*Ae. fascipalpis*	Southern	Zomba	Chilore	S 15.3755, E 35.5314	December 2011	LT	in	M55	LC473687	
101	*Ae. fascipalpis*	Southern	Zomba	Matawale	S 15.5133, E 35.3605	January 2012	LT	out	M56	LC473688	
102	*Ae. fascipalpis*	Southern	Zomba	Chilore	S 15.3755, E 35.5314	December 2011	LT	in	M60	LC473689	
103	*Ae. fascipalpis*	Central	Mchinji	Mkanda	S 13.6861, E 33.0125	February 2012	LT	in	M63	LC473690	
104	*Ae. fascipalpis*	Southern	Mangochi	Chipalamawamba	S 14.5702, E 35.4055	March 2012	LT	in	M65	LC473691	
105	*Ae. mcintoshi*	Central	Mchinji	Mkanda	S 13.7758, E 33.1494	February 2012	LT	out	M69	LC473692	
106	*Ae. mcintoshi*	Central	Nkhotakota	Illovo	S 12.5147, E 34.1777	February 2012	LT	out	M71	LC473693	
107	*Ae. mcintoshi*	Southern	Zomba	Kachulu	S 15.6033, E 35.6830	January 2012	LT	out	M89	LC473694	
108	*Ae. mcintoshi*	Southern	Zomba	Kachulu	S 15.6033, E 35.6830	January 2012	LT	out	M90	LC473695	
109	*Ae. mcintoshi*	Central	Mchinji	Mkanda	S 13.7627, E 33.1825	February 2012	LT	in	M107	LC473696	
110	*Lutzia tigripes*	Central	Mchinji	Mkanda	S 13.6861, E 33.0125	February 2012	LT	out	M16	LC473697	

(*continued on next page*)

Table 1 (*continued*)

Serial no.	Species	Region	Collection details of specimens Locality	Site	GPS coordinates	Date	Method	in/ out	Specimen code	GenBank accession no.	R.M.S
111	*Lt. tigripes*	Central	Salima	Chinyamunyamu	S 13.8836, E 34.5452	February 2012	LT	in	M19	LC473698	
112	*Lt. tigripes*	Southern	Blantyre	Mpemba	S 15.8908, E 35.1341	September 2012	LT	out	M21	LC473699	
113	*Lt. tigripes*	Central	Kasungu	Chitete	S 13.1586, E 33.5525	July 2012	LT	out	M241	LC473700	
114	*Mansonia africana*	Southern	Zomba	Kachulu	S 15.5386, E 35.7961	January 2012	LT	in	M3	LC473701	
115	*Ma. africana*	Southern	Zomba	Kachulu	S 15.4694, E 35.6588	January 2012	LT	out	M4	LC473702	
116	*Ma. africana*	Central	Kasungu	Mtunthama	S 13.1127, E 33.7372	February 2012	LT	in	M5	LC473703	
117	*Ma. africana*	Southern	Nsanje	Nsanje	S 17.1938, E 35.3838	April 2012	LT	out	M8	LC473704	
118	*Ma. uniformis*	Southern	Zomba	Kachulu	S 15.4694, E 35.6588	January 2012	LT	out	M2	LC473705	
119	*Ma. uniformis*	Southern	Nsanje	Nsanje	S 17.1938, E 35.3838	April 2012	LT	out	M11	LC473706	
120	*Ma. uniformis*	Southern	Nsanje	Nsanje	S 17.1938, E 35.3838	April 2012	LT	out	M12	LC473707	
121	*Coquillettidia metallica*	Northern	Karonga	Kaporo	S 9.9950, E 34.0252	March 2012	LT	in	M22	LC473708	
122	*Cq. metallica*	Southern	Zomba	Chilore	S 15.3755, E 35.5314	December 2011	LT	out	M40	LC473709	
123	*Cq. metallica*	Central	Nkhotakota	Illovo	S 12.5394, E 34.1194	February 2012	LT	out	M41	LC473710	
124	*Cq. metallica*	Central	Nkhotakota	Chia	S 13.2683, E 34.4305	February 2012	LT	out	M44	LC473711	
125	*Cq. fuscopennata*	Central	Nkhotakota	Illovo	S 12.5147, E 34.1777	July 2012	LT	out	M38	LC473712	
126	*Cq. microannulata*	Central	Nkhotakota	Chia	S 13.2211, E 34.5130	February 2012	LT	out	M36	LC473713	
127	*Cq. microannulata*	Northern	Karonga	Kaporo	S 9.7902, E 34.0072	July 2012	LT	out	M37	LC473714	
128	*Mimomyia splendens*	Southern	Zomba	Kachulu	S 15.4694, E 35.6588	January 2012	LT	out	M111	LC473715	
129	*Mi. splendens*	Southern	Nsanje	Nsanje	S 16.9672, E 35.4052	April 2012	LT	out	M112	LC473716	
130	*Mi. mimomyiaformis*	Southern	Mangochi	Chipalamawamba	S 14.5702, E 35.4055	August 2012	LT	out	M126	LC473717	
131	*Mi. mimomyiaformis*	Central	Nkhotakota	Illovo	S 12.4786, E 34.1536	February 2012	LT	out	M145	LC473718	
132	*Mi. mimomyiaformis*	Central	Nkhotakota	Chia	S 13.2211, E 34.5130	February 2012	LT	out	M146	LC473719	
133	*Mi. plumosa*	Central	Nkhotakota	Chia	S 13.3597, E 34.3736	February 2012	LT	out	M99	LC473720	
134	*Mi. mediolineata*	Central	Nkhotakota	Illovo	S 12.5080, E 34.1197	February 2012	LT	out	M116	LC473721	
135	*Mi. mediolineata*	Central	Nkhotakota	Illovo	S 12.5080, E 34.1197	February 2012	LT	out	M117	LC473722	
136	*Mi. mediolineata*	Southern	Nsanje	Nsanje	S 16.9391, E 35.4477	April 2012	LT	in	M120	LC473723	
137	*Mi. mediolineata*	Southern	Zomba	Kachulu	S 15.6033, E 35.6830	January 2012	LT	out	M121	LC473724	
138	*Aedeomyia africana*	Southern	Mangochi	Chipalamawamba	S 14.5702, E 35.4055	August 2012	LT	out	M104	LC473725	
139	*Ad. africana*	Southern	Chikwawa	Masanduko	S 16.5350, E 35.1388	September 2012	LT	out	M105	LC473726	
140	*Ad. furfurea*	Central	Nkhotakota	Illovo	S 12.5147, E 34.1777	July 2012	LT	out	M102	LC473727	
141	*Uranotaenia philonuxia*	Central	Nkhotakota	Illovo	S 12.5777, E 34.1416	February 2012	LT	out	M141	LC473728	
142	*Ur. bilineata*	Northern	Mzimba	Muyanjagha Bota	S 11.4611, E 33.5966	March 2012	LT	in	M233	LC473729	
143	*Ur. apicotaeniata*	Southern	Blantyre	Chigumula	S 16.0808, E 35.2327	January 2012	LT	out	M134	LC473730	
144	*Toxorhynchites brevipalpis*	Southern	Zomba	Zomba	S 15.3750, E 35.3275	April 2012	SW	in	M14	LC473731	

Preliminary mosquito collections conducted using BG-Sentinel mosquito trap (Biogents) which was placed indoor house and sweep net collection at in/outdoor house. LT: Light trap collection, BG: BG-Sentinel mosquito trap collection, SW: Sweep net collecttion.

Table 2
The mean, standard deviation, range of nucleotide sequence divergence calculated using the Kimura 2-parameter (K2P) model and relation diseases.

	Species	Number of		K2P divergence (%)					Related diseases*
		Specimens	Site	Mean	SD	Range			
1	*Anopheles coustani*	3	3	0.5	0.1	0.5	-	0.6	Bwamba virus, Lymphatic filariasis
2	*An. demeilloni*	5	3	0.8	0.8	0.0	-	1.9	
3	*An. arabiensis*	3	3	0.3	0.2	0.2	-	0.5	Malaria, Lymphatic filariasis, O'nyong-nyong virus (*An. gambiae* s.l.)
4	*An. maculipalpis*	3	3	0.4	0.2	0.2	-	0.6	
5	*An. pretoriensis*	2	2	2.3	-	-	-	-	
6	*An. rufipes*	3	3	1.5	0.9	0.5	-	2.2	
7	*An. squamosus*	2	2	1.4	-	-	-	-	
8	*Culex rubinotus*	5	3	1.4	0.6	0.5	-	2.2	Banzi virus, Ndumu virus, Germiston virus, Witwatersrand virus,
9	*Cx. rima*	2	2	0.3	-	-	-	-	
10	*Cx. cinereus*	2	2	0.0	-	-	-	-	
11	*Cx. poicilipes*	2	1	0.0	-	-	-	-	Rift Valley fever
12	*Cx. ethiopicus*	2	2	0.2	-	-	-	-	
13	*Cx. aurantapex*	3	2	0.0	0.0	0.0	-	0.0	
14	*Cx. annulioris*	4	4	1.3	1.1	0.3	-	2.3	Kamese virus
15	*Cx. duttoni*	2	2	0.0	-	-	-	-	
16	*Cx. argenteopunctatus*	3	2	0.1	0.1	0.0	-	0.2	
17	*Cx. univittatus*	5	4	3.9	1.5	0.2	-	5.1	Bagaza virus, Ustu virus, Wesselsbron virus, West Nile virus, Sindbis virus, Rift Valley fever
18	*Cx. striatipes*	1	1	-	-	-	-	-	
19	*Cx. mirificus*	4	2	0.2	0.2	0.0	-	0.3	
20	*Cx. terzii*	1	1	-	-	-	-	-	
21	*Cx. quinquefasciatus*	14	9	0.0	0.1	0.0	-	0.3	Ustu virus, West Nile virus, Lymphatic filariasis
22	*Cx. antennatus*	2	2	0.0	-	-	-	-	Ustu virus
23	*Cx. perfuscus*	3	2	0.7	0.5	0.2	-	1.1	
24	*Aedes scatophagoides*	1	1	-	-	-	-	-	
25	*Ae. aegypti*	2	2	0.8	-	-	-	-	Dengue virus, Yellow fever virus, Zika virus, Chikungunya virus, Rift Valley fever
26	*Ae. luteocephalus*	2	2	1.1	-	-	-	-	Yellow fever virus, Chikungunya virus
27	*Ae. simpsoni*	1	1	-	-	-	-	-	Yellow fever virus, Babanki virus
28	*Ae. argenteopunctatus*	2	2	0.0	-	-	-	-	Semliki Forest virus
29	*Ae. alboventralis*	1	1	-	-	-	-	-	
30	*Ae. ochraceus*	2	1	2.2	-	-	-	-	Babanki virus, Ndumu virus, Rift Valley fever
31	*Ae. quasiunivittatus*	1	1	-	-	-	-	-	
32	*Ae. dalzieli*	5	5	0.6	0.3	0.3	-	1.1	
33	*Ae. hirsutus*	6	6	0.4	0.2	0.2	-	0.6	
34	*Ae. fascipalpis*	5	4	0.5	0.2	0.2	-	0.8	
35	*Ae. mcintoshi*	5	3	1.5	1.1	0.3	-	2.8	Wesselsbron vitus, Babanki virus, Ndumu virus, Rift Valley fever, Bunyamwera virus, Ngari virus, Pongola virus
36	*Lutzia tigripes*	4	4	0.8	0.6	0.0	-	1.2	Kamese virus
37	*Mansonia africana*	4	3	0.5	0.2	0.2	-	0.8	Spondweni virus, Ustu virus, Middelburg virus, Rift Valley fever
38	*Ma.uniformis*	3	2	0.5	0.4	0.0	-	0.8	Spondweni virus, Zika virus, Ndumu virus, O'nyong-nyong virus, Rift Valley fever, Bwamba virus, Lymphatic filariasis
39	*Coquillettidia metallica*	4	4	0.7	0.6	0.0	-	1.4	
40	*Cq. fuscopennatus*	1	1	-	-	-	-	-	Sindbis virus,
41	*Cq. microannulata*	2	2	0.0	-	-	-	-	
42	*Mimomyia splendens*	2	2	0.3	-	-	-	-	
43	*Mi. mimomyiaformis*	3	3	0.2	0.1	0.2	-	0.3	
44	*Mi. plumosa*	1	1	-	-	-	-	-	
45	*Mi. mediolineata*	4	3	1.6	1.6	0.0	-	3.3	
46	*Aedeomyia africana*	2	2	6.5	-	-	-	-	
47	*Ad. furfurea*	1	1	-	-	-	-	-	
48	*Uranotaenia philonuxa*	1	1	-	-	-	-	-	
49	*Ur. bilineata*	1	1	-	-	-	-	-	
50	*Ur. apicotaeniata*	1	1	-	-	-	-	-	
51	*Toxorhynchites brevipalpis*	1	1	-	-	-	-	-	

* The related disease was modified Merero-lobo (2003) and Braack et al. (2018).
SD: standard devision.
Means were calculated for specimens for which the sequences were examined in more than two individuals.
SDs were calculated for specimens for which the sequences were examined in more than three individuals.

and a final extension at 72°C for 10 min. PCR products were confirmed with MultiNA (Shimadzu) and a DNA 12000 reagent kit. The resultant amplification products were purified with ExoSAP-IT (Affymetrix). The sequencing samples were prepared with BigDye Terminator Ver1.1 (Life Technologies), and the base sequences were decoded with ABI PRISM 3100-Avant Genetic Analyzer (Life Technologies) and edited with ATGC Ver.7 for Windows (GENETYX). The 658 bp fragment of the COI gene was determined, and 144 obtained sequences were then registered in the GenBank database.

2.2. Construction of a phylogenetic tree and investigation of intraspecific variation based on genetic distance

To construct a phylogenetic tree, Molecular Evolutionary Genetics Analysis software Ver. 5.2 (Tamura et al., 2011) was used. Nucleotide sequence divergences were calculated using the Kimura 2-parameter distance model (Kimura, 1980). A phylogenetic tree was drawn in accordance with the neighbor-joining method (Saitou & Nei, 1987). For the outgroup, *Chironomus riparius* Meigen (Diptera: Chironomidae; GenBank accession no. HM137925 and HM137890) was used, with the reliability of the tree form represented by a bootstrap value after 1,000

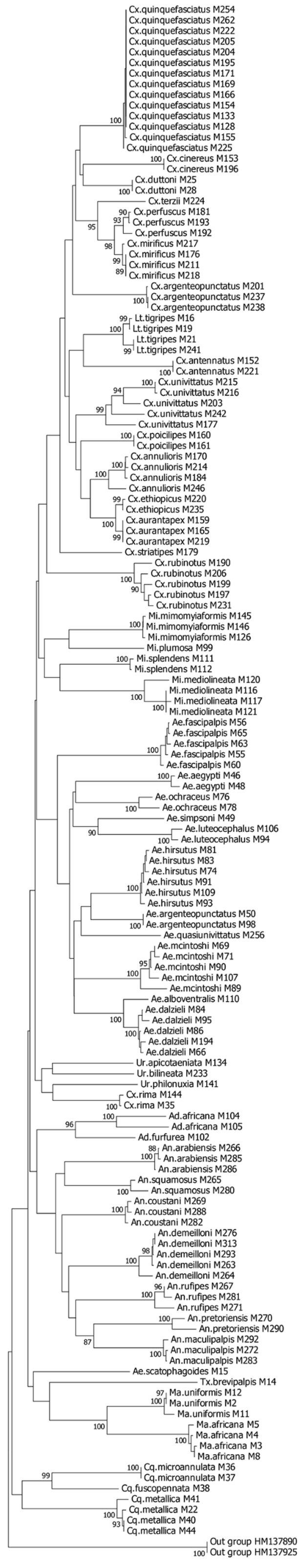

Figure 2. A neighbor-joining tree with 1000 bootstrap replicates constructed using the Kimura 2-parameter calculated from COI sequences (658 bp) of 144 Malawian mosquitoes and 2 outgroup samples, *Chironomus riparius* Meigen (Diptera: Chironomidae). The specimens are labeled with species name and specimen code number listed in Table 1.

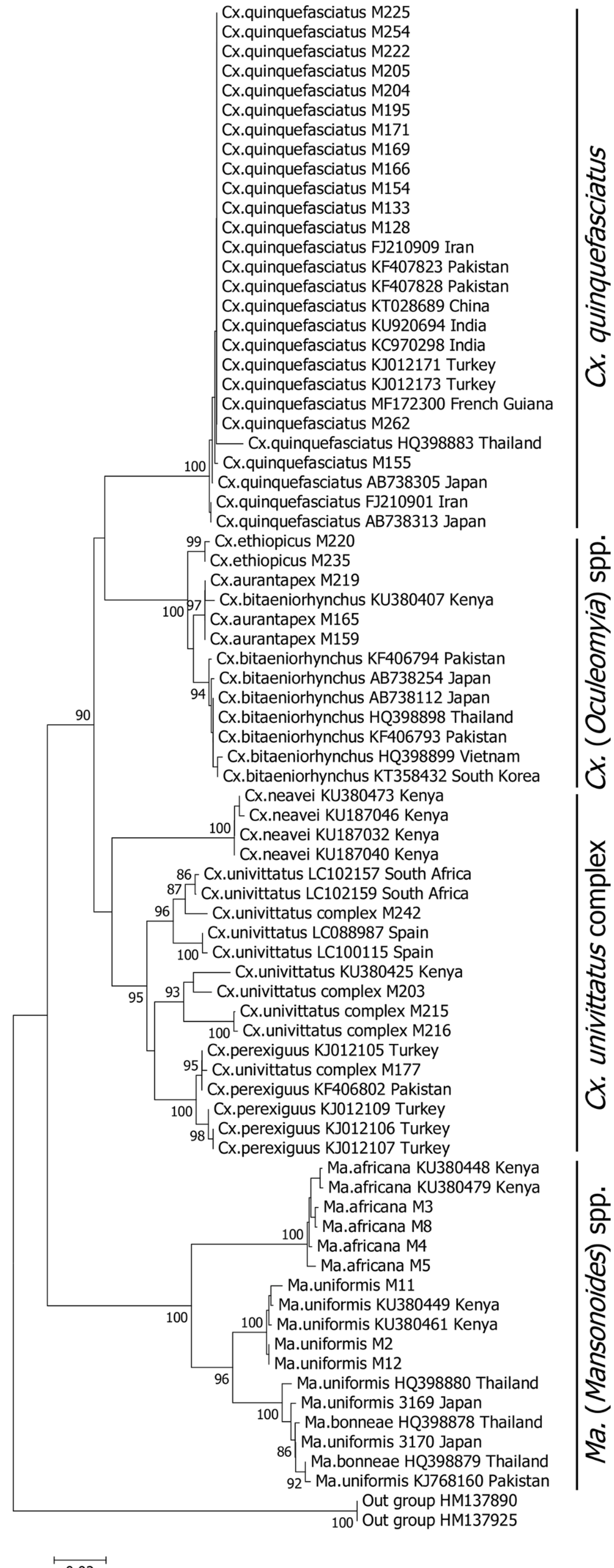

Figure 3. Phylogenetic tree derived from COI sequences (582 bp) using GenBank accessions and specimens of *Cx. quinquefasciatus*, *Cx.* (*Oculeomyia*) spp., *Cx. univittatus* complex, and *Ma.* (*Mansonoides*) spp. The tree was constructed by the neighbor-joining method with 1000 replicates using the Kimura 2-parameter. Specimens collected in this study are labeled with the species name and specimen code listed in Table 1. Specimens found in GenBank are labeled with species name, GenBank accession number and country.

Table 3
Percent pairwise divergence among 19 *Cx. univittatus* complex, calculated using the K2P model.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*Cx. univittatus* complex_M177																			
2	*Cx. univittatus* complex_M203	4.3																		
3	*Cx. univittatus* complex_M215	5.2	3.4																	
4	*Cx. univittatus* complex_M216	5.4	3.5	0.2																
5	*Cx. univittatus* complex_M242	4.8	4.3	5.4	5.2															
6	*Cx. univittatus*_KU380425_Kenya	4.1	2.1	3.4	3.5	5.0														
7	*Cx. univittatus*_LC102157_South_Africa	4.6	3.7	5.0	5.2	1.2	4.5													
8	*Cx. univittatus*_LC102159_South_Africa	4.8	3.9	5.2	5.4	1.4	4.6	0.2												
9	*Cx. univittatus*_LC088987_Spain	4.4	4.3	6.0	6.2	2.3	5.0	2.1	2.3											
10	*Cx. univittatus*_LC100115_Spain	4.6	4.5	6.2	6.4	2.5	5.2	2.3	2.5	0.2										
11	*Cx. perexiguus*_KJ012105_Turkey	0.2	4.1	5.0	5.2	4.6	3.9	4.4	4.6	4.3	4.4									
12	*Cx. perexiguus*_KJ012106_Turkey	1.0	4.3	5.9	6.1	5.2	4.4	4.6	4.8	4.8	4.6	0.9								
13	*Cx. perexiguus*_KJ012107_Turkey	1.0	4.3	5.9	6.1	5.2	4.4	4.6	4.8	4.8	4.6	0.9	0.0							
14	*Cx. perexiguus*_KJ012109_Turkey	0.9	4.1	5.7	5.9	5.0	4.3	4.4	4.6	4.6	4.8	0.7	0.2	0.2						
15	*Cx. perexiguus*_KF406802_Pakistan	0.2	4.1	5.0	5.2	4.6	3.9	4.4	4.6	4.3	4.4	0.0	0.9	0.9	0.7					
16	*Cx. neavei*_KU380473_Kenya	8.0	8.2	9.7	9.9	8.6	9.0	8.4	8.6	9.0	9.2	7.8	8.2	8.2	8.0	7.8				
17	*Cx. neavei*_KU187040_Kenya	7.8	8.0	9.5	9.7	8.4	8.8	8.2	8.4	8.8	9.0	7.6	8.0	8.0	7.8	7.6	0.2			
18	*Cx. neavei*_KU187032_Kenya	7.8	8.0	9.5	9.7	8.4	8.8	8.2	8.4	8.8	9.0	7.6	8.0	8.0	7.8	7.6	0.2	0.0		
19	*Cx. neavei*_KU187046_Kenya	8.2	8.4	9.9	10.1	8.8	9.1	8.6	8.8	9.2	9.4	8.0	8.4	8.4	8.2	8.0	0.2	0.3	0.3	

Specimens collected in this study are labeled with the species name, specimen code and country listed in Table 1.
Specimens found in GenBank are labeled with species name, GenBank accession number and country.
The mean intraspecific variation within the same mosquito species is less than 2%, and if a mean intraspecific variation is over 2% may possibly have included multiple genetically different populations.

repetitions. The mean intraspecific variation (nucleotide sequence divergence) was calculated for specimens of 38 species examined with more than two individuals, and standard deviation (SD) was calculated for the mosquito species with more than three individuals. It has been previously reported that the mean intraspecific nucleotide sequence divergence for same mosquito species is less than 2% (Kumar et al., 2007; Taira et al., 2012). Therefore, given that species with a mean intraspecific variation of more than 2% may possibly have included multiple genetically different populations, the constructed phylogenetic tree was examined to see if it contained any obvious clusters. Whenever several clusters were observed within the same species, a pairwise divergence between the clusters was calculated—bearing in mind the possibility that they belonged to unknown species or subspecies. Additionally, for globally distributed medically important species (*Cx. quinquefasciatus, Ma. uniformis* and *Cx. bitaeniorhynchus*), the GenBank-registered COI gene sequences were compared with the sequences obtained in this study to investigate geographical differentiation and other related factors.

3. Results

A total of 144 individuals belonging to 51 species in 10 genera were registered in GenBank (Table 1). Of the 51 species subjected to gene analysis, the mean, SD, minimum, and maximum values of nucleotide sequence divergence by species were calculated in 38 species (Table 2). The mean intraspecific variation was $<$1.6% in 34 species and $>$2% in the following 4 species: *An. pretoriensis* (Theobald), *Cx. univittatus* complex, *Aedes ochraceus* (Theobald), and *Aedeomyia africana* Neveu-Lemaire. This result was consistent with those of previous studies reporting that the mean intraspecific variation within the same species was $<$2% (Kumar et al., 2007; Taira et al., 2012). Of the four species with intraspecific variation $>$2%, *Cx. univittatus* complex had a larger mean intraspecific divergence of 3.9% (min: 0.2%; max: 5.1%) (Table 2). A few subclades in the phylogenetic tree were distinguished in the cluster of *Cx. univittatus* complex and we inferred that genetically different populations were contained therein (Fig. 2). Accordingly, we performed a phylogenetic analysis using the GenBank-registered COI gene sequence (582 bp) for the *Cx. univittatus* complex distributed in Africa, which revealed three distinct clusters classified as *Cx. univittatus* Theobald, *Cx. perexiguus* and *Cx. neavei* Theobald (Fig. 3). However, *Cx. neavei*, registered from Kenya, falls outside a clade comprising two species of *Cx. univittatus* complex (*Cx. univittatus* and *Cx. perexiguus*). The pairwise divergence between 4 Kenyan *Cx. neavei* and 15 *Cx. univittatus* complex was 7.6–10.1%, showing that *Cx. neavei* and *Cx. univittatus* complex are highly divergent populations (Table 3).

Culex quinquefasciatus, Ma. uniformis, and *Cx. bitaeniorhynchus* are known to be important disease vector mosquitoes that are widely distributed in tropical and subtropical regions. These species are thought to have undergone regional differentiation at progressive levels. Using the GenBank-registered COI gene sequence of these species and related species belonging to the same subgenus, we conducted a phylogenetic analysis (Fig. 3) and calculated pairwise divergence between specimens. The mean pairwise divergence of *Cx. quinquefasciatus* was 0.2% (min: 0.0%; max: 1.9%) (Table 4). *Culex quinquefasciatus,* registered in GenBank from Thailand, showed a higher pairwise divergence (1.2%–1.9%) with other *Cx. quinquefasciatus*. The pairwise divergences were low between other populations (0.0%–0.6%), demonstrating that they are a genetically homogeneous population from Africa to Asia. Taira et al. (2012) reported that low divergence (0.2%–0.5%) was observed in *Cx. quinquefasciatus* between populations from Ryukyus, Japan, and Iran. Therefore, the specimen from Thailand (HQ398883) might be genetically different from other populations. Intensive gene studies are required for *Cx. quinquefasciatus* populations from Thailand.

The African specimens of *Ma. uniformis* were grouped into a different clade from the Asian specimens (Fig. 3). Therefore, we conducted a phylogenetic analysis to confirm the obtained result using the COI gene

Table 4
Percent pairwise divergence among 27 *Cx. quinquefasciatus* collected from 9 countries, calculated using the K2P model.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	*Cx. quinquefasciatus*_M128_Malawi																											
2	*Cx. quinquefasciatus*_M133_Malawi	0.0																										
3	*Cx. quinquefasciatus*_M154_Malawi	0.0	0.0																									
4	*Cx. quinquefasciatus*_M155_Malawi	0.2	0.2	0.2																								
5	*Cx. quinquefasciatus*_M166_Malawi	0.0	0.0	0.0	0.2																							
6	*Cx. quinquefasciatus*_M169_Malawi	0.0	0.0	0.0	0.2	0.0																						
7	*Cx. quinquefasciatus*_M171_Malawi	0.0	0.0	0.0	0.2	0.0	0.0																					
8	*Cx. quinquefasciatus*_M195_Malawi	0.0	0.0	0.0	0.2	0.0	0.0	0.0																				
9	*Cx. quinquefasciatus*_M204_Malawi	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0																			
10	*Cx. quinquefasciatus*_M205_Malawi	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0																		
11	*Cx. quinquefasciatus*_M222_Malawi	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0																	
12	*Cx. quinquefasciatus*_M225_Malawi	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2																
13	*Cx. quinquefasciatus*_M254_Malawi	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2															
14	*Cx. quinquefasciatus*_M262_Malawi	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0														
15	*Cx. quinquefasciatus*_MF172300_French_Guiana	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0													
16	*Cx. quinquefasciatus*_FJ210901_Iran	0.5	0.5	0.5	0.3	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.5	0.5	0.5												
17	*Cx. quinquefasciatus*_FJ210909_Iran	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.5											
18	*Cx. quinquefasciatus*_KJ012171_Turkey	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.5	0.0										
19	*Cx. quinquefasciatus*_KJ012173_Turkey	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.5	0.0	0.0									
20	*Cx. quinquefasciatus*_KC970298_India	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.5	0.0	0.0	0.0								
21	*Cx. quinquefasciatus*_KU920694_India	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0							
22	*Cx. quinquefasciatus*_KF407823_Pakistan	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0						
23	*Cx. quinquefasciatus*_KF407828_Pakistan	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0					
24	*Cx. quinquefasciatus*_KT028689_China	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
25	*Cx. quinquefasciatus*_AB738305_Japan	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2			
26	*Cx. quinquefasciatus*_AB738313_Japan	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.5	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2		
27	*Cx. quinquefasciatus*_HQ398883_Thailand	1.4	1.4	1.4	1.5	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.2	1.4	1.4	1.4	1.9	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.5	1.7	

Specimens collected in this study are labeled with the species name, specimen code and country listed in Table 1.
Specimens found in GenBank are labeled with species name, GenBank accession number and country.
The mean intraspecific variation within the same mosquito species is less than 2%, and if a mean intraspecific variation is over 2% may possibly have included multiple genetically different populations.

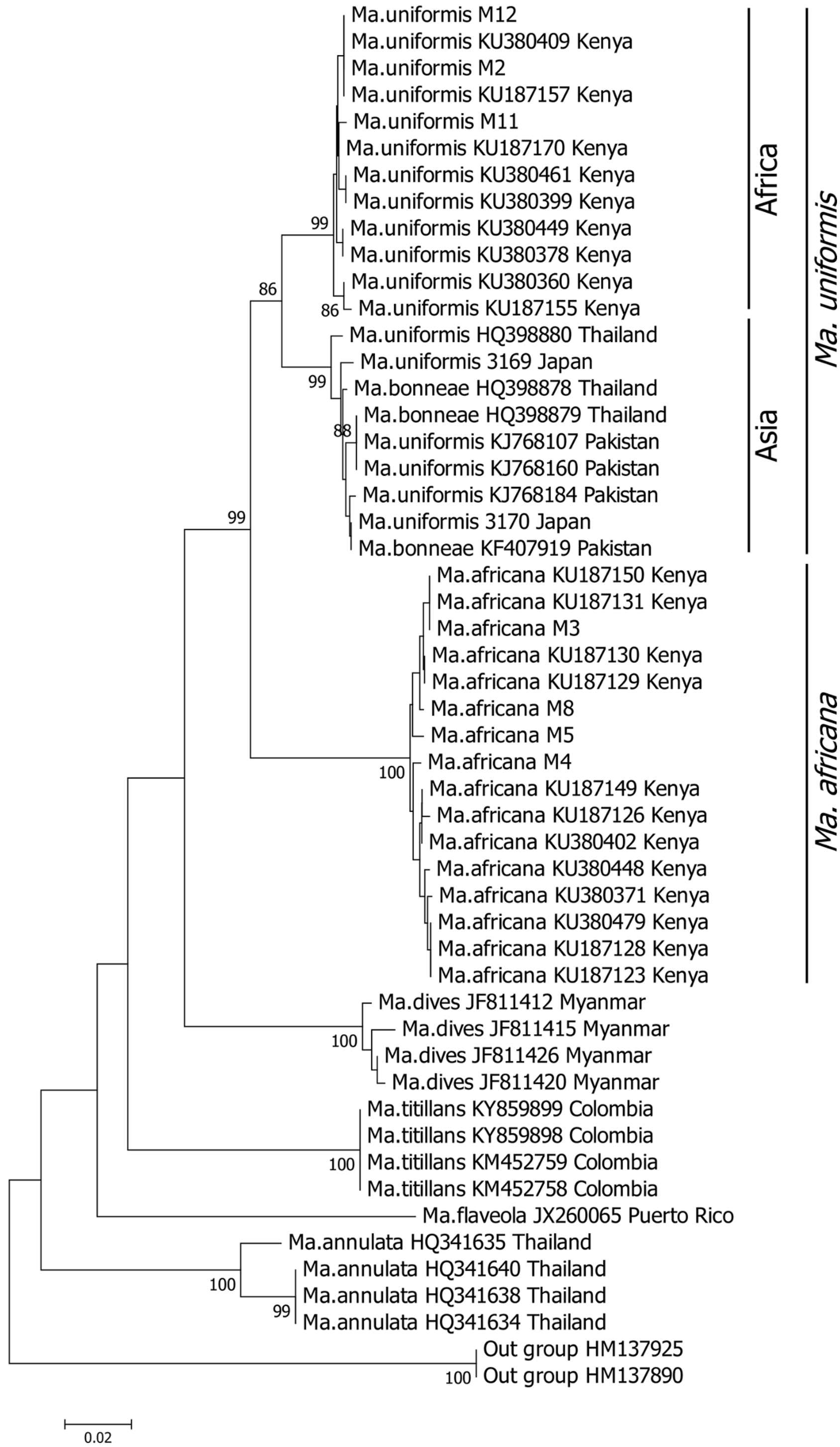

Figure 4. Phylogenetic tree derived from COI sequences (468 bp) using GenBank accessions and specimens of *Ma.* (*Mansonoides*) species. The tree was constructed by the neighbor-joining method with 1000 replicates using the Kimura 2-parameter. Specimens collected in this study are labeled with the species name and specimen code listed in Table 1. Specimens found in GenBank are labeled with species name, GenBank accession number and country.

sequence (468 bp) of *Ma.* (*Mansonoides*) species from GenBank and found that the African and Asian specimens of *Ma. uniformis* were grouped into distinctly different clades (Fig. 4). While the Malawian and Kenyan individuals were genetically homogeneous, with a mean pairwise divergence of 0.4% (min: 0.0%; max: 0.9%) (Table 5), those from Pakistan, Thailand, and Japan were highly divergent populations with a mean pairwise divergence of 3.7% (min: 3.7%; max: 4.0%).

Currently, *Cx. ethiopicus* Edwards is categorized as a synonym of *Cx. bitaeniorhynchus* (Harbach, 1988). However, Malawian specimens identified as *Cx. ethiopicus* were grouped into a different clade from *Cx. bitaeniorhynchus* in our phylogenetic analysis (Fig. 3). Using the COI gene sequence (430 bp) of four species belonging to the genus *Cx.* (*Oculeomyia*), including *Cx. ethiopicus* and *Cx. bitaeniorhynchus*, we calculated the pairwise divergences and performed a phylogenetic analysis. The results showed species-specific clades (Fig. 5). The mean pairwise divergence was 2.3% (min: 1.9%; max: 2.9%) between *Cx. ethiopicus* and Asian *Cx. bitaeniorhynchus* (Table 6). Meanwhile, the mean pairwise divergence of Asian *Cx. bitaeniorhynchus* was 0.6% (min:

Table 5
Percent pairwise divergence among 18 *Ma. uniformis* from 5 countries, calculated using the K2P model.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*Ma. uniformis*_M2_Malawi																		
2	*Ma. uniformis*_M11_Malawi	0.4																	
3	*Ma. uniformis*_M12_Malawi	0.0	0.4																
4	*Ma. uniformis*_KU380360_Kenya	0.6	0.6	0.6															
5	*Ma. uniformis*_KU380378_Kenya	0.4	0.4	0.4	0.6														
6	*Ma. uniformis*_KU380399_Kenya	0.4	0.4	0.4	0.6	0.4													
7	*Ma. uniformis*_KU380409_Kenya	0.0	0.0	0.4	0.6	0.4	0.4												
8	*Ma. uniformis*_KU380449_Kenya	0.4	0.4	0.4	0.6	0.0	0.4	0.4											
9	*Ma. uniformis*_KU380461_Kenya	0.4	0.4	0.4	0.6	0.4	0.0	0.4	0.4										
10	*Ma. uniformis*_KU187155_Kenya	0.9	0.9	0.9	0.2	0.9	0.9	0.9	0.9	0.9									
11	*Ma. uniformis*_KU187157_Kenya	0.0	0.0	0.4	0.6	0.4	0.4	0.0	0.4	0.4	0.9								
12	*Ma. uniformis*_KU187170_Kenya	0.2	0.2	0.2	0.4	0.2	0.2	0.2	0.2	0.2	0.6	0.2							
13	*Ma. uniformis*_KJ768107_Pakistan	3.7	3.7	4.2	4.4	4.2	4.2	3.7	4.2	4.2	4.7	3.7	4.0						
14	*Ma. uniformis*_KJ768160_Pakistan	3.7	3.7	4.2	4.4	4.2	4.2	3.7	4.2	4.2	4.7	3.7	4.0	0.0					
15	*Ma. uniformis*_KJ768184_Pakistan	4.0	4.0	4.4	4.2	4.0	4.4	4.0	4.0	4.4	4.4	4.0	4.2	0.6	0.6				
16	*Ma. uniformis*_HQ398880_Thailand	3.5	3.5	4.0	3.7	3.5	4.0	3.5	3.5	4.0	4.0	3.5	3.7	1.1	1.1	0.4			
17	*Ma. uniformis*_3170_Japan	3.7	3.7	4.2	4.0	4.2	4.2	3.7	4.2	4.2	4.2	3.7	4.0	0.4	0.4	0.2	0.6		
18	*Ma. uniformis*_3169_Japan	3.7	3.7	4.2	4.0	4.2	4.2	3.7	4.2	4.2	4.2	3.7	4.0	0.9	0.9	0.6	1.1	0.4	

Specimens collected in this study are labeled with the species name, specimen code and country listed in Table 1.
Specimens found in GenBank are labeled with species name, GenBank accession number and country.
The mean intraspecific variation within the same mosquito species is less than 2%, and if a mean intraspecific variation is over 2% may possibly have included multiple genetically different populations.

0.0%; max: 2.4%), indicating homogeneity of the population (Table 6). These results suggest that *Cx. ethiopicus* and *Cx. bitaeniorhynchus* are genetically independent species.

4. Discussion

In this study in Malawi, we analyzed the COI gene sequences of 144 individual mosquitoes from 51 species and obtained new findings relating to *Cx. univittatus* complex, *Cx. bitaeniorhynchus*, and *Ma. uniformis*.

The *Cx. univittatus* complex distributed in Africa consists of three species (all of which transmit the West Nile virus in Africa (Harbach, 2011; Mixão et al., 2016)): *Cx. univittatus, Cx. perexiguus*, and *Cx. neavei*. They are distributed allopatrically; thus, their morphological similarities make it difficult to distinguish between them. *Culex perexiguus* has been reported as being distributed in arid areas of northern Africa and southwestern Asia, extending eastward into India (Harbach, 1988; Jupp & Harbach, 1990), but was not believed to inhabit southeastern areas in Africa. However, between the *Cx. univittatus* complex from this study and those registered in GenBank, comparisons of the COI gene sequences showed that specimen code M177 was grouped into the same clade as *Cx. perexiguus* that was reported in Pakistan and Turkey (Fig. 3). The mean pairwise divergence of the clade was 0.7% (min: 0.2%; max: 1.0%)—i.e., the clade is extremely homogeneous (Table 3). Given these results, we compared the potentially diagnostic characteristics of the *Cx. univittatus* complex suggested by Harbach (1988) with the characteristics of the individuals observed in this study (Table 7). Based on the pale area of the ventral surface of the proboscis and the scaling at the bases of the wing costa, the samples were classified into two groups (specimen codes M177/M242 and M203/M215/M216). The postspiracular scales of M177 were crescent shaped, slightly creamy to yellowish in color, and were distinctly different from other specimens collected in Malawi. Although the number of individuals analyzed was low, differences were observed both morphologically and genetically. Therefore, it is reasonable to regard M177 as an individual specimen of *Cx. perexiguus*. Studies report that *Cx. perexiguus* is widely distributed in northern Africa, southwestern Asia, and India (Harbach, 1988; Jupp & Harbach, 1990). The results of this study confirm, for the first time, the presence of this species in Malawi, suggesting that its distribution extends south of the Sahara. The remaining four individuals formed a *Cx. univittatus* clade (Fig. 3). M242 was included in groups registered from South Africa and Spain, whereas M203, M215, and M216 were included in groups registered from Kenya. M242 differed from the other three individuals in that the white part below the proboscis was wider, the postspiracular scales were white and narrow, and the wing costa bases had clear, short, white scale lines. These features are similar to those of *Cx. univittatus* (Table 7). The pairwise divergence between two individuals collected in South Africa was less than 2%. The two individuals collected in Spain showed a pairwise divergence exceeding 2% compared with the Malawian and South African individuals, indicating a larger genetic difference (Table 3). As a result, it was showed that M242 was likely *Cx. univittatus,* based on the designated clade and similar characteristics with *Cx. univittatus*. The remaining three (M203, M215, and M216) were morphologically alike in that they had a weak and narrow pale area in the middle of the ventral surface of the proboscis, with a few pale-grayish scales at the base of the costa. The postspiracular scales of M215 were white with a width a third to half that of the prealar scales. The area of the postspiracular region covered by the white scales was significantly different from that of M203. Phylogenetic analysis grouped M203 in the same clade as the individual reported from Kenya (KU380425); however, the pairwise divergence was 3.0% (min: 2.1%; max: 3.5%) between populations from Malawi and Kenya. Among the three *Cx. univittatus* complex species listed in Table 7, these morphological characteristics are suggested to be similar to those of *Cx. neavei*.

Of the five *Cx. univittatus* complex specimens obtained in this study, it was recognized that M177 was *Cx. perexiguus* and M242 was *Cx. univittatus.* The remaining three (M203, M215, and M216) had similar morphological features to *Cx. neavei*, and formed a clade adjacent to *Cx. perexiguus* and *Cx. univittatus*. However, the clade and genetic divergence of the three specimens were distinct from the *Cx. neavei* registered from Kenya (Fig. 3, Table 3). Therefore, it is possible that these three specimens from Malawi are undescribed sibling species of the *Cx. univittatus* complex. Four of the Kenyan *Cx. neavei* were shown to be genetically different from the *Cx. univittatus* complex, as shown in Fig. 3 and Table 3. Therefore, if the Kenyan *Cx. neavei* does not belong to the *Cx. univittatus* complex, it may have been misidentified or it could belong to an undescribed sibling species. *Culex neavei* is largely distributed in the lowlands of subtropical and tropical zones to the south and east of the Sahara (Jupp & Harbach, 1990); thus, it is reasonably likely that this species is present in Malawi as well. To resolve the uncertainties concerning the taxonomic placement of *Cx. neavei*, additional morphological and molecular studies should be conducted on mosquitoes collected from more African countries.

The results of the phylogenetic and genetic analyses suggest that

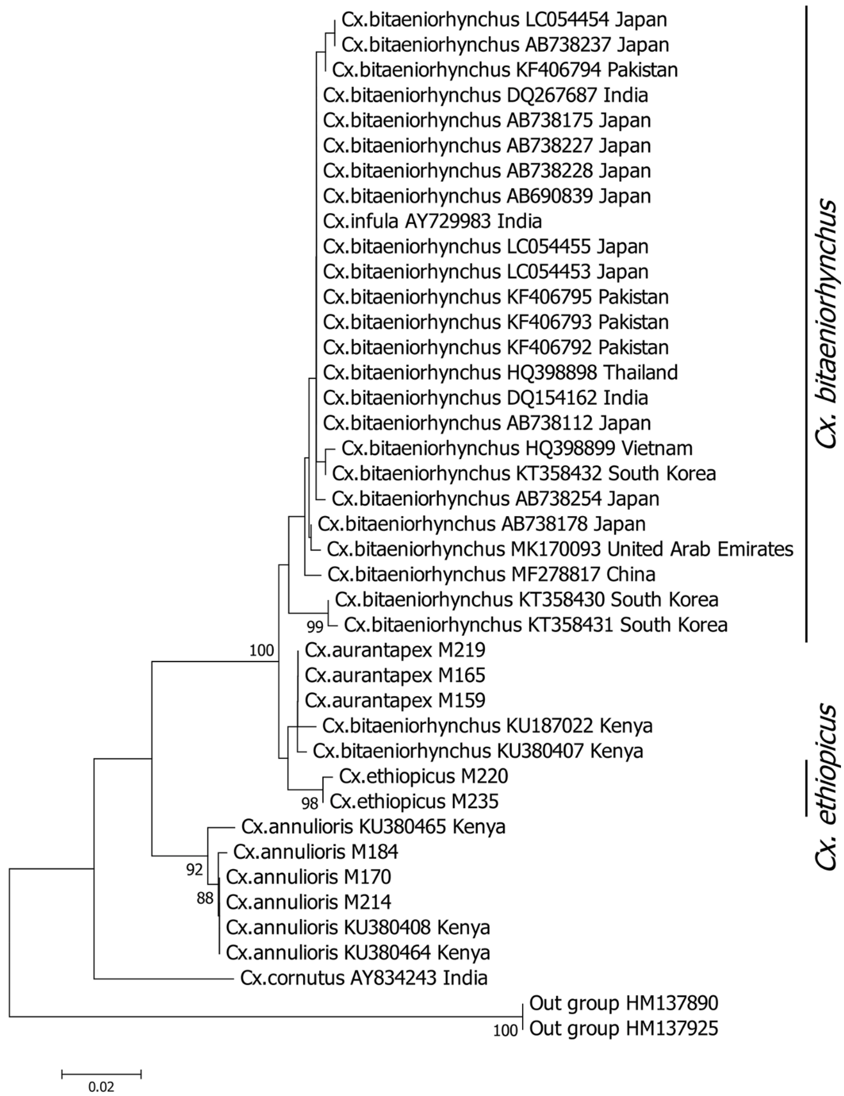

Figure 5. Phylogenetic tree derived from COI sequences (430 bp) using GenBank accessions and specimens of *Cx.* (*Oculeomyia*) species. The tree was constructed by the neighbor-joining method with 1000 replicates using the Kimura 2-parameter. Specimens collected in this study are labeled with the species name and specimen code listed in Table 1. Specimens found in GenBank are labeled with species name, GenBank accession number and country.

African and Asian *Ma. uniformis* are different species (Fig. 4, Table 5). In Africa, two species of *Ma.* (*Mansonoides*), *Ma. uniformis* and *Ma. africana* (Theobald), have been reported (Edwards, 1941; Service, 1990; Jupp, 1996), while only *Ma. uniformis* has been reported in Japan (Tanaka et al., 1979). We compared the morphology of specimens identified as *Ma. uniformis* from Malawi (n = 6) and Japan (n = 6) and found distinct differences in the pale patches on the foretibia and hind femur. The pale patch pattern on the hind femur of Japanese *Ma. uniformis* was similar to *Ma. africana,* as shown by Edwards (1941). Where Japanese *Ma. uniformis* had five or six clear pale patches on the hind femur, the patches of the Malawi specimen were fused and formed a pale stripe-like pattern on the basal half (or a little more posterior or anterior) of the surface of the hind femur. Furthermore, the Japanese *Ma. uniformis* had a clear pale patch on the foretibia, while the Malawi specimen had a pale stripe-like pattern. These findings indicate that the African and Asian *Ma. uniformis* are different species both morphologically and genetically.

Culex bitaeniorhynchus is widely distributed in the Afrotropical region, eastern and southern areas of the Palearctic region, and the Oriental and Australian regions (Harbach, 1988). Harbach (1988) mentioned that it is possible that *Cx. bitaeniorhynchus* consists of more than one species, but there is no indication of geographical differentiation. The results of our phylogenetic analysis using the COI gene sequence (430 bp) of *Cx.* (*Oculeomyia*) species registered in GenBank showed that the Malawian specimens that were morphologically identified as *Cx. ethiopicus* were grouped into a different clade from that of the Asian *Cx. bitaeniorhynchus* (Fig. 5). The mean pairwise divergence exceeded 2% between *Cx. ethiopicus* and Asian *Cx. bitaeniorhynchus* (Table 6), suggesting that they are genetically distinct species. Thus, in this study, we treated *Cx. ethiopicus* as an independent species based on the results of phylogenetic analysis, even though *Cx. ethiopicus* is currently considered a synonym of *Cx. bitaeniorhynchus* (Harbach, 1988). We compared the morphologies of the Malawian *Cx. ethiopicus* and the Japanese *Cx. bitaeniorhynchus* and found a noticeable difference in the wing scaling. In general, two kinds of scale (squame and plume) are distinguishable on mosquito wings (Christophers, 1960; Harbach & Knight, 1980). There were differently colored squame scales but almost no plume scales on the wings of the Malawian specimens, matching the wings of *Cx. ethiopicus* illustrated by Edwards (1941). On the other hand, the wings of Japanese *Cx. bitaeniorhynchus* have plume scales as well as squame scales, and the plume scales are particularly prominent on veins R2+3, R2, R3, R4, and R6. This difference in wing scaling between Malawian and Japanese specimens was not mentioned by Edwards (1941), Tanaka et al. (1979), or Harbach (1988). Although the number of samples examined in this study was low, morphological as well as

Table 6
Percent pairwise divergence between Cx. ethiopicus and Cx. bitaeniorhynchus, calculated using the K2P model.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	*Cx. ethiopicus*_M235_Malawi																										
2	*Cx. ethiopicus*_M220_Malawi	0.2																									
3	*Cx. bitaeniorhynchus*_MK170093_United_Arab_Emirates	2.1	2.4																								
4	*Cx. bitaeniorhynchus*_KF406792_Pakistan	2.1	2.4	0.5																							
5	*Cx. bitaeniorhynchus*_KF406793_Pakistan	2.1	2.4	0.5	0.0																						
6	*Cx. bitaeniorhynchus*_KF406794_Pakistan	2.4	2.6	0.7	0.2	0.2																					
7	*Cx. bitaeniorhynchus*_KF406795_Pakistan	2.1	2.4	0.5	0.0	0.0	0.2																				
8	*Cx. bitaeniorhynchus*_DQ154162_India	2.1	2.4	0.5	0.0	0.0	0.2	0.0																			
9	*Cx. bitaeniorhynchus*_DQ267687_India	2.1	2.4	0.5	0.0	0.0	0.2	0.0	0.0																		
10	*Cx. bitaeniorhynchus*_HQ398898_Thailand	2.1	2.4	0.5	0.0	0.0	0.2	0.0	0.0	0.0																	
11	*Cx. bitaeniorhynchus*_HQ398899_Vietnam	2.6	2.8	0.9	0.5	0.5	0.7	0.5	0.5	0.5	0.5																
12	*Cx. bitaeniorhynchus*_MF278817_China	1.9	2.1	0.7	0.7	0.7	0.9	0.7	0.7	0.7	0.7	1.2															
13	*Cx. bitaeniorhynchus*_KT358431_South_Korea	1.9	2.1	2.4	1.9	1.9	2.1	1.9	1.9	1.9	1.9	2.4	2.1														
14	*Cx. bitaeniorhynchus*_KT358430_South_Korea	1.7	1.9	2.1	1.6	1.6	1.9	1.6	1.6	1.6	1.6	2.1	1.9	0.2													
15	*Cx. bitaeniorhynchus*_KT358432_South_Korea	2.4	2.6	0.7	0.2	0.2	0.5	0.2	0.2	0.2	0.2	0.2	0.9	2.1	1.9												
16	*Cx. bitaeniorhynchus*_AB738112_Japan	2.1	2.4	0.5	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.5	0.7	1.9	1.6	0.2											
17	*Cx. bitaeniorhynchus*_AB738254_Japan	2.4	2.6	0.7	0.2	0.2	0.5	0.2	0.2	0.2	0.2	0.7	0.9	2.1	1.9	0.5	0.2										
18	*Cx. bitaeniorhynchus*_LC054453_Japan	2.1	2.4	0.5	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.5	0.7	1.9	1.6	0.2	0.0	0.2									
19	*Cx. bitaeniorhynchus*_LC054454_Japan	2.6	2.9	0.9	0.5	0.5	0.2	0.5	0.5	0.5	0.5	0.9	1.2	2.4	2.1	0.7	0.5	0.7	0.5								
20	*Cx. bitaeniorhynchus*_LC054455_Japan	2.1	2.4	0.5	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.5	0.7	1.9	1.6	0.2	0.0	0.2	0.0	0.5							
21	*Cx. bitaeniorhynchus*_AB690839_Japan	2.1	2.4	0.5	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.5	0.7	1.9	1.6	0.2	0.0	0.2	0.0	0.5	0.0						
22	*Cx. bitaeniorhynchus*_AB738237_Japan	2.6	2.9	0.9	0.5	0.5	0.2	0.5	0.5	0.5	0.5	0.9	1.2	2.4	2.1	0.7	0.5	0.7	0.5	0.0	0.5	0.5					
23	*Cx. bitaeniorhynchus*_AB738228_Japan	2.1	2.4	0.5	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.5	0.7	1.9	1.6	0.2	0.0	0.2	0.0	0.5	0.0	0.0	0.5				
24	*Cx. bitaeniorhynchus*_AB738227_Japan	2.1	2.4	0.5	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.5	0.7	1.9	1.6	0.2	0.0	0.2	0.0	0.5	0.0	0.0	0.5	0.0			
25	*Cx. bitaeniorhynchus*_AB738178_Japan	1.9	2.1	0.2	0.2	0.2	0.5	0.2	0.2	0.2	0.2	0.7	0.5	2.1	1.9	0.5	0.2	0.5	0.2	0.7	0.2	0.2	0.7	0.2	0.2		
26	*Cx. bitaeniorhynchus*_AB738175_Japan	2.1	2.4	0.5	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.5	0.7	1.9	1.6	0.2	0.0	0.2	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.2	

Specimens collected in this study are labeled with the species name, specimen code and country listed in Table 1.
Specimens found in GenBank are labeled with species name, GenBank accession number and country.
The mean intraspecific variation within the same mosquito species is less than 2%, and if a mean intraspecific variation is over 2% may possibly have included multiple genetically different populations.

Table 7
Comparison of morphological characters for *Cx. univittatus* complex (Harbach, 1988) and 5 specimens collected in Malawi.

Character	Harbach (1988)			Malawi specimens				
	Cx. univittatus	*Cx. perexiguus*	*Cx. neavei*	M177	M203	M215	M216	M242
Ventral surface of proboscis	pale in middle	pale except at base, weakly pale on distal 0.25	inconspicuously pale in middle	widely pale in middle	weakly pale in middle, not widely	weakly pale in middle, not widely	weakly pale in middle, not widely	widely pale in middle
Postspiracular area	tendency for scales to cover less than dorsal 0.5	tendency for scales to cover more than dorsal 0.5	tendency for scales to occur in small patch near spiracle	less than 0.5, very narrow creamy scales as 1/4 width of pre-alar scales	less than 0.5, white scale and same size of pre-alar scales	small patch near spiracle, narrow white scales as 1/3 to 1/2 width of pre-alar scales	lacked or without scales	less than 0.5, narrow white scales as 1/3 width of pre-alar scales
Forefemur	sometimes with indistinct anterior pale stripe	usually with indistinct anterior pale stripe	no anterior pale stripe	no anterior pale stripe	weakly indistinct anterior pale stripe	rather indistinct pale stripes	rather indistinct pale stripes	indistinct anterior stripe
Midfemur	with complete distinct or indistinct anterior pale stripe	with or without incomplete faint or distinct anterior pale stripe	normally without anterior pale stripe, weakly indicated when present	indistinct anterior pale stripe	weakly indistinct anterior pale stripe	rather indistinct pale stripes	rather indistinct pale stripes	indistinct anterior pale stripe
Hind tibia	with distinct anterior and posterior pale stripes on proximal 0.8, separated ventrally by complete dark stripe; with distinct apical pale spot	with distinct anterior and posterior pale stripes on proximal 0.8, partly separeted on proximal 0.5 or less by weak ventral dark stripe; with distinct apical pale spot	with rather indistinct anterior and posterior pale stripes ending before base; with rather indistinct apical pale spot	distinct pale stripes on proximal 0.8, less weak ventral dark stripe, with distinct apical pale spot	distinct pale stripes on proximal 0.8, with distinct apical pale spot	rather indistinct pale stripes, distinct apical pale spot	rather indistinct pale stripes, indistinct apical pale spot	no legs
Wing; Costa	with short line of pale scales at base	with short line of pale scales at base	with pale scales at base	short line of pale scales at base	few pale scales at base	few pale scales at base	few pale scales at base	short line of pale scales at base
Wing; Vein 2A	usually with line of scales	occasionally with few scales	female occasionally with few scales	line of scales	line of scales	line of scales	line of scales	line of scales
Abdomen; pale bands on terga	normal	normal	reduced or absent	normal	patch on 2 -3 and normal band	reduced or lack band	reduced or lack band	normal

genetic differences were found between *Cx. ethiopicus* from Malawi and *Cx. bitaeniorhynchus* from Japan. To confirm our findings, additional entomological studies would be required.

O'nyong-nyong fever (1959–1962) and Chikungunya fever (1987–1989) spread to east African countries. These outbreaks have also been reported in Malawi, and the Chikungunya virus antibody was detected in patients at the Kamuzu Central Hospital in Lilongwe, Malawi. (Lutwama et al., 1999; van den Bosch and Lioyd, 2000; Powers and Logue, 2007; Rezza et al., 2017). In recent years, an increasing number of cases of mosquito-borne viral infectious diseases have been reported in the countries surrounding Malawi. The invasion of the pathogens into Malawi with humans and animals are highly possible. However, there is nearly no reports on clinical cases nor mosquito-borne viral infectious diseases in Malawi. Because the cases of febrile illness are usually regarded by physicians as malaria, typhoid fever, or common flu, due to the limited use of proper diagnostic tests. In many areas of sub-Saharan Africa, most health facilities lack the capacity to conduct diagnostics for arboviral infections on patients with "undifferentiated febrile illnesses" or "fevers of unknown origin," and physicians are restricted to treatment based on symptoms (Sule et al., 2018). Within the borders of Malawi, at least 18 species of mosquitoes transmit pathogens that cause human and animal diseases (Table 2), and if viral pathogens were to invade Malawi, the infectious diseases could rapidly become more widespread. To prevent and control the invasion and spread of pathogens in Malawi, detecting the pathogens in humans and animals—particularly mosquitoes—is vital importance. Therefore, the system and accuracy of testing in hospitals and other medical institutions need to improve, and, simultaneously, regular surveys on epidemics and vector mosquitoes need to be conducted throughout the country. We recommend the introduction of a system that accumulates and analyzes data on disease cases and vector mosquitoes, and for that information to be regularly disseminated nationally.

Author contributions

Yoshihide Maekawa: Conceptualization, Investigation, Data Curation, Writing- original Draft, Dylo Pemba: Resources, Project administration, Conceptualization, Justin Kumala: Investigation, Steve Gowelo: Investigation, Yukiko Higa: Supervision, Methodology, Investigation, Kyoko Futami: Supervision, Methodology, Investigation, Kyoko Sawabe: Conceptualization, Funding acquisition, Yoshio Tsuda: Writing-Reviewing and Editing, Supervision.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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