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Original Article

Identification of Three Distinct Groups of *Anopheles lindesayi* in Japan by Morphological and Genetic Analyses

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SUMMARY: *Anopheles* (*Anopheles*) *lindesayi* Giles consists of 5 subspecies. In Japan, only one subspecies, *An. l. japonicus* Yamada, has been reported. Its geographical populations are morphologically diverse; however, they are regarded as a single subspecies. In this study, we re-evaluated the taxonomic status of *An. l. japonicus* in Japan, and that of another subspecies, *An. l. pleccau*, distributed in Taiwan, by comparative morphological and molecular analyses based on the gene sequences of mitochondrial DNA cytochrome c oxidase I (COI) and ribosomal DNA internal transcribed spacer 2 (ITS2). Nucleotide sequence divergence was calculated using the Kimura-two-parameter (K2P) distance model. Phylogenetic trees based on COI and ITS2 sequences showed 3 distinct clades: Eastern Japan, Western Japan, and the Ryukyus. The sequences of the Ryukyu specimens were located within the same clade as that of the sequences of the Taiwanese specimens. Regarding the COI sequences, the 3 geographical groups in Japan were genetically distinct. The following morphological characteristics distinguished the groups: larval seta 1-S, pupal setae 5 through segments IV–VII, and pupal setae 6 on segments IV–VII. Based on these results, it was revealed that *An. l. japonicus* included 3 genetically and morphologically distinct groups: 2 groups of *An. l. japonicus* and a group in the Ryukyus, which was a synonym of *An. l. pleccau*.

INTRODUCTION

Malaria was completely eradicated from Japan in the early 1960s. Nevertheless, resurgence remains a great concern owing to increased global human traffic (1–4). However, less than 80 imported cases have been reported each year since 2010 (5), and vector mosquitoes are still commonly observed throughout Japan.

In Japan, 12 anopheline species have been reported (6). Three species, *An. sinensis* Wiedemann, *An. lesteri* Baisas and Hu, and *An. yaeyamaensis* Somboon and Harbach (formerly *An. minimus* Theobald species E), are considered to be the main malarial vectors in Japan (7,8). However, recent molecular techniques have modified the overall phylogenetic picture of these vectors, revealing misidentifications, synonyms, and new geographical distributions (9–12).

Anopheles lindesayi Giles is a species whose taxonomic status needs to be re-evaluated. This species includes 5 subspecies (*lindesayi*, *japonicus*, *pleccau*, *cameronensis*, and *benguetensis*) (13–16), specimens of which were collected from the ground or rock pools beside streams in mountainous areas (14,16–19). Of these, one subspecies in Taiwan, *An. l. pleccau* Koidzumi, is capable of forming sporozoites of *Plasmodium falciparum*, *P. vivax*, and *P. malariae* (20). Another subspecies, *An. l. japonicus* Yamada is distributed throughout Japan. It is also capable of forming sporozoites of *P. vivax* and *P. falciparum* (21). Vector competence of other subspecies is unknown.

Despite their potential importance as vectors, no valid identification key for the 5 subspecies is available. For instance, the morphology of the legs (the length of the basal pale band and the middle pale band on the hind femur) and wings (the patterns of the apical pale spots on the veins) were used for distinguishing the subspecies of *An. lindesayi* (13–15,22). However, Tanaka et al. (15) showed that the characteristics used in previous studies were not useful to distinguish the subspecies because of their broad-ranged variations. They also indicated that "it is still possible that more or less discrete local population of *lindesayi* may have evolved, as its distribution ranges from the tropics and northern temperate districts, and its habitat is usually restricted to mountainous area (15)."

An. l. japonicus is the only subspecies of *An. lindesayi* reported in Japan (19). It was first described in 1918 as *An. japonicus*, and was distinguished from the Indian specimens of *An. lindesayi* (23). However, Christophers (24) suggested that *An. japonicus* was a variety of *An. lindesayi*, because of the lack of obvious morphological differences between them. Furthermore, Reid (14) and

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Harrison (16) considered the taxon as a subspecies of *An. lindesayi*.

There are extensive morphological variations. For instance, the *An. l. japonicus* population in the Tokara Archipelago (a part of the Ryukyu Islands) had a characteristic "pale spot on the tips of the veins cu_1 and r_{4+5}" that are uncommon in any other *An. l. japonicus* population in Japan (25). Morphological variations among *An. l. japonicus* populations in Japan strongly suggest that there are multiple groups of this subspecies. Therefore, the current taxonomic status of *An. l. japonicus* as a subspecies needs to be investigated.

In the present study, we re-evaluated the taxonomic status of *An. l. japonicus* populations in Japan using molecular analyses and morphological characteristics. Based on the results of the morphological study, we present a summary of morphological differences among *An. l. japonicus* populations in Japan.

MATERIALS AND METHODS

Source of specimens: **Mosquito collection** Adult specimens were obtained from individually reared larvae collected from Japan and Taiwan from 2014 to 2016. The collections were conducted at 4 locations in Japan (Hokkaido, Fukushima, Nagasaki, and Kumamoto), and 2 locations in Taiwan (Hsinchu and Taichung), indicated as "Newly field collected +" in Table 1. The map summarizes the collection sites, along with other information including year, country, region, locality, sex, stage, GenBank accession No., and the place of sample deposit, as shown in Fig. 1 and Table 1. The adults were identified to the species level using the characteristics provided by Tanaka et al. (15) and Lien (26). The adults were pinned and their associated larval and pupal exuviae were mounted using Euparal (Waldeck GmbH & Co. KG, Münster, Germany). The specimens were deposited in the Department of Vector Ecology and Environment, Institute of Tropical Medicine, Nagasaki University. We then used the region name shown in Table 1 to indicate the specimens from Japan, whereas the country name shown in Table 1 was used for specimens from other countries. **Specimens examined** All specimens collected from Japan (11 females, 8 males, 4 whole larvae, 10 larval exuviae, and 9 pupal exuviae) and 20 adults from Taiwan (2 females and 1 male in good condition were selected from each of the 5 collection sites), and the associated exuviae of 15 females were examined for morphological analyses. These adults were used for the examination of the body (head, thorax, and abdomen), wings, and legs, as denoted by "B," "W," and "L," respectively, in Table 1. Then, 10 specimens from Japan and 8 from Taiwan in good condition were used for molecular analyses after at least 1 specimen was selected from each collection site.

We also examined museum-deposited specimens (45 females, 12 males, 10 larval exuviae, and 10 pupal exuviae) for morphological analyses. The adults were used to examine the wings and legs, denoted as "W" and "L" in Table 1, respectively. These deposited specimens were used for the examination of the wings and legs only owing to the lack of time available to access them. As these morphological characteristics have often been used in previous studies, these characteristics were

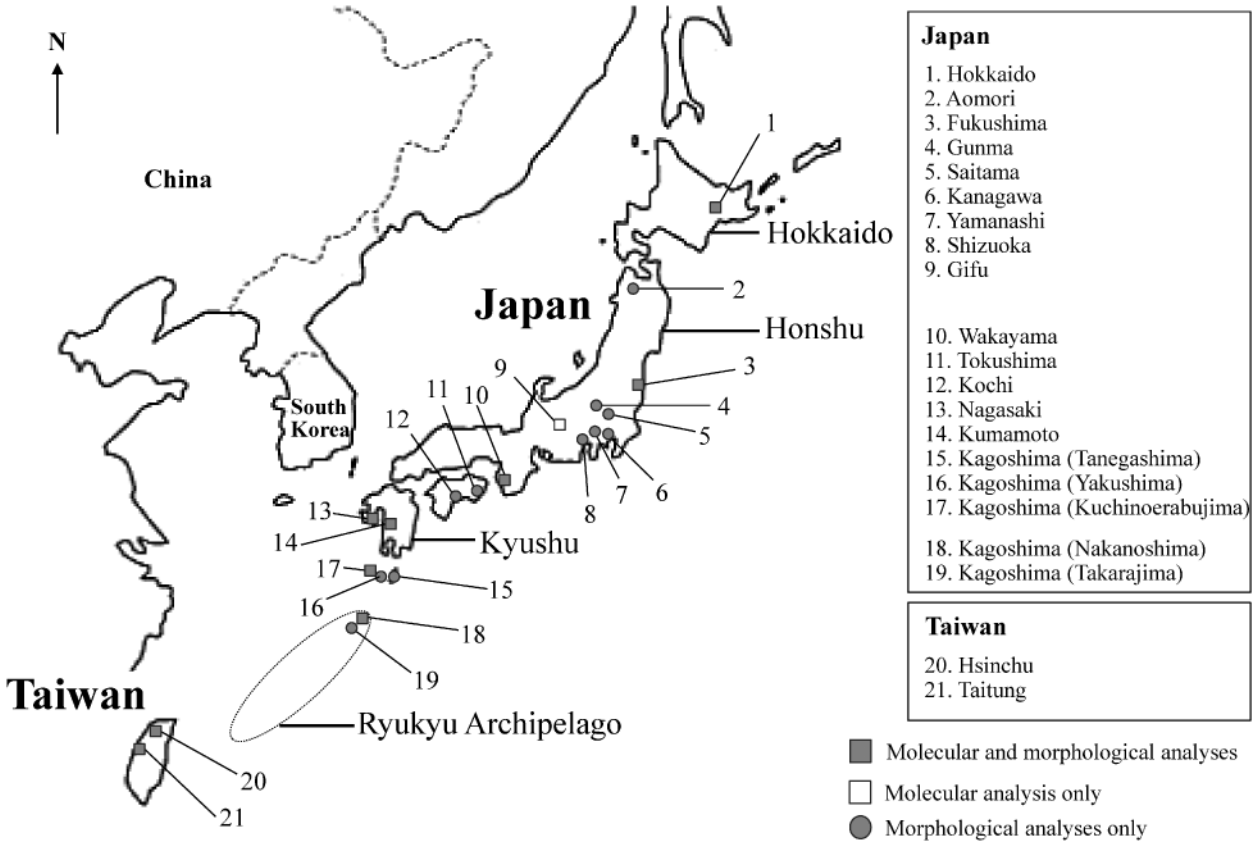

Fig. 1. Collection sites of specimens for this study.

Table 1. Specimens of *Anopheles lindesayi* examined in this study

Sample ID	Newly field collected	Species/ subspecies	Year	Country	Region	Locality	Sex	Stage[1)]				GenBank accession No.		
								A[2)]	L	Le	Pe	COI	ITS2	Deposit[3)]
42		*An. l. japonicus*	1970	Japan	Hokkaido	Shiretoko	female	BW						NMNS
43		*An. l. japonicus*	1970	Japan	Hokkaido	Shiretoko	female	BWL						NMNS
113	+	*An. l. japonicus*	2014	Japan	Hokkaido	Onneto	female	BWL		+	+	LC330870	LC330894	NU
114	+	*An. l. japonicus*	2014	Japan	Hokkaido	Onneto	male	BL		+	+	LC330871	LC330895	NU
140	+	*An. l. japonicus*	2014	Japan	Hokkaido	Onneto	female	BWL		+	+			NU
142	+	*An. l. japonicus*	2014	Japan	Hokkaido	Onneto	−		+					NU
143	+	*An. l. japonicus*	2014	Japan	Hokkaido	Onneto	−		+					NU
144	+	*An. l. japonicus*	2014	Japan	Hokkaido	Onneto	−		+					NU
145	+	*An. l. japonicus*	2014	Japan	Hokkaido	Onneto	−		+					NU
23		*An. l. japonicus*	2013	Japan	Aomori	Mt. Iwaki	female	WL						YM
24		*An. l. japonicus*	2013	Japan	Aomori	Mt. Iwaki	female	WL						YM
25		*An. l. japonicus*	2013	Japan	Aomori	Mt. Iwaki	male	L						YM
26		*An. l. japonicus*	2013	Japan	Aomori	Mt. Iwaki	female	WL						YM
27		*An. l. japonicus*	2013	Japan	Aomori	Mt. Iwaki	female	WL						YM
115	+	*An. l. japonicus*	2013	Japan	Fukushima	Date	female	BWL		+	+	LC330872	LC330896	NU
35		*An. l. japonicus*	1972	Japan	Gumma	Mt. Kashozan	female	L						NMNS
46		*An. l. japonicus*	1918	Japan	Gumma	Mt. Myogi	male	L						NMNS
18		*An. l. japonicus*	1982	Japan	Saitama	Arakawa	female	WL						NIID
34		*An. l. japonicus*	1972	Japan	Saitama	Kawamata	female	WL						NMNS
37		*An. l. japonicus*	1972	Japan	Saitama	Kawamata	female	W						NMNS
38		*An. l. japonicus*	1972	Japan	Saitama	Kawamata	female	L						NMNS
39		*An. l. japonicus*	1972	Japan	Saitama	Kawamata	female	WL						NMNS
44		*An. l. japonicus*	1972	Japan	Saitama	Kawamata	female	WL						NMNS
19		*An. l. japonicus*	1973	Japan	Kanagawa	Nakatsu valley	female	WL						NIID
20		*An. l. japonicus*	1973	Japan	Kanagawa	Nakatsu valley	male	L						NIID
21		*An. l. japonicus*	1973	Japan	Kanagawa	Nakatsu valley	female	L						NIID
40		*An. l. japonicus*	1966	Japan	Kanagawa	Tanzawa	female	W						NMNS
36		*An. l. japonicus*	1973	Japan	Yamanashi	Mt. Fuji	male	L						NMNS
59		*An. l. japonicus*	2005	Japan	Yamanashi	Mt. Fuji	female	BL						KH
41		*An. l. japonicus*	1971	Japan	Shizuoka	Akashi-onsen	female	WL						NMNS
29		*An. l. japonicus*	2014	Japan	Gifu	Shirakawa	female	WL						YM
30		*An. l. japonicus*	2014	Japan	Gifu	Shirakawa	male	L						YM
31		*An. l. japonicus*	2014	Japan	Gifu	Shirakawa	female	WL						YM
32		*An. l. japonicus*	2014	Japan	Gifu	Nagawa	female	WL						YM
33		*An. l. japonicus*	2014	Japan	Gifu	Nagawa	female	WL						YM
28		*An. l. japonicus*	2014	Japan	Wakayama	Kakiuchi	female	WL						YM
14		*An. l. japonicus*	1981	Japan	Tokushima	Tokushima	male	L						NIID
15		*An. l. japonicus*	1981	Japan	Tokushima	Tokushima	female	WL						NIID
16		*An. l. japonicus*	1981	Japan	Tokushima	Tokushima	female	WL						NIID
17		*An. l. japonicus*	1981	Japan	Tokushima	Tokushima	female	WL						NIID
45		*An. l. japonicus*	1921	Japan	Kochi	Murotosaki	female	WL						NMNS
47		*An. l. japonicus*	1921	Japan	Kochi	Murotosaki	female	L						NMNS
12	+	*An. l. japonicus*	2014	Japan	Nagasaki	Isahaya	female	BWL						NU
13	+	*An. l. japonicus*	2014	Japan	Nagasaki	Isahaya	female	BWL						NU
58	+	*An. l. japonicus*	2014	Japan	Nagasaki	Isahaya	female	BWL				LC330873	LC330897	NU
65		*An. l. japonicus*	1960s	Japan	Nagasaki	Isahaya	female	BL						NU
66		*An. l. japonicus*	1960s	Japan	Nagasaki	Isahaya	female	BL						NU
68	+	*An. l. japonicus*	2014	Japan	Nagasaki	Isahaya	male	BL						NU
69	+	*An. l. japonicus*	2014	Japan	Nagasaki	Isahaya	male	BL				LC330874	LC330898	NU

[1)]: A, adult; L, whole larva; Le, associated larval exuvia; Pe, associated pupal exuvia.
[2)]: B, body; W, wing analyses; L, leg analyses.
[3)]: NMNS, the National Museum of Nature and Science, Japan; YM, Dr. Yoshihide Maekawa; NU, the museum in Nagasaki University, NEKKEN, Japan; TY, Dr. Takeo Yamauchi; IM, Dr. Ichiro Miyagi; RU, The museum in the University of Ryukyus, Japan; KH, Dr. Keita Hoshino; TCDC, Taiwan CDC; GenBank, molecular analyses only using GenBank data.

prioritized. Three of the females from Japan (Kuchino-erabu, Nakanoshima, and Takarajima) were used for further molecular analyses (Table 1).

Molecular analysis: DNA extraction, amplification, and sequencing Total DNA was extracted from one leg of individual adult mosquitoes using either REDExtract-N-Amp™ Tissue PCR kit (Sigma-Aldrich, St. Louis,

Table 1. (continued) Specimens of *Anopheles lindesayi* examined in this study

Sample ID	Newly field collected	Species/ subspecies	Year	Country	Region	Locality	Sex	Stage[1]				GenBank accession No.		
								A[2]	L	Le	Pe	COI	ITS2	Deposit[3]
71	+	*An. l. japonicus*	2016	Japan	Nagasaki	Kinkai	male	BL			+	LC330875	LC330899	NU
72	+	*An. l. japonicus*	2016	Japan	Nagasaki	Kinkai	male	BL			+	LC330876	LC330900	NU
73	+	*An. l. japonicus*	2016	Japan	Nagasaki	Kinkai	male	BL		+	+	LC330877	LC330901	NU
74	+	*An. l. japonicus*	2016	Japan	Nagasaki	Kinkai	female	BWL		+	+	LC330878	LC330902	NU
75	+	*An. l. japonicus*	2016	Japan	Nagasaki	Kinkai	female	BWL		+	+			NU
76	+	*An. l. japonicus*	2016	Japan	Nagasaki	Kinkai	female	BWL		+				NU
77	+	*An. l. japonicus*	2016	Japan	Nagasaki	Kinkai	male	BL						NU
78	+	*An. l. japonicus*	2016	Japan	Nagasaki	Kinkai	female	BWL		+				NU
79	+	*An. l. japonicus*	2016	Japan	Nagasaki	Kinkai	female	BWL		+				NU
67	+	*An. l. japonicus*	2014	Japan	Kumamoto	Hirata	male	BL				LC330879	LC330903	NU
60		*An. l. japonicus*	1993	Japan	Kagoshima	Tanegashima	female	WL				−	−	TY
61		*An. l. japonicus*	1993	Japan	Kagoshima	Tanegashima	female	WL						TY
70		*An. l. japonicus*	1993	Japan	Kagoshima	Tanegashima	female	WL						TY
62		*An. l. japonicus*	1993	Japan	Kagoshima	Yakushima	female	WL						NU
63		*An. l. japonicus*	2006	Japan	Kagoshima	Kuchinoerabujima	female	WL				LC330880	−	TY
64		*An. l. japonicus*	2006	Japan	Kagoshima	Kuchinoerabujima	female	WL						TY
98		*An. l. japonicus*	1981	Japan	Ryukyus	Nakanoshima	male	BL						IM
99		*An. l. japonicus*	1981	Japan	Ryukyus	Nakanoshima	female	BWL						IM
100		*An. l. japonicus*	1981	Japan	Ryukyus	Nakanoshima	male	BL						IM
101		*An. l. japonicus*	1981	Japan	Ryukyus	Nakanoshima	male	BL						IM
102		*An. l. japonicus*	1981	Japan	Ryukyus	Nakanoshima	male	BL						IM
103		*An. l. japonicus*	1981	Japan	Ryukyus	Nakanoshima	female	BWL				LC330882	−	IM
104		*An. l. japonicus*	1981	Japan	Ryukyus	Nakanoshima	female	BWL						IM
93		*An. l. japonicus*	1981	Japan	Ryukyus	Takarajima	female	BWL				LC330883	−	IM
94		*An. l. japonicus*	1981	Japan	Ryukyus	Takarajima	female	BWL						IM
95		*An. l. japonicus*	1981	Japan	Ryukyus	Takarajima	female	BWL						IM
96		*An. l. japonicus*	1981	Japan	Ryukyus	Takarajima	female	BWL						IM
97		*An. l. japonicus*	1981	Japan	Ryukyus	Takarajima	male	BL						IM
154		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−			+				RU
155		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−			+				RU
156		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−			+				RU
157		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−			+				RU
158		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−			+				RU
108		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−			+				RU
109		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−			+				RU
110		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−			+				RU
111		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−			+				RU
112		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−			+				RU
159		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−				+			RU
160		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−				+			RU
161		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−				+			RU
162		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−				+			RU
163		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−				+			RU
108		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−				+			RU
109		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−				+			RU
110		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−				+			RU
111		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−				+			RU
112		*An. l. japonicus*	1971	Japan	Ryukyus	Takarajima	−				+			RU

[1]: A, adult; L, whole larva; Le, associated larval exuvia; Pe, associated pupal exuvia.
[2]: Examined part. B, body; W, wing; L, leg.
[3]: NMNS, the National Museum of Nature and Science, Japan; YM, Dr. Yoshihide Maekawa; NU, the museum in Nagasaki University, NEKKEN, Japan; TY, Dr. Takeo Yamauchi; IM, Dr. Ichiro Miyagi; RU, The museum in the University of Ryukyus, Japan; KH, Dr. Keita Hoshino; TCDC, Taiwan CDC; GenBank, molecular analyses only using GenBank data.

MO, USA) or DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). The cytochrome c oxidase I (COI) barcoding region (658 bp) was amplified by polymerase chain reaction (PCR) using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAAT CA-3′) (27) and TaKaRa Ex Taq Hot Start Version (Takara Bio Inc., Shiga, Japan). For 6 specimens that could not be amplified using the method above, we used PreCR® Repair Mix (New England BioLabs Inc.,

Table 1. (continued) Specimens of *Anopheles lindesayi* examined in this study

Sample ID	Newly field collected	Species/ subspecies	Year	Country	Region	Locality	Sex	Stage[1]				GenBank accession No.		
								A[2]	L	Le	Pe	COI	ITS2	Deposit[3]
80	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	female	BWL		+	+			NU
81	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	male	BL						NU
82	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	female	BWL		+	+			NU
83	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	female	BWL		+	+			NU
84	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	female	BWL		+	+			NU
85	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	female	BWL		+	+			NU
86	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	female	BWL		+	+	LC330884	LC330904	NU
87	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	male	BL						NU
88	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	female	BWL		+	+	LC330885	LC330905	NU
89	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	female	BWL		+	+	LC330886	LC330906	NU
90	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	female	BWL		+	+			NU
91	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	male	BL						NU
150	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	female	BWL		+	+	LC330890	LC330909	NU
151	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	male	BL				LC330891	LC330910	NU
152	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	female	BWL		+	+	LC330892	LC330911	NU
153	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	female	BWL		+	+	LC330893	LC330912	NU
165	+	*An. l. pleccau*	2016	Taiwan	Hsinchu	Jianshi Twonship	female	BWL		+	+			NU
164	+	*An. l. pleccau*	2016	Taiwan	Taichung	Heping District	female	WL		+	+			NU
168	+	*An. l. pleccau*	2016	Taiwan	Taichung	Heping District	male	L						NU
92	+	*An. l. pleccau*	2016	Taiwan	Taichung	Heping District	female	WL		+	+	LC330889	LC330908	NU
107		*An. l. pleccau*	1953	Taiwan	Taitung	−	female	WL						TCDC
105		*An. l. pleccau*	1961	Taiwan	I lan	−	female	WL						TCDC
106		*An. l. pleccau*	1954	Taiwan	Pingtung	Wutai	male	L						TCDC
−		*An. l. japonicus*	−	Japan	Aomori	−	−					LC104317	−	GenBank
−		*An. l. japonicus*	−	Japan	Aomori	−	−					LC104318	−	GenBank
−		*An. l. japonicus*	−	Japan	Fukushima	−	−					LC054422	−	GenBank
−		*An. l. japonicus*	−	Japan	Fukushima	−	−					LC054423	−	GenBank
−		*An. l. japonicus*	−	Japan	Tokyo	Nishitama	−					AB690833	−	GenBank
−		*An. l. japonicus*	−	Japan	Gifu	−	−					LC054420	−	GenBank
−		*An. l. japonicus*	−	Japan	Gifu	−	−					LC054421	−	GenBank
−		*An. l. japonicus*	−	Japan	Wakayama	−	−					LC054424	−	GenBank
−		*An. l. japonicus*	−	Japan	Wakayama	−	−					LC104319	−	GenBank
−		*An. l. japonicus*	−	South Korea	−	−	−					−	AJ620898	GenBank
−		*An. l. japonicus*	−	South Korea	−	−	−					−	DQ398773	GenBank
−		*An. lindesayi*	−	China	Shaanxi	−	−					KF830750	−	GenBank
−		*An. lindesayi*	−	China	Shaanxi	−	−					−	JX944708	Genbank
−		*An. nilgiricus*	−	India	−	−	−					KR872408	−	GenBank

[1]: A, adult; L, whole larva; Le, associated larval exuvia; Pe, associated pupal exuvia.
[2]: W, wing analyses; L, leg analyses.
[3]: NMNS, the National Museum of Nature and Science, Japan; YM, Dr. Yoshihide Maekawa; NU, the museum in Nagasaki University, NEKKEN, Japan; TY, Dr. Takeo Yamauchi; IM, Dr. Ichiro Miyagi; RU, The museum in the University of Ryukyus, Japan; KH, Dr. Keita Hoshino; TCDC, Taiwan CDC; GenBank, molecular analyses only using GenBank data.

Ipswich, MD, USA). We performed primary PCRs using the PreCR® Repair Mix with the primer sets LCO1490 and HCO2198. Subsequently, we conducted secondary PCRs using the first PCR products as the template DNA with the primers "intraLCO" (5′ CCTGATATAGCATT TCCTCG 3′) and "intraHCO" (5′ TACTGCCCCTAA AATTGAAG 3′), which amplified 265 bp of intra-sequences within the target region. Approximately, 350 bp of the internal transcribed spacer 2 (ITS2) region was amplified according to Cornel et al. (28). Direct sequencing was carried out following the methods of Linton et al. (29) and Walton et al. (30), with "intraLCO" and "intraHCO" primers using ABI PRISM BigDye Terminator version 3.1 in the Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA,USA). Sequences were aligned using ClustalW of the MEGA version 5.2 package (31). **Molecular analysis for COI and ITS2 region** We compared the obtained DNA sequences with the sequences of *An. lindesayi* from Japan, China, and South Korea available in GenBank (Table 1). *An. nilgiricus* Christophers was used as an outgroup for COI analysis. Phylogenetic trees with 1,000 bootstrap tests were built using the neighbor-joining (NJ) algorithm in MEGA version 5.2 (32). Nucleotide sequence divergence was calculated using the Kimura-two-parameter (K2P) distance model (33).

Morphological examinations: We examined the morphological characteristics of the adults and chaetotaxy of the fourth-instar larvae and pupae according to previous studies (14,15,22,25). **Body** The morphological characteristics of the head, thorax, and abdomen were examined and measured using a stereoscopic

microscope. **Wing** The absence or presence of an apical pale spot on the wing vein of r_3, r_{3+4}, m_{1+2}, m_{3+4}, cu_1, and cu_2 was examined under a stereoscopic microscope. The right wings of individual specimens were observed. **Leg** The hind femur of individual specimens was observed and images were captured. The length of the middle pale band, basal pale band at the ventral side, and hind femur were measured from the images using a video-micrometer in the NIS-Elements D 3.00 software (Nikon Instech Co., Ltd., Tokyo, Japan). We calculated the ratio of the length of the middle pale band to the length of hind femur and the ratio of the length of the basal pale band to the length of the hind femur. **Chaetotaxy of larva and pupa** The number of seta branches on the larval exuviae and pupal exuviae were counted using an optical microscope. One side (left or right) of the exuviae was examined. The terminology and abbreviations used by Tanaka et al. (15) were adopted for morphological characterization.

RESULTS

Molecular analysis: The COI and ITS2 sequences obtained in the present study were submitted to GenBank, under the accession numbers LC330870-LC330912.

The NJ phylogenetic tree based on 571 bp of the COI barcoding region sequences showed 3 distinct clades (Fig. 2A). The first clade consisted of specimens from Eastern Japan, comprising Hokkaido, Aomori, Fukushima, Tokyo, and Gifu (named as E-Japan hereafter). The second clade consisted of the specimens of the Ryukyu Archipelago (the Ryukyus, hereafter) and Taiwan (Taiwan, hereafter). The third clade consisted of specimens from Western Japan, comprising Wakayama, Nagasaki, Kumamoto, and Kagoshima (W-Japan, hereafter). In addition, a COI sequence from mainland China showed a close relationship with the W-Japan clade.

The NJ phylogenetic tree based on 345 bp of ITS2 (Fig. 2B) also showed 3 distinct clades: E-Japan, W-Japan, and Taiwan, similar to that observed with COI. The ITS2 sequences of Nagasaki and Kumamoto (Western Japan) were close to those from South Korea (AJ620898.1 and DQ398773.1) and China (JX944708.1), with > 99% similarity, as compared with the 97% similarity with the sequences of the specimens from Taiwan. The ITS2 sequences of Hokkaido and Fukushima (E-Japan) showed 81.1% and 81.7% similarity with those from W-Japan and Taiwan, respectively. Sequence data of the ITS2 region could not be obtained for the Ryukyus specimens, as insufficient DNA was extracted for sequencing ITS2 from the damaged specimens collected in 1971.

We estimated the K2P distances between the 3 clades (Table 2). For the COI sequences, the K2P distance between W-Japan and the Ryukyus was 3.07%–4.73%. Similarly, the distance between W-Japan and Taiwan was 3.04%–4.92%. The K2P distance between E-Japan and the other clades (> 8%) was much higher than that between each W-Japan, the Ryukyus, and Taiwan. The

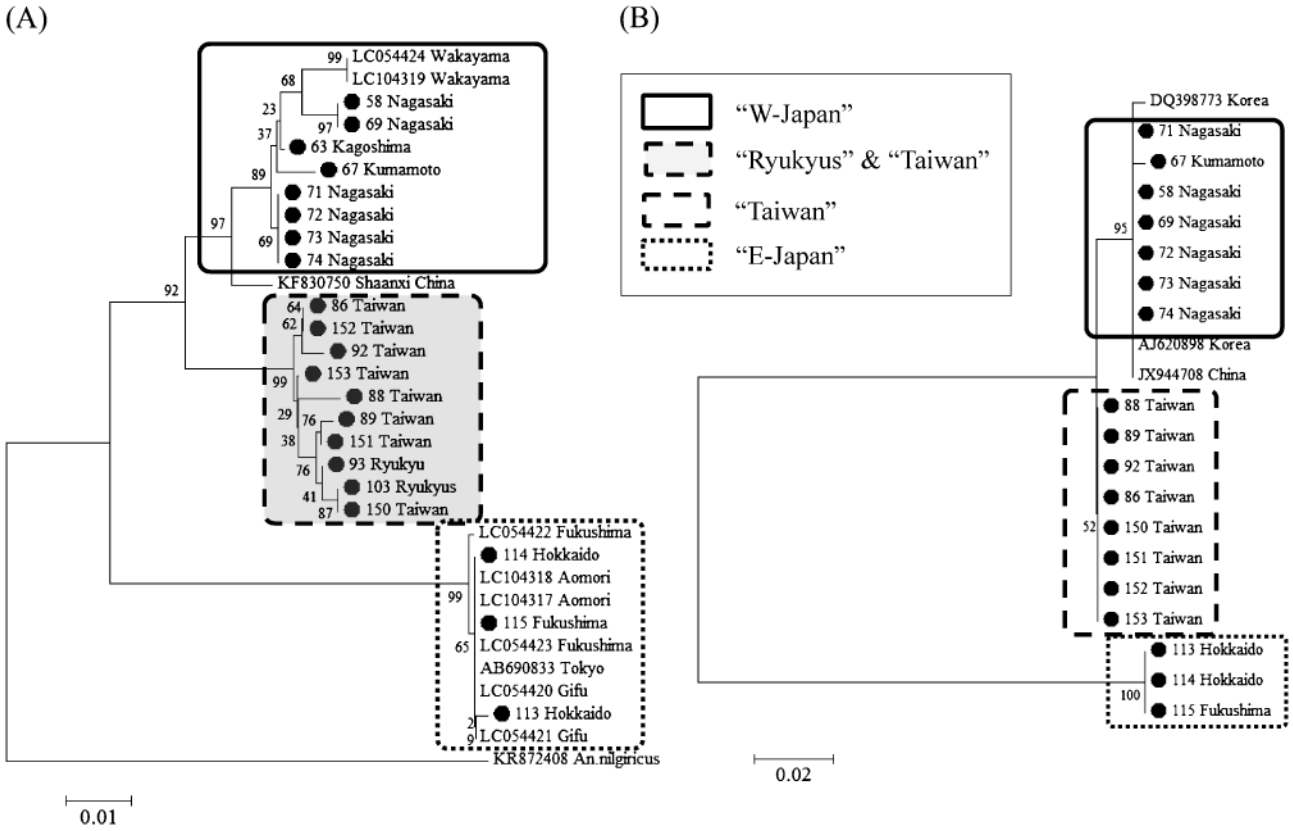

Fig. 2. Neighbor-joining tree with 1,000 bootstrap replicates constructed using the Kimura-two-parameter model based on (A) COI sequences (571 bp) and (B) ITS2 sequences (320 bp) of *Anopheles lindesayi.* The sequences are labeled as "sample ID or accession number in GenBank" and "collection site." Circles indicate sequences obtained in this study.

ITS2 sequences of E-Japan showed a K2P distance of > 20% from each of the other groups. Conversely, W-Japan showed 0.0%–0.6%, 0.0%, and 0.9%–1.3% K2P distance from the sequences of South Korea, China, and Taiwan, respectively.

Morphological analysis: The results of the morphological examination are shown in Table 2–4, grouping data by 3 geographical groups in Japan ("E-Japan," consisted of specimens from Hokkaido to Gifu; "W-Japan," consisted of specimens from Wakayama to Kuchinoerabujima; and "the Ryukyus," consisted of specimens from Nakanoshima and Takarajima) and *An. l. pleccau* in Taiwan.

The presence or absence of an apical pale spot on the wing vein in each geographical group was recorded and shown in Table 3. The apical pale spot on r_{4+5} appeared on some specimens (4/12) of E-Japan, whereas most specimens presented the morphology in W-Japan (16/18) and Taiwan (11/12). Pale spot on vein cu_1 did not appear in all the specimens of the Ryukyus and Taiwan, and rarely in E-Japan (3/22) and W-Japan (1/22). The specimens from Shikoku Island (Tokushima and Kochi, $n = 4$) were included in W-Japan in Table 3, because of the geographical location of the island. They showed the same tendency, viz., r_3: absence; r_{4+5}: presence; m_{1+2}: absence; m_{3+4}: presence; cu_1: absence; cu_2: presence. Contrarily, the specimens from Nagasaki and Kumamoto (W-Japan) generally showed the presence on vein r_{4+5}.

The morphological measurements of the legs showed overlapping values among the geographical region (data not shown).

Important morphological characteristics for differentiating the 3 geographical groups in Japan and *An. l. pleccau* in Taiwan are shown in Table 4. **Adult. The ratio of antennal segments in female** The ratio of segments 1 and 2 ranged between 1.79–1.86 in E-Japan; however, it was smaller in the other groups (1.13–1.70 in W-Japan, 1.15–1.41 in the Ryukyus, and 1.21–1.66 in Taiwan). **Larva. The number of branches on seta 1-S** E-Japan showed a lower value (3–4 branches) than that of the other groups (4–8 branches in W-Japan, 4–7 branches in the Ryukyus and 7–8 branches in Taiwan). **Pupa. The total number of branches on seta 5 through segments IV–VII** E-Japan showed a lower value (< 20) than that of W-Japan (> 24). **Seta 6 on segments IV–VII** Branched for the Ryukyus; single for E-Japan, W-Japan, and Taiwan.

Table 2. Percent pairwise divergence among 5 clades (3 clades in Japan, one in Taiwan, and one in China) of *Anopheles lindesayi* based on 650 bp of COI sequences

	Eastern Japan ($n = 10$)	Western Japan ($n = 10$)	Ryukyus ($n = 2$)	Taiwan ($n = 8$)
Western Japan	7.94–9.13%			
Ryukyus	8.43–9.13%	3.07–4.73%		
Taiwan	8.73–9.73%	3.04–4.92%	0.35–1.24%	
China ($n = 1$)	7.75–8.14%	1.24–2.15%	3.07–3.79%	3.04–3.79%

The numbers of base substitutions per site averaged over all sequence pairs between groups are shown. Analyses were performed using the Kimura-two-parameter model.
Eastern Japan: Hokkaido, Aomori, Fukushima, Tokyo, and Gifu; Western Japan: Wakayama, Nagasaki, Kumamoto, and Kagoshima; Ryukyus: Nakanoshima and Takarajima; Taiwan: Hsinchu and Taichung.

Table 3. The presence of apical pale spot on the veins of the right wing of *Anopheles lindesayi* among 3 geographical groups from Japan (Eastern Japan, Western Japan, Ryukyus) and one population from Taiwan

Population	The number of presence (+)/The number of examined specimens											
	r_3		r_{4+5}		m_{1+2}		m_{3+4}		cu_1		cu_2	
	N	(%)	*N*	(%)	*N*	(%)	*N*	(%)	*N*	(%)	*N*	(%)
Eastern Japan	1/12	8	4/12	33	0/22	0	21/22	95	3/22	14	21/21	100
Western Japan	6/18	33	16/18	89	9/21	43	19/21	90	1/22	5	18/22	82
Ryukyus	0/7	0	6/7	86	2/7	29	7/7	100	0/7	0	6/7	86
Taiwan	1/12	8	11/12	92	2/12	17	12/12	100	0/12	0	12/12	100

N: Number of examined specimens.

Table 4. Important morphological characteristics of *Anopheles lindesayi* for differentiating 3 geographical groups from Japan and one population from Taiwan

	Eastern Japan	Western Japan	Ryukyus	Taiwan
Female: Flm1/Flm2*	1.79–1.86 ($n = 4$)	1.13–1.70 ($n = 7$)	1.15–1.41 ($n = 7$)	1.21–1.66 ($n = 15$)
Female: Cell R_2/vein r_{2+3}	2.29–2.65 ($n = 4$)	1.32–2.54 ($n = 8$)	1.88–2.10 ($n = 7$)	1.33–1.99 ($n = 15$)
Male: hind tarsomere 1/tibia	1.14–1.34 ($n = 4$)	1.15–1.19 ($n = 5$)	1.19–1.30 ($n = 5$)	1.22–1.65 ($n = 5$)
Pupa: the No. of branch on seta 1–V	Single ($n = 4$)	2–3 ($n = 5$)	Single ($n = 10$)	5–11 ($n = 15$)
Pupa: the total No. of branch on setae 5 through segments IV–VII	< 20 ($n = 4$)	> 24 ($n = 5$)	12–22 ($n = 10$)	20–50 ($n = 15$)
Pupa: the No. of branch on setae 6 on segments IV–VII	Single ($n = 4$)	Single ($n = 5$)	2 ($n = 10$)	Single ($n = 15$)
Larva: the No. of branch on Seta 1–S	3–4 ($n = 6$)	4–8 ($n = 8$)	4–7 ($n = 10$)	7–8 ($n = 15$)

*: Flm1/Flm2: ratio of the length of flagellum segment 1 to the length of flagellum segment 2.
Eastern Japan: Hokkaido and Fukushima; Western Japan: Nagasaki, Kumamoto, and Kagoshima; Ryukyus: Nakanoshima and Takarajima; Taiwan: Hsinchu and Taichung.

DISCUSSION

This study revealed that *An. lindesayi* in Japan consisted of 3 genetically distinct groups: "E-Japan," ranging from Hokkaido to central Honshu, which was regarded as an *An. l. japonicus* population because it included Hokkaido, a type locality of the subspecies; "W-Japan," ranging from the middle of Honshu to Kyushu; and "the Ryukyus," the specimen from the Ryukyu Archipelago.

A previous molecular study based on the COI barcoding region showed that *An. l. japonicus* species of Honshu were divided into 2 populations, Eastern Japan (Hokkaido, Fukushima, and Gifu) and Western Japan (Wakayama, in the middle of Honshu) (34). The present study compared the COI barcoding region, adding further DNA alignments from *An. japonicus* of Tokyo, Kyushu, and the Ryukyu Archipelago and *An. l. pleccau* of Taiwan. Phylogenetic analysis (Fig. 2A) showed that *An. l. japonicus* from the Ryukyu Archipelago could be the third population, which was located in the same clade as *An. l. pleccau* from Taiwan. Moreover, specimens from Kyushu were added to the Western Japan population, which was located in the same clade as the populations of China and South Korea.

In terms of the K2P divergence of the COI barcoding region, the 3 geographical groups of Japan were genetically well isolated (> 3%) from each other at the species level. Notably, the largest K2P distance (9.13%) observed between the specimens of Hokkaido (E-Japan) and Nagasaki (W-Japan) was much more than that of the intra-specific K2P divergence (< 2%) based on the COI barcoding region of mosquitoes (35,36). Maekawa et al. (34) also reported a high K2P distance (8.5%) between the specimens of Hokkaido (E-Japan) and Wakayama (W-Japan). Contrarily, K2P distance between the specimens of the Ryukyus and Taiwan was relatively low (0.35%–1.24%).

In the present study, ITS2 sequences obtained from Hokkaido and Fukushima (E-Japan) specimens showed a K2P distance > 20% from any of the other groups (Nagasaki, Kumamoto, Taiwan, South Korea, and China). Conversely, relatively smaller values of K2P distance were observed (0.0%–1.3%) among the specimens of Nagasaki (W-Japan), Taiwan, South Korea, and China. Fang et al. (37) reported that sequence divergence between the species averaged 0.48% in the ITS2 sequences of the *An. hyrcanus* group members (approximately 550 bp) based on the K2P distance model. Compared to this value, the genetic distance between E-Japan and other groups was much more.

We identified 7 characteristics that could distinguish the 3 genetically distinct *An. lindesayi* groups in Japan. Our results suggested that the combination of the pupal characteristics, seta 1-V, seta 6-IV–VII, and seta 5-IV–VII could separate the 4 groups. While the branch number of seta 5-IV, V, VI, or VII alone was not able to separate the 3 groups clearly, the total number of branches of seta 5 through segments IV–VII distinguished "E-Japan" from "W-Japan."

The morphology of the legs and wings have been commonly used to distinguish the subspecies of *An. lindesayi* (13–15,22). However, the morphological measurements on the legs and wings showed overlapping values among the 3 geographical groups of Japan and "Taiwan," as indicated by Tanaka et al. (15) in their broad-ranged variations.

Morphological and genetic characteristics of the specimens of the Ryukyus and Taiwan were similar and overlapped with a few differences on the number of branches of pupal setae. For example, the apical pale spot on vein cu_1 showed similarities between the specimens of the Ryukyus and Taiwan. Tanaka et al. (15) indicated that pupal seta 1-V and larval seta 1-S could be used to distinguish *An. lindesayi* of Japan and Taiwan. We confirmed that the number of pupal seta 1-V could distinguish "Taiwan" from the other 3 geographical groups in Japan. However, the larval seta 1-S separated "Taiwan" only from "E-Japan."

Geographical barriers might be important for the diversification of *An. l. japonicus* species. According to the "dual origin" model that describes the formation of the Japanese Islands (38), the northeastern and the southwestern portions of the Japanese Islands separated independently from the Eurasian continent around 21–11 Ma. The 2 portions were moved by plate tectonics, forming the shape of the current Japanese Island, at approximately 5 Ma. The junction area has a very diverse and complex geological structure, known as the Japanese Alps. The greatest depression in Honshu is known as the Fossa Magna (39). This dynamic topography is regarded as a geographical barrier and is believed to play a role in the low dispersibility of some organisms (39). This diverse and complex topography could be a strong barrier for *An. l. japonicus*, because of its low dispersibility and isolated distribution in the forest/mountainous areas, and would be a possible factor involved in the speciation between "E-Japan" and "W-Japan."

Sames et al. (40) suggested that the subspecies of *An. lindesayi* may be elevated to the species level by confirmation with molecular analysis. The current study strongly suggested that a group of W-Japan was possibly a novel species or subspecies distributed in China, South Korea, and Western Japan. This group could be distinguished from the others by several morphological characteristics. However, this group needs further comparison with other subspecies of *An. lindesayi* (*benguetensis* and *cameronensis*, mainly reported from the Philippines and Malaysia, respectively) to confirm its taxonomic status. In addition, specimens from Shikoku (included in W-Japan in the current study) must be re-examined to reveal the distribution of the "W-Japan" population. We could not conduct molecular analyses for the specimens. The specimens from Shikoku and Nagasaki showed different patterns of the apical pale spot on wing vein.

In conclusion, the present study demonstrated that the previously identified populations of *An. l. japonicus* in Japan consisted of 3 morphologically and genetically distinct groups: "E-Japan" as *An. l. japonicus*, "the Ryukyus" as a synonym of *An. l. pleccau*, and "W-Japan" as an unrecognized group. The present study reported that *An. l. pleccau* from Japan could be added as a species to the list of potential malarial vectors in the country.

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Conflict of interest None to declare.

REFERENCES

1. Centers for Disease Control and Prevention. Local transmission of *Plasmosdium vivax malaria* – Palm Beach County, Florida, 2003. Morb Mortal Wkly Rep. 2003;52:908-11.
2. Cuadros J, Calero MJ, Benito A, et al. *Plasmodium ovale* malaria acquired in central Spain. Emerg Infect Dis. 2002;8:1506-8.
3. Loupa CV, Tzanetou K, Kotsantis I, et al. Autochthonous *Plasmodium vivax* malaria in a Greek schoolgirl of the Attica region. Malar J. 2012;11:52.
4. Kimura M, Suzaki A, Matsumoto Y, et al. Epidemiological and clinical aspects of malaria in Japan. J Travel Med. 2003;10:122-7.
5. National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases Control Division, Ministry of Health, Labour and Welfare, Japan. The characteristics of reported malaria cases in Japan during 2006–2014. Infectious Agents Surveillance Report. Available at <https://www.niid.go.jp/niid/ja/malaria-m/malaria-iasrd.html>. Accessed October 30, 2017. Japanese.
6. Kamimura K. A list of Japanese mosquitoes (Diptera: Culicidae). Proceedings of the Research Group of Pestology. 2009;6:24-43.
7. Toma T. Studies on the *Anopheles* mosquitoes in the Ryukyu Archipelago, Japan. J Med Entomol Zool. 2002;53:7-19. Japanese.
8. Kurihara T. Malaria vectors in Japan (except Nansei Islands). Med Entomol Zool. 2002;53:1-28. Japanese.
9. Wilkerson RC, Li C, Rueda LM, et al. Molecular confirmation of *Anopheles* (*Anopheles*) *lesteri* from the Republic of South Korea and its genetic identity with *An.* (*Ano.*) *anthropophagus* from China (Diptera). Zootaxa. 2003;378:1-14.
10. Hwang UW, Yong TS, Ree HI. Molecular evidence for synonymy of *Anopheles yatsushiroensis* and *An. pullus*. J Am Mosq Control Assoc. 2004;20:99-104.
11. Rueda LM. Two new species of *Anopheles* (*Anopheles*) *hyrcanus* group (Diptera: Culicidae) from the Republic of South Korea. Zootaxa. 2005;941:1-26.
12. Hwang UW. Revisited ITS2 phylogeny of *Anopheles* (*Anopheles*) Hyrcanus group mosquitoes: reexamination of unidentified and misidentified ITS2 sequences. Parasitol Res.2007;101:885-94.
13. Christophers SR. The Fauna of British India, Including Ceylon and Burma. Diptera. Vol. IV. Family Culicidae. Tribe Anophelini. London: Taylor and Francis; 1933.
14. Reid JA. Anopheline mosquitoes of Malaysia and Borneo (Kuala Lumpur, Malaysia. Institute for Medical Research. Studies, no.31). Kuala Lumpur : Govt. of Malaysia;1968.
15. Tanaka K, Mizusawa K, Sangstad ES. A revision of the adult and larval mosquitoes of Japan (including the Ryukyu Archipelago and the Ogasawara Islands) and Korea (Diptera: Culicidae). Contrib Am Entomol Inst. 1979;16:1-987.
16. Harrison BA, Rattanarithikul R, Peyton EL, et al. Taxonomic changes, revised occurrence records and notes on the Culicidae of Thailand and neighboring countries. Mosq Syst. 1990;22:196-227.
17. Koidzumi M. The research on preventive medicine of mosquitoes in Taiwan. In: Research Institute of the Governor-General of Taiwan, editors. Report of the Research Institute of the Governor-Generol of Taiwan. vol. 8. Taipei: Research Institute of the Governor-General of Taiwan; 1921. p. 34-8. Japanese. Available at <http://iss.ndl.go.jp/books/R100000039-I001767881-00>. Accessed October 23, 2018.
18. King WV. The Philippine varieties of *Anopheles gigas* and *Anopheles lindesayi*. Philipp J Sci. 1931;46:751-7. Available at <https://www.cabdirect.org/cabdirect/abstract/19321000164>. Accessed October 23, 2018.
19. LaCasse WJ, Yamaguti S. Mosquito Fauna of Japan and Korea. Kyoto: Surgeon, 8th U.S. Army; 1950. Available at <https://ci.nii.ac.jp/ncid/BA12483288>. Accessed October 23, 2018.
20. Anazawa K. Experimental research on malaria infection of *Anopheles* species in Taiwan (Part 4). J Formos Med Assoc. 1931;30:609-32. Japanese. Available at <http://iss.ndl.go.jp/books/R100000074-I000366002-00>. Accessed October 23, 2018.
21. Morishita K. Malaria: epidemiology and prevention-reports and researches in Taiwan during Japanese rule. Tokyo: Kikuya Shobo; 1976. Japanese. Available at <http://iss.ndl.go.jp/books/R100000002-I000001141652-00>. Accessed October 23, 2018.
22. Ma S, Xu B. Studies on the morphological variation of the *Anopheles* (*A*) *lindesayi* GILES in China. Acta Entomol Sinca. 1983;26:93-8. Chinese. Available at <http://en.cnki.com.cn/Article_en/CJFDTOTAL-KCXB198301016.htm>. Accessed October 23, 2018.
23. Yamada S. A new species of *Anopheles* from Hokkaido. J Hyg Infect Dis. 1918;13:689-91. Japanese.
24. Christophers SR. *A.* (*Myzomyia*) *pattoni*, a new *Anopheles* from Shan-Tung, North China; with notes on some other species of *Anopheles* from the same locality. Indian J Med Res. 1926;13:871-7.
25. Miyagi I, Toma T, Suzuki H, et al. Mosquitoes of the Tokara Archipelago, Japan. Mosq System. 1983;15:18-27. Available at <http://agris.fao.org/agris-search/search.do?recordID=US8209030>. Accessed October 23, 2018.
26. Lien JC. Pictorial Keys to the Mosquitoes of Taiwan. Taipei: Yi Hsien Publishing Co., Ltd.; 2004. Available at <http://wwwlibuser.nmns.edu.tw/content.cfm?id=M000215703&uuid=&list_num=10&current_page=1>. Accessed October 23, 2018.
27. Folmer O, Black M, Hoeh W, et al. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294-9.
28. Cornel AJ, Porter CH, Collins FH. Polymerase chain reaction species diagnostic assay for *Anopheles quadrimaculatus* cryptic species (Diptera: Culicidae) based on ribosomal DNA ITS2 sequences. J Med Entomol. 1996;33:109-16.
29. Linton YM, Samanidou-Voyadjoglou A, Harbach RE. Ribosomal ITS2 sequence data for *Anopheles maculipennis* and *An. messeae* in northern Greece, with a critical assessment of previously published sequences. Insect Mol Biol. 2002;11:379-83.
30. Walton C, Somboon P, O'Loughlin SM, et al. Genetic diversity and molecular identification of mosquito species in the *Anopheles maculatus* group using the ITS2 region of rDNA. Infect Genet Evol. 2007;7:93-102.
31. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673-80.
32. Tamura K, Peterson D, Peterson N, et al. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731-9.
33. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980;16:111-20.
34. Maekawa Y, Ogawa K, Komagata O, et al. DNA barcoding for molecular identification of Japanese mosquitoes. Med Entomol Zool. 2016;67:183-98. Japanese.
35. Wang G, Li C, Guo X, et al. Identifying the main mosquito species in China based on DNA barcoding. PLoS One 2012;7:e47051.
36. Taira K, Toma T, Tamashiro M, et al. DNA barcoding for identification of mosquitoes (Diptera: Culicidae) from the Ryukyu Archipelago, Japan. Med Entomol Zool. 2012;63:289-306.
37. Fang Y, Shi WQ, Zhang Y. Molecular phylogeny of *Anopheles hyrcanus* group members based on ITS2 rDNA. Parasit Vectors. 2017;10:417.
38. Otofuji Y, Matsuda T, Nohda S. Opening mode of the Japan Sea inferred from paleomagnetism of the Japan Arc. Nature. 1985;317:603-4.
39. Tojo K, Sekiné K, Takenaka M, et al. Species diversity of insects in Japan: their origins and diversification processes. Entomol Sci. 2017;20:357-81.
40. Sames WJ, Kim HC, Chong ST, et al. *Anopheles lindesayi japonicus* Yamada (Diptera: Culicidae) in Korea: comprehensive review, new collection records, and description of larval habitats. J Vector Ecol. 2008;33:99-106.