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A checklist of the *Anopheles* mosquito species (Diptera: Culicidae) in Bhutan

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ABSTRACT

The present paper records, for the first time, the *Anopheles* fauna of Bhutan, determined from surveys conducted from 2007 to early 2018. Adult mosquitoes were collected mainly on cattle bait and occasionally in human landing catches. Collections of immature stages were performed in various aquatic habitats. Larvae were preserved or reared to adults. Identification was based on morphological characters using available keys. A total of 30 species were identified, including nine species of subgenus *Anopheles* and 21 species of subgenus *Cellia*. Distribution and collection data are provided with notes on the locations and habitats of the species. *Anopheles pseudowillmori* is suspected to be a vector of malarial parasites in the plains and hilly forested areas of the country because it is widely distributed and the most common species collected in human landing catches. Notes also include observed morphological variation observed in *An. baileyi* and *An. lindesayi*, which differ from the type forms. Corrections are made for previous reports of *Anopheles* in Bhutan. The need for further surveys and molecular identification of members of species complexes and morphological variants is emphasized.

1. Introduction

The Kingdom of Bhutan is a landlocked country situated in the Eastern Himalayan Mountains. It is located to the north of India and south of Tibet, lying between latitudes 26° and 29 °N and longitudes 88° and 93 °E. It is subdivided into 20 districts (Fig. 1), and Thimphu is the capital of the country. Bhutan has a population of about 800,000 people (United Nations, Department of Economic and Social Affairs, Population Division, 2017). Geographically, most of the land consists of steep and high mountains criss-crossed by a network of swift rivers, which form deep valleys before draining into the Indian plains in the south of the country. There are limited plains areas along the Indian border. Elevation rises from 100 m in the southern foothills to more than 7000 m in the north. The climate in Bhutan varies with elevation, from subtropical in the south to temperate in the highlands, and with polar-like climate in the north with snow throughout the year. Sixty percent of Bhutan consists of well-conserved forests with a great diversity of plants and animals. Diseases caused by vector-borne pathogens, including malaria, leishmaniasis, scrub typhus, dengue, chikungunya and Japanese encephalitis, occur in subtropical areas of the country (https://thebhutanese.bt/the-threat-of-vector-borne-diseases-in-bhutan/).

Perennial malaria transmission in Bhutan occurs in seven of the 20 Dzongkhag (Districts) bordering India, i.e. the Samtse, Chhukha, Dagana, Sarpang, Zhemgang, Pemagatshel and Samdrup Jongkhar Districts (Tobgay et al., 2011). The malaria-endemic districts are mostly covered with forests, having a subtropical climate with hot and humid conditions and abundant rainfall during the monsoon period, which lasts up to three months (July to September). Four northern districts, Paro, Thimphu, Gasa and Bumthang, experience no malaria transmission due to their higher elevation. The nine other districts experience seasonal transmission during the summer months. *Plasmodium falciparum* and *P. vivax* are the main species of human malarial protozoa in Bhutan. Because there is no requirement for visas, movement of Indian and Bhutanese people across border areas is very common. Malaria control in the Indian border areas is relatively inadequate and malaria transmission is much more intense (Wangdi et al., 2015). People who travel into endemic areas in India may acquire malaria from the bites of local mosquito vectors, and Indian people infected with malaria may introduce the parasites to local people in Bhutan.

Historically, malaria was a dreadful disease in Bhutan. According to a malaria survey conducted in 1962, malaria prevalence in children ranged from 10.7 to 55.5% in endemic villages (Vector-borne Disease Control Programme, 2007). The national malaria eradication

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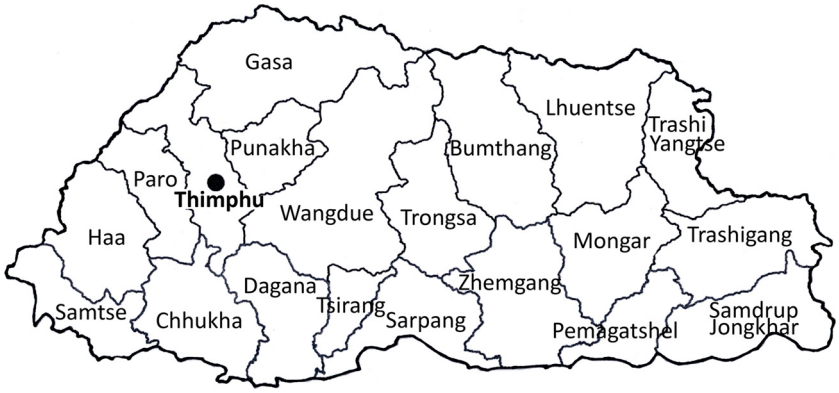

Fig. 1. Map of Bhutan showing the 20 districts. Thimphu, marked as bold, is the capital.

programme was established in 1964 by the Vector-borne Disease Control Programme (VDCP) of the Ministry of Health. Malaria eradication activities included extensive use of indoor residual spraying of DDT and establishment of malaria centers in southern districts for active case surveillance. However, the progress of malaria eradication was slow due to the lack of human resources, budget and difficulties with transportation. In addition, a security problem along the southern border areas in the early 1990s resulted in a dramatic increase in the incidence of malaria, with the highest incidence of about 40,000 cases reported in 1994 (Tobgay et al., 2011). In 2006, long-lasting insecticidal nets (LLIN) and artemisinin-based combination therapies were introduced. Over 90% of households in areas with perennial transmission received the LLIN (The Global Fund, 2009). This program resulted in a significant reduction of malaria incidence during the past decade. In 2016, there were 15 reported cases (World Malaria Report, 2017). Bhutan aims to eliminate malaria in 2018 to support the goal of an Asia-Pacific region free of malaria by 2030. To achieve the goal of malaria elimination, understanding of malaria epidemiology and vector biology is essential. Prior to 2007, little was known about *Anopheles* species and vectors of malarial protozoa in Bhutan due to limited entomological staff. According to the Walter Reed Biosytematics Unit (WRBU), Systematic Catalog of Culicidae (http://www.mosquitocatalog.org), only six species are recorded from Bhutan, two species of *Aedes* and four species of *Anopheles*, i.e. *An.* (*Anopheles*) *peditaeniatus* (Leicester), *An.* (*Cellia*) *pseudowillmori* (Theobald), *An.* (*Cel.*) *vagus* Dönitz and *An.* (*Cel.*) *willmori* (James). In 2007, extensive systematic surveys of mosquitoes were undertaken. Since then, training and higher education on the epidemiological and entomological aspects of malaria have been promoted. This has gradually built up the capacity of VDCP staff to perform studies of the Bhutanese mosquito fauna. The present study reports, for the first time, the composition of the *Anopheles* fauna, and the habitats and distributions of the species in the country gleaned from routine surveys conducted during 2007 to early 2018.

2. Materials and methods

2.1. Mosquito collection

Surveys of adult mosquitoes were usually carried out during March to November each year, and about one to two weeks during each month. No collections were made during December to February due to cold winter weather in Bhutan. In addition, in some months when dengue outbreaks occurred, all staff of VDCP focused on dengue vector control activities. Collections of adult mosquitoes were made mainly from cowsheds. Human-landing catches were seldom performed along the border and forest areas due to security reasons (e.g. kidnapping, curfew and wild animals). Nonetheless, human-landing catches (two persons per site) were carried out for a few hours after dusk in Meritsemo and Phuntsholing of the Chuukha District, Chuzargang and Gelephu of the Sarpang District, Sipsu and Tempaling of the Samtse District, and Rangjung of the Trashigang District. Mosquitoes were preserved by pinning or mounting on triangular points, or kept in pools in small containers. They were dried in an oven at 42–45 °C, or dried over silica gel.

Surveys for mosquito larvae and pupae were carried out in various aquatic habitats. Early instar larvae were reared to fourth-instars or adults. Fourth-instar larvae were killed by briefly placing them in hot water (60–65 °C) and preserved in 80% ethanol; some larvae, and also pupae, were reared to adults, which were preserved as above. The associated larval and pupal exuviae of some adults were preserved in 80% ethanol. Whole larvae and larval and pupal exuviae were mounted in Hoyer's medium (Neo-shigaral, Shiga Konchu Fukyusha, Tokyo, Japan) or Euparal (Waldeck, Germany).

Since collections of mosquitoes were the task of VDCP staff, there was no requirement for ethical approval in Bhutan. However, heath inspections were advised for those who felt ill after returning from field work.

2.2. Identification of mosquitoes

Since there is no specific key for morphological identification of mosquitoes that occur in Bhutan, specimens were identified using morphological keys for areas of southern and south-eastern Asia, including Christophers (1933); Reid (1968); Harrison and Scanlon (1975); Harrison (1980); Rao (1984) and Rattanarithikul et al. (2006). The morphological terminology used herein is defined in the Anatomical Glossary of the Mosquito Taxonomic Inventory (http://mosquito-taxonomic-inventory.info/). Most of the specimens collected are retained in the VDCP, Gelephu, Bhutan; some are housed in the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand.

3. Results

The *Anopheles* species collected, and the locations and habitats surveyed during 2007 to early 2018, are listed in Table 1. A total of 30 species were identified. It should be noted that their distributions are based on a limited number of collection sites.

Anopheles (*Anopheles*), Anopheles Series. Four species of this Series were collected, including two species of the Aitkenii Group, *An. aberrans* Harrison & Scanlon and *An. bengalensis* Puri, and two species of the Lindesayi Group, *An. baileyi* Edwards and *An. lindesayi* Giles. The larvae of *An. aberrans* are distinct from the larvae of *An. bengalensis* in having setae 11-II, 5-II and 14-P as described in Rattanarithikul et al. (2006). Unlike *An. aberrans* in Thailand, however, abdominal segments IV and V of larvae and adults of Bhutanese *An. aberrans* are usually not pale. Both species were often found in the same collection sites.

Table 1
List of the *Anopheles* species collected in Bhutan during 2007 to early 2018.

Species	Districts	Villages/Towns	Habitats	Available specimens
An. aberrans	Sarpang	Maugang,Tshachu	Highland forest stream margins, marshy pools, irrigation margins	Larvae/adults
	Trongsa	Kuengarabten, Yourmong		
	Wangdue	Mesena, Thinleygang	marshy pools	
	Zhemgang	Nanglam		
An. aconitus	Sarpang	Chuzargang, Gelephu, Umling	Stream margins, rice fields	Larvae/adults
An. annularis	Chhukha	Pekazhing	Hilly forest, spring shores, rice fields	Larvae/Adults
	Sarpang	Chuzargang, Umling		
	Trashigang	Rangjung		
	Tsirang	Patala		
An. baileyi	Bumthang	Chumed	Highland roadside pools, marshes, ground pools, stream margins	Larvae/adults
	Mongar	Charshingzor, Korila		
	Paro	Chumphu, Jichu, Kuduphu, Sendona, Shaba		
	Sarpang	Chabikhola, Maugang, Rongri		
	Thimphu	Bjemina, Lamperi Park, Ludrong Park, Tango		
	Trongsa	Kuengarabten, Samcholing		
	Wangdue	Ruirechu		
An. barbirostris s.l.	Sarpang	Bisty, Chuzargang, Singye, Sisty, Umling	Hilly forest, fisheryponds, paddy fields	Larvae/Adults
An. barbumbrosus	Sarpang	Rongri, Sarpangtar, Tshachu	Marshy pools, banana leaf axils, old tanks	Larvae/adults
	Pemagatshel	Nanglam, Peling Tso		
	Mongar	Yangbari		
An. bengalensis	Mongar	Korila	Highland forest stream margins, marshy pools, roadside pools	Larvae/adults
	Sarpang	Maugang, Surey, Tshachu		
	Pemagatshel	Nanglam, Peling Tso		
	Wangdue	Mesena, Ruirechu		
	Zhemgang	Bobsar, Pangtang		
An. crawfordi	Pemagatshel	Nanglam, Peling Tso	Marshes, rice fields	Larvae/adults
An. culicifacies s.l.	Chhukha	Pekazhing	Sandy riverbeds, rain pool, river seepage, river shores, fish ponds	Larvae/adults
	Paro	Shaba, Sendona		
	Samdrupjongkhar	Rongchuthang		
	Samtse	Tempaling		
	Sarpang	Bhur, Bisty, Chaskar, Dekiling, Singye, Sisty, Umling		
	Tashigang	Phongme, Jonlapam		
An. dravidicus	Chhukha	Pekazhing, Pasakha	Road side ditches, springs	Larvae/adults
	Samtse	Tempaling		
An. fluviatilis s.l.	Chhukha	Pekazhing	Marshy pools, rice fields	Larvae/adults
	Pemagatshel	Peling Tso		
	Trashigang	Rangjung		
	Samdrupjongkhar	Samdrupcholing		
	Sarpang	Singye		
An. interruptus	Tsirang	Pemathang	Tree hole	Larvae/adults
An. jamesii	Chhukha	Pakazhing	Ground pools	Larvae/adults
	Sarpang	Chuzargang, Gelephu, Umling		
An. jeyporiensis	Samdrupjongkhar	Belamsharang, Pemathang, Samdrupcholing	Stream margins, irrigation canals, rice fields	Adults
An. karwari	Sarpang	Chuzargang	Hilly forest	Larvae/adults
An. kochi	Samdrupjongkha	Belamsharang, Samdrupcholing, Pemathang	Ground pools, wheel tracks, ground ditches	Larva/adults
	Sarpang	Dawathang, Gelephu,Umling		
An. lindesayi	Bumthang	Chume	Highland forest stream margins, roadside pools, marshy pools	Larvae/adults
	Chhukha	Meritsemo		
	Mongar	Kilikhar, Korila		
	Paro	Bondey, Kuduphu		
	Sarpang	Maugang, Tamala		
	Thimphu	Semtokha, Tango		
	Zhemgang	Buli, Dakphai, Pangtang		
An. maculatus s.l.	Chhukha	Pekazhing	Stream margins, roadside pools	Larvae/adults
	Mongar	Telung		
	Samdrupjongkhar	Belamsharang, Martsala		
	Samtse	Sipsu, Tendu		
	Sarpang	Gelephu, Norbuling, Singye, Tareythang		
	Zhemgang	Nanglam		
An. minimus s.l.	Samtse	Tempaling	Hilly forests	Adults
An. nivipes s.l.	Samdrupjongkhar	Bangtar, Belamsharang	Rice fields, hilly forests	Adults
An. philippinensis	Samdrupjongkhar	Samdrupcholing, Umling	Rice fields, hilly forests	Adults
	Samtse	Tempaling		
An. peditaeniatus	Chhukha	Meritsemo, Pekazhing	Rice fields, marshes, streams	Larvae/adults
	Pemagatshel	Panbang, Peling Tso		
	Samdrupjongkhar	Belamsharang, Samdrupcholing, Samrang		
	Samtse	Sipsu		
	Sarpang	Bisty, Chuzargang, Gelephu, Ludarai, Norbuling, Singye, Sisty, Umling		
	Trashigang	Rangjung		
	Zhemgang	Panbang		
An. pseudojamesi	Sarpang	Dawathang, Umling	Hilly forest, fish pond	Adults

(*continued on next page*)

Table 1 (*continued*)

Species	Districts	Villages/Towns	Habitats	Available specimens
An. pseudowillmori	Chhukha	Arekha, Meritsemo, Pakazhing, Pasakha	Streams, roadside pools, riverbed pools, wheel tracks, irrigation channels, rice fields, marshes, hilly forest	Larvae/adults
	Haa	Dorithasa		
	Lhuentse	Autso		
	Mongar	Jaiposhing, Lingmithang, Yangbari, Gaskhaibali		
	Pemagatshel	Nanglam, Peling Tso		
	Samdrupjongkhar	Belamsharang, Pemathang, Samdrupcholing, Samrang		
	Samtse	Samtse, Sipsu, Tempaling, Ugyentse, Yuseltse		
	Sarpang	Bhur, Chuzargang, Dekiling, Dovan, Gelephu, Maugang, Norbuling, Singye, Tshachu, Umling		
	Trashigang	Lungtenzam, Rangjung		
	Tsirang	Dunglagang, Sunkosh		
	Wangdue	Bajo, Kamichu, Sha		
	Zhemgang	Manas, Panbang		
An. splendidus	Sarpang	Chuzargang, Gelephu, Umling	Hilly forest	Adults
	Trashigang	Rangjung		
An. subpictus	Sarpang	Chuzagang, Gelephu, Lhamoizingkha	Rice fields	Adults
An. tessellatus	Sarpang	Norbuling	Rice fields	Larvae/adults
An. vagus	Chhukha	Phuntsholing, Piping	Rice fields, streams, ground pools, wheel tracks	Larvae/adults
	Lhuentse	Autso		
	Samdrupjongkhar	Belamsharang, Samrang, Pemathang, Samdrupcholing		
	Samtse	Sipsu, Norboogang		
	Sarpang	Chuzargang, Gelephu, Sershong, Singye, Umling		
	Trashigang	Phongme, Rangjung		
	Zhemgang	Panbang		
An. varuna	Mongar	Yangbari	Stream margins	Larvae
	Samdrupjongkhar	Khaurong		
	Sarpang	Chuzargang		
An. willmori	Chhukha	Arekha, Meritsemo	Highland forest stream margins, marshy pools, road side drains	Larvae/adults
	Mongar	Kilikhar, Traling, Yangbari, Yarsaywong		
	Paro	Shaba		
	Pemagatshel	Nanglam, Peling Tso		
	Sarpang	Ludarai, Maugang, Singye, Surey, Tshachu		
	Trashigang	Rangjung		
	Trongsa	Dangdung, Kuengarabten, Rephey		
	Tsirang	Rilangthang, Sunkosh		
	Zhemgang	Berti, Buli, Paibang, Panbang, Pangtang, Praling,Tali		

The adult females of *An. baileyi* in Bhutan conform to the description of Christophers (1933) in having vein 1 A of the wing all dark, the midfemur with a large pale spot dorsally toward the apex and the wing fringe dark except for the usual large pale fringe spot between the apices of veins CuA and 1 A. However, seta 4-C of the Bhutanese larvae is usually branched (2–5) whereas this seta is usually single in Thai specimens (Rattanarithikul et al., 2006).

The morphology of *An. lindesayi* in Bhutan varies considerably. The wings of nine females from the Mongar District and Meritsemo in the Chhukha District usually have a pale spot at the tips of veins R_1, R_2, R_{4+5}, M_2, CuA and 1 A, occasionally also at the tips of veins R_3 and M_1 (Fig. 2a). Seven of these specimens have hindfemora with pale scaling covering the proximal third of the ventral surface, and a line of dark scales on the dorsal surface extending from or near the trochanter (Fig. 2c), whereas the hindfemora of the other two specimens have a basal pale band about two-thirds as long as the subapical pale band (Fig. 2d). Two females from Tango in the Thimphu District have a pale spot only at the tips of veins R_1, M_2, CuA and 1 A (Fig. 2b) and hindfemora with a basal pale band about two-thirds as long as the subapical white band (as shown in Fig. 2d). All specimens examined have dark scales on the remigium.

The typical larval habitats of *An. aberrans*, *An. bengalensis*, *An. baileyi* and *An. lindesayi* in Bhutan are ground-water habitats in high mountainous, forested areas, including ground pools, streams margins, marshes and roadside ditches. The first two species were usually found at or above 600 m whereas the latter two were common above 1000 m. These species were often found in the same habitats. A female of *An. lindesayi* was collected from human bait in a forest area of Meritsemo in the Chhukha District.

Anopheles (*Anopheles*) Lophoscelomyia Series. Larvae of one species in the Asiaticus Group, *An. interruptus* Puri, were collected from a tree hole in the Tsirang District (780 m elevation).

Anopheles (*Anopheles*), Myzorhynchus Series. Only four species of this Series have been found in Bhutan, including two of the Barbirostris Group and two of the Hyrcanus Group. The Barbirostris Group includes one species of the Barbirostris Subgroup, *An. barbirostris* s.l. and one species of the Vanus Subgroup, *An. barbumbrosus* Strickland & Chowdhury. Both species are rare but the latter appears to be more common than the former. The Hyrcanus Group includes two species of the Lesteri Subgroup, *An. peditaeniatus* and *An. crawfordi* Reid. *Anopheles peditaeniatus* is the most abundant and most widely distributed species of the Series, whereas *An. crawfordi* is rare. The species are found commonly in rice paddies, ground pools, marshes and small streams.

Anopheles (*Cellia*), Myzomyia Series. Mosquitoes in this Series in Bhutan belong to the Funestus Group, including *An. jeyporiensis* James (not placed in a subgroup), two species of the Aconitus Subgroup, *An. aconitus* Dönitz and *An. varuna* Iyengar, *An. culicifacies* s.l. of the Culicifacies Subgroup and *An. fluviatilis* s.l. and *An. minimus* s.l. of the Minimus Subgroup.

Members of the Myzomyia Series are generally found in low numbers. Adults were collected from cattle and occasionally landing on humans. The common habitats of larvae are stream margins, ground pools, marshes, ditches and rice paddies. *Anopheles culicifacies* s.l. larvae are abundant in small sandy pools of riverbeds in the late rainy season in some areas of the Sarpang District. In Rongchuthang, Samdrupjongkhar District, and Singye, Sarpang District, many *An. culicifacies* s.l. females were found resting indoors, but it is not clear if this was related to outbreaks of malaria in those areas. A few *An. minimus* s.l. females were collected from human bait in a border area of Samtse

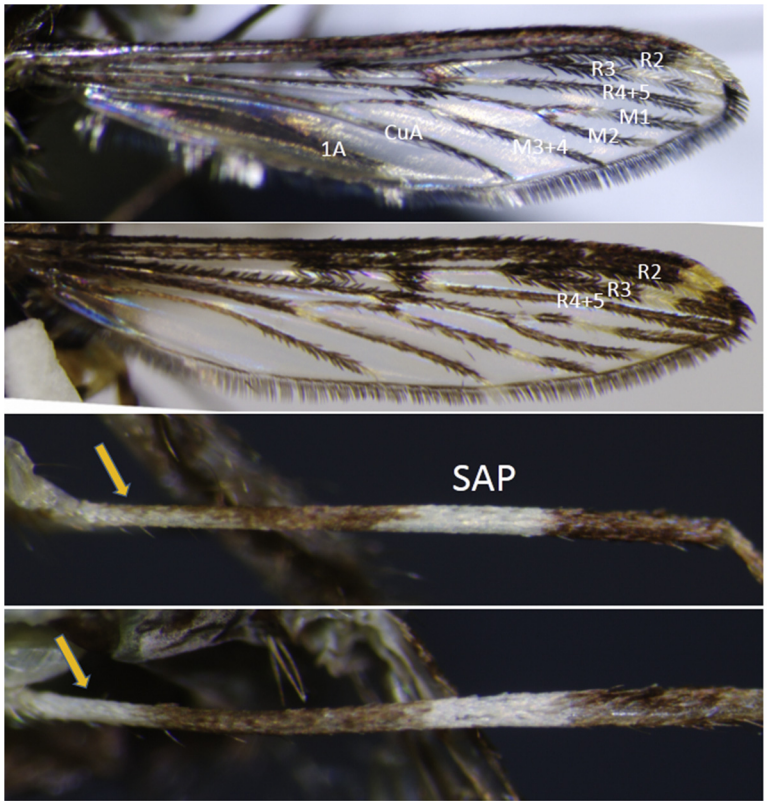

Fig. 2. a, b, Wings of *An. lindesayi* from Mongar and Tango, respectively, showing variation in the tip of veins R_2, R_3 and R_{4+5}. c, d, Variation in scaling of the hindfemur of specimens from Mongar showing a line of dark scales basally on the dorsal surface (c) and a basal pale band (d) indicated by arrows. SAP = subapical pale band.

District, but no larvae were found. Morphologically, the females have a presector pale spot on the costa of the wings, which is a common character of *An. minimus* Theobald.

Anopheles (*Cellia*), Neocellia Series. Mosquitoes of this Series include *An. karwari* James (not placed in a subgroup); three species of the Annularis Group, *An. annularis* van der Wulp, *An. nivipes* s.l. and *An. philippinensis* Ludlow, and three species of the Jamesii Group, *An. jamesii* Theobald, *An. pseudojamesi* Strickland & Chowdhury and *An. splendidus* Koidzumi. These species are usually found in low numbers. Four species of the Maculatus Group, *An. dravidicus* Christophers, *An. maculatus* Theobald, *An. pseudowillmori* and *An. willmori*, were collected. *Anopheles pseudowillmori* is the most abundant and widely distributed species of the Series collected in the country, from the plains to an altitude of 1900 m. It is the most common *Anopheles* species collected in all sites of human-landing catches (65.8%, 27 out of 41), and was abundant in cattle sheds. Larvae were found in a wide variety of habitats, including stream margins, rice paddies, marshes, ground pools, sand pools, rock pools and wheel tracks. *Anopheles willmori* is also a common species in forest in mountainous areas, at altitudes between 300–2000 m. It is also the second most common species obtained in human-landing catches in the Chuukha District (27.8%, 5 out of 18). *Anopheles maculatus* is relatively uncommon and was rarely collected landing on human bait, whereas *An. dravidicus* is rare and zoophilic. Both species were found in the plains and at altitudes up to 900 m.

Anopheles (*Cellia*), Neomyzomyia Series. Only two species of this Series were found, including one species of the Kochi Group, *An. kochi* Dönitz, and one species of the Tessellatus Group, *An. tessellatus* Theobald. Larvae of these species were found in rice paddies, ground pools and wheel tracks. Adults were occasionally collected from cattle bait. Previously, one female of the Leucosphyrus Group, *An. dirus* s.l. was collected in a cowshed in Gelephu of the Sarpang District bordering India, but this mosquito species has not been collected since. The single female may have come from nearby forest in India. *Anopheles dirus* s.l. is therefore not considered to occur in Bhutan.

Anopheles (*Cellia*), Pyretophorus Series. Only two species of the Subpictus Group of the Series have been found in Bhutan, *An. subpictus* s.l. and *An. vagus*. The former is rare, whereas the latter is abundant and widely distributed; both are strongly zoophilic. Larvae of *An. vagus* are typically found in ground pools, wheel tracks and rice paddies.

4. Discussion

The present study reports the occurrence of 30 species of *Anopheles* (nine of subgenus *Anopheles* and 21 of subgenus *Cellia*) collected in Bhutan to date. This number is about half of the number of *Anopheles* species recorded in India, including 26 species of subgenus *Anopheles* and 35 of subgenus *Cellia* (Bhattacharyya et al., 2014). The *Anopheles* fauna of Bhutan is abundant and highly diverse mainly at lower elevations of the country, which are subtropical, hilly forested border areas with rice paddies. The diversity appears to be negatively associated with elevation where a few common species, i.e. *An. baileyi*, *An. bengalensis* and *An. lindesayi*, can withstand extremely cold-weather conditions (over 3000 m elevation).

The larvae of Bhutanese *An. baileyi* have seta 4-C with 2–5 branches whereas this seta is usually single in Thai specimens (Rattanarithikul et al., 2006). It is not known if this difference reflects a population or specific distinction. A morphological comparison of the adult and pupal stages could not be made due to the limited number of available specimens. At present, *An. baileyi* is one of four members in the Gigas Complex (Harrison et al., 1990); the other three species are *An. gigas* Giles, *An. sumatrana* Swellengrebel & Rodenwaldt and *An. prachongae* Rattanarithikul & Harrison (Harbach et al., 2017). Additionally, *An. gigas* comprises eight subspecies that occur in various areas from the western Himalayas to Sri Lanka, Malaysia, the Philippines and Sumatra: *An. g. crockeri* Colless, *An. g. danaubento* Mochtar & Walandouw, *An. g. formosus* Ludlow, *An. g. gigas*, *An. g. oedjalikalah* Nainggolan, *An. g. pantjarbatu* Waktoedi Koesoemawinangoen, *An. g. refutans* Alcock and *An. g. simlensis* James (Harbach et al., 2017). None of the taxa of the Gigas Complex are known to be vectors of human malarial protozoa.

Anopheles lindesayi is a species of the Lindesayi Complex (Harrison

et al., 1990), which also includes *An. mengalangensis* Ma, *An. nilgiricus* Christophers and *An. wellingtonianus* Alcock. *Anopheles lindesayi* comprises five subspecies: *An. l. benguetensis* King (the Philippines), *An. l. cameronensis* Edwards (Malaysia, Thailand), *An. l. japonicus* Yamada (Japan, Korea), *An. l. lindesayi* (India) and *An. l. pleccau* Koidzumi (Taiwan) (Harrison et al., 1990). Identification of the subspecies is based principally on the presence or absence of a pale spot at the tips of different wing veins and a basal pale area on the hindfemur, which allows the taxa be divided into two groups (Christophers, 1933; Reid, 1968). Taxa of the first group have the tips of veins R_1, R_2 and 1 A, and occasionally CuA, pale, and the base of the hindfemur with a short pale band (*An. nilgiricus*) or about two-third the length of the preapical pale band (*An. l. cameronensis*). Taxa of the other group have at least one other vein with a pale tip, usually vein M_2, and the base of the hindfemur more extensively pale ventrally than dorsally (Fig. 2c). We found that the wing characters of *An. lindesayi* from the Mongar District and Muagan in the Sarpang District fall into the second group (Fig. 2a), but a few individuals have the base of the hindfemur similar to that of *An. l. cameronensis* (Fig. 2d). Specimens of *An. lindesayi* from Tango of Thimphu District have the tip of veins R_2 and R_{4+5} dark (Fig. 2b) and the hindfemur similar to that of *An. l. cameronensis*.

Anopheles lindesayi and *An. nilgiricus* have been found in India. However, *An. nilgiricus* is restricted to southern India (Christophers, 1933) and differs from the Bhutanese specimens of *An. lindesayi* in having yellow scales on the remigium and a short basal pale band on the hindfemur. *Anopheles lindesayi* was first described from Bakloh, western Himalayas, India (Giles, 1900), which is about 1400 km from Bhutan. The morphological characters of Bhutanese specimens do not fully agree with the type form. It would be interesting to determine whether the variation reflects a population or specific distinction, since a recent study revealed that *An. l. japonicus* comprises cryptic species with differences in morphological characters and DNA sequences (Imanishi et al., 2018). None of the members of the Lindesayi Complex are known to be vectors of human malaria plasmodia.

Other nominal *Anopheles* species that occur in Bhutan, i.e. *An. barbirostris* van der Wulp, *An. culicifacies* Giles, *An. fluviatilis*, *An. minimus* Theobald, *An. nivipes* (Theobald) and *An. subpictus* Grassi, are also known to be species complexes (Harbach, 2004). More studies are required for exact identification of these taxa. These complexes include vectors of malarial parasites in other countries, but whether they play a role in malaria transmission in Bhutan is not known.

Prior to 2007, the vectors of malaria in Bhutan were presumed to be *An. minimus*, *An. fluviatilis* and *An. dirus* (Yangzom et al., 2012). This was probably based on vector information derived from neighboring countries, India (Assam) and Bangladesh. In addition, *An. culicifacies*, *An. maculatus*, *An. philippinensis* and *An. annularis* were listed as "major" *Anopheles* species in Bhutan (World Malaria Report, 2017). It is uncertain about the source of the data contained in that report, which is not in agreement with the results of the current study because all four species were not commonly collected.

So far, however, there is no direct evidence (e.g. sporozoite detection) to show which species is(are) the vector(s) of malaria in Bhutan. In the present situation, where the incidence of malaria is very low (15 cases in 2016), detection of a sporozoite-positive mosquito may be extremely difficult. Therefore, no definitive incrimination of malaria vectors could be made at this stage. However, it is suspected that *An. pseudowillmori*, which appears to be the most common *Anopheles* species widely attacking humans in the plains and mountainous areas, may be an important vector of malaria parasites in the country. This species is the primary vector in areas of southern Tibet bordering India and Myanmar (Wu et al., 2009), and a secondary vector in north-western Thailand (Green et al., 1991). It is also the most common species of the Maculatus Group found in north-eastern areas of India adjacent to Bhutan (Singh et al., 2012). Another member of the Maculatus Group, *An. willmori*, might also play a role in local transmission, such as in the Chhukha District where it was also collected landing on human bait. *Anopheles willmori* is the vector of malaria in Nepal (Pradhan et al., 1970). Other members of the Maculatus Group, *An. sawadwongporni* Rattanarithikul & Green and *An. rampae* Harbach & Somboon (Somboon et al., 2011), have been reported from north-eastern India (Singh et al., 2012), but they have not been found in Bhutan.

Recently, *An. minimus* was collected from human bait in a border area in the Samtse District. It is not certain if the species occurs in Bhutan, as no larvae have been found in the country, or came from India. Only *An. minimus* Theobald (as *An. minimus* species A) has been reported in north-eastern India (Dutta et al., 2014). This species is a malaria vector in Assam, India and many hilly forested areas in mainland Southeast Asia and southern China (Rattanarithikul et al., 2006). Its vector status in Bhutan requires further investigation. Other members in the Minimus Complex include *An. harrisoni* Harbach & Manguin, which is found in southern China, Myanmar, Thailand, Laos and Vietnam (Manguin et al., 2008) and *An. yaeyamaensis* Somboon & Harbach, which is only found in the Rykyu Archipelago, Japan (Somboon et al., 2010), but neither has been recorded in southern Asia. *Anopheles culicifacies* is an important vector in India. In Bhutan, it might play a role in malaria transmission, particularly in the Sarpang and Samdrupjongkhar Districts where it was abundant indoors, which warrants further investigation.

Further surveys and more studies are needed in Bhutan, despite sometimes challenging field conditions. For examples, human-bait collections are difficult to perform in forest areas along the Bhutan-Indian border due to wild elephants and security issues. Accessibility to mountainous and remote forested areas in the rainy season is often hampered by flooded rivers, road blocks and landslides. In addition, entomological teams are in short supply. Therefore, capacity building and human resource development are needed to advance research and ensure sustainability of the malaria elimination programme in Bhutan.

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