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MOSQUITO BIODIVERSITY PATTERNS AROUND URBAN ENVIRONMENTS IN SOUTH-CENTRAL OKINAWA ISLAND, JAPAN

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ABSTRACT. Okinawa is the largest, most urbanized, and densely populated island in the Ryukyus Archipelago, where mosquito species diversity has been thoroughly studied. However, the south-central Okinawa mosquito fauna has been relatively poorly studied. Here, we present results from a mosquito faunal survey in urban environments of Nishihara city, south-central Okinawa. Mosquitoes were sampled biweekly, from April 2007 to March 2008, at 3 different environments: a forest preserve, an animal farm, and a water reservoir. We employed 4 mosquito collection methods: 1) oviposition traps; 2) light traps; 3) sweep nets; and 4) larval surveys of tree holes, leaf axils, and artificial water containers. We collected a total of 568 adults and 10,270 larvae belonging to 6 genera and 13 species, including 6 species of medical importance: *Aedes albopictus*, *Armigeres subalbatus*, *Anopheles* Hyrcanus group, *Culex bitaeniorhynchus*, *Cx. quinquefasciatus*, and *Cx. tritaeniorhynchus*. Mosquito species composition was similar to data from previous studies in Okinawa Island. The flattening of the species accumulation curve suggests that our diversity sampling was exhaustive with light and oviposition traps, as well as the coincidence between the species richness we found in the field and estimates from the Chao2 index, a theoretical estimator of species richness based on species abundance. This study highlights the importance of combining several sampling techniques to properly characterize regional mosquito fauna and to monitor changes in the presence of mosquito species.

KEY WORDS Mosquito biodiversity, mosquito fauna, Ryukyu Archipelago, Okinawa Island

INTRODUCTION

Mosquitoes have a rich biodiversity in tropical and subtropical regions (Foley et al. 2007). Okinawa, as the largest and most densely populated island in the subtropical Ryukyu Archipelago, consists of approximately 200 islands (Ota 1998). Several studies have shown the mosquito fauna of the Ryukyus to be extremely diverse (Miyagi 1976, Toma and Miyagi 1986, Miyagi et al. 1990, Okudo et al. 2004), with more than half of Japan's mosquito species present in the archipelago, which accounts for $<1\%$ of Japan's territory (Toma 2002). The Ryukyu Archipelago islands are continental and were connected with East Eurasia during the last glaciation by means of a land bridge connecting the island of Kyushu to southeastern China via Taiwan (Ota 1998). It is likely that this complex history, a relatively stable subtropical climate, widespread natural vegetation, and the abundance and diversity of natural habitats underpin the extraordinary patterns of mosquito species diversity in the archipelago (Toma and Miyagi 1986).

Mosquitoes also are medically important insects since the discovery of their role as disease vectors and are one of the major nuisance pests worldwide (Silver 2008). Risk assessment and control of mosquito-borne diseases, as well as nuisance pest control, require a comprehensive knowledge of mosquito species diversity, and environmental factors, in and around human settlements (WHO 1975, Silver 2008). In some urban settings, mosquito diversity has been associated with arbovirus infection patterns (Chaves et al. 2011, Thongsripong et al. 2013). Currently, mosquito-borne diseases are rare in Japan (NIID 2010). Nevertheless, severe malaria and dengue outbreaks occurred in the Ryukyu Archipelago during World War II, and the vectors are still present (Miyagi et al. 1996, Toma et al. 2003). This highlights the vulnerability of this area to the introduction of vector-borne diseases. Studies that consider whole mosquito communities, beyond species with known vectorial roles, therefore have the potential to identify ecological interactions that may prove useful in controlling vector abundance and disease transmission (Chaves et al. 2011). For example, based on the ecological interactions of mosquitoes as part of ecological communities, one study evaluated natural mosquito predators as an ecologically sound method for vector control in Okinawa (Miyagi et al. 1992). This demonstrates the need for the ecological study of mosquito species, especially in urban environments where mosquito-borne diseases can emerge and/or mosquito nuisance might be considered an important issue.

In this study, we describe diversity patterns of mosquito species in the south-central part of Okinawa Island, where urban areas are embedded in highly forested environments.

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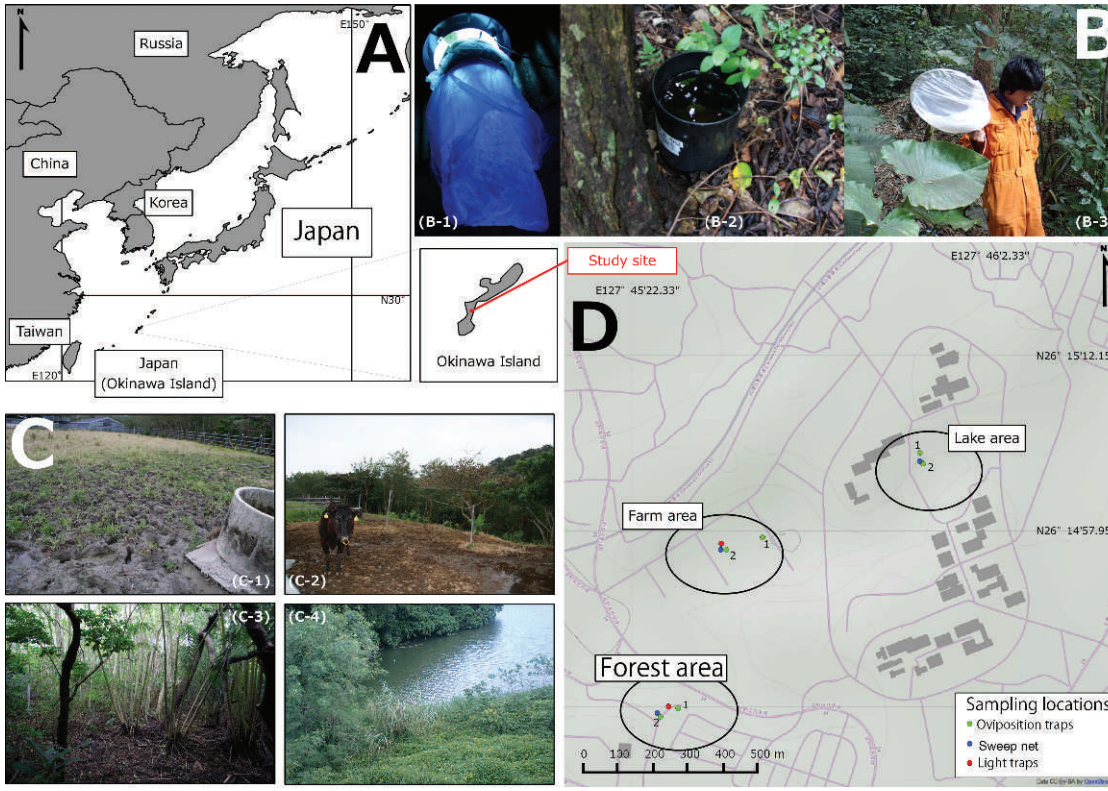

Fig. 1. Study locations in Okinawa Island and sampling methods. (A) Okinawa location in East Asia; (B) mosquito sampling methods: (B-1) light trap, (B-2) oviposition trap (ovitrap), and (B-3) sweep net; (C) sampling location habitats: (C-1) farm area where animals stay during the day, (C-2) farm area where animals stay during the night, (C-3) forest area, and (C-4) lake area; and (D) locations of each sampling method and habitats. Larval surveys of tree holes, leaf axils, and artificial containers were conducted near each ovitrap site.

MATERIALS AND METHODS

Study site

Mosquitoes were collected biweekly (i.e., every 2 wk) at the University of the Ryukyus campus, south-central Okinawa Island, from April 2007 to March 2008 (26°26′N, 127°77′E). The University of the Ryukyus campus extends over 1,033 m^2, and includes a forest, a lake, and a small animal farm. We selected sampling sites in each of these 3 habitats to consider habitat heterogeneity in an urban environment (Fig. 1). Briefly, the farm has several types of domestic animals: cows, goats, dogs, chickens, and rabbits. The animal farm sporadically had wild taro growing, which was systematically removed by the farm staff. The forest has a small stream; and the lake is a retention basin to prevent flooding with water present all year-round.

Mosquito collection

Mosquitoes were sampled with light traps (LTs), sweep nets (NSw), and oviposition traps (OTs). In addition, pipettes were occasionally used to suck the content from tree holes, leaf axils, and artificial containers near trap sites (OS). The LTs and NSw were used for adult collection, and OTs and OS for larval collection. Trap locations are shown in Fig. 1.

Light traps: Electrically operated LTs (Fig. 1; MC-8200; Ishizaki Electronic Manufacturing Co., Tokyo, Japan) powered by 30-W ultraviolet fluorescent lights were placed 1 night every 2 wk at 1.5 m above ground level and operated from 1600 h to 0800 h in the farm and forest sites (Hoshi et al. 2014). The lack of electricity sources prevented the use of LTs at the lake site. We selected this sampling period aiming to collect mosquitoes with day and night activity. Our traps had no dry ice. Mosquitoes collected were brought to the laboratory and killed by placing them in a refrigerator at low temperature (−40°C) for 10 min.

Sweep nets: Adult mosquitoes were also collected every 2 wk using a sweep net (36-cm diam; Model 61-B; Shiga Insect Co., Tokyo, Japan) (Miyagi and Toma 1980). Collections were made for 10 min between 1000 h and 1500 h, near the OTs (Silver 2008). The collector did not

actively sweep the vegetation to capture mosquitoes but rather stood at fixed points at each sampling site, collecting active mosquitoes. Mosquitoes collected were transferred into a vial with chloroform and subsequently put inside a plastic container (3 cm diam, 6 cm high; Fuji® Film roll case), using cotton to protect mosquitoes from damage before transporting them to the laboratory. The plastic tubes were placed in a freezer to kill any surviving mosquitoes.

Oviposition traps: Two black plastic buckets (15 cm × 20 cm, 3.53 liters, dustbin; Daiso Co., Hiroshima, Japan) were filled with water (2.5 liters) and set on the ground (Fig. 1) near vegetation at each site (Hoshi et al. 2014). Although OTs are most frequently used to sample eggs (Reisen and Basio 1972, Chaves and Kitron 2011, Nguyen et al. 2012), we used ovitraps for larval collections (Moriya 1974). Each bucket had 2 holes at a height of 15 cm from the base to prevent flooding in the event of rain. The contents of the traps were transferred to a plastic tray (37.4 cm × 27.3 cm × 6.4 cm, 4.6 liters, Sanbat2gou; Sanko Co., Tokyo, Japan), and mosquito larvae were sieved and placed in vials (7.5 cm × 9.2 cm, 0.3 ml; 5-026-01; Sankoukasei Co., Osaka, Japan) before transporting them to the laboratory for enumeration and species identification. This procedure took an average of 20 min per trap and was performed in the morning from 0900 h to 1100 h. Traps were continuously operated during the study, i.e., not removed.

Tree hole, leaf axil, and artificial container larval surveys: Tree holes, leaf axils, and artificial water containers near our OTs were sampled for mosquito larvae, using a 23-cm Komagome pipette (Komagome type pipette, 3 ml, Model 6-275-03; Maruemu Co., Osaka, Japan). Mosquito larvae were transferred to a plastic container (3 cm × 6 cm, film roll case) for each habitat sampled together with a small amount of habitat water, and transported to the laboratory. Only 25 samples, out of 78, were positive for mosquito larvae.

Mosquito identification

Mosquitoes were identified according to the keys for mosquitoes in the Ryukyu Archipelago (Toma and Miyagi 1986, Tanaka 2003). Adult mosquitoes were identified to species under a stereoscopic microscope. Nonpredaceous larvae were separated by species and placed in white trays (32.2 cm × 23.1 cm × 5.2 cm, 2.8 liters; Sanbat3gou, Sanko Co.) and reared to 4th instars/adults in the laboratory (27 ± 1°C). Approximately half of the 4th instars for each species were preserved in 70% ethanol. The remaining half were reared to adults. Predaceous *Lutzia* spp. larvae were separated into individual Fuji film roll cases (3 cm × 6 cm) and reared to adults by feeding them with chironomid larvae collected from the same habitat. We did this because *Lutzia* spp. can only be reliably identified in the adult stage (Bram 1967, Toma and Miyagi 1986). Voucher specimens are preserved in the Museum of Nagasaki University Institute of Tropical Medicine and the Entomological Museum of The Ryukyus University.

Statistical analysis

We estimated species accumulation curves (SAC) for all samples collected by each method (Gotelli and Colwell 2010, Chaves et al. 2011). A flattening SAC implies a comprehensive sampling of species richness for a given method. We also used the Chao2 species richness index to estimate the total number of mosquito species (i.e., species richness) by sampling method (Chao et al. 2006, Chaves et al. 2011). For these analyses, we only used data from OTs and LTs given the low number of species collected by NSw and OS. We used the Sørensen index of dissimilarity (beta diversity) to measure the similarity in species composition (Doi and Okamura 2011) across the study sites for OTs, since it was the only method with enough sampling locations warranting a valid analysis. The Sørensen index ranges from 0 to 1, with higher values indicating a high similarity in species composition. A hierarchical cluster analysis was used to display results from the Sørensen similarity index. For the analyses, we used R (version 3.0.3; Vienna, Austria) and its vegan package (version 2.0-10) and cluster package (version 1.15.2).

RESULTS

A total of 568 adults and 10,270 larvae, belonging to 6 genera and 13 species, were collected from the 3 habitats surveyed (animal farm, forest, and lake), using 4 mosquito collection methods from April 2007 to March 2008 (Tables 1–4).

Species collection using OTs and LTs were comprehensive as indicated by the flattening SAC curves (Fig. 2). The Chao2 species richness estimate for LTs (± SE) was 11.00 ± 0.01 and for OTs was 10.00 ± 3.74. These estimates were similar to the number of species we found in the field with each method, thus supporting the comprehensiveness of the sample surveys. The heterogeneity of our sampling sites was reflected in the species composition across our sampling locations, showing 2 clusters in LTs (Fig. 3). One of those clusters had a higher species richness (7 versus 8 species) but lower mosquito abundance (n = 2,299 versus 7,512). Similarity in species from the forest sites was split between one cluster for the lake area and another for the animal farm

Table 1. Total number of mosquitoes collected by oviposition traps in 3 different habitats: animal farm, forest, and lake. Two traps were operated in each habitat.

Species	Farm		Forest		Lake		Total
	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2	
Aedes (*Stegomyia*) *albopictus*	124	74	287	27	115	252	879
Armigeres (*Armigeres*) *subalbatus*	0	0	0	0	0	56	56
Culex (*Culex*) *quinquefasciatus*	0	435	0	0	0	0	435
Cx. (*Cux.*) *tritaeniorhynchus*	18	0	0	0	0	0	18
Cx. (*Culiciomyia*) *pallidothorax*	144	316	0	287	302	0	1,049
Cx. (*Cui.*) *ryukyensis*	15	8	79	89	197	174	562
Lutzia (*Metalutzia*) *fuscana*	10	1	0	0	2	0	13
Lt. (*Mlt.*) *vorax*	71	143	38	91	152	45	540
Uranotaenia (*Pseudoficalbia*) *novobscura ryukyuana*	162	0	2,734	284	960	2,119	6,259
Total	544	977	3,138	778	1,728	2,646	9,811

(Fig. 3). This may reflect the possibility that mosquito fauna at the forest acts as a "source" for most species present in the study area, i.e., larval habitats and species are more diverse in the forest, and then adults from the forest, a habitat "source," disperse into the animal farm and lake area, thus the forest sites clustering between the more different fauna of the lake and the animal farm.

The largest number of species per genus were observed in *Culex* (5 species, $n = 2{,}397$), followed by *Aedes* (2 species, $n = 1{,}344$) and *Lutzia* (2 species, $n = 560$), *Armigeres* (1 species, $n = 108$), *Anopheles* (1 species, $n = 55$), *Uranotaenia* (1 species, $n = 6{,}347$) and *Malaya* (1 species, $n = 22$). *Uranotaenia novobscura ryukyuana* Tanaka, Mizusawa, and Saugstad was the most abundant species, probably reflecting its stable density-dependent abundance (Hoshi et al. 2014). The highest number of mosquitoes were collected using OTs ($n = 9{,}811$, 9 species), and LTs had the richest species composition among the 4 sampling methods ($n = 294$, 11 species) used. Two species not collected by LTs were *Lt. fuscana* (Weidemann), collected by OT, and *Ml. genurostris* Leicester, collected by OS (Tables 1, 2, and 4).

DISCUSSION

The mosquito fauna at our study site has not been previously described, but 74 mosquito species have been recorded on the Ryukyu Archipelago (Toma and Miyagi 1986). Okinawa Island has a total of 48 mosquito species (Toma and Miyagi 1986) and 13 (27%) of these species appeared in our samples. Our mosquito survey was done in south-central Okinawa, where the last entomological survey was conducted >40 years ago, using only LTs (Intermill 1967). A comparison between the 2 surveys showed that 8 of the 10 reported species were collected during both studies: *Ae. albopictus* (Skuse), *Ae. vexans nipponii* (Theobald), *Ar. subalbatus* (Coquillett), *Anopheles* Hyrcanus group, *Cx. bitaeniorhynchus* Giles, *Cx. quinquefasciatus* Say, *Cx. tritaeniorhynchus* Giles, and *Lt. vorax* Edwards. Nevertheless, *Cx. sitiens* (Wiedemann) and *Mansonia uniformis* (Theobald), commonly found in coastal regions but not alongside lakes (Toma and Miyagi 1986), were not collected, in contrast with the 1967 survey by the US Army (Intermill 1967).

While *Ma. uniformis* larvae are found attached to emergent vegetation in swamps and lakes

Table 2. Total number of mosquitoes collected by light traps in 2 habitats: animal farm and forest. One trap was placed in each habitat.

Species	Farm		Forest		Total (male, female)
	Male	Female	Male	Female	
Aedes (*Aedimorphus*) *vexans nipponii*	0	26	0	3	29 (0, 29)
Ae. (*Stegomyia*) *albopictus*	7	22	18	58	105 (25, 80)
Armigeres (*Armigeres*) *subalbatus*	14	26	4	0	44 (18, 26)
Anopheles (*Anopheles*) Hyrcanus group	0	2	2	0	4 (2, 2)
Culex (*Oculeomyia*) *bitaeniorhynchus*	0	1	0	0	1 (0, 1)
Cx. (*Culex*) *quinquefasciatus*	0	0	8	2	10 (8, 2)
Cx. (*Cux.*) *tritaeniorhynchus*	0	12	2	1	15 (2,13)
Cx. (*Culiciomyia*) *pallidothorax*	0	0	7	0	7 (0, 7)
Cx. (*Cui.*) *ryukyensis*	0	0	2	0	2 (0, 2)
Lutzia (*Metalutzia*) *vorax*	1	0	1	3	5 (2, 3)
Uranotaenia (*Pseudoficalbia*) *novobscura ryukyuana*	12	49	0	11	72 (12, 51)
Total individuals	34	138	44	78	294 (78, 216)

Table 3. Total mosquito species abundance collected by sweep net in 3 different habitats: animal farm, forest, and lake. Each collection was conducted for 10 min.

Species	Farm		Forest		Lake		Total (male, female)
	Male	Female	Male	Female	Male	Female	
Aedes (*Stegomyia*) *albopictus*	7	21	39	126	17	56	266 (63, 203)
Armigeres (*Armigeres*) *subalbatus*	0	0	0	3	0	5	8 (0, 8)
Total	7	21	39	129	17	61	274

(Goma 1966, Silver 2008), similar to the lake in our study area, adults of this species were not collected during our study. This may be due to a shift in agricultural production from rice to sugarcane and pineapple, which suggests that environmental changes reduced populations of this species (McDonald and Savage 1972).

Five species had not been previously collected from our study area: *Cx. pallidothorax* Theobald, *Cx. ryukyensis* Bohart, *Ur. novobscura ryukyuana*, *Lt. fuscana*, and *Ml. genurostris*. It is probable that, in addition to environmental changes that might have altered species composition, our diverse and intensive sampling strategy effectively disclosed previously unreported mosquito species in the studied area, since these species have been reported elsewhere in Okinawa (Toma and Miyagi 1986). For example, the detection of *Ml. genurostris* was only possible because of larval collections in taro leaf axils, the natural habitat for *Malaya* spp. (Toma and Miyagi 1986). In addition, the distance of approximately 1.5 km between ours and the previous sampling site could be another possible explanation for differences in species composition. Although these 2 major differences between the present and past study (i.e., study location and sampling method) made a direct comparison difficult, we believe that our study environment has a richer species composition because of the more heterogeneous environmental setting.

Ecological studies on the *Anopheles* Hyrcanus group of Okinawa (Rueda et al. 2005, Taira et al. 2012) suggest that the species we found was *An. sinsensis* Weidemann. The 1st element to make this suggestion is that, from all the species in the *Anopheles* Hyrcanus group, only *An. lesteri* Baisas and Hu and *An. sinensis* have been found in Okinawa (Toma and Miyagi 1986, Rueda et al. 2005). These 2 species can be morphologically separated based on the presence of a fringe pale spot in the wings and the presence of abundant scales on the midcoxa (Tanaka et al. 1979, Toma and Miyagi 1986), which according to DNA barcoding are highly precise and accurate identification characters unique to *An. sinensis* (Taira et al. 2012). Thus, we are certain our samples do not belong to *An. lesteri*, given the presence of both the fringe pale spot in the wings and abundant scales on the midcoxa of our samples. However, additional molecular tests (Li et al. 2005, Rueda et al. 2007) are required to see if our samples

Table 4. Total number of mosquitoes collected by sampling of tree holes, leaf axils, and artificial water containers. Surveys were conducted in the animal farm, forest, and lake habitats.

Species	Site	Date	Larvae	Egg rafts	Habitat
Aedes (*Aedimorphus*) *vexans nipponii*	Farm	Apr. 1, 2008	56	—	Ground pool
Ae. (*Stegomyia*) *albopictus*	Lake	Jun. 15, 2007	5	—	Bamboo
	Forest	Oct. 2, 2007	1	—	Garbage can
	Farm	Sep. 11, 2007	3	—	Water vase
Anopheles (*Anopheles*) Hyrcanus group	Farm	Sep. 8, 2007	25	—	Water vase
	Farm	Sep. 11, 2007	26	—	Water vase
Culex (*Culex*) *tritaeniorhynchus*	Farm	Sep. 8, 2007	97	—	Water vase
	Farm	Sep. 11, 2007	186	—	Water vase
	Farm	Feb. 22, 2008	—	7	Water vase
Cx. (*Culiciomyia*) *ryukyensis*	Forest	Apr. 1, 2008	15	—	Water vase
Lutzia (*Metalutzia*) *vorax*	Farm	Sep. 11, 2007	2	—	Water vase
	Farm	Feb. 22, 2008	—	4	Water vase
Uranotaenia (*Pseudoficalbia*) *novobscura*	Lake	Jun. 15, 2007	16	—	Bamboo
Malaya genuirostris	Lake	Oct. 13, 2007	2	—	Taro leaf axil
	Lake	Nov. 3, 2007	1	—	Taro leaf axil
	Lake	Nov. 13, 2007	2	—	Taro leaf axil
	Lake	Dec. 23, 2007	4	—	Taro leaf axil
	Lake	Jan. 24, 2008	6	—	Taro leaf axil
	Lake	Jan. 24, 2008	6	—	Taro leaf axil
	Forest	Oct. 1, 2007	6	—	Taro leaf axil

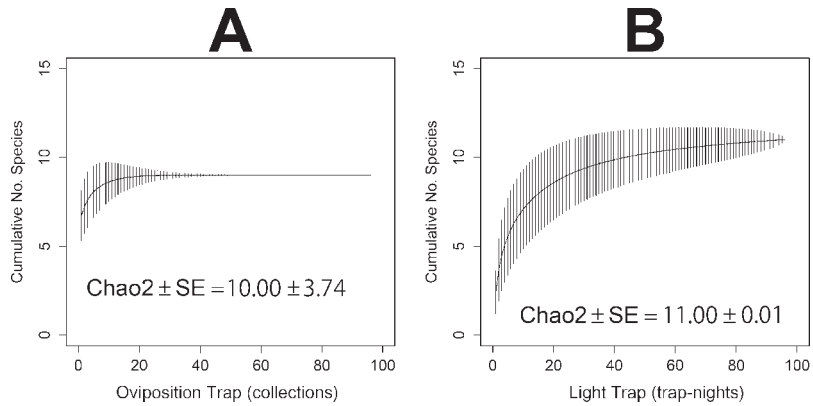

Fig. 2. Mosquito species richness in the south-central part of Okinawa Island. (A) Species accumulation curve (SAC) using ovitraps. The vertical axis shows cumulative number of species and the horizontal axis shows the number of ovitrap collections. (B) SAC using light traps. The vertical axis shows cumulative number of species and the horizontal axis shows the number of collections using light traps.

belong to *An. belenrae* Rueda, *An. kleini* Rueda, or *An. pullus* Yamada, species morphologically indistinguishable from *An. sinensis* (Li et al. 2005), which have not been found in Okinawa (Rueda et al. 2005, Taira et al. 2012).

We collected 6 species that are vectors of medically important pathogens: *Ae. albopictus* (dengue and chikungunya), *Anopheles* Hyrcanus group (malaria), *Ar. subalbatus* (filariasis), *Cx. quinquefasciatus* (West Nile, filariasis), *Cx. tritaeniorhynchus* (Japanese encephalitis), and *Cx. bitaeniorhynchus* (filariasis). West Nile, dengue, and chikungunya viruses are potential health threats to Japan (Kasai et al. 2007, Mizuno et al. 2011, Takasaki 2011). The Ryukyu Archipelago, as a subtropical region, is especially vulnerable to vector-borne disease transmission, as has been historically recorded (Miyagi et al. 1996). Therefore, monitoring mosquito biodiversity is an important task in this area. Finally, our results suggest that although LTs sample most of the species in a mosquito community (Brown et al. 2008), only the combination of several sampling methods provides a comprehensive description of a local mosquito faunal composition.

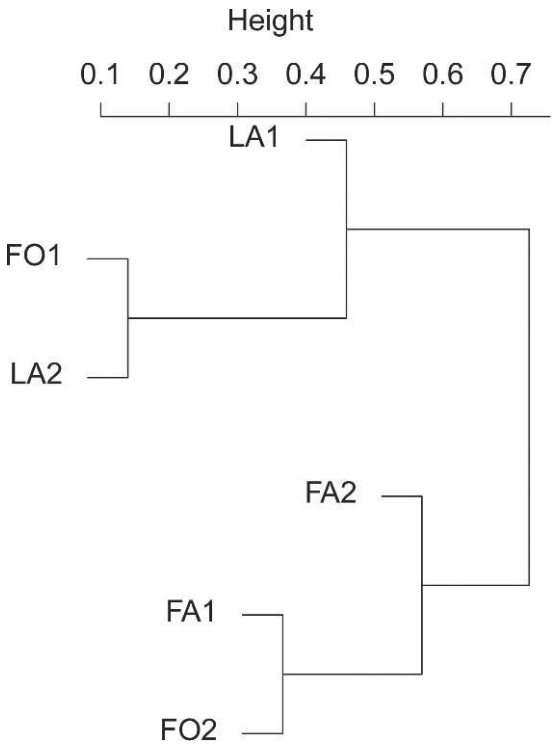

Fig. 3. The multisite Sørensen index of dissimilarity (beta diversity) for ovitraps. In the plot height indicates the difference between sites, i.e., the lower the height the more similar are species between each pair of sites. FO, forest; FA, animal farm; LA, lake.

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