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Original Article

Spatial Distribution and Pyrethroid Susceptibility of Mosquito Larvae Collected from Catch Basins in Parks in Nagasaki City, Nagasaki, Japan

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SUMMARY: We investigated the spatial distribution and pyrethroid susceptibility of the mosquito larvae belonging to *Aedes albopictus* and *Culex pipiens* group in catch basins located in parks in Nagasaki city, Nagasaki, Japan. Among the 308 parks located in the central regions of the city, 194 were investigated. *Cx. pipiens* group larvae were collected from 31 sites; larvae of *Ae. albopictus*, from 34 sites. The *Cx. pipiens* group larvae were identified by PCR: 93.4% were found to belong to *Cx. pipiens pallens*, and 0.9%, to *Cx. pipiens* form *molestus*. A bioassay was performed by observing the knockdown of larvae during 30-min exposures to 0.4- and 0.1-ppm solutions of *d*-allethrin. High tolerance to *d*-allethrin (susceptibility index = 36) was observed in only 1 colony of *Cx. pipiens pallens* across 24 sites. On the other hand, *Ae. albopictus* showed high tolerance (susceptibility index > 30) in 8 of 22 sites; this indicated that *Ae. albopictus* populations tolerant to pyrethroids were spreading widely in Nagasaki city. The organized and massive larvicidal treatment of graveyard containers with DDT in the 1950s was thought to be one of the main causes for the development of pyrethroid resistance in *Ae. albopictus*.

INTRODUCTION

Dengue fever and dengue hemorrhagic fever have recently been categorized in the list of "neglected tropical diseases"; they have not received adequate attention although they are still among the most important diseases in tropical and subtropical regions. The main vectors of these diseases are *Aedes aegypti* (L.) and *Ae. albopictus* (Skuse). *Ae. aegypti* is distributed in tropical regions across the world, such as South and Central America and South and Southeast Asia. It has been a target of vector control programs because of its medical importance as a vector of tropical diseases such as yellow fever and dengue fever. *Ae. aegypti* has often been reported to show insecticide resistance. *Ae. albopictus* is thought to have originated in the eastern hemisphere. Its distribution expanded to the Hawaiian and South Pacific islands in the last 20 centuries (1). It was first recorded in the southeastern region of North America in the 1980s (2) and is, at present, a common species in the central and southern regions of North America (3). The invasion of this species into South and Central America, Oceania, and Africa was confirmed by the end of the 1980s. The first report of the species in Europe was in Albania in 1979; since then, it has been detected in other countries such as Bosnia and Herzegovina, Croatia, Greece, France, Italy, Montenegro, the Netherlands, Serbia, Slovenia, Spain, and Switzerland (4). The shipment of used tires is one of the major causes of the expansion of *Ae. albopictus* throughout the world (2). Intrinsic aspects of *Ae. albopictus* such as high ecological plasticity and strong competitive aptitude and extrinsic factors such as globalization have caused the change in vectors, and this species has become an important vector of Chikungunya fever (5). There seem to be fewer reports on the insecticide resistance of *Ae. albopictus* than on that of *Ae. aegypti*; this indicates that insecticide resistance is not a serious concern in the case of this species, since the above expansions have taken place in recent years. Many studies have reported the insecticide resistance of *Culex pipiens pallens* Coquillet and *Cx. pipiens* form *molestus* Forskal in areas where these species are commonly found, especially China and Japan (6–11). Kasai et al. reported that larvae of *Cx. pipiens pallens* and *Cx. pipiens* form *molestus* collected from several places in Japan showed high resistance to etofenprox, a unique pyrethroid that can be administered in aqueous environments (10). The use of such pyrethroids for larval control will enhance their resistance and might severely reduce the effectiveness of spatial repellent devices such as mosquito coils and other popular and long-standing formulations using pyrethroids belonging to knockdown agents, such as allethrin, pyrethrin, and prallethrin. In particular, *d*-allethrin continues to be used in such devices, without any operational difficulties or problems of resistance. Pyrethroids are used as spatial repellents for preventing mosquito bites and are believed to be biorational agents since they do not kill the affected insects and exert no selection pressure on insect populations; they therefore induce minimum physiological resistance.

In order to effectively manage pyrethroid resistance, it is essential to establish a feasible insecticide management system and a regular system for monitoring pyrethroid susceptibility. We performed a pilot study for monitoring pyrethroid resistance in mosquitoes by investigating the distribution of mosquito larvae and their susceptibility to pyrethroids in the catch basins located in parks in Nagasaki city, Nagasaki, Japan.

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MATERIALS AND METHODS

Collection of mosquito larvae: Mosquito larvae were collected from parks located in the east-central, west-central, north-central, and south-central regions of Nagasaki city from June 12 to September 5, 2007. All catch basins in the parks were investigated unless they were locked or inaccessible, and mosquito larvae were collected by a metal dipper (13 cm in diameter). The number of parks listed in the above regions is 308 according to the homepage of Nagasaki city (http://www1.city.nagasaki.nagasaki.jp/kouen/search/retrieval.php [in Japanese]). We obtained prior permission for this investigation from the Road and Park section of the Nagasaki City Office. The geographical positions (determined using a global positioning system [GPS]) of the parks and the presence of catch basins and water works were recorded. The larvae collected were brought to the laboratory for bioassay and species identification. Identification of larvae was done according to the identification keys by Tanaka et al. (12).

Simplified knockdown bioassay and species identification: The bioassay for the detection of susceptibility to knockdown was carried out on the day of collection using the mosquito larvae obtained from the collection sites from which we could procure an adequate number of samples. The larvae collected from each site were identified on the day of collection. The fourth-instar larvae of the *Cx. pipiens* group, which occasionally comprised a mixed population of *Cx. pipiens pallens* and *Cx. pipiens* form *molestus*, and the *Ae. albopictus* larvae were subjected to the susceptibility test. Among the samples collected, the eggs or larvae in early instar stages were reared in laboratory conditions (25°C, 60% relative humidity) until they fully matured to the fourth-instar stage. A simplified knockdown bioassay was performed according to the methods described by Kawada et al. (13). Each larva was placed in a glass vial containing 20 ml of water. An emulsifiable concentrate of 90% *d*-T_{80}-allethrin was diluted with water to obtain a 250-ppm solution. After the larvae were released to the vials, 32 or 8 μl of the solution was added to each vial to obtain a concentration of 0.4 or 0.1 ppm, respectively. Twenty larvae from each site were used for each concentration regime. Knockdown of the larvae was observed for 30 min. The larvae that sank to the bottom of the glass vial and could not swim or float or were paralyzed were judged to be knocked down, and the time to knockdown was recorded for each larva. After the test, each larva was placed in a 1.5-ml plastic vial containing an ethanol solution until identification. After identification, the knockdown data were summarized for each mosquito species. The median knockdown times (KT_{50}s), i.e., the time required for 50% knockdown, were scored on a 6-point scale as follows: 1, <5 min; 2, 5–10 min; 3, 10–15 min; 4, 15–20 min; 5, 20–30 min; and 6, >30 min. The susceptibility index was calculated as the product of the scores at 0.1 and 0.4 ppm. Thus, mosquitoes with a susceptibility index of 1 were considered to be the most susceptible and those with a susceptibility index of 36 were considered to be the least susceptible to *d*-allethrin.

The species belonging to the *Cx. pipiens* group were identified using the PCR method described by Kasai et al. (14). After the bioassay, the mosquito samples (larvae immersed in ethanol) were lightly dried on a paper towel and then placed in a 1.5-ml PCR-reaction tube. The sample was homogenized in a mixture containing an extraction solution (40 μl) and a tissue-preparation solution (10 μl) (REDExtract-N-Amp™ Tissue PCR Kit; Sigma, St. Louis, Mo., USA) for extracting DNA. The solution was heated at 95°C for 3 min and then neutralized. Fragment amplification was carried out using 4 primers (F1457, GAGGAGATGTGGAATCCCAA; B1246s, TGGAGCCTCCTCTTCACGG; ACEpip2, GTGGAAACG CATGATACCAG; ACEpall7, CTCAGTTAGTTCTCATATT CATGCG). The PCR mixture contained 4 μl of REDExtract-N-Amp™ ReadyMix (Sigma), 0.5 μM of each primer, and 1 μl of the DNA template in a total volume of 10 μl. PCR was performed under the following conditions: 95°C for 5 min and 40 cycles of 95°C for 30 s, 63°C for 60 s, 72°C for 60 s; and 72°C for 5 min. Electrophoresis was performed and the samples showing bands of 280 bp and 500 bp were identified as belonging to *Cx. pipiens pallens* and *Cx. pipiens* form *molestus*, respectively.

Determination of the collection-site locations and analysis of the geographical and environmental information: We used an aerial photograph (GEOTIFF format; acquired on March 30 and 31 at 50-cm ground resolution; NTT-Neomeit Corp., Osaka, Japan) and a spatial data framework (Digital Map 2500; Geographical Survey Institute, Tokyo, Japan) of the center of Nagasaki city for determining the location of the collection sites. The year of establishment and the areas of the parks were verified from the homepage of Nagasaki city (http://www1.city.nagasaki.nagasaki.jp/kouen/search/retrieval.php [in Japanese]). Data on the population in the towns where the parks located were obtained from the statistical information on Nagasaki city for 2006 (http://www1.city.nagasaki.nagasaki.jp/toukei_data/ [in Japanese]). The relationships between the distribution of mosquito larvae and the geographical and environmental factors were statistically determined using ArcGIS 9.2 (ESRI Japan, Tokyo, Japan). We examined the relationships between the variables by stepwise multinomial logistic regression analyses using the presence of larvae of the *Cx. pipiens* group (*Cx. pipiens pallens* or *Cx. pipiens* form *molestus*) and *Ae. albopictus* in catch basins (assigning scores of 1 for presence, 0 for absence in catch basins) as the dependent variable, while altitude, presence of water works (assigning scores of 1 for presence, 0 for absence in catch basins), and population densities of the towns where parks are located as the independent variables. Multinomial logistic regression analysis was executed with backward steps; at each step a parameter was excluded according to a high *P*-value ($P > 0.05$). All statistical tests were conducted using JMP 7.0 (SAS Institute Japan, Tokyo, Japan).

RESULTS AND DISCUSSION

In the present study, we investigated 194 of the 308 parks in the selected region (Fig. 1). The average area and number of years after establishment of the parks were 11,025 m^2 (SD 74302; range, 217–2,140,000 m^2) and 30.3 years (SD 13.2; range, <1–58 years), respectively. Larvae belonging to the *Cx. pipiens* group were collected from 31 sites, and those belonging to *Ae. albopictus*, from 34 sites. These 2 species were found to cohabit in 14 sites (Fig. 2). Larvae belonging to *Ochlerotatus japonicus* (Theobald), *Cx. pallidothorax* Theobald, *Cx. sasai* Kano, and *Lutzia vorax* Edwards were also collected, in addition to the other larvae, from catch basins. Among the 768 larvae belonging to the *Cx. pipiens* group that were used for the bioassay, 717 were identified as belonging to *Cx. pipiens pallens* (93.4%), and 7, as belonging to *Cx. pipiens* form *molestus* (0.9%); 44 (5.7%) could not be identified because the samples deteriorated. Larvae belong-

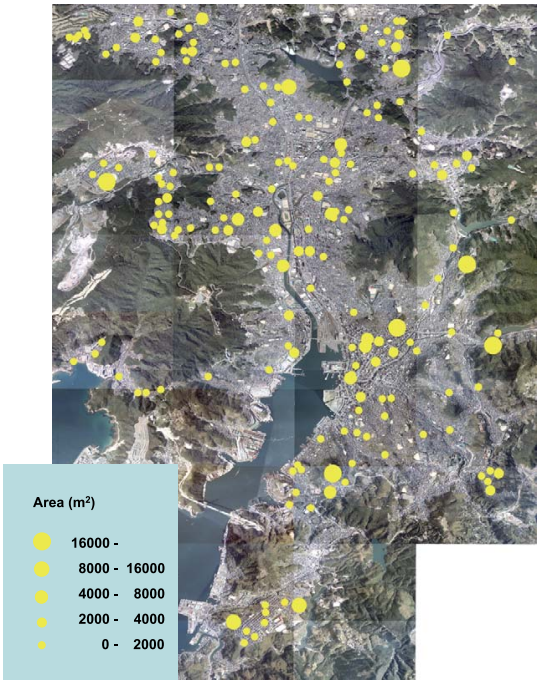

Fig. 1. The location and area of the parks investigated in Nagasaki city. The size of the circles indicate relative area size of the parks.

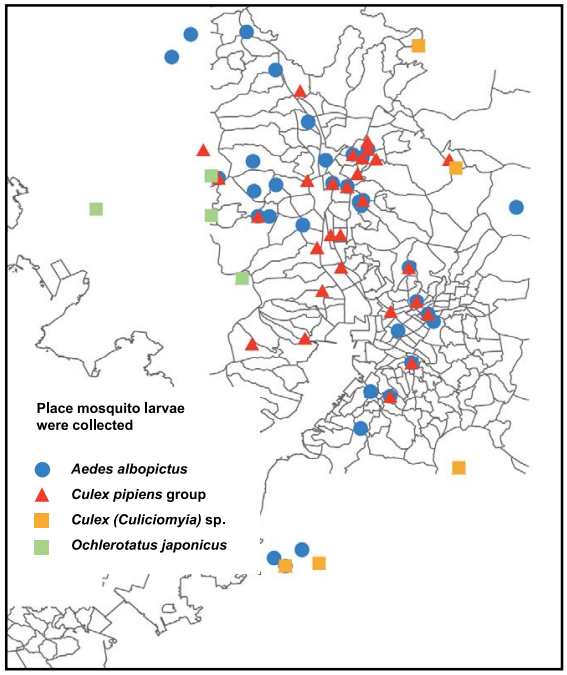

Fig. 2. The location of the parks where mosquito larvae were collected.

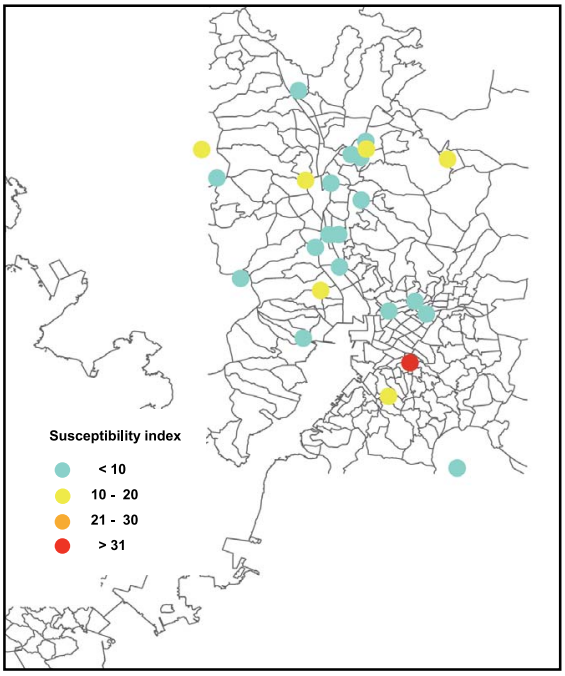

Fig. 3. The location of the parks the bioassay for *Culex pipiens pallens* was performed and the susceptibility index observed at each site.

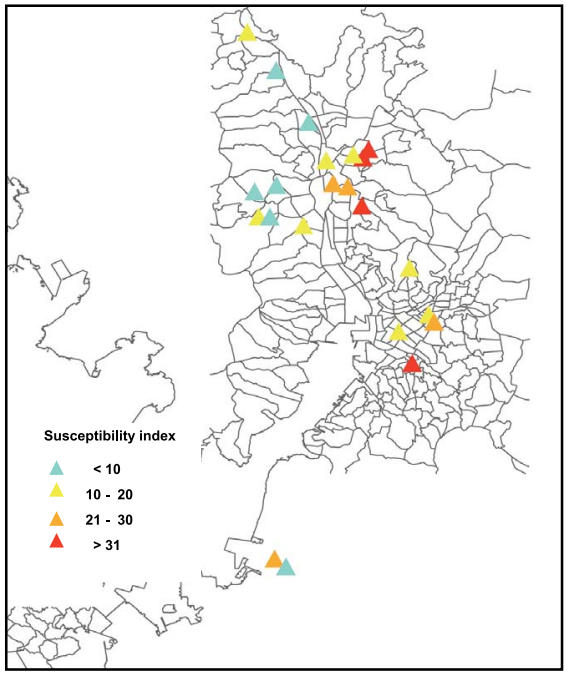

Fig. 4. The location of the parks the bioassay for *Aedes albopictus* was performed and the susceptibility index observed at each site.

ing to *Cx. pipiens* form *molestus* were collected from only 2 sites; this indicates that it is a minor species in catch basins. A stepwise multinomial logistic regression analysis revealed that the presence of water works affected the presence of *Cx. pipiens* group. For the presence of *Ae. albopictus*, the presence of water works and altitude were significant effects (Table 1). In contrast, population density of the towns where parks located was excluded from the model. This result suggests that the presence of water works might provide good breeding conditions for the 2 species of mosquito larvae. In addition, the presence of *Ae. albopictus* increased with altitude, indicating that this species prefer suburban areas to urbanized areas. The distribution of mosquitoes in Nagasaki city cannot be simply discussed by the results in the present paper, since there are variety of breeding sites in the study area. Further studies on the seasonal changes in mosquito populations and the investigation of other breeding sites in parks and their surrounding areas will be necessary for a more precise analysis.

The susceptibility indices of larvae belonging to *Cx. pipiens pallens* and *Ae. albopictus* are shown in Table 2, and their distribution patterns are shown in Figs. 3 and 4, respectively.

Table 1. Multinomial logistic regression results for the presence of mosquito in catch basins in parks in Nagasaki city

Mosquito species	Source	Parameter estimate	SE	χ^2	*P*
Culex pipiens pallens	Intercept	1.3711	0.3554	14.88	0.0001
	Altitude	0.0046	0.0035	1.65	0.1995
	Presence of water works	**0.6372**	**0.2175**	**8.59**	**0.0034**
	Overall fit			11.29	0.0035
Aedes albopictus	Intercept	0.9605	0.3375	8.10	0.0044
	Altitude	**0.0078**	**0.0037**	**4.47**	**0.0346**
	Presence of water works	**0.4735**	**0.2027**	**5.46**	**0.0195**
	Overall fit			11.15	0.0038

Bold typeface factors are significant at overall $\alpha = 0.05$.

Table 2. Geographical information, established year, area of the parks and susceptibility indices of mosquito larvae in Nagasaki city

Park (ID No.)	Latitude	Longitude	Elevation (m)	Established year	Area (m^2)	Susceptibility index	
						Ae. albopictus	*Culex pipiens pallens*
E001	32.7533	129.8807	55.9	1959	29,041	–	2
E003	32.7513	129.8755	22.5	1949	5,074	–	2
E007	32.7507	129.8830	24.4	1949	1,447	12	2
E010	32.7473	129.8770	27.3	1965	3,142	12	–
E017	32.7600	129.8792	177.2	1972	39,306	18	–
E022	32.7492	129.8841	22.7	1974	714	30	–
E034	32.7818	129.8873	203.9	1981	843	–	12
Gubiro[1)]	32.7725	129.8696	54.4	–	–	36	–
ITM[1)]	32.7736	129.8700	45.1	–	–	–	5
N001	32.7667	129.8635	12.4	1951	2,493	–	6
N002	32.7667	129.8654	11.7	1950	2,480	–	8
N003	32.7686	129.8579	39.1	1955	993	12	–
N006	32.7776	129.8588	16.5	1955	1,123	–	12
N007	32.7817	129.8625	20.3	1954	955	12	–
N009	32.7764	129.8668	40.3	1951	3,765	30	–
N0102	32.7770	129.8639	37.9	1951	156,700	30	10
N011	32.7602	129.8656	19.8	1958	1,617	–	6
N014	32.7767	129.8525	29.9	1962	672	6	–
N016	32.7822	129.8698	53.2	1963	2,732	36	6
N017	32.7704	129.8489	48.2	1965	1,858	18	–
N019	32.7828	129.8679	35.9	1965	1,781	12	8
N020	32.7854	129.8709	34.7	1965	6,849	–	3
N021	32.7704	129.8512	53.7	1965	2,200	2	–
N024	32.7838	129.8379	151.1	1971	1,204	–	12
N026	32.7839	129.8710	51.3	1972	1,519	36	12
N033	32.7781	129.8410	180.1	1977	1,234	–	3
N051	32.7754	129.8481	56.1	1984	285	6	–
O008	32.7016	129.8521	78.0	1987	12,861	30	–
O012	32.7000	129.8544	106.4	1987	1,536	6	–
S001	32.7409	129.8797	30.6	1957	1,209	36	36
S004	32.7197	129.8892	307.0	1967	90,000	–	3
S017	32.7342	129.8754	65.0	1975	854	–	12
U005	32.7893	129.8589	49.4	1969	896	6	–
U039	32.7998	129.8524	54.7	1980	194	6	–
U044	32.8075	129.8466	75.3	1981	4,618	12	–
U098	32.7956	129.8573	87.4	NA	NA	–	2
W001	32.7459	129.8583	14.1	1950	1,916	–	6
W0041	32.7579	129.8457	245.5	1951	927,600	–	4
W005	32.7555	129.8618	34.3	1953	2,196	–	12
W006	32.7641	129.8608	13.6	1952	7,687	–	4

[1)]: These two sites are not park but the places in the campus of Faculty of Medicine, Nagasaki University, Nagasaki, Japan.
NA, not available.

The larvae subjected to bioassay included *Cx. pipiens pallens* larvae from 24 sites (768 individuals) and *Ae. albopictus* larvae from 22 sites (846 individuals). The susceptibility data for *Cx. pipiens* form *molestus* were not analyzed since the sample number was not sufficient. With regard to *Cx. pipiens pallens*, only 1 population collected from Maruyama Park (S001) showed a high susceptibility index (36), thus indicating that this population had a high tolerance to pyrethroids. However, the susceptibility indices of this species in all the other sites were low (less than 12). On the other hand, with regard to *Ae. albopictus*, 8 populations collected from 22 sites showed high susceptibility indices (>30), thus indicating the existence of populations tolerant to pyrethroids across a wide area.

This study highlights the wide distribution of pyrethroid tolerance in *Ae. albopictus* in Nagasaki city. The history of mosquito control in Nagasaki city as obtained by a review of the literature and interviews with officials from the Nagasaki

Table 3. History of insecticide treatment for controlling mosquitoes in Nagasaki city

Year	Event
1952	Nationwide movement for establishment of healthy and clean town without flies and mosquitoes.
	Unscheduled treatment of DDT 5% oil to the breeding places where mosquito larvae are recognized.
1953	Fortnightly regular treatment of DDT 5% oil to the breeding places where mosquito larvae are recognized.
1954	Fortnightly regular treatment of BHC 3% oil to the breeding places where mosquito larvae are prospected to be recognized.
	Treatment of DDT (100 ppm) into containers in all cemeteries at the rate of 3 to 4 times per year (12).
1962	Investigation study of residual activity of DDT in containers in cemeteries (14).
1973	Ban on DDT treatment.
1974	Study on the availability of diazinon and temephos (14).
	Start of treatment of temephos into containers in cemeteries.
1985	First finding of DDT resistance in *Ae. albopictus* colony collected from the containers in cemeteries (47).
1987–	Start of treatment of fenthion 5% EC into containers in cemeteries.
	Treatment of fenitrothion, diazinon, and fenthion EC to roadside gutters.
	Treatment of DDVP oil to underground channels.
2000	Cancellation of insecticide treatment according to the revision of the Infectious Disease Prevention Law.

City Office is shown in Table 3. In the first half of the 1950s, an oil formulation of DDT [4,4′-(2,2,2-trichloroethane-1,1-diyl)bis(chlorobenzene)] or a dust formulation of BHC [1,2,3,4,5,6-hexachlorocyclohexane] was sprayed at the peripheries of graveyards or into flower vases on gravestones (15,16). Following the ban of DDT use in the 1970s, organophosphates such as diazinon, temephos, fenthion, and fenitrothion were used instead of DDT (17), and since then, no pyrethroid insecticide has been used for mosquito control. Moreover, an organized system for mosquito control in Nagasaki city has been discontinued according to the revision of the Infectious Disease Prevention Law in 2000. The pyrethroid resistance in *Ae. albopictus* populations in Nagasaki city might therefore be attributable to the massive and organized treatment with DDT in the 1950s as a larval control measure, since the common target site for pyrethroids and DDT is the same voltage-gated sodium channel and cross-resistance between them often occurs with the target-site mechanisms (38). Kawada et al. reported widespread pyrethroid resistance in *Ae. aegypti* in the southern regions of Vietnam using the same evaluation measures as those used in the present study (13), and they indicated the possibility that the above resistance was strongly related to the massive use of pyrethroids and DDT for malaria control. Kawada et al. also reported a recently discovered point mutation in the voltage-gated sodium channel (F1269C) associated with pyrethroid resistance in these resistant populations (18). In the above case, a strong selection pressure may be exerted by insecticides on adults since no chemical control in the larval breeding places, such as water jars and cisterns, is permitted in Vietnam. In contrast, in the present case, selection pressure may have been exerted by DDT on *Ae. albopictus* larvae. Further studies should be performed on the mechanism underlying pyrethroid resistance, the relationship between the resistance mechanisms of DDT and pyrethroid, and the difference in the resistance mechanisms in adults and larvae.

The history of insecticide resistance is closely linked to the history of insecticide development. Before the 1980s, DDT treatment was the most common method for controlling *Ae. aegypti*, and many studies on DDT resistance were reported worldwide (19–24). A number of studies have reported the resistance of *Ae. aegypti* to organophosphate and carbamate insecticides from the 1980s to the 2000s (25–29), and the number of reports on pyrethroid resistance in this species increased in the 1990s (30–35); they have been a mainstream of the resistance studies in 2000s (13,18,36–49). On the other hand, there are fewer reports on the insecticide resistance of *Ae. albopictus* than on that of *Ae. aegypti*, mainly because of the relatively recent invasion of this species. Nevertheless, it is noteworthy that several studies on the DDT resistance of *Ae. albopictus* were reported by Japanese researchers before the 1980s (19,50,51). In the latter half of the 1980s, organophosphate resistance in *Ae. albopictus* was reported in North America (26,52); this indicated frequent treatment with organophosphate insecticides for controlling this new invader. Although the number of reports on the pyrethroid susceptibility of *Ae. albopictus* increased in the 2000s, there has been no report on the pyrethroid resistance of this species to date (13,37,41,44,46,53–55). Pyrethroid resistance in *Ae. albopictus* is not a serious problem at present. This might be partly because of their ecological traits: adults have lower anthropophily and their breeding sites are more diverse than those of *Ae. aegypti*; both of these factors as well as extrinsic factors such as lack of surveillance and poor control (4) reduce the chances of exposure to insecticides. So-called "carpet bombing" of breeding sites with insecticides, however, will accelerate the development of resistance. Use of insecticides having the same mode of action as adulticides will aggravate the situation.

Pyrethroids provide one of the most promising countermeasures for controlling mosquitoes. Pyrethroid resistance, therefore, will be a major hindrance to mosquito control programs, since at present, there are no suitable chemical substitutes for pyrethroids. A regular monitoring system for insecticide susceptibility, including a simple biochemical evaluation system that can elucidate the modes of resistance at the mosquito population level, should be a priority.

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