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Distribution, abundance and blood-feeding behavior of *Aedes albopictus* (Diptera: Culicidae) in Korea

국내 흰줄숲모기의 분포, 발생 및 흡혈행동 연구

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February, 2017

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ABSTRACT

Aedes albopictus is an invasive mosquito that can be found in all continents. This species, considered as a secondary vector of Dengue virus, has recently been suggested to play a role in the transmission of Zika virus in several countries bordering Brazil. This mosquito originated in the forests of Southeast Asia. However, this species has spread throughout the world by increased intercontinental trade during the 20th century.

In this study, I surveyed the distribution, abundance and main micro habitats of *Ae. albopictus* in Korea. *Ae. alobpictus* accounted for 4% of a total of 99,625 mosquitoes examined. *Ae. albopictus* was first collected in May, its number increased slowly throughout August and reached the greatest number in September, then

followed by rapid decrease in number during October. The larval habitats were found within 500 m distance from the bamboo forest. Most larvae were found mainly in tires (44.4% in Iksan-si, 63.6% in Damyang-gun) and artificial containers (55.6% in Iksan-si, 45.5% in Damyang-gun) including a plastic wash basin, bowl, can, styrofoam-box within the range of adult mosquito activity.

Ae. albopictus activity began around sunrise with peaks in late morning (08:00–09:00) and early evening (16:00−19:00) and ended with sunset. Light intensity appears to be a major factor affecting mosquito activity: if light intensity is over some threshold, *Ae. albopictus* activity decreased.

The main bloodmeal source of *Ae. albopictus* was mammals (71%) followed by birds (26%), amphibians (2%) and fish (1%). The main mammalian blood source was human (86%). This results showed that *Ae. albopictus* feed exclusively on human, representing the potential of this mosquito as a major vector of dengue virus, once this virus becomes domestic.

In flavivirus detection, no virus was detected in the specimens of *Ae. albopictus*, but a total of six Japanese encephalitis virus (JEV)-positive pools were detected from *Culex orientalis* and *Cx. pipiens* except *Cx tritaeniorhynchus*, the main vector mosquitos of JEV. All the detected JEVs were identified as genotype V by phylogenetic analysis of the envelope gene. Our findings confirmed that a new

genotype of JEV was introduced into Korea and suggested that the two mosquito species may play a role in JEV transmission.

To investigate the possibility of using *Wolbachia*, as one of biological control strategy, I investigated the distribution of *Wolbachia* infection in *Ae. albopictus* according to geographical distribution in Korea. Over 99% of the collected mosquitoes harbored *Wolbachia*, and the sequence homologies of the WSP gene showed more than 98% similarity within the mosquito species. *Ae. albopictus* was found to be infected with two *Wolbachia* strains, *w*AlbA and *w*AlbB. Regional distribution analysis indicated that the *w*AlbA strain of *Wolbachia* showed more than 98% sequence similarity among *Ae. albopictus* collected from different regions. This study would support further functional and biocontrol-related studies of *Wolbachia*.

Additionally, I also investigated the mosquito species composition by employing the DNA barcoding method based on the mitochondrial cytochrome c oxidase subunit I (mtCOI) gene sequence. To this end, mtCOI genes from individual mosquitoes of 25 species were sequenced, and their phylogenetic relationship was analyzed. Phylogenetic analysis showed that most mosquito species were clustered according to morphological characteristics, except for certain *Anopheles* species. DNA barcoding using mtCOI genes successfully identified mosquito species, and it can be used as an effective technique to complement morphological identification.

Key words: *Aedes albopictus*, distribution, abundance, seasonal prevalence, habitats, breeding sites, diel activity, bloodmeal source, Japanese encephalitis virus (JEV), *Wolbachia*, *w*AlbA, *w*AlbB

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INTRODUCTION

The Asian tiger mosquito, *Aedes albopictus,* is characterized by black and white stripes on its legs and body as first described as “the banded mosquito of Bangla” by Skuse (1894) (Gratz, 2004). This species is also an invasive species that can be found in Asia, Europe, North America, South America, and Africa (Paupy et al., 2009). The spread of this species has occurred during recent thirty years by human activities (Lounibos, 2002). As an important vector of dengue fever (DENV), chikungunya (CHIKV), yellow fever, and also Zika in recent, *Ae. albopictus* has considered as a global public health threat (Rezza, 2012; Schuffenecker et al., 2006).

As *Ae. albopictus* is usually found at the edges of forests and breeds in natural habitats such as tree holes and bamboo stumps, it used to be considered as a rural mosquito (Higa, 2011). However, *Ae. albopictus* has adopted to urban environments with ecological plasticity. *Ae. albopictus* has exploited alternative blood sources such as human and domestic animals and adaped to use artificial container such as waste tires and plastic water containers as their breeding habitats (Caputo et al., 2012). This process of “domestication” has leaded *Ae. albopictus* to become a main mosquito species occuring in both rural and suburban areas (Paupy et al., 2009).

Ae. albopictus is also known as a daytime mosquito. Unlike other mosquitoes, this species prefers to bite in the early morning and late afternoon (Estrad-Franco,

1995; Hawley, 1988). Depending upon region and biotype, they showed different active peak time, but for the most part, they rest during the night hours.

Ae. albopictus not only prefers to bite mammals including humans, but also birds, reptiles, and amphibians (Hawley, 1988). Due to its opportunistic behavior, *Ae. albopictus* was previously considered as having limited opportunity to acquire or transmit arboviruses such as dengue virus (Richards et al., 2006). While the preferences of *Ae. albopictus* for animals versus humans were shown to be highly variable according to the geographic origin of mosquito population, when given the choice between human and animal bait, *Ae. albopictus* chose the human priory (Paupy et al., 2009). These traits of feeding behavior make *Ae. albopictus* particularly an efficient bridge vector that can transmit pathogens from animal-to-animal and from animal-to-human.

The ability of *Ae. albopictus* transmitting disease as a vector mosquito has been experimentally demonstrated for a wide range of arboviruses. *Ae. albopictus* have shown to be capable of transmitting 26 viruses that belong to the *Flaviviridae* (genus *Flavivirus*), *Togaviridae* (genus *Alphavirus*), *Bunyaviridae* (genus *Bunyavirus* and *Phlebovirus*), *Reoviridae* (genus *Orbivirus*) and *Nodaviridae* (genus *Picornavirus*) family. In addition, viral isolation or detection in the field conditions has been reported, including six flaviviruses (DENV-1, -2, -3, -4, *West Nile virus*, *Japanese encephalitis virus*), two alphaviruses (CHIKV and *Eastern equine encephalitis virus*)

and six bunyaviruses (Potosi virus, Tensaw virus, Keystone virus, La Crosse virus and Jamestown Canyon virus) (Paupy et al., 2009).

The primary vector of the DENV is known to be *Ae. aegypti*, which is present in most areas where the disease is endemic, whereas *Ae. albopictus* has been considered as a secondary vector, particularly in the areas where both species co-exist. However, the report of major epidemics in the areas where *Ae. albopictus* occurs alone demonstrates the potential role of *Ae. albopoictus* in DENV transmission and outbreaks (Kutsuna et al., 2015b).

Because there are no vaccines or drugs against DENV and CHIKV, vector control is considered as a principal method for the prevention and control of these disease. The control of *Ae. albopictus* has conventionally relied on the reduction of the larval source by cleaning the water-holding containers that serve as larval habitats and by using larvicides in breeding sites (Hawley, 1988). Recently, a novel form of biological control strategy has been implemented to control mosquitoes. *Wolbachia* is a maternally transmitted bacterial symbiont of many insects (Werren, 1997; Zug and Hammerstein, 2012). Many *Wolbachia* species manipulate host reproductive systems, leading to male-killing, cytoplasmic incompatibility (CI), parthenogenesis, and the feminization of genetic males, with large impacts on host ecology and evolution in arthropod (Harris and Braig, 2003; Iturbe-Ormaetxe and O'Neill, 2007; Walker et al., 2011; Werren et al., 1995). *Wolbachia* are particularly prevalent in the female germline throuthout adulthood, ensuring high-fidelity *Wolbachia* transmission to the

eggs produced by infected females (Dedeine et al., 2005; Dedeine et al., 2001; Serbus et al., 2008). Many studies have focused on characterization of the CI phenotypes of *Wolbachia*; endosymbiont *Wolbachia* shows similar phylogenetic diversity between bacterial and their hosts (Werren et al., 2008). Thus, investigation of *Wolbachia* infection status and the identification of genetic diversity in natural populations can be crucial for *Wolbachia*-based vector control strategies (Werren et al., 2008; Werren et al., 1995).

Recently, research on *Wolbachia* has focused on the control of the population of disease vector mosquitoes belonging to the genera *Aedes* and *Anopheles*, which carry dengue fever and malaria, respectively (Cook et al., 2008; Kitrayapong et al., 2002; Lambrechts et al., 2006).

In this study, to evaluate the distribution, abundance and the main micro habitats of *Ae. albopictus* in Korea, adult mosquitoes were collected on a national scale. In addition, mosquito larvae and adults were collected in and around two regions having well-developed bamboo forest, which is the major habitats of *Ae. albopictus*. As a potential flavivirus vector mosquito, *Ae. albopictus* has unique behavioral features of host search, host reference and vectoral capacity. In this study, I also investigated when this mosquito searches its host, what host it prefers, and what kinds of viruses it carries. Finally, I investigated the distribution of *Wolbachia* in *Ae. albopictus* according to geographical distribution in an attempt to evaluate the possibility of using *Wolbachia* as a potential control agent for vector mosquito.

Additionally, to facilitate the identification of mosquitoes, DNA barcoding was attempted. Mitochondrial cytochrome oxidase subunit I (mtCOI) genes from individual mosquitoes were sequenced and phylogentic analysis was conducted.

Chapter I.

Abundance and distribution of *Aedes albopictus*

I-1. Distribution and Abundance of *Aedes albopictus* in Korea

Abstract

Aedes albopictus is an invasive mosquito that can be found in all continents. This species, considered as a secondary vector of Dengue virus, has recently been suggested to play a role in the transmission of Zika virus in several countries bordering Brazil. In this study, I surveyed the distribution, abundance and main micro habitats of *Ae. albopictus* in Korea. Mosquitoes were collected in 10 sites using the BG-Sentinel and black light traps from April to October in 2015. Also, I collected adult and larval mosquitoes in two villages (Iksan-si and Damyang-gun) adjascent to a huge bamboo forest, which is considered as a main habitat in Korea. As results, the composition of *Ae. albpictus* was 4% out of total 99,625 mosquitoes. *Ae. albopictus* was first collected in May, slowly increased throughout August, and reached a peak in September, then followed by rapid decrease in number during October. Most larvae were found mainly in tires (44.4% in Icksan-sin, 63.6% in Damyang-gun) and artificial containers (55.6% in Icksan-si, 45.5% in Damyang-gun) including a plastic wash basin, bowl, can, styrofoam-box within the range of adult mosquito activity. The larval habitats were found within 500 m from the bamboo forest in Iksan-si. The

information obtained from this study on the main habitats and breeding sites would be useful for the control of *Ae. albopictus* in Korea.

Key words: *Aedes albopictus*, distribution, abundance, larval habitats

1. Introduction

Aedes albopictus is an aggressive and strongly anthropophilic mosquito. As an important vector of dengue fever, zika, chikungunya, and yellow fever, *Ae. albopictus* has emerged as a global public health threat (Rezza, 2012). *Ae. albopitus* is indigenous to both tropical and temperate regions of Southeast Asia and islands of the western Pacific and Indian Oceans, but it has recently expanded its range to every continent except Antarctica (Benedict et al., 2007; Caminade et al., 2012). *Ae. albopictus* is difficult to locate and control because this species utilizes small, different types of habitats, including small containers and spare tires (Bagny et al., 2009; Benedict et al., 2007).

Ae. albopictus has been known to be usually found at the edges of forest and breed in natural habitats (e.g., tree holes, bamboo stumps, and rack pools) and thus previously considered as a rural vector (Hawley, 1988). However, this species has adapted well to urban environments with larvae now breeding in artificial containers (e.g., tires, cemetery urns, and water storage containers) and has become the most important and sometimes sole vector in urban areas (Caputo et al., 2012). *Ae. albopictus* is found almost everywhere, especially in suburban park of which borders are connected to mountains. As increasing the population of enjoying leisure nowadays, every local government develops their walking trail embracing hillside

and villages to attract tourists. These developments would increase the encounter with human and mosquitoes. In this study, I investigated the seasonal distribution, abundance and main habitats of *Ae. albopicuts* in Korea.

2. Materials and method

2.1. Seasonal prevalence of *Ae. albopictus*

2.1.1. Mosquito collecting

Mosquitoes were collected from 10 sites (Gangwon, Gyeonggi,, Gyeongnam, Gyeongbuk, Incheon, Jeonnam, Jeonbuk, Jeju, Chungnam, and Chungbuk) from April through October in 2015 (Fig. 1). Each sites were subdivided into three collecting habiats (downtown area, migratory bird refuges and port or airport). A black light trap (Yoshizawa type, black light FL-6W, Shinyoung Co., Seoul, Korea) and a BG-Sentinel trap (Biogents, Regenburg, Germanmy) were placed in each collecting site and operated weekly from 4:00 PM to 10:00 AM. The collected mosquitoes were killed by freezing and then transferred to laboratory and identified under a dissecting microscope using morphological characters (REE, 2003b; Tanaka et al., 1979).

Trap indices (TI: mean number of female mosquitoes collected per trap per night) were determined for each site.

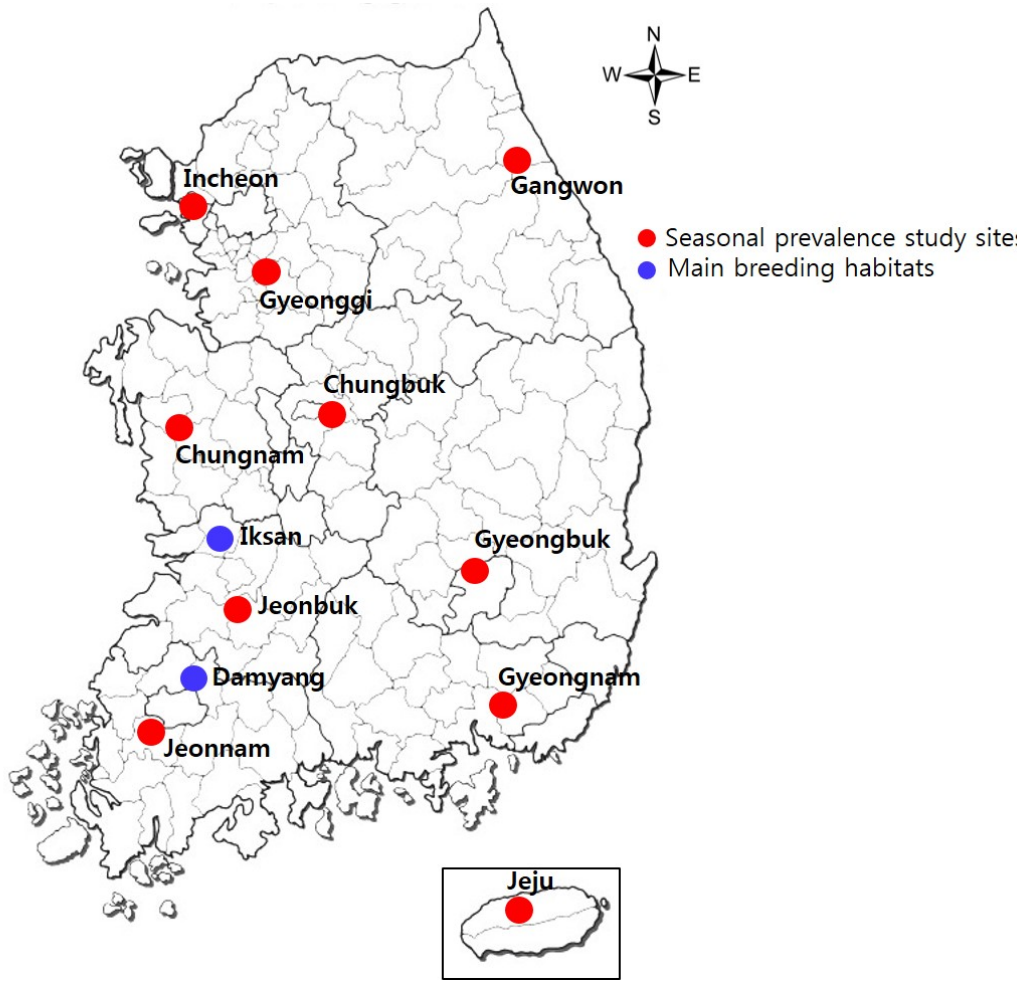

Figure 1. Mosquito collecting and study sites. Red circles are indicating the mosquito collecting sites of seasonal prevalence of *Ae. albopictus*. Blue circles are the study sites of *Ae. albopictus* main breeding sites, having well-developed bamboo forests.

2.1.2. ArcGIS geographical analysis

To compare the geographical distribution of *Ae. albopictus*, distribution maps were drawn by interpolation using the IDW (Inverse Distance Weighted) tequnique among Spatial Analyst Tool in ArcGIS 9.0 (2004, Environmental Research System Institute, Rdlands, CA, USA).

2.2. Survey of main breeding habitats of *Ae. albopictus*

2.2.1. Study sites

As study site, I selected Iksan-si (Gooryong village) and Damyang-gun (Woongyo-ri), which have high density of bamboo forest, the main habitat of *Ae. albopictus* in Korea. Gooryong village in Iksan-si is characterized by well-developed bamboo forest, rice field, orchard, reservoir, rice paddy and houses are surrounded by mountains. In Damyang-gun, a river passes the center of town. From the river, there are well-developed bamboo forest, livestock farm, college, sport stadium, park, residential area, shopping area forest and rice paddy.

2.2.2. Mosquito collection

Ikans-si

Adult mosquito

The BG-Sentinel trap with lure was used for adult surveillance. In Icksan-si, 11 BG-Sentinel traps were placed in various environmental habitats: bamboo forest, a

house in forest, farmland, residential area, cowshed, reservoir, rice paddy, abandoned lot, and urbanized commercial district (Figure 2 and Table 1). Traps were operated from 14:00 to 10:00 (next morning), and collected mosquitoes were transported to laboratory for species identification.

Figure 2. Sites of BG-Sentinel trap setup in Iksan-si. The red dot indicates the site of BG Sentinel trap setup. The number represents a habitat as this: 1. Residential area; 2. Abandon lot (near residential area); 3. Construction site; 4. Rice paddy; 5. Farm (field); 6. Orchard; 7. Forests; 8. Vacant lot; 9. Bamboo forest (control); 10. Cowshed; 11. Reservoir

Table 1. Site of BG Sentinel trap setup and their GPS coordinate in Iksan-si

No	Habitats	GPS coordinate
1	Residential area	N: 36° 00′ 51.0″ E: 127° 02′ 42.5″
2	Abandon lot (near residential area)	N: 36° 00′ 46.7″ E: 127° 02′ 40.8″
3	Construction site (near residential area)	N: 36° 00′ 50.4″ E: 127° 02′ 36.4″
4	Rice paddy	N: 36° 00′ 40.8″ E: 127° 02′ 41.8″
5	Farm (field)	N: 36° 00′ 38.3″ E: 127° 02′ 37.7″
6	Orchard	N: 36° 00′ 34.4″ E: 127° 02′ 43.8″
7	Forests	N: 36° 00′ 49.5″ E: 127° 02′ 51.6″
8	Vacant lot	N: 36° 00′ 39.6″ E: 127° 02′ 50.2″
9	Bamboo forest (control)	N: 36° 00′ 48.6″ E: 127° 02′ 45.2″
10	Cowshed	N: 36° 00′ 33.1″ E: 127° 03′ 16.5″
11	Reservoir	N: 36° 00′ 24.5″ E: 127° 03′ 5.6″

Larval mosquito

Laval mosquitoes were collected using a glass pipette and aquatic net or dipper. I surveyed all aquatic containers in study areas. The GPS coordinates were recorded. I searched 9 aquatic containers in Icksan-si (Figure 2 and Table 2). Collected mosquitoes were transported to laboratory, where they were reared until emergence for species identification.

Figure 3. Sites of larval mosquitoes collected in Iksan-si. The red dot indicates the site of BG Sentinel trap setup. The number represents a habitat as this: 1. Vacant lot, near house (tire); 2. Residential area, wayside (tire); 3. Residential area (tire); 4. Residential area (tire); 5. Residential area, an alley (plastic bowl); 6. Residential area, an alley (stone bowl); 7. Residential area (plastic bag); 8. Residential area, rest room (tin can); 9. Farm, vinyl green house (Styrofoam-box).

Table 2. The sites of larva mosquito surveyed in Iksan-si

No.	Collecting site	Micro habitats	GPS coordiate
1	Vacant lot (near house)	Tire	N: 36° 00′ 37.1″ E: 127° 02′ 49.1″
2	Residential area (wayside)	Tire	N: 36° 00′ 35.1″ E: 127° 02′ 38.7″
3	Residential area	Tire	N: 36° 00′ 41.4″ E: 127° 02′ 40.2″
4	Residential area	Tire	N: 36° 00′ 44.5″ E: 127° 02′ 41.0″
5	Residential area (an alley)	Artificial continer (plastic bowl)	N: 36° 00′ 51.7″ E: 127° 02′ 36.8″
6	Residential area (an alley)	Artificial continer (storn bowl)	N: 36° 00′ 52.6″ E: 127° 02′ 38.1″
7	Residential area	Artificial continer (plastic bag)	N: 36° 00′ 52.6″ E: 127° 02′ 38.1″
8	Residential area (rest room)	Artificial continer (tin can)	N: 36° 00′ 53.1″ E: 127° 02′ 40.1″
9	Farm (vinyl green house)	Artificial continer (Styrofoam-box)	N: 36° 00′ 51.7″ E: 127° 02′ 47.8″

Damyang-gun

Adult mosquito

The BG-Sentinel trap with lure was used for adult surveillance. 12 BG-Sentinel traps were placed in various environmental habitats: bamboo forest, a house in forest, farmland, residential area, cowshed, reservoir, rice paddy, abandoned lot, and urbanized commercial district (Figure 4 and Table 3). Traps were operated from 14:00 to 10:00 (next morning), and collected mosquitoes were transported to laboratory for species identification.

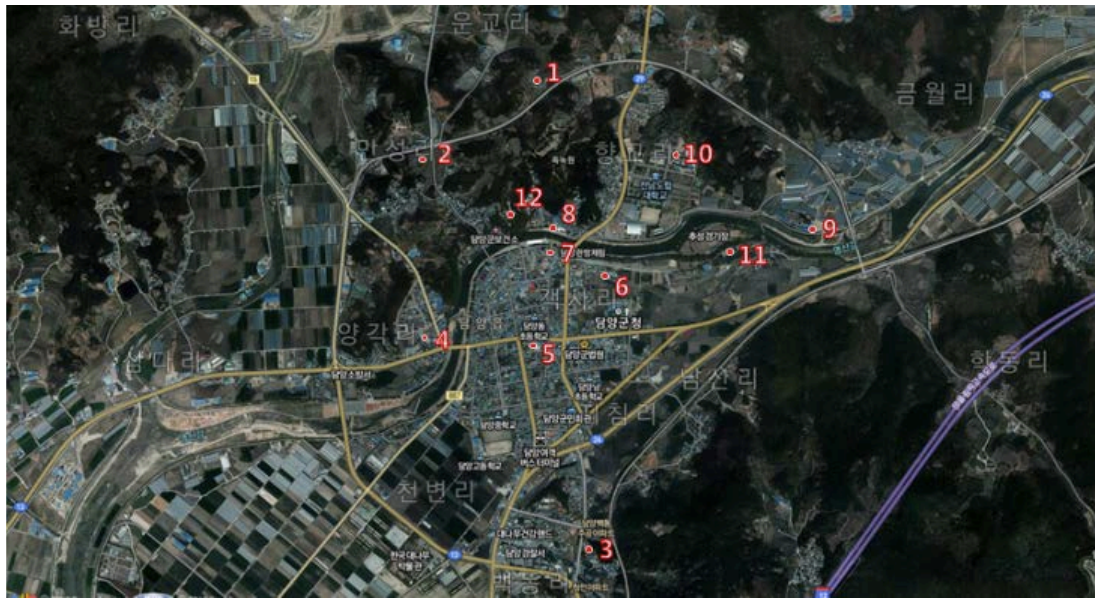

Figure 4. Sites of BG-Sentinel trap placed in Damyang-gun. The red dot indicates the site of BG Sentinel trap setup. The number represents a habitat as this: 1. Animal farm; 2. Rice paddy; 3. Apartment; 4. Field; 5. Park; 6. Junkyard; 7. Vacant lot; 8. Stream side; 9. Cowshed; 10. School; 11. Stadium; 12. Forest.

Table 3. The sites of BG Sentinel trap placed and GPS coordinate in Damyang-gun.

No.	Habitats	GPS coordinate
1	Animal farm	N: 35° 19′ 55.1″ E: 127° 59′ 03.8″
2	Rice paddy	N: 35° 19′ 39.5″ E: 127° 58′ 37.7″
3	Apartment	N: 35° 18′ 32.5″ E: 126° 59′ 10.8″
4	Field	N: 35° 19′ 11.1″ E: 126° 58′ 36.0″
5	Park	N: 35° 19′ 09.6″ E: 126° 58′ 58.6″
6	Junkyard	N: 35° 19′ 22.6″ E: 126° 59′ 10.4″
7	Vacant lot	N: 35° 19′ 24.5″ E: 126° 59′ 01.9″
8	Stream side	N: 35° 19′ 30.5″ E: 126° 59′ 02.6″
9	Cowshed	N: 35° 19′ 29.7″ E: 126° 59′ 55.1″
10	School	N: 35° 19′ 44.1″ E: 127° 59′ 32.4″
11	Stadium	N: 35° 19′ 23.9″ E: 126° 59′ 31.4″
12	Forest	N: 35° 19′ 30.9″ E: 126° 58′ 56.9″

Larval mosquito

Laval mosquitoes were collected using a glass pipette and aquatic net or dipper. I surveyed all aquatic containers in study areas (Figure 4 and Table 4). The GPS coordinates were recorded. I searched 23 aquatic containers in Damyan-gun. Collected mosquitoes were transported to laboratory, where they were reared until emergence for species identification.

Figure 5. Sites of larval mosquitoes collected in Damyang-gun. The red dot indicates the site of BG Sentinel trap setup. The number represents a habitat as this: 1. Bamboo processing plant (tire); 2. Residential area (tire); 3. Residential area, wayside (tire); 4. Funeral hall (pot); 5. Restaurant (pot); 6. Restaurant (bowl); 7. House (plastic container); 8. Field (tin can); 9, Field (paint pot); 10. Vacant lot (tire); 11. Wayside (tire); 12. Bamboo experience center (pot); 13. Butcher's shop (tire); 14. Animal farm (stone bowl); 15. Wayside (tire); 16. Wayside (tire); 17. Residential area (tire); 18. Residential area (tire), 19. Junkyard (tire); 20. House (tire); 21. Village entrance (tire); 22. Car repair shop (tire); 23. Car repair shop (tire).

Table 4. Sites of larval mosquitoes collected and their GPS coordinate in Damyang-gun.

No.	Collecting sites	Micro habitats	GPS coordinate
1	Bamboo processing plant	Tire	N: 35° 19′ 58.5″ E: 126° 59′ 17.6″
2	Residential area	Tire	N: 35° 19′ 35.2″ E: 126° 58′ 38.4″
3	Residential area (wayside)	Tire	N: 35° 19′ 48.6″ E: 126° 58′ 39.3″
4	Funeral hall	Pot	N: 35° 19′ 43.6″ E: 126° 58′ 34.1″
5	Restaurant	Pot	N: 35° 19′ 40.8″ E: 126° 58′ 44.4″
6	Restaurant	Bowl	N: 35° 19′ 40.8″ E: 126° 58′ 44.4″
7	House	Plastic container	N: 35° 19′ 36.1″ E: 126° 58′ 47.0″
8	Field	Tin can	N: 35° 19′ 35.3″ E: 126° 58′ 45.2″
9	Field	Paint pot	N: 35° 19′ 35.3″ E: 126° 58′ 45.2″
10	Vacant lot	Tire	N: 35° 19′ 50.6″ E: 126° 58′ 31.1″
11	Wayside	Tire	N: 35° 20′ 35.5″ E: 126° 58′ 31.1″
12	Bamboo experience center	Pot	N: 35° 19′ 58.0″ E: 126° 58′ 35.1″

Table 4. Sites of larval mosquitoes collected and their GPS coordinate in Damyang-gun (continued)

No.	Collecting sites	Micro habitats	GPS coordinate
13	Butcher's shop	Tire	N: 35° 19′ 57.7″ E: 126° 58′ 38.3″
14	Animal farm	Stone bowl	N: 35° 19′ 55.1″ E: 126° 59′ 05.6″
15	Wayside	Tire	N: 35° 19′ 24.4″ E: 126° 58′ 34.9″
16	Wayside	Tire	N: 35° 19′ 17.2″ E: 126° 58′ 59.1″
17	Residential area	Tire	N: 35° 19′ 21.1″ E: 126° 59′ 03.1″
18	Residential area	Pot	N: 35° 19′ 23.6″ E: 126° 58′ 57.0″
19	Junkyard	Tire	N: 35° 19′ 22.6″ E: 126° 59′ 10.1″
20	House	Tire	N: 35° 19′ 21.7″ E: 126° 59′ 15.6″
21	Village entrance	Tire	N: 35° 19′ 23.1″ E: 126° 59′ 08.0″
22	Car repair shop	Tire	N: 35° 19′ 02.1″ E: 126° 59′ 06.9″
23	Car repair shop	Tire	N: 35° 19′ 13.2″ E: 126° 59′ 07.5″

3. Results

3.1. Seasonal prevalence of *Ae. albopictus*

A total of 99,625 mosquitoes belonging to 9 genera and 19 species were collected at 10 cities. Representing as TI, there were a mean of 125.1 mosquitoes collected from each trap. The overall TI for all sites ranged from a low of 21.7 (Kangwon) to a high of 416.8 (Chungnam). The overall monthly TI ranged from 3.2 (May) to 268.2 (August). Details are represented at Table 5. The most frequently collected species were *Ae. vexans* (TI 34.9, 27.9%), followed by *Cx. pipiens* (TI 34.8, 27.8%), *An. spp*. (TI 17.8, 14.3%), *Cx. tritaeniorhynchus* (TI 17.7, 14.2%), *Armigeres subalbatus* (TI 6.6, 5.3%), *Ae. albopictus* (TI 4.9, 4.0%), and *Ochlerotatus koreicus* (TI 4.6, 3.7%) (Table 6).

Ae. albopictus was first collected in May (TI 0.7), slowly increased throughout August (TI 8.2), and reached peak in September (TI 10.9), then followed rapidly decrease in number during October (TI 3.6) (Table 6, and Figure 6). According to the collecting sites, the most *Ae. albopcitus* was collected in Gyeongbuk, followed by Jeju and Chungnam (Figure 7).

The number of collected mosquitoes was different depending on the traps used (Table 7). While more mosquitoes were collected by black light traps (TI 128.5) than

by BG-Sentinel traps (TI 119.9), *Ae. albopictus* was more collected by BG-Sentinel traps (TI 11.0, 9.2%) than by black light traps (TI 1.2, 0.9%) (Figure 8).

Based on the number of collected mosquitoes by collecting habitats, the site with most mosquitoes collected was port or airport (mean TI 228.9), followed by migratory bird refuge (TI 150.5), and downtown area (TI 81.7) (Table 8). As for just *Ae. albopictus*, most mosquitoes were collected fromt downtown area (TI 7.3, 8.9%), followed migratory bird refuge (TI 2.8, 1.9%), and port or airport (TI 1.7, 0.7%) (Figure 9).

Table 5. Monthly trap indices[†] of collected mosquitoes according to collecting sites

Cities	Apr	May	Jun	Jul	Aug	Sep	Oct	Mean
Gangwon	1.8	13.2	22.3	30.1	26.3	40.9	17.3	21.7
Gyeonggi	0.8	9.1	30.2	30.8	39.0	35.6	9.6	22.2
Gyeongnam	0.3	4.8	111.4	486.7	906.0	103.7	24.3	233.9
Gyeongbuk	3.2	15.7	105.5	104.0	70.1	127.0	54.7	68.6
Incheon	6.6	42.8	97.7	281.9	189.4	133.6	52.9	115.0
Jeonnam	1.5	15.6	330.7	233.5	117.3	126.6	26.8	121.7
Jeonbuk	4.4	106.9	185.0	352.3	167.1	290.9	6.4	159.0
Jeju	6.5	25.8	60.5	39.9	45.9	66.0	63.8	44.1
Chungnam	3.8	222.6	356.2	832.1	989.6	458.0	55.7	416.8
Chungbuk	2.6	38.3	80.0	91.3	87.1	83.8	26.9	58.6
Mean	3.2	48.0	136.7	243.1	268.2	141.2	35.6	125.1

[†] Trap index (TI) = total number of captured mosquitoes / (number of traps x number of nights)

Table 6. Monthly trap indices of collected according to collected species

Species	Apr	May	Jun	Jul	Aug	Sep	Oct	Mean (%)
Aedes albopictus	0.0	0.7	3.6	7.6	8.2	10.9	3.6	5.0 (4.0)
Ae. vexans	0.3	25.1	42.2	68.7	83.3	23.8	1.0	34.9 (27.9)
Anopheles spp.‡	0.0	1.0	25.0	47.5	28.2	22.0	1.3	17.9 (14.3)
Armigeres subalbatus	0.0	0.5	2.4	7.2	18.1	12.1	6.0	6.6 (5.3)
Coquillettidia ochracea	0.0	0.0	0.0	0.5	0.6	0.4	0.0	0.2 (0.2)
Culiseta bergrothi	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0 (0.0)
Culex bitaeniorhynchus	0.0	0.0	0.3	0.4	0.3	0.2	0.0	0.2 (0.1)
Cx. inatomii	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0 (0.0)
Cx. orientalis	0.1	0.1	0.8	2.0	1.4	0.8	0.1	0.8 (0.6)
Cx. pipiens complex	2.6	17.0	58.0	61.0	33.1	50.6	21.2	34.8 (27.8)
Cx. tritaeniorhynchus	0.0	0.0	0.6	33.4	78.5	10.9	0.6	17.7 (14.2)
Cx. vagans	0.0	0.3	0.3	1.2	0.1	0.1	0.2	0.3 (0.3)
Mansonia uniformis	0.0	0.0	0.5	4.0	4.6	1.6	0.0	1.5 (1.2)
Ochlerotatus dorsalis	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0 (0.0)
Oc. hattorii	0.0	0.0	0.0	0.4	0.0	0.2	0.2	0.1 (0.1)
Oc. koreicus	0.1	2.9	2.5	7.7	10.9	6.8	0.9	4.6 (3.7)
Oc. nipponicus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.0)
Oc. togoi	0.1	0.2	0.4	1.2	0.9	0.7	0.4	0.6 (0.4)
Total	3.2	48.0	136.7	243.1	268.2	141.2	35.6	125.1 (100.0)

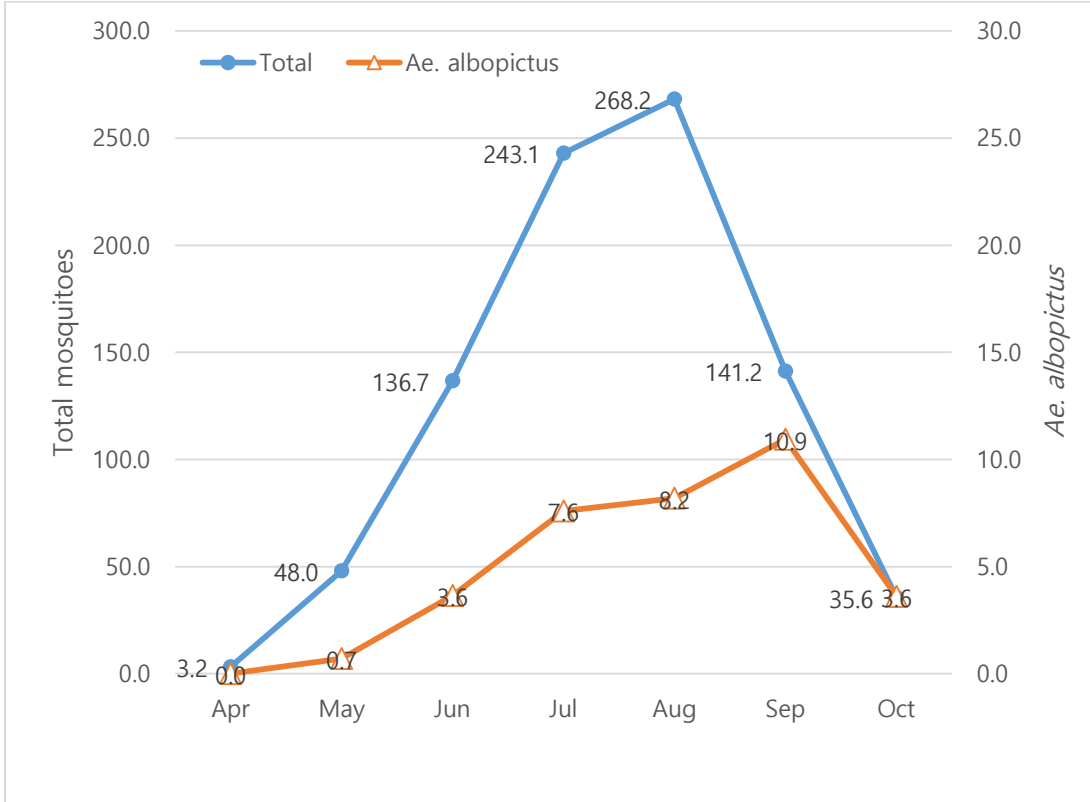

Figure 6. Seasonal prevalence of *Ae. albopcitus* and total mosquitoes. Mosquitoes were collected using Black light traps and BG Sentienl traps at 10 cities in Korea. Collecting numbers are represented by trap index (number of mosquito / nights x traps).

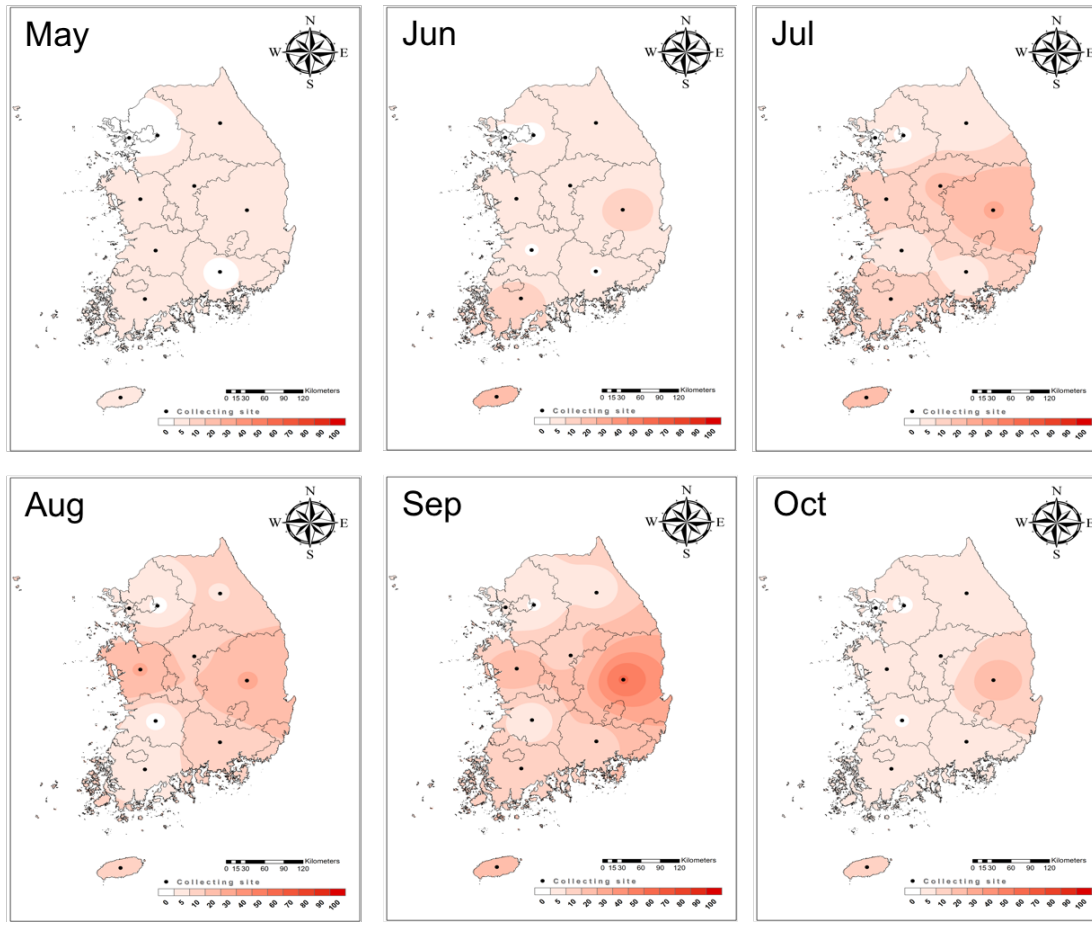

Figure 7. The geographical distritution of *Ae. albopcitus* according to seasonal changing from May to October 2015 in Korea.

Table 7. Monthly trap indices[†] of collected mosquitoes according to the collecting traps

Species	Apr		May		Jun		Jul		Aug	
	BG[*]	BL[**]	BG	BL	BG	BL	BG	BL	BG	BL
Aedes albpictus	0.0	0.0	1.6	0.1	8.2	0.8	16.4	2.0	18.2	1.9
Ae. vxans	0.8	0.0	26.7	24.2	10.6	62.1	8.9	106.2	9.3	129.8
Anopheles spp.[‡]	0.0	0.0	0.7	1.2	1.4	39.8	24.5	61.9	1.4	45.0
Armigeres subalbatus	0.0	0.0	1.0	0.3	5.1	0.6	18.0	0.4	45.0	1.2
Coquillettidia ochracea	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.3	1.1	0.2
Culiseta bergrothi	0.0	0.0	0.0	0.0	0.1	0.0	0.3	0.0	0.0	0.0
Culex bitaeniorhynchus	0.0	0.0	0.1	0.0	0.6	0.1	0.7	0.2	0.4	0.1
Cx inatomii	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0
Cx. orientalis	0.1	0.1	0.0	0.1	0.6	0.9	2.5	1.7	1.5	1.4
Cx. pipiens	4.0	1.7	34.1	6.3	96.5	33.7	109.5	30.4	56.8	18.3
Cx. tritaeniorhynchus	0.0	0.0	0.0	0.1	0.2	1.0	0.8	53.9	7.1	123.3
Cx. vagans	0.0	0.1	0.3	0.3	0.6	0.1	0.0	1.9	0.1	0.1
Mansonia uniformis	0.0	0.0	0.0	0.0	0.1	0.8	1.0	5.9	1.8	6.3
Ochlerotatus dorsalis	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.1
Oc. hattorii	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0
Oc. koreicus	0.1	0.1	6.5	0.7	4.3	1.4	18.3	1.1	25.8	1.6
Oc. nipponicus	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Oc. togoi	0.1	0.0	0.3	0.2	0.7	0.2	1.2	1.3	0.9	0.9
Total	5.1	2.0	71.2	33.5	129.0	141.6	204.3	267.4	169.5	330.2

[†] Trap index (TI) = total number of captured mosquitoes / (number of traps x number of nights)

[‡] *Anopheles spp*.: includes *An. sinensis, An. lesteri, An. lindesai, An. pullus, An. sineroides, An. belenrae, An. kleini.*

[*]BG: BG Sentinel trap, [**]BL: Black light trap

Table 7. Monthly trap indices[†] of collected mosquitoes according to the collecting traps (continued)

Species	Sep		Oct		Mean (%)			
	BG	BL	BG	BL	BG		BL	
Aedes albpictus	24.0	2.7	8.5	0.6	11.0	(9.2)	1.2	(0.9)
Ae. vxans	1.9	37.6	0.8	1.0	8.4	(7.0)	51.6	(40.1)
Anopheles spp.	4.9	32.7	0.2	2.0	4.7	(4.0)	26.1	(20.3)
Armigeres subalbatus	27.9	2.2	13.0	1.7	15.7	(13.1)	0.9	(0.7)
Coquillettidia ochracea	0.6	0.2	0.0	0.0	0.4	(0.3)	0.1	(0.1)
Culiseta bergrothi	0.1	0.0	0.0	0.0	0.1	(0.1)	0.0	(0.0)
Culex bitaeniorhynchus	0.5	0.1	0.0	0.0	0.3	(0.3)	0.1	(0.1)
Cx inatomii	0.0	0.0	0.0	0.0	0.0	(0.0)	0.0	(0.0)
Cx. orientalis	1.1	0.5	0.1	0.2	0.8	(0.7)	0.7	(0.5)
Cx. pipiens	102.6	18.0	43.4	7.3	63.8	(53.3)	16.5	(12.5)
Cx. tritaeniorhynchus	7.2	13.2	0.2	0.8	2.2	(1.9)	27.5	(21.4)
Cx. vagans	0.2	0.1	0.0	0.3	0.2	(0.1)	0.4	(0.3)
Mansonia uniformis	2.2	1.3	0.0	0.0	0.7	(0.6)	2.0	(1.6)
Ochlerotatus dorsalis	0.0	0.0	0.0	0.0	0.0	(0.0)	0.0	(0.0)
Oc. hattorii	0.5	0.0	0.5	0.0	0.3	(0.2)	0.0	(0.0)
Oc. koreicus	16.1	1.0	1.7	0.5	10.4	(8.7)	0.9	(0.7)
Oc. nipponicus	0.0	0.0	0.0	0.0	0.0	(0.0)	0.0	(0.0)
Oc. togoi	1.4	0.3	0.4	0.4	0.7	(0.6)	0.5	(0.4)
Total	191.3	109.8	68.8	14.7	119.9	(100.0)	128.5	(100.0)

[†] Trap index (TI) = total number of captured mosquitoes / (number of traps x number of nights)

[‡] *Anopheles spp.*: includes *An. sinensis*, *An. lesteri*, *An. lindesai*, *An. pullus*, *An. sineroides*, *An. belenrae*, *An. kleini.*

[*]BG: BG Sentinel trap, [**]BL: Black light trap

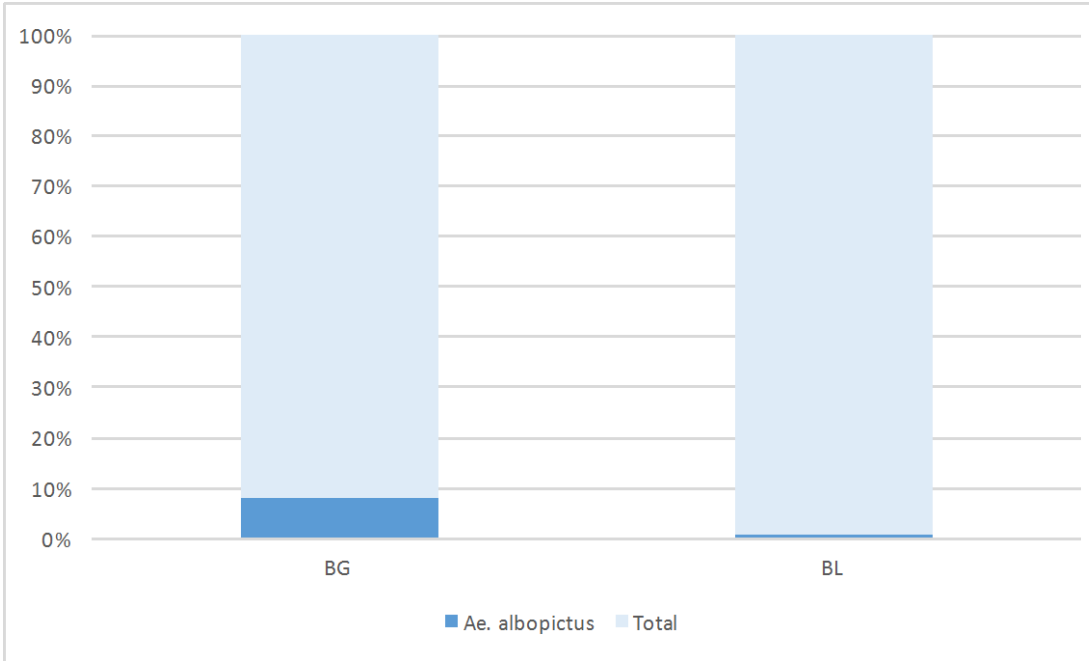

Figure 8. The different ratio of collected *Ae. albopictus* according to the traps used mosquito collection. Mosquitoes were collected at three different habitats (downtown areas, migratory bird refuges and port and airport areas) in 10 sites in Korea. BG: BG Sentinel trap, BL: Black light trap.

Table 8. Trap indices[†] of collected mosquitoes at downtown, migratory bird refuge and port or airport areas

Species	Downtowns (%)	Migratory bird refuges (%)	Port /airpots (%)
Aedes albopictus	7.3 (8.9)	2.8 (1.9)	1.7 (0.7)
Ae. vexans	7.9 (9.6)	80.7 (53.6)	9.5 (4.2)
Anopheles spp.[‡]	5.1 (6.2)	32.5 (21.6)	26.8 (11.7)
Armigeres subalbatus	8.7 (11)	5.8 (3.9)	0.6 (0.2)
Coquillettidia ochracea	0.0 (0.0)	0.5 (0.3)	0.2 (0.1)
Culiseta bergrothi	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)
Culex bitaeniorhynchus	0.1 (0.1)	0.3 (0.2)	0.0 (0.0)
Cx. inatomii	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Cx. orientalis	0.5 (0.6)	1.3 (0.9)	0.1 (0.1)
Cx. pipiens complex	44.5 (54.0)	16.3 (10.8)	49.9 (21.8)
Cx. tritaeniorhynchus	0.3 (0.4)	4.4 (2.9)	129.6 (56.6)
Cx. vagans	0.5 (0.6)	0.2 (0.1)	0.1 (0.0)
Mansonia uniformis	0.0 (0.0)	1.2 (0.8)	8.7 (3.8)
Ochlerotatus dorsalis	0.0 (0.0)	0.0 (0.0)	0.1 (0.1)
Ochlerotatus hattorii	0.1 (0.1)	0.1 (0.1)	0.0 (0.0)
Oc. koreicus	5.7 (7.0)	4.2 (2.8)	1.0 (0.4)
Oc. nipponicus	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Oc. togoi	0.9 (1.1)	0.0 (0.0)	0.6 (0.2)
Total	81.7 (100.0)	150.5 (100.0)	228.9 (100.0)

[†] Trap index (TI) = total number of captured mosquitoes / (number of traps x number of nights)

[‡] *Anopheles* spp.: includes *An. sinensis, An. lesteri, An. lindesai, An. pullus, An. sineroides, An. belenrae, An. kleini.*

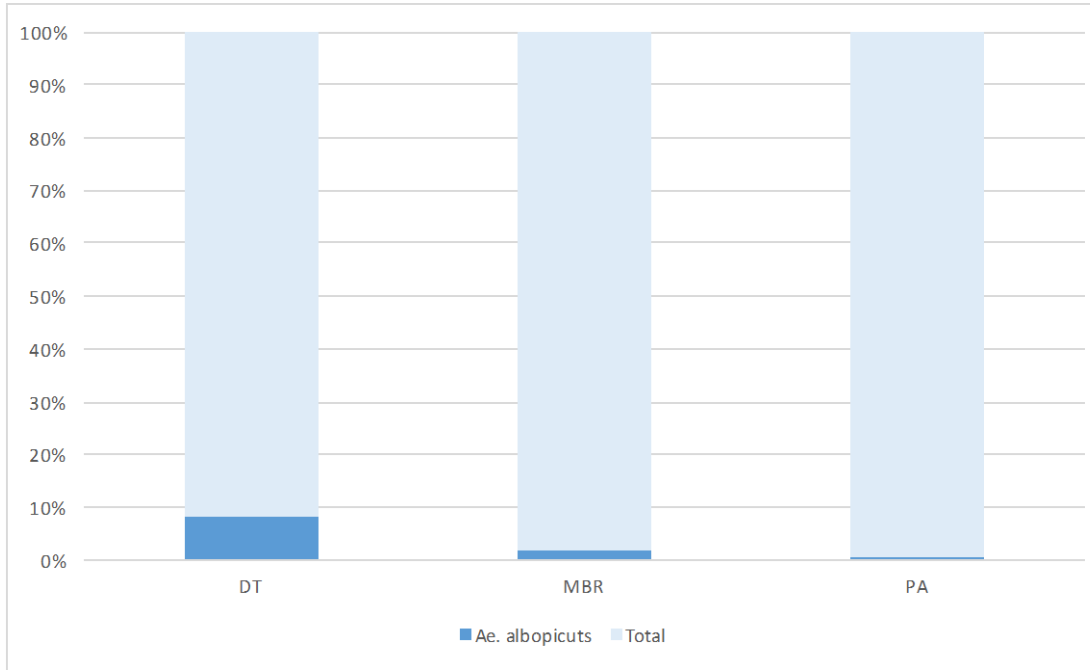

Figure 9. The different ratio collected *Ae. albopictus* according to mosquito collecting habitats. Mosquitoes were collected by Black light traps and BG Sentienel traps. DT: Downtown area, MBR: migratory bird refuge, PA: Port or airport

3.2. Survey of main breeding habitats of *Aedes albopictus*

Iksan-si (Kowloogn village)

Adult mosquito of *Ae. albopictus* were captured from 10 (exception of around cowshed) out of the 11 of micro habitats on Kowloogn village in Iksan-si (Table 12). A large number of adult mosquitoes were captured in a bamboo forest and a forest adjascent it. In contrast, fewer mosquitos were captured in rice paddy, field, orchards, and vacant lot proportional to increased distance from bamboo forest. Therefore, the bamboo forest and adjascent forest were considered as the main habitats of *Ae. albopictus* in Kowloogn willage in Iksan-si.

The larval mosquito of *Ae. albopucts* were collected from eight micro habitats out of nine (Table 13). The collected mosquito larvae were found mainly in tires (44.4%) and artificial containers (55.6%) including a plastic wash basin, bowl, can, styrofoam-box within the range of adult mosquito activity (Figure 9). The tires were found to be the preferred habitat for mosquito larvae (Table 13). The larval habitats were found within 500 m from the bamboo forest (Figure 10).

Table 9. The micro habitats of mosquitoes collected by BG Sentinel trap at Iksan-si

No.	Habitats	GPS coordinate	Species	Number of mosquitoes.
1	Residential area	N: 36° 00′ 51.0″ E: 127° 02′ 42.5″	*Ae. albopictus*	12
2	Abandon lot (near residential area)	N: 36° 00′ 46.7″ E: 127° 02′ 40.8″	*Ae. albopictus*	75
3	Constrction site (near residential area)	N: 36° 00′ 50.4″ E: 127° 02′ 36.4″	*Ae. albopictus*	157
4	Rice paddy	N: 36° 00′ 40.8″ E: 127° 02′ 41.8″	*Ae. albopictus*	2
5	Farm (field)	N: 36° 00′ 38.3″ E: 127° 02′ 37.7″	*Ae. albopictus* *Ae. vexans* *Ar. subalbatus*	20 2 24
6	Orchard	N: 36° 00′ 34.4″ E: 127° 02′ 43.8″	*Ae. albopictus*	11
7	forests	N: 36° 00′ 49.5″ E: 127° 02′ 51.6″	*Ae. albopictus* *Ae. vexans*	34 1
8	Vacant lot	N: 36° 00′ 39.6″ E: 127° 02′ 50.2″	*Ae. albopictus*	7
9	Bamboo forest (control)	N: 36° 00′ 48.6″ E: 127° 02′ 45.2″	*Ae. albopictus* *Tr. bambusa* *Ar. subalbatus*	548 30 10
10	cowshed	N: 36° 00′ 33.1″ E: 127° 03′ 16.5″	*Ae. vexans*	1
11	Reservior	N: 36° 00′ 24.5″ E: 127° 03′ 5.6″	*Ae. albopictus*	2

Table 10. The micro habitats of *Ae. albopictus* larvae collected in Iksan-si

No.	Collecting site	Micro habitats	GPS coordinate	Mosquito
1	Vacant lot (near house)	Tire	N: 36° 00′ 37.1″ E: 127° 02′ 49.1″	o
2	Residential area (wayside)	Tire	N: 36° 00′ 35.1″ E: 127° 02′ 38.7″	o
3	Residential area	Tire	N: 36° 00′ 41.4″ E: 127° 02′ 40.2″	o
4	Residential area	Tire	N: 36° 00′ 44.5″ E: 127° 02′ 41.0″	o
5	Residential area (an alley)	Artificial container (plastic bowl)	N: 36° 00′ 51.7″ E: 127° 02′ 36.8″	o
6	Residential area (an alley)	Artificial container (stone bowl)	N: 36° 00′ 52.6″ E: 127° 02′ 38.1″	o
7	Residential area	Artificial container (plastic bag)	N: 36° 00′ 52.6″ E: 127° 02′ 38.1″	o
8	Residential area (rest room)	Artificial container (tin can)	N: 36° 00′ 53.1″ E: 127° 02′ 40.1″	o
9	Farm (vinyl green house)	Artificial container (Styrofoam-box)	N: 36° 00′ 51.7″ E: 127° 02′ 47.8″	x

Figure 10. The micro habitats of *Ae. albopictus* larvae collected in Icksan-si. A: Vacant lot (tire); B: Residential area (tire); C: Residential area (tire); D: Residential area (tire); E: Residential area (plastic bowl); F: Residential area (stone bowl); G: Residential area (plastic container); H: Residential area (plastic container); I Farm (Styrofoam-box).

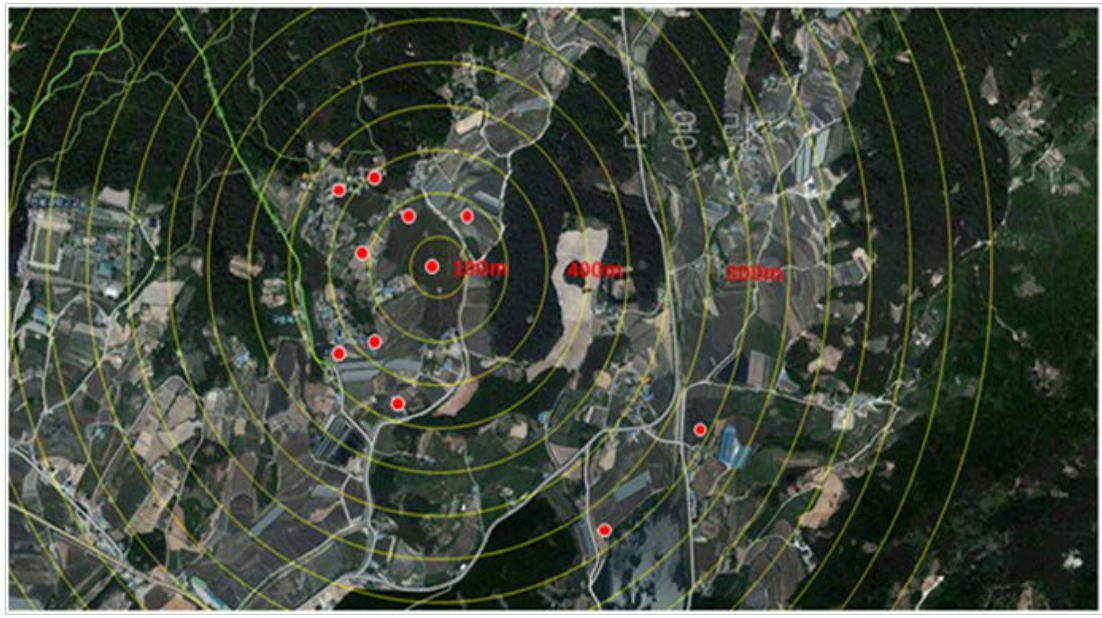

Figure 11. The distance of larvae collected micro habitats form the bamboo forest, the main habitat of *Ae. albopictus*. The red dots indicate the micro habitats of Ae. albopictus larvae collected. The yellow circles represent 100 m distance from the bamboo forest, the main habitat of *Ae. albopictus.*

Damyang-gun

In Damyang-gun, adult *Ae. albopictus* were captured in all 12 collecting sites, and larvae were collected from 22 micro habitats out of 23 (Table 14). Similar to Icksan-si, the collected larvae were found mainly in tires (63.6%) and artificial container (45.4%) (Table 15) (Figure 11). The larval habitats were found within 1,000 m from the bamboo forest (Figure 12).

Table 11. The micro habitats of *Ae. albopictus* collected by BG Sentinel trap in Damyang-gun

No.	Habitats	GPS coordinate	Species	Nmb. Mosq.
1	Animal farm	N: 35° 19′ 55.1 E: 127° 59′ 03.8″	*Ae. albopictus* *Ar. subalbatus*	21 1
2	Rice paddy	N: 35° 19′ 39.5″ E: 127° 58′ 37.7″	*Ae. albopictus* *Ae. vexans*	47 1
3	Apartment	N: 35° 18′ 32.5″ E: 126° 59′ 10.8″	*Ae. albopictus*	17
4	Field	N: 35° 19′ 11.1″ E: 126° 58′ 36.0″	*Ae. albopictus*	75
5	Park	N: 35° 19′ 09.6″ E: 126° 58′ 58.6″	*Ae. albopictus* *Ar. subalbatus*	33 1
6	Junkyard	N: 35° 19′ 22.6″ E: 126° 59′ 10.4″	*Ae. albopictus*	155
7	Vacant lot	N: 35° 19′ 24.5″ E: 126° 59′ 01.9″	*Ae. albopictus*	32
8	Strem side	N: 35° 19′ 30.5″ E: 126° 59′ 02.6″	*Ae. albopictus*	8
9	Cowshed	N: 35° 19′ 29.7″ E: 126° 59′ 55.1″	*Ae. albopictus* *Ae. vexans* *Cx. pipiens*	1 14 4
10	School	N: 35° 19′ 44.1″ E: 127° 59′ 32.4″	*Ae. albopictus* *Ae. vexans* *Tr. bambusa* *Ar. subalbatus*	18 2 8 2
11	Stadium	N: 35° 19′ 23.9″ E: 126° 59′ 31.4″	*Ae. albopictus* *Ae. vexans* *Ar. subalbatus* *Cx. pipiens*	63 2 8 2
12	Forest	N: 35° 19′ 30.9″ E: 126° 58′ 56.9″	*Ae. albopictus* *Ar. subalbatus*	22 3

Table 12. The micro habitats of *Ae. albopictus* larvae collected in Damyang-gun

No.	Collecting sites	Micro habitats	GPS coordinate	Inhabitoation *Ae. albopictus*
1	Bamboo processing lot	Tire	N: 35° 19′ 58.5″ E: 126° 59′ 17.6″	○
2	Residential area	Tire	N: 35° 19′ 35.2″ E: 126° 58′ 38.4″	○
3	Residential area (wayside)	Tire	N: 35° 19′ 48.6″ E: 126° 58′ 39.3″	○
4	Funernal hall	Pot	N: 35° 19′ 43.6″ E: 126° 58′ 34.1″	○
5	Restaurant	Pot	N: 35° 19′ 40.8″ E: 126° 58′ 44.4″	○
6	Restaurant	Bowl	N: 35° 19′ 40.8″ E: 126° 58′ 44.4″	○
7	House	Plastic container	N: 35° 19′ 36.1″ E: 126° 58′ 47.0″	○
8	Field	Tin can	N: 35° 19′ 35.3″ E: 126° 58′ 45.2″	○
9	Field	Paint pot	N: 35° 19′ 35.3″ E: 126° 58′ 45.2″	○
10	Vacant lot	Tire	N: 35° 19′ 50.6″ E: 126° 58′ 31.1″	○
11	Wayside	Tire	N: 35° 20′ 35.5″ E: 126° 58′ 31.1″	○
12	Bamboo experience center	Pot	N: 35° 19′ 58.0″ E: 126° 58′ 35.1″	○

Table 12. The micro habitats of *Ae. albopictus* larvae collected in Damyang-gun (continued)

No.	Collecting sites	Micro habitats	GPS coordinate	Inhabitoation *Ae. albopictus*
13	Butcher's shop	Tire	N: 35° 19′ 57.7″ E: 126° 58′ 38.3″	○
14	Animal farm	Stone bowl	N: 35° 19′ 55.1″ E: 126° 59′ 05.6″	○
15	Wayside	Tire	N: 35° 19′ 24.4″ E: 126° 58′ 34.9″	○
16	Wayside	Tire	N: 35° 19′ 17.2″ E: 126° 58′ 59.1″	○
17	Residential area	Tire	N: 35° 19′ 21.1″ E: 126° 59′ 03.1″	○
18	Residential area	Pot	N: 35° 19′ 23.6″ E: 126° 58′ 57.0″	×
19	Junkyard	Tire	N: 35° 19′ 22.6″ E: 126° 59′ 10.1″	○
20	House	Tire	N: 35° 19′ 21.7″ E: 126° 59′ 15.6″	○
21	Village entrance	Tire	N: 35° 19′ 23.1″ E: 126° 59′ 08.0″	○
22	Car repair shop	Tire	N: 35° 19′ 02.1″ E: 126° 59′ 06.9″	○
23	Car repair shop	Tire	N: 35° 19′ 13.2″ E: 126° 59′ 07.5″	○

Figure 12. The micro habitats of *Ae. albopictus* larvae collected in Damyang-gun. A: Bamboo processing lot (tire); B: Residential area (tire); C: Residential area, wayside (tire); D: Funeral hall (pot); E: Restaurant (pots); F: Restaurant (bowl); G: House (plastic container); H: Field (tin can); I: Field (paint pot); J: Way side (tire); K: Way side (tire); L: Bamboo experience center (pot); M: Butcher's shop (tire); N: Animal farm (stone bowl); O: Wayside (tire); P: Wayside (tire); Q: Residential area (tire); R: Residential area (pot); S: Junkyard (tire); T: House (tire); U: Village entrance (tire); V: Car repair shop (tire); W: Car repair shop (tire).

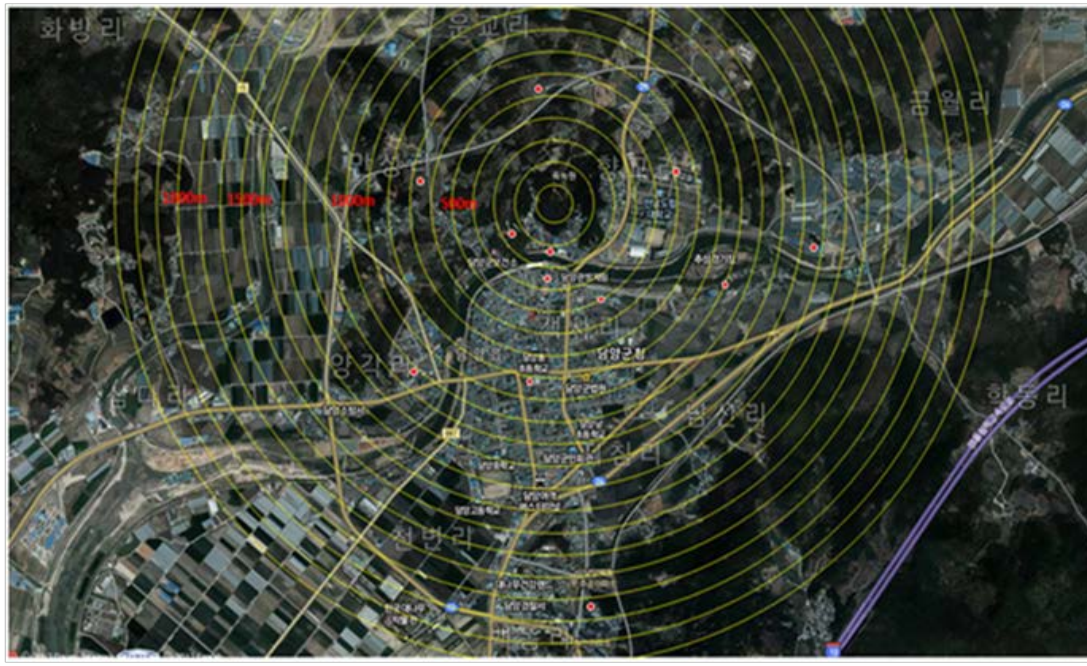

Figure 13. The distance of larvae collected micro habitats form the bamboo forest, the main habitat of *Ae. albopictus*. The red dots indicate the micro habitats of *Ae. albopictus* larvae collected. The yellow circles represent 100 m distance from the bamboo forest, the main habitat of *Ae. albopictus.*

4. Discussions

Ae. albopictus has a strong ecological plasticity that allows for its rapid adaptation to a very large range of habitats (Paupy et al., 2009). For instance, even though *Ae. albopicuts* originated in Asian forests, it has now adapted to human environments and preferentially occurs in suburban environments, while also being identified in highly dense urban areas (Dalla Pozza and Majori, 1992).

In this study, adult mosquitoes were collected in 10 sites, and larval and adult mosquito were collected in and around two regions having well-developed bamboo forest, which is the major habitats of *Ae. albopictus*. *Ae. albopictus* collected as the density of 4% out of total mosquitoes. Unlike seasonal prevalence of total mosquitoes, the population of *Ae. albopictus* was increased from late June and reached the high peak in September, showing some delayed occurring time when compared with other mosquitoes (about one month). Also, *Ae. albopictus* was collected in a higher rate in urban area than in refuge of migratory birds and in area of port and airport. In general, *Ae. albopicuts* is known as a forest mosquito species. In this study, however, *Ae. albopictus* seems to prefer suburban environments. In survey of main habitats, the larval breeding sites of *Ae. albopictus* were very broad and ranged from natural sites to artificial containers. Along with the preferred bamboo forest, *Ae. albopictus*was

also found in many artificial containers scattered around residential area of suburban environments.

Ae. albopictus is known to originate from the forests of Southeast Asia, where it was likely zoophilic (feeding on wildlife). However, the species progressively adapted to anthropogenic changes to the environment, which provided alternative blood sources (domestic animals and man) and water collections for larval habitats, which is known as domestication (Paupy et al., 2009). Urbanization refers to the increasing population of urban areas. Urbanization results in the physical growth of urban areas, leading to environmental changes. These changes in environmental conditions may directly or indirectly affect the ecology of mosquitoes, e.g., larval habitat availability and suitability, development, and survivorship (Li et al., 2014). These two factors, domestication and urbanization, results in increasing number of people who contact with mosquitoes. Consequently, this contact with mosquitoes causes increasing the risk of transmission and spread of viruses.

Because there are no vaccines against DENV and CHIKV, the main disease transmitted by *Ae. albopictus*, vector control remains the critical method for the prevention and control of these diseases. The information obtained from this study on the main habitats and breeding sites would be useful for control *Ae. albopictus* in Korea.

Chapter II.

Blood-feeding behavior of *Aedes albopictus*: host searching activityt, host preference, virus infection rate and *Wolbachia* infection rate

II-1. Daily activity of *Aedes albopicus* for searching hosts

Abstract

Aedes albopictus, known as the Asian tiger mosquito or forest day mosquito, is characterized by black and white stripes on its legs and body. It is a vector for many viral diseases such as dengue fever and Chikungunya fever. Unlike other mosquitoes, this mosquito attacks people mainly during the daytime. This study aimed to determine the exact active times of *Ae. albopictus*. Mosquitos were collected on three occasions from August to September 2009 in a bamboo forest in Damyang-gun, Joullanam-do, South Korea. Mosquitoes were collected for 25 h; captured mosquitoes were identified, counted and then released at every hour. *Ae. albopictus* activity began around sunrise (about 08:00) with peaks in late morning (08:00–09:00) and early evening (16:00−19:00) and ended with sunset at 20:00. Light intensity appears to be a major factor affecting mosquito activity: if light intensity is over some threshold, *Ae. albopictus* activity decreases.

Keywords: *Aedes albopictus*, Asian tiger mosquito, diel activity, light intensity, bamboo forest

1. Introduction

Aedes albopictus (Diptera: Culicidae), commonly referred to as the Asian tiger mosquito, is characterized by black and white stripes on its legs and body (Hawley, 1988). This mosquito is native to tropical and subtropical regions of Southeast Asia. However, in the early 20th century this species spread to Africa, the Middle East, Europe, and the Americas (North and South) by extending its range eastwards across Pacific islands through cargo transport and increasing international travel (Hawley et al., 1987; Knudsen, 1995).

Ae. albopictus is a vector for many virus-borne diseases: laboratory studies have shown that more than two dozen viruses can use it for reproduction (Enserink, 2008). Typical diseases caused by these viruses include dengue fever, which causes severe muscle and joint pain and can lead to dengue hemorrhagic fever (Estrad-Franco, 1995), and Chikungunya, which showed a massive outbreak in the Indian Ocean islands (Bellini et al., 2012; Bonilauri et al., 2008; Kumar et al., 2012).

In the areas where the closely related yellow fever mosquito, *Aedes aegypti*, is the main vector for dengue virus, dengue fever tends to be epidemic because *Ae. aegypti* feeds almost exclusively on human blood in tropical regions (Enserink, 2008). However, a 2014 outbreak of dengue fever in Tokyo, Japan indicated that *Ae.*

albopictus may also have an important role in transmitting dengue virus (Kutsuna et al., 2015a).

Ae. albopictus, also sometimes called the forest day mosquito, is, as this common name implies, most active in forests in the daytime. It is attracted to humans based not only by host factors such as carbon dioxide, moisture, and organic chemicals, but also by visual factors including movement (Estrad-Franco, 1995; Kawada et al., 2005).

Several studies on the diurnal biting cycle have indicated that *Ae. albopictus* has a general bimodal activity pattern, with one activity peak in early morning and another in the evening (Estrad-Franco, 1995; Hawley, 1988), suggesting that this mosquito has relatively low activity during most of daytime.

In this study, I have attempted to explain this discrepancy between observations and experimental data. These features of attraction to humans may be a key to understanding the pathogens transmitted by mosquitoes and developing strategies to block this transmission. Because this biting activity is influenced by geographical conditions, local studies are urgently needed to establish control strategies focusing on personal protection.

2. Materials and Methods

2.1. General Description of Study Site

To study the daily activity of *Ae. albopictus*, I selected a bamboo forest (approximately 48,563.30 m^2) in Damyang-gun, Joulanam-do, South Korea, a primary habitat of this species of mosquito (Figure 1). The majority of mosquitoes in this forest are *Ae. albopictus*. To evaluate the amount of light as a factor affecting mosquito activity, I divided the study area into three sites according to the distance from the edge of the forest. Site A was the edge of the forest, site B was 10 m into the forest, and site C was 15 m into the forest (Figure 1). The farther from the edge, the less light is available. Using a photometer (Digital Light Meter 2, Taiwan), I measured the light intensity every hour at each site.

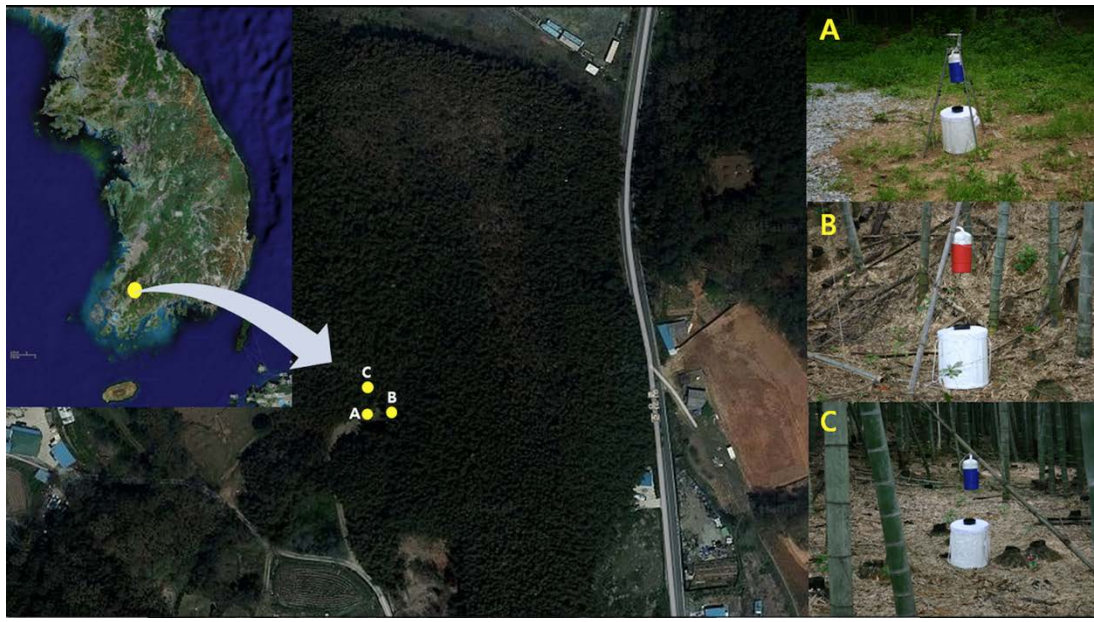

Figure 1. Location of this study, Damyang-gun, Korea. A) Site A, bamboo forest edge; B) Site B, 10 m into the forest; C) Site C, 15 m into the forest.

2.2. Mosquito collection

Mosquito collections were conducted on August 5, August 31, and September 9, 2009. Mosquitoes were collected using BG-Sentinel traps (Biogents, Germany) with dry ice. These traps were operated for 25 h on each sampling day. The traps were turned on every hour, each for 30 min, then stopped for identification and counting of collected mosquitoes before the next hour's collection began. All mosquitoes were released each time not to affect the population by sampling. Mmosquito population in the area was assumed to remain stable within the sampling period, and so use the number of mosquitoes collected as a measure of mosquito activity.

When the forest was disturbed by expermenter, the mosquitos greatly increased their activity. To minimize the disturbance caused by such activity, each collection day began at a different time, and to ensure all hours of the day were included, I operated the traps for 25 h, discarding the first hour's data.

3. Results

3.1. Mosquito collecting

I collected 10,806 mosquitoes representing six genera and 10 species: four species of *Culex*, two species of *Aedes*, and one species of each *Ochlerotatus*, *Armigeres*, *Tripteroides*, and *Anopheles*. Among these collected mosquitoes, *Ae. albopictus* was the most abundant, representing 83.4% of all individuals (Table 1). The largest number of mosquitoes was collected at Site C (5,285), followed by Site B (3,707), and Site A (1,814). Details are presented in Table 1.

3.2. Activity of *Ae. albopictus*

Although I collected mosquitoes for a full day and night, *Ae. albopictus* was found only from sunrise (about 08:00) to the evening (about 20:00), which confirms that *Ae. albopictus* has a diurnal activity cycle. They had a bimodal activity pattern, with a minor peak of activity at 09:00, and a major peak at 17:00. This pattern was less pronounced deeper in the forest (i.e., from site A to site C). In contrast, the number of collected mosquitoes increased with distance inside the forest (Fig. 2).

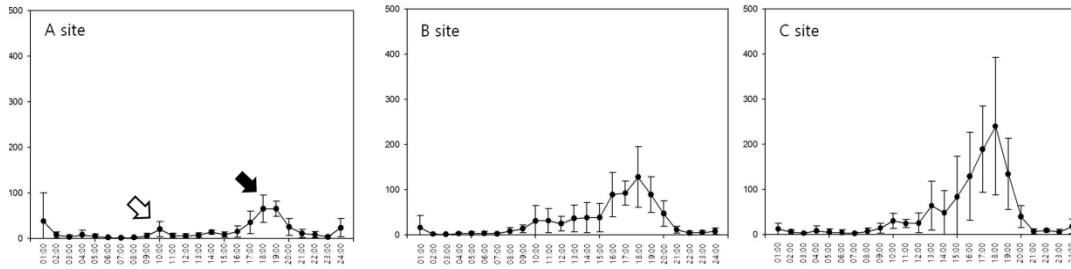

Figure 2. Number of collected mosquitoes by time. Site A shows bimodality, with two peaks in mosquito activity. The minor peak (white arrow) is at around 09:00, followed by the major peak (dark arrow) at around 17:00-18:00. Site C, where most mosquitoes were collected overall, has one broad peak. Activity at Site B was intermediate to that at Sites A and C.

Table 1. Total number of mosquitoes collected by species and sites

Species	Site A	Site B	Site C	Total	(%)
Aedes albopictus	684	1020	1,482	3,186	(84.4)
Ae. vexans	8	4	8	20	(0.5)
Culex bitaeniorhynchus	1	-	-	1	(0.0)
Cx. orientalis	1	-	-	1	(0.0)
Cx. pipiens	1	4	5	10	(0.3)
Cx. rubensis	1	-	-	1	(0.0)
Anopheles spp. ‡	-	2	1	3	(0.1)
Armigeres subalbatus	25	56	54	135	(3.6)
Ochlerotatus dorsalis	1	-	-	1	(0.0)
Tripteroides bambusa	45	155	216	416	(11.0)
Total	767	1241	1,766	3,774	(100.0)

‡ *Anopheles* spp.: includes *An. sinensis, An. lesteri, An. lindesai, An. pullus, An. sineroides, An. belenrae, An. kleini.*

3.3. The effect of light intensity on *Ae. albopictus* activity

At Site A, the first detected light intensity was 174.7 lux at 06:00 (Table 2). It increased to 82,266.7 lux at 13:00 then decreased until reaching to 0 lux at 20:00. At Sites B and C, light intensity measurements started at 80 lux and 18 lux respectively at 06:00, increasing to 10,277.0 lux and 1,240.7 respectively at 15:00. The number of mosquitoes collected at Sites B and C increased with the light intensity. In contrast, at Site A there was a different pattern: the number of mosquitoes collected increased with light intensity only until 09:00, then decreased and remained low even as light intensity continued to increase. The number of mosquitoes increased again as light began to decrease at 16:00, then decreased once again when the sun set (Fig. 3). In other words, the activity of *Ae. albopictus* at site A is induced by increasing light intensity in the early morning but is reduced by the high light intensity in the middle of the day.

At Site C, where the most mosquitoes were collected, the light intensity was much lower than at the other sites. More mosquitoes (average 239.7) were collected at 17:00, when the light intensity was 245.3 lux, than any other time (Fig. 3 and Table 2). Interestingly, this light intensity is similar to that at 09:00 (276.3 lux), when only 29.7 mosquitoes were collected on average.

From these results, we estimate a rough threshold value of light intensity at 200–300 lux. If light intensity is below this threshold, *Ae. albopictus* activity will increase with light intensity until the light intensity surpasses the threshold, at which

point their activity will decline until light intensity drops below the threshold (Figure 4).

Table 2. Temperature, relative humidity, and light intensity at the mosquito collection sites

Time	Temp (°C)	Lux, Site A	No. mosq. Site A	Lux, Site B	No. mosq. Site B	Lux, Site C	No. mosq Site C
24:00-01:00	17.9	0.0	38	0.0	16	0.0	12
01:00-02:00	17.7	0.0	7	0.0	1	12.0	6
02:00-03:00	17.1	0.0	3	0.0	1	5.7	2
03:00-04:00	16.7	0.0	7	0.0	2	0.0	8
04:00-05:00	16.4	0.0	5	0.0	3	0.0	5
05:00-06:00	16.2	0.0	2	0.0	3	0.0	4
06:00-07:00	15.6	174.7	1	80.0	2	18.0	2
07:00-08:00	16.5	2007.3	2	147.7	8	82.7	7
08:00-09:00	18.4	2946.7	5	220.0	13	149.0	14
09:00-10:00	20.5	3726.7	20	302.3	31	276.3	30
10:00-11:00	22.9	35900.0	6	431.3	31	430.0	24
11:00-12:00	24.8	63200.0	5	673.3	24	550.7	25
12:00-13:00	25.1	80166.7	7	848.7	36	648.0	63
13:00-14:00	26.6	82266.7	13	1002.3	38	814.3	48
14:00-15:00	28.1	70833.3	8	9208.3	38	792.0	83
15:00-16:00	28.3	62433.3	15	10277.0	89	1240.7	129
16:00-17:00	28.6	42500.0	35	1309.3	92	472.7	189
17:00-18:00	26.8	6920.0	65	1222.7	128	245.3	240
18:00-19:00	24.6	2163.3	65	359.7	89	91.7	134
19:00-20:00	22.5	45.7	25	3.7	47	0.3	39
20:00-21:00	21	0.0	11	0.0	11	0.0	6
21:00-22:00	20.4	0.0	8	0.0	4	0.0	9
22:00-23:00	19.3	0.0	3	0.0	4	0.0	5
23:00-24:00	18.7	0.0	23	0.0	8	0.0	18

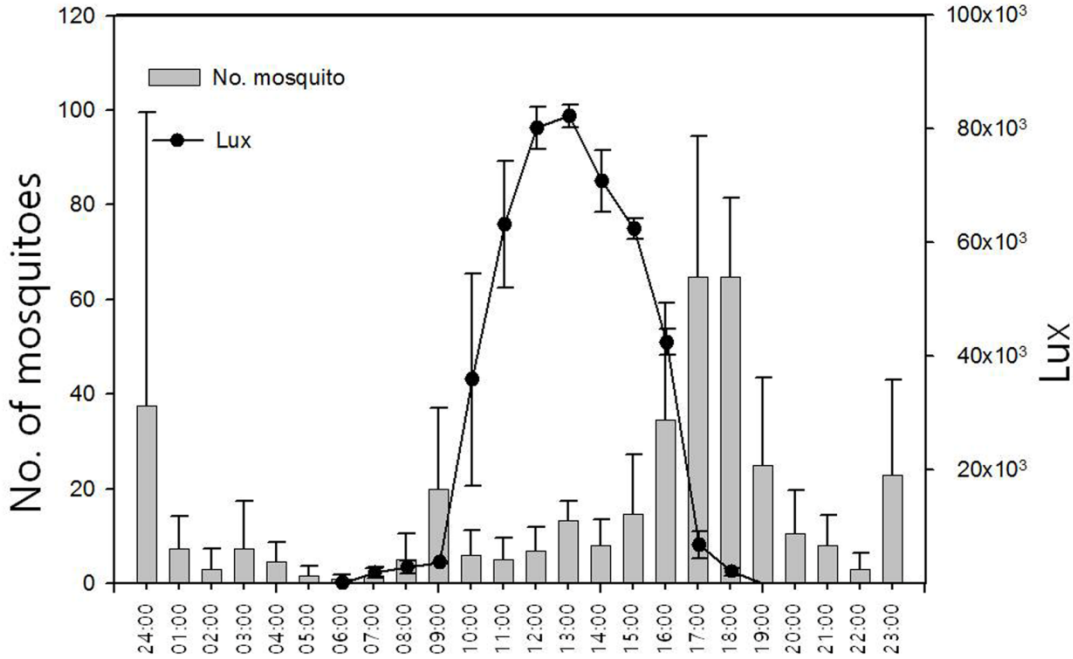

Figure 3. The effect of light intensity on the number of *Ae. albopictus* mosquitoes collected at Site A. Mosquito activity decreased under very high light intensity (from 10:00 to 15:00).

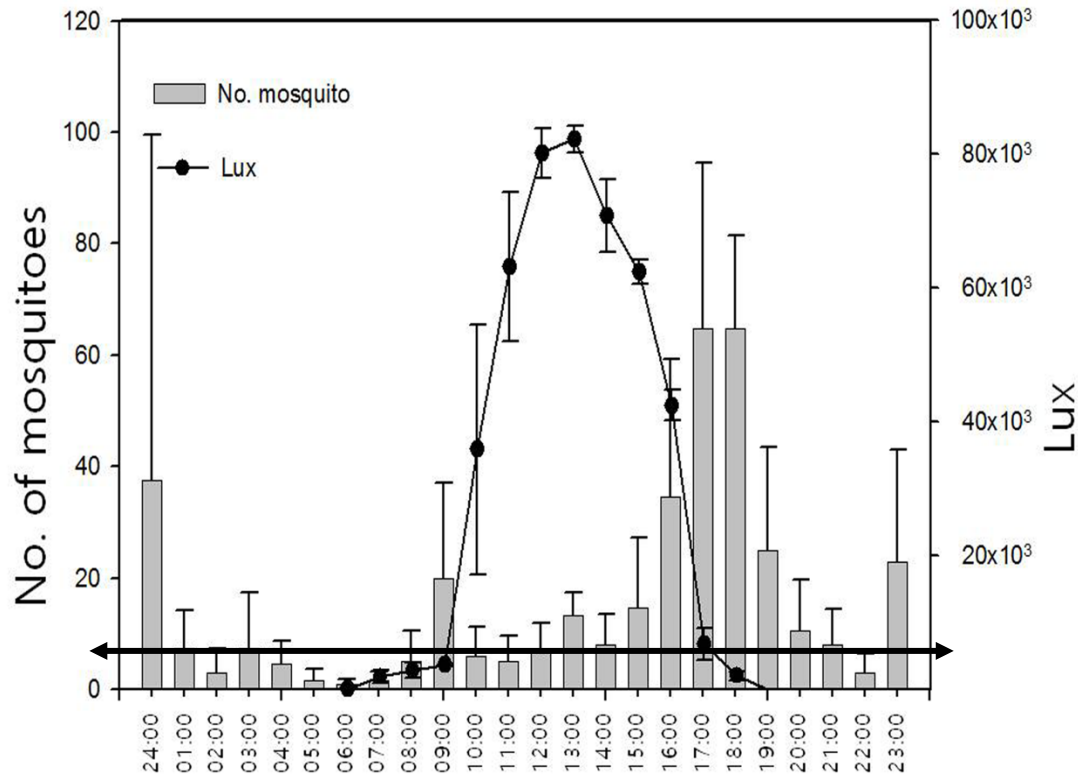

Figure 4. Estimating a hypothetical threshold of light intensity for *Ae. albopictus* at Site A. Arrow line representing the hypothetical threshold. Both the major (at 17:00) and minor activity peaks (at 09:00) are under the threshold.

3.4. The effect of temperature on *Ae. albopictus* activity

The light intensity was almost thesame at both the minor and major peaks in mosquito abundance, as 276.3 lux and 245.3 lux respectively, at Site C. However, the collected mosquitoes were significantly different. To explain the difference, we compared the temperatures at those two times. The average temperature at 09:00 was 20.5°C and at 19:00 was 26.8°C, showing 6.3°C difference (Figure 5). This temperature difference could explain the inconsistency in mosquito activity at times with similar light levels. Although temperature and the activity of *Ae. albopictus* seem to correspond well, temperature alone does not explain the bimodality of this mosquito's activity.

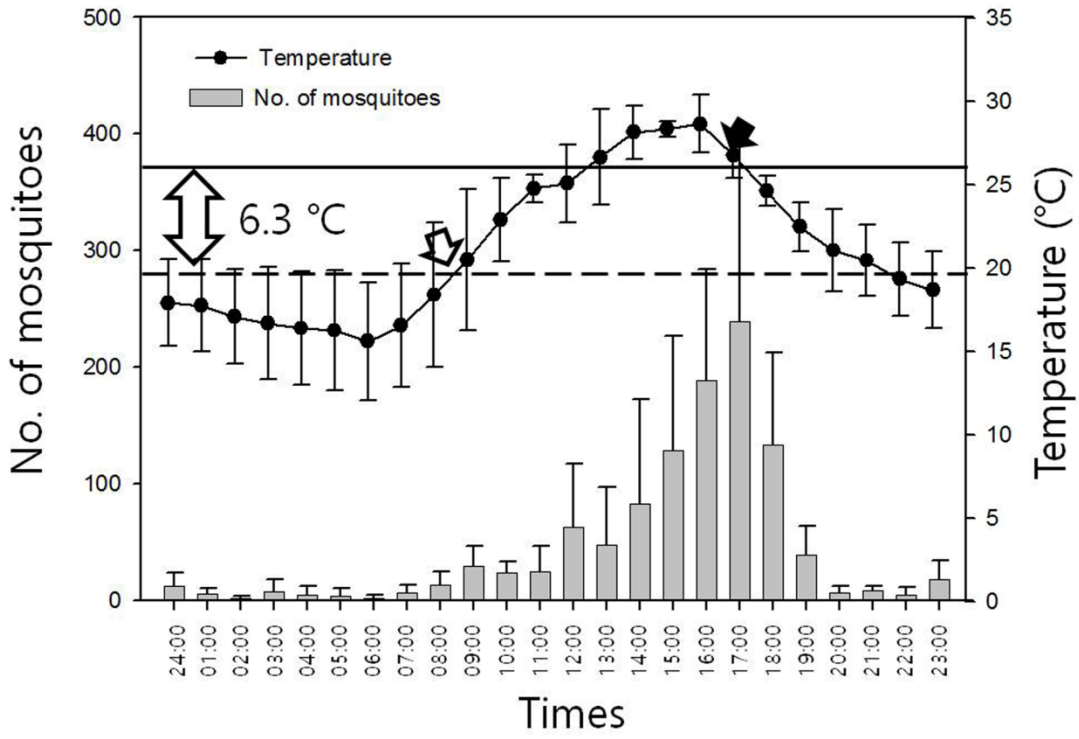

Figure 5. The effect of temperature on *Ae. albopictus* activity. The 6.3°C difference in temperature may cause the difference between mosquito activity at the two peaks (white arrow indicate the time of minor peak; dark arrow is major peak).

4. Discussion

The purpose of this study was to determine when *Ae. albopictus* seeks hosts for blood feeding, and what influences this activity pattern. Season, weather conditions, and other factors play roles in mosquito diel flight and biting activities. Most *Culex* and other common mosquito species have bimodal activity periods: from 05:00 to 06:00 and from 18:00 to 20:00 (Burkett et al., 2002). In this study, *Ae. albopictus* showed a similar diel activity pattern, with two peaks (one 08:00–09:00 and a larger one 16:00−18:00). This bimodal activity is less apparent in deep forest. This is similar to Wang's (1962) finding that an indoor population showed only a single broad period of activity in the afternoon. Per (Hawley, 1988), Macdonald and Traub (1960) found a similar pattern in forested areas.

Potentially the most significant factor is temperature, which showed an effect on *Ae. albopictus* activity. However, several studies on the behavior of *Aedes* mosquitoes have shown that visual information plays an important role in behaviors such as resting, host seeking, and oviposition (Kawada et al., 2005). Consistent with this finding, light intensity influenced the number of mosquitoes collected in this study. At site A, at the edge of the bamboo forest and therefore exposed to the most light, I see the bimodal pattern with a minor peak at 09:00 and a major peak at 16:00–18:00. I hypothesize that light may cause *Ae. albopictus* to become active, but that

when light intensity is over some threshold it may act as a deterrent. Our equipment was unable to detect small changes in light intensity. Further study of the correlation between the intensity of light and *Ae. albopictus* activity will require instruments that are more precise.

When I entered the bamboo forest, no matter the time of day or night, the number of *Ae. albopictus* attracted to me was increased for the first hour. This indicates that *Ae. albopictus* could attack animal hosts for blood feeding at any time regardless of the typical pattern of diel activity. Therefore, knowing diel activity of *Ae. albopictus* may not be enough to create strategies to avoid being bitten by this mosquito.

Finally, I consider the non-specific activity of *Ae. albopictus* at night. This non-specific activity may be related to the stage of the moon. (Davies, 1975) reported that the mosquitoes' biting activity has a relativity with moonlight. The nocturnal host-seeking activity of both *Ae. aegypti* and *Ae. albopictus* may be correlated with increasing light intensity (Kawada et al., 2005). Therefore, the non-specific activity of *Ae. albopictus* at night may be caused by the light intensity in a forest under a full moon.

Recently, there have been several reports that dengue fever has spread in downtown parks in Japan. They suspected that *Ae. albopictus* may have an important role in transmitting dengue virus. The feeding behavior and feeding preferences of vector mosquito play a key role in transmitting pathogens in vector-borne disease

(Childs, 2004). Therefore, understanding of the daily activity patterns of *Ae. albopictus*—known as a vector mosquito of dengue fever—will be a critical step in establishing strategies to prevent disease.

II-2. Host feeding patterns of *Aedes albopictus* in an urban park

Abstract

Aedes albopictus is well known for transmitting dengue and chikungunya viruses. Since it has been reported recently that Zika virus was transmitted by this *Ae. albopictus*, it has drawn the great attention worldwide. To understand the virus transmission dynamics thorough blood feeding behavior of *Ae. albopictus*, I investigated bloodemal source and detected the dengue virus infection rate from *Ae. albopictus*. I conducted this study at urban park in Seoul as one of metropolitans with high population density and many floating population, which has the possibility of dengue virus transmission. Mosquitoes were collected twice a month from May through October 2015 using BG Sentinel trap (Biogents, Germany) and InsectaZooka, (BioQuip, CA, USA). Of collected mosquitoes, all of *Ae. albopictus* were used for bloodmeal analysis and dengue virus detection. Among the other mosquito species, only blood-fed mosquitoes were used for bloodmeal source analysis. A total of 54,682 mosquitoes representing 7 genera and 12 species were collected. The primary bloodmeal source of *Ae. albopictus* was mammals (71%) followed by birds (26%), amphibians (2%) and fish (1%). The main mammalian blood source was human

(86%). This results showed that *Ae. albopictus* feed exclusively on human, representing the potential of this mosquito as a major vector of dengue virus, once this virus becomes domestic.

Key words: *Aedes albopictus*, dengue virus, bloodbmeal source

1. Introduction

Aedes albopicut, known as Asian tiger mosquito, is originated from the forest of Southeast Asia (Paupy et al., 2009). This species has spread many countries through international travel and transport of goods, particularly via the international trade of used tires in the past twenty years (Reiter and Sprenger, 1987). At the stage of establishment of this species, mosquito may be considered as a displeased pest (Benedict et al., 2007). However, this mosquito has become a significant vector for the transmission of many viral pathogens. Viral isolation and vector competence studies have shown that this mosquito has a potential of vectoring more than 20 arbovirus (Paupy et al., 2009). In place where *Ae. albopictus* and *Ae. aegypti* co-exist, *Ae. albpictus* was considered as second or regional vector of dengue and chikungunya (Hawley, 1988; Knudsen, 1995). However, its importance of vector came to light during the recent global outbreak of chikungunya (Tsetsarkin et al., 2007), autochthonous Dengue fever in Japan (Kutsuna et al., 2015b), and the possibility of Zika virus transmission (Wong et al., 2013). The concern about the possibility of dengue virus spread throughout Korea, where *Ae. albopictus* is rampant. This highlights the need for a deeper understanding of its ecological role, which is essential in determining its efficiency as a vector of pathogens.

To control this vector-borne disease, a better understanding of dynamics of these viral transmissions is crucial (Egizi et al., 2013). Vector mosquitoes usually

acquire these pathogens from feeding on an infected host, and transmit the pathogen to a naive host during subsequent feeding events. Blood feeding patterns of mosquito vector provide insight into the ecological transmission cycles of pathogens and lead to more efficient disease and vector control measures for the benefit of animal and human health (Rasgon, 2008).

Several techniques have been used to identify blood meal sources, such as hemoglobin crystallization, precipitation, enzyme-linked immunosorbent assay or ELISA (Boakye et al., 1999). However, because of the methodological limitation, including a high percentage of false positives and low specificity, PCR based techniques have been developed (Kent, 2009).The present study aims to identify blood meal sources of mosquitoes captured in urban parks surrounded by metropolitan of Seoul and estimate the risk of spreading dengue virus through the country.

2. Materials and Methods

2.1. Study sites

The study site, an urban park, was located in Seoul, Korea, a city with high population density and many floating populations. The presence of immigrants from other countries in areas around the park significantly increased the possibility of introducing pathogens such as dengue virus from abroad. Mosquitoes collected in a bamboo forest, the major habitat of *Ae. albopictus*, located in Damyang-gun, Jeonnam Province, Korea, were used as a control.

2.2. Mosquito collection and identification

Mosquitoes were collected using the BG sentinel traps with dry ice. Eight traps were placed in bushes. Trap operation stared at 14:00 and they were retrieved the following morning (about 10:00 AM). Supplementary collections were made using InsectZooka aspirators (BioQuip Products, Rancho Dominguez, CA, USA). Captured mosquitoes were placed in plastic icebox containing dry ice during transport to avoid degradation of virus during transport. The collected mosquitoes were identified using morphological characters (REE, 2003b; Tanaka et al., 1979) under a dissecting microscope, and classified into groups by species and presence or absence of blood in the abdomen. Blood-fed females were placed individually in a 2-mL tube containing four 2.5 mm-diameter glass beads. Since it was difficult to identify the level of blood in *Ae. albopictus,* the abdomen of the mosquitos was cut out with scissors and

transferred into 2-ml tubes for identification of blood meal source. To avoid cross-contamination, the scissors were sterilized with 100% ethanol after each use. Female mosquitoes that did not feed on blood were placed in another 2-mL tube and labeled by date and geographic region of their capture. Each mosquito pool (up to 50 mosquitoes) was placed in a 2-mL sterile tube containing four glass beads 2.5 mm in diameter.

2.3. Blood meal analysis

DNA was isolated from individual mosquitos and the abdomen of *Ae. albopictus* using a G-spin Total DNA Extraction kit (iNtRON Biotechnology, Sungnam, Korea) according to the manufacturer's instructions. Isolated DNA served as template in PCR reactions. PCR primers, L14841 (5′-CCA TCC AAC ATC TCA GCA TGA TGA AA-3′) and H15149 (5′-GCC CCT CAG AAT GAT ATT TGT CCT CA-3′), were designed based on mitochondrial *cytochrome b* (*cyt b*) sequences of vertebrates (Kocher et al., 1989). Amplification conditions consisted of initial denaturation for 5 min at 95°C followed by 30 cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The reaction was completed with a 10 min extension at 72°C. PCR products were visualized by electrophoresis on 2% agarose gels. The amplified PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced in both directions using the ABI PRISM BigDye Terminator Cycle Sequencing kit and an ABI 3730 xl sequencer (Applied Biosystems,

Foster City, CA, USA) at Marcrogen (Seoul, South Korea). Obtained nucleotide sequences were subjected to BLAST search to identify the origin of blood meals.

2.4. Dengue virus detection

Viral RNAs were extracted from the homogenate of pooled mosquito samples using a QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. The dengue viral RNA were analyzed using oasig Lyophilsed OneStep qRT-PCR MasterMix and genesig Dengue standard kit (PrimerDesign Ltd., Chandler's Ford, UK) (Gurukumar et al., 2009). Real time PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR Systems (Applied Biosystems, USA).

3. Results

3.1. Mosquito collection

A total of 54,682 females, representing 7 genera and 12 species, were collected in the urban park (Table 1). The predominant species was *Culex pipiens* (90.1%), followed by *Ae. albopictus* (3.6%), *Ochlerotatus koreicus* (3.5%), and *Cx. orientalis* (1.6%). In bamboo forest, a total of 1,094 females, comprising 6 genera and 9 species, were collected (Table 2). The dominant species was *Ae. albopictus* (80.5%), followed by *Tripteroides bambusa* (7.1%) and *Armigeres subalbatus* (7.1%).

Table 1. The number of collected mosquitoes at urban park in Seoul

Species	Total (%)	TI†	Blood fed mosquitoes
Culex pipiens complex§	49,317(90.1)	587.1	870
Aedes albopictus	1,995(3.6)	23.8	0*
Ochlerotatus koreicus	1,903(3.5)	22.7	6
Culex orientalis	875(1.6)	10.4	11
Coquillettidia ochracea	352(1>)	4.2	32
Culex bitaeniorhynchus	77(1>)	0.9	0
Aedes vexans	62(1>)	0.7	3
Anopheles spp.‡	31(1>)	0.4	0
Culex tritaeniorhynchus	24(1>)	0.3	1
Armigeres subalbatus	19(1>)	0.2	0
Mansonia uniformis	18(1>)	0.2	1
Culex vagans	9(1>)	0.1	0
Total	54,682	651.0	924

† Trap indecies: the number of collected mosquitoes / nights/ traps
§ *Culex pipiens* complex: includes *Cx pipiens pallens*, *Cx pipiens molestus.*
‡ *Anopheles spp*.: includes *An. sinensis, An. lesteri, An. lindesai, An. pullus, An. sineroides, An. belenrae, An. kleini.*
* Do not identify the status of blood fed. All *Ae. albopuctus* were subjected to blood meal identification experiment.

Table 2. The number of collected mosquitoes at bamboo forest in Damyang

Species	Total(%)	TI[†]	Blood fed mosquitoes
Aedes albopictus	881(80.5)	36.7	9*
Tripteroides bambusa	78(7.1)	3.3	0
Armigeres subalbatus	77(7.1)	3.2	0
Aedes vexans	29(2.6)	1.2	0
Culex tritaeniorhynchus	9(1>)	0.4	0
Ochlerotatus koreicus	9(1>)	0.4	0
Culex orientalis	8(1>)	0.3	0
Culex pipiens complex[§]	2(1>)	0.1	0
Anophels spp.[‡]	1(1>)	0.0	0
Total	1,094	45.6	9

[†] Trap indices: the number of collected mosquitoes / nights / traps

* All collectied *Aedes albopictus* were subjected to blood meal identifying experiment.
[§] *Culex pipiens* complex*:* includes *Cx pipiens pallens, Cx pipiens molestus.*
[‡] *Anopheles spp.:* includes *An. sinensis, An. lesteri, An. lindesai, An. pullus, An. sineroides, An. belenrae, An. kleini.*

3.2. Bloodmeal source identification

Among the 54,682 mosquitoes collected in the urban park, blood meal was analyzed in 925 blood fed mosquitoes and 1,995 individuals of *Ae. albopictus*. The blood meal source was identified in 388 mosquitoes (Table 3). These mosquitos belonged to *Cx. pipiens* (75.8%), *Ae. albopictus* (23.2%), *Coquillettidia ochracea* (0.5%), *Cx. orientalis* (0.3%), and *Oc. koreicus* (0.3%). The blood meal sources were of birds (73.0%), mammal (24.8%), amphibian (1.0%), and fish (1.3%) origin. Among the examined *Ae. albopictus* specimens, the blood meal source belonged to mammals (71.1%), birds (25.6%), amphibians (2.2%), and fish (1.1%). The blood meal obtained from *Cx. pipiens* originated from mammals (10.2%), birds (87.8%), amphibians (1.0%), and fish (1.0%).

In bamboo forest, 1,094 mosquitoes were collected and blood meal analysis was conducted in 881 samples of *Ae. albopictus*. Blood meal source was identified in 58 samples—it originated from mammals (humans) (96.6%) and birds (3.4%).

Table 3. Host feeding preference of mosquitoes collected in urban park in Seoul

Blood meal source			Species of mosquitoes					Total
Classify	Family	Common name	*Ae. alb*	*Cx. pip*	*Cx. ori*	*Coq. och*	*Oc. kor*	
Mammal	Human	Human	55	8	1	-	1	65
	Felinae	Cat	5	19	-	-	-	24
	Murinae	Rat	3	2	-	-	-	5
	Vespertilioninae	Bat	1	-	-	-	-	1
	Caprinae	Goat	-	1	-	-	-	1
			64	**30**	**1**	**-**	**1**	**96**
Avian	Corvidae	Crow	1	182	-	-	-	183
	Passeridae	Sparrow	7	39	-	1	-	47
	Phasianidae	Pheasant	-	3	-	1	-	4
	Columbidae	Pigeon	6	12	-	-	-	18
	Muscicapidae	Flycatcher	-	5	-	-	-	5
	Ardeidae	Heron	-	7	-	-	-	7
	Anatidae	Duck	6	-	-	-	-	6
	Pycnonotidae	Brown-Eared bulbul	1	5	-	-	-	6
	Emberizidae	Beadow bunting	-	2	-	-	-	2
	Paridae	A great tit	-	2	-	-	-	2
	Strigidae	Owl	1	-	-	-	-	1
	Picidae	Woodpeeker	-	1	-	-	-	1
	Cettiidae	A bush warbler	1	-	-	-	-	1
			23	**258**	**-**	**2**	**-**	**283**

Table 3. Host feeding preference of mosquitoes collected in urban park in Seoul (continued)

Blood meal source			Species of mosquitoes					Total
Classify	Family	Common name	*Ae. alb*	*Cx. pip*	*Cx. ori*	*Coq. och*	*Oc. kor*	
Amphibian	Hynobiidae	Salamander	2	2	-	-	-	4
	Ranidae	Frog	-	1	-	-	-	1
			2	3	-	-	-	5
Fish	Siluridae	Catfish	-	2	-	-	-	2
	Cyprinidae	Carp	-	1	-	-	-	1
	Cobitidae	Loach	1	-	-	-	-	1
			1	**3**	**-**	**-**	**-**	**4**
Total			**90**	**294**	**1**	**2**	**1**	**388**

3.3. Dengue virus detection

qRT-PCR failed to detect any virus in all of the 112 pools of mosquitoes (79 pools from the urban park, 33 pools from the bamboo forest) (Table 4).

Table 4. The results of Dengue virus detection

Sites	Month	Nm. of mosquitoes	Pool	Positive
Urban park in Seoul	May	37	6	0
	Jul	174	15	0
	Jun	315	16	0
	Aug	684	16	0
	Sep	641	14	0
	Oct	144	12	0
Bamboo forest in Damyang	Jun	189	9	0
	Aug	597	15	0
	Sep	95	9	0
Total		2,876	112	0

4. Discussion

In the present study, I identified the blood feeding pattern of *Ae. albopictus* collected in an urban park. Revealing the blood meal sources of mosquitoes is crucial for understanding the transmission network of infectious diseases and estimating the risk of pathogen transmission to humans. Studies on host preference of *Ae. albopictus* are often limited by low sample numbers of blood-fed mosquitoes. This is so because blood-fed *Ae. albopictus* have been difficult to collect (Faraji et al., 2014). I used BG-Sentinel traps, which are generally used for surveillance of host-seeking *Ae. albopictus*, but they also captured blood-fed mosquitoes. However, since it is difficult to ascertain whether *Ae. albopictus* were fully blood-fed or not, I cut the body into two parts—the abdomen was used in blood meal analysis and the other part of the body was used in virus detection.

The results of the present study represent the first data on the feeding pattern of *Ae. albopictus* in Korea. The results suggested that *Ae. albopictus* feeds predominantly on humans, which contradicts the opportunistic and zoophilic feeding behavior reported by (Paupy et al., 2009). This discrepancy in feeding behavior of *Ae. albopictus* is thought to be due to the differences in species composition of animals present in studied habitats. In deep forests with lower chance of human contact, *Ae. albopictus* is more likely to attack various animals, whereas in the presence of

humans, this species prefers people (Richards et al., 2006). In my preliminary study on blood meal source in bamboo forest (Damyoang, the right site of the present study) conducted 5 years ago, small mammals (raccoons, dogs, rats; data not show) were the main blood meal source, whereas in the present study only human blood was identified as a source blood in *Ae. albopictus*. This shift in blood meal source might be the result of the large-scale development of bamboo forest for tourism, resulting in increased contact between mosquitoes and humans. Conversely, in urban parks surrounded by the metropolitan area, the diversity of animals that mosquitos can feed on is very low, while the number of people is very high. My data indicate that in urban parks within metropolitan areas such as Seoul, where both human and mosquito populations reach very high density, *Ae. albopictus* feed almost exclusively on humans, showing the potential of this mosquito to become a major vector of dengue virus, once this virus enters the domestic territory.

The blood meals source analysis of mosquitos captured in an urban park revealed that humans were the main food source for *Ae. albopictus*. Although dengue virus was not detected in *Ae. albopictus* in the present study, several recent reports announced that dengue fever has spread in downtown parks in Japan. One of the reports suspected that *Ae. albopictus* may have had an important role in transmitting dengue virus (Kutsuna et al., 2015b). Because the city landscape and climate in Korea are similar to those in Japan, the likelihood of dengue fever appearing and spreading in Korea might be high. Therefore, continuous monitoring of this mosquito and the

rate of virus infection is important to prevent the occurrence of autochthonous dengue fever or Zika patients in Korea.

II-3 Detection of Japanese encephalitis virus (JEV) in *Aedes albopictus* collected from the high risk area

Abstract

Japanese encephalitis virus (JEV) causes significant viral encephalitis and is distributed throughout the Asian countries. The virus is known to be transmitted by *Culex tritaeniorhynchus*, which mainly breeds in rice paddies in Korea. In this study, I investigated the presence of other mosquito species that can transmit JEV as a second or regional vector. I selected five cities where patients have experienced JE in the last 5 years as mosquito-collecting locations and subdivided them into four collection sites according to the mosquito habitats (cowshed, downtown area, forest, and swamp). Mosquitoes were caught using the BG-Sentinel trap, CDC black-light trap, Fay-Prince trap, and Gravid trap. A total of 993 pools from 22,774 mosquitoes were prepared according to their species, collection date, and site. I performed a SYBR Green 1 based real-time RT-PCR assay to detect JEV from the mosquito pools. A total of six JEV-positive pools were detected from *Culex orientalis* and *Culex pipiens* caught in the Gangwon-do and Gyeonngi-do provinces. All the detected JEVs were revealed as genotype V by phylogenetic analysis of the envelope gene. Our

findings confirm that a new genotype of JEV was introduced in Korea and suggest that two mosquito species may play a role in JEV transmission.

Keywords: Japanese encephalitis virus, Korea, phylogenetic analysis, real-time RT-PCR

1. Introduction

Japanese encephalitis virus (JEV) is a mosquito-borne RNA virus in the genus *Flavivirus* (family Flaviviridae) and causes approximately 30,000–50,000 human encephalitis cases each year throughout Asian countries (Chambers et al., 1990; Solomon et al., 2003). JEV is of a circulating nature, forming a transmission cycle from mosquitoes to ardeid birds and swine, which together form the virus's reservoir, and the swine act as an amplifying host. Thereafter, the cycle continues to mosquitoes, and then to humans or some incidentally infected vertebrates. JEV has five genotypes (I–V) based on the genetic distances of the envelope gene or complete genome sequences when it has only one serotype (Solomon et al., 2003).

JEV is distributed in temperate and tropical areas of eastern and southern Asia, extending to India and Pakistan in the west. In these areas, rice irrigation is a common agricultural method. This ecosystem provides a good habitat for paddy-breeding mosquitoes such as *Culex tritaeniorhynchus*, the major vector mosquito of JEV in most parts of Asia, and other *Culex* mosquitoes that play roles as secondary or regional vectors (van den Hurk et al., 2009). Since these species share a similar ecological niche in irrigated rice paddies, JE is largely associated with rural areas (Chen et al., 2000).

In South Korea, JE has been well controlled after vaccine importation in the late 1970s. An extensive surveillance program, the JE epidemic forecast program, has been conducted since 1975 (Sohn, 2000). Through this program, vector mosquito density was monitored on a weekly basis, and the JEV was isolated from the mosquitoes. Antibody levels in unvaccinated pigs were also monitored in order to predict an epidemic. As a result, annual JE cases have been below ten after the last epidemic in 1983 (139 cases). We experienced an abrupt increase in JE cases in 2010, with five out of 26 cases occurring in Gangwon-do where *Cx. trtitaneniorhyncus* mosquitoes are rarely distributed during the JE season (August to October) (Lee et al., 2012). While health authorities investigated the reason for this increase based on mosquito density and antibody levels in pigs, they could not provide a clear explanation for the JE outbreak in an unexpected province. A possible answer was given in an article published by a US military research group in South Korea (Takhampunya et al., 2011). They detected JEV genotype V from *Culex bitaeniorhynchus* mosquitoes in north Gyeonggi-do in 2010, which was the third case following the previous reports in Malaysia and China (Li et al., 2011; Mohammed et al., 2011). In Taiwan, Chen et al suggested that the detection of a JEV antibody on a rice-free islet might be related to another potential vector mosquito found in similar ecological conditions (Chen et al., 2000). These data indicate that JEV is rampant in nature and suggest the possibility of new vector-mosquito involvements in the natural cycle of JEV.

I also reviewed the current mosquito trapping methods and collection sites. In the JE epidemic forecast program, black-light traps were mainly used to capture mosquitoes, and the collection sites are primarily near cowsheds in villages. This condition would be acceptable for the purpose of the program (to detect JEV activity near human habitats and to take timely preventive measures) but does not reflect the distribution of mosquito species in various habitats. This makes it difficult to investigate the virus activity in nature.

In this study, I investigated the presence of new mosquito species that may transmit JEV in a variety of different habitats, and using various mosquito traps.

2. Materials and Methods

2.1. Mosquito collection

Mosquitoes were collected from May through October 2012 in five cities (Ansan, Cheongju, Hwachon, Nonsan, and Yeoju) where JE patients occurred from 2007 to 2011 in South Korea (Fig. 1). Each city was subdivided into four collecting sites (cowshed, downtown area, forest, and swamp). Four different types of traps: A CDC black-light trap (John W. Hock, USA), BG-Sentinel trap (Biogents AG, Germany), Fay-Prince trap (John W. Hock, USA), and CDC-Gravid trap (John W. Hock, USA) were placed in each habitat and operated once a month from 4:00 PM to 10:00 AM. Trap indices (TI: mean number of female mosquitoes collected per trap per night) were determined for each site. Collected mosquitoes were killed by freezing and stored in an icebox containing dry ice. They were then transported to the laboratory.

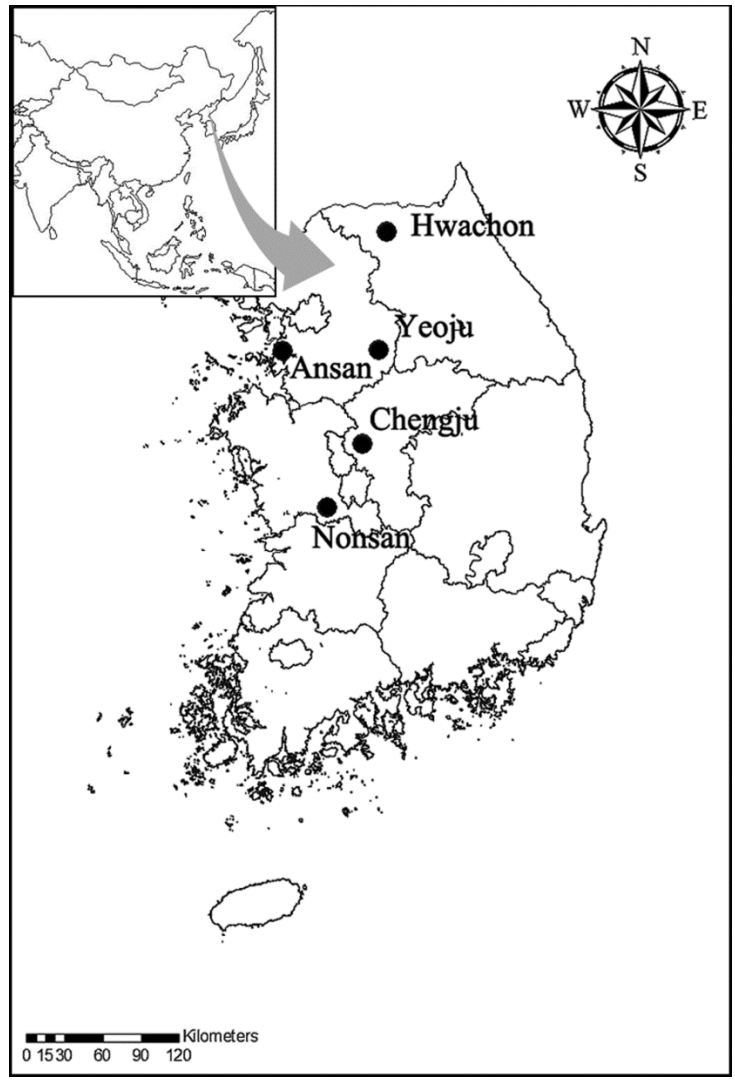

Figure 1. Locations of mosquito collection in South Korea. Mosquitoes were caught in five cities (four sites per city) once a month from May through October 2012.

2.2. Virus detection

Female mosquitoes were identified morphologically and labeled based on the date and geographic region of their capture. Each mosquito pool (up to 50 mosquitoes) was placed in a 2-ml sterile tube containing four to six 2.5-mm-diameter glass beads. Different amounts of sterile phosphate-buffered saline (PBS) were then added to each tube according to the number of mosquitoes contained therein (0.3 ml PBS for up to 10 mosquitoes, 0.6 ml PBS for 11–20 mosquitoes, and 1.2 ml PBS for 21–50 mosquitoes). Mosquitoes were homogenized using automatic equipment, FastPrep (MP Biomedicals, Solon, OH, USA), 2 times for 20 s at 5000 rpm. Homogenates were placed on ice for at least 5 min, clarified by brief centrifugation, and used for RNA extraction.

The viral RNAs were extracted from the clarified supernatant of the homogenates using a QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The RNAs were analyzed using one-step SYBR Green 1-based real-time RT-PCR with flavivirus group-specific primers detecting the NS 5 partial gene (Yang et al., 2010). Verso SYBR Green 1-Step QRT-PCR ROX (Thermo, Waltham, MA, USA) and StepOnePlus^{TM} instruments (Applied Biosystems, Foster City, CA, USA) were used for the RT-PCR assay. The reaction mixture (25 µl) contained 12.5 µl of 1-Step QRT-PCR SYBR ROX mix, 1.75 µl of each of the three primers (FL-F1, FL-R3, and FL-R4, 1µM stock), 1.25 µl of RT enhancer, 0.25 µl of verso enzyme, 0.75 µl of RNase/DNase free water, and 5 µl of

the RNA sample. The thermal cycling consisted of reverse transcription at 50°C for 30 min, activation of *Taq* polymerase at 95°C for 15 min followed by 45 cycles of PCR (94°C for 15 s, 58°C for 20 s, 72°C for 30 s). After amplification, a melting-curve analysis was performed to verify each product by its specific melting temperature. Positive reactions, including for dengue virus, Japanese encephalitis virus, yellow fever virus, and West Nile virus showed melting temperatures ranging from 81°C to 85°C. The expected size of the positive product was approximately 212 bp in agarose gel electrophoresis.

The positive products were extracted from the gels using the QIAquick gel extraction kit (QIAGEN) and sequenced with primers FL-F1, FL-R3, and FL-R4. PCR direct sequencing was done in both directions using the ABI PRISM BigDye Terminator Cycle Sequencing kits and an ABI 3730xl sequencer (Applied Biosystems) at Macrogen (Seoul, South Korea). The resulting nucleotide sequences were done via an NCBI-BLAST search to identify the exact pathogens involved.

2.3. Nucleotide sequencing and phylogenetic analysis

I designed five set of primer to obtain the complete envelope gene for JEV-positive pools (Table 1). The primers were designed manually by comparing two sequences of JEV genotype V (XZ0934 and 10-1827) available from GenBank. The eight microliters of RNA extracted from mosquito homogenate were reverse transcribed with a random hexamer using the SuperScript III First-Strand Synthesis

System (Invitrogen, CA, USA) according to the manufacturer's protocol. Then, synthesized cDNA was amplified with each primer set using the DyNAzyme^{TM} EXT DNA Polymerase. The composition of reaction mixture was 5 µl of 10× optimized buffer, 1 µl of dNTP mix, 2.5 µl of each of the forward and reverse primers (10 µM stock), 1 µl of DNA Polymerase, 36 µl of RNase/DNase free water, and 2 µl of the cDNA template. The thermal profile for the PCR was as follows: pre-denaturation at 94°C for 2 min, 40 cycles of 94°C for 30 s, 60°C for 20 s (55°C for primer set of 1240F and 1734R), 72°C for 20 s, and a final extension cycle at 72°C for 3 min. The amplified fragments were visualized in agarose gel and extracted for nucleotide sequencing. PCR direct sequencing was done at Macrogen (Seoul, South Korea) as described in the section above. The resulting sequence files were compiled using the SeqMan program in the Lasergene software version 8.0 (DNASTAR, WI, USA) and the final 1,500 bp of the envelope gene sequences were obtained. The nucleotide sequences were compared with those of 38 other JEV strains deposited in the GenBank (Table 2). The phylogenetic analyses were performed using MEGA 6.06 (Tamura et al., 2013). A multiple alignment was generated with the MUSCLE program (Edgar, 2004) in MEGA and the best evolutionary model for estimating genetic distances between sequences was estimated using the 'Models' function in MEGA. Phylogenetic trees were inferred by both distance- and character-based methods. For the distance method, a Neighbor Joining (NJ) tree was constructed using the evolutionary model of Tamura-Nei and the rate variation among sites was

estimated from a gamma distribution (shape parameter = 5). The reliability of the tree was tested by bootstrap methods with 1,000 replications. For the character-base method, a Maximum Likelihood (ML) tree was constructed using the same substitution model and gamma distribution. The envelope gene sequence of the West Nile virus was used as the out-group (B956strain, GenBank accession number: NC_001563).

Table 1. Primers for amplification of the complete envelope gene of JEV genotype V

Primer	Sequence (5' -3')	Position†	Polarity	Size (bp)
69F	TGCAAACCCACGGAGAA	763-779	Forward	501
550R	GCCTTGCTTGCAGACATAG	1262-1244	Reverse	
373F	CCTCACTATCATGGCGAACGACA	1067-1089	Forward	557
908R	CGCGATGGACTAGGAACGACTTA	1623-1601	Reverse	
819F	AGCTTGGAGATTACGGAGAGGTCA	1513-1536	Forward	572
1370R	CTTCGAATTGGCGGTGGATGT	2084-2064	Reverse	
1240F	TGGTACGGTTGTCATAGAA	1934-1952	Forward	512
1734R	CACCTCCTGTAGCAAGAA	2445-2428	Reverse	
1622F	GGAGCTTTCAGAACCCTTTTTG	2316-2337	Forward	378
1980R	CCTGACGGCTTCCCACATTT	2693-2674	Reverse	

† The nucleotide position was based on the JEV XZ0934 strain (Genotype V, GenBank accession number, JF915894).

Table 2. Details of the Japanese encephalitis viruses used in the phylogenetic analysis

Strain	**Geno type**	Location	Sampling year	GenBank accession number.
YN86-B8639	**1**	China	1986	DQ404133
SH80	**1**	China	2001	JN381848
3XG009	**1**	China	2011	JX514950
IND/11/WB/JEV45	**1**	India	2011	KC526872
99P104	**1**	Japan	1999	FJ943474
09P123	**1**	Japan	2009	GU108334
K94P05	**1**	South Korea	1994	U34929
K01-JN	**1**	South Korea	2001	FJ938222
K05-GS	**1**	South Korea	2005	FJ938223
A10.825	**1**	South Korea	2010	JN587259
K10CT661	**1**	South Korea	2010	JX018150
TC2009-11	**1**	Taiwan	2009	JF499801
CY2010-3	**1**	Taiwan	2010	JF499824
ThCMAr4492	**1**	Thailand	1992	D45362
JE_CM_1196	**1**	Thailand	2005	DQ238602
90VN70	**1**	Vietnam	1990	HM228921
VN88	**1**	Vietnam	2001	AY376464
07VN310	**1**	Vietnam	2007	HM228922
FU	**2**	Australia	1995	AF217620
JKT5441	**2**	Indonesia	1980-Jun	JQ429306
BN19	**3**	China	1982	FJ185038
YN03-A151	**3**	China	1998	DQ404136
SCDJY01	**3**	China	2011	JX045833
GP78	**3**	India	1978	AF075723
IND/12/WB/JEV50	**3**	India	2012	KC526871
JaOArS982	**3**	Japan	1982	M18370
JaNAr0290	**3**	Japan	1990	AY427794
K88A071	**3**	South Korea	1988	FJ938228
K94A071	**3**	South Korea	1994	FJ938217
CH1392	**3**	Taiwan	1990	AF254452
YL0506a	**3**	Taiwan	2005	GQ260611
HL0805a	**3**	Taiwan	2008	GQ260628
VN207	**3**	Vietnam	1986	AY376461
04VN75	**3**	Vietnam	2004	HQ009263
JKT6468	**4**	Indonesia	1981	AY184212
XZ0934	**5**	China	2009	JF915894
Muar	**5**	Malaysia	1952	HM596272
10-1827	**5**	South Korea	2010	JN587258

3. Results

3.1. Mosquito collection

A total of 20,774 mosquitoes representing 9 genera and 20 species were collected, including 9 species of *Culex*, 2 species of *Aedes*, 3 species of *Ochlerotatus*, and *Armigeres*, *Coquillettidia*, *Culiseta*, *Mansonia*, *Tripteroides*, and *Anopheles* complex. The predominant species were *Cx. pipiens* (44.7%) and *Ae. vexans* (18.0%). Details are presented in Table 3.

The TI of all traps (Table 4) during the study period was 49.1, ranging from a low of 0.9 (Hwachon, at GV) to a high of 337.5 (Ansan, at BG) female mosquitoes per trap night.

Table 3. Total number of mosquitoes collected at 5 cities in South Korea

Species	Cheongju (%)		Nonsan (%)		Ansan (%)		Yeoju (%)		Hwacheon (%)		Total (%)	
Culex bitaeniorhynchus	6	(0.1)	2	(0.1)	34	(0.4)	8	(0.4)			50	(0.2)
Culex hayshii							3	(0.1)	1	(0.1)	4	(<0.1)
Culex inatomii			2	(0.1)	468	(4.9)					470	(2.3)
Culex mimeticus			1	(<0.1)							1	(<0.1)
Culex orientalis	83	(2.1)	73	(2.3)	31	(0.3)	264	(12)	47	(2.6)	498	(2.4)
Culex pipiens	1,155	(28.7)	757	(23.8)	6,254	(65.5)	917	(41.5)	208	(11.5)	9,291	(44.7)
Culex rubensis	1	(<0.1)									1	(<0.1)
Culex tritaeniorhyncus	2	(<0.1)	3	(0.1)	1	(<0.1)	4	(0.2)			10	(<0.1)
Culex vagans	3	(0.1)			2	(<0.1)					5	(<0.1)
Aedes albopictus	110	(2.7)	180	(5.7)	191	(2)	73	(3.3)	9	(0.5)	563	(2.7)
Aedes vexans	1,135	(28.2)	928	(29.1)	164	(1.7)	602	(27.3)	917	(50.8)	3,746	(18)
Ochlerotatus dorsalis					6	(0.1)					6	(<0.1)
Ochlerotatus koreicus	544	(13.5)	5	(0.2)	63	(0.7)	7	(0.3)	7	(0.4)	626	(3)
Ochlerotatus nipponicus									2	(0.1)	2	(<0.1)
Anopheles spp.†	353	(8.8)	846	(26.6)	84	(0.9)	210	(9.5)	552	(30.6)	2,045	(9.8)
Armigeres subalbatus	551	(13.7)	373	(11.7)	31	(0.3)	117	(5.3)	60	(3.3)	1,132	(5.4)
Coquillettidia ochracea	11	(0.3)			104	(1.1)					115	(0.6)
Culiseta bergrothi					1	(<0.1)					1	(<0.1)
Mansonia uniformis	59	(1.5)			2,116	(22.2)	1	(<0.1)			2,176	(10.5)
Tripteroides bambusa	15	(0.4)	14	(0.4)			2	(0.1)	1	(0.1)	32	(0.2)
Total	4,028	(100)	3,184	(100)	9,550	(100)	2,208	(100)	1,804	(100)	20,774	(100)

†*An. spp*: includes *An. sinensis, An. lesteri, An. lindesai, An. pullus, An. sineroides, An. belenrae, An. kleini*

Table 4. Total number of female mosquitoes collected at mosquito collecting cities with four traps during May to October in 2012

Cities	Name of traps	No. trap	Mosquitoes	No. species	Trap nights	TI†
Cheongju	BL	4	250	9	63	10.4
	BG	4	1,821	13	455	75.9
	FP	4	1,913	12	478	79.7
	GV	4	41	5	10	1.7
Nonsan	BL	4	1,352	9	338	56.3
	BG	4	972	11	243	40.5
	FP	4	801	9	200	33.4
	GV	4	59	6	15	2.5
Ansan	BL	3	65	9	22	3.6
	BG	3	6,075	15	2,025	337.5
	FP	3	3,363	12	1,121	186.8
	GV	3	47	5	16	2.6
Yeoju	BL	4	96	5	24	4.0
	BG	4	957	10	239	39.9
	FP	4	1,125	11	281	46.9
	GV	4	31	6	8	1.3
Hwachon	BL	4	1,130	7	283	47.1
	BG	4	306	8	77	12.8
	FP	4	349	9	87	14.5
	GV	4	21	6	5	0.9
Total		76	20,774	20	273	49.9

†TI (Trap Index): Average number of mosquitoes per trap night.

BL: Black light trap, BG: BG sentinel trap, FP: Fay prince trap, GV: Gravid trap

3.2. Virus detection and phylogenetic analysis

Of the 933 pools of mosquitoes tested by real-time RT-PCR, 6 JEVs and 2 chaoyang viruses were detected from 2 species of *Culex* and *Aedes vexans* mosquitoes, respectively (Table 5). In detail, JEVs were detected in *Cx. orientalis* and *Cx. pipiens* mosquitoes caught in mid August to early September in Hwacheon, Ansan, and Yeoju cities (Table 6). The JEV-positive mosquitoes were caught at all the four habitats (cowshed, downtown area, forest, and swamp) using three collecting traps except the Gravid trap. It is notable that the RT-PCR protocols used in this study allowed the detection of JEV harbored in only one of five mosquitoes (Table 6). The chaoyang virus was detected in the *Ae. vexans* mosquito, which is the dominant species throughout the country. I did not further analyze this virus because it was outside the scope of this study.

The nucleotide sequences of six JEVs showed sequence similarity ranging from 99.5% to 100% based on the NS 5 gene (180–200 nucleotides). When an NCBI-BLAST search was done for each sequence, the highest hit was the sequence of JEV genotype V. To confirm the genotype, I amplified five fragments covering the complete envelope gene using genotype specific primers. I named the resultant sequences according to the country, year, location and sample number. For example, in the sequence named K12HC959, K means Korea, 12 is a two-digit marking of the sampling year, HC is the abbreviation of location and 959 is the sample number. Among the six JEVs, four pools were successful in having their complete envelope

gene sequenced. Only partial sequences around 490 nucleotides were obtained for K12YJ1174 and K12YJ1182 (Table 6). Instead of the two partial sequences, I used K12YJ1203 as a representative sequence which was from the same mosquito species, but acquired on 6 September in Yeoju. An NCBI-BLAST search showed all the envelope gene sequences were best matched to those of JEV genotype V.

When phylogenetic analyses were performed with other JEV strains representing each genotype and geography, Korean JEVs were able to be divided into genotypes I, III, and V on the NJ tree (Figure 2). Both the NJ and ML trees showed the same genotype classification, albeit with differences in branch lengths and bootstrap values. All the JEVs detected in this study were grouped into genotype V, together with the findings of Muar (1952, Malaysia), XZ0934 (2009, China) and 10-1827(2010, Korea). I edited the original tree to make the relationship between the Korean JEV genotype V sequences clear. Because branch length from K12AS1151to its ancestral node was zero in the original tree, I moved the descendant node (K12AS1151 and 10-18927) back to its ancestral node (Figure 2). Genotype changes in Korean JEVs were shown in 1994 (genotype III → I) and in 2010 (genotype I → V) in the tree. Four JEV sequences obtained in this study showed a nucleotide sequence similarity of 99.1%–99.7% and an amino acid sequence similarity of 99.2%–100% (Table 7). When all seven genotype V sequences in the world were compared to each other, the sequence similarity was 89.9%–99.9% and 95.2%–100% at the nucleotide and amino acid sequence levels, respectively. The genotype V group showed mean

sequence divergence ranging from 21.9%–22.8% against the other four genotypes, while those genotypes showed mean sequence divergence of 10.8 – 18.2 % each other.

Table 5. Results of flavivirus detection from field-caught mosquitoes

Species	Total	(%)	Tested pool*	JEV	Other Flavivirus
Culex bitaeniorhynchus	50	(0.2)	16	0	0
Culex hayshii	4	(0.0)	2	0	0
Culex inatomii	470	(2.3)	16	0	0
Culex mimeticus	1	(0.0)	1	0	0
Culex orientalis	498	(2.4)	83	5	0
Culex pipiens	9,295	(44.7)	264	1	0
Culex rubensis	1	(0.0)	1	0	0
Culex tritaeniorhyncus	10	(0.0)	7	0	0
Culex vagans	5	(0.0)	2	0	0
Aedes albopictus	564	(2.7)	64	0	0
Aedes vexans	3,744	(18.0)	168		2‡
Ochlerotatus dorsalis	6	(0.0)	4	0	0
Ochlerotatus koreicus	625	(3.0)	70	0	0
Ochlerotatus nipponicus	2	(0.0)	NT	-	-
Anopheles spp.†	2,045	(9.8)	NT	-	-
Armigeres subalbatus	1,132	(5.4)	145	0	0
Coquillettidia ochracea	115	(0.6)	14	0	0
Culiseta bergrothi	1	(0.0)	1	0	0
Mansonia uniformis	2,176	(10.5)	66	0	0
Tripteroides bambusa	30	(0.1)	9	0	0
Total	20,774		933	6	2

†*Anopheles* spp.: Includes *An. sinensis, An. lesteri, An. lindesai, An. pullus, An. sineroides, An. belenrae, An. kleini; An. sinensis* was not used in the virus survey.
*Tested pools: ≤50/1pool, separated by locality and time.
‡ Identified as Chaoyang virus by sequencing analysis and NCBI-BLAST search (data not shown).
NT: Not tested

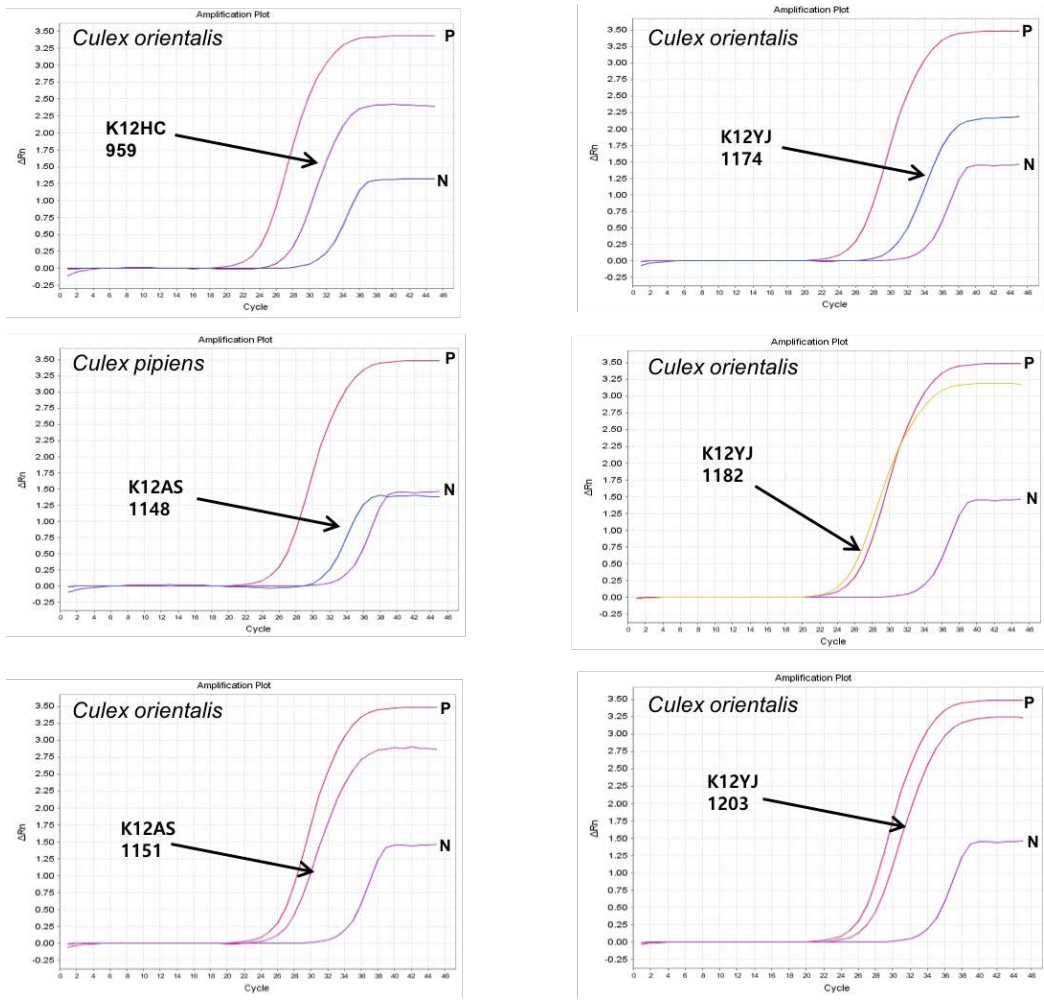

Figure 2. Results of real-time PCR amplification of Japanese encephalitis viruses (from field collected mosquitoes. K12HC959 is indicating the *Cx. orientalis* collected at Hwacheon; K12AS1148 is *Cx. pipiens* at Ansan; K12AS1151 is *Cx. orientalis* at Ansan; K12YJ1174 is *Cx. orientalis* at Yeoju; K12YJ1182 is *Cx. orientalis* at Yeoju; K12YJ1203 is *Cx. orientalis* at Yeoju. P is indicating positive control and N is negative control.

Table 6. Japanese encephalitis viruses detected from mosquitoes in this study

Code	Mosquito species (numbers/pool)	Collection date	Location	Trap †	GenBank Accession no.‡
K12HC959	*Culex orientalis* (12)	2012-08-16	Hwacheon (swamp)	BL	KJ420589
K12AS1148	*Culex pipiens* (50)	2012-08-29	Ansan (swamp)	BG	KJ420590
K12AS1151	*Culex orientalis* (5)	2012-08-29	Ansan (swamp)	BG	KJ420591
K12YJ1174	*Culex orientalis* (46)	2012-09-06	Yeoju (cowshed)	FP	KJ420593
K12YJ1182	*Culex orientalis* (30)	2012-09-06	Yeoju (forest)	BG	KJ420594
K12YJ1203	*Culex orientalis* (1)	2012-09-06	Yeoju (downtown)	BG	KJ420592

† BL: CDC Black-Light trap, BG: BG Sentinel trap, FP: Fay-Prince trap
‡ K12YJ1174 and K12YJ1182 are partial length of envelope gene. Others are complete length of 1,500 nts.

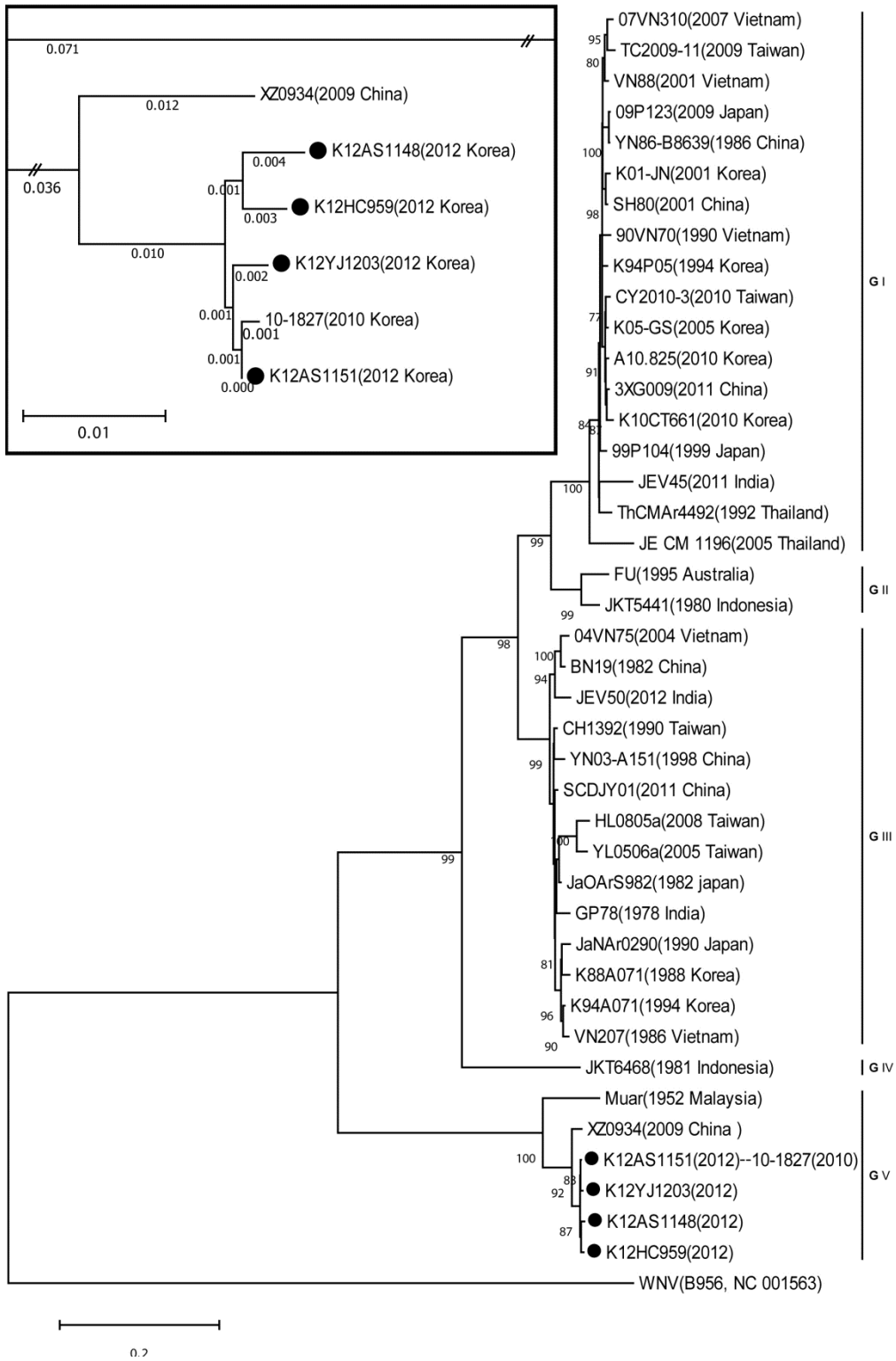
0.071
XZ0934(2009 China)
0.012
K12AS1148(2012 Korea)
0.004
0.036
0.001
K12HC959(2012 Korea)
0.003
0.010
K12YJ1203(2012 Korea)
0.002
0.001
10-1827(2010 Korea)
0.001
0.001
K12AS1151(2012 Korea)
0.000
0.01
07VN310(2007 Vietnam)
95
TC2009-11(2009 Taiwan)
80
VN88(2001 Vietnam)
09P123(2009 Japan)
YN86-B8639(1986 China)
100
K01-JN(2001 Korea)
SH80(2001 China)
98
90VN70(1990 Vietnam)
K94P05(1994 Korea)
CY2010-3(2010 Taiwan)
77
K05-GS(2005 Korea)
A10.825(2010 Korea)
91
3XG009(2011 China)
K10CT661(2010 Korea)
84
83
99P104(1999 Japan)
JEV45(2011 India)
100
ThCMAr4492(1992 Thailand)
JE CM 1196(2005 Thailand)
99
FU(1995 Australia)
JKT5441(1980 Indonesia)
99
98
04VN75(2004 Vietnam)
100
BN19(1982 China)
94
JEV50(2012 India)
CH1392(1990 Taiwan)
99
YN03-A151(1998 China)
SCDJY01(2011 China)
HL0805a(2008 Taiwan)
100
YL0506a(2005 Taiwan)
99
JaOArS982(1982 japan)
GP78(1978 India)
JaNAr0290(1990 Japan)
81
K88A071(1988 Korea)
96
K94A071(1994 Korea)
VN207(1986 Vietnam)
90
JKT6468(1981 Indonesia)
Muar(1952 Malaysia)
XZ0934(2009 China)
100
K12AS1151(2012)--10-1827(2010)
88
92
K12YJ1203(2012)
87
K12AS1148(2012)
K12HC959(2012)
WNV(B956, NC 001563)
0.2
G I
G II
G III
G IV
G V

Figure 3. Phylogenetic analysis of Japanese encephalitis virus (JEV) based on the complete envelope gene (1,500 nt) from 42 strains representing each genotypes and countries. The Maximum-Likelihood tree was constructed with a substitution model of Tamura-Nei plus gamma distribution using MEGA software 6.06. West Nile virus (WNV, B956 strain, NC_001563) was used as the outgroup in the tree. Branch reliability is indicated by the percentage of bootstrap values at each node (1,000 replications). The scale bar indicates the number of base substitutions per site. JEVs detected in this study were marked with a closed circle. The left side of the tree is omitted for ease of understanding. In a small rectangle, the topology of genotype V sequences in the original tree is presented. The branch length between K12AS 1154 and its ancestral node is zero and is edited to make the ancestor-descendant relationship clear.

Table 7. Nucleotide sequence similarity and divergence of envelope gene among JEV genotype V

	Muar	XZ0934	10-1827	K12AS1148	K12AS1151	K12HC959	K12YJ1203
Muar		90.2	89.9	90.1	90.1	89.9	89.9
XZ0934	9.8		97.3	97.0	97.5	97.1	97.3
10-1827	10.1	2.7		99.1	99.9	99.3	99.5
K12AS1148	9.9	3.0	0.9		99.3	99.2	99.1
K12AS1151	9.9	2.5	0.1	0.7		99.4	99.7
K12HC959	10.1	2.9	0.7	0.8	0.6		99.2
K12YJ1203	10.1	2.7	0.5	0.9	0.3	0.8	

The upper right of diagonal shows percentage sequence similarity and the lower left of diagonal shows sequence divergence (p-distance).

4. Discussion

In this study I detected six JEVs from *Cx. orientalis* and *Cx. pipiens* mosquitoes. These results may provide answers to the question of why JE patients have increased abruptly since 2010 and why the outbreaks have occurred in an unexpected province where the density of *Cx. tritaeniorhynchus* mosquitoes was very low throughout the JE season. Recently, two research groups in Italy and Korea have reported that *Cx. pipiens* mosquitoes may transmit JEV (Ravanini et al., 2012; Seo et al., 2013). In addition, JEV was isolated from *Cx. pipiens* which were collected during winter in Korea in the early 1970s. Despite the low vector competence that *Cx. pipiens* displays in laboratory studies (Turell et al., 2006), our study strongly supports the previous finding that this mosquito is infected by JEV, and may transmit the virus.

Here, the name *Cx. pipiens* is used to represent *Cx. pipiens pallens* Coquillett. In Korea, the *Cx. pipiens* complex consists of two species of mosquitoes, *Cx. pipiens pallens* and *Cx. pipiens molestus* Forskal. However, it is agreed that *molestus* is not a subspecies, but rather a strain of *Cx. pipiens pallens* (Ree, 2003a).

This is the first report of JEV detection in a *Cx. orientalis* mosquito that was distributed in Far-East Asia, including China, Japan, Korea, Siberia, and Taiwan. However, this mosquito is not likely to cause a large impact JE outbreak because of its bionomics. The larvae of this species live strictly in fresh water, such as slowly

moving ponds and streams, on mountains in Korea, and the adults apparently do not feed on humans (Tanaka et al., 1974). However, *Cx. pipiens* is the most common mosquito in human dwelling areas in Korea. The larvae of this mosquito occur in a very wide variety of artificial containers, or other types of stagnant water, such as ditches, gutters, ground pools, etc. They prefer polluted water containing abundant organic matter (Tanaka et al., 1974). The adult mosquito is commonly considered to be an avian blood feeder, but a recent study has shown that the mammal feeding rate was comparatively high (Hamer et al., 2008). Therefore, the role of *Cx. pipiens* and *Cx. orientalis* in the transmission of JEV should be studied urgently.

In this study virus isolation was not attempted because dead mosquitoes were stored for several weeks before the RT-PCR. According to our long-term experience, and another paper (Johansen et al., 2002), virus recovery could not be expected in such a circumstance. Instead, I sequenced the complete envelope gene using genotype specific primers. Interestingly, all the JEVs detected in this study belonged to genotype V, which was the second report in Korea to do so, after a US military research group detected it in 2010 in north Gyeonggi-do province (Takhampunya et al., 2011). Historically, genotype III was the dominant strain worldwide until the latter part of the 20th century. Then, a JEV genotype shift from type III to I was reported in many regions, and genotype I became recognized as the dominant strain in many countries (Nga et al., 2004). Genotype V, on the other hand, is a very rare strain that was first reported from an encephalitis patient in Malaysia in 1952 (Mohammed

et al., 2011) and then in 2009 in China (Li et al., 2011). In Korea, JEV genotype III strains were also dominant until the genotype I was first isolated in 1994. Since then, only genotype I strains were isolated until 2010 (Yun et al., 2010). The pathogenic properties of the genotype V strain have only rarely been documented, and there is no relevant information on whether current genotype III-based vaccines could protect against this new genotype. Thus, I could not determine the effects of the emergence of this new genotype on the current JE outbreak in South Korea.

JEV vector surveillance is one of the most important tools for providing information on the distribution, intensity, and abundance of circulating viruses that can be used to create strategies for public health (Seo et al., 2013). As a mosquito collection tool, we traditionally used a black-light trap in cowsheds or downtown areas. However, this trap attracts only some of the dominant species of mosquitoes, and consequently failed to collect the full diversity of species distributed in the natural ecosystem. Therefore, I tried four different trapping methods in various habitats. The number of collected mosquito species has showed a strong contrast by trap type (Table 4). This strategy proved to be a success in that JEV-positive pools were collected in both forests and swamps as well as the traditional sampling sites, and four out of six positive pools were collected with a BG sentinel trap. To accurately understand the circulation of viruses in nature, I recommend the collection of mosquitoes in various habitats.

In conclusion, I found that two new *Culex* mosquitoes, *Cx. orientalis* and *Cx. pipiens* may transmit JEVs as a secondary or regional vector. Additionally, I confirmed that JEV genotype V strains were disseminated in at least the Gangwon-do and Gyeonggi-do provinces. These findings broaden our knowledge on the vector diversity of JEV and highlight the need to review the current vector surveillance protocol in Korea.

II-4. *Wolbachia* infection rate in *Aedes albopictus* collected from different geographical locations in Korea

Abstract

Recently, several studies of the intracellular endosymbiont bacteria of the genus *Wolbachia* have focused on the control of disease vector mosquitoes, such as those of the genera *Aedes* and *Anopheles*. In this study, I aimed to analyze the regional distribution of *Wolbachia* in the Republic of Korea. I distinguished nine different regions of the Republic of Korea based on mountain chains and waterways, and collected *Aedes albopictus* vector mosquitoes from each region. Whole genomic DNA was extracted from each collected specimen, and PCR analysis and sequencing were conducted for detection and identification of *Wolbachia* based on WSP and *w*AlbA, respectively. Over 99% of the collected mosquitoes harbored *Wolbachia*, and the sequence homologies of the WSP gene showed more than 98% similarity within the mosquito species. *Ae. albopictus* was found to be infected with two *Wolbachia* strains, *w*AlbA and *w*AlbB. Regional distribution analysis indicated that the *w*AlbA strain of *Wolbachia* showed more than 98% sequence similarity in *Ae. Albopictus* from different regions. To our knowledge, this study represents the first report of

Wolbachia infection and strains in Korea, and demonstrates that the endosymbiont possesses high sequence homology.

Keywords: *Wolbachia*, *Aedes albopictus*, vector mosquito, regional distribution

1. Introduction

Dengue fever is the most important arboviral disease in humans; 40% of the world population, in more than 100 countries, is at risk of infection, and an estimated 50–100 million cases occur annually (Guzman and Kouri, 2002; Iturbe-Ormaetxe et al., 2011; Kyle and Harris, 2008). Dengue virus (DENV) is primarily transmitted by the bite of female *Aedes aegypti*, and to a much lesser extent, *Aedes albopictus* mosquitoes (Lambrechts et al., 2010). Additionally, the Chikungunya virus (CHIKV) is a major public health problem, which is an alphavirus transmitted by *Ae. albopictus* mosquitoes (Nasci, 2014; Tsetsarkin et al., 2014).

The intracellular endosymbiont bacterium *Wolbachia* is currently considered most abundant in arthropods, but it has also been isolated from nematodes, amphipods, isopods, mites, and spiders (Werren, 1997; Zeh et al., 2005). Many *Wolbachia* species manipulate host reproductive systems, leading to male-killing, cytoplasmic incompatibility (CI), parthenogenesis, and the feminization of genetic males, with large impacts on host ecology and evolution in arthropods (Harris and Braig, 2003; Iturbe-Ormaetxe and O'Neill, 2007; Walker et al., 2011; Werren et al., 1995). *Wolbachia* are particularly prevalent in the female germline throughout adulthood, ensuring high-fidelity *Wolbachia* transmission to the eggs produced by infected females (Dedeine et al., 2005; Dedeine et al., 2001; Serbus et al., 2008).

Many studies have focused on characterization of the CI phenotypes of *Wolbachia*; endosymbiont *Wolbachia* shows similar phylogenetic diversity between bacterial and their hosts (Werren et al., 2008).

Thus, investigation of *Wolbachia* infection status and the identification of genetic diversity in natural populations can be crucial for *Wolbachia*-based vector control strategies (Werren et al., 2008; Werren et al., 1995). Recently, research on *Wolbachia* has focused on the control of the population of disease vector mosquitoes belonging to the genera *Aedes* and *Anopheles*, which carry dengue fever and malaria, respectively (Cook et al., 2008; Kitrayapong et al., 2002; Lambrechts et al., 2006).

Wolbachia strains are classified by their places in molecular phylogenies, which define 14 different groups (Fenn and Blaxter, 2006; Lo et al., 2002). The majority of arthropod *Wolbachia* belong to groups A and B. Groups E, F, and G have been described from only a restricted set of crustacean, chelicerate, hexapod, and nematode hosts (Fenn and Blaxter, 2006). The *Wolbachia* Genome Consortium, held in 1999, developed a plan to sequence representative genomes covering the diversity of *Wolbachia* (Slatko et al., 1999). The other supergroups seem to be related with their host lineages, such as supergroup H from termites, and M and N from aphids (Augustinos et al., 2011; Wang et al., 2014).

To evaluate *Wolbachia* strains, genes encoding the surface protein of *Wolbachia* (wsp) have been used for sequencing analysis with different *Wolbachia* strains (Baldo and Werren, 2007; Zhou et al., 1998). Population of natural *Ae.*

albopictus was superinfected with two *Wolbachia* strains such as *w*AlbA and *w*AlbB (Werren et al., 1995; Zhou et al., 1998). The *w*AlbA strain has previously been reported to show a lower rate of maternal transmission because of *w*AlbB strain was known as higher density than *w*AlbA (Dutton and Sinkins, 2004). The whole *Wolbachia w*AlbB genome has previously been reported, consisting of 165 contigs encompassing 49 scaffolds. The genome contains 1,239,814 bp and has an average GC content of 33.7%. It contains 1,209 predicted protein-coding sequences with an average length of 849 bp, 34 tRNA genes, and one rRNA copy split into two operons (16S and 5S-23S). Based on the phylogenetic analysis of 52 orthologous ribosomal proteins retrieved from four complete *Wolbachia* genome sequences, strain *w*AlbB appears to be most closely related to *Wolbachia w*Pip, an endosymbiont of the mosquito *Culex pipiens* (Klasson et al., 2008; Klasson et al., 2009; Mavingui et al., 2012).

In this study, we investigated the distribution of *Wolbachia* infection in *Ae. albopictus* according to geographical distribution in Korea.

2. Materials and Methods

2.1. Mosquito collection

To survey *Wolbachia* infection according to geographical separation, I identified nine different areas in South Korea based on mountain chains and waterways (Figure 1). I collected *Ae. albopictus* individuals from each area using human baiting and two different traps (CDC black light trap and BG Sentinel trap, Bioquip). Sampling was conducted from May to October 2013. The collected mosquitoes were fresh-frozen with dry-ice and stored in a freezer at -20°C. A total of 739 females *Ae. albopictus* were collected from the nine geographical areas over a total of 17 regions, with at least 30 specimens collected in each area (Table 1).

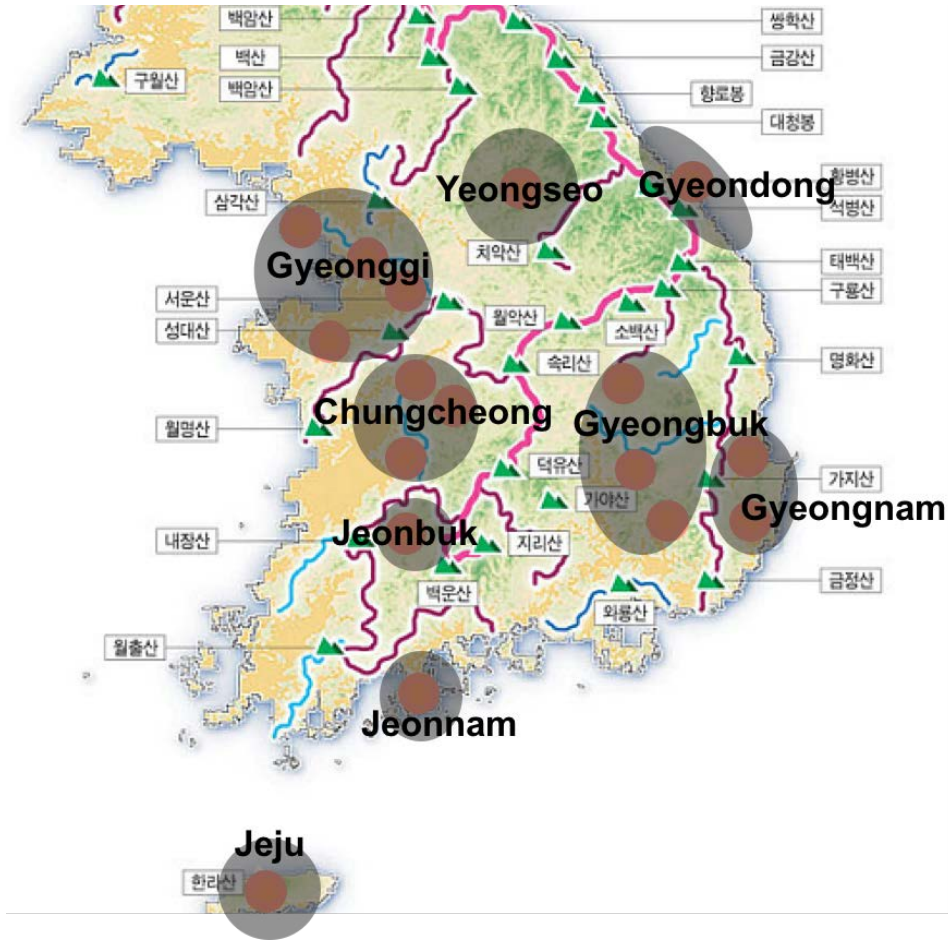

Figure 1. Mosquito collecting sites. Used map is for indicating the mountain chains and waterways.

Table 1. *Wolbachia* infection status according to mosquito sampling sites in Korea

Sampling site		Latitude	Longitude	Number of samples (positive %)[b]
Gyeonggi	Pyeongtaek	N 37° 02′ 40.26″	E 127° 02′ 00.74″	117 (100)
	Ansan[a]	N 37° 16′ 56.58″	E 126° 50′ 13.44″	
	Gangwha	N 37° 44′ 10.05″	E 126° 18′ 31.08″	
Chungcheong	Cheonan[a]	N 36° 47′ 26.30″	E 127° 14′ 23.46″	72 (97.2)
	Seosan	N 36° 39′ 21.50″	E 126° 32′ 40.92″	
Yeongseo	Gangneung[a]	N 37° 44′ 03.93″	E 128° 58′ 48.69″	53 (98.1)
Yeongdong	Chuncheon[a]	N 37° 52′ 44.83″	E 127° 42′ 43.75″	49 (95.9)
Gyeongnam	Ulsan[a]	N 35° 33′ 03.78″	E 129° 16′ 55.84″	112 (98.2)
	Gyeongju[a]	N 35° 51′ 52.29″	E 129° 11′ 43.78″	
	Pohang	N 35° 56′ 70.71″	E 129° 19′ 56.52″	
Gyeongbuk	Andong	N 36° 32′ 16.21″	E 128° 31′ 05.68″	126 (99.2)
	Daegu[a]	N 35° 55′ 68.61″	E 128° 38′ 31.67″	
Jeonbuk	Jeonju	N 35° 44′ 43.57″	E 127° 02′ 51.70″	73 (100)
Jeonnam	Damyang[a]	N 35° 19′ 19.01″	E 126° 58′ 37.05″	72 (100)
	Goheung[a]	N 34° 27′ 25.05″	E 127° 31′ 09.07″	
Jeju	Bomok dong[a]	N 33° 14′ 48.91″	E 126° 33′ 16.05″	65 (98.5)
	Cheonjiyeon fall	N 33° 14′ 44.66″	E 126° 36′ 03.77″	

[a] Sequence analysis for *w*AlbA.
[b] Wolbachia infection rate of *Aedes albopictus*

2.2. DNA extraction and Polymerase Chain Reaction (PCR)

Whole genomic DNA was extracted from the collected mosquitoes. A DNA Extraction Solution kit (G-spin, iNtRON) was used for DNA extraction, and the manufacturer's recommended protocol was followed. Briefly, each mosquito abdomen was homogenized in lysis buffer using a homogenizer. The homogenized samples were incubated at 65°C for 15 min, and 250 μl binding buffer was added and completely mixed by pipetting after incubation. The cell lysates were loaded on columns and centrifuged at 13,000 rpm for 1 min. Two wash steps using 500 μl wash buffer and elution with 30–50 μl distilled water were then conducted. The *Wolbachia* infection status of the mosquitoes was determined by PCR amplification with primers for WSP, and β-actin was used as an internal control. The primers and primer specifications were as follow: β-actin_forward 5′-AGA TCA TGT TCG AGA CCT TC-3′, β-actin_reverse 5′-TCA GGA TCT TCA TCA GGT AA-3′, WSP_forward 5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′, and WSP_reverse 5′-AAA AAT TAA ACG CTA CTC CA-3′.PCR consisted of an initial denaturation at 95°C for 5 min, then 29 cycles of 20 sec at 95°C, 30 s at 55°C, and 1 min at 72°C, followed by a final extension at 72°C for 5 min. The PCR products were confirmed using 1.5% agarose gel electrophoresis and visualized by Safepinky DNA gel staining solution.

2.3. Nucleotide sequencing and phylogenetic analysis

The positive products were subjected to direct PCR sequencing using the primers WSP_forward, WSP_reverse, *w*AlbA_forward, *w*AlbA_reverse, *w*AlbB_forward, and *w*AlbB_reverse, procured from Macrogen (Seoul, Korea). When the product concentrations were insufficient, I further amplified the fragments with the same primers using DyNAzyme^{TM}EXT DNA Polymerase (Thermo, Waltham, MA, USA). The reactions were performed in a 50 µl reaction mixture containing 5 µl of 10× optimized buffer, 1 µl of dNTP mix (10 mM), 2 µl of each primer (10 pM stock), 1 µl of DNA polymerase (200 U), 38 µl of distilled water (RNase/DNase free), and 3 µl of the template. The thermal profile for PCR was 94°C for 2 min, then 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 40 s, followed by a final extension at 72°C for 5 min. For *Wolbachia*-specific WSP gene sequence analysis, partial nucleotide sequences from *Ae. albopictus* were obtained and compared. Reported *Drosophila melanogaster* (GenBank accession nos. DQ412100, AF020063, and AY888034) and *Cx. pipiens* (GenBank accession no. AF301010) sequences were used as controls. The partial WSP gene sequence of *Wolbachia* from *Bactericera cockerelli* was used as an out-group (isolate Coahuila 2, GenBank accession no. AY971948).

For sequence analysis of the *Wolbachia* strains *w*AlbA and *w*AlbB, the partial nucleotide sequences were obtained from 38 *Ae. albopictus* individuals from different regions and compared. I employed at least three samples from each region. A multiple alignment was generated with the MUSCLE program implemented in

MEGA 5.0 (Egizi et al., 2013; Tamura et al., 2011), and a neighbor-joining tree was constructed with the substitution model of maximum composite likelihood. The reliability of the tree was tested by bootstrap methods with 2,000 replications.

3. Results

3.1. Survey of *Wolbachia* in *Ae. albopictus* using the WSP gene

Ae. albopictus was screened and analyzed using PCR and sequencing methods with a general WSP gene primer to identify endosymbiont *Wolbachia*. After the PCR results were obtained, WSP was amplified from *Ae. albopictus* obtained from Daegu. These results indicated that nearly all of *Ae. albopictus* samples were infected with *Wolbachia*. To evaluate the sequence similarity between the known and detected WSP genes, I performed sequence analysis. Sequence alignment of the selected WSP gene was performed by a neighbor-joining algorithm (Saitou and Nei, 1987). For phylogenetic analysis, *Ae. albopictus* (GeneBank accession no. KF725080) was used as reference, and *Drosophila melanogaster* (GenBank accession nos. DQ412100, AF020063, and AY888034) and *Culex pipiens* (GenBank accession no. AF301010) were used outgroups (Figure 2). The WSP genes showed 98–100% similarities within the same species, including the reported sequence (GeneBank accession no. KF725080). Between the known *Cx. pipiens* and collected *Ae. albopictus* sequences, the WSP genes showed approximately 85% sequence homology. Interestingly, the WSP from *D. melanogaster* showed higher sequence homologies with those from *Ae. albopictus* (92–94%) than those from *Cx. pipiens* (81–82%).

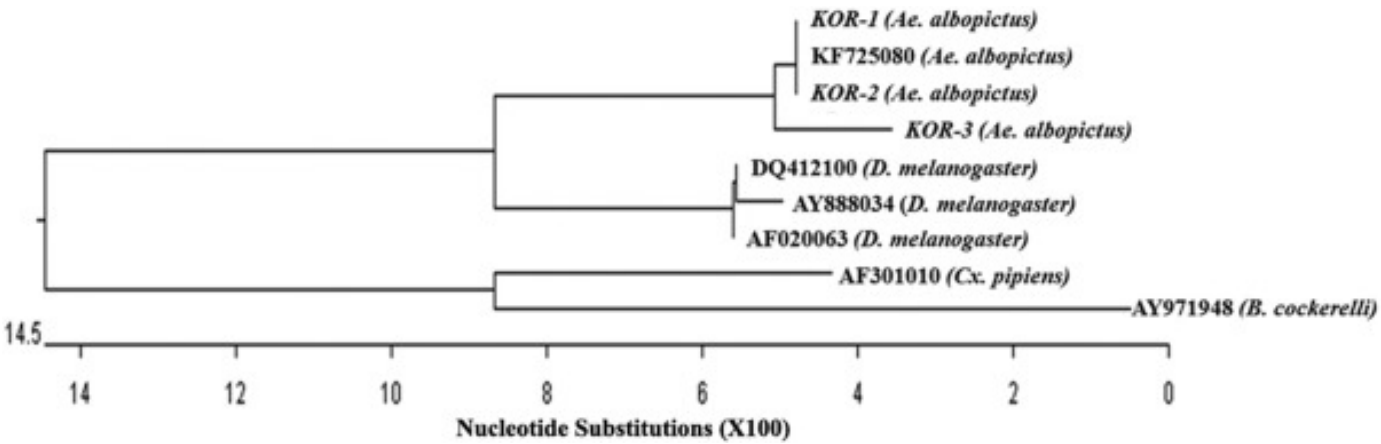

Figure 2. Phylogenic tree constructed based on partial WSP gene sequences. Sequence analysis and phylogenic tree construction were conducted using the MUSCLE program implemented in MEGA 5.0. WSP from the enodsymbiont *Wolbachia* of *Aedes albopictus* (GenBank accession no.: KF725080), *Drosophila melanogaster* (GenBank accession no.: AY888034, AF020063, DQ412100), and *Culex pipiens* (GenBank accession no.: AF301010) were used as reference *Wolbachia* sequence. KOR-1, KOR-2, and KOR-3 were detected from *Ae. albopictus* in Korea. *Bactericera cockerelli* (GenBank accession no.: AY971948) was selected as an out-group.

3.2. Classification of *Wolbachia* in *Ae. albopictus* according to geographical distribution

The *Wolbachia* infection rate differed between geographical regions. Based on the WSP gene information, Cheonbook, Cheonnam, and Gyeonggi each presented a 100% infection rate, with infection becoming increasingly less prevalent in Gyeongbook (99.2%), Jeju (98.5%), Gyeongnam (98.2%), Yeongseo (98.1%), Chungchung (97.2%), and Yeongdong (95.9%) (Table 1 and Figure 3). By PCR analysis, all *Wolbachia*-infected mosquito samples were found to be infected with *w*AlbA strain but 98% of samples were found to be infected with wAlbB strain. Double infection with both *w*AlbA and *w*AlbB occurred in 98% of *Wolbachia*-infected mosquitoes (Supplementary Figure 4). According to the PCR results, the rate of double infection with both *w*AlbA and *w*AlbB differed between areas. Individuals infected only by *w*AlbA were detected at a very low rate, approximately 2%. No individual in this survey was found to be infected only by *w*AlbB. To confirmation of regional distribution with *Wolbachia* strains, I analyzed 38 samples of *w*AlbA from 11 different areas using PCR. I also performed phylogenic analysis using *w*AlbA target primers to create a phylogenic tree. Despite the regional differences, the *w*AlbA sequences showed high homologies of 99–100% (Figure 5).

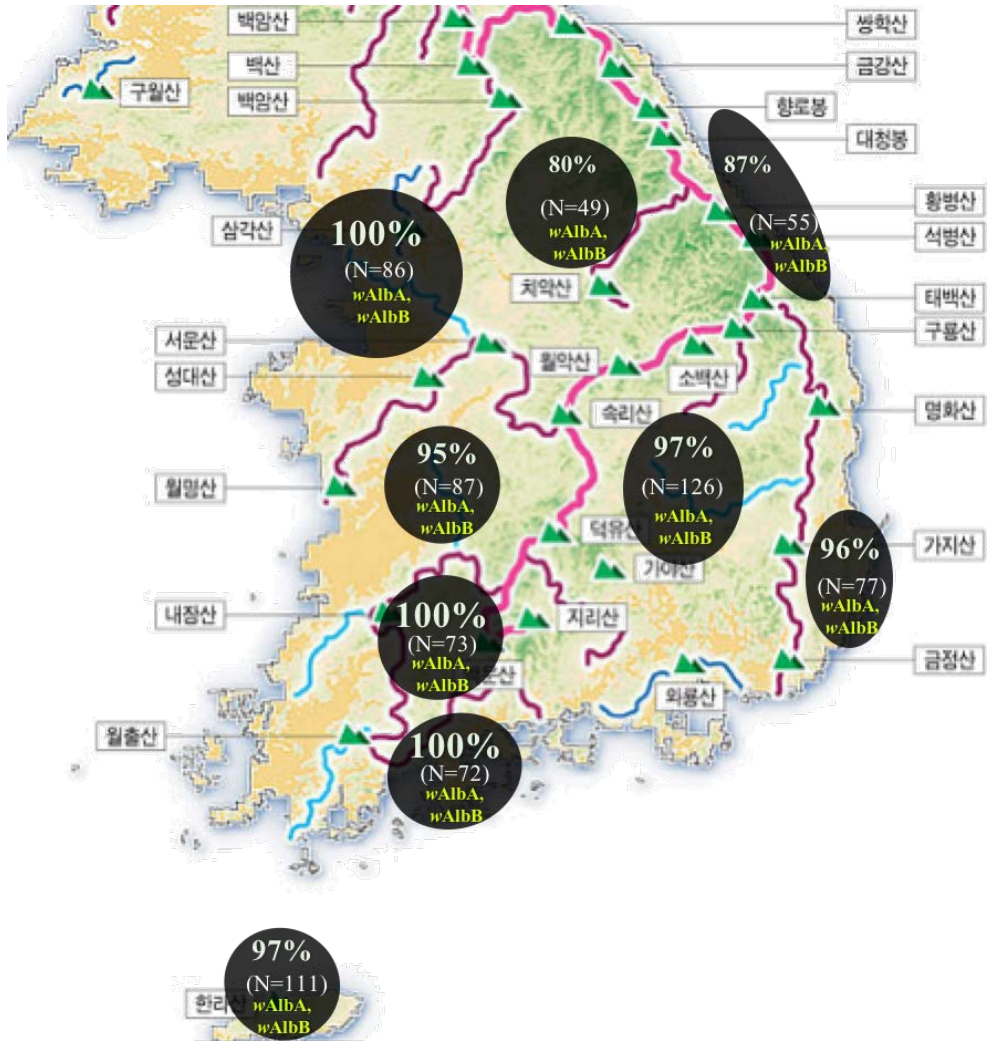

Figure 3. *Wobachia* infeation rate in *Ae. albopictus* according to the geographical regions. N is indicating the number of collected *Ae. albopictus*; *w*AlbA, *w*AlbB is *Wolbachia* strains found in *Ae. albopictus*

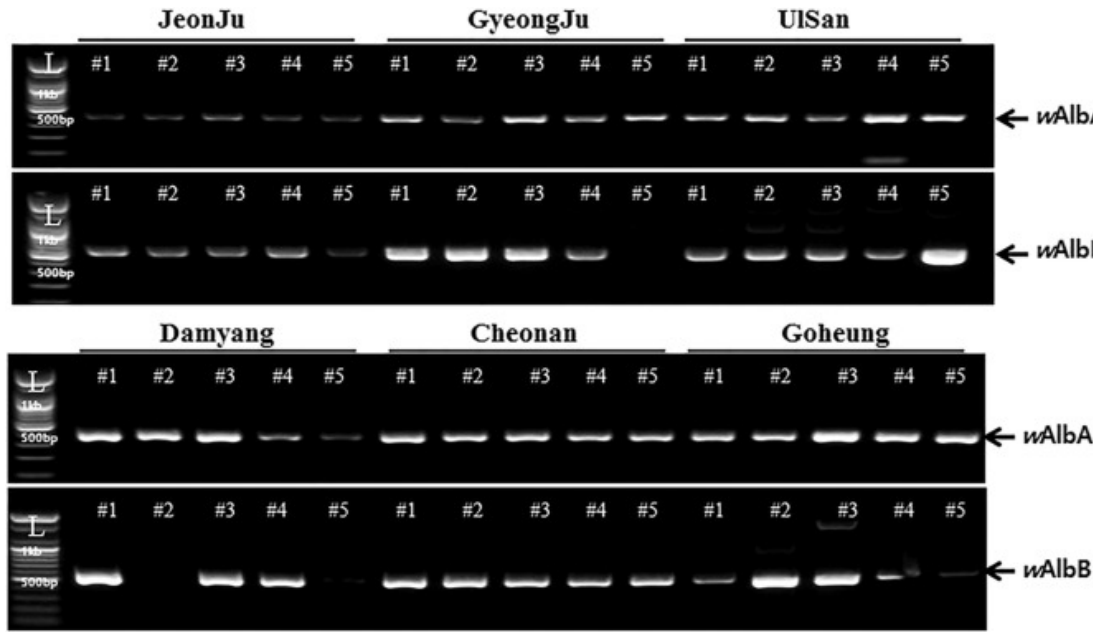

Figure 4. Confirmation of *Wolbachia* strains according to geographical distribution. According to PCR analysis, all samples were found to be infected with the *w*AlbA strain, but 98% of samples were found to be infected with the *w*AlbB strain, regardless of geographical distribution.

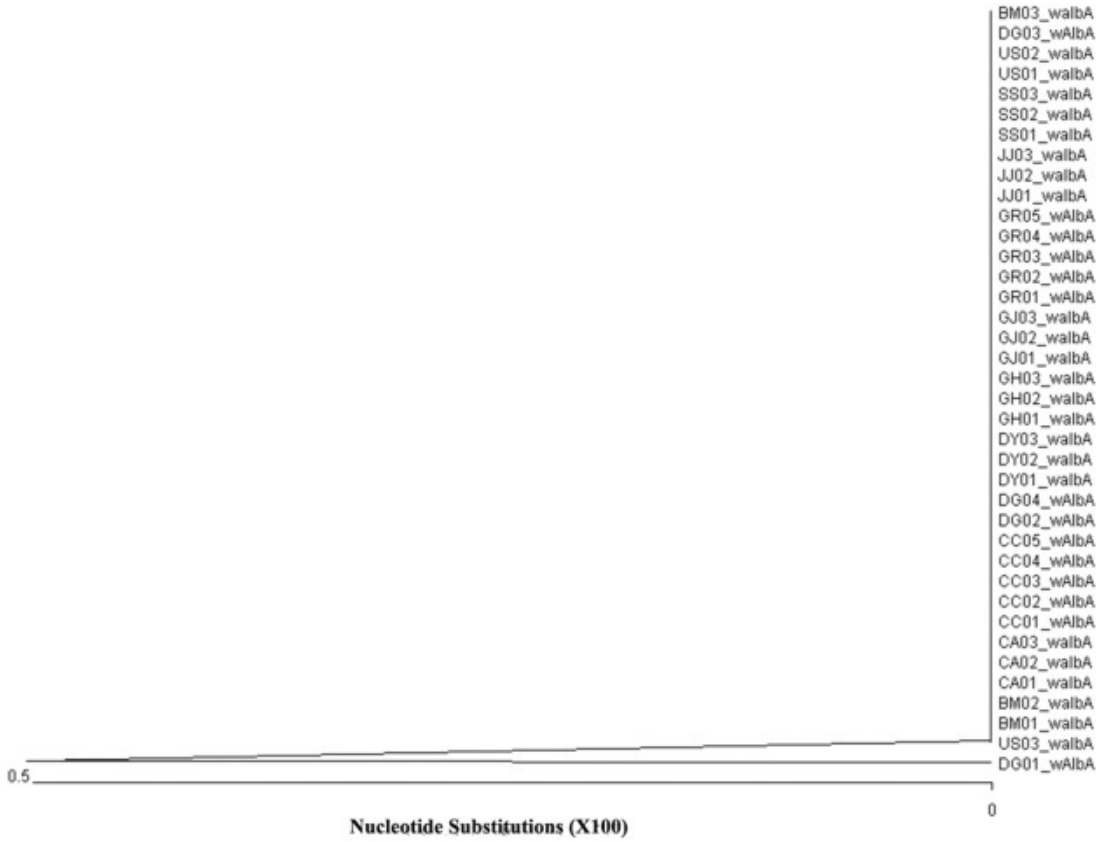

Figure 5. Phylogenic tree constructed based on partial *w*AlbA. Sequence analysis and phylogenic tree construction were conducted by DNASTAR software. DG (Daegu), BM (Bomok), GH (Goheung), GJ (Gyeongju), GN (Gangneung), US (Ulsan), SS (Seosan), JJ (Jeonju), CC (Chuncheon), DY (Damyang), and CA (Cheonan) indicate collection areas in South Korea.

4. Discussion

Recent research on the endosymbiont bacteria of the genus *Wolbachia* has focused on their symbiotic relationships with hosts rather than biological characteristics such as growth and reproduction. In this study, we present, to our knowledge, the first survey of *Wolbachia* infection in vector mosquitoes.

Various species of vector mosquitoes have been reported to be infected with different strains of *Wolbachia* (Kittayapong et al., 2000). Our results examined the natural infection of the vector mosquito *Ae. albopictus* with *Wolbachia*. For the survey of *Wolbachia*, PCR was performed with a known WSP gene sequence, and a consensus tree was constructed based on analysis of the aligned WSP gene sequences. Sequence analysis of the WSP genes revealed different groups of *Wolbachia* depending on the vector species (Figure 1). Previously, Ruang-Areerate et al. (2003) reported that *Wolbachia* from *Ae. albopictus* and *D. melanogaster* were members of Group A, but that from *Cx. pipiens* was associated with Group B (Ruang-Areerate et al., 2003). These results are similar to patterns observed in vector mosquitoes in Korea. Although I used WSP genes for this survey of *Wolbachia* infection, many studies have reported that WSP and *ftsZ* detection results are identical (Kitrayapong et al., 2002; Ruang-Areerate et al., 2003; Werren et al., 1995).

My analysis indicated that more than 98.8% *Ae. albopictus* were super-infected under natural conditions, and that 100% of infected mosquitoes harbored the *w*AlbA strain. Interestingly, single infection with the *w*AlbB strain was not detected in the test samples. These results are similar to infection patterns reported previously (Kitrayapong et al., 2002).

I performed sequence analysis with more than three *Wolbachia w*AlbA samples from each area to determine regional differences in infection. However, no differences in node positions were observed between vector mosquitoes from different areas in the most parsimonious tree (Figure 2). The PCR sequences were analyzed using the NCBI BLAST, as well as invertebrate nucleotide sequences, with megablast optimization and a cutoff e-value smaller than $2e^{-100}$. BLAST analysis identified the *Wolbachia* endosymbiont of *Ae. albopictus* wolbachia surface protein (wsp) with 99% identity.

In conclusion, in this study, I provide, to our knowledge, the first report of *Wolbachia* infection and strains in Korea, and distinguish them according to vector mosquitoes and region. This study would support further functional and biocontrol-related studies of *Wolbachia*

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Appendix

Identification of mosquito species using DNA barcoding method in Korea

Table 1. List of mosquito species, collection sites, and Genbank numbers

Mosquito species	Collection site	GenBank number
Aedes albopictus	Incheon (2009)	KT358457, KT358458, KT358459
Aedes flavopictus	Boeun (2014)	KT358463, KT358464, KT358465
Aedes lineatopennis	Ansan (2014)	KT358466, KT358467, KT358468
Aedes vexans	Ansan (2014)	KT358460, KT358461, KT358462
Anopheles belenrae	Dora (2013)	KT358454, KT358455, KT358456
Anopheles kleini	Dora (2013)	KT358443, KT358444, KT358445
Anopheles lesteri	Dora (2013)	KT358451, KT358452, KT358453
Anopheles pullus	Dora (2013)	KT358449, KT358450
Anopheles sinensis	Dora (2013)	KT358446, KT358447, KT358448
Armigeres subalbatus	Ansan (2014)	KT358440, KT358441, KT358442
Coquillettidia ochracea	Ansan (2014)	KT358437, KT358438, KT358439
Culex bitaeniorhynchus	Ansan (2014)	KT358430, KT358431, KT358432
Culex inatomii	Ansan (2014)	KT358435, KT358436
Culex mimeticus	Daejeon (2009)	KT358433, KT358434
Culex orientalis	Ansan (2014)	KT358427, KT358428, KT358429
Culex pipiens	Ansan (2014)	KT358422, KT358423, KT358424, KT358425, KT358426
Culex tritaeniorhynchus	Ansan (2014)	KT358419, KT358420, KT358421
Culex vagans	Cheongju (2015)	KT358417, KT358418

Table 1. List of mosquito species, collection sites, and Genbank numbers (continued)

Mosquito species	Collection site	GenBank number
Culiseta nipponica	Ansan (2014)	KT358414, KT358415, KT358416
Mansonia uniformis	Ansan (2014)	KT358411, KT358412, KT358413
Ochlerotatus dorsalis	Ansan (2014)	KT358408, KT358409, KT358410
Ochlerotatus koreicus	Ansan (2014)	KT358407
Ochlerotatus nipponicus	Boeun (2014)	KT358404, KT358405, KT358406
Ochlerotatus togoi	Jeju (2014)	KT358401, KT358402, KT358403
Tripteroides bambusa	Boeun (2014)	KT358398, KT358399, KT358400

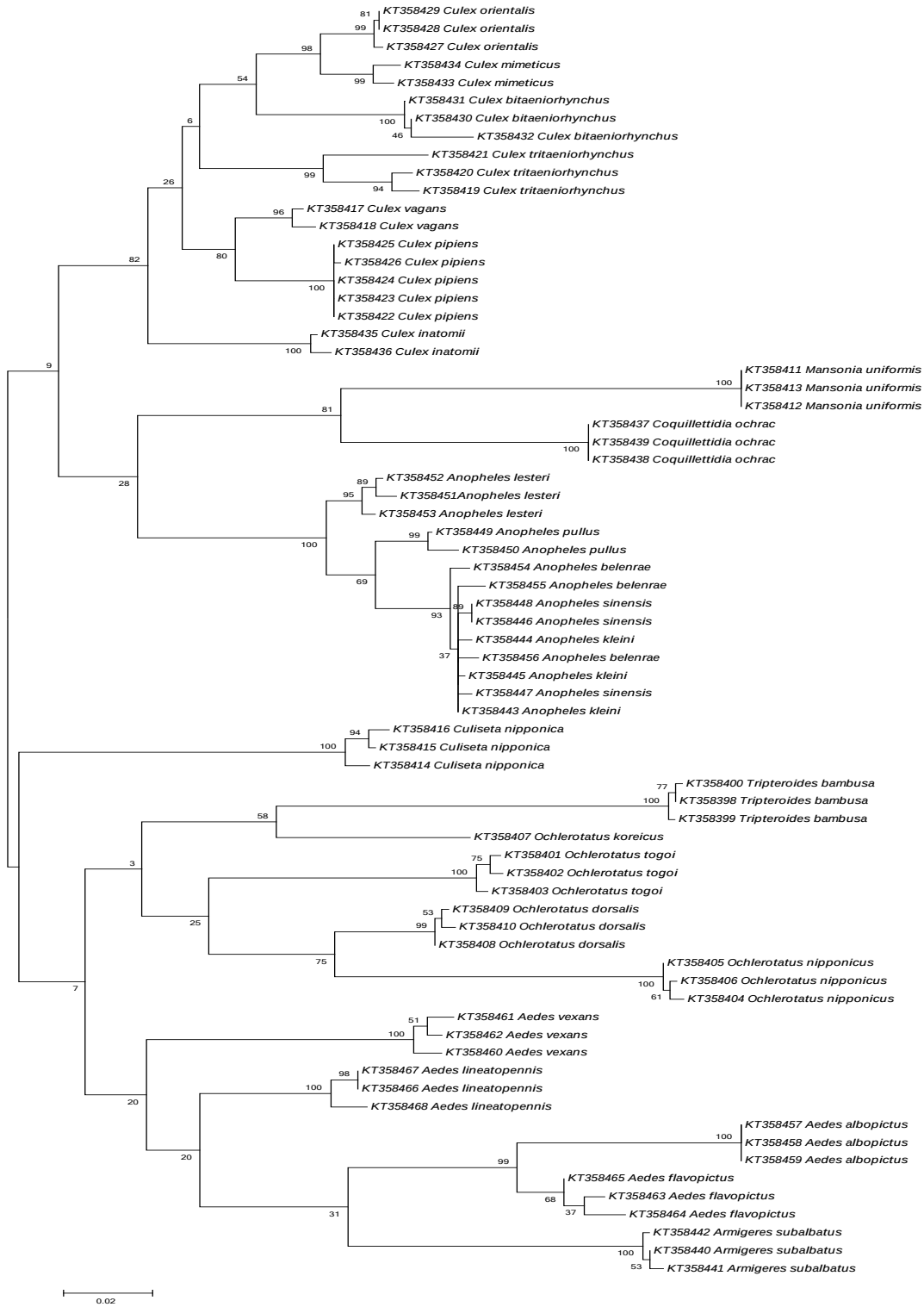
KT358429 Culex orientalis
KT358428 Culex orientalis
KT358427 Culex orientalis
KT358434 Culex mimeticus
KT358433 Culex mimeticus
KT358431 Culex bitaeniorhynchus
KT358430 Culex bitaeniorhynchus
KT358432 Culex bitaeniorhynchus
KT358421 Culex tritaeniorhynchus
KT358420 Culex tritaeniorhynchus
KT358419 Culex tritaeniorhynchus
KT358417 Culex vagans
KT358418 Culex vagans
KT358425 Culex pipiens
KT358426 Culex pipiens
KT358424 Culex pipiens
KT358423 Culex pipiens
KT358422 Culex pipiens
KT358435 Culex inatomii
KT358436 Culex inatomii
KT358411 Mansonia uniformis
KT358413 Mansonia uniformis
KT358412 Mansonia uniformis
KT358437 Coquillettidia ochrac
KT358439 Coquillettidia ochrac
KT358438 Coquillettidia ochrac
KT358452 Anopheles lesteri
KT358451Anopheles lesteri
KT358453 Anopheles lesteri
KT358449 Anopheles pullus
KT358450 Anopheles pullus
KT358454 Anopheles belenrae
KT358455 Anopheles belenrae
KT358448 Anopheles sinensis
KT358446 Anopheles sinensis
KT358444 Anopheles kleini
KT358456 Anopheles belenrae
KT358445 Anopheles kleini
KT358447 Anopheles sinensis
KT358443 Anopheles kleini
KT358416 Culiseta nipponica
KT358415 Culiseta nipponica
KT358414 Culiseta nipponica
KT358400 Tripteroides bambusa
KT358398 Tripteroides bambusa
KT358399 Tripteroides bambusa
KT358407 Ochlerotatus koreicus
KT358401 Ochlerotatus togoi
KT358402 Ochlerotatus togoi
KT358403 Ochlerotatus togoi
KT358409 Ochlerotatus dorsalis
KT358410 Ochlerotatus dorsalis
KT358408 Ochlerotatus dorsalis
KT358405 Ochlerotatus nipponicus
KT358406 Ochlerotatus nipponicus
KT358404 Ochlerotatus nipponicus
KT358461 Aedes vexans
KT358462 Aedes vexans
KT358460 Aedes vexans
KT358467 Aedes lineatopennis
KT358466 Aedes lineatopennis
KT358468 Aedes lineatopennis
KT358457 Aedes albopictus
KT358458 Aedes albopictus
KT358459 Aedes albopictus
KT358465 Aedes flavopictus
KT358463 Aedes flavopictus
KT358464 Aedes flavopictus
KT358442 Armigeres subalbatus
KT358440 Armigeres subalbatus
KT358441 Armigeres subalbatus
0.02

Figure 1. Phylogenetic analysis of mtCOI sequences from mosquitoes collected in South Korea. An alignment of mtCOI sequences was performed using the clustalX method, and the phylogenetic tree was constructed using the maximum likelihood method in the MEGA6 program. Bootstrap values with 1,000 replications were displayed on branches.

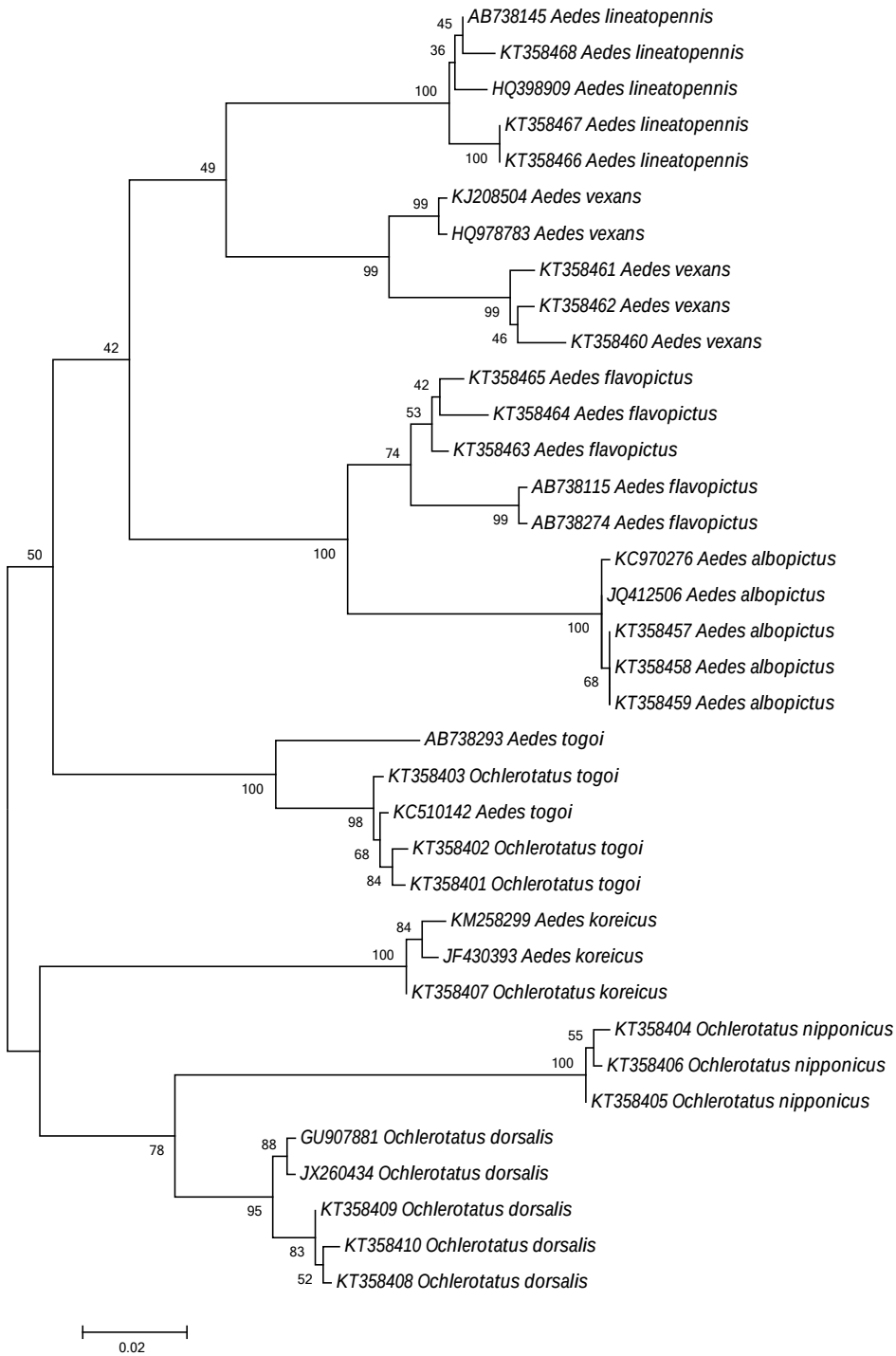
AB738145 Aedes lineatopennis
KT358468 Aedes lineatopennis
HQ398909 Aedes lineatopennis
KT358467 Aedes lineatopennis
KT358466 Aedes lineatopennis
KJ208504 Aedes vexans
HQ978783 Aedes vexans
KT358461 Aedes vexans
KT358462 Aedes vexans
KT358460 Aedes vexans
KT358465 Aedes flavopictus
KT358464 Aedes flavopictus
KT358463 Aedes flavopictus
AB738115 Aedes flavopictus
AB738274 Aedes flavopictus
KC970276 Aedes albopictus
JQ412506 Aedes albopictus
KT358457 Aedes albopictus
KT358458 Aedes albopictus
KT358459 Aedes albopictus
AB738293 Aedes togoi
KT358403 Ochlerotatus togoi
KC510142 Aedes togoi
KT358402 Ochlerotatus togoi
KT358401 Ochlerotatus togoi
KM258299 Aedes koreicus
JF430393 Aedes koreicus
KT358407 Ochlerotatus koreicus
KT358404 Ochlerotatus nipponicus
KT358406 Ochlerotatus nipponicus
KT358405 Ochlerotatus nipponicus
GU907881 Ochlerotatus dorsalis
JX260434 Ochlerotatus dorsalis
KT358409 Ochlerotatus dorsalis
KT358410 Ochlerotatus dorsalis
KT358408 Ochlerotatus dorsalis
0.02

Figure 2. Phylogenetic analysis of mtCOI sequences from *Aedes* and *Ochlerotatus* spp. mosquitoes. An alignment of mtCOI sequences from *Aedes* and *Ochlerotatus* spp. mosquitoes was used to construct the phylogenetic tree using the maximum likelihood method in the MEGA6 program. mtCOI sequences from other mosquitoes obtained from NCBI and Genbank sequences identified during the present study have the prefix KT.

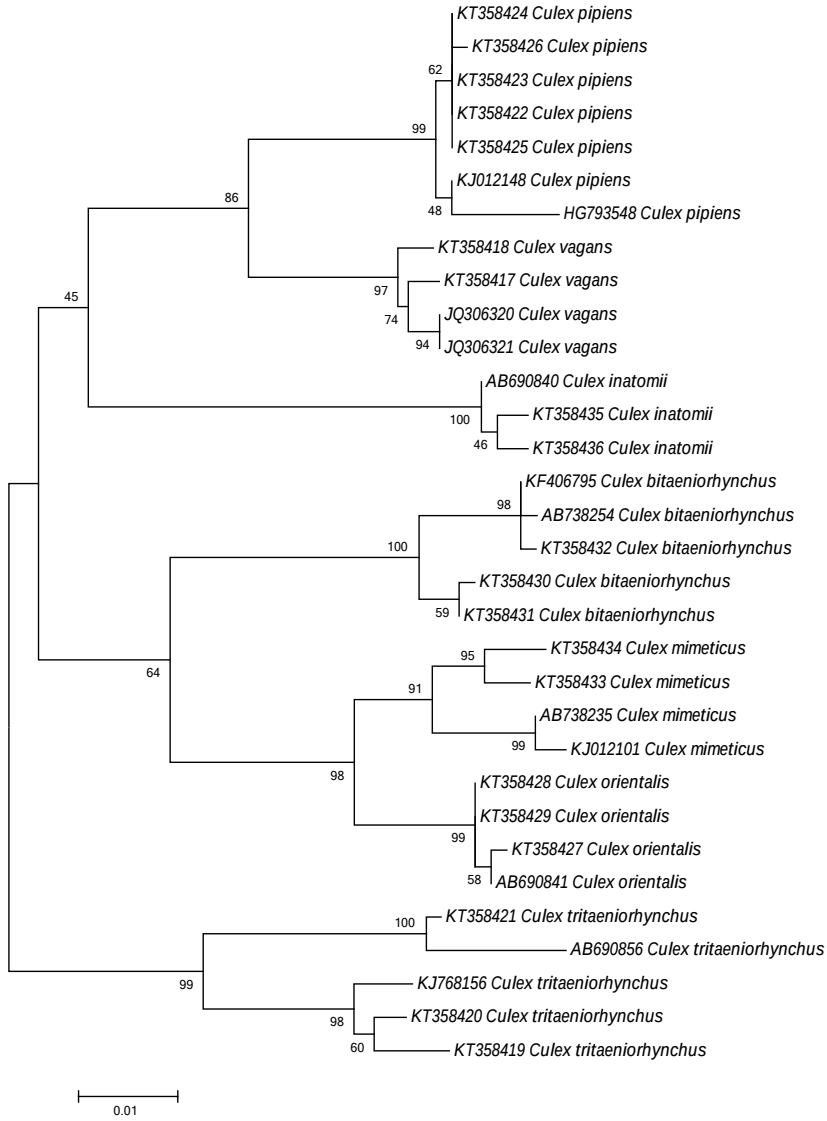
KT358424 Culex pipiens
KT358426 Culex pipiens
KT358423 Culex pipiens
KT358422 Culex pipiens
KT358425 Culex pipiens
KJ012148 Culex pipiens
HG793548 Culex pipiens
KT358418 Culex vagans
KT358417 Culex vagans
JQ306320 Culex vagans
JQ306321 Culex vagans
AB690840 Culex inatomii
KT358435 Culex inatomii
KT358436 Culex inatomii
KF406795 Culex bitaeniorhynchus
AB738254 Culex bitaeniorhynchus
KT358432 Culex bitaeniorhynchus
KT358430 Culex bitaeniorhynchus
KT358431 Culex bitaeniorhynchus
KT358434 Culex mimeticus
KT358433 Culex mimeticus
AB738235 Culex mimeticus
KJ012101 Culex mimeticus
KT358428 Culex orientalis
KT358429 Culex orientalis
KT358427 Culex orientalis
AB690841 Culex orientalis
KT358421 Culex tritaeniorhynchus
AB690856 Culex tritaeniorhynchus
KJ768156 Culex tritaeniorhynchus
KT358420 Culex tritaeniorhynchus
KT358419 Culex tritaeniorhynchus
62
99
86
48
45
97
74
94
100
46
98
100
59
64
95
91
99
98
99
58
100
99
98
60
0.01

Figure 3. Phylogenetic analysis of mtCOI sequences from *Culex* spp. mosquitoes. An alignment of mtCOI sequences from *Culex* spp. mosquitoes was used to construct the phylogenetic tree using the maximum likelihood method in the MEGA6 program. mtCOI sequences from other mosquitoes obtained from NCBI and Genbank sequences identified during the present study have the prefix KT.

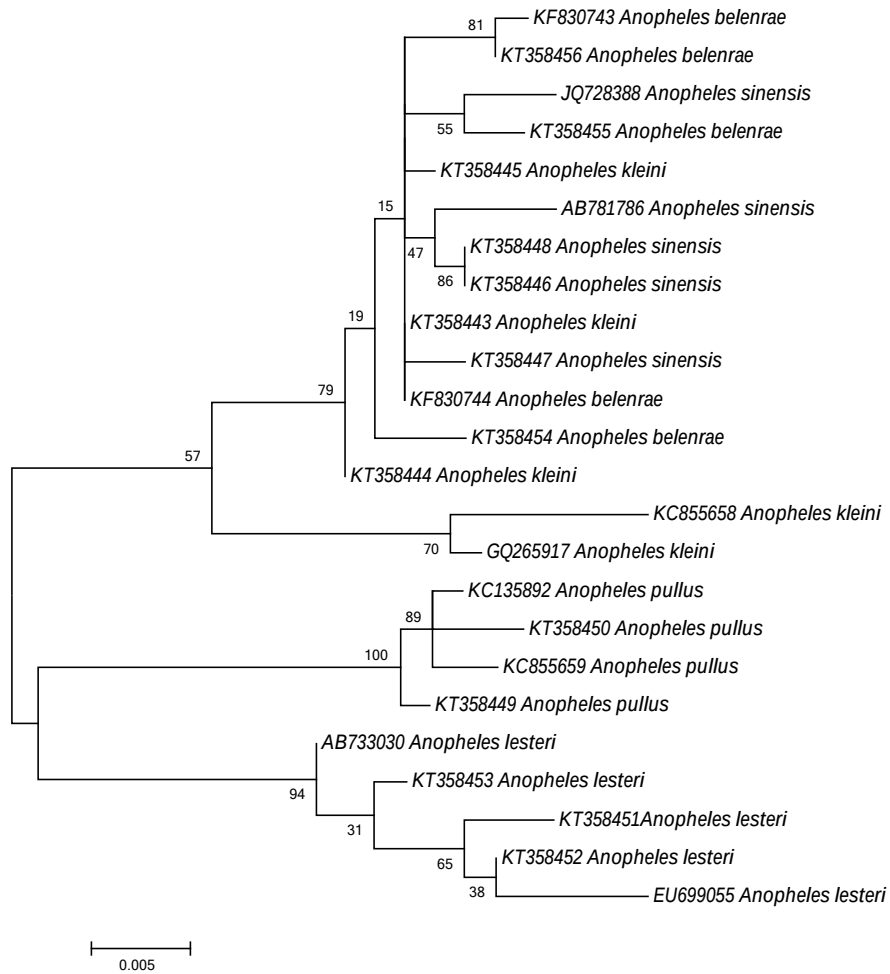

Figure 4. Phylogenetic analysis of mtCOI sequences from *Anopheles* spp. mosquitoes. An alignment of mtCOI sequences from *Anopheles* spp. mosquitoes was used to construct the phylogenetic tree using the maximum likelihood method in the MEGA6 program. mtCOI sequences from other mosquitoes obtained from NCBI and Genbank sequences.

KOREAN ABSTRACT

흰줄숲모기의 분포, 발생 및 흡혈행동 연구

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김현우

초록

흰줄숲모기(*Aedes albopictus*)는 뎅기열병과 치쿤구니아병과 같은 감염병을 매개하는 주요한 모기이다. 최근 지카바이러스의 전파에 관여하는 것으로 알려지면서 전세계적인 관심을 받고 있다. 이런 흰줄숲모기는 주로 동남아시아의 숲에 자생하던 모기이지만 최근 국제무역과 사람들의 이동이 왕성해지면서 미국과 유럽, 아프리카를 포함해 전세계적으로 그 서식 범위를 확대하고 있어 그 위험성이 날로 더해가고 있다.

본 연구에서는 이러한 흰줄숲모기의 분포와 연간 발생량, 흡혈 습성 특성과 매개하는 바이러스를 확인하였다. 흰줄숲모기는 국내에서 채집되는 전체모기 중 4%를 차지하였으며, 5 월 처음 채집된 이후 서서히 증가하다 9 월에 이르러 최고의 개체수가 채집된 후 10 월에 급격히 줄어 드는 월별 발생 양상을 보였다. 흰줄숲모기 유충은 이 모기의 주요 발생지점인

대나무숲을 중심으로 500 m 이내의 인공용기와 폐타이어에서 주로 발견되었다.

흰줄숲모기는 주로 이른 아침(08:00-09:00)과 이른 저녁(16:00-19:00) 두 번에 걸쳐 먹이 탐색하는 것으로 나타났으며, 이런 특징은 온도와 광량에 의해 영향을 받는 것으로 판단되었다.

흰줄숲모기의 흡혈원으로는 포유동물(71%), 조류(26%), 양서류(2%), 그리고 물고기(1%)로 나타났으며, 포유동물로 나타난 흡혈원의 대부분이 사람(86%)이었다. 흰줄숲모기는 그들의 흡혈대상으로 주로 사람을 공격하는 것으로 나타났다. 이는 만일 뎅기열 바이러스가 국내에 들어올 경우 흰줄숲모기에 의한 바이러스 확산이 쉽게 일어날 수 있다는 위험성을 시사한다.

다행스럽게도, 바이러스 검출 실험에서는 흰줄숲모기로부터 아무런 바이러스가 검출되지 않았다. 그러나 일본뇌염바이러스가 기존 알려진 매개체인 작은빨간집모기(*Culex tritaeniorhynchus*)가 아닌 빨간집모기(*Cx. pipiens*)와 동양집모기(*Cx. orientalis*)에서 검출되었다. 또한 검출된 일본뇌염바이러스의 계통분석을 통해 genotype V 임을 확인하였다. 이를 통해 국내 일본뇌염바이러스의 새로운 genotype 과 일본뇌염바이러스 전파에 빨간집모기와 동양집모기가 중요한 역할을 함을 제안해 본다.

최근 이집트숲모기(*Ae. aegypti*)와 흰줄숲모기 방제를 위해 곤충 공생미생물인 볼바카아(*Wolbachia*)가 활발히 연구되고 있다. 본 연구에서는 볼바키아를 이용한 국내 흰줄숲모기의 방제 가능성을 검토하고자 흰줄숲모기를 지역별로 채집하여 볼바키아의 감염율과 감염된 볼바키아의 strain 을 확인하였다. 채집된 흰줄숲모기의 99%에서 볼바키아 감염율을 확인하였으며, WSP 유전자의 서열비교로부터 기존 알려진 볼바키아

strain 과 98% 정도의 유사도를 보였다. 또한 두 종류의 볼바키아 strain 인 wAlbA 와 wAlbB 에 동시에 감염되어 있음을 확인하였다. 이러한 결과는 앞으로 수행할 볼바키아 관련된 생물학적 방제의 중요한 기초자료로 이용될 것이다.

끝으로 정확한 모기종 동정을 위해 DNA 바코드의 적용 가능성을 부록으로 나타냈다. 국내 서식하는 25 종의 모기로부터 cytochrome c oxidase subunit I (COI) 유전자를 추출하여 염기서열 분석 후 계통도를 그려 종을 분류하였다. 얼룩날개모기속을 제외하고는 정확하게 형태적 특징에 따라 분류가 됨을 확인하였다.

검색어: 흰줄숲모기, 분포, 발생량, 계절적 발생 소장, 서식지, 유충서식지, 활동시간, 흡혈원, 일본뇌염바이러스, 볼바키아, *w*AlbA, *w*AlbB

학번: 2011-30350