

## Global Environment Research Account for National Institutes

### Assessment of global warming by arboviruses, bacteria in water, and vector mosquitoes in the natural environment (Abstract of the Final Report)

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**Key Words**      arbovirus, bacteria, vector mosquitoes, monitoring, assessment of global warming

#### 1. Introduction

Vector-borne diseases are transmitted to humans by blood-feeding arthropods such as mosquitoes, ticks, and fleas. These cold-blooded animals are easily influenced by climate change. High temperatures during winter season may increase survival rate among arthropod vectors such as mosquitoes and ticks in Temperate Zone. It is also known that high temperature caused by warming accelerates the propagation speeds of several viruses in the vector mosquitoes. The global warming also may influence the genetic variation of arboviruses. The expansion of the distribution area of mosquito vector, *Aedes albopictus* caused an outbreak of Chikungunya fever in north Italy in 2007. Global warming, extreme climate change, changing physical distribution, and an increase in oversea travel are also expected to influence the epidemiology of infectious diseases caused by arboviruses and arthropod vector<sup>1,2)</sup>.

The activities of Japanese encephalitis virus (JEV) in Japan is still active in summer between mosquitoes and pigs. However, human JE cases are less than 10 because of vaccination and improvement of the living environment. The ecology of JEV in winter is unclear. Some papers reported that JEV sometimes moves to Japan from China, JEV genotype 5 were isolated in China and Korea, we should prepare the invasion of genotype 5<sup>3,4)</sup>. On the other hand, there are possibilities that JEV overwinter in wild animals.

Diverse prokaryotes including bacteria and archaea co-exist in the microflora of various environments including environmental water. The microflora in such environmental water forms the basis of the food chain, and changes in microflora biomass or its composition will influence the entire water ecosystem. Composition of the microflora is dependent on environmental conditions such as temperature, salinity, organic or inorganic nutrients, and human activities<sup>5)</sup>. Although *in vitro* culture and isolation of each organism were employed in an attempt to determine the microbial composition of environmental samples, recently it became evident that most environmental bacteria cannot be cultured. As an alternative, whole genomic DNA was extracted from samples of marine water, fresh water, and soil for the analysis of DNA sequences. The analysis of marine water in the Sargasso Sea found 148 novel phylotypes and 1.2 million genes among 1.6 Gbp of sequence. These metagenomic data were integrated with those of a global ocean sampling expedition from the Atlantic Ocean near the Nova Scotia peninsula in Canada to the

Pacific Ocean near French Polynesia via the Gulf of Panama. Comparative metagenomic analysis of samples collected from marine, hyper saline, fresh, and coral reef water uncovered the differences between the metabolic profiles in each environment. Thus, it is important to know the effect of climate change on microbes flora in ocean, lakes, rivers and marshes. To demonstrate such bacteria, analysis of bacterial DNA in the environmental samples have been employed.

Does JEV overwinter in Japan? or migrate from overseas areas? It has been discussing for a long time, but is still unclear. For the former, the possibility of overwintering of JEV in vector mosquito *Culex tritaeniorhynchus* females, was negated<sup>6)</sup>. The gonotrophic dissociation (phenomenon of blood feeding without development of mature eggs) occurs commonly in this mosquito under experimental conditions of short day-length, but in nature this phenomenon is observed only at a low rate, even under short day-length in autumn. Therefore, only a very few females will overwinter after showing the gonotrophic dissociation. Also, parous females just having emerged from hibernation are seldom collected in early spring. It was suggested that only an extremely small number of females in the overwintering population has the experience of blood-feeding in autumn. Accordingly, it is not likely for JEV to overwinter in this mosquito. On the other hands, prediapause migration of *Cx. tritaeniorhynchus* reported one year previously was observed again in the autumn of 2008 in the same park in urban Tokyo. The time course of prediapause migration in 2007 and 2008 was similar starting in mid-/late September, peaking in mid-October and ending in December. In this study, we conducted laboratory experiments and field trials to evaluate the overwintering ability of *Cx. tritaeniorhynchus*.

It is known that high temperature caused by warming accelerates the propagation speeds of several viruses in the vector mosquitoes. The global warming also may influence the genetic variation of arboviruses. It may also accelerate the expanding of mosquito distribution of *Culex pipiens* group in the northern parts in temperate zone countries including Japan, East Asia and European countries. Members of the *Cx. pipiens* complex are the representing vectors of many human pathogens. Among this group, *Cx. quinquefasciatus* the important primary vector of West Nile virus (WNV) and St. Louis encephalitis virus, are previously reported from Kyushu and Shikoku of Japan<sup>7)</sup> but a few information is available in recent days. So, we investigate the tolerance to cold temperature during winter season using several incubators to check the physiological responses to low temperature.

## 2. Research Objectives

Because of increases in the density of atmospheric carbon dioxide, the mean air temperature rose 0.6°C in the last century alone, and it has become clear that the temperature increase in the Northern Hemisphere is greater than that in other area. The average global temperature is expected to rise by 1.4°C to 5.8°C by 2100, potentially raising the sea level to rise a maximum of 88 centimeters. The 4th IPCC Assessment Report forecasts a worst-case scenario if warming continues at the current pace. Indirect effects of global warming include the possible expansion of the habitats of vector mosquitoes and ticks that transmit diseases to human beings in increasingly higher latitudes. This project has a three sub-themes including the assessment of recent global warming against the arbovirus, bacteria in lakes or rivers and vector mosquitoes. Recent report pointed out that some vector mosquitoes migrate to higher latitudes, the risk of contracting infectious diseases related to these vector insects would increase. Effects of global warming on ecosystems may substantially affect an activity of arboviruses, bacteria in the lakes or rivers and vector arthropods. The propagation of contagious diseases associated with or without vector arthropods are influenced by complex environmental factors<sup>8)</sup>.

The recent Japanese encephalitis virus (JEV) isolates were analyzed genetically and compared with JEV isolates from mosquitoes in Kyoto during 1980-1993. Wild boars often appear near around the resident area in Japan. We investigated the possibilities as an amplifier of JEV on wild boars, because boars and pigs are congeneric. JEV genotype 5 were recently isolated in China and Korea. There are some reports that some of JEV moved from South East Asia and China. The

current JE vaccine were manufactured using genotype 3 (Beijing-1), the efficacy was measured against genotype 5.

Several macroscopic indexes, such as temperature and the elevations of water levels, have been employed to estimate global warming and other global changes in climate and/or environment. Based on such data, progression of global warming, including the occurrence of more distractive tropical cyclones, changes in water circulation, decrease in fishery, extinction of species, spreading of infectious disease, is predicted. However, the effects of climate changes on the lives and ecosystems have not been clearly shown despite having many efforts. Recently, public database for bacterial gene is established. In addition, techniques of DNA microarray analyses and whole genome amplification have been developed. These progress enabled bioinformatics analysis of genomic information in the environmental samples without cultivating bacteria *in vitro*. We have employed these technologies and aimed to establish a novel means to monitor global warming. We collect environmental water from all over Japan, purify DNA and perform DNA microarray analysis to demonstrate the entire bacterial flora in such water. We apply this array to metagenomic analysis of microflora from water samples around Japan in order to evaluate its usefulness to reflect environmental changes.

JEV vector mosquito, *Cx. tritaeniorhynchus*, distributes from Southeast Asia to Japan. In this project we focus the capability of overwintering of JEV vector in Tohoku, Kanto and Chubu areas to test the cold hardness in the laboratory. Recent report pointed out that some vector mosquitoes migrate to higher latitudes, the risk of contracting infectious diseases related to these vector insects would increase. Particularly, mean temperature of winter season seems to be related to the survival of overwintering mosquitoes in Temperate areas. In this study, we focused on the overwintering ability for *Cx. tritaeniorhynchus*, and thus conducted laboratory experiments and field trials. *Cx. pipiens* complex were checked a tolerance to the low temperature in winter season in Japan. Effects of global warming on ecosystems was also monitored by genetic variation of JEV and responsibility to the vaccine for the JEV isolated from the wild boar in 2009 in Hyogo Prefecture. The propagation of contagious diseases associated with or without vector arthropods are influenced by complex environmental factors<sup>8)</sup>. This project has a three sub-themes including the assessment of recent global warming against the arbovirus, bacteria in sea and river water and vector mosquitoes. Effects of global warming on ecosystems may substantially affect an activity of arboviruses, bacteria in the lake and river water and vector arthropods.

### 3. Research Methods

(1) JEV isolated from wild boar and the relationship between JEV prevalence and climatological factors.

Present available vaccine for Japanese encephalitis virus was produced by using genotype 1 virus (Beijing 1). Recently genotype V strain of JE virus was isolated in China. It is generally accepted that JE virus was carried by some vehicle, such as migratory birds or vector mosquito from China to Japan. JE vaccine was inoculated to mice (DDY, 4 and 5 weeks) and checked neutralizing antibodies against Beijing strain and newly isolated JE virus from wild boar.

Viral isolation: Mosquitoes were homogenized and inoculated onto Vero cells of C6/36 cells, and sera of wild boars were inoculated onto those cells. 140 wild boars were caught in Nishinomiya city and around there, Hyogo prefecture. The supernatant of Vero cells were collected 5~6 days after inoculation. RNAs were extracted from the supernatant and applied to realtime RT-PCR (TaqMan methods).

Viral sequence analysis: The amplified products by RT-PCR were sequenced directly on both stands using BigDye Terminator Version 3.1 and analyzed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystem). Sequence analysis was performed using MEGA 4 gene analysis software [<http://www.megasoftware.net/>]. Sequence alignment and construction of phylogenetic tree were performed using MEGA 4. A phylogenetic tree was constructed using the neighbor-joining method.

Neutralizing Assay (Plaque Reduction Neutralizing Test): Mice were immunized with JE inactivated vaccine and their sera were pooled. The JEV neutralizing antibody titers of mouse serum were tested against JEV genotype 3, genotype 1 and genotype 5<sup>9)</sup> by plaque reduction neutralizing assay.

### (2) Evaluation of microflora from water samples around Japan by metagenomic analysis

To establish a simplified protocol for metagenomic analysis of environmental water, we have developed a novel DNA microarray to detect prokaryotes based on 16S rRNA sequences registered in the DNA Data Bank of Japan. Multiple DNA probes were designed for each of the 97,927 sequences in the database and mounted on a glass chip in duplicate. The microarray was expected to identify about 64,000 bacterial and archaeal species. According to information supplied by the Japan Meteorological Agency ([www.jma.go.jp](http://www.jma.go.jp)), the Black Current flows from the south of Japan along the Pacific side of the archipelago and partially into the Sea of Japan. The frigid Oyashio Current flows from north of Japan (Hokkaido) along the Pacific coast and runs into the Black Current. These two currents create the basis of warm and cold sea surface temperatures around Japan. Therefore, one liter of environmental water



Fig. 1 Location of water sampling sites in 2008- 2012

samples were collected respectively from 13 sites (Wakkanai and Nemuro in Hokkaido, Yamada and Otsuchi in Iwate prefecture, Choshi in Chiba prefecture, Hachijojima in Tokyo, Kaka in Shimane prefecture, Naruto and Minami in Tokushima prefecture, Ikeda-ko and Yoron in Kagoshima prefecture, Nanjo and Kouri-jima in Okinawa prefecture) in Japan (Fig. 1). The samples were passed through a 0.22  $\mu\text{m}$  filter, then whole DNA was extracted and uniformly amplified using whole genome amplification (WGA). The DNA sample was randomly digested and adapter sequences were ligated to both ends to make a DNA library. All the genome was amplified uniformly with an adapter specific PCR primer. DNA was labeled with Cy3-conjugated 16S rRNA specific primers and subjected to microarray analysis.

### (3) Evaluation of the global warming by vector mosquitoes.

JEV vector mosquitoes, *Cx. tritaeniorhynchus*, was collected by CDC type light traps and black light type traps from Tohoku and northern Kanto districts, western parts of prefecture from Tokyo. Mean numbers of vector mosquitoes collected by two types of traps and climatological factors were analyzed using correlation coefficient. The average of minimum low temperature in January was processed to prepare the climate chart with 1 km grid using 4 categories of minimum temperature (above 5, 3-5, 1-3, and below 1°C). The areas which *Cx. tritaeniorhynchus* are able to survive during winter season were analyzed. *Cx. tritaeniorhynchus* collected from Rinshinomori park in Tokyo was checked a tolerance to cold temperature in the laboratory. Conditioned adult mosquitoes for overwintering were also tested to cold hardness using the incubators with different kinds of temperature and day-length (25°C16L:8D, 20°C11L:13D, 15°C11L:13D, 10°C10L:14D,

5°C darkness). Survival rates of vector mosquitoes kept in different condition were compared. More than 3,000 females were released into the cage presented on the residential area of Tokyo in October of 2010 and 2011. We observed their activities during winter and survival until each next April. Finally, the possibility of overwintering of *Cx. tritaeniorhynchus* under natural condition of outdoor in Kanto area by the 2-years field trails.

*Culex* mosquitoes were collected by net-trap or Jhonson-trap, which was used for collection of agricultural pests showing long-distance flight from China to Japan. A total of 1,361 mosquitoes were analyzed for 2-years and a PCR based method (Kasai *et al.* 2008) was undertaken to identify the mosquito species *Cx. pipiens* complex. *Cx. p. pallens* and *Cx. p. form molestus*, which were laboratory colonies were kept in the different temperature and photoperiod (25°C16L:8D, 20°C11L:13D, 15°C11L:13D, 10°C10L:14D) and checked the survival rates. To know the meteorological data in the winter season in Kyushu, Shikoku, Chugoku districts, the average of minimum low temperature in 3 months (January, February and March) was processed to prepare the climate chart with 1 km grid using 3 categories (below 4°C, 4-10°C and above 10°C).

#### 4. Results

The deletion located in 3'-NTR of JEV recent isolates was confirmed in the genotype 1 strain. However, the function of these deletions in the viral cycle of JEV genome, (including receptor binding and membrane fusion) was not clear. JEV(JaNBo37) was isolated from a boars caught in resident area of Nishinomiya city on December, 2008. It belongs to genotype 1. According to the sequence analysis, it is autochthonous type not from mainland China. JEV genomes were detected from 3 boars. Two JEV genome belong to genotype 1 and another belongs to genotype 3. The current JE vaccine produced by genotype 3 was provably effective for genotype 1 (JanBo37), however low effective for genotype 5. JEV genotype 5 and West Nile viruses were not detected in 120 wild boars in Nishinomiya city, Hyogo prefecture.

Clustering analysis was performed using samples having signal intensities greater than mean + 3SD of the signal derived from all samples. As a result, we could detect several bacterial species commonly found in many of the sampling points, as well as several other bacterial species that are unique to each sampling point, which represent the differences in the environmental water. Pathogenic bacteria were also identified by the analysis. Of interest, several clusters that represent sampling seasons rather than sampling points were identified. Seawater samples collected about six months after the Great East Japan Earthquake contained many soil bacterial species including *Azotobacter*, suggesting that large amounts of earth and sand were delivered into the sea after the Tsunami. Thus, our analysis could differentiate common and unique bacterial species in the environmental water. The analysis also detected bacterial species that clearly reflected the changes of marine environment. Therefore, it was strongly suggested that the present method will be useful in monitoring the changes of bacterial flora that reflect global warming, changes in ocean environment as well as natural disasters. Furthermore, the proportion of each phylum in the samples showed unique composition patterns, reflected by the composition of the probes designed and mounted on the array chip. Thus, it was shown that this microarray could distinguish prokaryotic species that are applicable to the analysis of environmental microflora, in results comparable to those obtained by pyrosequencing.

The mean number of *Cx. tritaeniorhynchus* collected by CDC traps in northern parts of Kanto and Tohoku districts was very few compared to the number from western or southern parts of Tokyo (Fig. 2). This big difference will be related to the cold hardness of this vector mosquito species. In the incubator with 5°C, female *Cx. tritaeniorhynchus* which were treated under overwintering condition, were quickly died. Both temperature with 20°C and 25°C also showed the short survival periods. When these treated mosquitoes were kept with the condition of 10°C or 15°C incubators, about 20-30 % of adult mosquitoes survived at least 4 months. This means that JE vector, *Cx. tritaeniorhynchus* are able to overwinter in the moderately high temperature during winter season, although we don't know the actual environment of overwintering of this mosquito

species in the field. Detailed analysis of meteorological data, particularly in the winter season, will be contributed to understand the overwintering condition of JE vector. During 2009 to 2010, over 3,000 of *Cx. tritaeniorhynchus* females were maintained under outdoor condition, but no mosquitoes survived until next April. During 2010 and 2011, 400 females were tested, but no *Cx. tritaeniorhynchus* was recovered. Both results from the field trails and laboratory experiments showed same tendency.

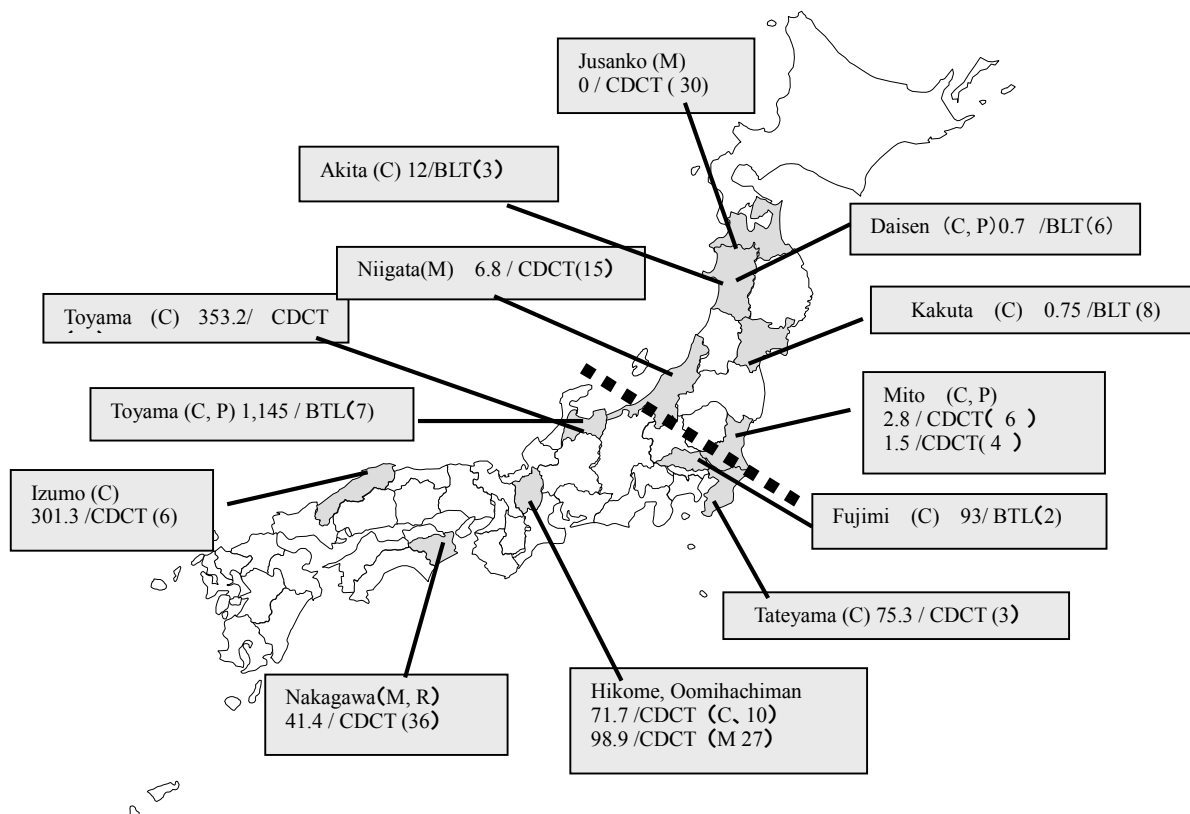


Fig. 2 Mean number of mosquitoes, *Culex tritaeniorhynchus* collected from various areas ( ): shows the number of traps. CDCT: CDC traps, BLT: Black light traps, M: Marsh, C: Cowshed, P: Pigpen, R: Rice paddy field.

In 2009 and 2010, mosquitoes were collected from four sites of the Kyushu district. A total of 1,361 mosquitoes collected in 2010 revealed that *Cx. p. pallens* and *Cx. p. form molestus* are widely distributed in all the surveillance areas whereas *Cx. quinquefasciatus* are found to inhabit in Minami-Satsuma city and Iki city. Since the route of *Cx. quinquefasciatus* and their dispersal in those places is really unclear, a further surveillance of the presence of this species in Japan should be monitored. Adult mosquitoes of *Cx. p. pallens* survived more longer than *Cx. p. form molestus*. Female mosquitoes of *Cx. p. pallens* survived longer than that of *Cx. p. form molestus* under the laboratory condition, and the maximum survival was for 209 days under the condition of 15°C and 11L:13D. Female of *Cx. p. form molestus* survived for 89 days under the condition of 20°C and 11L:13D. The data revealed that *Cx. p. pallens* and *Cx. p. form molestus* are widely distributed in Japan. *Cx. quinquefasciatus* are found to inhabit Minami-Satsuma, Kagoshima Prefecture and Iki, Nagasaki Prefecture. Since the route of migration of JE vector species, such as *Cx. tritaeniorhynchus*, *Cx. quinquefasciatus* and other *Culex* mosquitoes, and their dispersal in those places is really unclear, a further surveillance of the presence of this species in Japan should be monitored. The meteorological data in the winter season in Kyushu, Shikoku, Chugoku districts, climate chart with 1 km grid was prepared in the average of minimum low temperature categorized by below 4°C, 4-10°C and above 10°C. The climate data in average minimum low temperature prepared by 1980-2000 in Minami-Satsuma and Iki showed 4-10 °C. At least, it seems that the these

areas do not freeze over in the winter season. There is a possibility to expand the distribution area of *Cx. quinquefasciatus* in southern parts of Japan.

## 5. Discussion

JEV were isolated from wild boar in winter season. It suggested that JEVs are overwintering in wild boars. JEV genotype 3 was detected in a wild boar. It means that genotype 1 is currently major genotype, however, genotype 3 is still circulating in Japan. JEV inactivated vaccine produced from genotype 3 was effective for genotype 1 (JaNB037), however low effective against genotype 5. The invasion of JEV genotype 5 should be monitored in Japan.

We designed a novel DNA microarray that can distinguish approximately 64,000 prokaryotic species, and applied it to a comprehensive analysis of microflora in the environmental waters around Japan. Cluster analysis found possible index species based on water temperature and differences in environment. In addition, pathogenic bacteria and tetrodotoxin-producing bacteria were detected. Sequencing analysis reads all of the metagenomic DNA sequences, most of which are not taxonomic markers. In comparison, microarray analysis is simple and free from the alignment step. The proportion of each phylum in the samples showed unique composition patterns, compared with the composition of the probes designed and mounted on the array chip. Thus, it is possible that this microarray can distinguish prokaryotic species that are applicable to the analysis of environmental microflora, in results comparable to those obtained by pyrosequencing. DNA microarray analysis identified some common and specific bacterial species in the environmental water samples in Japan. This method will allow comprehensive detections of prokaryotes in a simple and cost-effective manner, making it possible to analyze clusters that include both known and novel index species. This DNA microarray will be a useful tool in the detection of pathogenic and temperature-sensitive bacteria in environmental samples collected in Japan. The microarray also detected potentially 'pathogenic' bacteria; however, it should be noted that this method cannot evaluate if they were actually pathogenic or even if they were viable.

The mean number of JEV vector mosquito collected in northern parts of Kanto and Tohoku districts using two types of traps was extremely different. It is generally known that *Cx. tritaeniorhynchus* is derived from subtropical or tropical area<sup>10,11</sup>. This big difference in number of collected mosquitoes between western and northern areas from Tokyo will be related to the cold hardness of this vector mosquito. In the condition of low temperature (5 °C) close to monthly mean temperature in January in Tokyo, all adult female mosquitoes were died during shot period. When these mosquitoes were incubated 15 °C under short day length, ca 30% of tested mosquitoes survived at least 4 months. These data show that high temperature will be needed to overwinter. It was shown that the mean temperature of daily lowest temperature in January will be a limited factor of overwintering of the vector mosquito. The present study demonstrated that *Cx. tritaeniorhynchus* did not survive under winter condition in the laboratory and outdoor of Tokyo. We thus concluded that the overwintering of *Cx. tritaeniorhynchus* is not negated, but the possibility is very low and the area is very limited.

However, after East Japan Great Earthquake the distribution *Cx. tritaeniorhynchus* and *Cx. inatomii* and abundance of the mosquitoes in tsunami affected rice-field in Miyagi Prefecture clearly changed<sup>12</sup>. It is important to know that the population density of *Culex* mosquitoes in these areas with natural disaster and global warming will be affected strongly. Distribution of *Cx. pipiens* complex in Japan was evaluated by using PCR method and 2 strains (*Cx. p. pallens* and *Cx. p. form molestus*) of the complex distributed from Kyushu to Hokkaido. *Cx. quinquefasciatus* distributes in southwest islands but the recent distribution was not fully known. From our analysis using PCR method, *Cx. quinquefasciatus* clearly distributes in south Kyushu and Iki. Adult mosquitoes of *Cx. p. pallens* survived more longer than *Cx. p. form molestus*. Recently, the culverts in the urban areas is one of overwintering sites of *Cx. p. pallens* in Japan<sup>13</sup>. From the collection of *Culex* mosquitoes from Kyushu to Hokkaido, *Cx. p. pallens* distributed in the whole country, but *Cx. p. form molestus* was not collected in Tohoku and Hokkaido districts. In Kyushu district, *Cx. quinquefasciatus* was

confirmed to be distributed in southern and northern Kyushu Island. Global warming will be contributed to expand *Cx. quinquefasciatus* distribution in the Shikoku and Chugoku districts in the future.

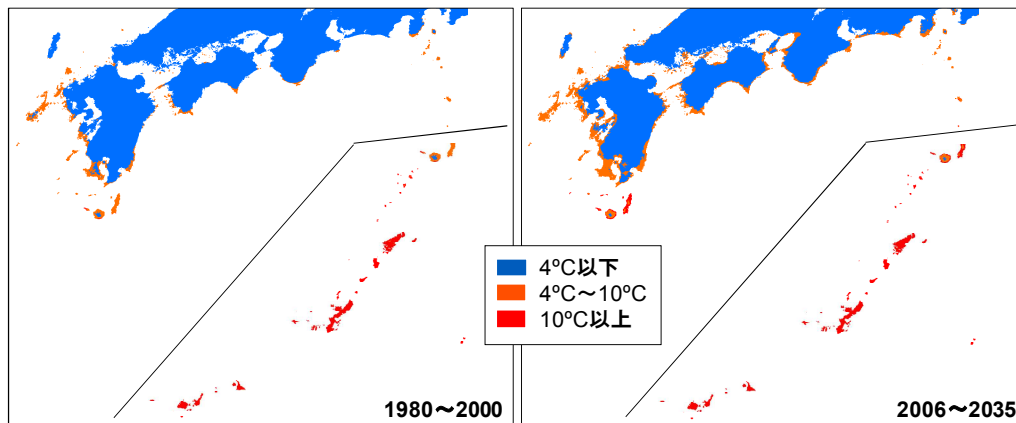


Fig. 3 One km mesh climate maps showing the area with below 4°C, 4-10°C and above 10°C monthly mean minimum temperature calculated for three months (December to February).



## Reference

- 1) Kobayashi, M., Komagata, O., Nihei, N. Global warming and vector-borne infectious diseases. *J. Disaster Research*, 3: 105-112, 2008.
- 2) Kobayashi, M., Kasai, S., Sawabe, K., Tsuda, Y. Distribution and ecology of potential vector mosquitoes of West Nile fever in Japan. *Global Environmental Research*, 12: 27-33, 2008.
- 3) Takhampunya, R., Kim, H.C., Tippayachai, B., Kengluetcha, A., Klein, T.A., Lee, W.J., Grieco, J., Evans, B.P. Emergence of Japanese encephalitis virus genotype V in the Republic of Korea. *Virology Journal*, 8: 449, 2011.
- 4) Li, M.H., Fu, S.H., Chen, W.X., Wang, H.Y., Guo, Y.H., Liu, Q.Y., Li, Y.X., Luo, H.M., Da, W., Duo, Ji.D.Z., Ye, X.M., Liang, G.D. Genotype V Japanese encephalitis virus is emerging. *PLoS Neglected Tropical Diseases*, 5: e1231, 2011.
- 5) Akama, T., Tanigawa, K., Nakamura, K., Kawashima, A., Wu, H., Sue, M., Yoshihara, A., Ishido, Y., Ishii, N., Suzuki, K. Whole Genome Tiling Array Analysis of Bacterial Genome. In: Campbell MJ (Eds): *DNA Microarrays, Synthesis and Synthetic DNA*. Nova Science Publishers, Hauppauge, NY, 2011.
- 6) Oda, T., Wada, Y., Hayashi, K. Considerations on the possibility of overwintering of Japanese encephalitis virus in *Culex tritaeniorhynchus* females. *Tropical Medicine*, 20: 153-155, 1978.
- 7) Tanaka, K., Mizusawa, K., Saugstad, S. A revision of the adult and larval mosquitoes of Japan (including the Ryukyu Archipelago and the Ogasawara Islands) and Korea (Diptera: Culicidae). *Contribution of American Entomology Institution*. 16: 1-987, 1979.
- 8) Tsuda, Y., Komagata, O., Kasai, S., Hayashi, T., Nihei, N., Saito, K., Mizutani, M., Kunida, M., Yoshida, M., Kobayashi, M. A mark-release-recapture study on dispersal and flight distance of *Culex pipiens pallens* in an urban area of Japan. *Journal of American Mosquito Control Association*, 24: 339-343, 2008.
- 9) Mohammed MA. et. al. 2011. Molecular phylogenetic and evolutionary analyses of Muar strain of Japanese encephalitis virus reveal it is the missing fifth genotype. *Infect. Genet. Evol.*, 11(5): 855-862
- 10) World Health Organization. Geographical distribution of arthropod-borne diseases and their principal vector. pp. 134, 1989.
- 11) LaCasse, W.J., Yamaguchi, S. Mosquito fauna of Japan and Korea. Office of the Surgeon, Hqs. 8<sup>th</sup> Army, APO343, pp. 213, 1955.
- 12) Tsuda, Y., Haseyama, M., Ishida, K., Niizuma, J., Kim, K.S., Yanagi, D., Watanabe, N., Kobayashi, M. After-effects of Tsunami on distribution and abundance of mosquitoes in rice-field areas in Miyagi Prefecture, Japan in 2011. *Medical Entomology and Zoology*, 63: 21-30, 2012.
- 13) Kobayashi, M., Kasai, S., Isawa, H., Hayashi, T., Sawabe, K., Tsuda, Y. Overwintering of *Culex pipiens pallens* in an urban environment of Saitama Prefecture in Japan, *Medical Entomology and Zoology*, 63: , 2012.