Two hidden taxa in the Japanese encephalitis vector mosquito, *Culex tritaeniorhynchus*, and the potential for long-distance migration from overseas to Japan

Satoru Arai1*, Ryusei Kuwata2*, Yukiko Higa3, Yoshiihide Maekawa3, Yoshio Tsuda3, Sudipta Roychoudhury3, Arlene Garcia Bertuso4, Tran Vu Phong5, Nguyen Thi Yen6, Tomoki Etoh6, Akira Otuka7, Masaya Matsumura8, Takeshi Nabeshima9, Keiko Tanaka Taya1, Nobuhiko Okabe10, Mutsuo Kobayashi7, Kyoko Sawabe3‡

1 Center for Surveillance, Immunization, and Epidemiologic Research, National Institute of Infectious Diseases, Tokyo, Japan, 2 Faculty of Veterinary Medicine, Okayama University of Science, Ehime, Japan, 3 Department of Medical Entomology, National Institute of Infectious Diseases, Tokyo, Japan, 4 Department of Parasitology, College of Public Health, University of the Philippines Manila, Manila, Philippines, 5 Department of Medical Entomology and Zoology, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, 6 Saga Fruit Tree Experiment Station, Saga, Japan, 7 Koshi Research Station, Institute for Plant Protection, National Agriculture and Food Research Organization, Kumamoto, Japan, 8 Institute for Plant Protection, National Agriculture and Food Research Organization, Ibaraki, Japan, 9 Department of Virology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan, 10 Kawasaki City Institute for Public Health, Kanagawa, Japan

* These authors contributed equally to this work.
‡ This author also contributed equally to this work.
* sawabe@niid.go.jp

Abstract

The *Culex vishnui* subgroups, particularly *Culex tritaeniorhynchus*, are considered the primary vectors of the Japanese encephalitis virus (JEV) in Asia. Recent molecular phylogenetic analyses of JEV isolates from Asian countries have shown that JEVs with diverse genetic variants are present in Asia. Furthermore, some JEV strains have been found to have crossed the East China Sea and been introduced into Japan. In this study, the possibility of overseas migration of the JE vector mosquito, *Cx. tritaeniorhynchus* was examined from the genetic, physical, and meteorological perspectives. Molecular phylogenetic analysis was performed based on both whole coding sequences and on the barcoding region of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene of *Cx. vishnui* subgroups collected from Asian countries. *Culex tritaeniorhynchus* was classified into two genetically independent taxa by *COI* sequences: the Japanese type (Ct-J), which inhabits Japan except for the Amami Islands of southern Japan, and the continental type (Ct-C), which inhabits the Asian region except for Japan. It was confirmed that approximately 10% of *Cx. tritaeniorhynchus* trapped during the summer in western Kyushu were Ct-C, and that they could fly for up to 38 h continuously. The meteorological analysis also confirmed that the atmospheric flow occurring over the continent coincided with the date of Ct-C capture. This is the first report showing the existence of two taxa in *Cx. tritaeniorhynchus*. Their physical and physiological characteristics suggest the possibility of long-distance migration from overseas.
regions to Japan across the East China Sea. Future efforts are expected to provide evidence to support the occurrence of long-distance migration of *Cx. tritaeniorhynchus* with JEV.

**Author summary**

We have been conducting research and studies on the importance of understanding the biological characteristics of each vector species in order to eradicate vector-borne diseases. In recent years, most reported cases of mosquito-borne diseases in Japan, such as dengue fever, malaria, and chikungunya fever, are imported cases from overseas; however, Japanese encephalitis (JE) is the only mosquito-borne disease for which autochthonous cases have been confirmed. The primary vector of JEV in Asia, a species called *Culex tritaeniorhynchus*, has long been recognized as a single species, however, this study is the first to report the existence of a cryptic species that is genetically distinct from *Culex tritaeniorhynchus*. Although long-distance migration of this species has been suspected for a long time, it has never been experimentally verified, and its contribution to the spread of JEV has not been investigated. The present study is also the first to confirm the capability of long-distance migration of this species biologically and physically, and to support it with meteorological data. Our objectives are to contribute to the prediction and minimization of the JE epidemic in Asia based on the results of experiments using the latest molecular genetic techniques combined with detailed physiological investigations.

**Introduction**

Japanese encephalitis (JE), the main cause of viral encephalitis, is an important human infectious mosquito-borne disease, with an estimated 68,000 clinical cases reported globally each year, and more than 30% of the cases result in death every year [1]. The occurrence of JE virus (JEV; family, Flaviviridae; genus, Flavivirus) has been reported throughout Asia [2–4], and this virus has recently spread to South India, Sri Lanka, and Northern Australia [5–7]. Twenty-four countries in the World Health Organization (WHO) South-East Asia and Western Pacific regions have endemic JEV transmission, and it has been estimated that more than three billion people are at risk of JEV infection [1]. Outside of these endemic areas, the emergence of JEV in Angola was reported in 2016 [8]. In Italy, viral RNA was detected in birds in 1997–2000 and *Culex pipiens* mosquitoes in 2010 [9,10]. JEVs have been isolated from more than 30 different species of mosquitoes, mainly the genus *Culex* [4]. However, three of these species, *Cx. vishnui*, *Cx. pseudovishnui*, and *Cx. tritaeniorhynchus*, which belong to the *Cx. vishnui* subgroup, have been recognized as the most important vectors of JEV in endemic countries. One of the main characteristics of this group is that the morphological features among its members are so similar that it is difficult to distinguish between species based on morphological classification alone. It is also recognized that among the members of this subgroup, it has been recognized that *Cx. tritaeniorhynchus* is arguably the most important JEV vector throughout Asia.

In Japan, a large JEV epidemic occurred in 1924, with more than 6,000 cases, and more than 60% of these cases resulted in death [11]. Vaccination against JEV began in the latter half of the 1960s. The number of patients infected with JEV has declined rapidly, with fewer than 10 cases per year since 1999 [12]. Furthermore, the management of paddy fields, which represent the main habitat of *Cx. tritaeniorhynchus* larvae, and breeding practices for domestic
animals, mainly pigs, have undergone extensive changes. Despite the decrease in JE cases due to these factors, the JEV becomes active every summer in both amplifying animals and vector mosquitoes [13,14]. Therefore, as the virus is still active, opportunities for autochthonous infections are not lost. Based on the analysis of JEV strains isolated in Asian countries, it was recently observed that virus strains with diverse genetic variations are present throughout Asia. For example, JEV strains isolated from Vietnam and China were found in Japan a few years later [15–17]. These findings strongly suggest that some JEV strains have been introduced into Japan from Southeast Asia. How these JEVs are brought into Japan remains an important question.

In the late 1960s, variety of insects including agricultural insects were captured on the weather-ship Tango in the East China Sea (29 N and 135 E), approximately 500 km off the islands of Japan [18–20]. Based on these findings, rice planthoppers, such as brown planthopper, *Nilaparvata lugens*, white-backed planthopper, *Sogatella furcifera*, and small brown planthopper, *Laodelphax striatellus*, were identified to frequently migrate to Japan from overseas each year [21]. As planthoppers are known to be harmful insects that damage and transmit viral disease to rice plants, studies to predict their migration patterns from continental Asia began to rapidly develop in the 1980s. Currently, models using highly precise three-dimensional simulations are being developed [22–24]. In this system, the migration dates of planthoppers were estimated based on the presence of a strong wind level of 850 hPa on the weather charts. This prediction program was developed by the Japan Plant Protection Association (JPP-NET) and is already in general use. During the survey in the late 1960s, several *Cx. tritaeniorhynchus* mosquitoes were identified among the insects captured at sea [18–20]. Furthermore, in Jiangsu Province, China, *Cx. tritaeniorhynchus* has also been observed returning from the north in October, covering an estimated distance of 200 km per night [25]. These studies provide evidence of long-distance migration of *Cx tritaeniorhynchus*. As such, the flight range of vector mosquitoes is an important factor in predicting the risk area of transmission of mosquito-borne pathogens to humans. It is necessary to physically verify the flight capability of this species. However, although there have been some reports of studies on forced flight experiments for *Aedes* mosquitoes [26–29] and *Culex pipiens pallens* [30], no studies have been conducted on *Cx. tritaeniorhynchus*.

In *Cx. tritaeniorhynchus*, a notable variant of the var. *siamensis* has been previously described based on morphological characteristics [31,32]. This variant was later recognized as a subspecies, *Cx. tritaeniorhynchus summorosus* [33,34] or variant [35]. Recently, *Cx. tritaeniorhynchus summorosus* was considered a variety of the subspecies *Cx. tritaeniorhynchus*, specifically in the Indian strain [36]. Today, genetic analysis such as using the mitochondrial cytochrome c oxidase subunit I (COI) have become popular, but results applied to this species have not yet been reported. Therefore, according to conventional taxonomy, the common perception has been that *Cx. tritaeniorhynchus* is a single species, though it is widely distributed throughout Asia. In this study, phylogenetic analysis was conducted based on COI sequences of *Cx. tritaeniorhynchus* collected in Asian countries, including Japan, and the results indicated that they can be divided into two genetically different taxonomic groups. We used the above indices to differentiate between mosquitoes captured using net traps for planthoppers in the Kyushu region during the summer season, and it was confirmed that some mosquitoes had flown from abroad. We conducted a forced flight experiment using a flight mill [37,38] and a meteorological analysis using the National Oceanic and Atmospheric Administration’s (NOAA) Hybrid Single Particle Lagrangian Integrated Trajectory Model (NOAA based on meteorological analysis using the NOAA HYSPLIT model [39], to estimate the flight
capabilities of the *Cx. tritaeniorhynchus* and the possibility of their participation in long-distance migration.

**Methods**

**Mosquito collection**

Mosquitoes were collected in Japan, Vietnam, Philippines, and Indonesia from 2007 to 2011. Adult mosquitoes were collected around livestock throughout the day using a Centers for Disease Control and Prevention (CDC) miniature light trap enhanced with dry ice throughout the day [40]. The aspirators were generally used to collect mosquitoes on livestock for 3 h each night between 18:00 and 21:00. Collected adult mosquitoes were transported in an icebox to the laboratory of each counterpart. Larval mosquitoes were collected from paddy fields using dippers. Larvae were transported alive to the office and reared to adults under laboratory conditions, then identified after their emergence. All field-collected adult mosquitoes were transported on ice to the laboratory for species identification according to established identification keys [41–44]. Following species identification, the mosquitoes were stored at −80°C until DNA extraction. All classified mosquito specimens were transferred individually into 1.8 mL microtubes (Eppendorf, Hamburg, Germany), and stored at -80°C until subsequent analyses by COI sequencing. The collectors always wore long-sleeved shirts, long pants, and hats, and applied repellent to the bare skin of their hands and faces to prevent mosquito bites.

The net trap (NT) and Johnson-Taylor suction trap (JT-ST) (Burkard Manufacturing Co. Ltd., Hertfordshire, UK) have often been employed as standard tools in agricultural investigations for monitoring long-distance migratory insects. Our NT consists of a net attached to a 1.5 meter deep, 1 meter diameter ring at the top of a pole 10 m above the ground. Insect collection using NTs was conducted at three locations in the western Kyushu area, namely, Saga, Iki, and Goto Cities. A JT-ST was used in Minami-Satsuma City, located in the southern Kyushu area. Mosquitoes trapped in NTs between June and September 2009 and 2010 were used for the analysis.

**Sequence analysis of genomic DNA of mosquito specimens**

Total genomic DNA was extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) or MagDEA DNA 200 (GC) (Precision System Science, Matsudo, Japan) according to the manufacturer’s protocol. Genomic DNA of mosquitoes was used to determine the nucleotide sequence of the entire 1,542-base-pair (bp) of the COI gene and the 658 bp of partial COI sequences, the DNA barcoding region [45,46]. The COI barcoding region was amplified using primer pairs LCO1490 and HCO2198 [45] and TaKaRa Ex Taq Hot Start Version (TaKaRa Bio Inc., Shiga, Japan) or a newly designed primer pair, MosMt-948F (5’-AGG WGG ATT ACC YCC ATT TYT AGG A-3’) and MosMt-3070R (5’-ATC CTA AAT TTG CTC AGG TTG CCA-3’) with the Phusion enzyme (New England Biolabs, Ipswich, MA, USA). The PCR reactions were carried out in a 10 μl volume containing 1.00 μl of 10x PCR buffer, 0.80 μl of 2.5 μM dNTP mixture, 0.05 μl of 5 U/μl Ex Taq HS or Phusion enzyme, 0.50 μl of each 2.5 μM primer, 6.15 μl of DDW and 1.00 μl of DNA template. Amplification conditions were as follows: initial denaturation at 95°C for 5 min, followed by 5 cycles of 94°C for 40 s (denaturation), 45°C for 1 min (annealing), 72°C for 1 min (extension), 35 cycles of 94°C for 40 s (denaturation), 51°C for 1 min (annealing), 72°C for 1 min (extension), and a final extension at 72°C for 10 min for Takara Ex Taq or stepdown program was used for the Phusion enzyme: initial denaturation at 95°C for 2 min was followed by two cycles each of denaturation at 95°C for 15 s, 2°C step-down annealing from 60°C to 50°C for 30 sec, and elongation at 68°C for 1 min 30 s, then 30 cycles of denaturation at 95°C for 15 sec, annealing
at 55˚C for 30 s, and elongation at 68˚C for 1 min 30 s. PCR products were confirmed with MultiNA (SHIMADZU Corporation, Kyoto, Japan) and a DNA 12000 reagent kit. The resultant amplification products were purified with ExoSAP (Affymetrix Inc., Santa Clara, CA, USA) or Centri-Sep columns (Princeton Separations, Adelphia, NJ, USA). Sequencing samples were prepared using the BigDye Terminator ver1.1 Cycle Sequencing Kit (Thermo Fisher Scientific K.K., Tokyo, Japan), and the base sequences were decoded with ABI PRISM 3100-Avant Genetic Analyzer (Thermo Fisher Scientific K.K.) and edited with ATGC-Win ver.14 (Genetyx Corp., Tokyo, Japan).

**Phylogenetic analysis**

Molecular phylogenetic analysis was performed based on the nucleotide sequences of the whole coding sequences and the barcoding region of the mtDNA COI gene. Multiple alignments of the sequences from selected mosquito strains were carried out using the Clustal W program [47]. GenBank accession numbers for the sequences used in the phylogenetic analysis are listed in S1 Table. The aligned matrix data were analysed using MrBayes software [48] for Bayesian phylogenetic inference under the Markov chain Monte Carlo algorithm with the GTR+I+G model evolution, as selected by jModelTest [49].

**Flight mill experiments**

The mosquitoes employed in this analysis were of the Cx. tritenniorhynchus Chiba strain (Ct-J) collected from Chiba Prefecture (35.60 N, 140.24 E) in 2009 and the Cx. pipiens pallens NIID strain (Cpp) collected from Tokyo Metropolis (35.42 N, 139.43 E) in 2008. Both strains were established as laboratory colonies at 25˚C and 70% relative humidity (RH), under a photoperiod of 16:8 (Light:Dark) h, and maintained after emergence under three different combinations of photoperiod and temperature: 8L:16D and 15˚C; 11L:13D and 20˚C; and 16L:8D and 25˚C, with 80% RH. The flight mill experiments were carried out under the same conditions used for mosquito maintenance. Ten females, 7–10 days after emergence, were used for the flight mill experiments. The experiments were replicated twice, in June 2010 and November 2011.

The rotor consisted of a thin steel line of 14 cm in total length as a horizontal arm, an insect pin of 4 cm as a rotating axis, and a small sponge rubber block to which the arm and the axis were fixed in a cross shape. Both outer parts of the arm were bent at 2 cm from each tip by 90˚ downward. The length of the right or left arm was approximately 5 cm, and the weight of the rotor was in average 81.6 mg. Twenty rotors were placed in an incubator and operated at 15˚C, 20˚C, and 25˚C with 80% RH. Immediately before the flight mill experiment, the tip of the arm was attached to the notum of mesothorax of a mosquito lightly anesthetized by placing it on ice, with a small amount of quick-drying adhesive (Wood glue, Konishi Co., Ltd., Osaka, Japan). Each rotor with an insect was set in the up and down directions attached to two parallel acrylic rods. Two pulse electric signals per 360˚ rotation were produced by a photo sensor attached to the rods. The number of pulses per 5 s was recorded automatically by specify software; fewer than four pulses per 5 s did not meet a standard for continuous flight, and as a result, were not included in a later analysis. Flight mills were continuously monitored for at least 48 h. Thereafter, the continuous flight time for each individual mosquito was calculated. A t-test was used for the statistical analysis. Statistical significance was set at $p < 0.05$. GraphPad Prism software version 7 and Microsoft Excel 2016 were used for the statistical analyses.

In this study, we could only evaluate the flight capability of Ct-J. Originally, we should have used a flight mill to estimate the flight capability of Ct-C. Unfortunately, as far as we know,
there are no maintained colonies of Ct-C in the world. Although whether Ct-C can fly as well as Ct-J needs to be clarified in future studies using Ct-C, it is known that in late autumn, long-distance domestic migration of Ct-J occurs suddenly, which is thought to be a pre-diapause seasonal migration from breeding sites to overwintering sites in Japan [50]. It is reasonable to assume that this ability to migrate long distances is a common characteristic of Cx. tritaeniorhynchus. Therefore, we considered that the flight capability of Ct-C could be evaluated based on the results of flight-mill experiments using Ct-J.

**Backward trajectory analysis using the NOAA’s HYSPRIT model**

HYSPRIT is a computer model used to compute air parcel trajectories and the deposition or dispersion of atmospheric pollutants. This tool was developed by NOAA and Australia’s Bureau of Meteorology [51,52]. HYSPRIT can be run in client-server mode from the NOAA website: https://www.ready.noaa.gov/HYSPLIT.php. The backward trajectory analysis of HYSPRIT can be combined with satellite imagery to infer how air pollution has been propelled by air masses. We analysed the atmospheric flow at 24–36 h before the day when Cx. tritaeniorhynchus was found, and determined a possible flying path from the continent to four locations in Kyushu District, western Japan, where NTs and JT-ST were installed.

**Results**

**Phylogenetic analyses of members of the Cx. vishnui subgroup**

A total of 86 mosquito specimens from the Cx. vishnui subgroup, comprised of 60 specimens from Japan and 26 from six Asian regions outside Japan, were used for phylogenetic analysis (Table 1).

The details of the specimens were as followed. Sixty-nine Cx. tritaeniorhynchus including 19 sequences from GenBank database, 10 Cx. pseudovishnui including 5 from GenBank database and 7 Cx. vishnui including 2 from GenBank database. The MrBayes tree was constructed based on 1542 complete nucleotide sequences of the COI gene with 26 corresponding strains from the GenBank database. Twenty-three specimens were outgroup sequences (21 corresponding sequences from GenBank databases and two specimens analysed in this study) (S1 Table).

The Cx. vishnui subgroup was classified into four robust taxa: Cx. vishnui, Cx. pseudovishnui, and two Cx. tritaeniorhynchus (Fig 1). The Cx. tritaeniorhynchus specimens were divided into two taxa. The phylogenetic relationship between these two taxa (clusters 1 and 2) was suggested to be as independent as that between Cx. vishnui and Cx. pseudovishnui. Cluster 1 of Cx. tritaeniorhynchus was composed of 19 specimens from overseas, but also included three specimens from Japan (two from Okinawa Prefecture and one from Kagoshima Prefecture). These three specimens were captured by NTs (two from Saga Prefecture) and a JT-ST (one from Kagoshima Prefecture). Cluster 2 of Cx. tritaeniorhynchus, consisting of 44 specimens, was composed entirely of 43 specimens from Japan, except for one specimen from South Korea. The remaining nine specimens captured by NTs were included in this cluster. The other MrBayes tree (Fig 2) was constructed based on 658 bp COI partial sequences of barcoding regions from the same specimens of the Cx. vishnui subgroup, as described previously. In this tree, a total of 22 specimens were outgroup sequences (20 corresponding sequences from GenBank databases, and two specimens analysed in this study) (S1 Table). There were four genetically independent taxa in the Cx. vishnui subgroup, which showed the same trend as the results shown in Fig 1. Surprisingly, even in a partial sequence of 658 nucleotides, this result
showed that *Cx. tritaeniorhynchus*, previously thought to be a single species, encompasses two genetically distinct taxa.

Based on the results of these two phylogenetic trees, cluster 1, consisting of specimens from the south of the Amami Archipelago and Asian countries other than Japan, was designated as the continental type of *Cx. tritaeniorhynchus* (*Ct*-C), and cluster 2, consisting of specimens from northern mainland Kyushu, was designated as the Japanese type of *Cx. tritaeniorhynchus* (*Ct*-J; Fig 3). As shown in the callout of Fig 3, it is clarified that there are not only *Ct*-J but also *Ct*-C specimens in the summer. This means that there are times and seasons when these two types coexist in western Kyushu areas.

Table 1. Mosquito specimens used in this study.

| Mosquito species          | Origine  | No. specimens analyzed |
|---------------------------|----------|------------------------|
|                           |          | Total  | Ct-C  | Ct-J  |
| **Culex vishnui subgroup**|          |        |       |       |
| *Culex tritaeniorhynchus* | Japan    | 49     | 6     | 43    |
|                           | South Korea | 2      | 1     | 1     |
|                           | China     | 2      | 2     | 0     |
|                           | Taiwan    | 3      | 3     | 0     |
|                           | Philippines | 1     | 1     | 0     |
|                           | Thailand  | 1      | 1     | 0     |
|                           | Vietnam   | 11     | 11    | 0     |
| *Culex pseudovishnui*     | Japan    | 10     | 10    | 0     |
| *Culex vishnui*           | India    | 1      | 1     | 0     |
|                           | Japan     | 1      | 1     | 0     |
|                           | Philippines | 5     | 5     | 0     |
| **Total**                 |          | 86     | 42    | 44    |

| Out group species         |          |        |       |       |
|---------------------------|----------|-------|-------|-------|
| *Aedes aegypti*           |          | 1     |       |       |
| *Aedes albopictus*        | Japan    | 1     |       |       |
| *Anopheles darlingi*      | Brazil   | 1     |       |       |
| *Anopheles gambiense*     |          | 1     |       |       |
| *Cneaphia dactotensis*    | Canada   | 1     |       |       |
| *Culex bitaeniorhynchus*  | Japan    | 1     |       |       |
| *Culex decens*            | Senegal  | 1     |       |       |
| *Culex gelidus*           | China    | 1     |       |       |
| *Culex infantulus*        | Japan    | 2     |       |       |
| *Culex mimeticus*         | Japan    | 1     |       |       |
| *Culex nigropunctatus*    | China    | 1     |       |       |
| *Culex orientalis*        | Japan    | 1     |       |       |
| *Culex pipiens*           | Japan    | 2     |       |       |
| *Culex quinquefasciatus*  |          | 1     |       |       |
| *Culex sasai*             | Japan    | 1     |       |       |
| *Culex tarsalis*          | USA      | 1     |       |       |
| *Culex torrentium*        | Russia   | 1     |       |       |
| *Culex vagans*            | South Korea | 2    |       |       |
| *Culiseta impatiens*      | Canada   | 1     |       |       |
| *Tipula cockerelliana*    | China    | 1     |       |       |
| **Total**                 |          | 23    |       |       |

https://doi.org/10.1371/journal.pntd.0010543.t001
Mosquitoes collected using net traps and Johnson-Taylor suction trap

Among the long-distance migratory insects captured between June 2009 and September 2010, a total of 1241 mosquitoes were trapped by NTs, and a JT-ST (Table 2). The mosquitoes were morphologically classified into three genera, namely, Culex, Aedes, and Anopheles, with five species. In 2009, a total of 32 and 222 specimens were captured in Saga and Minami-Satsuma Cities, while in 2010, 99 and 888 specimens were captured in Goto and Iki Cities, respectively. Among the captured specimens, Cx. tritaeniorhynchus was the most abundant species (47.8%). The 593 specimens of Cx. tritaeniorhynchus were classified into two types: Cx. tritaeniorhynchus Cx. tritaeniorhynchus Cluster 1 (Ct-C) and Cx. tritaeniorhynchus Cluster 2 (Ct-J) using the COI partial sequences.

Net traps (NTs) were used in the Western Kyushu, Saga, Goto and Iki Cities. Johnson-Taylor suction trap (JT-ST) was used in the south Kyushu, Minami-Satsuma City.
Culex tritaeniorhynchus was discriminated into Japanese type (Ct-J) and Continental type (Ct-C) by the partial COI sequences. The shaded area marks total number of planthoppers collected at each trap. The discovery date of Ct-C between June and September is shown in Fig 4. In Saga City in 2009, three out of the 27 specimens were classified as Ct-C (11.1%). Each was found on July 8, 10 and 23. In Minami-satsuma City, only one Ct-C out of 81 specimens was captured, on 3 August 2009 (1.2%). In Goto City in 2010, one of the 14 Ct-C specimens was captured on 30 June and the other on 22 July (14.3%). In Iki City in 2010, one each of the 471 Ct-C specimens was captured on 17, 22, 24, 27, and 31 July (0.9%). The number of Ct-C specimens was particularly high in July in both 2009 and 2010. Of the total specimens captured, Ct-C specimens were
12.5% (3/24), 8.3% (1/12), and 11.1% (5/45) in the Saga, Goto, and Iki Cities, respectively. These results indicate that approximately 10% of the total trapped *Cx. tritaeniorhynchus* were *Ct*-C individuals that migrated to western Kyushu from overseas regions. When a JT-ST was used in Minami-Satsuma City in 2009, only one specimen of *Ct*-C was trapped on 1 August. This trend was different from that of the other three collection sites where NTs were installed.

![Map of mosquito collection sites in Kyushu District](https://doi.org/10.1371/journal.pntd.0010543.g003)

**Fig 3.** Locations of the mosquito collection sites in this study are shown here and details are given in S1 Table. The phylogenetic analysis indicated the *Cx. tritaeniorhynchus* was categorized into two taxa, *Ct*-J (open circles) and *Ct*-C (closed circles). The *Cx. tritaeniorhynchus* were captured in net-traps (NTs) and a Johnson-Taylor suction trap (JT-ST) installed at four localities on the Kyushu District (shaded area). Open and closed triangles with blue indicate capture of *Ct*-J and *Ct*-C of the *Cx. tritaeniorhynchus*, respectively. Double circles indicate specimens not used in the phylogenetic analysis, but are plotted on the map as the *Ct*-C referred from the COI sequences of the GenBank database. A map was created using free materials downloaded from the following websites: https://power-point-design.com/ppt-design/world-map-for-powerpoint/ and https://power-point-design.com/ppt-design/japan-map-available-for-powerpoint/.

Based on the geographical positions recorded using a geographical positioning system (GPS: GPSMAP64, Garmin, USA) (S1 Table), the collection sites were plotted on this map.
Flight capability of *C. tritaeniorhynchus* female based on flight mill experiments

*Culex pipiens pallens* (*Cpp*) was the most abundant species caught in the NTs, after *C. tritaeniorhynchus*, in western and southern Kyushu (Table 2). Therefore, *Cpp* was considered the most suitable comparison in evaluating the flight capability of *C. tritaeniorhynchus*. Each 20 mosquitoes were used for the flight mill experiments. There was a large variation between individuals, and a few mosquitoes recorded no flight or less than three cycle rotations in 5 s. Therefore, the average flight times were calculated using mosquitoes that rotated over three cycles. These flight times were 10.05 h (0.95–25.05 h, *n* = 19) and 5.49 h (0.01–15.00 h, *n* = 19) at 15˚C; 5 h (0.03–11.08 h, *n* = 19) and 1.28 h (0.01–5.2 h, *n* = 16) at 20˚C; and 8.61 h (0.001–19.31 h, *n* = 17) and 4.45 h (0.02–16.18 h, *n* = 18) at 25˚C for *Ct*-J and *Cpp*. The *Ct*-J specimens could fly longer than *Cpp* under all experimental conditions (*t*-test, *p* < 0.05 at 15˚C, *p* < 0.001 at 20˚C, and *p* < 0.1 at 25˚C) (Fig 5). When the rearing and flight temperatures were the same, *Ct*-J had a longer continuous flight time than *Cpp* under all experimental conditions except *Ct*-J at 20˚C. *Ct*-J females were reared at 25˚C, but flight experiments were conducted at 20˚C. Both species flew the longest at the lowest temperature of 15˚C. Particularly *Ct*-J at 20˚C showed the longest total flight time at 10.14 h (0.2–37.97 h, *n* = 20). A significant difference was found between *Ct*-J and *Ct*-J at 20˚C (*t*-test, *p* < 0.05). Surprisingly, one *Ct*-J specimen was found to fly continuously for 38 h.

Some of the *Cpp* specimens flew continuously for > 20 h; however, a remarkable difference in flight patterns was found between *Ct*-J and *Cpp*. The number of rotations in 5 s was compared (Fig 6). The results suggested that *Ct*-J tended to rotate for longer periods for a smaller number of rotations than *Cpp* under all conditions at 15˚C (*Cpp* Fig 6A and *Ct*-J Fig 6B), 20˚C (Fig 6C and 6D), and 25˚C (Fig 6F and 6G). Particularly for *Ct*-J (Fig 6E), three to five rotations were very common. Fig 7 shows the differences in flight patterns between each specimen that exhibited the longest flight time, *Cpp* (Fig 7A, shown in bold in Fig 6A) and *Ct*-J (Fig 7B, shown in bold in Fig 6E). Although the *Cpp* fluttered their wings powerfully, it was for a short time, after which their wings tended to stop flapping immediately (Fig 7A). For *Ct*-J, in contrast to *Cpp*, the flights lasted for a long period and their wings continued to flap very slowly.

### Table 2. Mosquitoes captured in net traps and a Johnson-Ta ylor suction trap in 2009 and 2010.

| Mosquitoes       | Locations | 2009 | 2010 | Total | (%)  |
|------------------|-----------|------|------|-------|------|
|                  | Saga      | Minami-Satsuma | Goto | Iki   |      |
| *Culex tritaeniorhynchus* | 27       | 81   | 14   | 471   | 593  | (47.8) |
| *Ct*-J           | 24       | 80   | 12   | 466   | 582  | (46.9) |
| *Ct*-C           | 3        | 1    | 2    | 5     | 11   | (0.9)  |
| *C. pipiens pallens* | 4       | 3    | 4    | 175   | 186  | (15.0) |
| *C. quinquefasciatus* | 0      | 69   | 0    | 2     | 71   | (5.7)  |
| *C. pseudovishnui* | 0       | 4    | 0    | 0     | 4    | (0.3)  |
| *Culex spp.*     | 0        | 19   | 2    | 0     | 21   | (1.7)  |
| *Aedes vexans*   | 0        | 0    | 0    | 60    | 60   | (4.8)  |
| *Aedes spp.*     | 1        | 20   | 36   | 5     | 62   | (5.0)  |
| *Anopheles spp.* | 0        | 20   | 0    | 106   | 126  | (10.2) |
| Un-identified    | 0        | 6    | 43   | 69    | 118  | (9.5)  |
| **Total**        | 32       | 222  | 99   | 888   | 1,241| (100)  |

https://doi.org/10.1371/journal.pntd.0010543.t002
throughout the flight (Fig 7B). The difference in flight patterns between Cpp and Ct-J+ was more pronounced than the difference in the total flight distance.

**Backward trajectory analysis by the NOAA’s HYSPRIT Model**

The results of the flight mill experiment showed that female Ct-J reared at 25˚C flew the longest during the flight at 20˚C (Ct-J+) (Figs 5 and 6E). This is important because the temperature at approximately 800–900 m above the ground is 20˚C when the ground surface is approximately 25˚C, and decreases by 0.6˚C every 100 m higher. Therefore, we analysed the atmospheric flow in the airspace 1000 m above the ground [23,53]. Because the speed in this airspace is more than 10 m/s, it was calculated that the planthoppers could fly in the lower jet stream in the airspace around 1500 m above the ground. It was suggested that the planthoppers could fly in the lower jet stream in the airspace around 1500 m above the ground [23,53].

Because the speed in this airspace is more than 10 m/s, it was calculated that the planthoppers were estimated to reach the western Kyushu areas within 24–36 h of departure. As the back trajectories in our study reached 1000 km within 36 h, we ran the HYSPLIT for 36 h alone. The HYSPLIT model was used to produce 10 back trajectories at 2-h intervals ending at 15:00 UTC on 8 July 2009 and 10 July 2009 in Saga City (Fig 8A and 8B), and on 30 June 2010 Goto...
City (Fig 8C). In these cases, the dates of Ct-C capture and the atmospheric flow coincided very well. The Ct-C specimens may have been carried by air flows originating from mainland China and the East China Sea up to 36 h before they arrived in Japan.

However, we found some cases where the date of Ct-C capture did not coincide with the onset of air flow. In these cases, air flows were observed from 36 h to several days earlier than the trap dates. The HYSPLIT model was used to produce 10 back trajectories at 2-h intervals ending at 00:00 UTC for each date. For example, it was estimated that the Ct-C specimen trapped in Saga City on 23 July 2009 may have been carried by air flows originating from South Korea or China on 21 July (S1A Fig), while the Ct-C specimen captured on 3 August 2009 in Minami-Satsuma City may have been carried by air flow from China on 29 July (S1B Fig), and the specimen captured on 13 July 2010 in Goto City may have been transported by air flow from China on 13 July (S1C Fig). In Iki City in 2010, it is possible that the Ct-C specimens captured on 17 July were carried by air flow from mainland China on 14 July (S1D Fig); those captured on 22 July likely departed from Korea on 15 July (S1E Fig); specimens captured

Fig 5. Comparison of a total flight time between Cx. tritaeniorynchus (Ct-J, shaded bars) and Cx. p. pallens (Cpp, open bars) under each flight condition. The bars represent the mean with standard deviations (SDs). An independent two sample t-test assuming unequal variances was applied to determine significant differences. Statistically significant differences are indicated shown by Roman numerals; a, \( p < 0.001 \); b, \( p < 0.01 \); c, \( p < 0.1 \).

https://doi.org/10.1371/journal.pntd.0010543.g005
on 24 and 27 July probably left Jeju Island, South Korea, on 26 and 27 July (S1F and S1G Fig), and those captured on 31 July most likely travelled on air flow generated in China on 30 July (S1H Fig). Considering the long-distance migratory planthoppers, as a reference, this data strongly suggested that the crossing of the East China Sea by *Cx. tritaeniorhynchus* is meteorologically possible.

**Discussion**

In the present study, we demonstrated for the first time the existence of two taxa of *Cx. tritaeniorhynchus*, which are referred to as Ct-J and Ct-C in this study for convenience, using COI sequence analysis. In the COI phylogenetic trees developed in this study, the genetic distance between Ct-J and Ct-C was approximately the same as that between *Cx. vishnui* and *Cx.*
Fig 7. Comparison of the flight pattern between *Cx. p. pallens* (*Cpp*) at 15°C (A) and *Cx. tritaeniorhynchus* (*Ct-J*) at 20°C (B). A and B are the flight patterns of the individuals with the longest total flight time in each experiment of Fig 6A and 6E, respectively. Flight patterns of *Cpp* and *Ct-J* were observed for 69 h and 72 h, respectively. The shaded area marks environmental darkness.

https://doi.org/10.1371/journal.pntd.0010543.g007

Fig 8. Computed back trajectories at 2-h intervals for the 36-h period ending at 1500 UTC of 8 and 10 July 2009 at Saga City (A and B), and 30 June 2010 at Goto City (C). Top portion shows the horizontal path, and the bottom portion shows the vertical path of the trajectories. Each figure is the result of a backward trajectory analysis of HYSPLIT using the NOAA website: https://www.ready.noaa.gov/HYSPLIT.php.

https://doi.org/10.1371/journal.pntd.0010543.g008
pseudovishnui. From this result, we concluded that the Cx. vishnui subgroup consists of four species, instead of three. The present study also revealed that Ct-J and Ct-C are genetically distinct not only by the full-length COI sequence, but also by partial COI sequences. DNA barcoding using partial COI sequences can be used to identify closely related species, as well as new species. COI barcode region sequences of Cx. tritaeniorhynchus are now being accumulated, and the discussion of both types will proceed more quickly in the future. To date, the common perception has been that Cx. tritaeniorhynchus is a single species, though it is widely distributed throughout Asia, according to conventional taxonomy. However, our results strongly suggest the possibility of the existence of a cryptic species or a new species in Cx. tritaeniorhynchus based on the genetic aspects. Although we compared the morphology of the male genitalia, one of the most important taxonomic keys, between Ct-J and Ct-C, unfortunately, any significant differences were found. It is expected that the morphological characteristics of both types will be studied in detail in the future.

The present study revealed the existence of Ct-J and Ct-C in Korea, and suggested the Amami Archipelago could be the boundary of the distribution of Ct-J and Ct-C. However, the actual boundary of distribution between the two types is unknown. In addition, although we could not confirm any sequences on the COI gene showing the characteristics of hybridization between the two types, we cannot deny the possibility of the existence of hybrid, as they coexisted in the same region, even temporarily. As a next step, it will be necessary to analyse not only the mitochondrial genes from the mother, but also the nuclear genes inherited in pairs from both parents. Based on these facts and questions, we hope that as soon as possible a discussion will begin on whether to support each type as an independent species.

Culex tritaeniorhynchus is a tropical/subtropical mosquito that does not have a high resistance to low temperatures. However, this species has previously been observed to overwinter in caves in the Izu Peninsula and Chiba Prefecture in eastern Japan [54], and sewage infrastructure (such as culverts), even in urban areas of Japan [50]. Owing to global warming, the areas in which the Cx. tritaeniorhynchus can overwinter have undeniably expanded; however, the areas in Japan where they can overwinter remain limited. Therefore, there is a high possibility of Cx. tritaeniorhynchus in Japan is temporarily mixed with individuals that migrate from the south every year. Several findings have been revealed regarding the long-distance migration of Cx. tritaeniorhynchus. In Jiangsu province, China, they have been observed flying back from the north in October, covering an estimated distance of 200 km per night [25]. Recently, sequence divergence results have shown that the Australian population of this species is likely to have originated in East Timor (99.7% nucleotide similarity) [55]. These results suggested that while the introduction pathways were unconfirmed, it is plausible that Cx. tritaeniorhynchus may have travelled to Australia from Timor-Leste via windblown adult mosquitoes, given the relatively short distance (465 km) between Timor-Leste and Melville Island near Darwin, and that Cx. tritaeniorhynchus has been previously recorded as flying 200–500 km over sea waters in the Northwest Pacific [55]. These findings support the hypothesis that the Cx. tritaeniorhynchus has ample potential to migrate across the East China Sea into Japan.

We were able to detect Ct-C migrating from overseas to Japan using the COI sequence in this study. It was clarified that Ct-C was also captured in the deployed NT and JT-ST. A total of 11 Ct-C specimens were trapped from June 2009 to August 2010 in the Kyushu region. In these areas, approximately 10% of the specimens captured in the traps, especially in July, were Ct-C. Interestingly, the dates when Ct-C were caught coincided well with the predicted migration date of the planthoppers [56]. The following analysis was conducted under the assumption that this species has the same capabilities as planthoppers. A backward trajectory analysis using the HYSPRIT model in an airspace 1000 m above the ground was conducted, based on
the weather data provided by the NOAA in the USA. The following three cases suggested that the migration date of the Ct-C and air flow coincided very well: specimens caught on 8 and 10 July in 2009 at Saga City, and on 30 June in 2010 at Goto City. In other cases, the migration of Ct-C did not coincide with air flow, but air flows that occurred before more than 36 h were observed. This suggests that Ct-C, which had already arrived in Japan and stayed for a while, may have been captured in the trap along with Ct-J.

The distance between mainland China and western Kyushu is approximately 1000 km. It was suggested that the planthoppers could fly in the lower jet stream in the airspace around 1500 m above the ground [23,53]. Because the speed in this airspace is more than 10 m/s, it is calculated that the planthoppers are estimated to reach the western Kyushu areas within 24–36 h. In our flight mill experiments, we simulated long-distance migration at 1000 m above the ground when the ground temperature was 25˚C (Ct-J"). In other words, a female Ct-J reared at 25˚C and flown at 20˚C was observed to be able to fly continuously for approximately 38 h (Ct-J"). In addition, two females of Ct-J were also observed continuously flying at 15˚C for up to 20 h (maximum 25 h). This result suggests that Ct-J can fly not only at 1000m above the ground, but also at 1500m in the airspace.

In this study, we only evaluated the flight capability of Ct-J. Although whether Ct-C can fly as well as Ct-J needs to be clarified in future studies using Ct-C, it is known that in late autumn, long-distance domestic migration of Ct-J occurs suddenly, which is thought to be a prediapause seasonal migration from breeding sites to overwintering sites in Japan [50]. It is reasonable to assume that this ability to migrate long distances is a common characteristic of Cx. tritaeniorhynchus. When the same mechanism of long-distance migration as that of the planthoppers can be applied to Cx. tritaeniorhynchus, then it is possible this species could physically migrate for more than 1000 km.

Previous reports on flight mill experiments in Aedes mosquitoes [26–29] and Cx. p. pallens (Cpp) [30] are known. For instance, the mean flight time for Cpp was 17,090.84 s (4.8 h) in 20-d-old females, and 14884.96 s (4.1 h) in 2-d-old. They also reported that their total flight time and distance tended to be shorter for 5- and 6-d-old females than any other age group, showing significant differences in flight capability between ages. This result suggested that young or aged females may be able to fly longer [30]. In our study using 7–10-d-old females, the longest flight time of Cpp was 5.49 h at 15˚C. Other d-old Cpp may be able to fly for a longer time. Although there was no information on the Cx. tritaeniorhynchus in the previous study [30], it would also be necessary to evaluate the flight capability of young or aged females.

At the start of the 1990s, JEV in Asian countries, including Japan, transitioned from genotype III (GIII) to genotype I (GI). Currently, GI is still the major endemic strain at least in China and Japan [57,58]. However, in recent years, GV JEV has been isolated from Cx. pipiens group from China [59]. Furthermore, in South Korea, GV JEV has also been isolated not only from Cx. Tritaeniorhynchus, but also from several culicine mosquitoes, such as Cx. pipiens group, Cx. bitaeniorhynchus, and Cx. orientalis, which prefers avian over human [60]. A shift from GI to GV may have already begun in some areas of Asia. Every year, JEV becomes active in the summer in Japan; however, its ecology during winter is virtually unknown. Whether JEV overwinters in Japan or migrates each year from abroad has long been debated, and this issue has not yet been resolved. Nevertheless, based on phylogenetic analyses carried out in recent years, viruses from abroad have been found in Japan. It is very possible that viruses native to Japan overwinter in wild animals, such as wild boars or other mosquito species, in addition to Cx. tritaeniorhynchus. In contrast to this, our results support the hypothesis that Cx. tritaeniorhynchus migrates annually from the continent to Japan via a low-level jet stream. It is highly likely that such mosquito migration will result in simultaneous changes in JEV genotypes in Asia. Several issues remain to be clarified regarding the two taxa within Cx.
tритаенiorхинус, which provided the basis for this study, including the distributional boundaries of the two types, the existence of hybrid offspring between the two types, the ability of Ct-C to migrate long distances, the possibility of Ct-C overwintering in Japan, and the JEV prevalence of the two types. We hope that future efforts will provide evidence to support the long-distance migration of Cx. tritaeniorhynchus with JEV.

Conclusions

This is the first report showing the existence of two genetically independent taxonomic groups in Cx. tritaeniorhynchus. The physical and physiological characteristics of Cx. tritaeniorhynchus suggest the possibility of long-distance flight from overseas regions, while meteorological studies confirmed the presence of atmospheric currents that make this possible. The epidemic strain of JEV might be influenced by several environmental conditions, including the weather phenomena of that particular year and the current epidemic strains of JE present in the regions of Asia, which might be the areas from which the mosquitoes migrate. It is thus important to consider multiple factors to effectively manage this disease, including the environment, vector mosquitoes, wild animals, humans, and the virus. In the future, we expect research to provide more information that will facilitate the management of this disease. For instance, summing up the results of surveys and studies from various fields, such as epidemiology and genetic information of JEV, mosquito ecology and physiology, and meteorology in Asia, will undoubtedly contribute to the prediction and control of JEV epidemics.

Supporting information

S1 Table. Details of the mosquito specimens used for phylogenetic analysis in this study. The geographical positions of the collection sites were obtained both from database and the current study. The geographic positions obtained in this study were recorded using a geographical positioning system (GPS: GPSMAP64, Garmin, USA).

S1 Fig. Computed back trajectories at 2-h intervals for the 36-h period. Computing was at 0000 UTC of 21 July 2009 at Saga City (A), 29 July 2009 at Minami-Satsuma City (B), 13 July 2010 at Goto City (C), and 14, 15, 26, 27 and 30 July at Iki City (D–H), respectively. Each figure is the result of a backward trajectory analysis of HYSPLIT using the NOAA website: https://www.ready.noaa.gov/HYSPLIT.php.

Acknowledgments

We express our thanks to Drs. Shaohong Lu of Institute of Parasitic Diseases, Zhejiang Academy of Medical Sciences, China, His-Chieh Wang of Center for Research & Diagnostics, Center for Disease Control, Taiwan, E-Hyun Shin of Division of Medical Entomology, Center for Disease Control, Korea, Shin-ichiro Shobu of Saga Agricultural Experiment Station, Reiko Ohtsu of Nagasaki Agricultural and Forestry Technical Development Center, Hideaki Inoue of Kagoshima Prefectural Institute for Agricultural Development, Toru Yanase of National Institute of Animal Health, NARO, Hiroyuki Matsuoka of Jichi Medical University, the staff members of NIHE, Vietnam, and the Department of Medical Entomology of NIID, for their kindness in making the arrangements for the field investigations, and also for their helpful suggestions and critiques. We also thank the late Dr. Sumio Tojo for encouragement and scientific advice in our research on long-distance migration of mosquitoes. The authors gratefully acknowledge the NOAA Air Resources Laboratory (ARL) for the provision of the HYSPLIT
transport and dispersion model and/or READY website (https://www.ready.noaa.gov) used in this study, and would like to thank Editage (www.editage.com) for English language editing.

Author Contributions

Conceptualization: Satoru Arai, Mutsuo Kobayashi, Kyoko Sawabe.

Formal analysis: Satoru Arai, Ryusei Kuwata, Sudipta Roychoudhury, Akira Otuka, Kyoko Sawabe.

Funding acquisition: Satoru Arai, Mutsuo Kobayashi, Kyoko Sawabe.

Investigation: Ryusei Kuwata, Yukiko Higa, Yoshihide Maekawa, Yoshio Tsuda, Arlene Garcia Bertuso, Tran Vu Phong, Nguyen Thi Yen, Tomoki Etoh, Mutsuo Kobayashi, Kyoko Sawabe.

Methodology: Satoru Arai, Tomoki Etoh, Akira Otuka, Masaya Matsumura, Kyoko Sawabe.

Project administration: Kyoko Sawabe.

Supervision: Takeshi Nabeshima, Keiko Tanaka Taya, Nobuhiko Okabe, Mutsuo Kobayashi.

Validation: Satoru Arai, Takeshi Nabeshima, Kyoko Sawabe.

Visualization: Satoru Arai, Ryusei Kuwata, Kyoko Sawabe.

Writing – original draft: Satoru Arai, Ryusei Kuwata, Akira Otuka, Kyoko Sawabe.

Writing – review & editing: Satoru Arai, Ryusei Kuwata, Yukiko Higa, Yoshihide Maekawa, Yoshio Tsuda, Sudipta Roychoudhury, Arlene Garcia Bertuso, Tran Vu Phong, Nguyen Thi Yen, Tomoki Etoh, Akira Otuka, Masaya Matsumura, Takeshi Nabeshima, Keiko Tanaka Taya, Nobuhiko Okabe, Mutsuo Kobayashi, Kyoko Sawabe.

References

1. World Health Organization (WHO), Japanese encephalitis. [cited 2021 Dec 1]. Available from: https://www.who.int/news-room/fact-sheets/detail/japanese-encephalitis.

2. Solomon T, Dung NM, Kneen M, Gainsborough M, Vaughn DW, Khanh VT. Japanese encephalitis. J Neurol Neurosurg Psychiatry 2000; 68: 405–415. https://doi.org/10.1136/jnnp.68.4.405 PMID: 10727474.

3. Solomon T, Ni H, Beasley DW, Ekkelenkamp M, Cardosa MJ, Barrett AD. Origin and evolution of Japanese encephalitis virus in southeast Asia. J Virol. 2003; 77: 3091–3098. https://doi.org/10.1128/jvi.77.5.3091-3098.2003 PMID: 12884335.

4. Van den Hurk AF, Ritchie SA, Mackenzie JS. Ecology and geographical expansion of Japanese encephalitis virus. Annu Rev Entomol. 2009; 54: 17–35. https://doi.org/10.1146/annurev.ento.54.110807.090516 PMID: 19067628.

5. Hanna JN, Ritchie SA, Phillips DA, Shield J, Bailey MC, Mackenzie JS, et al. An outbreak of Japanese encephalitis in the Torres Strait, Australia, 1995. Med J Aust. 1996; 165: 256–260. https://doi.org/10.5694/j.1326-5377.1996.tb124690.x PMID: 8816682.

6. Pyke AT, Williams DT, Nisbet DJ, van den Hurk AF, Taylor CT, Johansen CA, et al. The appearance of a second genotype of Japanese encephalitis virus in the Australasian region. Am J Trop Med Hyg. 2001; 65: 747–753. https://doi.org/10.4269/ajtmh.2001.65.747 PMID: 11791969.

7. Tewari SC, Thennozhi V, Arunachalam N, Samuel PP, Tyagi BK. Desiccated vector mosquitoes used for the surveillance of Japanese encephalitis virus activity in endemic southern India. Trop Med Int Health. 2008; 13: 286–290. https://doi.org/10.1111/j.1365-3156.2008.02038.x PMID: 18304277.

8. Simon-Loriere E, Faye O, Prot M, Casademont I, Fall G, Fernandez-Garcia MD, et al. Autochthonous Japanese encephalitis with yellow fever coinfection in Africa. N Engl J Med. 2017; 376: 1483–1485. https://doi.org/10.1056/NEJMoa1701600 PMID: 28402771.

9. Platonov A, Rossi G, Karan L, Mironov K, Busani L, Rezza G. Does the Japanese encephalitis virus (JEV) represent a threat for human health in Europe? Detection of JEV RNA sequences in birds
collected in Italy. *Euro Surveill.* 2012; 17: 20241. https://doi.org/10.2807/esr.17.32.20241-en PMID: 22913940.

10. Preziuso S, Mari S, Mariotti F, Rossi G. Detection of Japanese encephalitis virus in bone marrow of healthy young wild birds collected in 1997–2000 in Central Italy. *Zoonoses Public Health* 2018; 65: 796–804. https://doi.org/10.1111/zph.12501 PMID: 29974677.

11. Hiroyama T. Epidemiology of Japanese encephalitis. *Saishin Igaku* 1962; 17: 1272–1280.

12. Infectious Diseases Weekly Report (IDWR). IDWR surveillance data table 2020 week 53. National Institute of Infectious Diseases, Japan, 2020. Local transmission of Japan. [cited 2021 Dec 1]. Available from: https://www.niid.go.jp/niid/ja/ydata/10067-report-ja2019-20.html.

13. Obara M, Yamauchi T, Watanabe M, Hasegawa S, Ueda Y, Matsuno K, et al. Continuity and change of Japanese encephalitis virus in Toyama Prefecture, Japan. *Am J Trop Med Hyg* 2011; 84: 695–708. https://doi.org/10.4269/ajtmh.2011.10-0188 PMID: 21540378.

14. Faizah AN, Kobayashi D, Isawa H, Amoa-Bosompet M, Murota K, et al. Deciphering the virome of *Culex vishnui* subgroup mosquitoes, the major vectors of Japanese encephalitis, in Japan. *Viruses* 2020; 12: 264. https://doi.org/10.3390/v12030264 PMID: 32121094.

15. Morita K. Molecular epidemiology of Japanese encephalitis in East Asia. *Vaccine* 2009; 27: 7131–7132. https://doi.org/10.1016/j.vaccine.2009.09.051 PMID: 19799848.

16. Nabeshima T, Loan HTK, Inoue S, Sumiyoshi M, Haruta Y, Phan TG, et al. Evidence of frequent introductions of Japanese encephalitis virus from south-east Asia and continental east Asia to Japan. *J Gen Virol.* 2009; 90: 827–832. https://doi.org/10.1099/vir.0.007617-0 PMID: 19264633.

17. Nabeshima T, Morita K. Phylogeographic analysis of the migration of Japanese encephalitis virus in Asia. *Future Virol.* 2010; 5: 343–354. CRID: 1010282257043738504.

18. Tsuruoka Y, Asahina S. Records of the insect which visited a weather ship located at the Ocean Weather Station ‘Tango’ on the Pacific. II. *Kontyu* 1966; 36: 190–202.

19. Asahina S, Tsuruoka Y. Records of the insect which visited a weather ship located at the Ocean Weather Station ‘Tango’ on the Pacific. III. *Kontyu* 1969; 37: 290–304.

20. Asahina S. Transoceanic flight of mosquitoes on the Northwest Pacific, Japan. *J Med Sci Biol.* 1970; 23: 255–258.

21. Kishimoto R. Planthoppers and leafhoppers in Japan. *Farming Japan* 1976; 10: 24–37.

22. Otsuka A, Watanabe T, Suzuki Y, Matsumura M. Estimation of the migration source for the white-backed planthopper *Sogatella furcifera* (Horváth) (Homoptera: Delphacidae) immigrating into Kyushu in June. *Jpn J Appl Entomol Zool* 2005; 49: 187–194. https://doi.org/10.1303/jjaez.2005.187.

23. Otsuka A. Three dimensional models for the migration analysis of rice planthoppers. *Plant Protection* 2006; 60: 14–17.

24. Otsuka A, Matsumura M, Watanabe T. The search for domestic migration of the white-backed planthopper, *Sogatella furcifera* (Horváth) (Homoptera: Delphacidae), in Japan. *Appl Entomol Zool.* 2009; 44: 379–386. https://doi.org/10.1303/aez.2009.379 CRID: 1390282681219300906.

25. Ming JG, Riley HJ, Reynolds DR, Smith AD, Wang RL, Cheng JY, et al. Autumn southward ‘return’ migration of the mosquito *Culex tritaeniorhynchus* in China. *Med Vet Entomol.* 2019; 33: 323–327. https://doi.org/10.1111/j.1365-2915.1993.tb00699.x PMID: 8268485.

26. Briegel H, Knüsel I, Timmermann SE. *Aedes aegypti*: size, reserves, and flight potential. *J Vector Ecol.* 2001; 26: 21–31. PMID: 11469181.

27. Briegel H, Waltiert A, Kuhn AR. Reproductive physiology of *Aedes* (Aedimorphus) vexans (Diptera: Culicidae) in relation to flight potential. *J Med Entomol.* 2001; 38: 557–565. https://doi.org/10.1603/0022-2585-38.4.557 PMID: 11476336.

28. Rojas-Araya D, Altu BW, Burkett-Cadena ND, Cummings DA. Impacts of fluorescent powders on survival of different age cohorts, blood-feeding success, and tethered flight speed of *Aedes aegypti* (Diptera: Culicidae) females. *Acta Trop.* 2020; 207: 105491. https://doi.org/10.1016/j.actatropica.2020.105491 PMID: 32283091.

29. Somerville AGT, Gleave K, Jones CM, Reimer LJ. The consequences of *Brugia malayi* infection on the flight and energy resources of *Aedes aegypti* mosquitoes. *Sci Rep.* 2019; 9: 18449. https://doi.org/10.1038/s41598-019-54819-2 PMID: 31804546.

30. Cui J, Li S, Zhao P, Zou F. Flight capacity of adult *Culex pipiens* p. pallens (Diptera: Culicidae) in relation to gender and day-age. *J Med Entomol.* 2013; 50: 1055–1058. https://doi.org/10.1603/me12078 PMID: 24180110.

31. Barraud PJ, Christophers SR. On a collection of anopheline and culicine mosquitoes from Siam. *Rec Malar Survey India* 1931; 2: 269–285.
32. Sucharit S, Surathin K, Shresta SR. Vectors of Japanese encephalitis virus (JEV): species complexes of the vectors. *Southeast Asian J Trip Med Public Health* 1989; 20: 611–621. PMID: 2576966.

33. Colless DH. Notes on the Culicine mosquitoes of Singapore—II. The Culex vishnui group (Diptera: Culicidae), with the description of two new species. *Ann Trop Med Parasitol.* 1957; 51: 87–101. PMID: 13425320.

34. Sirivanakarn S. Medical entomology studies—III. A revision of the subgenus Culex in the Oriental region (Diptera: Culicidae). *Contrib Am Entomol Inst.* 1976; 12: 1–272.

35. Kaur S, Airi M, Tewari PK. Intraspecific variations in three vector species of Culex vishnui (Diptera: Culicidae) based on male genitalia. *Entomol.* 2010; 34: 123–135.

36. Airi M, Kaur S. Confirmation of Culex (Culex) tritaeniorhynchus summorosus (Diptera: Culicidae) as a separate species. *J Vector Borne Dis.* 2015; 52: 219–223. PMID: 26418652.

37. Ohkubo N. Experimental studies on the flight of planthoppers by the tethered flight technique. I. Characteristics of flight of the brown planthopper Nilaparvata lugens STAl and effects of some physical factors. *Appl Entomol Zool.* 1973; 17: 10–18.

38. Sato Y. Flight ability of the Podocarp Bark Borer, Hirticlytus comosus (Matsushita) (Coleoptera: Cerambycidae). *Jpn For Soc.* 2005; 87: 247–250. https://doi.org/10.4005/jfs.87.247

39. National Oceanic Atmospheric Administration (NOAA), Air Resources Laboratory. HYSPLIT [cited 2021 Dec 1]. Available from: https://www.ready.noaa.gov/HYSPLIT.php.

40. Tsuda Y, Higa Y, Kurahashi H, Hayashi T, Hoshino K, Komagata O, et al. Dry-ice trap collection of mosquitoes at urban areas surrounding Tokyo, Japan in 2003 and 2004. *Med Entomol Zool.* 2006; 57: 75–82. https://doi.org/10.7601/mez.57.75_1

41. Stojanovich CJ, Scott HG. Illustrated Key to Mosquitoes of Vietnam. Atlanta, GA: U. S. Department of Health, Education, and Welfare, Public Health Service. 1968.

42. Tanaka K, Mizusawa K, Saugstad ES. A revision of the adult and larval mosquitoes of Japan (including the Ryuku Archipelago and the Ogasawara Islands) and Korea (Diptera: Culicidae). *Contrib Amer Entomol Inst.* 1979; 16: 1–987.

43. Reuben R, Tewari SC, Hiriyan J, Akiyama J. Illustrated keys to species of *Culex* (Diptera: Culicidae) associated with Japanese encephalitis in southeast Asia (Diptera: culicidae). *Mosq Systematics* 1994; 26: 75–96.

44. Reinert JF. New classification for the composite genus Aedes (Diptera: Culicidae: Aedini), elevation of subgenus Ochlerotatus to generic rank, reclassification of the other subgenera, and notes on certain subgenera and species. *J Am Mosq Control Assoc.* 2000; 16: 175–188. PMID: 11081644.

45. Folkler O, Black MB. DNA primers for amplification of mitochondrial Cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol.* 1994; 3: 294–299. PMID: 7881515.

46. Hebert PD, Cywinska A, Ball SL, deWaard JR. Biological identification through DNA barcodes. *Proc Biol Sci.* 2003; 270: 313–321. https://doi.org/10.1098/rspb.2002.2218 PMID: 12614582.

47. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994; 22: 4673–4680. https://doi.org/10.1093/nar/22.22.4673 PMID: 7984417.

48. Ronquist FM, Teslenko M, van der Mark P, Ayres DL, Darling A, zHöhna S, et al. MrBayes 3.2: Efficient bayesian phylogenetic inference and model selection across a large model space. *Syst Biol.* 2012; 61: 539–542. https://doi.org/10.1093/sysbio/sys028 PMID: 22357727.

49. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods* 2012; 9: 772. https://doi.org/10.1038/nmeth.2109 PMID: 22647109.

50. Stein AF, Draxler RR, Rolph GD, Stunder BJB, Cohen MD, Ngan F. NOAA’s HYSPLIT atmospheric transport and dispersion modeling system. *Bull Amer Meteor Soc.* 2015; 96: 2059–2077. https://doi.org/10.1175/BAMS-D-14-00110.1

51. Rolph G, Stein A, Stunder B. Real-time environmental applications and display sYstem: READY. *Environmental Modelling & Software* 2017; 95: 210–228. https://doi.org/10.1016/j.envsoft.2017.06.025 [cited 2021 Aug 1]. Available from: http://www.sciencedirect.com/science/article/pii/S1364815217302360.

52. Šogawa K, Watanabe T, Tsurumachi M. Emigration areas and meteorological factors inducing overseas migration of the brown planthopper. *Proc Assoc Pl Prot Kyushu* 1988; 34: 79–82.

53. Wada Y, Miura T, Kamiya M, Toyokawa K. Mosquitoes overwintering in Izu Peninsula and Mt. Nokogiri. *Med Entomol Zool.* 1968; 19: 82–83. ID 110003820899.

54. Tsuda Y, Kim KS. Sudden autumnal appearance of adult Culex tritaeniorhynchus (Diptera: Culicidae) at a park in urban Tokyo: First field evidence for prediapause migration. *J Med Entomol.* 2008; 45: 610–616. https://doi.org/10.1603/0022-2585(2008)45[610:saoac]2.0.co;2 PMID: 18714859.
55. Lessard BD, Kurucz N, Rodriguez J, Carter J, Hardy CM. Detection of the Japanese encephalitis vector mosquito Culex tritaeniorhynchus in Australia using molecular diagnostics and morphology. *Parasites Vectors* 2021; 14: 411. https://doi.org/10.1186/s13071-021-04911-2 PMID: 34407880.

56. National Agriculture and Food Research Organization (NARO). [cited 2021 dec 1]. Available from: https://www.naro.affrc.go.jp/project/results/laboratory/narc/2008/narc08-04.html.

57. Phan TN, Del Carmen Parquet M, Vuong D.C, Ma SP, Hasebe F, Inoue S, et al. Shift in Japanese encephalitis virus (JEV) genotype circulating in northern Vietnam: implications for frequent introductions of JEV from Southeast Asia to East Asia. *J Gen Virol.* 2004; 85: 1625–1631. https://doi.org/10.1099/vir.0.79797-0 PMID: 15166447.

58. Erlanger TE, Weiss S, Keiser J, Utzinger J, Wiedenmayer K. Past, present, and future of Japanese encephalitis. *Emerg Infect Dis.* 2009; 15: 1–7. https://doi.org/10.3201/eid1501.080311 PMID: 19116041.

59. Li MH, Fu SH, Chen WX, Wang HY, Guo YH, Liu QY, et al. Genotype V Japanese encephalitis virus is emerging. *PLoS Negl Trop Dis.* 2011; 5: e1231. https://doi.org/10.1371/journal.pntd.0001231 PMID: 21750744.

60. Kim H, Cha GW, Jeong YE, Lee WG, Chang KS, Roh JY, et al. Detection of Japanese encephalitis virus genotype V in Culex orientalis and Culex pipiens (Diptera: Culicidae) in Korea. *PLoS One* 2015; 10: e0116547. https://doi.org/10.1371/journal.pone.0116547 PMID: 25658839.