Genetic analysis of *Aedes aegypti* captured in two international airports serving the Greater Tokyo area during 2012—2015

Kentaro Itokawa[1], Jinping Hu[2], Nayu Sukehiro[3], Tsuda Yoshio[4], Osamu Komagata[4,5], Shinji Kasai[4], Takashi Tomita[4], Noboru Minakawa[2] and *Kyoko Sawabe[4]

1. Pathogen Genomics Center, National Institute of Infectious Diseases, Tokyo, Japan
2. Department of Vector Ecology and Environment, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan
3 Fukuoka Quarantine station, Fukuoka, Japan
4. Department of Medical Entomology, National Institute of Infectious Diseases, Tokyo, Japan
5. Antimicrobial Resistance Research Center, National Institute of Infectious Diseases, Tokyo, Japan

*To whom correspondent should be addressed: sawabe@nih.go.jp

Abstract

During 2012—2015, live larvae and an adult of the yellow-fever mosquito, *Aedes aegypti*, were captured in two international airports serving the Greater Tokyo area of Japan. Because this species does not naturally distribute in this country, those mosquitoes were considered to be introduced from some foreign countries *via* air-transportation. To infer the places of origin of those mosquitoes, we genotyped 12 microsatellite loci for which the most comprehensive population genetic reference is available. Although clustering by Bayesian and multivariate methods both suggested all those airport mosquitoes belong to Asia/Pacific population, they did not form a single population cluster. There was variation in mitochondrial *Cox1* haplotypes between airport mosquitoes collected in different incidents, indicating there were multiple maternal origins for those mosquitoes. Whereas we conclude there is little evidence to support overwintering of *Ae. aegypti* in the airports in this study, special attention is still desired to prevent the invasion of this prominent arbovirus vector.

Keywords: Aedes aegypti, microsatellite, airport, exotic, mosquito

Background

Aedes aegypti, dengue-yellow fever mosquito, distributes in the most part of the tropical and subtropical regions. This species shows strong biting preference to humans and adaptation to urbanized environments, which make them an extremely effective vector of numerous arthropod-borne viral diseases including dengue, yellow fever and Zika. The global distribution of this species is a result of intercontinental movement of the mosquitoes along with trades and traffic by human. Recent population genetic studies indicate existence of two major genetic clusters of *Ae. aegypti* world-wide (1,2). The African cluster which exclusively distribute in Africa is considered representing the ancestral population of this species. On the other hand, *Ae. aegypti* population distributing in all regions outside of Africa as well as some parts of Africa is a monophyletic population lineage. The "out-of-Africa" is more domesticated and well adapted to human inhabitation than "Africa" population. This cluster is believe to be introduced from Africa to the other part of the world probably around the 16th century along with transatlantic traffic (3).

Modern global transportation may accelerate spread of exotic insect pests across continents. For disease transmitting mosquitoes, aircrafts is one of the most important pathway for its daily volume and speed (4,5). In Japan, the number of international scheduled flights increases constantly in these years (6) partly due to the expansion of low-cost carriers. Thus, reinforcement of surveillance system for the exotic mosquitoes in airports is highly demanded. In 2012, *Ae. aegypti* larvae were discovered in a single oviposition trap placed in a passenger terminal of Narita International Airport (NRT), Chiba, Japan, which was the first detection of this mosquito species in a building of international airport, this country (7). Insecticide was sprayed around the area soon after the discovery, and the following intensive survey did not detect *Ae. aegypti* further in that season (7). After

the incident in 2012, however, the mosquitoes were sporadically trapped again by ovitraps in NRT in August and September 2013, September 2014 and June, September and November 2015 (Table 1). Also, in September 2013, single *Ae. aegypti* adult was captured in Tokyo International Airport (aka Haneda Airport: HND) which locates approximately 60 km south-west away from NRT (Fig. 1). Although the continuous discoveries of *Ae. aegypti* in NRT may indicate repetitive introductions from some foreign regions, the possibility for existence of a source *Ae. aegypti* population which is stable and overwintering somewhere in the airport buildings cannot be excluded.

Preceding studies using genotype on 12 microsatellite loci have revealed the hierarchical structure of *A. aegypti* population (2,8). According to the result of those studies, the world-wide *Ae. aegypti* population is divided into Africa and out-of-Africa clusters, as mentioned above. The out-of-Africa cluster is further divided into the New-world and the Asia/Pacific cluster (8). We considered such a hierarchical structure and existing comprehensive microsatellite genotype table of world-wide population (8) allow us to narrow down the origin of the exotic mosquitoes discovered in airports. In this study, we analyzed genotypes of the 12 microsatellite loci and the sequence of mitochondrial cytochrome oxidase 1 (*Cox1*) gene haplotypes in those mosquito samples captured in airport buildings during 2012—2015.

Material and methods

Mosquitoes

The routine surveillance for mosquitoes during 2012—2015 conducted by the airport quarantine station stuffs discovered *Ae. aegypti* larvae and adults in two international airports close to the Tokyo capital city (Fig. 1) (9). Among those incidents, the incident in

NRT, 2012 was detailed in Sukehiro et al. (2013). Those mosquitoes were identified as *Ae. aegypti* morphologically. Some of the larvae were kept in laboratory and allowed to be developed to adults before being provided to us. For some mosquitoes, we obtained only one or few legs from the quarantine office after the rest of bodies was subjected to flaviviruses and Chikungunya virus detection by RT-PCR.

DNA extraction

DNA was extracted individually using MagExtractor™ -Genome- (Toyobo) from whole body mosquitoes and eluted in 50 μl TE buffer (pH 8.0). If only single or few legs were available, modified alkaline lysis method (10) was used to prepare PCR template. In our modified alkaline lysis method, legs were homogenized in 10 μl of NaOH solution (0.2 M) in each well of 8-stripped PCR tubes by shaking with a zirconia bead (2 mm in diameter, Nikkato, Japan) in TissuLyser II (Qiagen) for 30 s at 30 Hz. The homogenate was incubated for 10 min on 75 ºC, then neutralized by adding 10 μl of neutralization buffer (360 mM tris-HCl, 10 mM EDTA, pH 8.0) and 90 μl of Milli-Q water.

Genotyping microsatellite loci

Twelve microsatellite loci developed in Brown et al. (2) and Slotman et al. (11) were amplified by PCR with labeled M13 primers as described in Brown et al. (2). Primers and fluorescent dye combinations are described in Table S1. A PCR mixture contained 1 ul of template DNA, 1× Type-it Multiplex PCR Master Mix (Qiagen), 0.2 μM of each locus specific reverse primers, 0.02 μM of each locus specific forward primers and 0.2 μM of fluorescent labeled M13 primers, and the PCR condition was 95 °C for 2 min, 40 cycles of 98 °C for 5 s, 55 °C for 90 s and 72 °C for 20 s, then final extension on 72 °C for 1 min. The resulted PCR

fragments were electrophoresed with GeneScan 500 LIZ size standard (Applied Biosystems, ABI) in ABI3130 (ABI) for fragment analysis. Allele sizes were scored in Peak Scanner Software v1.0 (Thermo Fisher Scientific). Five DNA samples previously analyzed by Brown et al. (2) was also genotyped in same manner to calibrate the consistency of allele-call between the different laboratories. .

Population clustering and assignment

The genotype data of airport populations were merged with the reference individual genotype table (VBP0000138 in Population Biology Project of VectorBase.org) (excluding the *Ae. mascarensis* and *Ae. queenslandensis* data) and formatted for analysis by STRUCTURE 2.3.4 (12,13). Each analysis was conducted with 200,000 burn-in followed by 500,000 sampling, without using prior information of collection locations and with allele frequency correlated model for ten independent runs as replication. The best K value was determined according to the Evanno's criteria (14). The replications at the best K were averaged by CLUMPP (15), and then visualized by DISTRUCT (16) using CLUMPAK server (17).

Discrimination analysis of principle component (DAPC) was conducted for microsatellite genotype data using adegenet v2.0.1 package (18) in R v3.3.2. Countries of origins (except Hawaii/USA, which were treated as separated regions) were used for predefinition of populations for reference genotype panels. For airport samples collected in Japan, samples collected in each different incidence are treated as distinct predefined populations.

Population assignment analyses were conducted in GeneClass2 (19). First, the reference genotypes were divided into African and out-of-Africa groups. As setting cutoff threshold probability to 0.8, GeneClass2 assigned 95.4% (42 / 918) African genotypes and 98.0%

(2661 / 2714) out-of-Africa genotypes back to each original group. Misassignment (assigning to wrong cluster with probability >0.8) rate, on the other hand, was 2.9 and 1% for African and out-of-Africa genotypes, respectively. Then, the out-of-Africa reference genotypes were divided into New-world and Asia/Pacific groups. In self-assignment test among these groups, GeneClass2 assigned 88.2% (1740 / 1972) New-world genotypes and 88.1% (654 / 742) Asia/Pacific genotypes were properly assigned back to each original group. Misassignments, on the other hand, occurred in 8.3 and 7.3 % for New-world and Asia/Pacific genotypes, respectively.

Sequencing cytochrome oxidase I gene (*Cox1*) of mitochondrial DNA

The fragments of the *Cox1* genes were amplified individually using primers COI-FOR 5□-GTAATTGTAACAGCTCATGCA-3'/COI-REV 5□-AATGATCATAGAAGGGCTGGAC-3' (20). The PCR mixture 10 µl reaction mixes containing 1 µl of 10× reaction buffer (Qiagen), 0.8 µl dNTP, 20 pmol of each primer and 1 U of Taq polymerase (Qiagen, USA) and 1 µl of the DNA template. PCR was performed under the following conditions: 94°C for 3 min and 35 cycles of 94°C for 15 s, 55°C for 30 s, 72°C for 30 s; and 72°C for 10 min. The amplified PCR product were cleaned using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and sequenced in 3730 DNA Analyzer (Applied Biosystems) using BigDye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems). The *Cox1* haplotype of NRT13_Sep was queried in BLASTN (21) (2019/05/09) search at NCBI Nucleotide collection (nt/rt) database restricted in *Ae. aegypti*, and hits with more than 95% query coverage were aligned using MUSCLE (22). Sequences retrieved were AF380835, AF390098 and AY056597 (23); AF425846 (24); AY432106 and AY432648 (25); EU352212; HQ688292-688298 (26), JQ926676-926684, JQ926686-926690, JQ926692-926696, JQ926698-926700 and

JQ926702, JQ926704 (27); KF909122 (28), KM203140-203248 (29); KT313642, KT313645, KT313648, KT313650-313653 (30); KT339661 and KT339679-339683 (31), KU186990; KX171382-171394. Haplotype network was drawn for the alignment using the *pegas* package (v0.11) in R (32).

Result

Population genetic analysis

The 12 microsatellite loci were genotyped for 40 *Ae. aegypti* samples collected in two international airports serving the Greater Tokyo area during 2012—2015. As already confirmed in preceding study (2,8), STRUCTURE separated the individual genotypes in whole data (samples in this study + references) into Africa and out-of-Africa genetic clusters at the best K-value=2 with some level of admixture in Kenyan and Argentina (Fig. 2A). All samples collected in the airports belonged to the out-of-Africa cluster. The genotypes from out-of-Africa countries plus the airport samples were further separated into two New-world clusters and one Asia/Pacific cluster at the best K-value=3 (Fig. 2B) as expected from the result of preceding study (8). Genotypes of the airport samples showed preferences to the Asia/Pacific cluster. The Asia/Pacific group plus the airport samples were separated at the best K-value=5 (Fig. 2C). The result showed weak population structure according to geographic locations/countries. The airport population in Japan showed affinities to several different clusters with some congruency within samples collected in the same incident. DAPC analysis of same genotype data also supported the affiliation of our airport samples to out-of-Africa group (Fig. 3A). Clustering genotypes excluding Africa data marginally separated New-world and Asia/Pacific genotypes with substantial overlap. All airport samples were contained in a range of Asia/Pacific cluster (Fig 3B) but were not completely

distinct from the New-world cluster. No more fine clustering was obtained from DAPC analysis within Asia/Pacific group (Fig. 3C).

The result of STRUCTURE and DAPC was cross validated by assigning the airport samples to *a priori* defined genetic group by GeneClass2. All airport samples, except one in NRT14 assigned to the out-of-Africa group for “Africa or out-of-Africa” selection panel. When using the “New-world or Asia/Pacific” selection panel, most individual genotypes in NRT samples showed Asia/Pacific origin. The only one sample from HND, on the other hand, was assigned to New-world group (Fig. S1).

Mitochondrial lineage

Mitochondria *Cox1* gene was sequenced for the 40 individuals captured in airports. Individuals captured in the same incident had an identical haplotype suggesting those mosquitoes were siblings from a single female mosquito. Fig 4 shows haplotype network graph for the representing *Cox1* haplotypes of the airport mosquitoes plus other entries registered in NCBI Nucleotide collection database. NRT13_Sep, NRT15_Jun and NRT15_Nov mosquitoes shared the same haplotype which was also identical to haplotypes already reported from Asia/Pacific region. Although other airport samples had each unique haplotype among all airport samples, the haplotypes in HND13 and NRT14 were identical to some reported haplotypes isolated from Asia/Pacific region and both New-world and Asia/Pacific region, respectively.

Discussion

Origin of *Ae. aegypti* captured in Narita and Haneda international airports

We analyzed *Ae. aegypti* sampled in two international airports serving the Greater Tokyo

area of Japan. Both Bayesian (STRUCTURE) and multivariate (DAPC) clustering methods supported all those samples belong to Asian/Pacific genetic group. Although there are some disagreements, genetic assignment test by GeneClass2 assigned most samples to the Asia/Pacific group by hierarchical clustering approach. During 2012 to 2015, more than half of total passenger planes arriving at Narita Airport originated from Asia/Pacific region every year, while direct flights originating from Africa or South America (most likely source in the New world) accounted for less than 0.3% of the total flights (33). Considering relatively high misassignment rate in GeneClass2 test and the high traffic volume from Asia/Pacific regions to Japan, we, at the moment, assume the origins of all airport samples are somewhere in Asia/Pacific region. To obtain more confidence and resolution to assign individuals into narrower local populations (i.e. country level), further expansion of reference panel to include more world-wide populations and utilization of richer genetic information such as genome-wide single nucleotide polymorphisms (SNPs) (34–36) would be required.

Are *Ae. aegypti* reproduce stably in airport?

The mosquitoes collected in same incident had all identical mitochondrial haplotypes with each other. This suggests individuals collected in same incident represented siblings from single female. On the other hand, mosquitoes collected in different incidents could had different mitochondrial haplotypes indicating that there were multiple different maternal lineages for entire *Ae. aegypti* collected in airports during 2012—2015. Furthermore, STRUCTURE analysis did not assign all airport individuals into single cluster within Asia/Pacific group. Considering the facts that the discovery were occasional and intensive surveys following each discovery did not find additional *Ae. aegypti*, there is so far little evidence to support establishment of stable *Ae. aegypti* population in airport.

While most region in Japan are not suitable for *Ae. aegypti* inhabitation, this species are

once established overwintering population in temperate zone in Japan within limited period after the World War II (1944—1952) (37). Overwintering of *Ae. aegypti* was also suspected in Washington, DC during 2011—2014, where the mosquito may be utilizing the subterranean habitat (38). In 2014, local infection of dengue occurred in Tokyo (39) for the first time in those 70-years, though the vector mosquito was *Ae. albopictus*. Nevertheless, continuous introduction of both vectors and pathogens pose an undesirable risk that would change epidemiological situation in this country. Thus, further intensive surveillance and preventive measure for exotic mosquito in airport are desired.

Conclusion

The *Aedes aegypti* discovered in the two international airports in Japan are suspected to be come from some countries in Asia/Pacific region. There is little evidence to support overwintering of *Ae. aegypti* in those airports.

List of abbreviations

CoxI: cytochrome oxidase 1

DAPC: Discrimination analysis of principle component

HND: Tokyo International Airport (aka Haneda Airport)

NRT: Narita International Airport

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: Microsatellite genotypes are provided as supporting material Table_S2. Sequences of each *CoxI* haplotype were deposited to the Data Bank of Japan (DDBJ) under accession number described in Table 1.

Funding: This research was supported by the Research Program on Emerging and Re-emerging Infectious Diseases from Japan Agency for Medical Research and development (AMED) and by Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) from Ministry of Education, Culture, Sports, Science and Technology in Japan and AMED.

Authors' contributions: KI, SK and KS designed this study and revised the manuscript. TY and NS participated in sample collection. KI, JH and OK performed experiments. KI, TT

and NM executed data analysis. KI and JH drafted the manuscript. All authors read and approved the final manuscript.

Competing interests: The authors declare that they have no competing interests.

Supportive information

Table_s1_primer_dye.xlsx: Table for sequences of primers, dyes and multiplex group used to amplify microsatellites in this study.

Table_s2_Genotypes.tsv: Table for microsatellite genotypes of each *Ae .aegypti* individuals.

FigS1.pdf: Result of GeneClass2 population assignment.

Acknowledgement

We acknowledge to staffs in the Quarantine Stations in Narita and Haneda airports for kindly providing insect samples. Also, we thank to Dr Jeff Powell and Dr. Andrea Gloria-Soria in Yale university for providing reference *A. aegypti* genomic DNAs for electrophoresis calibration.

1. Gloria-Soria A, Brown JE, Kramer V, Hardstone Yoshimizu M, Powell JR. Origin of the dengue fever mosquito, Aedes aegypti, in California. PLoS Negl Trop Dis. 2014/08/01. 2014;8(7):e3029.
2. Brown JE, McBride CS, Johnson P, Ritchie S, Paupy C, Bossin H, et al. Worldwide patterns of genetic differentiation imply multiple "domestications" of Aedes aegypti, a major vector of human diseases. Proc Biol Sci. 2011/01/14. 2011;278(1717):2446–54.
3. Powell JR, Gloria-Soria A, Kotsakiozi P. Recent History of Aedes aegypti: Vector Genomics and Epidemiology Records. Bioscience. 2018 Nov 1;68(11):854–60.
4. Gratz NG, Steffen R, Cocksedge W. Why aircraft disinsection? Bull World Heal Organ. 2000/09/20. 2000;78(8):995–1004.
5. Ibañez-Justicia A, Gloria-Soria A, den Hartog W, Dik M, Jacobs F, Stroo A. The first detected airline introductions of yellow fever mosquitoes (Aedes aegypti) to Europe, at Schiphol International airport, the Netherlands. Parasit Vectors. 2017 Dec 8;10(1):603.
6. Japan Ministry of Land, Infrastructure, Transport and Tourism [Internet]. [cited 2019 Sep 4]. Available from: https://www.mlit.go.jp/koku/koku_fr19_000005.html

295 7. Sukehiro N, Kida N, Umezawa M, Murakami T, Arai N, Jinnai T, et al. First Report
296 on Invasion of Yellow Fever Mosquito, Aedes aegypti, at Narita International Airport,
297 Japan in August 2012. Jpn J Infect Dis. 2013;66(3):189–94.
298 8. Gloria-Soria A, Ayala D, Bheecarry A, Calderon-Arguedas O, Chadee DD, Chiappero
299 M, et al. Global genetic diversity of Aedes aegypti. Mol Ecol. 2016/10/28.
300 2016;25(21):5377–95.
301 9. Quarantine Information Office_ Ministry of Health_ Labor and Welfare Japan.
302 Vector Surveillance Reports [Internet]. [cited 2019 Jul 4]. Available from:
303 https://www.forth.go.jp/ihr/fragment2/index.html
304 10. Rudbeck L, Dissing J. Rapid, simple alkaline extraction of human genomic DNA from
305 whole blood, buccal epithelial cells, semen and forensic stains for PCR. Biotechniques.
306 1998/10/30. 1998;25(4):588-590,592.
307 11. Slotman MA, Kelly NB, Harrington LC, Kitthawee S, Jones JW, Scott TW, et al.
308 Polymorphic microsatellite markers for studies of Aedes aegypti (Diptera: Culicidae),
309 the vector of dengue and yellow fever. Mol Ecol Notes. 2007;7(1):168–71.
310 12. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using
311 multilocus genotype data. Genetics. 2000/06/03. 2000;155(2):945–59.
312 13. Falush D, Stephens M, Pritchard JK. Inference of population structure using
313 multilocus genotype data: linked loci and correlated allele frequencies. Genetics.
314 2003/08/22. 2003;164(4):1567–87.
315 14. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals
316 using the software STRUCTURE: a simulation study. Mol Ecol. 2005;14(8):2611–20.
317 15. Jakobsson M, Rosenberg NA. CLUMPP: a cluster matching and permutation
318 program for dealing with label switching and multimodality in analysis of population

319 structure. Bioinformatics. 2007 Jul 15;23(14):1801–6.
320 16. Rosenberg NA. distruct: a program for the graphical display of population structure.
321 Mol Ecol Notes. 2003 Dec 10;4(1):137–8.
322 17. Kopelman NM, Mayzel J, Jakobsson M, Rosenberg NA, Mayrose I. Clumpak: a
323 program for identifying clustering modes and packaging population structure
324 inferences across K. Mol Ecol Resour. 2015 Sep;15(5):1179–91.
325 18. Jombart T, Devillard S, Balloux F. Discriminant analysis of principal components: a
326 new method for the analysis of genetically structured populations. BMC Genet.
327 2010/10/19. 2010;11:94.
328 19. Piry S, Alapetite A, Cornuet JM, Paetkau D, Baudouin L, Estoup A. GENECLASS2:
329 a software for genetic assignment and first-generation migrant detection. J Hered.
330 2004/10/12. 2004;95(6):536–9.
331 20. Paupy C, Le Goff G, Brengues C, Guerra M, Revollo J, Barja Simon Z, et al. Genetic
332 structure and phylogeography of Aedes aegypti, the dengue and yellow-fever
333 mosquito vector in Bolivia. Infect Genet Evol. 2012 Aug 1;12(6):1260–9.
334 21. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search
335 tool. J Mol Biol. 1990 Oct 5;215(3):403–10.
336 22. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high
337 throughput. Nucleic Acids Res. 2004/03/23. 2004;32(5):1792–7.
338 23. Morlais I, Severson DW. Complete mitochondrial DNA sequence and amino acid
339 analysis of the cytochrome C oxidase subunit I (COI) from Aedes aegypti. DNA Seq.
340 2002 Apr;13(2):123–7.
341 24. Mitchell A, Sperling FAH, Hickey DA, Mitchell A, Sperling FAH&, Hickey DA.
342 Higher-level phylogeny of mosquitoes (Diptera: Culicidae): mtDNA data support a

343 derived placement for Toxorhynchites Insect Syst Evol.
344 25. Bartholomay LC, Cho W-L, Rocheleau TA, Boyle JP, Beck ET, Fuchs JF, et al.
345 Description of the Transcriptomes of Immune Response-Activated Hemocytes from
346 the Mosquito Vectors Aedes aegypti and Armigeres subalbatus. Infect Immun. 2004
347 Jul 1;72(7):4114–26.
348 26. Fort P, Albertini A, Van-Hua A, Berthomieu A, Roche S, Delsuc F, et al. Fossil
349 Rhabdoviral Sequences Integrated into Arthropod Genomes: Ontogeny, Evolution,
350 and Potential Functionality. Mol Biol Evol. 2012 Jan 1;29(1):381–90.
351 27. Paupy C, Le Goff G, Brengues C, Guerra M, Revollo J, Barja Simon Z, et al. Genetic
352 structure and phylogeography of Aedes aegypti, the dengue and yellow-fever
353 mosquito vector in Bolivia. Infect Genet Evol. 2012 Aug;12(6):1260–9.
354 28. Seixas G, Salgueiro P, Silva AC, Campos M, Spenassatto C, Reyes-Lugo M, et al.
355 Aedes aegypti on Madeira Island (Portugal): genetic variation of a recently
356 introduced dengue vector. Mem Inst Oswaldo Cruz. 2013;108(suppl 1):3–10.
357 29. Jaimes-Dueñez J, Arboleda S, Triana-Chávez O, Gómez-Palacio A. Spatio-Temporal
358 Distribution of Aedes aegypti (Diptera: Culicidae) Mitochondrial Lineages in Cities
359 with Distinct Dengue Incidence Rates Suggests Complex Population Dynamics of the
360 Dengue Vector in Colombia. Aguilar P V., editor. PLoS Negl Trop Dis. 2015 Apr
361 20;9(4):e0003553.
362 30. Calvez E, Guillaumot L, Millet L, Marie J, Bossin H, Rama V, et al. Genetic Diversity
363 and Phylogeny of Aedes aegypti, the Main Arbovirus Vector in the Pacific. Turell MJ,
364 editor. PLoS Negl Trop Dis. 2016 Jan 22;10(1):e0004374.
365 31. Vadivalagan C, Karthika P, Murugan K, Panneerselvam C, Paulpandi M,
366 Madhiyazhagan P, et al. Genetic deviation in geographically close populations of the

367 dengue vector Aedes aegypti (Diptera: Culicidae): influence of environmental barriers
368 in South India. Parasitol Res. 2016 Mar 2;115(3):1149–60.
369 32. Paradis E. pegas: an R package for population genetics with an integrated-modular
370 approach. Bioinformatics. 2010 Feb 1;26(3):419–20.
371 33. Sukehiro N, Ishihara H, Enomoto M, Nakagawa E, Kida N, Jinnai T, et al. The
372 Invasion Risk of Yellow Fever Mosquito [Japanese]. J Japan Quar Med Assoc.
373 2016;18:52–9.
374 34. Rasic G, Filipovic I, Weeks AR, Hoffmann AA. Genome-wide SNPs lead to strong
375 signals of geographic structure and relatedness patterns in the major arbovirus
376 vector, Aedes aegypti. BMC Genomics. 2014/04/15. 2014;15:275.
377 35. Schmidt TL, van Rooyen AR, Chung J, Endersby - Harshman NM, Griffin PC, Sly A,
378 et al. Tracking genetic invasions: Genome - wide single nucleotide polymorphisms
379 reveal the source of pyrethroid - resistant *Aedes aegypti* (yellow fever mosquito)
380 incursions at international ports. Evol Appl. 2019 Mar 18;eva.12787.
381 36. Evans BR, Gloria-Soria A, Hou L, McBride C, Bonizzoni M, Zhao H, et al. A
382 Multipurpose, High-Throughput Single-Nucleotide Polymorphism Chip for the
383 Dengue and Yellow Fever Mosquito, Aedes aegypti. G3. 2015/02/28. 2015;5(5):711–8.
384 37. Tanaka K, Mizusawa K, Saugstad ES. Mosquitoes of Japan and Korea. In:
385 Contribution of the American Entomological Institute, Vol 16. 1979.
386 38. Lima A, Lovin DD, Hickner P V., Severson DW. Evidence for an overwintering
387 population of aedes aegypti in capitol hill Neighborhood, Washington, DC. Am J Trop
388 Med Hyg. 2016;94(1):231–5.
389 39. Kutsuna S, Kato Y, Moi ML, Kotaki A, Ota M, Shinohara K, et al. Autochthonous
390 Dengue Fever, Tokyo, Japan, 2014. Emerg Infect Dis. 2015 Mar;21(3):517–20.

Table 1 Description for incidents in which *Ae. aegypti* were captured in the two international airports, Japan, during 2012—2015

ID	Location	Period	Description*	N	*Cox1* Access. No.
NRT12	NRT	2012 Aug	Pupae and larvae were discovered in single ovitrap. Intensive survey after the discovery in area 400 m around did not detect additional mosquitoes (Sukehiro et al., 2013).	4	LC482631
HND13	HND	2013 Sep	Single adult male was captured in CDC light trap placed in cargo terminal. Intensive survey after the discovery in area 400 m around did not detect additional mosquitoes	1	LC482636
NRT13_Aug	NRT	2013 Aug	Pupae and larvae were discovered in single ovitrap set in airplane arrival terminal which located 1.5 km depart from NRT13_Sep_Aug discovered spot. Intensive survey after the discovery in 400 m area around the spot did not detect additional mosquitoes .	8	LC482632
NRT13_Sep	NRT	2013 Sep	Pupae and larvae were discovered in single ovitrap set in cargo terminal which located 1.5 km depart from NRT13_Sep_Aug discovered spot. Intensive survey after the discovery in 400 m area around the spot did not detect additional mosquitoes .	10	LC482633
NRT14	NRT	2014 Sep	Larvae were discovered in single ovitrap. Intensive survey after the discovery in 400 m area around the spot did not detect additional mosquitoes .	7	LC482630
NRT15_Jun	NRT	2014 Jun	Larvae were discovered in single ovitrap. Intensive survey after the discovery in 400 m area around the spot did not detect additional mosquitoes.	4	LC482634
NRT15_Sep	NRT	2014 Sep	Larvae were discovered in single ovitrap. Intensive survey after the discovery in 400 m area around the spot did not detect additional mosquitoes.	2	LC482629
NRT15_Nov	NRT	2014 Nov	Larvae were discovered in single ovitrap. Intensive survey after the discovery in 400 m area around the spot did not detect additional mosquitoes.	4	LC482635

*From Vector Surveillance Reports by Quarantine Information Office, Ministry of Health, Labor and Welfare Japan: https://www.forth.go.jp/ihr/fragment2/index.html)

Figure lengends

Fig. 1 Locations of Narita-airport (NRT) and Haneda airport (HND) on map including Tokyo and peripheral cities.

The map was reproduced from Geospatial Information Authority of Japan website (https://www.gsi.go.jp).

Fig. 2 Bayesian clustering by STRUCTURE

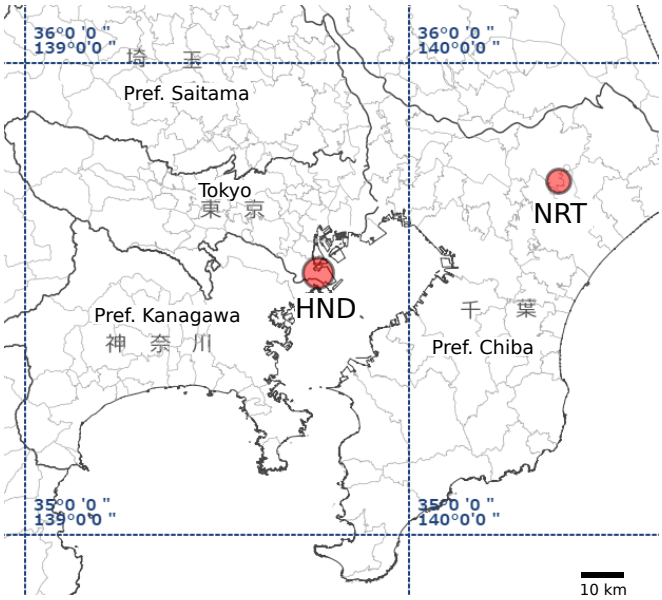
Result of multiple STRUCTURE runs were averaged by CLUMPP. Only results for the best K-values in Evanno's method are shown. Magnified views for the airport samples are shown at the right end of each figure. (A) Clusters of all *Ae. aegypti* genotypes + airport samples in Japan at the best k-value 2. (B) Clusters of out-of-African genotypes + airport samples in Japan at the best k-value 3. There were two distinct clustering results. (C) Clusters of Asia/Pacific genotypes + airport samples in Japan at the best k-value 5.

Fig. 3 DAPC analysis by Adegenet

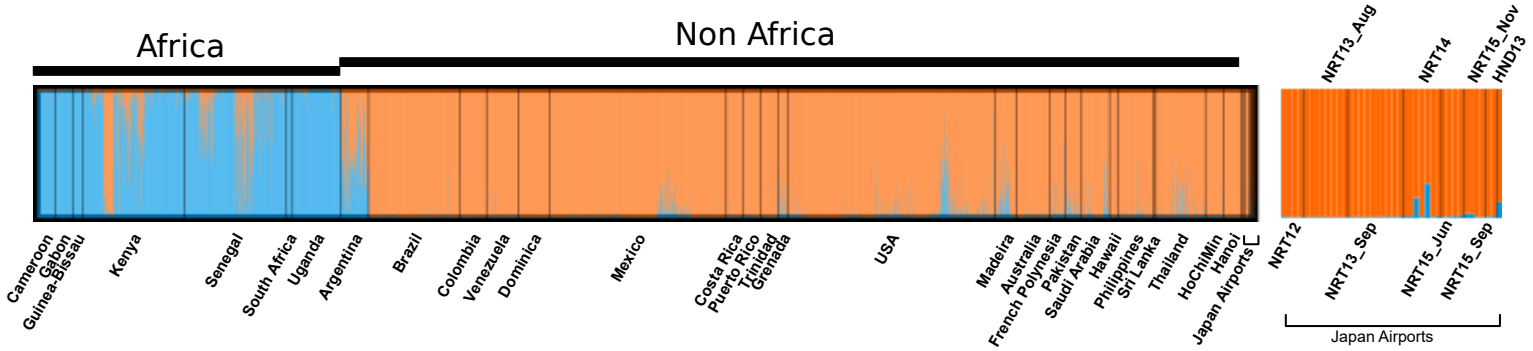
The results of Discriminative Analysis of Principle Components (DAPC) are shown. X- and Y- axes indicate 1st and 2nd principal component of DAPC, respectively. Right and left insects show scree plots of PCA and DA eigenvalues, respectively. Labels and individual points for airport samples in Japan are drown in black. First, we conducted clustering using whole genotype data along with airport samples in Japan (A). Next, out-of-African genotypes along with airport samples in Japan were clustered. (B). Finally, Asia/Pacific genotypes along with airport samples in Japan were clustered (C).

Fig. 4 Haplotype network graph for *Cox1* gene

Each node indicates distinct *Cox1* haplotype. Number of ticks on each edge show number of mutations. AF: Africa, NW: New-world, AP: Asia/Pacific, NA: Information unavailable.

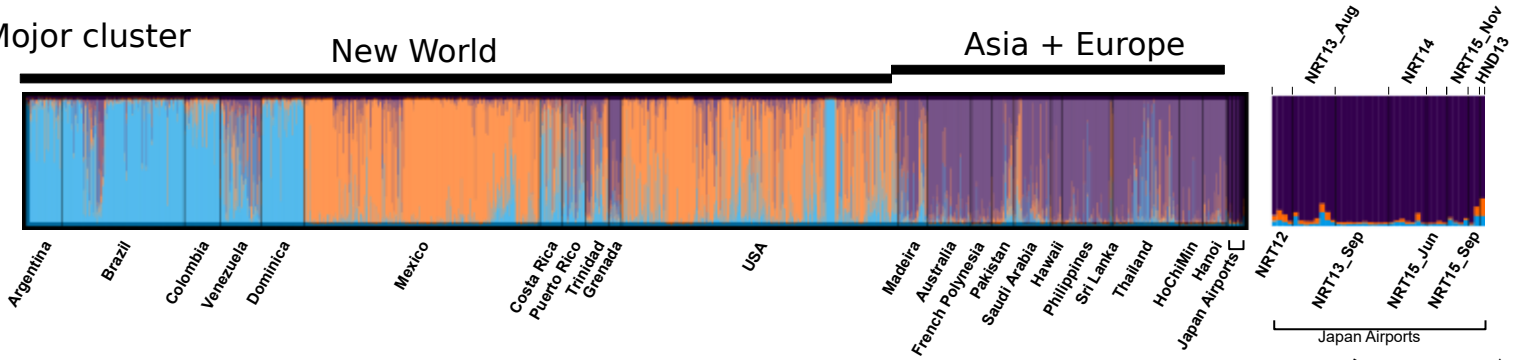

36°0 '0 "
139°0'0 "
埼 玉
Pref. Saitama
36°0 '0 "
140°0'0 "
Tokyo
東 京
NRT
HND
Pref. Kanagawa
神 奈 川
千 葉
Pref. Chiba
35°0 '0 "
139°0'0 "
35°0 '0 "
140°0'0 "
10 km

A) All countries (K = 2)

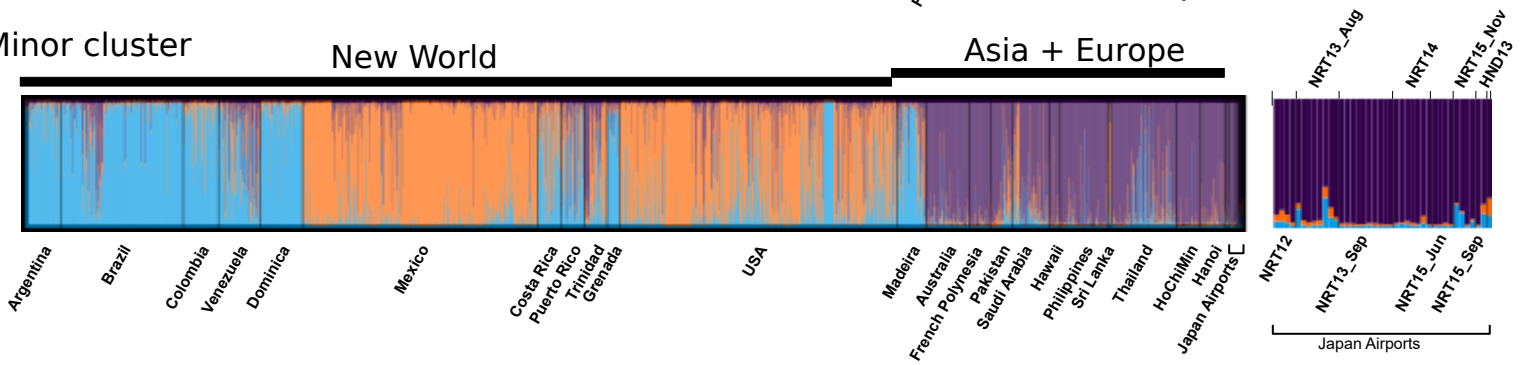


B) Non-Afica (K = 3)

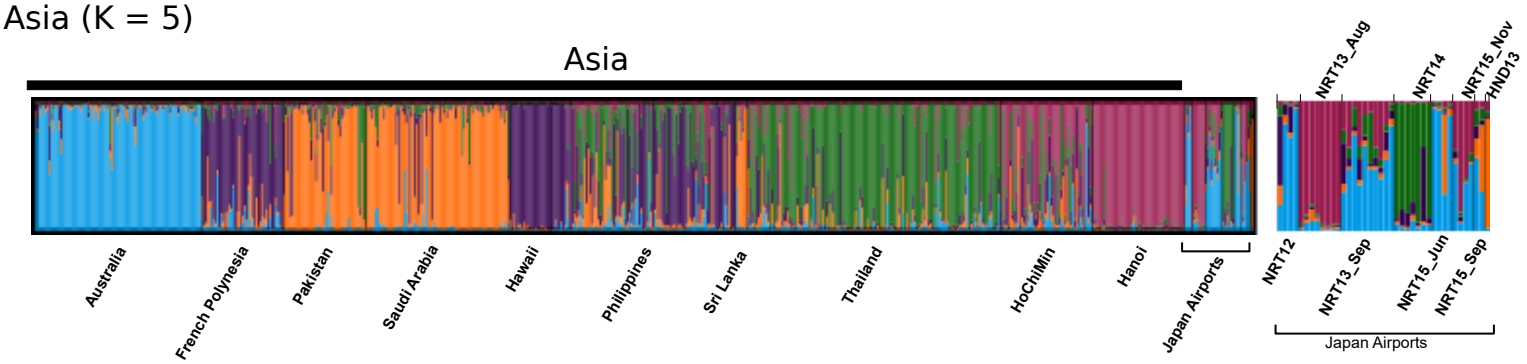
Mojor cluster

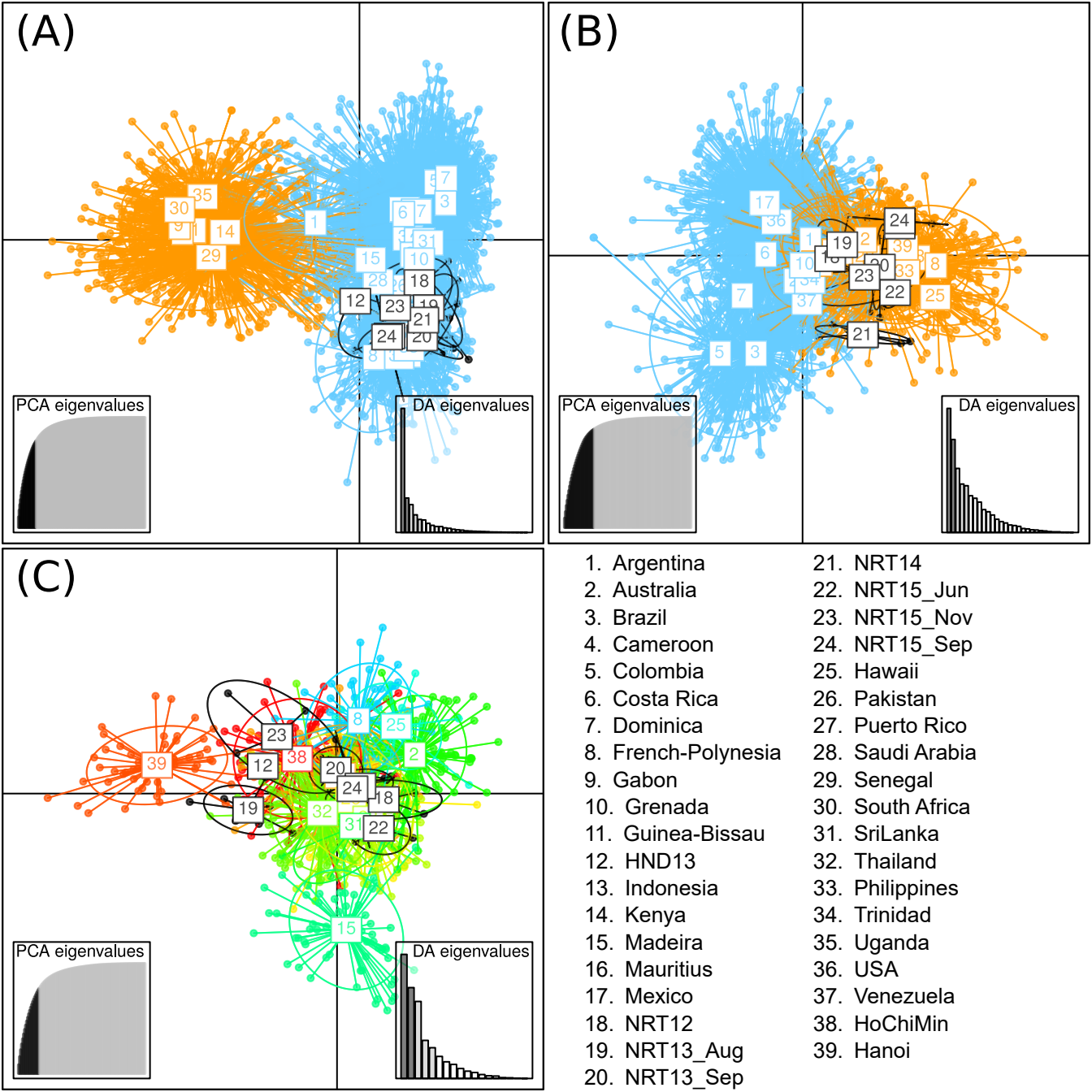


Minor cluster

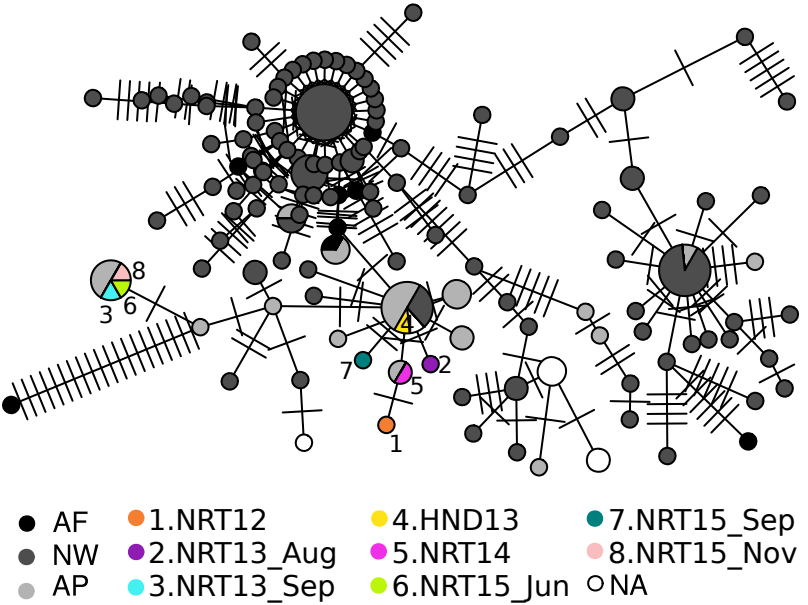


C) Asia (K = 5)





1. Argentina
2. Australia
3. Brazil
4. Cameroon
5. Colombia
6. Costa Rica
7. Dominica
8. French-Polynesia
9. Gabon
10. Grenada
11. Guinea-Bissau
12. HND13
13. Indonesia
14. Kenya
15. Madeira
16. Mauritius
17. Mexico
18. NRT12
19. NRT13_Aug
20. NRT13_Sep
21. NRT14
22. NRT15_Jun
23. NRT15_Nov
24. NRT15_Sep
25. Hawaii
26. Pakistan
27. Puerto Rico
28. Saudi Arabia
29. Senegal
30. South Africa
31. SriLanka
32. Thailand
33. Philippines
34. Trinidad
35. Uganda
36. USA
37. Venezuela
38. HoChiMin
39. Hanoi


8
3
6
4
7
2
5
1
AF
NW
AP
1.NRT12
2.NRT13_Aug
3.NRT13_Sep
4.HND13
5.NRT14
6.NRT15_Jun
7.NRT15_Sep
8.NRT15_Nov
NA