

# Detection and persistence of environmental DNA (eDNA) of a vector mosquito, *Culex pipiens pallens*

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## Research Article

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## Abstract

Introduction: Preventing mosquito-borne infectious diseases requires that vector mosquitoes are monitored and controlled. Targeting immature mosquitoes (eggs, larvae, and pupae), which have less mobility than adults, is an effective management approach. However, conducting these surveys is often difficult due to the limitations of morphological classification and survey costs. The application of environmental DNA (eDNA) analysis can solve these issues because it allows easy estimation of species distribution and morphology-independent species identification. Although a few previous studies have reported mosquito eDNA detection, there is a gap in knowledge regarding the dynamics of mosquito eDNA during the developmental stages of immature mosquitoes.

Methods: We used *Culex pipiens pallens*, a vector of West Nile fever, as a model species. First, we developed a species-specific detection assay and confirmed its specificity using in silico and in vitro tests. Next, we conducted laboratory experiments using breeding tanks. Water samples were collected at each developmental stage. In addition, water samples were collected daily until the seventh day after emergence from the pupae. We quantified eDNA using real-time PCR with the developed assay to investigate the dynamics of mosquito eDNA.

Results: The specificity of the developed assay was confirmed by in silico and in vitro tests. Mosquito eDNA was detected at all developmental stages and detected up to seven days after emergence of pupae. In particular, high concentrations of eDNA were detected immediately after hatching from eggs and after emergence from pupae. Highly frequent positive eDNA signals were continuously detected between egg hatching and pupa hatching.

Conclusions: Mosquito eDNA was detected immediately after the eggs were introduced, and eDNA-positive detections continued until pupae emergence, suggesting that eDNA analysis is useful for monitoring mosquito larvae. The results show that eDNA analysis provides valuable information about the water sources inhabited by immature mosquitoes in mosquito control. In the future, monitoring immature mosquitoes using eDNA analysis will aid in preventing mosquito-borne infectious diseases.

## Introduction

Mosquitoes are insects of the family Culicidae, with various species acting as vectors of numerous human infectious diseases such as malaria, dengue fever, Zika fever, yellow fever, and West Nile fever [1–3]. These mosquito-borne infectious diseases are an important cause of mortality and morbidity in at least one million people each year [4]. Therefore, controlling mosquito populations is an effective method for preventing the spread of these infections, and insecticides have been widely used to control adult mosquito populations [1]. However, due to the long-term use of chemical insecticides, resistance to different classes of insecticides has been increasingly reported [5]. Regarding mosquito population control strategies, targeting the larval stage could be more effective because unlike adults, mosquito larvae have limited mobility and are not able to change their habitat, making them more susceptible to

control methods [6,7]. Therefore, monitoring immature mosquito habitats is an important task for effective and ecological mosquito management [8]. As a strategy to target larvae, the use of an insect growth regulator (IGR) has been very effective for controlling dengue fever. The use of simple traps to monitor the egg-laying of vector mosquitoes has also been used as a monitoring tool for many years. However, the vectors of other insect-borne diseases such as malaria are diverse, and the source of each larva is different. Thus, there is currently no facile method for monitoring larvae, such as in the case of dengue mosquitoes.

Ecological monitoring of the immature stages of Culicidae is usually performed by surveying and identifying eggs, larvae, or pupae [9]. However, because immature mosquitoes live in widely distributed and diverse water environments, such as pools (e.g., hoof prints and puddles), large and complex water bodies such as river or lake margins, and rice fields, conducting physical sampling of immature stages of mosquitoes requires significant human labor, which can be costly depending on the area to be surveyed [9]. In particular, targeting mosquitoes that prefer large water bodies makes conducting surveys of immature mosquitoes more difficult [9]. In addition, some mosquito larvae are sometimes difficult to morphologically identify [10]. Therefore, although mosquito monitoring is an important task for mosquito control, it is difficult to accurately identify the habitats of each species in a large area.

Evaluating environmental DNA (eDNA) is a relatively new approach in parasite ecoepidemiology that consists of detecting target DNA in environmental samples to determine the presence of a given species [11]. This ecological surveying approach has been used to determine the distribution of waterborne human parasite populations [12]. The eDNA analysis method has some advantages, such as the ability to detect organisms with high sensitivity without requiring direct collection and the ability to easily identify species without morphological knowledge [11,13]. In addition, because of the importance of mosquitoes as vectors of several infectious diseases [14], the Culicidae DNA database is extensive, facilitating the design of eDNA-based detection assays and analysis. Therefore, this method could be helpful for monitoring immature mosquito stages in ecological surveys.

To date, few studies have used eDNA analysis to detect mosquito species. Schneider et al. [15] first reported that eDNA analysis could be applied to identify mosquito habitats. Odero et al. [16] revealed that eDNA from mosquito larvae can be detected six hours after releasing larvae to tanks under experimental conditions, and eDNA could be detected even at low larval densities. However, there is no knowledge of the detectable period of eDNA according to the developmental stage of mosquitoes. Here, we investigated when and how long *Culex pipiens pallens* eDNA could be detected from the release of eggs to the emerging adult stages under laboratory conditions. Our study provides new and important knowledge related to the eDNA detection period along mosquito life stages in an ecotope, which will be useful for surveying and monitoring water sources for immature stages of mosquitoes under natural conditions.

## Materials And Methods

We set *Culex pipiens pallens* as a model species in this study because this species is a major mosquito in Japan, a vector of West Nile virus in the world, and a type of mosquito that inhabits large water bodies that are difficult to survey<sup>3</sup>. In addition, the management of the immature stages of this species is required because adults are difficult to eradicate because of their high resistance to pyrethroid insecticides<sup>5</sup>. We conducted a tank experiment to verify the detectable period of eDNA according to the developmental stage. The experiment was conducted twice at the Research and Development Laboratory of Dainihon Jochugiku Co., Ltd. over two periods: August 21–September 28, 2017 and July 3–29, 2018. Per experimental period, two 6 L tanks were prepared with one egg patch of *C. pipiens pallens* released in each tank. Water samples were collected at each developmental stage of the mosquitoes, and water was added after each sampling to keep the amount of water in the tanks stable during the experiments.

A total of 14 water samples were collected from each tank in plastic bottles with a volume of 120 mL for each sample (for details, see Fig. 2). Benzalkonium chloride at a final concentration of 0.1% was added to each bottle to prevent the degradation of eDNA<sup>17</sup>. The collected water samples were transported in frozen conditions to the dedicated eDNA laboratory and stored at -28 °C until further use.

After melting in a water bath at room temperature (ca. 20 °C), the water samples were filtered with a glass fiber filter with a nominal pore size of 0.7 µm (GF/F; GE Healthcare Life Science). To monitor potential contamination during the filtration and eDNA extraction process, 120 mL of reverse osmosis membrane water was used as a negative control for each filtration day. eDNA on the filters was extracted using the Salivette (Sarstedt) and DNeasy Blood & Tissue Kit (QIAGEN Science, Hilden, Germany) and stored at -25 °C according to the methods described in a previous study<sup>18</sup>.

Sequences of the mitochondrial cytochrome c oxidase subunit I (CO1) gene of the target species *C. pipiens pallens* and closely related species (non-target species), *C. bitaeniorhynchus*, *Aedes albopictus*, and *A. aegypti*, potential sympatric species in Japan, were downloaded from the database of the National Center for Biotechnology Information (NCBI: <https://www.ncbi.nlm.nih.gov>) (Table 1). Based on these sequences, we designed species-specific primers satisfying three conditions: 1) melting temperature around 60 °C, 2) at least one each of species-specific base within the five bases at the 3' end of forward and reverse primers, and 3) a small target fragment (50–150 bp)<sup>13,19</sup>.

Potential cross-reactivity of the assay was checked in silico (i.e., Primer-BLAST was performed on all databases; [https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK\\_LOC=BlastHome](https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome)). Then, an in vitro specificity test was performed by real-time PCR using target and non-target tissue DNA templates (*C. bitaeniorhynchus*, *Aedes albopictus*, and *A. aegypti*). Real-time PCR was carried out in triplicate using morphologically identified specimens extracted DNA from each species as a template. Each reaction mixture (20 µl final volume) contained 900 nM primers and 125 nM TaqMan probe (Fig. 1) in 1× Environmental Master Mix 2.0 (Life Technologies) and 100 pg DNA of each species. The real-time PCR conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 55 2-step cycles of 15 s at 95 °C and 60 s at 60 °C. To detect false positives due to contamination during the real-time PCR procedures, ultrapure water was used instead of DNA in three reaction mixtures (non-template negative controls). The limit of

detection (LOD) and limit of quantification (LOQ) of the assay were 1 and 3 copies per reaction, respectively.

The concentration of eDNA in *C. pipiens pallens* was quantified by TaqMan real-time quantitative PCR (qPCR) targeting the CO1 region of *C. pipiens pallens* using primers and a probe (Fig. 1). The qPCR conditions were the same as above, including 5 µL of the DNA template. The qPCR also included a dilution series (30,000, 3,000, 300, and 30 copies) of standard DNA derived from PCR amplicons. All qPCRs for eDNA extracts, standards, and negative controls were performed in triplicate, and the eDNA concentrations were calculated by averaging the triplicates. PCR-negative replicates (indicating non-detection) and samples with less than one copy were regarded as containing zero copies<sup>20</sup>. The copy number detected from the negative control was subtracted from the corresponding samples according to the previously described method<sup>13,21</sup>.

## Results

The assay with primers Cpi-pa-CO1-F (5'-GTTTAGTAGAAAATGGAGCTGCGA-3') and Cpi-pa-CO1-R (5'-TGCACCTAAAATTGATGAAATTCCCTG-3') and a probe Cpi-pa-CO1-P (5'-FAM-TGAACAGTGTATCCCCCTCT-MGB-3') was developed (Fig. 1). The melting temperatures of Cpi-pa-CO1-F and Cpi-pa-CO1-R were 59.76°C and 60.79°C, respectively. The developed primers and probe each contain a base specific to the target species on the 3' side of primers and the center of the probe (shown in Fig. 1). The result of Primer-BLAST showed amplification of only the target species. The in vitro test using tissue DNA revealed amplification of the target species only and no amplification of closely related species.

The results of the laboratory experiment showed that the fluctuation in eDNA concentration along with changes in the mosquito developmental stage, and the range of eDNA concentrations detected was 1.0–754.9 copies/reaction. (Fig. 2; Table S1). In particular, high concentrations of eDNA were detected immediately after hatching from eggs and immediately after emergence from the pupae. Before installing mosquitoes (sample ID = 1), one sample showed eDNA amplification. The period when the eggs had not yet hatched after installation (sample ID = 3) showed little eDNA detection (only one tank was positive). From hatching eggs to just after emergence of pupae, many positive eDNA signals were detected (sample ID = 4 to 8). In particular, eDNA was detected in all tanks immediately after and up to 1 d after hatching (sample ID = 7 and 8). The mosquito eDNA could be detected up to seven days after the emergence of all pupae (Fig. S1).

## Discussion

Our findings revealed that eDNA can be detected for up to seven days after mosquito emergence and the organism disappears from the water body. eDNA detection with no living mosquitoes in the water after emergence from pupae is expected to facilitate the identification of inhabited water areas. Therefore, this finding will contribute to mosquito management. In the application of eDNA analysis to monitor the immature stages of mosquitoes, the period for which eDNA is detectable after a mosquito has flown off

as an adult is important [22]. In addition, this remaining period for which eDNA can be identified seems to be similar to the known eDNA dynamics of vertebrates [23]. In general, eDNA is affected by water state, temperature, sunlight (UV), and pH [24]. However, it is necessary to accumulate more basic knowledge related to mosquito eDNA because the dynamics of insect eDNA may not necessarily be comparable to that of vertebrates. The application of mosquito eDNA analysis to field surveys requires careful consideration of the dynamics of mosquito eDNA. In addition, because the baseline information essential for determining the areas of larval control includes larval habitat preferences [25], eDNA analysis makes wide-range surveys easier and help to obtain such information, contributing to efficient mosquito control.

We developed a species-specific real-time PCR assay for evaluating *C. pipiens pallens*. The specificity of our assay was confirmed through in silico and in vitro specificity checks. Although we considered only closely related species inhabiting Japan when developing this assay, it can be used not only in Japan but also worldwide based on the results of in silico tests. Therefore, our assay will contribute to monitoring the immature stages of *C. pipiens pallens*, which is a known vector of West Nile fever.

As a control management approach for targeting immature mosquito, the larval source management (LSM) method has been employed [26], which can potentially overcome problems of adult insecticide resistance [7]. In LSM, permanent or temporary reduction of the availability of larval habitats (habitat control) and adding substances to standing water that either kill or inhibit the development of larvae (larvae control) have been conducted [26]. In addition, IGRs have been applied to the management of larvae instead of insecticides because they affect other insects and the environment [27]. Considering the mechanism by which LSM functions and the application of IGRs, which requires that larval habitats are identified, eDNA analysis can contribute to LSM. Accumulation of basic information on the eDNA of immature mosquitoes is expected for future eDNA-based LSMs.

After eggs hatched and pupae emerged, the eDNA concentration was relatively high. Continuous sampling revealed that this strong eDNA signal may be useful for inferring the developmental stage of the target species [28]. Such eDNA signals would be useful for evaluating mosquito habitats. Although it is difficult to make a simple comparison because of the difference in taxonomic groups, some studies reported increased eDNA concentrations after eggs hatched [29]. However, eDNA is rarely detected during the egg period. In the future, experiments focused on each developmental stage should be conducted to better investigate the dynamics of mosquito eDNA.

In conclusion, eDNA analysis of mosquitoes will enable more efficient monitoring of the immature stages of mosquitoes. In addition, several mosquito species can be detected simultaneously using the eDNA metabarcoding assay reported in a previous study [15]. Such monitoring methods can be applied not only to this target species but also to other mosquito species, which are vectors of malaria and yellow fever, and we are planning to apply this approach to other mosquito species. We conducted experiments in the laboratory to examine the detectable period of eDNA in mosquitoes, and we will need to demonstrate whether this finding is similar in the field for application to field monitoring in the future. As an example, in areas that are difficult to survey such as tropical forests, it may be applicable to investigate mosquito

distribution by performing water sampling using unmanned aerial vehicles and eDNA analysis, as demonstrated in a previous study [30]. The use of eDNA analysis is desirable for improving the control of infectious diseases.

## References

1. Smith, L. B., Kasai, S. & Scott, J. G. Pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus*: Important mosquito vectors of human diseases. *Pesticide Biochemistry and Physiology* **133**, 1–12 (2016).
2. Benelli, G. & Mehlhorn, H. Declining malaria, rising of dengue and Zika virus: insights for mosquito vector control. *Parasitology Research* **115**, 1747–1754 (2016).
3. Campbell, G. L., Marfin, A. A., Lanciotti, R. S. & Gubler, D. J. West Nile virus. *Lancet Infectious Diseases* **2**, 519–529 (2002).
4. Tolle, M. A. Mosquito-borne Diseases. *Current Problems in Pediatric and Adolescent Health Care* **39**, 97–140 (2009).
5. Wang, Y. *et al.* Investigation of mosquito larval habitats and insecticide resistance in an area with a high incidence of mosquito-borne diseases in Jining, Shandong Province. *PLOS ONE* **15**, e0229764 (2020).
6. Fivelman, Q. L., Butcher, G. A., Adagu, I. S., Warhurst, D. C. & Pasvol, G. Malarone treatment failure and in vitro confirmation of resistance of *Plasmodium falciparum* isolate from Lagos, Nigeria. *Malaria Journal* **1**, 1–4 (2002).
7. Fillinger, U. & Lindsay, S. W. Larval source management for malaria control in Africa: Myths and reality. *Malaria Journal* **10**, 353 (2011).
8. Hemingway, J. The role of vector control in stopping the transmission of malaria: threats and opportunities. *Philosophical Transactions of the Royal Society B: Biological Sciences* **369**, 20130431 (2014).
9. Silver, J. B. *Mosquito Ecology-Field Sampling Methods 3rd Edition*. Springer (2008). doi:10.1007/978-1-4020-6666-5
10. Walton, C., Sharpe, R. G., Pritchard, S. J., Thelwell, N. J. & Butlin, R. K. Molecular identification of mosquito species. *Biological Journal of the Linnean Society* **68**, 241–256 (1999).
11. Minamoto, T., Yamanaka, H., Takahara, T., Honjo, M. N. & Kawabata, Z. Surveillance of fish species composition using environmental DNA. *Limnology* **13**, 193–197 (2012).

12. Sato, M. O. *et al.* Usefulness of environmental DNA for detecting *Schistosoma mansoni* occurrence sites in Madagascar. *International Journal of Infectious Diseases* **76**, 130–136 (2018).
13. Goldberg, C. S. *et al.* Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution* **7**, 1299–1307 (2016).
14. Wang, G. *et al.* Identifying the Main Mosquito Species in China Based on DNA Barcoding. *PLoS ONE* **7**, e47051 (2012).
15. Schneider, J. *et al.* Detection of invasive mosquito vectors using environmental DNA (eDNA) from water samples. *PLoS ONE* **11**, e0162493 (2016).
16. Odero, J., Gomes, B., Fillinger, U. & Weetman, D. Detection and quantification of *Anopheles gambiae* sensu lato mosquito larvae in experimental aquatic habitats using environmental DNA (eDNA). *Wellcome Open Research* **3**, 26 (2018).
17. Yamanaka, H. *et al.* A simple method for preserving environmental DNA in water samples at ambient temperature by addition of cationic surfactant. *Limnology* **18**, 233–241 (2017).
18. Minamoto, T., Hayami, K., Sakata, M. K. & Imamura, A. Real-time polymerase chain reaction assays for environmental DNA detection of three salmonid fish in Hokkaido, Japan: Application to winter surveys. *Ecological Research* **34**, 237–242 (2019).
19. Sakata, M. K., Maki, N., Sugiyama, H. & Minamoto, T. Identifying a breeding habitat of a critically endangered fish, *Acheilognathus typus*, in a natural river in Japan. *The Science of Nature* **104**, 100 (2017).
20. Ellison, S. L. R., English, C. A., Burns, M. J. & Keer, J. T. Routes to improving the reliability of low level DNA analysis using real-time PCR. *BMC Biotechnology* **6**, 33 (2006).
21. Barnes, M. A. *et al.* Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science and Technology* **48**, 1819–1827 (2014).
22. Mauvisseau, Q., Troth, C., Young, E., Burian, A. & Sweet, M. The development of an eDNA based detection method for the invasive shrimp *Dikerogammarus haemobaphes*. *Management of Biological Invasions* **10**, (2019).
23. Sansom, B. J. & Sassoubre, L. M. Environmental DNA (eDNA) Shedding and Decay Rates to Model Freshwater Mussel eDNA Transport in a River. *Environmental Science and Technology* **51**, 14244–14253 (2017).
24. Barnes, M. A. & Turner, C. R. The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics* **17**, 1–17 (2016).

25. Ondiba, I. M., Oyieke, F. A., Athinya, D. K., Nyamongo, I. K. & Estambale, B. B. A. Larval species diversity, seasonal occurrence and larval habitat preference of mosquitoes transmitting Rift Valley fever and malaria in Baringo County, Kenya. *Parasites and Vectors* **12**, (2019).
26. Tusting, L. S. *et al.* Mosquito larval source management for controlling malaria. *Cochrane Database of Systematic Reviews* **2013**, (2013).
27. Batra, C. P., Mittal, P. K., Adak, T. & Ansari, M. A. Efficacy of IGR compound Starycide 480 SC (Triflumuron) against mosquito larvae in clear and polluted water. *Journal of Vector Borne Diseases* **42**, 109–116 (2005).
28. Spear, S. F., Groves, J. D., Williams, L. a & Waits, L. P. Using environmental DNA methods to improve detectability in a hellbender monitoring program. *Biological Conservation* **183**, 38–45 (2014).
29. Takeshita, D. *et al.* Projection range of eDNA analysis in marshes: a suggestion from the Siberian salamander ( *Salamandrella keyserlingii* ) inhabiting the Kushiro marsh, Japan . *PeerJ* **8**, e9764 (2020).
30. Doi, H. *et al.* Water sampling for environmental DNA surveys by using an unmanned aerial vehicle. *Limnology and Oceanography: Methods* **15**, 939–944 (2017).

## Tables

Table 1. Accession numbers of nucleotide sequences used for designing eDNA assays.

Species name	Accession No.
<i>Culex pipiens</i>	MF278809.1, KT851543.1
<i>pallens</i>	
<i>Culex</i>	LC054455.1, AB738112.1, AB738175.1, AB738178.1, AB738227.1, AB738228.1,
<i>bitaeniorhynchus</i>	AB738237.1, AB738254.1, HQ398898.1, HQ398899.1, LC054451.1, LC054452.1, LC054453.1, LC054454.1
<i>Aedes aegypti</i>	KT630484.1, KM280573.1, KM280574.1, KT630395.1, KT630396.1, KT630397.1, KT630398.1, KT630399.1, KT630402.1, KT630403.1, KT630404.1, KT630405.1, KT630406.1, KT630407.1, KT630408.1, KT630409.1, KT630410.1, KT630411.1, KT630412.1, KT630413.1, KT630414.1, KT630415.1, KT630416.1, KT630417.1, KT630418.1, KT630419.1, KT630424.1, KT630425.1, KT630426.1, KT630427.1, KT630428.1, KT630429.1, KT630430.1, KT630431.1, KT630432.1, KT630433.1, KT630434.1, KT630435.1, KT630436.1, KT630437.1, KT630438.1, KT630439.1, KT630440.1, KT630441.1, KT630442.1, KT630443.1, KT630444.1, KT630445.1, KT630446.1, KT630447.1, KT630448.1, KT630449.1, KT630450.1, KT630451.1, KT630452.1, KT630453.1, KT630454.1, KT630455.1, KT630456.1, KT630457.1, KT630458.1, KT630459.1, KT630460.1, KT630461.1, KT630462.1, KT630463.1, KT630464.1, KT630465.1, KT630466.1, KT630467.1, KT630468.1, KT630469.1, KT630470.1, KT630471.1, KT630472.1, KT630473.1, KT630474.1, KT630475.1, KT630476.1, KT630477.1, KT630478.1, KT630479.1, KT630480.1, KT630481.1, KT630482.1, KT630483.1
<i>Aedes albopictus</i>	KY817566.1, KY765450.1, KY765451.1, KY765452.1, KY765453.1, KY765454.1, KY765455.1, KY765456.1, KY765457.1, KY765458.1, KY765459.1, KY765460.1, KY765461.1, KY765462.1, KY765463.1, KY765464.1, KY765465.1, KY765466.1, KY765467.1, KY765468.1, KY765469.1, KY765470.1, KY765471.1, KY765472.1, KY765473.1, KY765474.1, KY765475.1, KY765476.1, KY765477.1, KY765478.1, KY765479.1, KY765480.1, KY765481.1, KY765482.1, KY765483.1, KY765484.1, KY765485.1, KY765486.1, KY765487.1, KY765488.1, KY765489.1, KY765490.1, KY765491.1, KY765492.1, KY765493.1, KY765494.1, KY765495.1, KY765496.1, KY765497.1, KY765498.1, KY765499.1, KY765500.1, KY765501.1, KY765502.1,

KY765503.1, KY765504.1, KY765505.1, KY765506.1, KY817523.1, KY817524.1, KY817525.1, KY817526.1, KY817527.1, KY817528.1, KY817529.1, KY817530.1, KY817531.1, KY817532.1, KY817533.1, KY817534.1, KY817535.1, KY817536.1, KY817537.1, KY817538.1, KY817539.1, KY817540.1, KY817541.1, KY817542.1, KY817543.1, KY817544.1, KY817545.1, KY817546.1, KY817547.1, KY817548.1, KY817550.1, KY817551.1, KY817553.1, KY817554.1, KY817555.1, KY817556.1, KY817557.1, KY817558.1, KY817559.1, KY817560.1, KY817561.1, KY817562.1, KY817563.1, KY817564.1, KY817565.1

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## Declarations

### Consent for publication

Not applicable.

### Ethical statement

Based on current laws and guidelines of Japan relating to animal experiments on insects, the collection of mosquito tissue for extracting DNA and the use of DNA samples do not require ethical approval. All experiments were performed according to Japanese standards and guidelines currently in place.

### Availability of data and materials

All raw data are included in the Supporting Information.

### Competing interest

TM is an inventor of the patent for the use of BAC to eDNA preservation. The other authors declare that they have no conflict of interest.

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Okinawa & formulation of research networking (PI: Jun Kobayashi).

## Author's Contributions

MKS, MS, MOS, JK, and TM conceived and designed the study. MKS, TW, HM, and TH performed the laboratory experiments and environmental DNA analysis. MKS, MS, MOS, and TM wrote and edited the first draft of the manuscript. All authors discussed the results and contributed to the development of the manuscript.

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## Figures

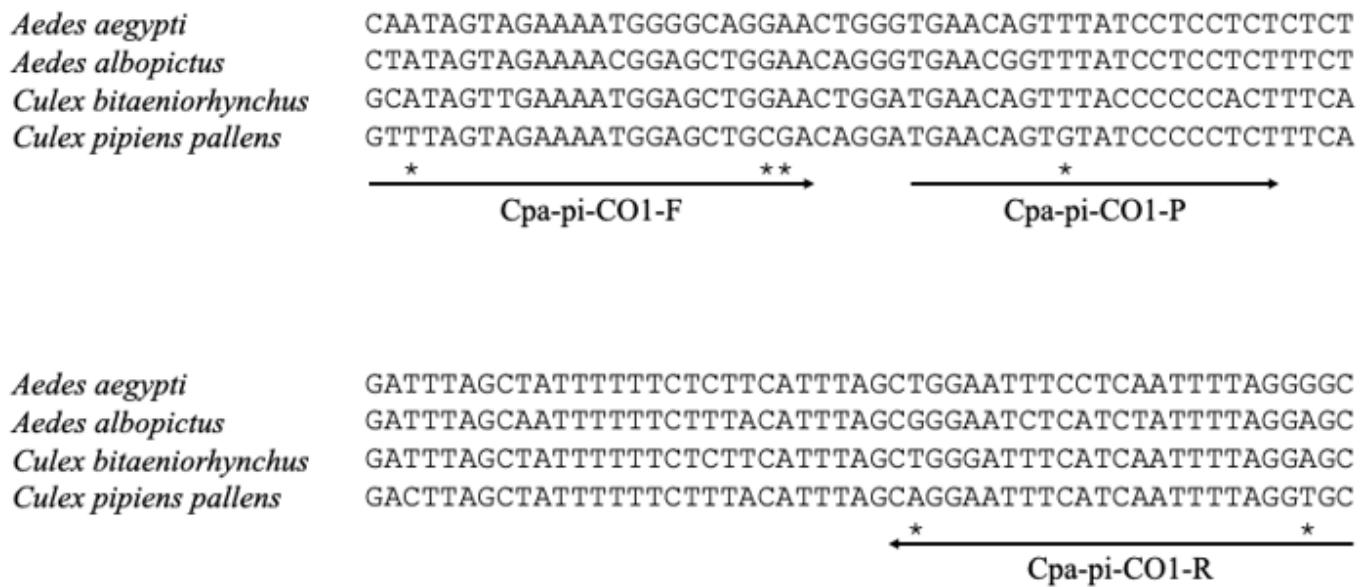
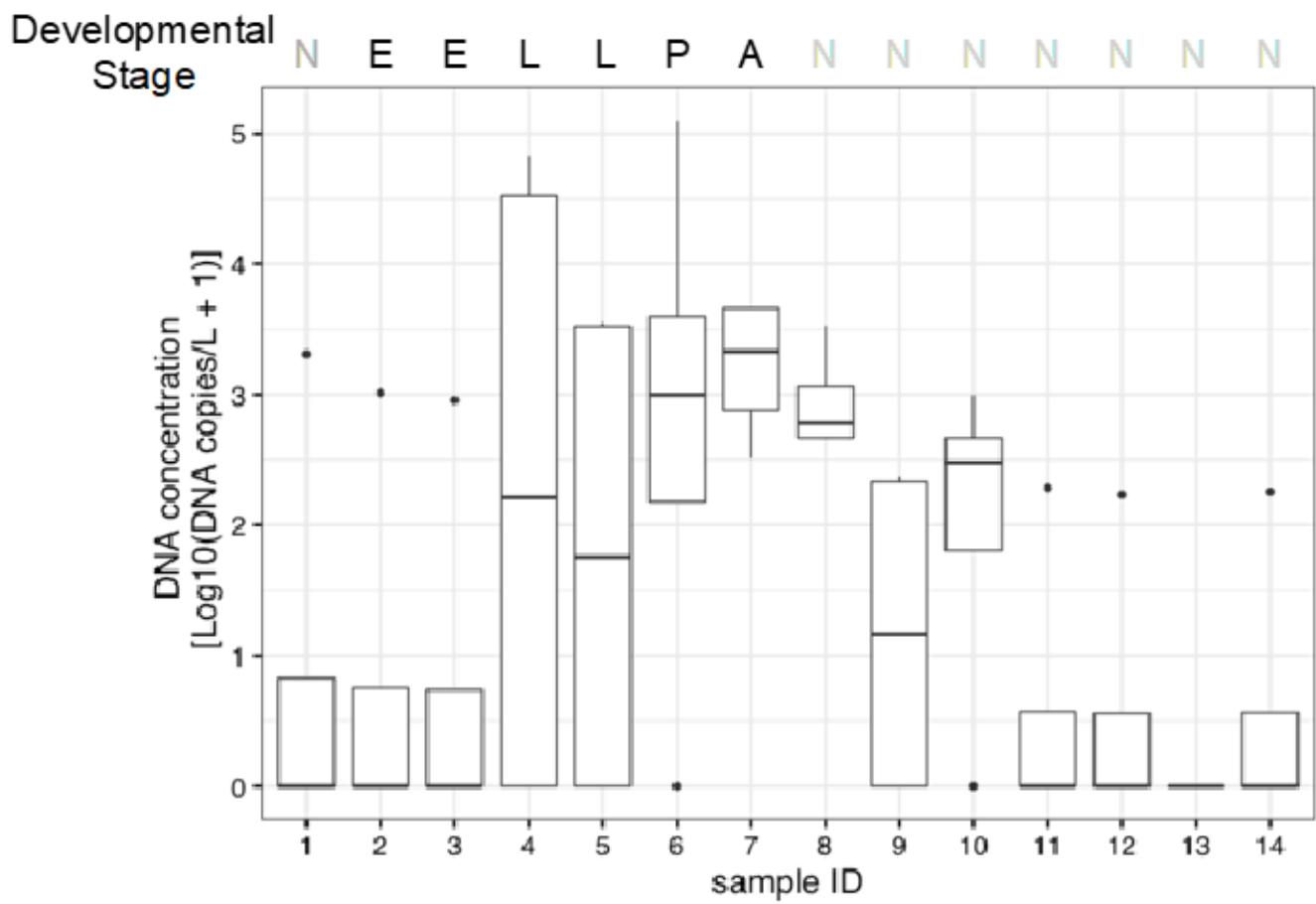


Figure 1

Alignment of the amplified region of mitochondrial CO1 gene of *C. pipiens pallens* and closely related, potentially sympatrically distributed species with *C. pipiens pallens* in Japan. Asterisks indicate *C. pipiens pallens*-specific nucleotides. The length of the amplicons was 136 bp.



**Figure 2**

Mosquito developmental stage and tank experiment results. eDNA can be detected three days after total mosquito emergence (sample ID = 10). Developmental stage was showed by alphabets; N: No mosquito in the tanks, E: Egg, L: Larvae. P: Pupa, A: Adult. Sample IDs: life stage, sample condition are; 1: no mosquitoes, before introducing mosquitoes; 2: egg, one hour after introducing mosquitoes; 3: egg, before the eggs hatched; 4: larvae, after the eggs hatched. Age is one to two days; 5: larvae, 3rd stage to 4th stage larvae; 6: pupa, all mosquitoes are pupa; 7: adult, just after total mosquito emergence (Day 0) ; 8: adult, one day after total mosquito emergence (Day 1) ; 9: adult, two days after total mosquito emergence (Day 2) ; 10: adult, three days after total mosquito emergence (Day 3); 11: adult, four days after total mosquito emergence (Day 4) ; 12: adult, five days after total mosquito emergence (Day 5); 13: adult, six days after total mosquito emergence (Day 6) ; and 14: adult, seven days after total mosquito emergence (Day 7).

## Supplementary Files

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