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Original Article

PCR-Based Identification of *Culex pipiens* Complex Collected in Japan

Shinji Kasai*, Osamu Komagata, Takashi Tomita, Kyoko Sawabe, Yoshio Tsuda, Hiromu Kurahashi, Takeshi Ishikawa[1], Mitsugu Motoki[2], Tomoya Takahashi[3], Tsutomu Tanikawa[4], Masahiro Yoshida[5], Goro Shinjo[6], Tomoyuki Hashimoto[6], Yukiko Higa[7] and Mutsuo Kobayashi

Department of Medical Entomology, National Institute of Infectious Diseases, Tokyo 162-8640;
[1]Shell Company Co., Ltd., Tokyo 104-0061; [2]APEX Industry Co., Ltd., Tokyo 105-0014;
[3]Fuji Environmental Service Co., Ltd., Shizuoka 422-8046; [4]Ikari Corporation, Chiba 260-0844;
[5]Ikimono Kenkyuusha, Osaka 537-0023; [6]Japan Environmental Sanitation Center, Kanagawa 210-0828; and
[7]Center of International Collaborative Research, Nagasaki University, Nagasaki 852-8523, Japan

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SUMMARY: The *Culex pipiens* complex consists of vector mosquitoes that transmit important human pathogens. In this study we established a simplified method to distinguish three members of the *Cx. pipiens* complex, *Cx. p. pallens* Coquillet, *Cx. p.* form *molestus* Forskal, and *Cx. quinquefasciatus* Say, collected in Japan. Sequence analysis of the *Drosophila Ace*-orthologous acetylcholinesterase (*Ace*) gene (668 to 680 bp) revealed that a single polymorphic region characterizes each species. Based on this region, specific primers that distinguish *Cx. p.* form *molestus* (ACEpip2) and *Cx. p. pallens* (ACEpall2) were newly designed. Polymerase chain reactions were performed with the genomic DNA of *Culex* mosquitoes as the template, and these primers clearly distinguished two *Culex* spp. The accuracy of the designed primers was evaluated with 38 colonies of mosquito samples collected from 9 prefectures of Japan. The testing revealed that the distribution of anautogenous *Cx. p. pipiens* has not been confirmed in Japan. It also revealed that the male of *Cx. p. pallens* possesses an *Ace* gene haplotype that is highly similar to the sequence of *Cx. quinquefasciatus*. This improved method allows the evaluation of vector competence of *Cx. p.* form *molestus*, which is the suspected vector of West Nile virus.

INTRODUCTION

Members of the *Culex pipiens* complex are representative vectors of human pathogens including lymphatic filaliasis, St. Louis encephalitis, and equine encephalitis. They are also expected to be the most important vector mosquitoes of West Nile virus if the pathogen is introduced to this country. In the United States, the West Nile fever epidemic has been ongoing since 1999, and the total numbers of patients has exceeded 20,000, with more than 900 fatalities in the past 8 years (1). The transmission cycle of West Nile virus is alternately rotated between birds and mosquitoes (2). An incidental bite from an infected mosquito causes viral infection in humans.

In Japan, *Cx. p. pallens*, *Cx. p.* form *molestus*, and *Cx. quinquefasciatus* are the dominant species of the *Cx. pipiens* complex. Although there are several notations for *molestus* (for example, *Cx. p. molestus*, *Cx. molestus*, autogenous *Cx. p. pipiens*, underground *Cx. p. pipiens*), here we follow the notation proposed by Tanaka et al. (3). *Cx. p. pallens* inhabits East Asia, including Japan, Korea, and North China (3). Historically, the taxonomical status of *Cx. p. pallens* had been uncertain, and some researchers considered it as a hybrid population between *Cx. p. pipiens* and *Cx. quinquefasciatus* (3). At present, however, it appears that *Cx. p. pipiens* does not exist in Japan and *Cx. p. pallens* is treated as a subspecies of *Cx. p. pipiens* (3). Although males of *Cx. quinquefasciatus* can be identified by the ratio of the dorsal to the ventral arm (D/V ratio) of the male genitalia (3), it is not easy to distinguish females of *Cx. quinquefasciatus* from those of the other two *Culex* complexes by morphological characteristics. The distribution of *Cx. quinquefasciatus* in Japan is limited to the southern parts of the country (Nansei islands and the southernmost tip of Kyushu and Shikoku) (3), while *Cx. p. pallens* and *Cx. p.* form *molests* are the primal *Culex pipiens* mosquitoes in most areas of Japan. The major habitat of the *Cx. p.* form *molestus* in Japan is enclosed dark spaces such as basements and subways, and there are several physiological distinctions between *Cx. p. pallens* and *Cx. p.* form *molestus*. First, *Cx. p.* form *molestus* mostly inhabit enclosed spaces under buildings, laying the first egg raft without blood ingestion (autogenous), while females of *Cx. p. pallens* oviposit only after hematophagia. The number of eggs in each raft of *Cx. p.* form *molestus* is relatively smaller than that of *Cx. p. pallens*. In addition, *Cx. p.* form *molestus* possesses the ability to copulate in a very narrow space (stenogamous) and to reproduce continuously, i.e., without diapose (homodynamous). On the other hand, *Cx. p. pallens* exhibits imaginal diapause. Since *Cx. p.* form *molestus*, among members of the *Cx. pipiens* complex, is the most common mosquito in basements, underground shopping areas, and subways in Japan, it tends to be the target of control by insecticides. Our recent study demonstrated that the *Cx. p.* form *molestus* collected in the Tokyo metropolitan area already presents high levels of resistance to pyrethroid insecticides (4). Furthermore, in addition to its underground habitat, *Cx. p.* form *molestus* is occasionally observed aboveground (5,6), allowing contact with both humans and birds and thus making this species a possible vector mosquito of West Nile virus. Therefore, the strategy of mosquito control in Japan is highly dependent on the vector competence of *Cx. p.* form *molestus*, but this

*Corresponding author: Mailing address: Department of Medical Entomology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111 ext. 2424, Fax: +81-3-5285-1147, E-mail: kasacin@nih.go.jp

vector competence has not been well evaluated in Japan, since it is quite difficult to distinguish *Cx. p. pallens*, *Cx. p.* form *molestus*, and *Cx. quinquefasciatus* morphologically, especially in the case of the females, as the reproductive organs are indistinguishable between these species. At present it is impossible to undertake studies on biting behavior, population density, and host preference of *Cx. p.* form *molestus*. Therefore, it is essential to establish a convenient and simple method for discriminating members of the *Culex pipiens* complex.

In 1998, Bourguet et al. identified several polymorphisms in the *Drosophila Ace*-orthologous acetylcholinesterase (*Ace*) gene and established a method to distinguish *Cx. p. pipiens* and *Cx. quinquefasciatus* by restriction enzyme digestion of polymerase chain reaction (PCR)-amplified DNA (7). In 2003, an assay to identify *Culex pipiens* complex by means of PCR was newly introduced (8). Thereafter, Smith and Fonseca further reported a rapid assay to separate the *Culex pipiens* complex (*Cx. quinquefasciatus*, *Cx. p. pipiens*, and *Cx. p. pallens*) and its sibling species by using diagnostic primers designed from the *Ace* gene (9). Since anautogenous *Cx. p. pipiens* does not inhabit Japan (3,10,11), we speculated that the *Cx. p.* form *molestus*, *Cx. p. pallens*, and *Cx. quinquefasciatus* could be discriminated using these primers, although so far little is known about the genetic differences between the anautogenous *pipiens* and *Cx. p.* form *molestus*.

In this study, we conducted sequence analysis of the *Ace* gene of three *Culex pipiens* complex members collected in Japan. In the process of the study, we found that primers introduced in the previous manuscript (9) have insufficient

Table 1. Localities of *Culex* colonies used for the verification of primers designed for the discrimination of *Culex* mosquitoes

Species or biotype	Locality	Prefecture (or country)	Code (in figures)	No. of larvae tested	Judgement[1)]
Cx. p. pipiens	Fort Collins, Colorado	(USA)	USP[2)]	–	–
autogenous	Udagawachou, Shibuya-ku (2003)	Tokyo	SBY	2	Cpmol
autogenous	Shinjuku, Shinjuku-ku (2004)	Tokyo	SNJ	2	Cpmol
autogenous	Shinjuku, Shinjuku-ku (1990)	Tokyo		4	Cpmol
autogenous	Yayoi, Bunkyo-ku	Tokyo		4	Cpmol
autogenous	Kichijouji, Musashino (2004)	Tokyo		4	Cpmol
autogenous	Hikawadai, Higashikurume (2003)	Tokyo		2	Cpmol
autogenous	Daimonchou, Higashikurume (2004)	Tokyo		4	Cpmol
autogenous	Takadanobaba, Shinjuku-ku	Tokyo		10	Cpmol
autogenous	Ochiai, Shinjuku-ku (2004)	Tokyo		2	Cpmol
autogenous	Otemachi, Chiyoda-ku (2004)	Tokyo	OTM	8	Cpmol
autogenous	Kameido, Koutou-ku (2004)	Tokyo		4	Cpmol
autogenous	Kawasaki-ku, Kawasaki (2004)	Kanagawa		4	Cpmol
autogenous	Shinkashiwa, Kashiwa (2003)	Chiba	KSW	2	Cpmol
autogenous	Nakayama, Ichikawa (2003)	Chiba	IKW	2	Cpmol
autogenous	Yokosuka, Kamogawa (2004)	Chiba		4	Cpmol
autogenous	Chuou-ku, Chiba (2004)	Chiba		4	Cpmol
autogenous	Aoi-ku, Shizuoka (1989)	Shizuoka		4	Cpmol
autogenous	Nishijin, Sawara-ku (2004)	Fukuoka		4	Cpmol
autogenous	Nakamachi, Nagasaki (2004)	Nagasaki		4	Cpmol
autogenous	Sakamoto, Nagasaki (2004)	Nagasaki		10	Cpmol
anautogenous	Shibasakichou, Tachikawa (2003)	Tokyo	TCK	2	Cppal
anautogenous	Honmachi, Hino (2003)	Tokyo		2	Cppal
anautogenous	Miyukichou, Fuchu (2003)	Tokyo	IRG	8	Cppal
anautogenous	Togoshi, Shinagawa-ku (2003)	Tokyo		3	Cppal
anautogenous	Motoizumi, Komae (2003)	Tokyo	KME	5	Cppal
anautogenous	Komazawa, Setagaya-ku (2003)	Tokyo		2	Cppal
anautogenous	Noge, Setagaya-ku (2003)	Tokyo		2	Cppal
anautogenous	Koyamadai, Shinagawa-ku (2003)	Tokyo	RNS	18	Cppal
anautogenous	Toyama, Shinjuku-ku (2001)	Tokyo	TYM	4	Cppal
anautogenous	Ohnuma, Kasukabe (2004)	Saitama		4	Cppal
anautogenous	Huchinobe, Sagamigahara (2004)	Kanagawa		4	Cppal
anautogenous	Yohoshiba, Sanbugun (2004)	Chiba		4	Cppal
anautogenous	Higashi-ku, Nagoya (2004)	Aichi		4	Cppal
anautogenous	Chuou-ku, Osaka (2004)	Osaka		4	Cppal
anautogenous	Higashinari-ku, Osaka (2004)	Osaka		2	Cppal
anautogenous	Aino, Minamikourai (2004)	Nagasaki		4	Cppal
anautogenous	Sakamoto, Nagasaki (2004)	Nagasaki		4	Cppal
anautogenous	Nakamachi, Nagasaki (2004)	Nagasaki		4	Cppal
Cx. quinquefasciatus	Jefferson Co., Florida	(USA)	USQ[3)]	–	–
Cx. quinquefasciatus	Jeddah	(Saudi Arabia)	JPL	–	–
Cx. quinquefasciatus	Chichijima, Ogasawara (1968)	Tokyo	OGS	–	–

[1)]: Cpmol and Cppal denote that the molecular diagnosis judged as *Cx. p. molestus* and *Cx. p. pallens*, respectively.
[2)]: Obtained from GenBank (accession no. is AY196910).
[3)]: Obtained from GenBank (accession no. is AY196911).

specificities to the *Culex* mosquitoes collected in Japan, so we modified the system and designed new primers. The utility of these primers was verified using mosquito samples collected from widely dispersed geographical areas of Japan.

MATERIALS AND METHODS

Collection of mosquitoes: The localities of the larval collection and origins of the strains used in this study are given in Table 1 and Fig. 1. The field collected mosquito larvae (2003 to 2004) were reared in the insectarium, and then the emerged adults were judged autogenous if the females deposited eggs without first feeding on blood. Species were preliminarily identified from morphological keys as described by Tanaka et al. (3) and from the potential for autogeny. In order to use only genuine mosquitoes, populations collected from open water and containing autogenous-type mosquitoes were not used.

Extraction of genomic DNA: Genomic DNA was isolated from individual fourth-instar larvae using REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, Mo., USA). The extraction solution and the tissue preparation solution were mixed, and each larva was homogenized in 125 μl of the mixture and incubated at room temperature for 10 min followed by incubation at 95°C for 3 min. One hundred microliters of the neutralization solution was added to the sample and mixed by vortexing. The resultant mixture was used directly for the genomic PCR.

PCR: PCR was performed using the REDExtract-N-Amp PCR Reaction Mix (10 μl; Sigma), which was mixed gently with a mixture of 10 μM primers (1 μl each), deionized ultra pure water (4 μl), and extracted DNA solution (4 μl). The PCR reaction mixture was heated to 94°C for 5 min and then put through 30-35 cycles of PCR amplification: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min. The amplified DNA was loaded onto an agarose gel (2%) with the 100-bp ladder loading marker (Bio-Rad, Richmond, Calif., USA), stained with ethidium bromide (Amresco Inc., Solon, Ohio, USA), and visualized on a UV trans-illuminator (TF-20C; Vilber Lourmat, Marne La Vallee, France).

Sequencing analysis: For the sequence analysis, a partial sequence of the *Ace* gene was amplified using F1457 and B1246s primers (Table 2), electrophoresed, and purified. The purified PCR products were cloned into the TA-cloning vector (Invitrogen, Carlsbad, Calif., USA), and multiple clones were sequenced with universal primers designed from the sequence of the vector. The genome sequences were aligned with the ClustalX program, version 1.81 (12,13).

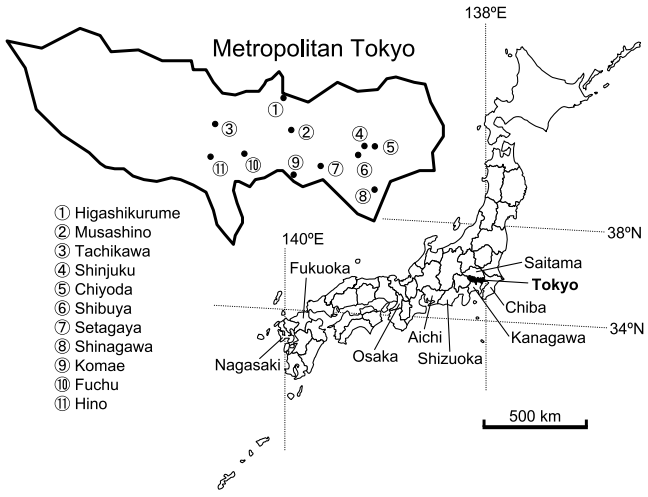

Fig. 1. Map of mosquito collection sites in Japan. The *Cx. pipiens* complex was collected from 9 prefectures. The map of metropolitan Tokyo is magnified to show each of the collection sites. The detailed collection information is shown in Table 1.

Table 2. Primer sequences used in this study

Primer name	Sequence (5′→3′)
F1457[1)]	GAGGAGATGTGGAATCCCAA
B1246s[1)]	TGGAGCCTCCTCTTCACGG
ACEpall[1)]	ATGGTGGAGACGCATGACG
ACEpall2	GTGGAGACGCATGACGCAT
ACEpip[1)]	GGAAACAACGACGTATGTACT
ACEpip2	GTGGAAACGCATGATACCAG
ACEquin[1)]	CCTTCTTGAATGGCTGTGGCA

[1)]: Introduced by Smith and Fonseca (9).

Verification of the newly designed primers: PCR was performed with genomic DNA isolated from F_1 or F_2 larvae of field-collected mosquitoes as the templates. For each individual, two kinds of PCR reactions were performed with different primer sets (ACEpip2/B1246s and ACEpall2/B1246s) (Table 2). The amplified PCR products were electrophoresed onto the 2% agarose gels, stained with ethidium bromide, and visualized as described above.

Male specific haplotype of the *Ace* gene in *Cx. p. pallens*: The larvae of *Cx. p. pallens* collected from Shinagawa, Tokyo (Table 1, Fig. 1), were reared in the insectarium, and the lack of autogeny was confirmed. Each pupa of the progeny was isolated in a 1.5-ml plastic tube with a small amount of water to avoid mating and left at 25°C until adult emergence. Genomic DNA was isolated as described above from each individual, and PCR was performed with the isolated DNA and two primer sets (ACEpall2/B1246s and ACEquin/B1246s). The PCR products were electrophoresed and visualized as described above. Forty adult mosquitoes (male:female = 20:20) were analyzed.

RESULTS AND DISCUSSION

Application of ACEpip, ACEpall, and ACEquin primers to Japanese mosquito colonies: To examine the availability of ACEpall, ACEpip, and ACEquin primers for discriminating the *Cx. p. pallens*, *Cx. p.* form *molestus*, and *Cx. quinquefasciatus* collected in Japan, we performed PCR with genomic DNA isolated from *Cx. p. pallens* (TYM and RNS colonies), *Cx. p.* form *molestus* (SNJ and OTM colonies), and *Cx. quinquefasciatus* (JPL and OGS strains) (Table 1 and Fig. 2). The ACEpall/B1246s primer set effectively amplified the *Cx. p. pallens Ace* gene, although a small amount of DNA amplification was also observed from *Cx. p.* form *molestus* and *Cx. quinquefasciatus*. The *Cx. p.* form *molestus Ace* gene was effectively amplified by the ACEpip/B1246s primer set, although these primers amplified the *Ace* gene of *Cx. p. pallens* and *Cx. quinquefasciatus* as well. No *Ace* gene of the *Cx. p.* form *molestus* was amplified by the primer set of ACEquin and B1246s. This primer set clearly discriminated *Cx. p.* form *molestus* and *Cx. quinquefasciatus*. However, PCR amplification was observed from several individuals of *Cx. p. pallens* (Fig. 2). These results suggested that primers previously designed for discriminating the *Culex pipiens* complex (9) are not specific enough to identify each species.

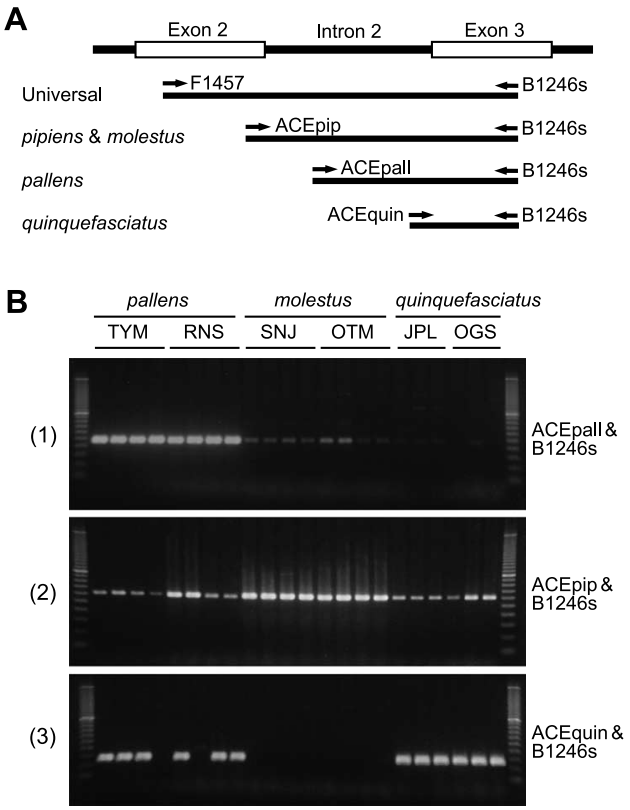

Fig. 2. PCR amplification of the *Ace* gene with the primers introduced by Smith and Fonseca (9). A, Diagram of the *Ace* gene and the position of the primers. B, Electrophoresis profiles of PCR with the ACEpall/B1246s primers (1), ACEpip/B1246s primers (2), and ACEquin/B1246s primers (3). The description of the three letter code for each collection site is shown in Table 1.

Sequence analysis of the *Ace* gene: Since the primers examined were not sufficiently specific to discriminate the three *Culex* spp., we conducted further sequence analysis of the *Ace* genes for *Cx. p. pallens*, *Cx. p.* form *molestus*, and *Cx. quinquefasciatus*, and based on this analysis the ACEpip, ACEpall, and ACEquin primers were designed (Figs. 3 and 4). The region in which the ACEpip primer was designed is quite similar among three sibling species, so it is easy to understand why the ACEpip/B1246s primer set generates nonspecific amplifications of the *Ace* gene from all samples of *Cx. p. pallens* and *Cx. qinquefasciatus* (Fig. 2). As was already described by Smith and Fonseca (9), there is only a single polymorphism between *Cx. p. pipiens* or *Cx. p.* form *molestus* and *Cx. p. pallens* within the ACEpip primer sequence (i.e., T in *pipiens* or *molestus* substitutes for A in *pallens*; Fig. 3), and it causes occasional amplifications in other species when PCR is conducted with the ACEpip and B1246s primer set (Fig. 2). Similarly, the ACEpall primer is also not specific enough to discriminate *Cx. p. pipiens* and *Cx. p. pallens*. Therefore, if we perform multiplex PCR for *Cx. quinquefasciatus* with the primer set of ACEpip/ACEpall/ACEquin/B1246s, it is likely that multiple bands will occasionally be generated (sometimes strongly, as shown in Fig. 2), even if the sample is inbred. And some of the inbred samples will possibly be incorrectly identified as a hybrid of *Cx. p. pipiens* and *Cx. quinquefasciatus*.

Notably, the sequence of the *Cx. p.* form *molestus* is very similar to the sequence of the anautogenous *Cx. p. pipiens*. The similarity of a haplotype of the *Cx. p.* form *molestus* from Kashiwa, Chiba Prefecture, Japan, to the sequence of *Cx. p. pipiens* from the United States was 99.9% (674/675). This value was much larger than the *Ace* gene similarity between *Cx. p. pipiens* and *Cx. p. pallens* (94-95%) or between *Cx. p. pipiens* and *Cx. quinquefasciatus* (93%). The region of intron 2 where the ACEquin primer was designed was found to be relatively unique to *Cx. quinquefasciatus*, except that some of the larvae of *Cx. p. pallens* possessed *Ace* gene haplotypes highly similar (98%) to the *Ace* gene of *Cx. quinquefasciatus* (Fig. 3). It seems that certain individuals of *Cx. p. pallens* have two types of *Ace* gene (i.e., a *pallens* type and a *quinquefasciatus* type), resulting in a non-specific amplification by PCR, as shown in Fig. 2. Sequence analysis of the *Ace* genes has revealed only a few nucleotide differences in 675-bp *Ace* gene fragments between the anautogenous *Cx. p. pipiens* and *Cx. p.* form *molestus*, so the two species are genetically too close to allow the discrimination of one from the other. Historically, the taxonomical status of *Cx. p.* form *molestus* has long been a topic of intense debate (14). One of the pieces of supporting evidence for *Cx. p.* form *molestus* being an independent subspecies has been the reports of the non-fertility between *Cx. p.* form *molestus* and *Cx. p. pipiens* (14-16). Thereafter, Harbach et al. revealed that this phenomenon is not due to reproductive isolation but rather to the rickettsial symbiont, *Wolbachia pipientis* (14). Accordingly, Harbach and others have insisted that the autogenous status of *Cx. p. pipiens* does not satisfy the definition of a subspecies advocated by Mayr (17) and that therefore the *Cx. p.* form *molestus* is unacceptable as either a species or a subspecies (14,18). A recent study also reported that genetic variation between *Cx. p. pipiens* and *Cx. p.* form *molestus* collected in New York, USA, was limited (only a single mutation in an approximately 300-bp SH60 gene fragment), and development of a restriction fragment length polymorphism diagnostic was unsuccessful (19). The results presented here possibly support this point of view, although further sequence comparison will be needed to back up this idea.

New primers for discriminating *Cx. p.* form *molestus* and *Cx. p. pallens*, and their verification: Since it appeared that the primers indicated in Fig. 2 were not specific enough to discriminate *Cx. p. pallens*, *Cx. p.* form *molestus*, and *Cx. quinquefasciatus*, we designed two new primers specific to *Cx. p. pallens* and *Cx. p.* form *molestus*. Of the approximately 700-bp gene fragments analyzed, the region where ACEpall was designed was rich in variety and in the characteristics of each species (Figs. 3 and 4), so we newly designed the ACEpip2 primer for *Cx. p.* form *molestus* and the ACEpall2 primer for *Cx. p. pallens* (Fig. 5A). Since non-specific gene amplifications were seen when the ACEpall/B1246s primer set was used (Fig. 2), we intended to intensify the specificity of the ACEpall primer by shifting it by three base pairs to the 3′ end, which resulted in the new primer ACEpall2 (Fig. 5A).

We attempted PCR using the same templates shown in Fig. 2 but with the newly designed primers. The ACEpall2/B1246s and ACEpip2/B1246s primer sets effectively amplified the *Ace* gene of *Cx. p. pallens* and *Cx. p.* form *molestus*, respectively. Further, since non-specific PCR amplification was minimized in *Cx. quinquefasciatus* with primer sets of ACEpall2/B1246s and ACEpip2/B1246s (Fig. 5B), PCR with these primer sets will be a good tool to distinguish *Cx. quinquefasciatus* as well to use in combination with the F1457/B1246s primer set (Fig. 5B). These primer sets appeared to clearly discriminate the three *Culex* spp., and non-specific amplification of the *Ace* gene was suppressed to a large extent. Subsequently, we verified the accuracy of the

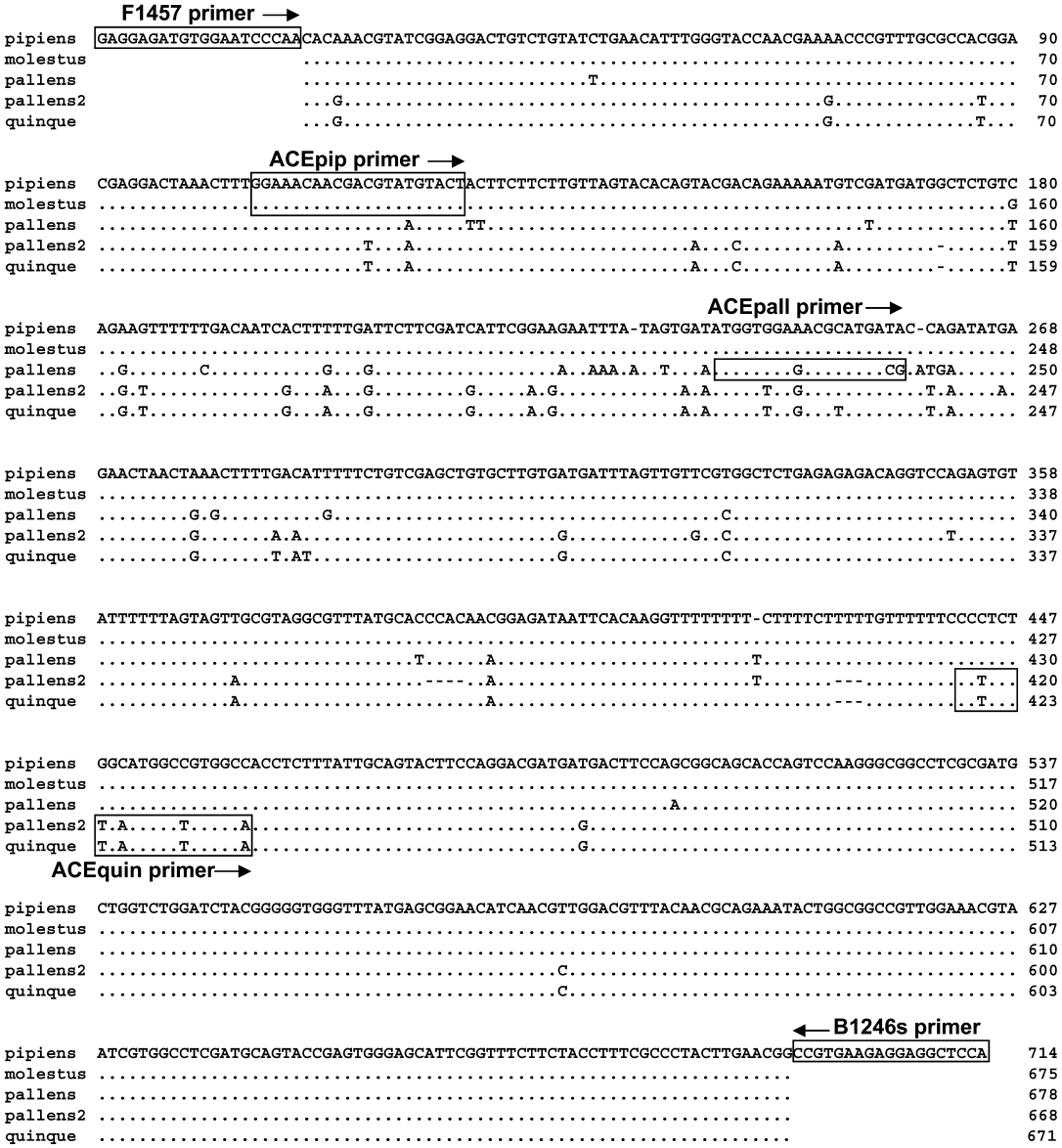

Fig. 3. Alignment of 5 *Ace* genes in *Culex pipiens* complex. Dots indicate nucleotides shared with the reference sequence (*Cx. p. pipiens*), wheres dashes indicate gaps. pipiens = *Cx. p. pipiens* (USP, collected in USA, accession no. is AY196910); molestus = *Cx. p.* form *molestus* (KSW, accession no. is AB294405); pallens = *Cx. p. pallens* (KME, accession no. is AB294403); pallens2 = *Cx. p. pallens* (RNS, accession no. is AB294404); quinque = *Cx. quinquefasciatus* (OGS, accession no. is AB294406). Each primer sequence is framed with a rectangle.

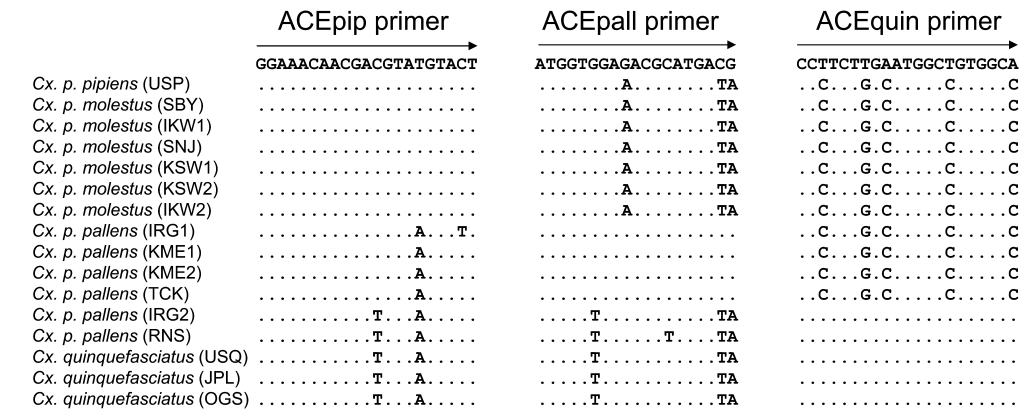

Fig. 4. Alignment of the three primer regions of the *Ace* gene. The abbreviations of the mosquito collection sites are listed in Table 1. Dots indicate nucleotides shared with primer sequences (ACEpip, ACEpall and ACEquin primers). The sequences of *Cx. p. pipiens* (USP, collected in USA) and *Cx. quinquefasciatus* (USQ, collected in USA) were obtained from GenBank (accession nos. AY196910 and AY196911, respectively).

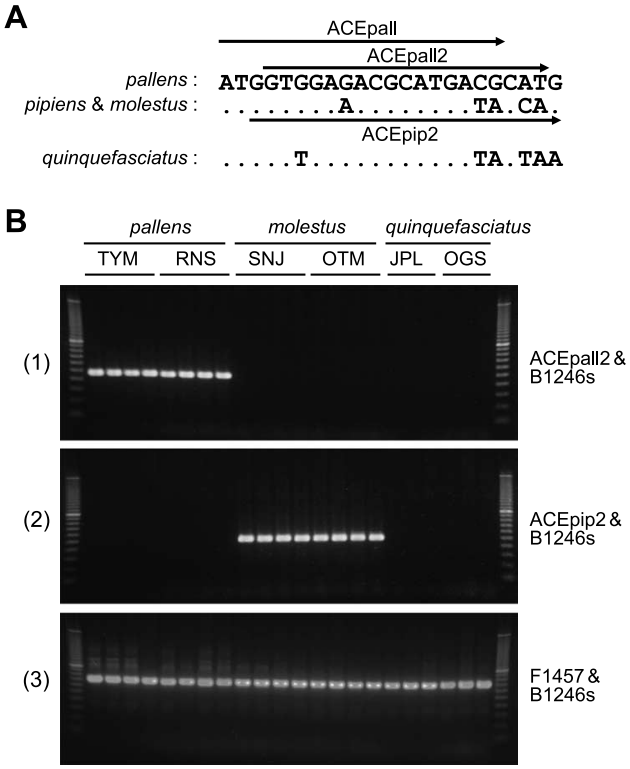

Fig. 5. PCR amplification of the *Ace* gene with the newly designed primers. A, Diagram of the positional relationship among ACEpall, ACEpall2, and ACEpip2. Dots indicate nucleotides shared with the sequence of *Cx. p. pallens*. B, Electrophoresis profiles of PCR with ACEpall2/B1246s primers (1), ACEpip2/B1246s primers (2), and F1457/B1246s primers (3). The description of the three letter code for each collection site is shown in Table 1.

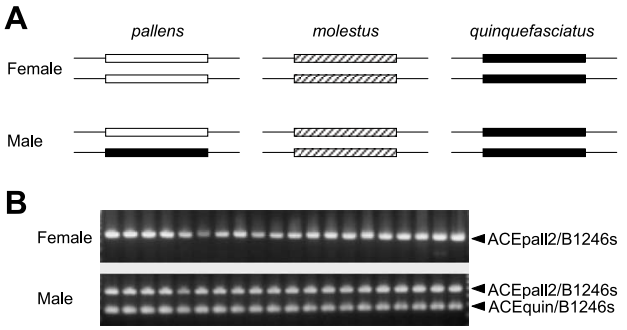

Fig. 6. Examination of the male specific haplotype in *Cx. p. pallens*. A, Diagram of two haplotypes of each mosquito species showing the male in *Cx. p. pallens* possesses a *Cx. quinquefasciatus*-like *Ace* gene haplotype. B, Electrophoresis profiles of PCR with ACEpall2/B1246s (for amplification of *pallens*-type *Ace* gene) and ACEquin/B1246s (for amplification of *Cx. quinquefasciatus*-type *Ace* gene) primer sets. After PCR was terminated, the same volume (8 μl each) of the two PCR mixtures was loaded into the same wells and electrophoresed. Twenty adult males and females collected from Shinagawa, Tokyo (RNS) were tested.

ACEpall2 and ACEpip2 primers with the *Culex pipiens* complex collected in Japan. Thirty-eight colonies of *Culex* mosquito were collected from 9 prefectures and identified by morphological criteria and potential for autogeny (Table 1). Eventually, 20 autogenous and 18 anautogenous colonies were tested for verification of the primers. The ACEpall2/B1246s primers amplified the *Ace* gene of all 84 of the anautogenous mosquitoes, and the ACEpip2/B1246s primers amplified the *Ace* gene of all 80 of the autogenous mosquitoes, suggesting that these primers are highly accurate in discriminating between *Cx. p. pallens* and the *Cx. p.* form *molestus*. As far as could be tested, a distribution of anautogenous *Cx. p. pipiens* was not confirmed in Japan.

Male specific haplotype of the *Ace* gene in *Cx. p. pallens*: The *Cx. p. pallens* collected from Tokyo were reared in the insectarium, and genomic DNA was individually isolated from virgin males and females. Overall, 40 adults were tested via PCR amplification with the ACEpall2/B1246s and ACEquin/B1246s primer sets. In males, all individuals possessed both *pallens* and *quinquefasciatus* type *Ace* genes, and in females, all individuals possessed only the *Cx. p. pallens* type *Ace* genes in a homozygous manner (Fig. 6). We further analyzed this for two more colonies of *Cx. p. pallens* from Nagasaki and Kanagawa Prefectures, and the same results were obtained for both colonies (data not shown). Therefore, we concluded that it is impossible to distinguish males of *Cx. p. pallens* and hybrids of *Cx. p. pallens* and *Cx. quinquefascaitus* using the primers introduced by Smith and Fonseca (9).

In Japan, the coexistence of *Cx. p. pallens* and *Cx. quinquefasciatus* is reported in the southern parts of Kyushu and Shikoku (3), and thus it is likely that the distinction of these two species collected from such locations would be difficult using the ACEquin/B1246s primer set. By any measure, however, why would only the male possess this unique gene? Recent investigations have revealed that the *Ace* gene is tightly linked to the locus of the sex determining factor (0.8 centimorgans) on chromosome 1 (20-22). The lack of the *Cx. quinquefasciatus*-like *Ace* gene in females supports the theory that this sex locus is related to the so-called *M* (male determining) factor, as described by Gilchrist and Haldane (23). The tested *Cx. p. pallens*, Rinshi strain, has been reared in the insectarium for more than 20 generations but yet no recombinant was observed, as shown in Fig. 6. The ACEquin primer may be a useful tool to distinguish the genders of *Cx. p. pallens* at the larval stage. It is uncertain whether the fact that the male *Cx. p. pallens* possesses the *quinquefasciatus*-like *Ace* gene is a trace of the evolutionary history such that this mosquito is a derivative species of *Cx. quinquefasciatus*. Taxonomically, *Cx. p. pallens* tends to be regarded as an intraspecies of the anautogenous *Cx. p. pipiens* (24), or alternatively, *Cx. p. pallens* might be judged an intermediate species between *Cx. p. pipiens* and *Cx. quinquefasciatus*, according to the DV/D ratios and larval siphonal index values (25,26). However, as far as can be seen from the gene structure of *Ace*, *Cx. p. pallens* seems to be much closer to *Cx. quinquefasciatus* than *Cx. p. pipiens*. Further investigation is needed to resolve this intriguing problem.

Significance of molecular discrimination on the study of vector competence: The significance of *Cx. p.* form *molestus* as the vector for Japanese encephalitis and West Nile viruses has been reported recently using mosquitoes collected in Uzbekistan (27) and Taiwan (28). One of these reports concluded with a comment that additional study to identify the host preference of the *Cx. p.* form *molestus* is essential to further evaluate the vector competence for West Nile and Japanese encephalitis viruses (27). The blood preference survey, however, would be quite difficult to accomplish at the locations where both *Cx. p.* form *molestus* and anautogenous *pipiens* type mosquitoes are distributed. In this regard, Japan is an ideal country for the study of *Cx. p.* form *molestus*, since *Cx. p. pallens* and *Cx. p.* form *molestus* are the major species of *Culex* mosquitoes collected in the field (29,30) and the anautogenous type of *Cx. p. pipiens* has

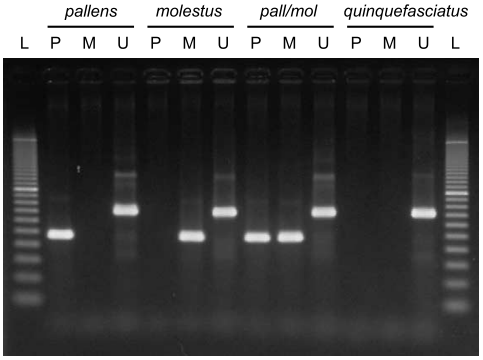

Fig. 7. Electrophoresis profiles of PCR with ACEpall2/B1246s primers (P), ACEpip2/B1246s primers (M), F1457/B1246s primers (U). 100-bp DNA marker was loaded (L). Genomic DNA was prepared from 4th instar larvae as described in Materials and Methods and used as the template. PCR for hybrid of *Cx. p. pallens* and *Cx. p.* form *molestus* was performed with the template DNA prepared by mixing equal volume of genomic DNA of both species (*pall*/*mol*).

yet to make an appearance in this country. Therefore, the primers newly designed in this study are of value for such physiological and ecological studies of the *Cx. p.* form *molestus* and will uncover valuable information towards gaining an understanding of the potential of this mosquito to transmit the West Nile and Japanese encephalitis viruses.

According to the laboratory tests for cross hybridizations, some crossings among *Cx. p. pallens*, *Cx. p.* form *molestus*, and *Cx. quinquefasciatus* have the potential to produce hybrid offspring (31). Fonseca and others reported that natural hybridizations induced a change in host preference of the *Cx. pipiens* complex in the United States, resulting in a change of the vector competence of these mosquitoes for the West Nile virus (32). Although it is uncertain whether natural hybridizations have occurred in the field in Japan among these mosquitoes, the primers designed in this study may be able to detect hybrids from the field-collected mosquitoes, as shown in Fig. 7.

In conclusion, we attempted to improve an assay that identifies members of the *Cx. pipiens* complex collected in Japan, and the reliability of the designed primers was confirmed. This method is convenient and useful for comparative ecological studies on the transmissibility of viral diseases in the *Cx. pipiens* complex.

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