Hybridization between sympatric populations of *Culex pipiens pallens* and *Culex pipiens f. molestus* (Diptera: Culicidae) in Nagasaki, Japan

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**Abstract:** The occurrence of natural hybridization between the mosquitoes *Culex pipiens pallens* and *Cx. pipiens f. molestus* in Northwestern Kyushu, Japan, was confirmed based on morphology and molecular methods. In order to assess the morphological difference in male genitalia of the two forms on the basis of the dorsal/ventral (D/V) ratio, mosquito egg rafts were collected in Nagasaki City using oviposition traps. The morphological parameters of male genitalia from *Cx. pipiens f. molestus* were discontinuous with those from *Cx. pipiens pallens*, while intermediate individuals could be found in a single egg raft. These individuals were identified as hybrids using a polymerase chain reaction (PCR)-based assay. The fitness of the family descended from hybrids was lower than that from typical *pallens* and *molestus* families, as measured by egg raft size and egg hatchability. The reduced fitness of the hybrids suggested that the genetic introgression between the two forms could be restricted.

Key words: *Culex pipiens* complex, D/V ratio, acetylcholinesterase gene, egg raft size, egg hatchability, vectorial capacity

**INTRODUCTION**

The *Culex pipiens* complex is distributed worldwide, and its members are important mosquito vectors for human and animal diseases. Among the species complex, *Culex pipiens* s.s. L. and *Culex quinquefasciatus* Say are ubiquitous in temperate regions and in tropical and subtropical regions, respectively. *Culex pipiens pallens* Coquillett is indigenous to northern China, Korea, and the main islands of Japan, and is recognized as a subspecies of *Cx. pipiens* s.s. (Tanaka et al., 1979). *Culex pipiens* form *molestus* Forskal is regarded as a physiological and ecological variant (a biotype) of *Cx. pipiens* s.s. because the two variants are morphologically indistinguishable from each other, but differ dramatically in their physiology and ecology (Harbach, 2012). Immature stages of *Cx. pipiens* s.s. (hereafter called form *pallens*) and *Cx. pipiens* *pallens* (hereafter called form *pallens*) breed in aboveground pools of water, and adults show unautogeny (oviposition requires a blood meal), eurygamous mating, winter diapause, and ornithophilic host preference (Harbach et al., 1985; Mogi, 2012). The form *pallens* showed both ornithophilic and mammalophilic host preference (Sawabe et al., 2010). In contrast, *Cx. pipiens f. molestus* inhabit artificial underground pools and exhibit autogeny (lay a first egg batch without a blood meal), stenogamous mating, non-diapause overwintering, and mammalophilic host preference (Harbach et al., 1984). The distinct behaviors and physiologies in the *Cx. pipiens* complex greatly influence the efficiency with which they transmit disease, known as their vectorial capacity (Farajollahi et al., 2011).

The form *pallens* was the primary vector of lymphatic filariasis in the past (Omori, 1962; Wada, 2011), and remains a common pest in Japan. The form *molestus* was first found in Tokyo in 1943 (Tanaka et al., 1979), and its occurrence in temperate Japan was well-recognized in the 1950s (Ikuzawa, 1955; Omori et al., 1955; Kamura, 1959; Noguchi, 1962; Wada and Ofuji, 1962). This form is suspected to have been accidentally introduced into cities in Japan (Vinogradova, 2000). Consequently, sympatric occurrence of the forms *pallens* and *molestus* is common in temperate Japan. The form *molestus*, which was unintentionally introduced by humans, may hybridize with indigenous populations of the form *pallens*. Hybridization in the *Culex pipiens* complex may influence their vectorial capacity through behavioral changes in the populations (Spielman, 2001; Fonseca et al., 2004; Kilpatrick et al., 2007).

However, there have been few studies on the occurrence of hybrids in the *Culex pipiens* complex in Japan, because of the difficulty in species identification.

To determine whether natural hybridization between
the two forms occurs, we used the morphological trait of male genitalia, in combination with polymerase chain reaction (PCR) assay, to detect hybridization in a natural population. In addition, the fitness of the hybrid strain was determined by assessing the egg raft size and egg hatchability, allowing us to evaluate the possible importance of the hybrids in the genetic introgression of these two forms.

MATERIALS AND METHODS

Study site
This study was performed at the campus of the School of Medicine, Nagasaki University (32°46′22.6″N, 129°52′9.0″E), Nagasaki City, located in Northwestern Kyushu, Japan. The campus is approximately 10.5 ha and is surrounded by a residential area. The climate is temperate with relatively mild winters and moderate rainfall. Annual mean temperature and precipitation from 1981 to 2010 were 17.2°C and 1858 mm/year, respectively (the Japan Meteorological Agency).

Egg collection and larval rearing
Plastic containers (15-L) containing 10 L of water were used as oviposition traps. To attract the gravid females, 10 g of hay was immersed in the water. Four oviposition traps were placed along four different buildings, and one trap was placed under a camphor tree. Egg rafts of the Cx. pipiens complex were collected from oviposition traps from March to December 2007 and brought to the laboratory. Egg rafts were allowed to hatch individually in 200-mL plastic cups containing 100 mL of water. A sample of 30–50 larvae was randomly selected from each egg raft and fed with a 1:1 mixture of powdered rat food (CE-2; CLEA Japan Inc., Tokyo) and dried yeast (Ebios®; Mitsubishi Tanabe Pharma Co., Osaka) in a cup under laboratory conditions. Eggs of Cx. quinquefasciatus were collected from the campus of Ryukyu University, Okinawa Island, Southern Japan.

Morphological measurement
Morphological measurements were performed by mounting the genitalia of the males that emerged from the eggs on a glass slide with mounting medium and observing under a microscope. The distances between the tips of dorsal (D) and ventral (V) arms were measured (Fig. 1). A D/V ratio of less than 1 is considered to be typical of form pallens, and a ratio greater than 1 is considered to be typical of form molestus (Sasa et al., 1967; Makiya, 1972). For better differentiation between the two forms, the maximum width of the sickle blade-shaped part (SB) of the ventral arms was recorded (Fig. 1). The relationship between the D/V ratio and SB value was used for morphological separation among the members of the Cx. pipiens complex (Makiya, 1972). One male randomly selected from each of the 56 egg rafts was observed, except in the case of hybrids, in which 5 males were measured from a single egg raft. Nine males from Cx. quinquefasciatus, collected in Okinawa Island, were also observed to allow comparison of their morphology to those from Cx. pipiens.

PCR identification
The specimens were identified using a multiplex PCR assay based on a polymorphic region in the acetylcholinesterase (Ace) gene, as described by Smith and Fonseca (2004) and Kasai et al. (2008). DNA extraction was performed using a REDExtract-N-Amp™ Tissue PCR kit (SIGMA, Saint Louis, MO, USA). Six legs from individual females were homogenized in a mixture containing an extraction solution (40 µL) and tissue-preparation solution (10 µL) for extracting DNA. The solution was incubated at room temperature for 10 min followed by incubation at 95°C for 3 min. A 40-µL measure of the neutralization solution was added to the sample and mixed by vortexing. The resultant mixture was used directly for PCR using REDExtract-N-Amp™ PCR Reaction Mix (SIGMA). The multiplex PCR amplifications were conducted using primers F1457 (GAG GAG ATG TGG AAT CCC AA) and ACEpal7 (CTC AGT TAG TTC TCA TAT TCA TGC G) for pallens identification, and ACEpip2 (GTGGAA

Fig. 1. Male genitalia of Cx. pipiens pallens (A) and Cx. pipiens f. molestus (B) collected in Nagasaki, Japan. D: distance between dorsal arms, V: distance between ventral arms, SB: the maximum width of the sickle blade-shaped part (SB) of the ventral arms.
ACG CAT GAT ACC AG) and B1246s (TGG AGC CTC TTC AGC) for molestus identification (Fig. 2A). The PCR mixture contained 4 µL of REDExtract-N-Amp ReadyMix, 0.5 µM of each primer, 3 µL of ultrapure water, and 1 µL of the DNA template in a total volume of 10 µL. The PCR reaction mixture was heated to 95°C for 5 min as a preheating step, followed by 40 cycles at 95°C for 30 s, 63°C for 1 min, 70°C for 1 min, and 72°C for 5 min as a final extension. The amplified PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and visualized using a UV transilluminator. This method allows forms pallens and molestus and their hybrids to be distinguished (Fig. 2B).

Reproductive capacity of hybrids

The number of eggs in a single egg batch (i.e., egg raft size) and egg hatchability of families from hybrids (F6 after the collection), typical form pallens (F4), and typical form molestus (F1) were recorded. The larvae and adults from each family were reared at 27 ± 1°C and under 16 L:8 D photoperiod conditions. The families were allowed to lay their autogenic eggs prior to feeding on a blood meal. After taking the blood meal, egg rafts were collected in a 200-mL cup with water and a small amount of hay. The number of eggs in each egg raft was counted on wet cotton under a binocular scope, and then individual egg rafts were placed in 50-mL cups with water to score for hatchability.

Statistical analysis

The number of eggs per egg raft and percentage of egg hatchability were compared using the Tukey-Kramer HSD test for multiple comparisons among families. Egg hatchability was analyzed after arcsine square-root transformation.

RESULTS

Morphological identification

Figure 3 shows the relationship between the D/V ratio and SB value in the male genitalia of the collected Cx. pipiens complex mosquitoes. The forms molestus and pallens could be morphologically differentiated from each other. Male genitalia of forms molestus and pallens were measured from 21 and 35 egg rafts, respectively. Five males from a single egg raft collected on May 30, 2007 showed morphologies intermediate between the two forms. This egg raft was hypothesized to be hybrids...
between the two forms. *Cx. quinquefasciatus* collected in Okinawa Island could also be differentiated from these two forms.

Genetic identification

The result of the multiplex PCR assay, using genomic DNA, is shown in Fig. 2B. The band of 730 bp is common for the *Cx. pipiens* complex. The samples showing bands of either 280 or 500 bp were identified as the form *pallens* or *molestus*, respectively. The sample showing both 280- and 500-bp bands can be recognized as a hybrid. The results of the PCR assay corresponded completely with the morphological identification.

Reproductive capacity of hybrids

The egg raft size of the family descended from the hybrids was significantly smaller than that from *pallens* (*P*<0.01) and almost the same size as those from *molestus* (Fig. 4A). Egg hatchability of the family from the hybrids was significantly lower than the hatchability observed in families from *pallens* and *molestus* (Fig. 4B, *P*<0.01).

**DISCUSSION**

Our study provides morphological evidence for the occurrence of natural hybridization between the *Culex pipiens* complex forms *pallens* and *molestus* in Northwestern Kyushu, Japan. Morphologically intermediate individuals that were observed were suggested to be hybrids of the two forms. This hypothesis was confirmed by the PCR results. Hybrids between forms *pallens* and *molestus* have been detected in Tokyo, Japan, as determined by PCR-based identification, but without morphological studies (Kim et al., 2009).

Morphological studies using the shape of the dorsal and ventral arms of the male genitalia have been conducted to identify the distribution of members of the *Cx. pipiens* complex throughout the world. The geographical distributions of temperate *Cx. pipiens* s.s. and tropical *Cx. quinquefasciatus* overlap across North America. In the overlap zone, the shape of the male genitalia shows intermediate characteristics, indicating that introgression occurs between the populations (Barr, 1957; Urbanelli et al., 1997; Sanogo et al., 2008). In Japan, Sasa et al. (1967) showed that the D/V ratio of the male genitalia has a latitudinal cline from south to north, but without overlap between *Cx. quinquefasciatus* and *Cx. pipiens pallens*. The D/V ratio of the form *molestus* was also discontinuous with form *pallens*, in spite of the overlap of their distribution ranges (Sasa et al., 1967). These studies are consistent with our results. Other studies based on the morphology of male genitalia have concluded that hybridization between forms *pallens* and *molestus* was non-existent (Asahina et al., 1963; Noguchi et al., 1965; Moriya et al., 1967; Yonemoto 1971) or rare (Oda and Ueda, 1979) in natural populations in Japan.

The hybrid rate was not high in our study site (1.8% out of 57 egg rafts investigated), nor was it high in Tokyo (0.1% out of 814 females, Kim et al., 2009). These results suggest that behavioral and/or physiological isolation mechanisms may exist. Consistent with this possibility, pre-mating isolation behavior has been observed between form *molestus* and other members of the *Cx. pipiens* complex in Australia (Miles, 1977a, b). Post-mating isolation has also been observed in crossing experiments among Japanese strains of the *Cx. pipiens* complex (Sasa et al., 1966; Suenaga, 1982). Progeny from crossings between male *pallens* and female *molestus* were compatible, while reciprocal crossings were not compatible (Noguchi et al., 1965; Sasa et al., 1966; Suenaga et al., 1993). Infection of a different strain of the endosymbiont Wolbachia *pipientis* is considered to be responsible for the cytoplasmic incompatibility in the *Cx. pipiens* complex (Barr, 1980; Suenaga, 1982).

The sympatric occurrence of breeding sites may provide a mating opportunity between forms *pallens* and *molestus*, resulting in the occurrence of natural hybrids. The main larval breeding site of the form *molestus* in Japan is artificial underground water pools in human residential areas. The adults of *molestus* lay their first eggs in underground pools without blood feeding, but they later escape from the underground habitat (Oda and Fujita, 1986) to take blood meals from birds and mammals (Sawabe et al., 2010). Thus, the form *molestus* lays its eggs in aboveground pools after blood feeding (Oda and Ueda, 1979). These eggs coexist in the same breeding sites with the larvae of form *pallens* (Asahina et al., 1963; Yonemoto, 1971; Oda and Ueda, 1979), and thus adults of form *molestus* that emerged from aboveground pools could encounter form *pallens*.

Hybridization between members of the *Cx. pipiens* complex has been considered a major factor influencing the transmission of the West Nile virus in North America ( Fonseca et al., 2004). Genetic studies have shown that form *pipiens* with higher genetic ancestry from *molestus* are more likely to feed on humans and mammals ( Kilpatrick et al., 2007; Huang et al., 2009). A similar tendency was also found in Western Europe ( Osorio et al., 2013). Thus, hybridization between the bird-biting form, *pipiens*, and the more mammalian-biting form, *molestus*, is considered to have caused an efficient bridge vector for the transmission of the West Nile virus from birds to humans (Farajollahi et al., 2011).

Although the hybrid colony was maintained for over six generations in this study, the fitness of the family descended from hybrids was lower than that from typical *pallens* and *molestus* families, as measured by egg hatchability. The viable hybrid offspring could lead to introgression of the two forms through backcrossing with one of its parent populations, although the reduced fitness of hybrids restricts the gene flow between them, and thus induces disruptive selection in the two forms.
Further investigations into the genetic introgression of these two forms and the influence it has on feeding habits are needed throughout Japan. This type of study will lead to a better understanding of the vectorial capacity of the Cx. pipiens complex.

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