Molecular Differentiation and Diversity of *Forcipomyia taiwana* (Diptera: Ceratopogonidae) Based on the Mitochondrial Cytochrome Oxidase II Sequence

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**ABSTRACT** *Forcipomyia taiwana* (Shiraki), a biting midge, is one of the most annoying blood-sucking pests in Taiwan. In this study, partial DNA sequences of cytochrome c oxidase II from 113 individuals collected from 11 locations around the island were analyzed to delineate the differentiation pattern and possible dispersal processes of *F. taiwana* in Taiwan. The uncorrected nucleotide divergences, composed of mostly transition substitutions, were high (up to 2.7%) among the samples. Average comparable variations (=0.7%) were found within and between populations. Phylogenetic analysis suggested that several distinct lineages exist and some can be found simultaneously in some populations. A relationship between sequence divergences among populations and their relative geographical distances was observed. Moreover, haplotype diversity was high in all populations, and low to middle levels (*Fst* = 0.004–0.288) of genetic differentiation were found among populations. Linearized calibration from sequence divergences and phylogenetic analysis showed that different ancestral lineages of *F. taiwana* possibly emerged as early as 0.6 million years ago. Taken together, genetic exchanges among these divergently ancestral lineages, likely caused by recent artificial events, have possibly led to the similarly diversified compositions of *F. taiwana* populations all around Taiwan nowadays.

**KEY WORDS** *Forcipomyia taiwana*, cytochrome oxidase II, genetic diversity

The blood-sucking *Forcipomyia taiwana* (Shiraki) midge, locally known as “little black fly” and considered one of the major nuisance insects in Taiwan, has been documented to occur in the plains and foothills on this island (Sun 1961) and southern China (Chen and Tsai 1962). The female adult requires a blood meal from human and possibly domestic animals to produce eggs. Although no midge-born diseases have been reported for humans in Taiwan, minor to severe type I immediate hypersensitivity responses of skin, including intense pruritus and swelling, are observed at biting sites. Welts and lesions may persist several days for some individuals (Chen et al. 2005).

The development of *F. taiwana* from eggs to adults takes \(21–26\) d, and the adults can survive for 26 d on average (Sun 1967, 1974; Chuang et al. 2000). The eggs, usually laid around 3–4 d after the blood meal, hatch within 3 d. *F. taiwana* larvae, feeding mainly on blue-green algae, are often found associated with green mosses in moist habitats around houses, stream banks, and shaded areas around cultivated fields in the vicinity of human communities (Liu et al. 1964, Chen et al. 1979, Lien et al. 1988). Various laboratory colonization methods using mixture of soil and nutrients, such as yeast extracts and blue-green algae, have been reported (Sun 1967, 1974; Lien et al. 1988, Yeh and Chuang 1996).

Adult midges, ranging from 1.4 to 1.5 mm in body length, are active from late morning until early afternoon (Chen et al. 1981). The population sizes of *F. taiwana* fluctuate annually and peak around July in the summer (Chen et al. 1982, Lee and Hou 1997, Chuang et al. 2000). The morphological characters at different developmental stages of *F. taiwana* are similar among populations, with some minor differences including the number of thoracic and abdominal setae in larvae as well as the number of metatibial and antennal setae in adults (Chen and Tsai 1962, Sun et al. 1971, Chen et al. 1982).

*F. taiwana*, distributed at altitudes <1,000 m in midwestern and eastern Taiwan, was rarely recognized in southern and northern Taiwan by 1979 (Chen et al. 1979). However, it has been recorded all around the island since 1999 (Chuang et al. 2000). Factors possibly contributing to its island-wide spread include changes in agricultural practices since the 1990s in Taiwan. An expansion of tea, bamboo, and betel nut cultivations closer to human communities in the lowlands has probably created more habitats for larval midges. The growing trend of organic gardening and increased areas of abandoned farmlands are addi-
tional possible factors. Meanwhile, various chemical control measures have not been able to bring the midge populations down significantly (Lee and Hou 1997).

The tectonic formations of the Central Mountain Range (CMR) and, to a lesser extent, the other mountains were potentially the isolating forces to interrupt gene flow of many insects in Taiwan. Low genetic variations have been detected among blowfly (Diptera: Calliphoridae) populations in Taiwan (Chen and Shih 2003, Chen et al. 2004); however, different genetic patterns were reported for Aedes aegypti populations (Su et al. 2003). Because the dispersal ability of F. taiwana is low by itself, the CMR, >3,000 m, should pose a major barrier to the expansion of F. taiwana in Taiwan. However, relocation of F. taiwana populations could easily be facilitated by various human activities on this heavily populated island.

The goals of this study were to address the genetic composition of F. taiwana populations and to elucidate whether any relationship exists between genetic and geographic distance of these populations. DNA sequences of maternally inherited mitochondrial DNA (mtDNA), such as the mt cytochrome oxidase genes, have served widely as markers to help differentiate closely related taxa and populations (Hebert et al. 2004, Ward et al. 2005, Lee et al. 2008). In this study, partial sequences of the cytochrome oxidase II (COII) gene of F. taiwana collected at 11 different locations around Taiwan and two individuals from China were determined and analyzed to help delineate its differentiation pattern and to tentatively reconstruct its dispersal processes in Taiwan.

Materials and Methods

Sample Collection. The distribution of F. taiwana correlates well with that of humans on this island, except that this biting midge has rarely been observed in the southern-most and southeastern areas. Specimens were collected from various locations around the Taiwan Island (Fig. 1). Two F. taiwana specimens were collected in the Yunnan Province, China. In addition, two individuals of Forcipomyia species characteristics were collected in Ilan and re-
main to be classified by conventional taxonomic methods.

**DNA Extraction.** Specimens were preserved in 95% ethanol. DNA extraction of individual specimen was carried out using the Genomic DNA Mini Kit (Geneaid, Sijhih City, Taiwan) following the manufacturer’s protocol. Briefly, the specimen was homogenized in 200 μl of CT buffer. After 20 μl of proteinase K (10 mg/ml) was added, the samples were incubated at 70°C for 20 min. Subsequently, the reaction was mixed with 200 μl of ethanol and applied to a GD column. After the column was washed twice with GB buffer, DNA was eluted with elution buffer preheated to 70°C.

**Polymerase Chain Reaction and DNA Sequencing.** Primers TL-J3033 (5’-TAATGCACTGACTATTGAA-3’) and C2-N3655 (5’-CCACAAATTTCTGAA-CATTG-3’) were used in the amplification of ceratopogonid DNA (Beckenbach and Borkent 2003) to amplify a DNA fragment containing partial sequences of COII and its 5’-flanking tRNAAsu genes of *F. taiwana* by polymerase chain reaction (PCR). The 20-μl PCR reaction solution contained 2 μl of genomic DNA extraction, 1× IQZyme buffer, 5 nmol of dNTP, 0.01 nmol of each primer, and 1 U of IQZyme DNA polymerase (Farming Intelligene, Taichung City, Taiwan). Cycling parameters were 35 cycles of denaturation at 94°C for 30 s, annealing at 49°C for 30 s, and elongation at 72°C for 1 min, followed by 7-min incubation at 72°C. After agarose gel electrophoresis analysis, the PCR products were purified from the gel using the GENECLEAN III kit (Q-BIOgene, Morgan Irvine, CA) before DNA sequencing. DNA products were sequenced in both directions using the Taq Dye Terminator Cycle Sequencing Kit (Perkim Elmer Applied Biosystems, Foster City, CA) with a model 377 A DNA sequencer (Perkim Elmer Applied Biosystems).

**Sequence Analysis.** Sequences of *F. taiwana* specimens were piled-up using the BioEdit program (Hall 1999) and aligned with three outgroup sequences using the AlignX program in the Vector NTI Software Package (Invitrogen, Carlsbad, CA), followed by manual refinement. To approximate the absolute age of divergence among distinct lineages, the linearized tree was compiled using the MEGA version 4 program (Tamura et al. 2007) under the assumption of evolutionary rate constancy. Haplotype diversity (*Hd*), nucleotide diversity (*π*), and *F*$_{st}$ values were calculated using DnaSP 3.5 (Rozas et al. 2003).

**Relationship Analysis.** Statistical significance of interpopulation differentiation and of the relative relationship between nucleotide divergence and geographical distance was evaluated using the F-test (Excel; Microsoft, Redmond, WA). After sequence variation estimation, the neighbor-joining (NJ) and unweighted pair-group method with arithmetic average methods were used in phylogeny reconstruction. NJ analysis was carried out by means of MEGA version 4 using the proportional estimate. Bootstrap analysis of 1,000 resampling replicates was carried out on the phylogenetic tree inferred from the NJ method. To deduce population relationship, average nucleotide differences among populations were used to generate the NJ tree. The minimum spanning network was analyzed by Program MINSNENET (Excoffier and Smouse 1994) on the basis of the output differences among haplotypes and plotted manually using PowerPoint software (Microsoft).

**Results**

The PCR primers described for members of family Ceratopogonidae (Beckenbach and Borkent 2003) were able to amplify a DNA fragment of 667 bp from *F. taiwana*. After alignment, amplified PCR products of 607 bp were analyzed. DNA sequences obtained from 113 *F. taiwana* individuals have been deposited in the NCBI database (accession numbers EU408705–EU408769, FJ184985–FJ184987). The compiled consenus DNA sequence showed 22–23% variation from the same region of its congeneric species, *F. cilipes* (accession number AF547673). Furthermore, significant levels of divergence (20–21%) were also found in this region between *F. taiwana* and the two individuals (FJ184986–FJ184987) of different *Forcipomyia* species collected in Ilan. These three individuals were therefore used as outgroups for the phylogenetic analysis.

**Composition and Variation of *F. taiwana* COII Sequences.** Within the 607-bp region, the average nucleotide composition among the 113 *F. taiwana* individuals was 11.6, 33.1, 40.7, and 14.6% for G, A, T, and C, respectively. The observed bias toward adenine and thymine is consistent with that of the corresponding COII DNA region of other ceratopogonids (Beckenbach and Borkent 2003) and dipteran flies (Roe and Sperling 2007). Furthermore, a total of 52 bases (8%) were found variable in this region among the samples (Fig. 2). Up to 16 interindividual variations were observed in general, whereas 1–3 intraindividual variations were found within 10 individuals (Fig. 2). Variations were not randomly distributed over the examined DNA region, with the majority being transition substitutions. Most of the nucleotide substitutions (75%) occurred at the third position of the codon, whereas 20 and 5% of the substitutions were found at the first and second position, respectively. The uncorrected nucleotide divergences among these individuals were from 0 to 2.7% (0.9% in average). Significant sequence divergences, ranging from 0.5 (between Taipei and Miaoli) to 1.4% (between Nan-tou and Hualien-Fonlin), existed among these populations ($F = 8.87; df = 10,519; P < 0.0001$). Furthermore, a relationship between the sequence divergences among individuals of different populations and their relative geographical distances was found ($F = 17.2; df = 1,4799; P < 0.0001$), although the relative coefficient (0.0011) was low (Fig. 3).

**Genetic Diversity and Differentiation Among Populations of *F. taiwana*.** Among the 43 haplotypes (determined by excluding the terminal gaps) found in the 113 *F. taiwana* individuals from 11 locations, most haplotypes consisted of only 1 individual and the 4
most common haplotypes (namely 1, 3, 6, 27) of ~10 individuals (Fig. 2). In other words, haplotype diversity was high (0.6–1) in all populations, with the greatest found in the Ilan sample (Table 1). Nucleotide diversity in each population ranged from 0.00134 to 0.00586, with the greatest nucleotide diversity occurring in the Sinju population (Table 1). Furthermore, low to middle levels \((F_{st} = 0.00 - 0.29)\) of genetic differentiation were found among the populations (Table 2). Negative \(F_{st}\) values were found in some cases. The greatest differentiation was found in Tainan where the \(F_{st}\) values against the other populations were from 0.14 to 0.29.

**Relationship Analysis of *F. taiwana*.** A minimum spanning network was used in our attempt to infer the colonization processes of *F. taiwana* in Taiwan. The 40 spanning groups separated by many segregating sites included three major groups (I, II, and III) branched out to the major interior and exterior haplotypes (Fig. 4). Groups I, II, and III had 20, 12, and 11 individuals, respectively, whereas several groups had only 1 individual. The three major groups include individuals mostly from northern, midwestern, and eastern Taiwan. The spanning group compositions in the network diagram also indicated that sequences within each population were highly diversified.

Phylogenetic construction derived from NJ analysis showed that the 113 *F. taiwana* individuals can be divided into several distinct lineages which do not correlate significantly with their geographical distribution (Fig. 5). The three major lineages (A, B, and C) contained individuals from all locations except those from midwestern, southern, and northern Taiwan, respectively.

<table>
<thead>
<tr>
<th>Location</th>
<th>No. individuals</th>
<th>Haplotype diversity ((h))</th>
<th>Nucleotide diversity ((\pi))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP</td>
<td>10</td>
<td>0.644</td>
<td>0.00148</td>
</tr>
<tr>
<td>SJ</td>
<td>10</td>
<td>0.911</td>
<td>0.000856</td>
</tr>
<tr>
<td>ML</td>
<td>9</td>
<td>0.417</td>
<td>0.000314</td>
</tr>
<tr>
<td>TC</td>
<td>11</td>
<td>0.555</td>
<td>0.00038</td>
</tr>
<tr>
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<td>0.000345</td>
</tr>
<tr>
<td>TN</td>
<td>9</td>
<td>0.667</td>
<td>0.000537</td>
</tr>
<tr>
<td>KS</td>
<td>14</td>
<td>0.502</td>
<td>0.000429</td>
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<tr>
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</tr>
<tr>
<td>IL</td>
<td>7</td>
<td>1</td>
<td>0.000479</td>
</tr>
</tbody>
</table>

*Abbreviations for these locations are described in Fig. 1.*

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**Fig. 2.** Haplotypes and the variable positions within the aligned COII sequences of *F. taiwana*. Several *F. taiwana* were found to contain heterogeneous mtDNA with different bases at certain nucleotide positions in the region analyzed. The sequence of TP10 individual is shown, and the number above each nucleotide indicates the position of the nucleotide. Dots indicate identical bases as the ones of TP10 and the individuals of each haplotype were shown in the right. See Fig. 1 for location abbreviations. Y, T and C, R, A and G, and S, G and C nucleotide.

**Fig. 3.** Distance plot of pairwise comparison based on sequence divergences between individuals from different populations and their relative geographical distance of *F. taiwana* samples collected from populations in western Taiwan and Hualien County.
spectively. Average nucleotide differences among populations (Table 2) were used to construct an NJ tree. The cluster with the least divergence group was found among the Taipei, Miaoli, and Nantou samples (Fig. 6). The relationship among the remaining populations that showed similar divergence in COII remains to be elucidated.

### Discussion

Studies of the genetic differentiation and insect demography in Taiwan have shown low or no genetic variations between/within populations of some species, including aphid (Hemiptera: Aphididae) (Yeh et al. 2005, 2006), whitefly (Bemisia argentifolii) (Wang et al. 2004a), blowfly (Diptera: Calliphoridae) (Chen and Shih 2003, Chen et al. 2004), rice weevil (Coleoptera: Curculionidae) (Peng et al. 2003) and cricket (Loxoblemmus appendicularis) (Yeh et al. 2004). However, for crickets, which characteristically often occupy particular geographical regions in Taiwan, genetic differentiation is high between populations separated by long distances but low between neighboring populations and within the same population (Yeh et al. 2004). Nevertheless, high genetic diversity has been found between/within populations for certain species on the island. For example, different random amplified polymorphic DNA (RAPD) patterns were reported for individuals of Aedes aegypti (Su et al. 2003), and two distinct 16S rDNA lineages were found in pear psyllid (Cacopsylla chinensis) (Lee et al. 2007, 2008). These genetic variations were spec-
Fig. 5. Phylogenetic relationship of *F. taiwana* individuals on the basis of COII sequences using the NJ method. Bootstrap values >80 are shown beneath the branches. Major lineages were marked. See Fig. 1 for abbreviations.
activities started to increase significantly. However, this hypothesis remains to be tested. Some negative $F_{st}$ values (Table 2) observed in this study might have originated from the occurrence of numerous low-frequency haplotypes. An alternative cause is sampling bias resulted from human activities, such as relocation of individuals through various forms of transportation and selection by the application of chemicals.

This study has provided preliminary information on the genetic differentiation and composition of *F. taiwana* in Taiwan, based on partial sequences of the mt COII and tRNA^Leu* genes. Sequence analysis of additional specific nuclear markers, such as the intergenic spacer 2, of samples collected in Taiwan, as well as the Chinese continent, is underway to gain further insight into the colonization, dispersal history, and hybridization of *F. taiwana* populations.

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**References Cited**


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