MORPHOLOGY, SYSTEMATICS, EVOLUTION

Cytogenetic and Molecular Evidence of Additional Cryptic Diversity in High Elevation Black fly *Simulium feuerborni* (Diptera: Simuliidae) Populations in Southeast Asia

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**ABSTRACT** *Simulium feuerborni* Edwards is geographically widespread in Southeast Asia. Previous cytogenetic study in Thailand revealed that this species is a species complex composed of two cytoforms (A and B). In this study, we cytologically examined specimens obtained from the Cameron Highlands, Malaysia, and Puncak, Java, Indonesia. The results revealed two additional cytoforms (C and D) of *S. feuerborni*. Specimens from Malaysia represent cytoform C, differentiated from other cytoforms by a fixed chromosome inversion on the long arm of chromosome III (IIIIL-5). High frequencies of the B chromosome (33–83%) were also observed in this cytoform. Specimens from Indonesia represent the cytoform D. This cytoform is differentiated from others by a fixed chromosome inversion difference on the long arm of chromosome II (IIIL-4). Mitochondrial DNA sequences support genetic differentiation among cytoforms A, B, and C. The pairwise $F_{ST}$ values among these cytoforms were highly significantly consistent with the divergent lineages of the cytoforms in a median-joining haplotype network. However, a lack of the sympatric populations prevented us from testing the species status of the cytoforms.

**KEY WORDS** polytene chromosome, Simuliidae, *Simulium*, species complex

Black flies (Diptera: Simuliidae) are significant blood-sucking insects. The adult females of many species are important pests and vectors of disease, such as human onchocerciasis or river blindness. This disease was classified as the second placed cause of infectious blindness (Adler et al. 2010). Black fly biting can reduce poultry and cattle productivity (Crosskey 1990, Adler et al. 2004). On the other hand, the larval stage of the black fly plays an important role in nutrient turnover in stream ecosystems (Malmqvist et al. 2004). Therefore, taxonomic knowledge about black flies is crucial for understanding all aspects of their biology.

Banding patterns of the salivary gland polytene chromosomes have an indispensable role in black fly taxonomy and systematics (Rothfels 1979, Adler et al. 2010). Cytological differentiation of the morphologically inseparable species is based on the following three criteria: 1) fixed chromosome inversion differences; 2) sex chromosome differences; and 3) different floating inversions (Rothfels 1956). Cytogenetic studies have often revealed cryptic diversity in many black fly species (e.g., Tangkawanit et al. 2009, Pramual and Wongpakam 2011, Pramual and Kuvangkadilok 2012). Despite a highly successful use of banding patterns in the polytene chromosome for black fly taxonomy and systematics, recent studies have also pointed out the necessity of using an integrated approach based on multiple characters (Ilmonen et al. 2009, Adler and Huang 2011, Pramual and Kuvangkadilok 2012, Diaz et al. 2015). Therefore, in addition to the cytogenetic level, information from molecular genetics is also requiring to fully understand black fly biodiversity.

*Simulium feuerborni* Edwards is geographically widespread, being recorded from Thailand, Malaysia, and Indonesia (Adler and Crosskey 2014). However, this species is ecologically restricted to high elevation streams (>700 m above sea level), which are usually small and slow flowing (Pramual and Wongpakam 2013). A previous cytogenetic and molecular population genetic study in Thailand revealed that this species is a species complex composed of two cytoforms (A and B) differentiated by several fixed chromosome inversion differences. The two cytoforms also show high levels of genetic differentiation based on mitochondrial cytochrome c oxidase I (COI) sequences (Pramual and Wongpakam 2013). In this study, we further examine the genetic diversity of *S. feuerborni* from two additional countries, namely, Malaysia and Indonesia.
Polystene chromosome banding sequences and the mitochondri al DNA COI gene were compared with those previously reported from Thailand.

**Materials and Methods**

**Sample Collection and Species Identification.** Black fly samples were collected from six collections in two countries, namely, Puncak, Java (one collection), Indonesia and the Cameron Highlands (five collections), Malaysia (Table 1). Larvae were preserved in Carnoy’s solution (3:1, 95% ethanol: glacial acetic acid) for cytogenetic and molecular genetic studies (Pramual et al. 2011). Species were identified morphologically using the most recent keys of the *Simulium* (Nevermannia) *feuerborni* species group (Takaoka and Srisuka 2011).

**Cytogenetic Study.** Salivary gland polystene chromosomes were prepared from penultimate-instar larvae. The abdominal segment of the larvae was cut transversely. The anterior portion of the larval body was placed in 80% ethanol for further molecular works. The abdominal segment that contained the salivary glands was slit ventrally and then stained using the Feulgen method of Rothfels and Dunbar (1953). Chromosomes were prepared from penultimate-instar lar-vae. The abdominal segment of the larvae was cut into the anterior portion of the larva following a procedure described previously (Pramual et al. 2011). Name of the chromosome inversions were numbered in order of their discovery beginning after the inversion reported by Pramual and Wongpakam (2013).

**DNA Extraction, Polymerase Chain Reaction (PCR), and Sequencing.** DNA was extracted from the anterior portion of the larva following a procedure described previously (Pramual et al. 2011). The barcoding region of the COI gene was amplified using the primers LCO1490 (5’-GCTCAACAATCATATTGAATAAGTTGG-3’) and HCO2198 (5’-TAAATCAGGGTGACAAAAATAT-3’; Folmer et al. 1994). PCR was performed in a total volume of 50 μl containing 5 μl of 10× reaction buffer, 3 μl of MgCl₂ (50 mM), 1.6 μl of dNTPs (10 mM each primer (10 μM), and 0.4 μl of Taq DNA polymerase (5 U/μl). Temperature profile was as follow: 96°C for 1 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 7 min (Rivera and Currie 2009). PCR products were checked on a 1% agarose gel containing 0.5 μg/ml of ethidium bromide and cleaned using the HiYield Gel/PCR fragment extraction kit (RBC Bioscience, Xindian City, Taiwan). Cleaned PCR products were sequenced by the Macrogen sequencing service (Seoul, Korea) using the same primers as in the PCR.

**Data Analysis.** Mitochondrial COI sequences were deposited in GenBank under accession numbers KP770123–KP770132. Haplotype diversity and nucleotide diversity were calculated in Arlequin v.3.5 (Excoffier and Lischer 2010). Genetic differentiations among cytotypes were estimated by population pairwise FST in Arlequin. Significant test statistics were obtained from 1,000 permutations. A median-joining (MJ) haplotype network (Bandelt et al. 1999) was calculated using Network v.4.6.1.0 (http://www.fluxus-engineering.com). The “best match” and “best close match” methods in the program TaxonDNA (Meier et al. 2006) were used to test the frequency of successful identification of the specimens into cytotypes.

**Results**

**Chromosome Polymorphisms.** In total, 158 lar-vae of *S. feuerborni* from three locations, two from the Cameron Highlands, Malaysia, and one from Puncak, Java, Indonesia, were cytologically examined. Overall, we found a low frequency of polymorphic chromosome inversion. There was only a single floating inversion (IS-4; Fig. 1) with relatively low frequency (6%) from the Cameron Highlands site (Table 2). However, a high frequency of the B chromosome was found in this loca-tion (Table 2). This B chromosome is a small metacentric (Fig 2A) where the location of the centromere was determined by the ectopic pairing to the A complement centromere (Brockhouse et al. 1989) (Fig. 2B). One arm of the B chromosome consists of fluffy end and another arm consists of two heavy bands near the centromere and one lighter band near the end (Fig 2A). No chromosome inversion polymorphism and

<table>
<thead>
<tr>
<th>Location</th>
<th>Code</th>
<th>Collection date</th>
<th>Latitude/longitude</th>
<th>Altitude (m)</th>
<th>Stream width (m)</th>
<th>Stream depth (m)</th>
<th>Water velocity (m/s)</th>
<th>pH</th>
<th>Dissolved oxygen (mg/L)</th>
<th>Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brinchang, Cameron Highlands, Malaysia</td>
<td>CH15-1</td>
<td>26 Dec. 2012</td>
<td>04° 31.461’ N, 101° 23.338’ E</td>
<td>1,813</td>
<td>0.75</td>
<td>0.1</td>
<td>0.25</td>
<td>7.36</td>
<td>5.5</td>
<td>15.9</td>
</tr>
<tr>
<td></td>
<td>CH15-2</td>
<td>21 Mar. 2013</td>
<td>04° 31.461’ N, 101° 23.338’ E</td>
<td>1,813</td>
<td>0.2</td>
<td>0.05</td>
<td>0.22</td>
<td>6.69</td>
<td>2.4</td>
<td>15.4</td>
</tr>
<tr>
<td>Tanah Rata, Cameron Highlands, Malaysia</td>
<td>CH11-1</td>
<td>24 Nov. 2012</td>
<td>04° 28.738’ N, 101° 22.979’ E</td>
<td>1,405</td>
<td>0.03</td>
<td>0.22</td>
<td>0.54</td>
<td>6.81</td>
<td>7.4</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>CH11-2</td>
<td>27 Dec. 2012</td>
<td>04° 28.738’ N, 101° 22.979’ E</td>
<td>1,405</td>
<td>0.03</td>
<td>0.22</td>
<td>0.54</td>
<td>6.81</td>
<td>7.4</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>CH11-3</td>
<td>25 Dec. 2013</td>
<td>04° 28.738’ N, 101° 22.979’ E</td>
<td>1,405</td>
<td>0.03</td>
<td>0.24</td>
<td>0.41</td>
<td>6.60</td>
<td>3.59</td>
<td>17.0</td>
</tr>
<tr>
<td>Puncak, Java, Indonesia</td>
<td>PUN</td>
<td>3 Apr. 2012</td>
<td>NA</td>
<td>1,200</td>
<td>0.30–0.40</td>
<td>0.20</td>
<td>NA</td>
<td>7.00</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
Fig. 1. Chromosome IS of *S. feuerborni* cytoform C from Cameron Highlands, Malaysia. Breakpoints of the IS-4 floating inversions are indicated with brackets. C, centromere; NO, nucleolar organizer.

Table 2. Chromosome characteristics of *S. feuerborni* in Malaysia and Indonesia compared with the standard polytene chromosome maps of this species from Thailand of Pramual and Wongpakam (2013)

<table>
<thead>
<tr>
<th>Code</th>
<th>Country</th>
<th>No. of specimens (male: female)</th>
<th>Chromosome inversion</th>
<th>B chromosome</th>
<th>Cytoform assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH15-1</td>
<td>Malaysia</td>
<td>6:14</td>
<td>IS-4 (6%) IIIL-5'</td>
<td>70%</td>
<td>C</td>
</tr>
<tr>
<td>CH15-2</td>
<td>Malaysia</td>
<td>20:22</td>
<td>IIIL-5'</td>
<td>62%</td>
<td>C</td>
</tr>
<tr>
<td>CH11-1</td>
<td>Malaysia</td>
<td>3:9</td>
<td>IIIL-5'</td>
<td>33%</td>
<td>C</td>
</tr>
<tr>
<td>CH11-2</td>
<td>Malaysia</td>
<td>5:7</td>
<td>IIIL-5'</td>
<td>83%</td>
<td>C</td>
</tr>
<tr>
<td>CH11-3</td>
<td>Malaysia</td>
<td>5:6</td>
<td>IIIL-5'</td>
<td>64%</td>
<td>C</td>
</tr>
<tr>
<td>PUN</td>
<td>Indonesia</td>
<td>24:37</td>
<td>IIIL-4*</td>
<td>–</td>
<td>D</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>63:95</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Fixed inversion.

Fig. 2. The metacentric B chromosome of *S. feuerborni*. (A) B chromosome of the larva of *S. feuerborni* from Cameron Highlands, Malaysia, showing the location of centromere (C) and fluffy end (F). (B) Ectopic pairing of the B chromosome with chromosome II.
B chromosome were recorded from Puncak, Java, Indonesia.

Comparisons of the polytene chromosome banding patterns between Malaysian and Indonesian populations with the standard polytene chromosome map of this species from Thailand (Pramual and Wongpakam 2013) revealed two additional cytotypes.

**S. feuerborni** Cytoform C. Cytoform C was characterized by a fixed chromosome inversion on the long arm of chromosome III (III-L-5), compared with the standard polytene chromosome-banding sequences (Fig. 3), and undifferentiated sex chromosome. Larvae (n = 97) were examined from two populations in the Cameron Highlands (CH11 and CH15), Malaysia (Table 2). In addition, these populations also possessed a high frequency of the B chromosome (Table 2), which ranged between 33 and 83% in each collection.

**S. feuerborni** Cytoform D. Cytoform D was characterized by a fixed chromosome inversion on the long arm of chromosome II (II-L-4) (Fig. 4) and undifferentiated sex chromosome. This inversion has break points very close to the III-2 inversion previously reported in cytoform B (Pramual and Wongpakam 2013). Larvae (n = 61) were examined from a population from Puncak, Java, (PUN) Indonesia (Table 2). No floating inversion or B chromosome was found in this population.

**Mitochondrial DNA Sequence Variation and Genetic Differentiation Among Cytotypes.** Ten specimens from the Cameron Highlands, Malaysia, were sequences for a 586-bp fragment of the COI gene. The PCR with the specimens from Indonesia was unsuccessful, despite various PCR conditions being tried. In total, 69 sequences (10 from Malaysia and 59 from Thailand) were included in the data analysis.

Nucleotide substitutions were detected in 72 positions (Fig. 5). No deletions or insertions were found.

Genetic diversity at the molecular level based on the COI gene revealed lower diversity in the *S. feuerborni* population from Malaysia compared with those from Thailand (Table 3). Seven haplotypes were recognized from 10 specimens with a nucleotide diversity of 0.008 and a haplotype diversity of 0.869 (Table 3).

Mitochondrial DNA genealogy between sequences of *S. feuerborni* revealed that 10 specimens from Malaysia that represent cytoform C were clustered...
together and well separated from the other cytoforms (Fig. 6). Population pairwise \( F_{ST} \) indicated a high level of genetic differentiation among the cytoforms (Table 4). This was supported by the analysis of the DNA barcode using the best match and best close match methods, which revealed that all specimens were perfectly assigned into cytoforms.

**Discussion**

Black fly species that are geographically widespread and capable of using diverse habitats are likely to be a species complex (Adler and McCreadie 1997). *S. feuerborni* is ecologically restricted into high-elevation habitats. However, this species is geographically widespread being recorded from Thailand, Malaysia, and Indonesia (Adler and Crosskey 2014). Previous cytogenetic study in Thailand revealed that this species is a species complex composed of two cytoforms differentiated by several fixed chromosome inversion differences. This species also possesses high levels of intraspecific genetic divergence based on the COI barcoding sequences (Pramual and Wongpakam 2013, Pramual and Adler 2014).

Our results revealed two additional cytoforms (C and D) of *S. feuerborni*. Cytoform C was found in the Cameron Highlands, Malaysia. This cytoform was separated from the other by a fixed chromosome inversion on the long arm of chromosome III (III-5). In addition, this cytoform was also characterized by a high frequency of the B chromosome. The B chromosome was found in each collection from the Cameron Highlands population ranging from 33 to 83%. A high frequency of the B chromosome in black fly populations is usually found in the temperate and arctic species (Procunier 1975, Brockhouse et al. 1989, Adler et al. 2010). B chromosomes have been reported in tropical black fly species such as *Simulium tani* Takaoka & Davies (Tangkawanit et al. 2009) and *Simulium nakhonense* Takaoka & Suzuki (Kuvangkadilok et al. 1999) but the frequencies are very low (0.5 and 7%, respectively). The high frequency of the B chromosome in *S. feuerborni* cytoform C, therefore, is a unique character of the tropical black fly species. As *S. feuerborni* cytoform C occurs in high elevation habitats (>1,400 m above sea level) where the climatic condition resembles that of the temperate region, it is likely that the climatic condition could play a role in the occurrence of the B chromosome in black flies. There are several possible explanations for the presence of the B chromosome in the populations (Camacho et al. 2000), but occurrence at a high frequency suggested that it might have a positive effect. The role of the B chromosome has not been fully determined in black fly, but there are some relationships between the presence of the B chromosome and biological activities. For example, Kachvoryan et al. (1996) found that the frequencies of the

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**Table 3 Genetic diversity indices of the cytoforms of *S. feuerborni* based on mitochondrial COI sequences**

<table>
<thead>
<tr>
<th>Cytoform</th>
<th>( n )</th>
<th>Haplotype diversity</th>
<th>Nucleotide diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>30*</td>
<td>0.869 (±0.059)</td>
<td>0.008 (±0.005)</td>
</tr>
<tr>
<td>B</td>
<td>29*</td>
<td>0.579 (±0.110)</td>
<td>0.015 (±0.008)</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>0.911 (±0.006)</td>
<td>0.007 (±0.004)</td>
</tr>
</tbody>
</table>

* Data from Pramual and Wongpakam (2013)

---

*Fig. 5.* Variable positions in 586 bp of the COI gene for the 36 haplotypes of *S. feuerborni* cytoforms A, B, and C.
B chromosome in Cnetha zakhariensis Rubzov and Tetisimulium condici were related to water pollution. Procunier (1982) found that the presence of the B chromosome in Cnephia dacotensis (Dyar and Shannon) increased rRNA production efficiency that resulted in faster development of the larva. S. feuerborni cytoform D was found in Puncak, West Java, Indonesia. Because S. feuerborni cytoform D was collected from the location ~800 km far from the type locality of East Java (Edwards 1934), thus we cannot conclude that this cytoform could represent the typical S. feuerborni. S. feuerborni cytoform D was characterized by a fixed chromosome inversion on the long arm of chromosome II (IIIL-4). Break points in this inversion were very close to those previously reported in cytoform B from Thailand (Pramual and Wongpakam 2013). No B chromosome was found in this cytoform.

Ecological comparisons among the larval habitats indicated that cytoforms of S. feuerborni seem to occur under different ecological conditions. S. feuerborni cytoform C was found at a relatively higher elevation (1,405–1,813 m) than the other cytoforms (cytoform A, 1,185–1,615 m; cytoform B, 950–1,320 m [Pramual and Wongpakam 2013]; cytoform D, 1,200 m). In addition, S. feuerborni cytoform C was also found in more acidic water (pH 6.60–7.36) than the other cytoforms (pH >7.00). S. feuerborni cytoforms C and D were found in deeper streams (0.05–0.24 m) compared with cytoform A and B (<0.07 m) (Pramual and Wongpakam 2013). Our results agree with several previous studies finding that different cytoforms of black flies are associated with different stream ecologies (Adler and McCreadie 1997, Kuvangkadilok et al. 2008, Tangkawarnit et al. 2009, Pramual and Kuvangkadilok 2012).

The high level of the population pairwise $F_{ST}$ and the clear separation of the haplotype in the MJ network suggest that there is a long historical isolation of these cytoforms (Avise 2000). The high level of genetic diversity among cytoforms may be a result of the genetic drift due to the colonization of new habitats. Further studies are needed to investigate the genetic basis of these differences and to understand how these differences have evolved over time.

### Table 4. Population pairwise $F_{ST}$ between cytoforms of S. feuerborni based on mitochondrial COI sequences

<table>
<thead>
<tr>
<th>Cytoform</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.684*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.684*</td>
<td>0.760*</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>0.760*</td>
<td>0.696*</td>
</tr>
</tbody>
</table>

* $P < 0.001$.  

**Fig. 6.** MJ network of the 69 COI sequences of S. feuerborni from six populations from Thailand, one population from Malaysia, and one population from Indonesia. Circles represent haplotypes and the sizes are relative to the number of individuals sharing the specific haplotype.
differentiation among cytoforms found in this study supports a previous finding (Pramual and Wongpakam 2013). We also found that the COI barcode could effectively differentiate the cytoforms of S. feuerborni. Therefore, the COI barcoding sequences could be used to identify cytoforms of S. feuerborni. This could be applied to the other life stages (e.g., pupa and adult), in which polytene chromosome study cannot.

In conclusion, our study revealed additional diversity in S. feuerborni, one of high elevation black flies in tropical Asia. Molecular data based on the COI barcoding sequences further support the cytoform differentiation. As this species occupies high elevation habitats, which were previously suggested as being refugium sites for black flies during the dry conditions of the Pleistocene glaciation (Pramual et al. 2005), phylogeographic study of this species would be interesting to examine the effect of the Pleistocene climatic change on high elevation faunas in this biodiversity hotspot region. In addition, our integrated method enables us to obtain information from both cytogenetic and molecular levels. Therefore, this method could be applied to the onchocerciasis vectors that are known to be cytologically complex. Because limitation of cytogeography of the onchocerciasis vectors could be applied to the other life stages (e.g., pupa and adult), in which polytene chromosome study cannot.

Acknowledgments

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