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Polytene chromosomes of *Simulium arakawae*, a pest species in the *Simulium venustum* group (Diptera: Simuliidae) from Japan

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Abstract. The chromosomal relationships of *Simulium arakawae*, a black fly of medical-veterinary importance, are resolved relative to other members of the *Simulium venustum* species group and to the standard reference map for the subgenus *Simulium*. *Simulium arakawae* differs from the subgeneric standard by eight fixed inversions, apparently none of which are shared with any of the other 17 chromosomally studied nominal members of the group. The chromosomal features that indicate group membership might have been lost in *S. arakawae*, particularly if they were polymorphic in an ancestor.

INTRODUCTION

The *Simulium venustum* group consists of 36 nominal species and is distributed throughout the Holarctic Region (Adler & Crosskey, 2011). It includes species of medical and veterinary significance in both the Old and New Worlds. *Simulium arakawae*, for instance, is an important biting pest of humans in Japan. It also is a vector of *Onchocerca lienalis* and *Onchocerca* sp. in cattle (Takaoka, 1994), as well as an experimental vector of *Onchocerca dewittei japonica*, a parasite of wild boar and a causative agent of zoonotic onchocerciasis in southern Japan (Fukuda *et al*., 2008).

The constituent species are readily assigned to the *S. venustum* group, based on a combination of morphological characters such as a negative larval head-spot pattern, 6–8 pupal gill filaments arranged in pairs, and a slipper-shaped cocoon without lateral apertures (Adler *et al*., 2004). The polytene chromosomes of 18 of the nominal species have been studied (Rothfels *et al*., 1978; Hadi *et al*., 1996; Adler & Mason, 1997; Adler *et al*., 1999, 2004; Chubareva & Petrova, 2008). Of the three members of the *S. venustum* group in Japan – *Simulium arakawae* Matsumura, 1915, *Simulium nipponense* Shiraki, 1935, and *Simulium tobetsuense* Ono, 1977 – only *S. arakawae* has been examined chromosomally. Its banding sequence was published and two samples were screened for sibling species, based on material from Yufu (as Hasama, Oita Prefecture) and Kikuchi (Kumamoto Prefecture) on Kyushu Island, Japan (Hadi *et al*., 1996).

The chromosomal relationships of *S. arakawae* to other species, however, have not been investigated. We, therefore, compare the banding patterns of the polytene chromosomes of *S. arakawae* with the *Simulium* subgeneric standard sequence (Rothfels *et al*., 1978; Adler & Kachvorian, 2001; Tangkawanit *et al*., 2009) and with the banding patterns of all other chromosomally studied members of the *S. venustum* group.

MATERIALS AND METHODS

Larvae of *S. arakawae* were collected from trailing grasses in a partially shaded, moderately flowing roadside stream, 1.5–2.0 m wide, in a hilly area (altitude ca. 550 m) of Takafusi, Taketa City, Oita Prefecture, Kyushu Island, Japan, 7–8 March 2009. Larvae were fixed in Carnoy's solution (1:3 acetic ethanol).

To prepare the polytene chromosomes, the abdomen of each of 28 larvae was cut anterior to segment VI. The posterior portion was opened ventrally with fine needles, placed in distilled water for 20 minutes, and moved sequentially into preheated (64°C) 1 N HCl for 10 minutes, Feulgen stain for 60 minutes, sulfur water for 10 minutes, and two rinses in cold water (Rothfels & Dunbar, 1953). Stained abdomens were refrigerated in water (nondistilled) for up to 5 days before analysis. The silk-gland chromosomes and one gonad (to allow gender identification; Adler *et al.*, 2004) were removed with fine needles from each abdomen, placed in a drop of 50% acetic acid on a microscope slide, and flattened under a coverslip, with firm thumb pressure. The stained abdomens and anterior portions of the body were placed in 80% ethanol and deposited in the Clemson University Arthropod Collection, South Carolina, USA.

We compared the chromosomal banding pattern of *S. arakawae* against the standard reference sequence for the subgenus *Simulium*, using the maps of Rothfels *et al.* (1978) for the IS, IL, IIL, and IIIS arms and of Adler & Kachvorian (2001) and Tangkawanit *et al.* (2009) for the IIS and IIIL arms. Each novel inversion was sequentially numbered, following the last numbered inversion listed in Table 1 of Rothfels *et al.* (1978). Fixed inversions are italicized in the text and underlined on the figures; floating inversions are given in Roman type. Chromosomal sections that differed by fixed inversions from the *Simulium* subgeneric standard or had autosomal rearrangements were photographed under oil immersion on a compound microscope. All chromosomal rearrangements, including inversion breakpoints, are shown on photographic maps.

RESULTS

All 28 larvae (15 females, 13 males) were scored for the entire banding sequence and all rearrangements. *Simulium arakawae* differs from the *Simulium* subgeneric standard by 8 unique, fixed inversions: 1 in IL (*IL-3*; Figure 1), 3 in IIS (*IIS-1*, *IIS-2*, *IIS-3*; Figure 2), and 4 in IIL (*IIL-16*, *IIL-17*, *IIL-18*, *IIL-19*; Figure 3). The remainder of the complement has the standard sequence for the subgenus *Simulium*.

The population was nearly monomorphic. We found one male heterozygous for an enhanced band in section 42 of IIS (frequency = 0.018; Figure 2) and one female heterozygous for IIL-20 (frequency = 0.018; Figure 3). The IIL region encompassed by sections 58–59 appeared sticky, knotting together in the nuclei of about 25% of the larvae, even though no inversion was apparent. Three male larvae had B chromosomes (Figure 4). Sex chromosomes were microscopically undifferentiated.

DISCUSSION

Although Hadi *et al.* (1996) suggested that *S. arakawae* is a species complex, given its occurrence in a wide variety of habitats over a broad geographic range in Japan, neither they nor we found evidence of sibling species. Nor did we find any differences when comparing the banding sequence in their published maps with that in our larval sample. However, future samples, especially from disparate habitats and different islands, might reveal sibling species.

A critical issue is whether *S. arakawae* shares any fixed inversions with other members of the *S. venustum* group. The only shared inversion might be that represented by the IIS C sequence of Rothfels *et al.* (1978). All other studied members of the *S. venustum* group share the C sequence, though it is often overlain with additional

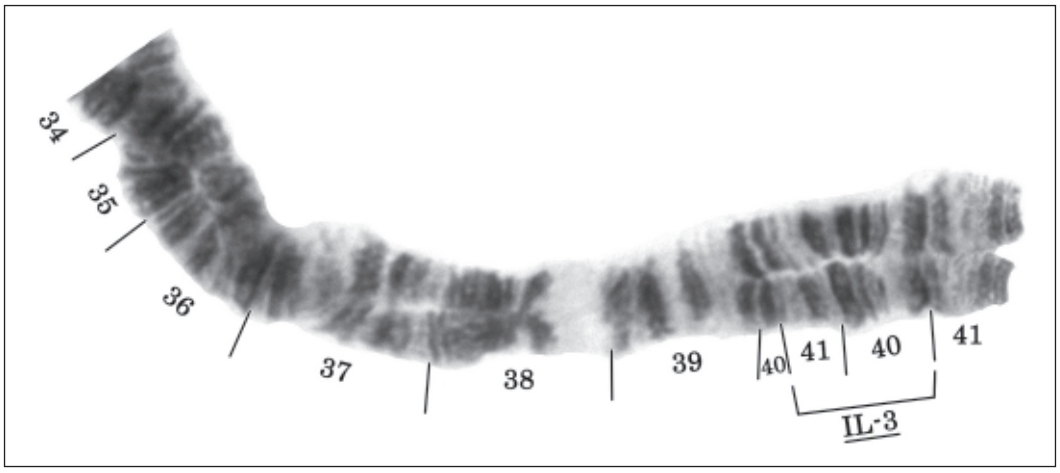

Figure 1. IL end of male larva of *Simulium arakawae* from Japan (Oita Prefecture, Taketa), showing inverted sequence for *IL-3*, relative to banding sequence of Rothfels *et al.* (1978)

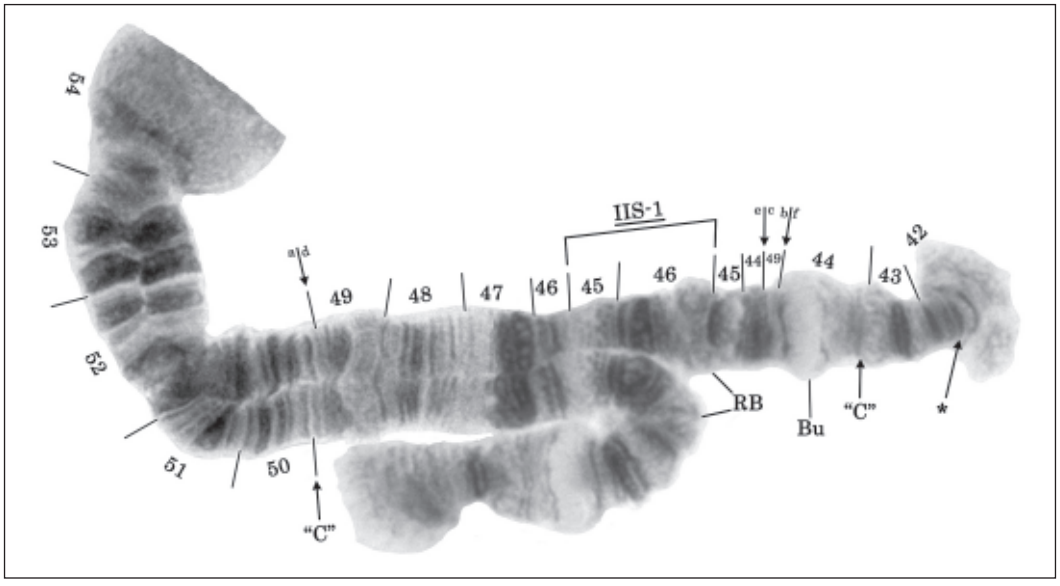

Figure 2. Photocomposite of IIS of female (sections 48–54) and male (sections 42–47) larvae of *Simulium arakawae* from Japan (Oita Prefecture, Taketa), showing fixed inversions *IIS-1*, *IIS-2*, and *IIS-3*; section numbers reflect standard banding sequence established by Adler & Kachvorian (2001) for subgenus *Simulium*. Arrows labeled "C" below the chromosome indicate breakpoints for the C inversion of Rothfels *et al.* (1978), hypothesized to be absent in *S. arakawae*. Asterisk indicates location of heteroband found in one male. Letters 'a' through 'f' represent a hypothesis for the order of sections required to generate the standard sequence from fixed inversions *IIS-2* and *IIS-3* (both represented by arrows above the chromosome), in concert with fixed inversion *IIS-1*. Breakpoints of *IIS-2* = 'a'/'d' and 'b'/'f'; breakpoints of *IIS-3* = 'd'/'f' (which results from inversion *IIS-2*) and 'e'/'c'. Bu = bulge, RB = ring of Balbiani

inversions (Rothfels *et al.*, 1978; Adler *et al.*, 1999, 2004; Chubareva & Petrova, 2008). The C sequence originally was proposed as the standard IIS sequence for the subgenus *Simulium* (Rothfels *et al.*, 1978), but a broader study of taxa suggested that the B sequence of Rothfels *et al.* (1978) is a more appropriate standard (Adler & Kachvorian,

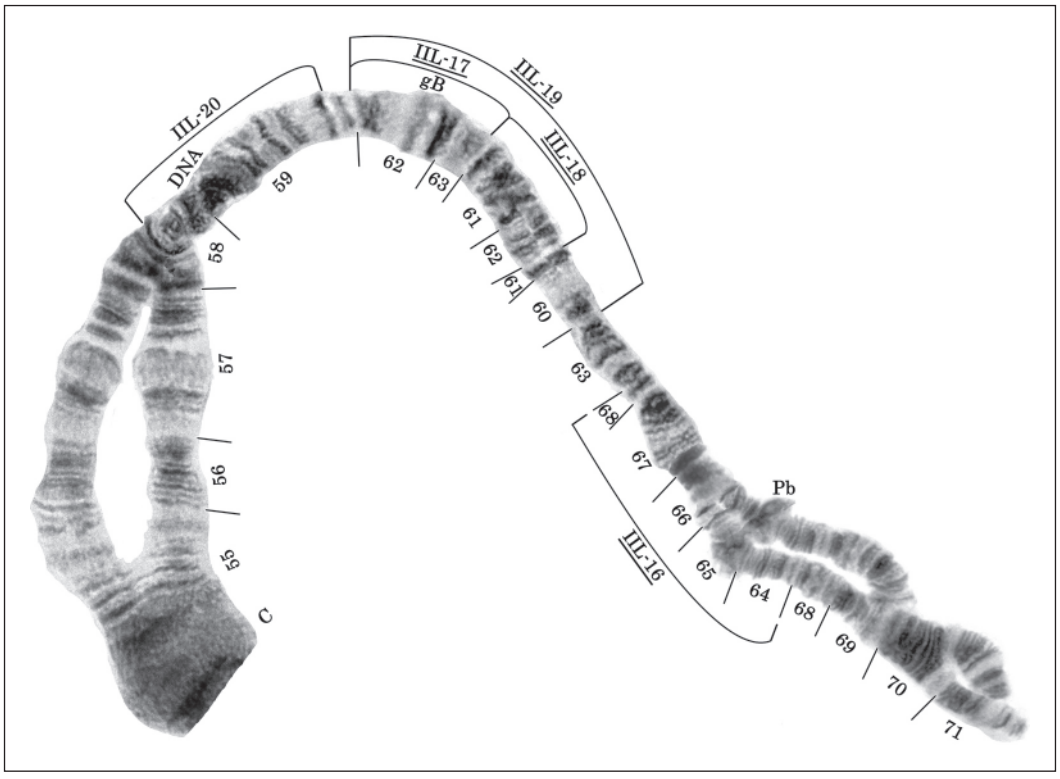

Figure 3. Photocomposite of IIL of female (sections 55–57) and male (sections 58–71) larvae of *Simulium arakawae* from Japan (Oita Prefecture, Taketa), showing fixed inversions *IIL-16*, *IIL-17*, *IIL-18*, and *IIL-19*, and limits of autosomal polymorphism IIL-20; section numbers reflect standard banding sequence established by Rothfels *et al.* (1978) for subgenus *Simulium*. C = centromere, DNA = DNA puff, gB = gray band, Pb = parabalbiani

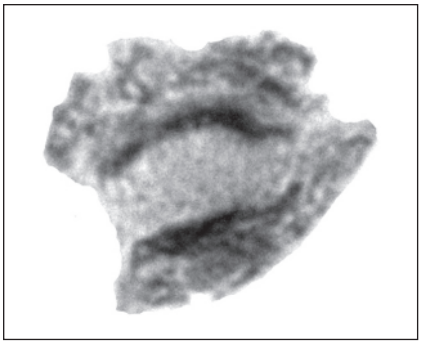

Figure 4. B chromosome of male larva of *Simulium arakawae* from Japan (Oita Prefecture, Taketa)

2001). The C sequence, therefore, is derived by one fixed inversion from the revised subgeneric standard IIS sequence of Adler & Kachvorian (2001), which is equivalent to the B sequence of Rothfels *et al.* (1978).

Whether IIS of *S. arakawae* is based on the C sequence of Rothfels *et al.* (1978) or the standard sequence of Adler & Kachvorian (2001) depends on the interpretation of fine bands in the distalmost fragments of section 44 and section 49 on either side of the bulge landmark (Figure 2). Deriving the sequence of *S. arakawae* from C requires four inversions, whereas deriving it from the subgeneric standard, without C, requires only three inversions. Although either alternative is possible, the latter scenario accounts for all bands in fewer inversion steps (Figure 2). Why would *S. arakawae* not share the C inversion with other members of the *S. venustum* group? One possibility is that *S. arakawae* is not a member of the *S. venustum* group. Morphologically, however, it has all of the diagnostic characters of the group and at one time was identified as *S. venustum* (Bentinck, 1955) because of the similarity

between the two species, although the shape of their ventral plates differs (Takaoka, 1977). *Simulium arakawae* also does not share any chromosomal rearrangements with other studied species groups in the subgenus *Simulium*. A second possibility is that the C sequence evolved in an ancestor of a subset of the group, after diverging from the lineage that includes *S. arakawae*. A third possibility is that the C inversion, though fixed in the ancestor of the *S. venustum* group, was lost in the line leading to *S. arakawae*. This loss, however, would require the original breaks for the C sequence to occur twice (once when gained, once when lost), which is considered unlikely, given the large number of possible breakage points in each arm (Rothfels, 1979). A fourth possibility, and the one we consider most plausible, is that the C sequence was polymorphic in an ancestor of the *S. venustum* group and assumed different fates in subsequent lineages, such as fixation or – relevant to *S. arakawae* – loss. This hypothesis would be strengthened if a polymorphism for the C sequence were discovered in another member of the *S. venustum* group.

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