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The *Simulium vernum* group (Diptera: Simuliidae) in Europe: multiple character sets for assessing species status

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The value of using characters from multiple sources – chromosomes, ecology, gene sequences, and morphology – to evaluate the species status of closely related black flies is demonstrated for three European members of the *Simulium vernum* group: *Simulium crenobium* (Knoz, 1961), *Simulium juxtacrenobium* Bass & Brockhouse, 1990, and *Simulium vernum s.s.* Macquart, 1826. *Simulium juxtacrenobium* is a chromosomally, molecularly, and morphologically distinct species that diverged from *S. crenobium* and *S. vernum s.s.* about 2 Mya. It is specialized for intermittent streams, is univoltine, and is recorded for the first time from northern Europe, based on collections from Finland and Sweden, representing a range extension of about 1800 km. In contrast, *S. crenobium*, although confirmed as a distinct species, differs from *S. vernum s.s.* by only a few larval and chromosomal characters, and by a breeding habitat restricted to mountain spring brooks. Whereas all four character sets independently support the specific distinctness of *S. juxtacrenobium* and *S. vernum s.s.*, multiple character sets are required to establish the specific validity of *S. crenobium*.

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INTRODUCTION

Black flies are as well known for their structural homogeneity as they are for their medical and veterinary importance (Crosskey, 1990). Despite the standard practice of describing species of black flies on the basis of larvae, pupae, males, females, and the breeding habitat, decisions about what constitutes a valid (i.e. reproductively isolated) species can be challenging. Black flies, however, are one of the few groups of organisms for which multiple character sets are routinely available. Giant polytene chromosomes are often mapped to facilitate species resolution (Adler, Currie & Wood, 2004), and the Bar Code of Life project has recently begun adding molecular sequences to the larger database of characters for discovering, discriminating, and identifying species of simuliids (http://www.barcodinglife.org/views/login.php).

The *Simulium vernum* group worldwide consists of more than 120 species (Crosskey & Howard, 2004), many of which are based on minute, if not questionable, structural differences. In Europe, the group includes about 35 species, some of which are widespread and ecologically important. The most recently described European member of the group is *Simulium juxtacrenobium* Bass & Brockhouse, 1990, which was first discovered in 1984 in southern England (Bass & Brockhouse, 1990). Since its discovery, the species has been recorded only from England and Ireland, with a questionable record from the Netherlands (Crosskey & Howard, 2004). *Simulium juxtacrenobium* breeds

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in small flows that are probably overlooked in typical stream surveys. We discovered *S. juxtacrenobium* in southern Finland in 2005 and in southern Sweden in 2006, representing the first records for northern Europe.

The discovery of *S. juxtacrenobium* roughly 1800 km from the nearest previously confirmed sites raises questions on the conspecificity of these disjunct populations. Furthermore, the minute structural and chromosomal differences previously recognized among *Simulium crenobium* (Knoz, 1961), *S. juxtacrenobium*, and *Simulium vernum s.s.* Macquart, 1826 (Bass & Brockhouse, 1990) raise the question of whether these entities are specifically distinct from one another. We use these closely related taxa as a platform for evaluating the use of multiple character sets – chromosomes, ecology, gene sequences, and morphology – in determining the species status of each nominal taxon.

MATERIAL AND METHODS

SIMULIID COLLECTIONS AND ECOLOGICAL MEASUREMENTS

Larvae and pupae were hand-picked from stones and trailing vegetation, and were then fixed in 95% ethanol, or in three changes of Carnoy's solution (1 : 3 acetic ethanol). Adults were reared or dissected from mature pupae, or were collected using emergence traps (site 3) or Malaise traps (site 4). Cytotyped larvae were deposited in the Clemson University Arthropod Collection. Specimens used for DNA analysis were deposited in the Royal Ontario Museum, Toronto, Ontario, Canada. Voucher specimens from Finnish localities were deposited in the Diptera collection of the Helsinki Museum of Zoology, and all other material was deposited in the personal collection of J. Ilmonen.

Stream pH and electrical conductivity were measured in the field using a portable device (WTW pH/Cond 340i). The substrate characteristics at each site were recorded, the width of the stream was measured, and associated simuliid species were collected and identified.

Simulium juxtacrenobium was collected from the localities described below in southern Finland and southern Sweden, representing new records for these two countries. The latitudes and longitudes are followed by the Finnish (KKJ 27°E) or Swedish (RT90) National grid coordinates.

Finland

1. Karkkila: 60°31.348′N, 24°8.415′E (Finnish grid, 67 158 : 33 431); 0.1-m wide ditch draining small mire, with detritus-dominated substrate and trailing sedges (*Carex* spp.); pH 4.6, conductivity 3.5–3.9 mS m^{-1} (measured 19 May 2005 and 30 April 2006); 31 larvae, 16 pupae, of which ten males and one female were reared (8–23 May 2005); 14 larvae, one pupa (30 April–20 May 2006); ten larvae, two pupae (15 May 2007); the only recorded black fly species.
2. Karkkila: 60°31.415′N, 24°8.319′E (Finnish grid, 67 159 : 33 430); 0.2–0.4-m wide stream draining small mire, with sand as the dominant substrate, and with patches of detritus and trailing vegetation (*Carex* spp.); pH 4.9–5.0, conductivity 2.9–3.0 mS m^{-1} (measured 19 May 2005 and 30 April 2006); 79 larvae, six pupae (8–13 May 2005); the only recorded black fly species.
3. Karkkila: 60°31.273′N, 24°8.817′E (Finnish grid, 67 156 : 33 435); 0.2–0.5-m wide stream draining mire, with gravel-dominated, swiftly flowing upper part, and slowly flowing lower part with peat, detritus, and trailing sedges; pH 4.9, conductivity 3.7 mS m^{-1} (measured 30 April 2006); 16 larvae (13–17 May 2005); ten males collected with emergence trap (30 May–27 June 2005); one larva (20 May 2006); collected with *Stegopterna trigonium* (Lundström, 1911), *Simulium lundstromi* (Enderlein, 1921), *S. vernum s.s.*, and *Simulium ornatum* group.
4. Karkkila: 60°31.686′N, 24°8.281′E (Finnish grid, 67 164 : 33 430); 0.1–0.2-m wide stream draining mire, with detritus substrate and trailing sedges; pH 4.92, conductivity 3.3 mS m^{-1} (measured 30 April 2006); four larvae, six pupae (1 June 2006); 24 males collected with Malaise trap (2–28 June 2006); collected with *S. vernum* group.
5. Nummi-Pusula: 60°31.222′N, 24°8.591′E (Finnish grid, 67 155 : 33 433); 0.3–0.4-m wide ditch, with substrate dominated by gravel and patches of liverwort (*Scapania* sp.); pH 4.8, conductivity 3.3 mS m^{-1} (measured 2 May 2006); 27 larvae (4–20 May 2006); ten larvae, three pupae (4 June 2006); collected with *S. vernum s.s.*, *Simulium intermedium* Roubaud, 1906, and *Simulium ornatum* Meigen, 1818 (all cytologically confirmed).
6. Nummi-Pusula: 60°31.099′N, 24°8.674′E (Finnish grid, 67 153 : 33 433); 0.3–0.6-m wide ditch, with substrate dominated by gravel, sand, and trailing sedges; pH 5.0, conductivity 3.4 mS m^{-1} (measured 2 May 2006); one larva (20 May 2006); collected with *S. vernum* group and *S. ornatum* group.
7. Espoo: 60°19.76′N, 24°41.517′E (Finnish grid, 66 931 : 33 726): 0.2–0.4-m wide ditch draining small mire, with substrate of detritus and clay; pH 4.6, conductivity 3.1 mS m^{-1} (measured 3 May 2006); eight larvae (3 May 2006); four pupae (2 June 2006); collected with *S. vernum* group.

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8. Espoo: 60°20.032′N, 24°40.702′E (Finnish grid, 66 936 : 33 719); 0.1–0.2-m wide stream draining small mire, with substrate dominated by detritus; pH 4.7, conductivity 2.1 mS m^{-1} (measured 3 May 2006); 30 larvae (3 May 2006); collected with *S. vernum* group.
9. Espoo: 60°19.844′N, 24°41.13′E (Finnish grid, 66 933 : 33 723); 0.2–0.5-m wide outlet stream draining small lake, with substrate dominated by detritus; pH 4.7, conductivity 2.6 mS m^{-1} (measured 3 May 2006); four larvae (3 May 2006); one larva, two pupae (2 June 2006); collected with *S. vernum s.s.* (cytologically confirmed).
10. Espoo: 60°19.976′N, 24°36.894′E (Finnish grid, 66 936 : 33 684); 0.2–0.5-m wide outlet stream draining small lake, with gravel-dominated, swiftly flowing upper part, and slowly flowing lower part with detritus substrate; pH 4.9, conductivity 2.4 mS m^{-1} (measured 5 May 2006); three larvae (5 May 2006); four pupae (2 June 2006); ten pupae and 65 larvae (25 April 2007); collected with *S. vernum s.s.*, and *S. intermedium* (all cytologically confirmed).
11. Espoo: 60°20.305′N, 24°40.533′E (Finnish grid, 66 941 : 33 718); 0.3–0.5-m wide stream draining mire, with substrate dominated by gravel; pH 4.9, conductivity 2.4 mS m^{-1} (measured 3 May 2006); three larvae (3 May 2006); collected with *S. vernum* group.

Sweden

12. Fäladshus: 55°37.000′N, 13°26.100′E (Swedish grid, 61 678 : 13 507); 0.4-m wide stream in young beech forest, with sandy bed, fine organic deposits, and trailing grasses; pH 7.0, conductivity 13.3 mS m^{-1}, current velocity 15 cm s^{-1}; three larvae (two cytotyped) (2 May 2006); collected with *S. vernum s.s.* (cytologically confirmed).
13. Åbjär: 55°52.309′N, 13°59.931′E (Swedish grid, 62 001 : 13 958); 0.3-m wide stony, shaded stream, with abundant organic matter, especially beech leaves; pH 5.2, conductivity 11.5 mS m^{-1}; 50 larvae (18 cytotyped) (3 May 2006); collected with *Simulium urbanum* Davies, 1966 (cytologically confirmed).
14. Fryele: 57°15.420′N, 14°10.601′E (Swedish grid, 63 489 : 14 018); 0.1–0.4-m wide ditch along road, with substrate dominated by the previous year's grasses and *Sphagnum*; pH 3.8, conductivity 5.0 mS m^{-1}; two larvae (6 May 2006); collected with *S. vernum s.s.* (cytologically confirmed).
15. Gotland, Lojsta: 57°19.161′N, 18°24.056′E (Swedish grid, 63 578 : 16 564); 0.9-m wide stream in agricultural landscape, with gravel bed and some trailing grasses. (Gotland is an island in the Baltic Sea, about 80 km from mainland Sweden); pH 8.4, conductivity 41.1 mS m^{-1}; one larva (25 May 2006); collected with *Simulium latipes* (Meigen, 1804) and *S. vernum* cytoform IIS-1 Brockhouse, 1985 (both cytologically confirmed).
16. Gotland, Stora Vede: 57°36.500′N, 18°23.479′E (Swedish grid, 63 900 : 16 545); 0.3-m wide, 2-cm deep, slow-flowing stream, with substrate of gravel and pebbles along former railway line; many small flows enter the stream; pH 7.9, conductivity 42.5 mS m^{-1}; four larvae, cytotyped, one pupa (24 May 2006); collected with *S. latipes* and *S. vernum* cytoform IIS-1 (both cytologically confirmed).

MORPHOLOGY

The studied material of *S. juxtacrenobium* included larvae, pupae, and reared adults from all 11 Finnish localities, larvae from five Swedish sites, four reared males (two with genitalia removed), and five last-instar larvae from the type locality and nearby populations in the UK [New Forest, 1986 and 2006, collected (leg.) and identified (det.) by J. Bass]. Larvae, pupae, and reared or pharate adults of *S. crenobium* were studied from three localities in Germany (Rhön Mountains, 1967 and 1986, leg. and det. by H. Zwick; Hinterer Bayerischer Wald, 1993 and 1999, leg. and det. by G. Seitz; Berchtesgadener Alpen, 1997, leg. and det. by G. Seitz) and one in Austria (Zillertaler Alpen, 2005, leg. and det. by G. Seitz). Type material of *S. crenobium* could not be found in the putative depository at the University of J. E. Purkyne (Brno, Slovakia), and is presumed lost (M. Kudela, pers. comm.). However, one larva, five pupae, and one female from the Jeseniky Mountain range in the Czech Republic, near or at the type locality (leg. and det. by J. Knoz, 1960), were studied. The German collection site in the Rhön Mountains, which is the spring source (sampling station F0A) of the river Fulda on the slope of Mt Wasserkuppe (Zwick, 1974), is the same locality from which Bass & Brockhouse (1990) examined material of *S. crenobium* (H. Zwick, pers. comm.). Finnish material of *S. vernum s.s.* was used for morphological comparisons.

Dissected parts of larvae and pupae were permanently slide-mounted in Euparal or Canada balsam, and adult genitalia were placed in a depression slide with glycerine. Measurements were made on the linear projections of the glycerine-mounted parts, using an ocular grid in a compound microscope at 100–200× magnification. The density of microtubercles on the pupal cuticle of each species was assessed along three cross-sections across the head, at

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100× magnification, by counting all microtubercles within a 0.02-mm-wide area covered by the ocular grid.

We used a multivariate analysis of variance (MANOVA) to test for overall interspecific differences in the measurements of the male ventral plate, followed by a Tukey's honest significant differences (HSD) test to identify pairwise differences among the significant dependent variables. Prior to the MANOVA, the presence of outliers and distributions of the variables were checked. No transformations were performed. The R 2.5.0 software package (R Development Core Team, 2007) was used to perform statistical analyses.

CYTOLOGY

The larval abdomens of material fixed in Carnoy's solution were stained with the Feulgen technique, as described by Rothfels & Dunbar (1953). The chromosomes of *S. juxtacrenobium* from three sites in Finland and five sites in Sweden were compared with those of *S. juxtacrenobium* described by Bass & Brockhouse (1990), and with the standard map for the *S. vernum* group (Brockhouse, 1985). We also examined the chromosomes of eight female and two male larvae of *S. crenobium* collected by G. Seitz from three sites: Ahornbach (28 July 2005), which is about 300 km south-west of the type locality of *S. crenobium*; Königstal (3 August 2005), 450 km south-west of the type locality; and Scheuereck (28 July 2005), 300 km south-west of the type locality. Our comparative material of *S. vernum* was based on the Knebworth cytoform of Brockhouse (1985), with IIIL-19 as an autosomal polymorphism. The Knebworth cytoform is generally accepted as being representative of *S. vernum s.s.*

To accommodate the discovery of new autosomal inversions, we followed the practice of Hunter (1987), who designated autosomal inversions by the relevant chromosome arm, an arbitrary number, and two letters of the species name; thus, the newly discovered autosomal inversions in our study were IIL-1ju, IIS-1ju, and IIIL-1ju for *S. juxtacrenobium*, and IIIL-1ce for *S. crenobium*. These inversions are described relative to the standard map from Brockhouse (1985) for the *S. vernum* group.

DNA

We applied molecular analyses to *S. juxtacrenobium* from Finland (ten larvae, site 1) and from the type locality in England (three larvae), to *S. crenobium* from Austria (three larvae, Zillertaler Alpen, ~500 km south-west of the type locality, 28 May 2005), and to *S. vernum s.s.* from Finland (three larvae, Sodankylä, 67°26′N, 26°41′E, 18 June 2005; two larvae, near Meltaus, 66°58′N, 25°28′E, 18 June 2005) and from Sweden (nine larvae, Öland, Penåsabäcken, 56°26′N, 16°27′E, 5 May 2006). We examined three mitochondrial markers: the 16S gene, the standardized barcode region of the cytochrome *c* oxidase I (COI) gene, and an additional 800-bp fragment of the COI gene adjacent to the standardized region. Past phylogenetic work often has focused on mitochondrial genes encoding ribosomal (12S, 16S) DNA, but their utility in taxonomic analyses is constrained by the prevalence of insertions and deletions (indels) that complicate the sequence alignments. The faster evolving COI gene, part of which is used as the standard molecular barcode region for species identification (http://barcoding.si.edu), has three important characteristics. First, indels are absent because most lead to a shift in the reading frame; second, the universal primers for this gene are robust, enabling recovery of its 5′ end from the representatives of most, if not all, animal phyla. Also, COI possesses a wider range of phylogenetic signal compared with other mitochondrial genes. In common with other protein-coding genes, its third-position nucleotides show a high incidence of base substitutions. However, changes in its amino acid sequence occur more slowly than those in any other mitochondrial gene.

Samples used for DNA extraction were obtained from small quantities of tissue (2–3-mm long) from the thoraxes of larvae fixed in 90% ethanol. For each individual, 30 μL of total DNA was extracted using the GeneElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich Co.). Three primer pairs were subsequently used in the study: (1) LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAAT CA-3′) (Folmer *et al.*, 1994), to amplify ~650-bp fragments at the 5′ terminus of the COI gene, which were later trimmed to 618 bp; (2) C1-J-2195 (5′-TTGATTTTTTGGTCATCCAGAAGT-3′; Simon *et al.*, 1994) and UEA10 (5′-TCCAATGCACTAATCTGCC ATATTA-3′; Lunt *et al.* 1996) for the 804-bp fragment, adjacent to the 'Folmer' standardized region of the same COI gene; and (3) 16SA-L (5′-CGCCTGT TTATCAAAAACAT-3′) and 16SB-H (5′-CCGGTCTGA ACTCAGATCACGT-3′) (Palumbi *et al.*, 1991) that amplify fragments of ~550 bp of the mitochondrial 16S rRNA gene, which were subsequently trimmed to 502 bp.

The PCR cocktail contained 2.3 μL of 10× PCR buffer, pH 8.3 (10 mM of Tris-HCl, pH 8.3, 50 mM of KCl, 0.01% NP-40), 1.3 μL of $MgCl_2$ (50 mM), 0.3 μL of deoxyribonucleotides (dNTP) (10 mM), 0.3 μL of Taq polymerase (5 U μL^{-1}), 0.25 μL of each primer (10 μM), 12 μL of 10% trehalose, 5 μL of DNA template (0.2–0.4 ng), and double-distilled H_2O to bring

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the volume up to 25 μL. The thermal profile was as follows: 1 cycle of 1 min at 95 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1.5 min at 72 °C; with a final cycle of 7 min at 72 °C. All PCR products (three PCR reactions per specimen) were subjected to dye terminator cycle sequencing reactions (30 cycles, 55 °C annealing), and were sequenced in both directions on ABI 3730 automated sequencers, using Big Dye v3.1.

Electropherograms for the 16S and COI genes were assembled and aligned using Sequencher (v4.5; Gene Codes Corporation), and were edited manually. Pairwise nucleotide-sequence divergences were calculated using the Kimura two-parameter (K2P) model (Kimura, 1980), whereas neighbour-joining (NJ) analysis (Saitou & Nei, 1987) in MEGA v2.1 was used to examine the relationships among taxa. The published sequences can be examined on the Barcoding Life website (http://www.barcodinglife.org/views/projectlist.php?& project name: Black flies (*S. vernum* group) from Europe), or in GenBank.

RESULTS

ECOLOGY

All streams in which *S. juxtacrenobium* were found were first-order, 0.1–0.9-m wide, and were typically associated with peatland-dominated catchments, brown water, and intermittent flow. Out of 16 streams, 13 were acidic (pH 3.8–5.2), one was neutral (site 12), and two (sites 15 and 16) – both on the lime-rich island of Gotland – were alkaline (pH 7.9–8.4). Conductivity ranged from 2.1 to 42.5 mS m^{-1}. The British records are all from acidic headwater streams with low summer flows (Bass & Brockhouse, 1990). The species, therefore, breeds in intermittent rivulets, where competition with other black-fly species is minimal. At two of the Finnish localities, *S. juxtacrenobium* was the only recorded black fly, and in other streams it was associated with between one and four species.

Simulium juxtacrenobium is among the earliest emerging black flies in Fennoscandia. Mature larvae were found soon after the streams had broken free of ice, and in southern Finland the latest pupae were found at the beginning of June. The species also tolerates drought. Site 2 had dried up by the end of May 2005, and remained dry until October 2005, which was similar to all of the other recorded localities for *S. juxtacrenobium* in Finland in 2005–2006. The summer of 2006 was particularly dry in southern Finland, leaving the smallest headwater streams dry from June to October/November. Still, an abundant population of *S. juxtacrenobium* was present at sites 1 and 10 in May 2007. *Simulium juxtacrenobium* probably oviposits either directly into the flow, with the eggs sinking and becoming covered in the sediments, or among mosses and other moist recesses in the stream bottom, where the eggs survive the dry summer period.

MORPHOLOGY

Males: The males of *S. juxtacrenobium* resemble those of *S. crenobium* and *S. vernum s.s.* Bass & Brockhouse (1990) noted that *S. juxtacrenobium* is a black, hairy member of the *S. vernum* group. Reared Finnish specimens have similar-sized hairs on the head and thorax, but are slightly less hairy than the British specimens. Compared with males of *S. juxtacrenobium*, males of *S. crenobium* (five reared specimens, collected in 1986) have variable quantities of body hair of similar size, albeit scarcer on the head and thorax.

The shape of the dorsal plate in the genitalia is similar among the three species, showing more intraspecific than interspecific variation. The body of the ventral plate also is similar: rather square, and without a conspicuous bulge posteriorly (Fig. 1). However, the overall range in measurements of the ventral plate differed significantly among species (MANOVA: Pillai's criterion = 0.910, multivariate $F = 2.78$, d.f. = 24, $P = 0.007$). The length of the basal arms differed significantly among species (univariate $F = 18.2$, d.f. = 24, $P < 0.001$), with the difference being greatest between *S. juxtacrenobium* and *S. vernum s.s.* (Tukey's HSD test, multiple comparisons; $P < 0.001$) and least between *S. crenobium* and *S. vernum s.s.* ($P = 0.031$) (Table 1). The length to maximum width ratio of the arms was significantly different (univariate $F = 12.0$, d.f. = 24, $P < 0.001$); pairwise comparisons indicated significant differences between *S. juxtacrenobium* and *S. vernum s.s.* ($P < 0.001$), and between *S. juxtacrenobium* and *S. crenobium* ($P = 0.008$), but not between *S. crenobium* and *S. vernum s.s.* ($P = 0.30$) (Table 1).

The most useful character for distinguishing males of *S. juxtacrenobium* from those of *S. crenobium* and *S. vernum s.s.* is the shape of the gonostylus. Viewed posteromedially, at a right angle to the flange, the medially directed subterminal flange is more elongated and acutely angled (~50–60°) than in *S. crenobium* (~70–75°) and *S. vernum s.s.* (~80–85°) (Fig. 1). Viewed posteriorly, it is tilted more dorsally. The number of subapical spinules on the gonostylus is not useful for separating these species. According to Knoz (1961) and Bass & Brockhouse (1990), two subapical spinules distinguish *S. crenobium* from *S. vernum s.s.* and *S. juxtacrenobium*, but only one of ten males (reared and pharate) of *S. crenobium* had one gonostylus with two spinules; all the others had gonostyli

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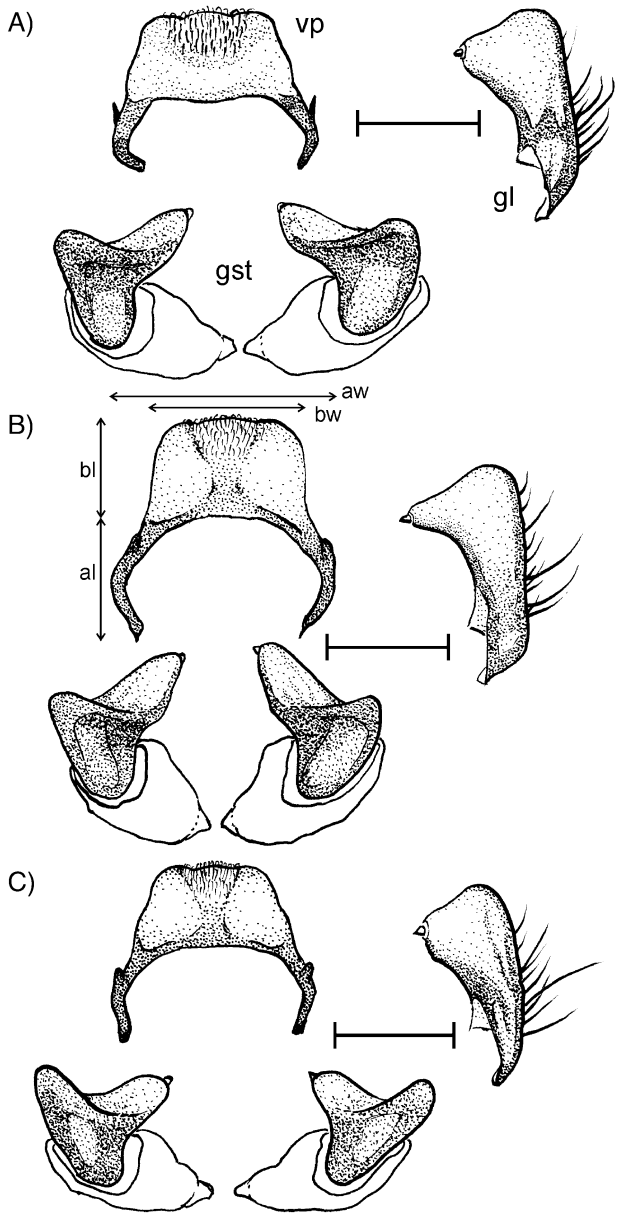

Figure 1. Ventral plate (vp; ventral view), left gonostylus (gl; posteromedial view), and gonostyli (gst; posterior view) of (A) *Simulium crenobium* (Knoz, 1961), (B) *Simulium juxtacrenobium* Bass & Brockhouse, 1990, and (C) *Simulium vernum s.s.* Macquart, 1826. Length and width measurements of basal arms (al, aw) and body (bl, bw) indicated in (b). Scale bar: 0.1 mm.

with single spinules. Occasional males of *S. juxtacrenobium* (2.2%, $N = 46$) and *S. vernum s.s.* (< 2% among Finnish specimens) also had at least one gonostylus with two subapical spinules.

Females: The females of the *S. vernum* group are difficult to identify, and only one reared female each of *S. juxtacrenobium* and *S. crenobium* was available for study. Based on our specimens, and the figures of Knoz (1961) and Bass & Brockhouse (1990), the genital fork of *S. crenobium* resembles that of *S. vernum s.s.*, having well-developed terminal plates, with conspicuous posteroventrally directed lobes. The terminal plates of *S. juxtacrenobium* are narrower, and lack a conspicuous lobe. However, variability in the shape of the genital fork makes reliable separation of these species difficult. Only one of four reared females of *S. crenobium* had cerci available for measuring: these were similar to those measured by Bass & Brockhouse (1990), instead of being shorter, as illustrated by Knoz (1961).

Pupae: The branching pattern of the gill filaments, especially the horizontal branching of the lower pair, distinguishes the pupae of *S. crenobium* and *S. juxtacrenobium* from those of *S. vernum s.s.*. Both *S. crenobium* and *S. juxtacrenobium* vary in the lengths of the common gill stalks. All Finnish pupae of *S. juxtacrenobium* (Fig. 2), as well as British pupae that we examined, had common stalks that were shorter than those illustrated by Bass & Brockhouse (1990), but were similar to those of *S. crenobium*. The shape and length of the anterodorsal projection of the cocoon is short and broadly triangular, or is abbreviated in *S. crenobium* and *S. juxtacrenobium*, but varies from short and triangular to long and narrow in *S. vernum s.s.* The Finnish and British pupae of *S. juxtacrenobium* have similar hemispherical thoracic microtubercles, approximately 0.04–0.06 mm in diameter. The cephalic and thoracic cuticle of *S. juxtacrenobium* is typically more densely covered by microtubercles than that of *S. crenobium* and Finnish *S. vernum s.s.*; the latter two species are similar, but are intraspecifically variable in the density of microtubercles. The number of microtubercles counted in three 0.02-mm-wide sections across the head of the pupae were 20–48, 25–41, and 14–35 for *S. crenobium* ($N = 3$ pupae); 38–48, 37–55, and 38–55 for *S. juxtacrenobium* ($N = 4$); and 27–45, 18–41, and 20–45 for *S. vernum s.s.* ($N = 3$) (Fig. 3). The microtubercles are slightly flatter in *S. crenobium* and *S. vernum s.s.*, although hemispherical microtubercles also occur frequently. They are also typically pale brown or yellow in *S. crenobium* and *S. vernum s.s.*, but are dark brown in *S. juxtacrenobium*, although within-species variation from pale yellow to dark brown also occurs in *S. crenobium* and *S. vernum s.s.* The pale muscle scars on the cephalic cuticle form six distinct groups in *S. juxtacrenobium* and *S. crenobium*, but form less distinct groups in *S. vernum s.s.* (Fig. 3). Given the variation, we regard the cuticular surface sculpture as unreliable for distinguishing the pupae of these three species.

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Table 1. Length (L), width (W), and length/width (%) of the body and arms of the ventral plates of *Simulium crenobium* (Knoz, 1961), *Simulium juxtacrenobium* Bass & Brockhouse, 1990, and *Simulium vernum s.s.* Macquart, 1826

		Body			Arms		
		L (μm)	W (μm)	L/W%	L (μm)	W (μm)	L/W%
S. crenobium	Mean	83.9	146.1	57.7	80.0a	184.4	43.6a
$N = 9$	SE	3.4	5.9	2.0	5.1	6.5	3.0
S. juxtacrenobium	Mean	83.9	139.4	60.3	98.3b	181.1	54.6b
$N = 9$	SE	1.3	2.8	0.9	2.1	5.2	1.6
S. vernum s.s.	Mean	81.7	137.2	59.7	65.0c	168.9	38.6a
$N = 9$	SE	2.1	3.1	1.8	3.6	3.6	2.1

Means followed by different lowercase letters are significantly different within colums.

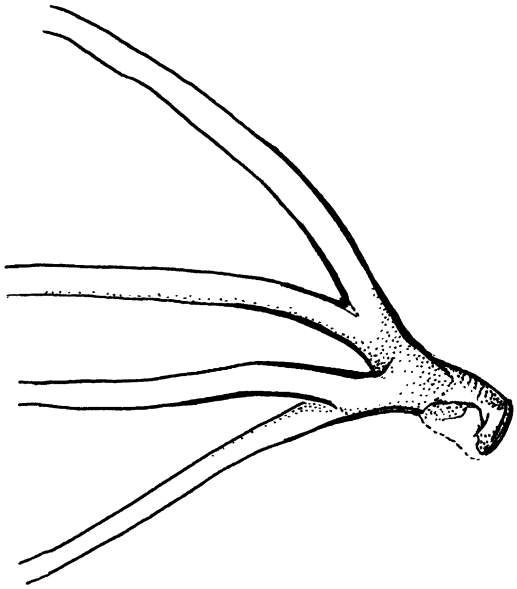

Figure 2. Base of a typical gill of a Finnish pupa of *Simulium juxtacrenobium* Bass & Brockhouse, 1990.

Larvae: The small, angular postgenal cleft distinguishes *S. juxtacrenobium* and *S. crenobium* from other sympatric species of the *S. vernum* group. However, the shape of the cleft varies in both species, as shown for *S. juxtacrenobium* (Fig. 4). The pigmentation patterns on the frontoclypeal apotome also vary within both species, but generally all markings of *S. crenobium* are obscure, whereas the posteromedial head spot is distinct and narrow in *S. juxtacrenobium* (Fig. 4). The head spots of *S. vernum s.s.* are typically dark and distinct. Most final-instar larvae of *S. juxtacrenobium* in Finnish samples (range 69–100%, mean 92%, SD 11%) have distinct pigmentation on a brown apotome, with a narrow posteromedian head spot, and a conspicuous eyebrow stripe (Fig. 4). Head patterns of final instars show little variation within or among populations: the posteromedial triangle is sometimes slightly broader (Fig. 4). The proximal antennal article is distinctly shorter in Finnish (mean = 215 μm, range 200–225, $N = 6$) and British (mean = 223 μm, range 200–235, $N = 5$) larvae of *S. juxtacrenobium*, as well as in those of *S. crenobium* (mean = 198 μm, range 175–215, $N = 8$), compared with the measurements of Bass & Brockhouse (1990). Given this intraspecific variation, the length of the proximal antennal article is not useful for distinguishing these species. However, the total length and dark dorsal pigmentation of the antenna distinguish larvae of *S. juxtacrenobium* from those of other species in the *S. vernum* group known from Fennoscandia (Fig. 4). The number of primary fan rays in *S. juxtacrenobium* overlaps with the numbers given by Bass & Brockhouse (1990). All larvae of *S. juxtacrenobium* (mean = 59, range 56–64, $N = 11$) have more rays than those of *S. crenobium* (mean = 44, range 39–47, $N = 8$), with Finnish larvae having more rays (mean = 61, range 58–64, $N = 6$) than British larvae (mean = 57, range 56–60, $N = 5$). Although the number of primary rays does not overlap between *S. juxtacrenobium* and *S. crenobium*, this character is subject to environmental influence (Zhang & Malmqvist, 1997). The number of mandibular teeth overlaps: 11–14 in *S. juxtacrenobium* and 9–13 in *S. crenobium*.

CYTOLOGY

The banding sequence of *S. juxtacrenobium* from Finland is identical to that described by Bass & Brockhouse (1990) from the type locality in Hampshire, England. Finnish larvae, like those at the type locality, are fixed for the IIIL-19 inversion, have the characteristic two different B (i.e. supernumerary) chromosomes, and possess a pseudochromocenter formed by ectopic pairing of the centromeres,

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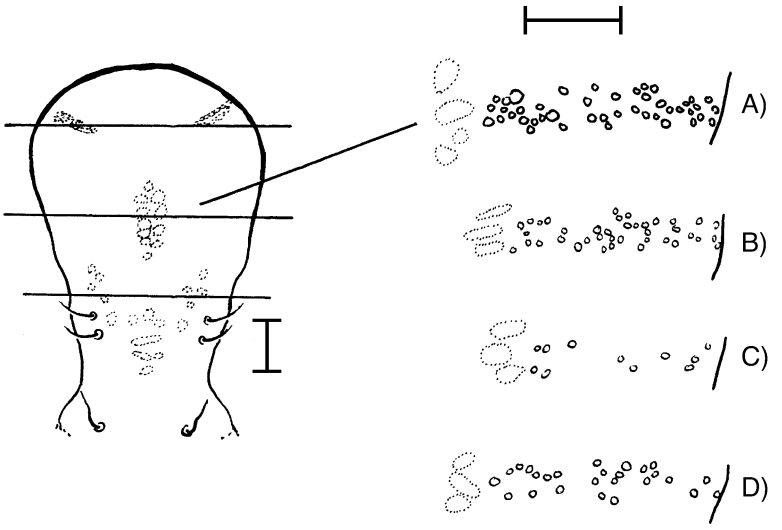

Figure 3. Cephalic cuticle of pupae, showing the location of the cross-sections for counting microtubercles (100×), and sections of the surface sculpture (200×) of (A) *S. juxtacrenobium* Bass & Brock., (B) *Simulium crenobium* (Knoz, 1961), and variations (C, D) of *Simulium vernum s.s.* Macquart, 1826 across the right-hand side of the middle transect. Scale bars: 0.1 mm.

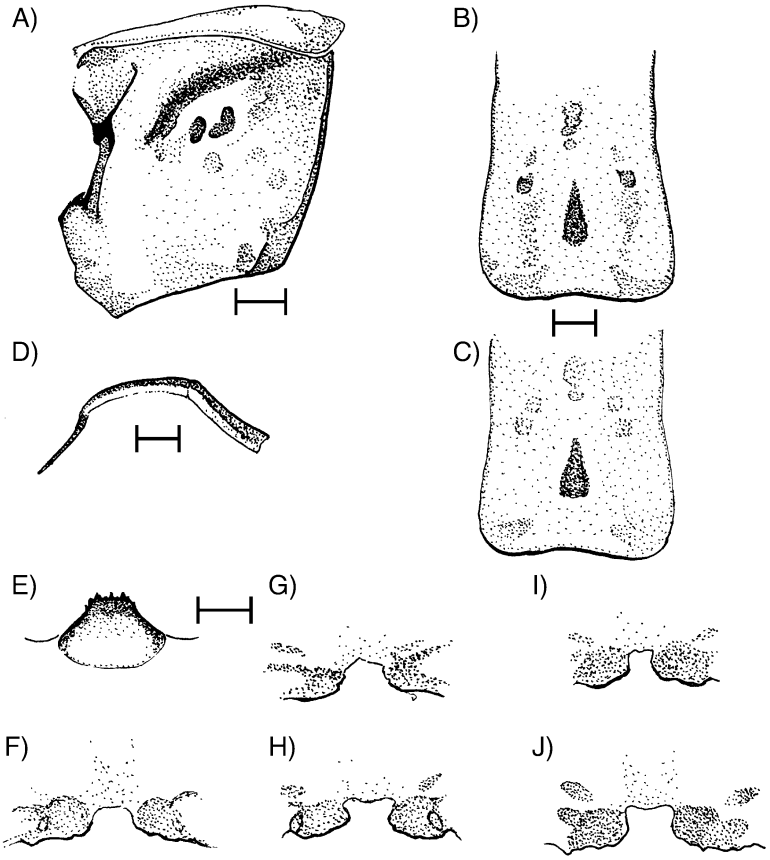

Figure 4. Larval head pigmentation viewed (A) laterally and (B, C) dorsally, and (D) antenna, (E) hypostoma, and (F–J) postgenal cleft of *Simulium juxtacrenobium* Bass & Brockhouse, 1990; examples from Finland show variation of apotome pigmentation (B, C), and variation in postgenal cleft shape and associated pigmentation (F–J). Scale bars: 0.1 mm.

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Table 2. Chromosomal polymorphisms of *Simulium juxtacrenobium* Bass & Brockhouse, 1990 in Finland (FIN) and Sweden (SWE)

Site, country, collection date	No. ♂ : ♀	Frequency of inverted constituent				Frequency of larvae with B chromosomes
		IIL-1ju	IIS-1ju	IIIL-1ju	IIIL-19	
1, FIN, 19 May 2005	0 : 11	0.32	0.00	0.14	1.00	0.54
5, FIN, 4 May 2006	2 : 2	0.62	0.00	0.00	1.00	0.25
9, FIN, 3 May 2006	2 : 1	0.67	0.17	0.17	1.00	1.00
12, SWE, 2 May 2006	2 : 0	1.00	0.00	0.00	0.50*	0.00
13, SWE, 3 May 2006	10 : 8	0.67	0.00	0.00	0.78*	0.00
14, SWE, 6 May 2006	0 : 2	0.00	0.00	0.00	1.00*	0.00
15, SWE, 25 May 2006	0 : 1	0.00	0.00	0.00	1.00*	1.00
16, SWE, 24 May 2006	1 : 3	0.50	0.00	0.00	0.88*	0.25

*IIIL-19 in these populations is linked to the X chromosome: all females are homozygously inverted for IIIL-19, whereas males are either heterozygously ($N = 11$) or homozygously inverted ($N = 2$) for IIIL-19.

including those of the B chromosomes (Fig. 5). They differ by lacking a secondary nucleolar organizer, and in having three unique autosomal polymorphisms, including IIL-1ju (Fig. 6), which inverts sections 64–65 in 44.4% of 36 chromosomal constituents (Table 2). Inversions IIS-1ju (sections 51A–55A, inclusive) and IIIL-1ju (sections 91C3–94C, inclusive) occurred heterozygously in single larvae. B chromosomes (Fig. 7) occurred in 55.6% of the 18 Finnish larvae, compared with 61.0% of topotypical larvae examined by Bass & Brockhouse (1990). In Finnish larvae, the sex chromosomes are microscopically undifferentiated, and the end of the IIIS chromosome is consistently splayed, more so than in Bass & Brockhouse (1990: fig. 22).

Swedish larvae of *S. juxtacrenobium* have a pseudochromocenter (often weak) and B chromosomes (7.4% of larvae), lack a secondary nucleolar organizer, and had IIL-1ju in 59.2% of the 54 chromosomal constituents. Males have two Y chromosomes: one undifferentiated from the X (= IIIL-19), and the other, which predominates (84.6%), carrying the standard IIIL sequence. The IIIS end of Swedish larvae is not as splayed as in Finnish larvae.

The chromosomal variation among populations of *S. juxtacrenobium*, based particularly on different sex-chromosome systems, might indicate the presence of sibling species. For the moment, however, we suggest that the shared floating inversion IIL-1ju, which is unknown in any other member of the *S. vernum* group, supports the conspecificity of Finnish and Swedish populations. Furthermore, we conservatively view Fennoscandian and topotypical populations of *S. juxtacrenobium* as conspecific, especially based on the pseudochromocenter and two different B chromosomes. We attribute the unique polymorphisms (e.g. IIL-1ju in Fennoscandia, IIIL-19 as an X chromosome in some Fennoscandian populations, and a secondary nucleolar organizer in England) to the geographic distance (~1000–2000 km) between the Fennoscandian and the British populations.

In mixed samples of *S. vernum* (excepting the IIS-1 cytoform) and *S. juxtacrenobium* from Finland and Sweden, the two species are immediately distinguishable, even at low magnification (100×), by the degree to which the homologues pair. The complement of *S. vernum s.s.* shows remarkably loose pairing, typically 75–90% unpaired, which is characteristic of *S. vernum s.s.* throughout Fennoscandia (Adler, Malmqvist & Zhang, 1999), whereas that of *S. juxtacrenobium* is less than 25% unpaired.

All ten larvae of *S. crenobium* that we examined were identical to those of *S. vernum* (Knebworth cytoform), except that they were homozygously inverted for IIIL-19. One larva had supernumerary chromosomes. Bass & Brockhouse (1990) examined the chromosomes of *S. crenobium* from the spring source of the river Fulda, on the slope of Mt Wasserkuppe, in Germany (H. Zwick, pers. comm.), about 500 km west of the type locality of *S. crenobium*. The chromosomes were identical to those of *S. vernum*, except that IIIL-19 acted as a sex chromosome in *S. crenobium* (Bass & Brockhouse, 1990: 79), although whether it was X- or Y-linked was not stated. Of the two males in our sample, one was chromosomally undifferentiated from the females, and the other was heterozygous for IIIL-1ce (sections 87A–92B, inclusive) on top of IIIL-19. Regardless of whether this inversion is autosomal or Y-linked, it has not been found among more than 1000 larvae of *S. vernum s.s.* examined by Brockhouse (1985), Hunter (1987), Adler *et al.* (1999), and P.H. Adler (unpubl. data). The occurrence of this unique inversion provides chromosomal evidence, albeit slim, supporting the species status of

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Figure 5. Total chromosome complement of *Simulium juxtacrenobium* Bass & Brockhouse, 1990 (female larva from Finland, site 9), showing the centromeric association of chromosomes with B chromosomes (B); chromosome arms are labelled with roman numerals; N.O., nucleolar organizer. The larva is heterozygous for IIL-1ju and homozygously inverted for IIIL-19.

S. crenobium vis-à-vis *S. vernum s.s.* The functional difference between IIIL-19 in our study (fixed) and that of Bass & Brockhouse (1990: 79; sex linked) indicates that *S. crenobium* is chromosomally variable over its range, perhaps owing to its possible isolation in mountain spring brooks or the existence of a sibling species, making its distinction from *S. vernum s.s.* more difficult at an individual level. Populations of *S. vernum* (Knebworth cytoform) can be standard, polymorphic, or X-linked for IIIL-19 (Brockhouse, 1985), also raising the possibility of sibling species. At a population level, *S. crenobium* differs

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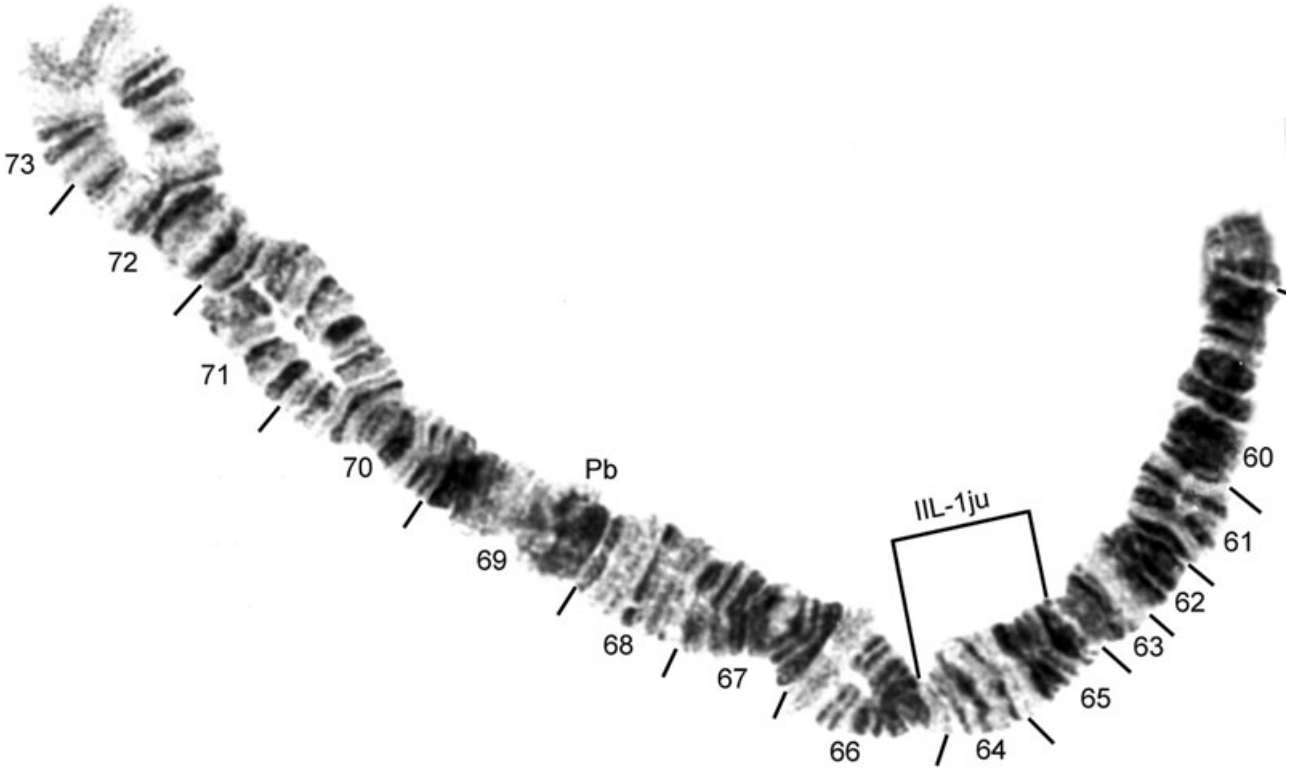

Figure 6. The IIL chromosome arm of *Simulium juxtacrenobium* Bass & Brockhouse, 1990 (male larva from Finland, site 5), showing IIL-1ju inversion; Pb, parabalbiani marker; sections 56–59 are omitted.

from *S. vernum* by presumed fixation of IIIL-19 in some populations, and by sex linkage of IIIL-19 (if Y-linked) in other populations.

Gene sequences

Simulium crenobium, *S. juxtacrenobium*, and *S. vernum s.s.* form a common cluster in the NJ analysis of the more slowly evolving 16S gene (Fig. 8). However, the other three NJ analyses based on: (1) the barcode COI gene (Fig. 9), (2) an additional 800-bp fragment from the COI gene (Fig. 10), and (3) the combined COI + 800-bp gene fragment (combination 1422-bp long; Fig. 11) show a separation of *S. juxtacrenobium* from both *S. crenobium* and *S. vernum s.s.* Pairwise comparisons of COI sequences between *S. crenobium–S. vernum s.s.* and *S. juxtacrenobium* show 4% K2P divergence and tight conspecific clusters, although the cluster of *S. juxtacrenobium* shows divergence among populations, with two or three distinct subgroups. Sequence divergences for other congeneric *Simulium* species are between 2.8 and 18.5%. Based on the typical calibration of the COI sequence (Knowlton *et al.*, 1993), i.e. a sequence divergence of 2% per million years, the analysis of a 618-bp fragment of the barcode COI in this study, showing on average 24 diagnostic substitutions between *S. crenobium–S. vernum s.s.* and *S. juxtacrenobium*, indicates that these two groups diverged about 2 Mya. The NJ analysis based on the 800-bp COI fragment shows even more significant genetic differences – 6.5% sequence divergence – between *S. crenobium–S.vernum s.s.* and *S. juxtacrenobium*. Moreover, this marker indicates some differentiation between *S. crenobium* and *S. vernum s.s.*, which is not visible with the use of the standard barcode COI diagnostics. The molecular taxonomic diagnosis of species becomes more difficult as the time since their reproductive isolation shrinks, as in the case of *S. crenobium* and *S. vernum s.s.* Our limited molecular data do not indicate that *S. vernum s.s.* and *S. crenobium* are separate species; more specimens of *S. crenobium* from other localities, however, are needed to evaluate the species status unambiguously, based on DNA.

DISCUSSION

Our study demonstrates that decisions regarding species status can be difficult, even when informed by multiple character sets. Morphologically, the males of *S. juxtacrenobium* are distinguishable from those of *S. crenobium* and *S. vernum s.s.*, based on newly

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Figure 7. Two morphologically different B chromosomes of *Simulium juxtacrenobium* Bass & Brockhouse, 1990 (female larva from Sweden, site 15).

recognized diagnostic characters of the genitalia, whereas males of *S. crenobium* and *S. vernum s.s.* are difficult to distinguish from one another. Immatures of *S. crenobium* and *S. juxtacrenobium* can usually be distinguished from those of *S. vernum s.s.*, although in mixed populations of *S. juxtacrenobium* and *S. vernum s.s.* in southern Finland, the intraspecific morphological variation (postgenal cleft, pupal gill) results in a number of larvae and pupae that cannot be reliably assigned to either species morphologically. The immatures of *S. crenobium* and *S. juxtacrenobium* are difficult to distinguish morphologically from one another. The only structural differences are in the number of primary fan rays and head pigmentation of the larva, and the surface sculpture of the pupa. These characters, however, vary intraspecifically, and fan structure is influenced by the environment (Zhang & Malmqvist, 1997).

It is possible that *S. juxtacrenobium* is conspecific with *Simulium erectum* (Rubtsov, 1959), based on Rubtsov's (1964) description and the type specimens studied in the collection of the Zoological Institute of the Russian Academy of Science (ZIN), St Petersburg (lectotype 5957, paralectotype 5956, pinned male pupae with genitalia mounted on plastic strips). The gill of *S. erectum* is similar to that of *S. crenobium* and *S. juxtacrenobium*, and the proportions of the ventral plate match those of *S. juxtacrenobium*. The diagnostic shape of the subterminal flange on the gonostylus, however, could not be seen adequately, and no larvae were found in the ZIN collections. Lacking information on the gonostyli, as well as on larval morphology, chromosomes, and DNA, we refrain at this time from synonymizing *S. juxtacrenobium* with *S. erectum*. However, the possibility of synonymy emphasizes the need to search for *S. juxtacrenobium* in continental Europe and western Russia. *Simulium erectum* has been recorded from central Russia and Siberia (Crosskey & Howard, 2004).

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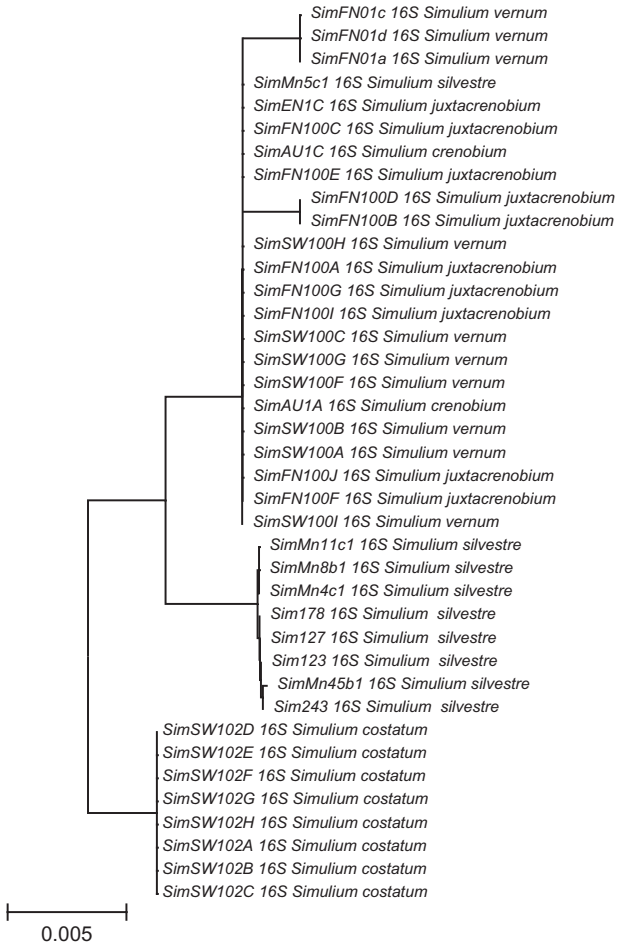

Figure 8. Neighbour-joining analysis of the Kimura two-parameter (K2P) distances of mitochondrial 16S sequences from *Simulium crenobium* (Knoz, 1961), *Simulium juxtacrenobium* Bass & Brockhouse, 1990, and *Simulium vernum s.s.* Macquart, 1826 in relation to *Simulium costatum* Friederichs, 1920 and *Simulium silvestre* (Rubtsov, 1956); AU, Austria; EN, England; FN, Finland; SW, Sweden; samples of *S. silvestre* are from Canada (Mn, Manitoba) and Wyoming (USA).

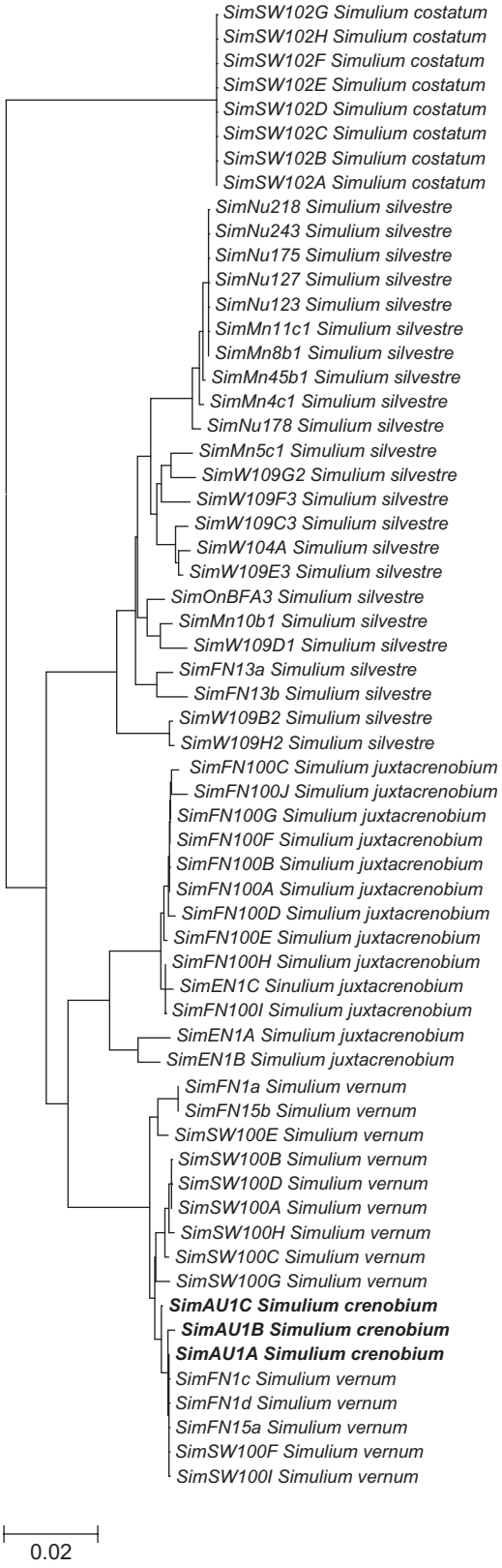

Figure 9. Neighbour-joining analysis of the Kimura two-parameter (K2P) distances of mitochondrial barcode cytochrome *c* oxidase I (COI) gene sequences from *Simulium crenobium* (Knoz, 1961), *Simulium juxtacrenobium* Bass & Brockhouse, 1990, and *Simulium vernum s.s.* Macquart, 1826 in relation to *Simulium costatum* Friederichs, 1920 and *Simulium silvestre* (Rubtsov, 1956); AU, Austria; EN, England; FN, Finland; Mn, Manitoba; Nu, Nunavut; On, Ontario (Canada); SW, Sweden; W, Wyoming (USA).

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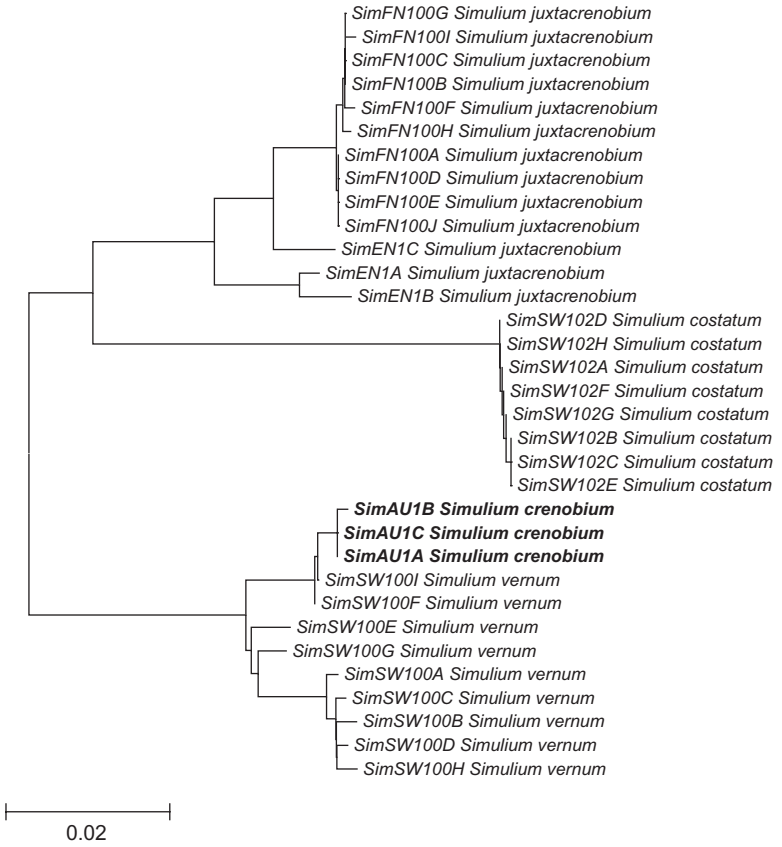

Figure 10. Neighbour-joining analysis of the Kimura two-parameter (K2P) distances of mitochondrial cytochrome *c* oxidase I (COI) 800-bp sequences from *Simulium crenobium* (Knoz, 1961), *Simulium juxtacrenobium* Bass & Brockhouse, 1990, and *Simulium vernum s.s.* Macquart, 1826 in relation to *Simulium costatum* Friederichs, 1920; AU, Austria; EN, England; FN, Finland; SW, Sweden.

Ecologically, *S. juxtacrenobium* has drought-resistant eggs and is a specialist in lowland, intermittent streams that dry out in summer, whereas *S. crenobium* occurs in temporally stable spring brooks in the mountains (Knoz, 1961, 1965; Zwick, 1974). *Simulium vernum s.s.* is found in streams of variable size and catchment characteristics, including spring brooks (Adler *et al.*, 1999; Hoffsten & Malmqvist, 2000) and intermittent streams. *Simulium juxtacrenobium* has one, early-emerging generation, whereas *S. crenobium* and *S. vernum s.s.* have two or three generations per year. Pupae of *S. vernum s.s.* are abundant from April to October in Germany (Zwick, 1974), and adults emerge from May to September in southern Finland (J. Ilmonen, unpubl. data).

Both larval polytene chromosomes and molecular sequences indicate that *S. crenobium* and *S. vernum s.s.* are closely related, whereas *S. juxtacrenobium* is distinct from both species. Moreover, the gene sequences imply that *S. juxtacrenobium* diverged from *S. crenobium* and *S. vernum s.s* around 2 Mya. This result, however, is not easily understood in the context of the geological history of the area, which includes the known range of the species. *Simulium juxtacrenobium* has been recorded mostly from recently deglaciated areas, whereas *S. crenobium* has only been recorded from areas that were free of the Fennoscandian ice sheet during the last glacial maximum (LGM), *c.* 18 Kyr BP (Crosskey & Howard, 2004).

The possibility of a species (*S. juxtacrenobium*) unique to the recently deglaciated area of northern Europe seems slight. During the Pleistocene, large ice sheets developed in the Northern Hemisphere, retracting and expanding as the climate changed from warm interstadial to cold glacial periods. During the LGM in the late Weichselian, *c.* 18 Kyr BP, northern Europe was covered by ice south to southern England, northern Germany, Poland, and north-western Russia. Smaller ice caps covered the Alps. Most of the presently known range of *S. juxtacrenobium*, therefore, was then uninhabitable. The results, however, can be brought into line if *S. juxtacrenobium* survived in refugia south of the ice cover, or if some records of

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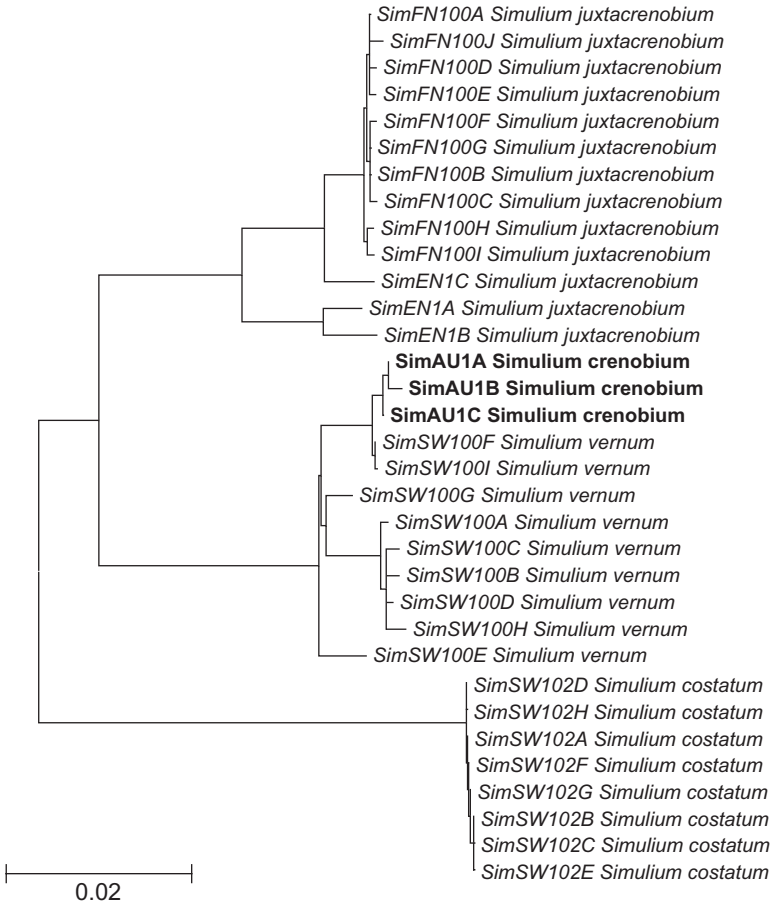

Figure 11. Neighbour-joining analysis of the Kimura two-parameter (K2P) distances of combined barcode cytochrome *c* oxidase I (COI) + COI 800-bp sequences from *Simulium crenobium* (Knoz, 1961), *Simulium juxtacrenobium* Bass & Brockhouse, 1990, and *Simulium vernum s.s.* Macquart, 1826 in relation to *Simulium costatum* Friederichs, 1920; AU, Austria; EN, England; FN, Finland; SW, Sweden.

S. crenobium are actually misidentifications of *S. juxtacrenobium*. The species might have inhabited small, lowland streams in north-western Europe, perhaps surviving in southern England during the LGM, and subsequently colonizing Scandinavia through Denmark after the ice sheet retracted. In light of its currently known distribution, *S. juxtacrenobium* should have been able to cross the Baltic Sea to reach southern Finland. However, it is more likely to have dispersed further in the east, which would have enabled colonization of Scandinavia from the east, which is another common dispersal route after the LGM (e.g. Crosskey, 1990).

Simulium crenobium has been recorded from springs in the mountains of Austria, Bosnia, Czech Republic, Germany, Hungary, Poland, Serbia, Slovakia, Slovenia, and Switzerland (Crosskey & Howard, 2004), although some of these records might be based on misidentifications of *S. juxtacrenobium*. Its present distribution is similar to that of some European high-altitude species (Jedlička, 2006), being absent from areas covered by the ice sheet during the LGM. In colder periods, when isolated ice caps covered the Alps, these species survived at altitudes below the ice caps, moving to higher elevations when the climate warmed. In isolated mountain ranges, this contraction and expansion of ranges resulted in disjunct geographical distributions for species adapted to cold environments. Some spring-dwelling insects, however, occur widely across Europe, also having colonized Scandinavia after the LGM (Ilmonen, 2008). These species include *Simulium carpathicum* Knoz, 1961, which inhabits spring-fed streams (Knoz, 1961, 1965), and is distributed across Europe (Crosskey & Howard, 2004), including northern Sweden (Adler *et al.*, 1999) and northern Finland (Ilmonen & Kuusela, 2006). The apparently restricted range of *S. crenobium* might be the result of specialization to cold environments or poor dispersal ability.

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Our study is one of the few to use four major character sources as a means of evaluating species status in the Simuliidae. Cytology, ecology, gene sequences, and morphology corroborate the specific distinctness of *S. juxtacrenobium* and *S. vernum s.s.* Less well defined, however, is the distinction between *S. crenobium* and *S. vernum s.s.*, which infrequently occur together in the streams of central Europe. We consider *S. crenobium* to be a valid species on the basis of the following features: (1) a highly specialized breeding habitat (crenal vs. rhithral/potamal), (2) the minute, angular postgenal cleft of the larva, (3) the poorly defined larval head-spot pattern, (4) the unique autosomal inversion, and (5) the different function of the IIIL-19 inversion. Any character set alone would be insufficient to assert species status for *S. crenobium* vis-à-vis *S. vernum s.s.* The combination of characters from independent sources, however, confirms the validity of *S. crenobium*.

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