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Chromosomal characteristics and evolutionary relationships of the Palearctic black fly *Simulium carthusiense* (Diptera: Simuliidae)

PETER H. ADLER[1] and GUNTHER SEITZ[2]

[1]Entomology Program, Clemson University, Clemson, SC 29634-0310 USA; e-mail: padler@clemson.edu
[2]District Government of Lower Bavaria, Regierungsplatz 540, 84028 Landshut, Germany; e-mail: gunther.seitz@reg-nb.bayern.de

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Abstract. The giant, polytene chromosomes of *Simulium carthusiense* Grenier & Dorier, 1959 were mapped, and all rearrangements were resolved relative to the standard banding sequence for the *S. vernum* group. The species is chromosomally cohesive from Austria to Spain, and is characterized by a chromocenter, two unique fixed inversions, 10 unique autosomal polymorphisms, and undifferentiated sex chromosomes. Rare individuals (3%) have two types of supernumerary chromosomes, representing the third example of a simuliid species that carries two different supernumeraries in the same individuals. Band-sequence comparisons with chromosomal outgroups indicate that *S. carthusiense* is the sister species of a clade that includes *S. beltukovae* (Rubtsov, 1956), the *S. cryophilum* complex, and *S. urbanum* Davies, 1966.

INTRODUCTION

The *Simulium vernum* group provides numerous examples of the taxonomic difficulties in the family Simuliidae (Ilmonen et al., 2009). It is the second largest species group of black flies in the world, consisting of about 130 nominal species, 80% of which are represented in the Palearctic Region (Adler & Crosskey, 2014). Members of the *S. vernum* group are structurally similar and often can be identified in only one or two life stages. The taxonomy and systematics of the group, however, have been aided through studies of the banding patterns in the giant chromosomes of the larval silk glands. These giant, polytene chromosomes have revealed cryptic species, defined species limits, and resolved evolutionary relationships in the group (Brockhouse, 1985; Hunter, 1987). About 26 Palearctic species in the *S. vernum* group have been examined chromosomally (e.g., Chubareva & Petrova, 2008), but only about half of these have been resolved relative to an established reference map (Brockhouse, 1985; Hunter, 1987; Bass & Brockhouse, 1990; Adler et al., 2004; Ilmonen et al., 2009).

The polytenes of the Simuliidae are expressed as 3, rarely 2, haploid submetacentric chromosomes (I, II, and III) each with a long (L) and short (S) arm on either side of the centromere (Rothfels, 1979). Chromosomal landmarks are consistently found within an arm and, therefore, aid arm recognition (Rothfels et al., 1978). A primary nucleolar organizer can appear anywhere in the complement, though its location is consistent at some taxonomic level (Adler et al., 2004). Chromosomal variability within and among species can be assessed by evaluating structural rearrangements, primarily inversions and bands of differential thickness (heterobands). These rearrangements can be fixed, autosomally polymorphic, or linked to the X or Y chromosome (Bedo, 1977; Adler et al., 2010). A standard chromosomal map for a taxon (e.g., species group) typically represents the most central banding sequence in the group (e.g., Brockhouse, 1985), allowing the banding patterns of all members of the taxon to be compared. Rearrangements, relative to the standard map, are considered to represent unique events (Rothfels, 1979), and can provide phylogenetic information when they are shown, by outgroup comparison, to be uniquely shared and derived from an ancestral condition (Adler & Huang, 2011).

Simulium carthusiense Grenier & Dorier, 1959, a typical member of the *S. vernum* group, was described from females, males, pupae, and larvae collected in the French Alps, and was considered conspecific with material from the Pyrenees of France and from Andalusia, Spain, collected at elevations of 300–2500 m (Grenier & Dorier, 1959). It subsequently has been found in Andorra, Austria, Czech Republic, Germany, Italy, Poland, Slovakia, Switzerland, and Ukraine (Adler & Crosskey, 2014). The preimaginal stages develop in swift mountain streams, and pupation occurs in June, producing one generation per year (Knoz, 1965).

We evaluate the polytene chromosomes of *Simulium carthusiense* against the standard map of Brockhouse (1985) for the *S. vernum* group, mapping all structural rearrangements, identifying unique features, and establishing evolutionary relationships with closely related species. The resulting cytophylogeny is the first, based on a cladistic approach, for any species in the *S. vernum* group.

MATERIAL AND METHODS

Larvae of *S. carthusiense* were hand collected from 4 streams in Austria and Spain (Table 1) and were fixed in 3 changes of 1 : 3 acetic ethanol. In Austria, larvae and pupae were collected with those of *Prosimulium rufipes* (Meigen, 1830) at Site 1 and with *P. rufipes* and *S. bertrandi* Grenier & Dorier, 1959 at Sites 2 and 3. These sites were in epirhithral, oligosaprobic, alpine, karstic streams with boulders. In Spain (Site 4), larvae and pupae were collected with those of *S. cryophilum* cytoform "A" (*IIIL-19 = rr* sequence of Hunter, 1987) and larvae of *S. urbanum* (chromo-

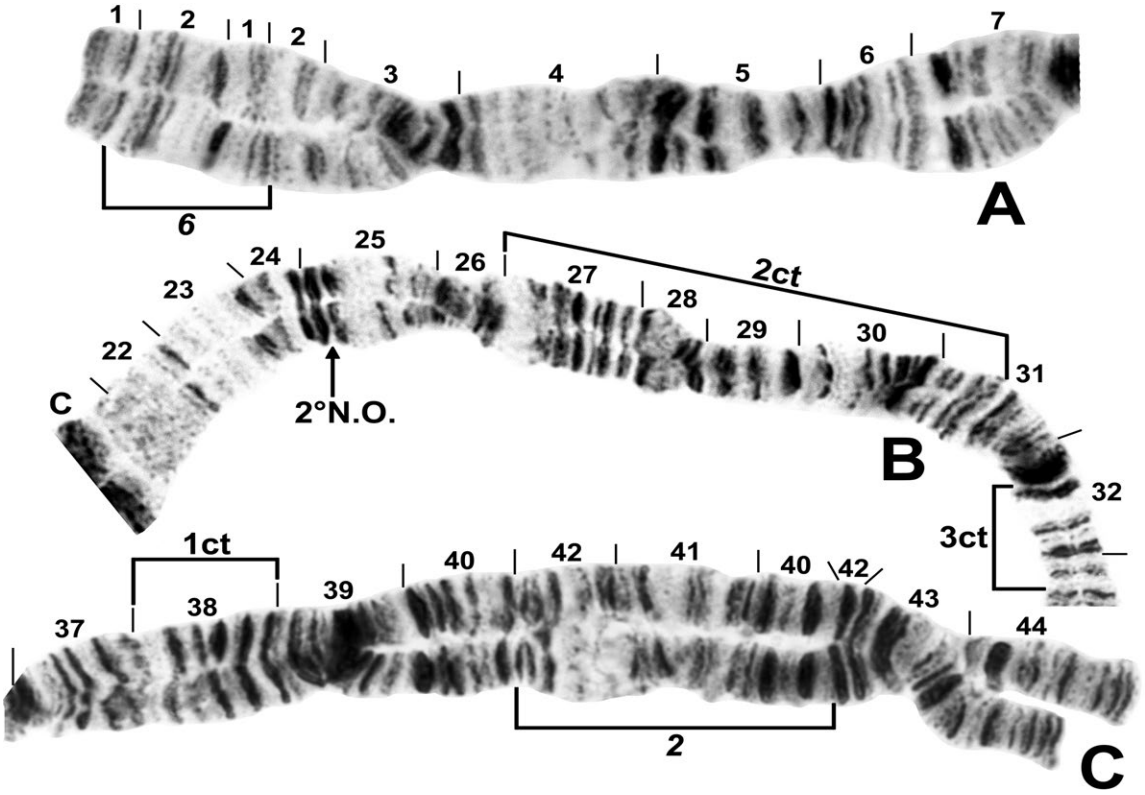

Fig. 1. Chromosome I of *Simulium* species. A – IS end of *S. carthusiense*, showing *IS-6* sequence. B – IL base of *S. cryophilum* cytoform "A" (female larva from Vindrarp, Sweden), representing the standard sequence with the banding pattern identical to that of *S. carthusiense*; breakpoints of IL-2ct and IL-3ct are indicated by brackets, and the location of a secondary nucleolar organizer (2°N.O.ct) by an arrow; C = centromere. C – IL end of *S. carthusiense*, showing *IL-2* sequence; breakpoints of IL-1ct are indicated by brackets.

somally typical, Adler et al., 1999), the latter species representing the first record for Spain and a southward range extension of about 1500 km. Site 4 was an aggregate of hypocrenal spring brooks in the Sierra Nevada in the vicinity of the Andalusian site where Grenier & Dorier (1959) found *S. carthusiense*.

The polytene chromosomes and gonads of larvae were prepared using the Feulgen procedure (Rothfels & Dunbar, 1953; Adler et al., 2004). Representative chromosomes from female larvae collected at Site 2 (Figs 1, 2A, 3) and Site 4 (Fig. 4) and male larvae collected at Site 1 (Fig. 2B) were photographed on an Olympus BX40 compound microscope and assembled into maps, using Adobe® PhotoShop® Elements 8. Larval carcasses from which chromosomes were prepared, plus photographs of selected chromosome preparations, were deposited in the Clemson University Arthropod Collection.

Section numbering on our maps follows that of Brockhouse (1985). Chromosomal sequences of all 33 larvae were compared band-for-band with the standard map established by Brockhouse (1985) for the *S. vernum* group. All fixed and common rearrangements and supernumerary (B) chromosomes were photographed. Infrequent rearrangements – those in 3% or less of all homologues (Table 2) – were mapped, with their precise breakpoints and locations indicated by brackets and arrows (Figs 1–3). Numbering of novel, fixed inversions follows the sequence of Hunter (1987) and Seitz & Adler (2009); unique polymorphisms are identified with a

Table 1. Collection sites for chromosomally analyzed larvae of *Simulium carthusiense*.

Site No.	Location	Latitude, longitude	Elevation (m a.s.l.)	Date
1	AUSTRIA, Haindlkarbach	47°34′31″N 14°36′51″E	785	28 June 2013
2	AUSTRIA, Moderbach	47°46′31″N 15°06′13″E	985	27 June 2013
3	AUSTRIA, Rothausbach	47°46′36″N 15°05′41″E	980	27 June 2013
4	SPAIN, Fuente Alta, Sierra Nevada	37°06′20″N 03°24′27″W	2200	15 May 2010

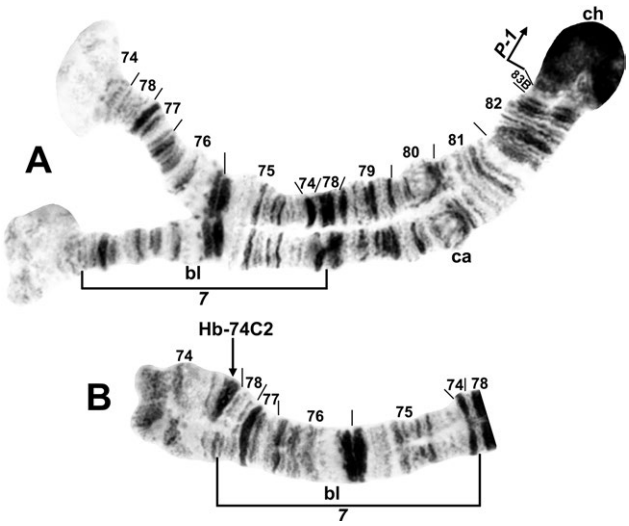

Fig. 2. IIIS of *Simulium carthusiense*. A – *IIIS-7* and *IIIP-1* sequences; bl = blister, ca = capsule, ch = chromocenter. B – IIIS end showing *IIIS-7* sequence and heterozygous expression of Hb-74C2 (arrow).

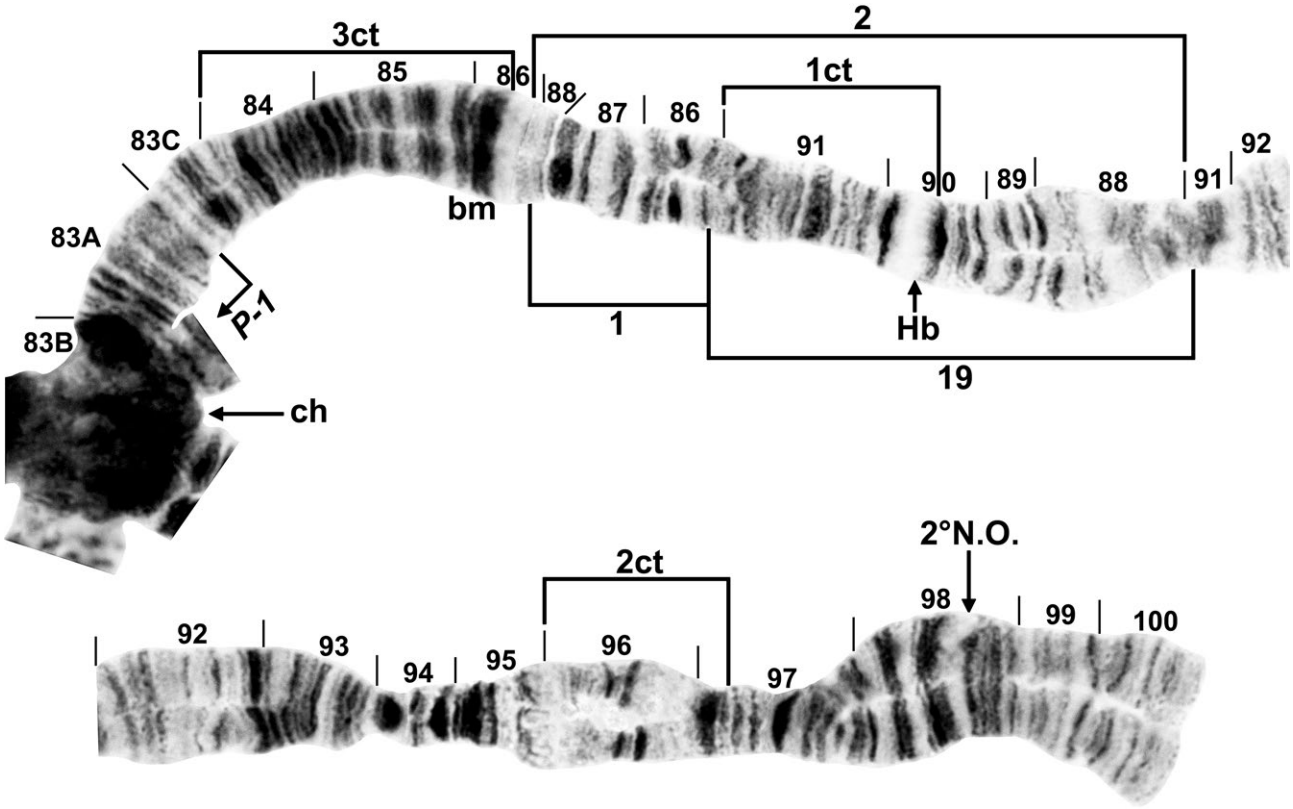

Fig. 3. IIIL of *Simulium carthusiense*, showing IIIL-19.1 (qq) and *IIIP-1* sequences. Breakpoints of IIIL-2 (t sequence), IIIL-1ct, IIIL-2ct, and IIIL-3ct are indicated by brackets, and locations of secondary nucleolar organizer (2°N.O.) and Hb-90C2ct (Hb) by arrows; bm = basal marker, ch = chromocenter.

Table 2. Frequency of all chromosomal rearrangements for *Simulium carthusiense* relative to the standard sequence for the *S. vernum* group.

	Site 1	Site 2	Site 3	Site 4
Female : Male	1 : 2	21 : 1	1 : 4	2[1] : 1
IS-6	1.00	1.00	1.00	1.00
IS 2°N.O.ct[2]		0.02		
IL-2	1.00	1.00	1.00	1.00
IL-1ct			0.10	
IL-2ct		0.02	0.20	
IL-3ct		0.02		
IL 2°N.O.ct		0.02		
IIIS-7	1.00	1.00	1.00	1.00
IIIS Hb-74C2	0.33	0.02	0.20	
III-P1	1.00	1.00	1.00	1.00
IIIL-19.1 (= q)	1.00	0.98	1.00	1.00
IIIL-2 (= t)		0.02		
IIIL-1ct		0.02		
IIIL-2ct		0.02		
IIIL-3ct		0.02		
IIIL 2°N.O.ct		0.02		
IIIL Hb-90C2ct		0.02		
B chromosomes				0.33
Chromocenter	1.00	1.00	1.00	1.00

[1] 1 female larva infected with the microsporidium *Amblyospora* possibly *bracteata*.

[2] Secondary nucleolar organizer (2°N.O.) in section 17 at the A/B junction of Brockhouse (1985).

species modifier (ct); thus, ct = *S. carthusiense* (e.g., IIIL-1ct). Italicized rearrangements were fixed; nonitalicized rearrangements were polymorphic. Terminology for B chromosomes follows that of Brockhouse et al. (1989).

To examine the evolutionary relationships of *S. carthusiense*, we compared its banding patterns with the sequences of related

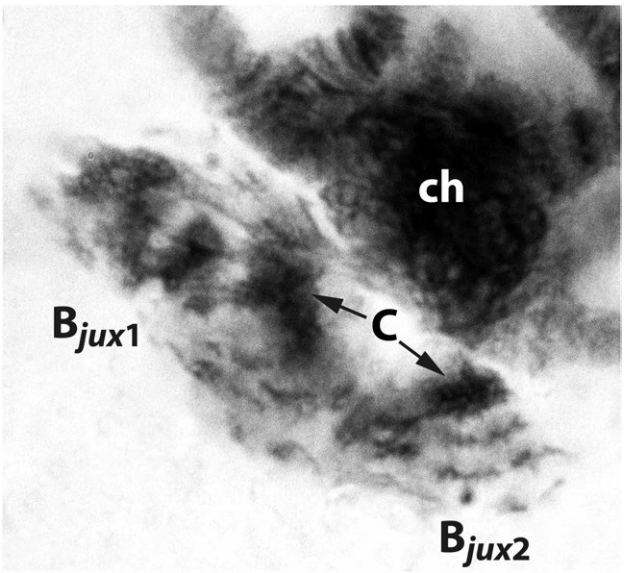

Fig. 4. Two types of supernumerary chromosomes (B_{jux1} and B_{jux2}) of *Simulium carthusiense*, attached by their centromeres (C) to the chromocenter (ch) of the 3 typical chromosomes.

species in the *S. vernum* group, specifically those species that share the pericentric *IIIP-1* fixed inversion first recognized by Hunter (1987). Where possible, we compared band sequences of these species with the relevant sequences in two outgroups, the *S. canonicolum* group (Golini & Rothfels, 1984) and *S. ruficorne* Macquart, 1838 (Bedo, 1989). Our cytophylogeny, therefore, is based on unique, shared chromosomal rearrangements derived from a common ancestor.

RESULTS AND DISCUSSION

Chromosomal characteristics

All larvae (25 females, 8 males) had the typical haploid complement of $n = 3$ chromosomes found in about 96% of the more than 500 cytologically examined species of simuliids (Adler et al., 2010). The chromosomal homologues were paired along 76–85% of their lengths, comparable to the degree of pairing seen in other temperate species of black flies in May and June (Rothfels & Featherston, 1981), and about three times the extent of pairing in *S. vernum* Macquart, 1826 (Adler et al., 1999). The three chromosomes were consistently united at their centromeres in a large, darkly staining chromocenter. More than 12% of all studied simuliids have a chromocenter (Adler et al., 2010), which aids rapid species diagnosis. The primary nucleolar organizer was located in the base of IS, the typical location for the *S. vernum* group (Brockhouse, 1985; Hunter & Connolly, 1986; Hunter, 1987). In addition to the homozygous primary nucleolus in all larvae, three secondary nucleolar organizers microscopically identical to the primary nucleolus occurred as rare heterozygotes – each in 1.5% of all homologues (Table 2). Secondary nucleolar organizers in the Simuliidae are frequent across species, but infrequent within species. Multiple nucleolar sites are postulated to occur in the genome, with one dominant to the exclusion of others in a species; incomplete suppression, however, might result in nucleolar expression at another site (Bedo, 1978).

All larvae were fixed for *IS-6*, *IL-2*, *IIIS-7*, and *IIIP-1* inversions (Figs 1–3). Thirteen additional rearrangements, all autosomal, were found among the 33 larvae (Table 2). IIIL-19.1 (q sequence of Hunter, 1987) was nearly fixed; one Moderbach female larva lacked IIIL-19.1 in one homologue, instead carrying IIIL-2 (t sequence of Hunter, 1987); thus, the larva was a IIIL-qt heterozygote. All but one of the 13 inversions were paracentric. Only *IIIP-1* was pericentric, resulting in shortening of the IIIS arm by about 6% and lengthening of the IIIL arm by about 2%. Three secondary nucleolar organizers, one heteroband (Hb-90C2ct), and six autosomal inversions were unique to *S. carthusiense*. Inversion heterozygosity per larva was low – 0.3 across all sites. Only three polymorphisms were found at more than one collection site: the nearly fixed IIIL-19.1 (Fig. 3); IIIS Hb-74C2 (Fig. 2B), which occurred heterozygously in two female and three male larvae; and IL-2ct (Fig. 1B), which was found at two sites less than 1 km apart. Of the two heterobands, only IIIS Hb-74C2 occurred in more than one larva. Heterobands, reflecting enhanced DNA content, are found in many species of the Simuliidae (Bedo, 1978), including other members of the *S. vernum* group (Hunter, 1987). None of the rearrangements in *S. carthusiense* were linked to the X or Y chromosomes; the sex chromosomes, therefore, were microscopically undifferentiated (X_0Y_0). About three-quarters of the 26 studied Palearctic species in the *S. vernum* group have undifferentiated sex chromosomes (Bass & Brockhouse, 1990; Ilmonen et al., 2009; Seitz & Adler, 2009).

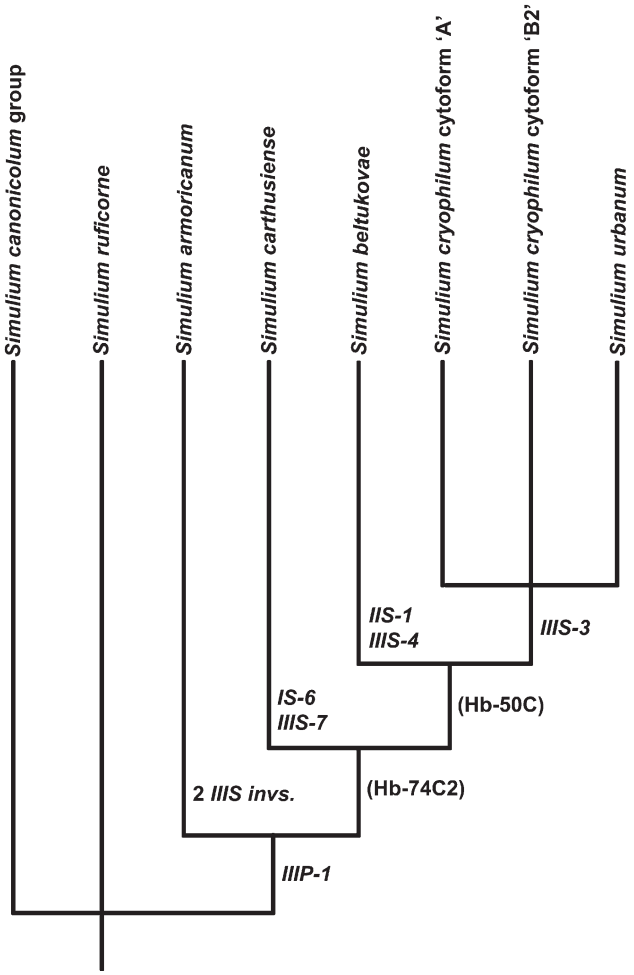

Fig. 5. Cytophylogeny of chromosomally studied members of the *S. vernum* group, which share the pericentric *IIIP-1* inversion of Hunter (1987), based only on uniquely shared rearrangements; parentheses indicate that the rearrangement is polymorphic; italics indicate that the inversion is fixed.

One female larva (Site 4) had polytenized supernumerary (B) chromosomes expressed as a large, flocculent mass. Two heavy centromere bands, each representing a distinct telocentric B chromosome, were attached to the chromocenter of the three chromosomes of the A complement (Fig. 4); the B chromosomes were detached from the chromocenter in about 10% of the nuclei. Thus, *S. carthusiense*, like *S. juxtacrenobium* Bass & Brockhouse, 1990 and *S. costatum* Friederichs, 1920, has two types of B chromosomes, one of which is equivalent to B_{jux2} and the other is either equivalent to B_{jux1} or unique (Brockhouse et al., 1989). The discovery of B chromosomes in *S. carthusiense*, which are shared with several other members of the *S. vernum* group (Brockhouse, 1985; Brockhouse et al., 1989), indicates a deeper common ancestry for the

Bs. Rothfels (in Brockhouse et al., 1989) suggested that all known B chromosomes in the family Simuliidae share a common origin.

We conclude that our samples of *S. carthusiense* represent a single species chromosomally distinct from all other cytologically analyzed species of the *S. vernum* group.

Phylogenetic relationships

IS-6 and *IIIS-7* are unique to *S. carthusiense*, and both sequences are derived, relative to the ancestral condition in our outgroups. *IL-2* and IIIL-19 are shared with many members of the *S. vernum* group in the Holarctic Region, whereas *IIIP-1*, IIIL-1, and IIIL-2 (Fig. 3) are shared with a restricted group of European species, including *S. armoricanum* Doby & David, 1961, *S. bavaricum* Seitz & Adler, 2009, *S. beltukovae* (Rubtsov, 1956) – chromosomally studied as *S. carpathicum* (Knoz, 1961) – the *S. cryophilum* complex, and *S. urbanum* Davies, 1966 – chromosomally studied as "B1" – (Hunter, 1987; Seitz & Adler, 2009). Of these five inversions, only *IIIP-1* could be determined by outgroup comparison as derived from the ancestral sequence; the remaining four inversions were in areas of the complement too scrambled for reliable comparison with outgroup sequences. We determined that all previously published rearrangements in IIS and IIIS (Hunter, 1987), which are used in our cytophylogeny, are derived from the ancestral condition (Fig. 5). IIIS Hb-74C2 of *S. carthusiense* is shared with *S. beltukovae*, *S. cryophilum* cytoforms "A" and "B2", and *S. urbanum* (Hunter, 1987). *Simulium carthusiense* is the sister species of a clade united by IIS Hb-50C of Hunter (1987), which includes *S. beltukovae*, the *S. cryophilum* complex, and *S. urbanum*.

Although the entire *S. vernum* group represents a well-defined clade based on structural characters, particularly the unique shape of the gonostylus, structural characters for inferring relationships within the group are limited (Adler et al., 2004). Our phylogeny reveals a clade of closely related species within the group, defined by the unique pericentric *IIIP-1* inversion, demonstrating that chromosomal characters can provide species-level resolution of relationships. Unlike a majority of closely related black flies (Rothfels, 1989), however, differentiation of sex chromosomes has not played a role in the evolution of the *IIIP-1* clade; only *S. armoricanum* has differentiated sex chromosomes (Bass & Brockhouse, 1990). The *IIIP-1* clade is biogeographically cohesive; all species are found in Western Europe, and are concentrated in the area from Spain to Germany.

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