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REPRODUCTIVE STATUS AND CONTINUITY OF TAXA OF THE *SIMULIUM ARCTICUM* COMPLEX (DIPTERA: SIMULIIDAE) AT THE CLEARWATER RIVER, MONTANA (2007, 2008, AND 2009)

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ABSTRACT.—We made 27 collections over nine years at the Clearwater River and conducted preliminary cytogenetic analysis on 232 individuals of the *Simulium arcticum* complex there. Based on these preliminary findings, we analyzed an additional 1825 larvae from Clearwater collections in 2007, 2008, and 2009 to investigate the reproductive statuses of the two prevalent taxa of the complex there and to determine whether cytogenetic diversity and frequency of types were similar from year to year. We describe a cytotype new to science and a potentially derivative cytotype of *S. arcticum* sensu stricto. We also determine that these two taxa are present in similar frequencies from year to year and are in genetic equilibrium, suggesting that they are not reproductively isolated. This study constitutes the fourth positive test of our Geographic Distribution/Taxon Age hypothesis.

Key words: black flies, cytogenetics, sex-linked inversions, divergence, genetic equilibrium, natal site fidelity.

The single morphospecies of black fly (Diptera: Simuliidae) of classical taxonomy usually reveals itself as any number of sibling species that are cytogenetically differentiable when polytene chromosomes of larvae are analyzed (Rothfels 1956, Shields and Procunier 1982, Adler 1987, Rothfels 1987). The existence of siblings among what were initially considered single morphospecies would have gone unrecognized were it not for cytogenetic analyses (Rothfels 1989). In most cases, differentiation may be augmented by the establishment of novel chromosomes that carry sex-linked, paracentric inversions (Shields and Procunier 1982, Adler et al. 2004). This pattern has been observed in numerous species complexes of black flies, including *Prosimulium hirtipes* (Rothfels 1956), *Twinnia* spp. (Rothfels and Freeman 1966), *Simulium pictipes* (Bedo 1975), *S. venustum*/*S. verecundum* (Rothfels et al. 1978), *S. vittatum* (Rothfels and Featherston 1981), *S. arcticum* (Shields and Procunier 1982), *Cnephia* (Procunier 1982), *Prosimulium* (*Helodon*) *onychodactylus* (Newman 1983), *Eusimulium pugetense* (Allison and Shields 1989), *E. aureum* (Leonhardt and Feraday 1989), and *S. tuberosum* (McCreadie et al. 1995).

At least 9 sibling species have been described in the *Simulium arcticum* complex (Shields and Procunier 1982, Adler et al. 2004). Additionally, we have described 20 cytotypes, taxa having unique paracentric inversions on their Y chromosomes (Shields et al. 2007a, 2007b, Shields unpublished data). The presence of an apparently derived type of the IIL-3 *S. arcticum* sensu stricto sibling and the newly discovered cytotype, *S. arcticum* IIL-22, at the Clearwater River, Missoula County, Montana, encouraged additional study. Since the phenomenon of sex-linked inversions associated with diverging taxa is so widespread in simulids, we reasoned that we might shed light on the mechanisms of differentiation in this diverse group of flies by investigating the reproductive statuses of these 2 taxa in sympatry.

In the present study, we monitored the Clearwater site for chromosomal diversity among 27 collections of larvae made from 2000 to 2009. We initially described cytogenetic diversity among 232 individuals from 8 dates and conducted a detailed analysis of an additional 579, 575, and 686 larvae from the same site in 2007, 2008, and 2009, respectively. Since observations on the year-to-year fidelity of gravid females to egg-laying sites are difficult to obtain (McCreadie et al. 1997, Hunter and Jain 2000), we compared the types and frequencies of sex chromosome and autosomal inversions for all three years. Finally, we determined whether the IS-1 autosomal

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TABLE 1. Preliminary cytogenetic analysis of taxa in the *Simulium arcticum* complex at the Clearwater River.

Date	Females	Males								Total
	X_0X_0	$X_0Y_{IIL\text{-}3,\ 23}$	$X_0Y_{IIL\text{-}3,\ 23,\ 24}$	$X_0Y_{IIL\text{-}22}$	$X_0Y_{IIL\text{-}22,\ 24}$	$X_0Y_{IIL\text{-}22,\ 23,\ 24}$	$X_0Y_{IIL\text{-}9}$	$X_0Y_{IIL\text{-}13}$	$X_0Y_{IIL\text{-}19}$	
28 February 2003	2	0	0	0	0	0	0	0	4	6
14 March 2006	5	0	0	0	0	0	3	0	2	10
30 March 2003	1	0	8	0	0	0	0	0	0	9
16 April 2001	4	1	3	0	2	1	0	0	0	11
20 April 2003	31	9	37	1	11	12	0	0	0	101
8 May 2006	29	4	20	0	4	0	0	0	0	57
18 May 2003	20	0	6	0	0	0	0	0	0	26
11 June 2000	6	0	0	0	0	0	0	4	0	10
2 July 2004	2	0	0	0	0	0	0	0	0	2
Total	100	14	74	1	17	13	3	4	6	232

polymorphism was randomly distributed between the two prevalent taxa of the complex for each year and all years combined. Since *S. arcticum* IIL-3 is geographically widespread (Adler et al. 2004) and since *S. arcticum* IIL-22 had been found previously only at the Clearwater River (Shields unpublished data), we hypothesized that the 2 taxa would not be reproductively isolated.

If chromosome change acts as an isolating barrier in speciation, comparative analyses at different stages of divergence are important to document (Coyne and Orr 2004). We believe that the present study provides the opportunity to investigate the transition from coexistence of interbreeding taxa to reproductive isolation (i.e., speciation).

METHODS

Larvae were removed with forceps from submergent trailing vegetation, branches, and large boulders from the Clearwater River, Missoula County, Montana (47°00′03″N, 113°22′94″W), and immediately fixed in freshly prepared acetic ethanol (1 volume glacial acetic acid to 3 volumes 200-proof ethyl alcohol). Collection vials (45 mL) were filled with larvae only to one-third capacity and held at wet-ice temperature (4 °C) in a portable cooler until our return to the laboratory. The initial fixative was replaced with fresh, cold acetic alcohol until the supernatant remained clear (usually after 4 changes). In the laboratory, larvae were sorted to morphospecies (Currie 1986) and stored in acetic alcohol at –20 °C until stained. We used the Feulgen method (Rothfels and Dunbar 1953) to stain polytene chromosomes and gonads of each larva, and we used the chromosome maps of the *S. arcticum* complex (Shields and Procunier 1982) to describe variation among individuals. We specifically studied the linear banding sequences of chromosomes IIL and IS, the latest stage of meiosis in males, the presence or absence of b chromosomes, and the polymorphism for centromere bands in chromosome II. We documented frequencies of the 3 genotypes for the IS-1 autosomal polymorphism and used the χ^2 statistic to test for equilibrium among the IIL-3 and IIL-22 larvae. We also tested for association of Y-linked inversions for all 3 years. Finally, we used a contingency analysis and the χ^2 statistic to determine whether the categories and

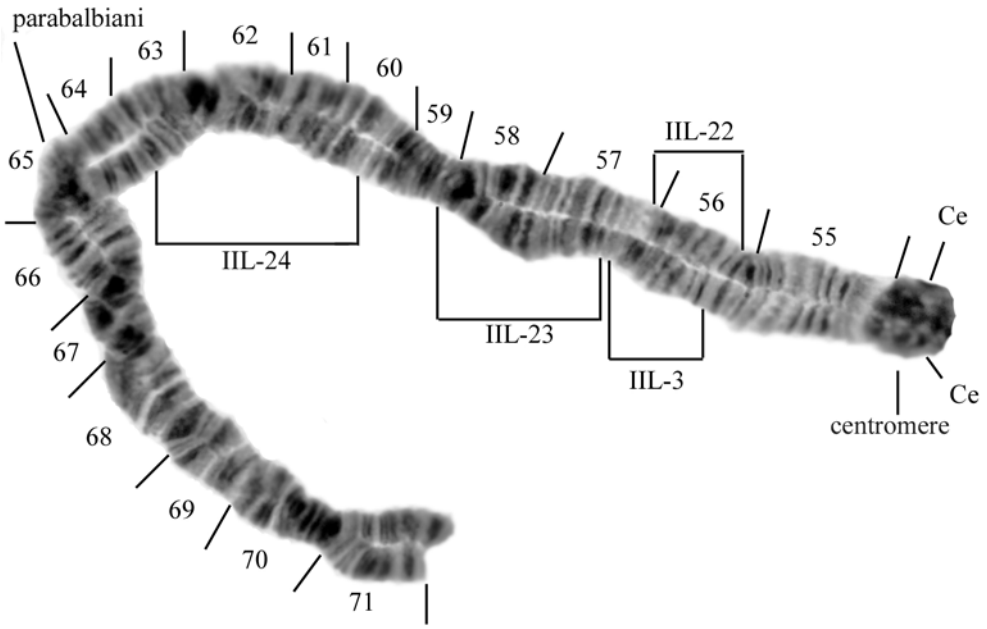

Fig. 1. The long arm of chromosome II of the *Simulium arcticum* complex indicating the IIL-3, IIL-22, IIL-23, and IIL-24 inversions. Brackets indicate the inclusive break points for the four inversions. Numbers above and below the chromosome indicate section limits (55–71). Ce = enhanced centromere band.

frequencies of the male genotypes were similar from year to year.

RESULTS

Preliminary cytogenetic analysis indicated small numbers of *S. arcticum* IIL-9 and IIL-19 in late February and March and *S. arcticum* IIL-13 in June. Abundant larvae of the IIL-3 and IIL-22 types (Figs. 1, 2, 3) occurred from mid-April to mid-May (Table 1). Since the former three taxa occurred only in small numbers, they were not considered further in this report.

Frequencies of IIL-3 and IIL-22 Types

We analyzed 579, 575, and 686 larvae of the *S. arcticum* complex in 2007, 2008, and 2009, respectively (Table 2). Because the 700 females in the total sample possessed the standard chromosome sequence, they could not be cytogenetically identified to taxon. The ratio of males to females differed from year to year (1.22:1 in 2007, 1.61:1 in 2008, and 2.05:1 in 2009). For each larva of the IIL-22 type, there were about 4 larvae of the IIL-3 type (Table 2). Nonetheless, we assumed that the frequency of the former taxon was sufficient to allow for tests of equilibrium (Table 3). Nearly all (99.7%) of the IIL-3 larvae also possessed either the IIL-23, the IIL-24 inversion, or both, while 69.3% of IIL-22 larvae possessed either the IIL-23 or the IIL-24 inversion, or both; thus, we specifically categorized each of these types for clarity of analysis (Fig. 1, Table 2).

Each type of male was present in each year and in remarkably similar frequencies in categories having large numbers of individuals (Table 2). All female larvae were homozygous for enhanced (Ce) centromere bands (Fig. 1) while all males had one enhanced and one thin (Ct) centromere band (Fig. 3). Ten male larvae possessed acrocentric b chromosomes: two IIL-3, 23, 24 larvae had two b's while one had one b; three IIL-3, 23 larvae had one b; one IIL-3, 23 larva had two b's; one IIL-22 larva had one b; and one IIL-22, 24 larva also had one b. One IIL-9 larva had one b, and a IIL-3, 23, 24 male larva from the 20 April 2003 collection had 4 acrocentric b chromosomes (data not shown).

Some genotypes (e.g., YIIL-22; YIIL-22, 23; and YIIL-22, 24) were significantly different from year to year ($\chi^2 = 50.9$, df = 14,

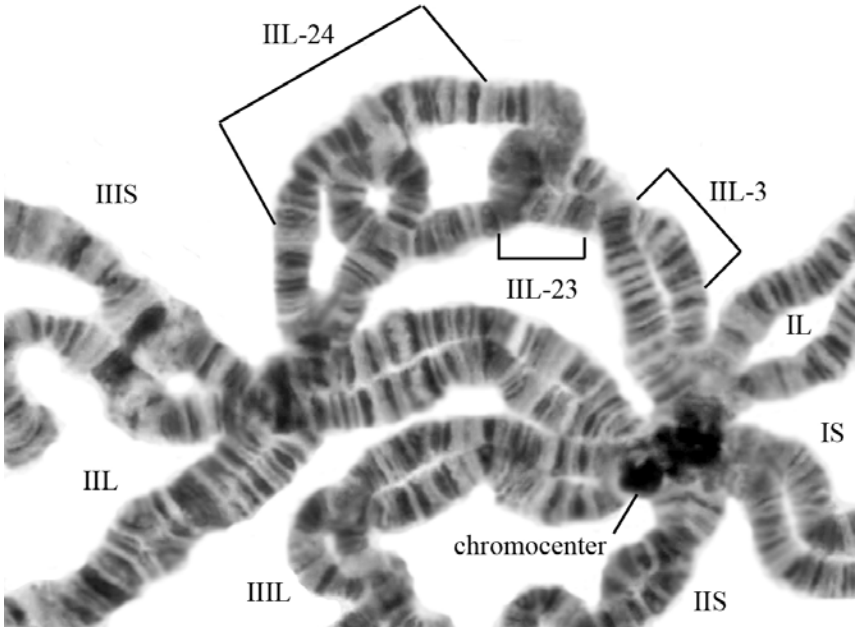

Fig. 2. Portions of the entire genome of a IIL-3, IIL-23, IIL-24 inversion heterozygote of *Simulium arcticum.* Supposedly, the IIL-23 and IIL-24 inversions are long enough to form reverse inversion loops while the IIL-3 inversion is not. The centromere is heterozygotic for the centromere dimorphism (Ce/Ct, where Ce = enhanced centromere band and Ct = thin centromere band).

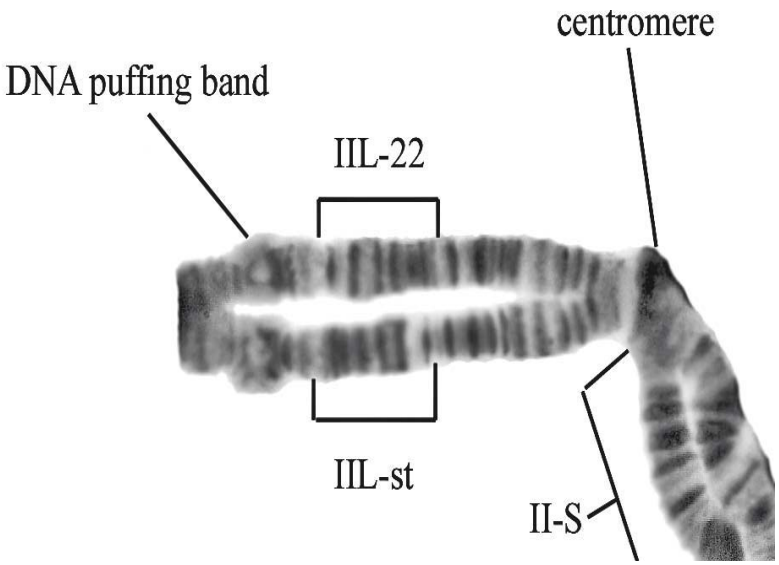

Fig. 3. Base of the IIL chromosome arm showing a IIL-22 heterozygote. As with IIL-3, the IIL-22 inversion may be too small to form a reverse inversion loop.

TABLE 2. Percentages (actual numbers in parentheses) of IIL-3 and IIL-22 sex chromosome types among two taxa of the *Simulium arcticum* complex, Clearwater River, Missoula County, Montana, 2007–2009. Total sample size was 1825 (700 for females and 1125 for males).

		Females	IIL-3 males (% of all males)					IIL-22 males (% of all males)				
Year	*n*	X_0X_0	$X_0Y_{IIL\text{-}3}$	$X_0Y_{IIL\text{-}3,\ 23}$	$X_0Y_{IIL\text{-}3,\ 24}$	$X_0Y_{IIL\text{-}3,\ 23,\ 24}$	Total IIL-3	$X_0Y_{IIL\text{-}22}$	$X_0Y_{IIL\text{-}22,\ 23}$	$X_0Y_{IIL\text{-}22,\ 24}$	$X_0Y_{IIL\text{-}22,\ 23,\ 24}$	Total IIL-22
2007[a,b]	568	45.1 (256)	0.6 (2)	24.7 (77)	0.3 (1)	54.2 (169)	79.8	0.3 (1)	3.8 (12)	14.7 (46)	1.3 (4)	20.2
2008[c]	571	38.4 (219)	0.9 (3)	22.7 (80)	0.3 (1)	55.7 (196)	79.5	3.6 (13)	2.0 (7)	14.9 (51)	0.2 (1)	20.5
2009[d]	686	32.8 (225)	0.9 (4)	24.9 (115)	0	55.3 (255)	81.1	6.9 (32)	0.4 (2)	11.5 (53)	0	18.9

[a]All data for 2007 are based on a single collection on 21 April 2007. Because peak development of *S. arcticum* larvae was somewhat later in 2008, collections were made on 17 April 2008, 27 April 2008, and 3 May 2008 and the data for those dates were pooled.

[b]In 2007, one each of *S. negativum*$_{IIL\text{-}3+4}$, *S. apricarium*$_{IIL\text{-}7}$, *S. arcticum*$_{XIIL\text{-}22\ YIIL\text{-}3,\ 23,\ 24}$, and *S. arcticum*$_{XIIL\text{-}3\ YIIL\text{-}3,\ 23,\ 24}$, and three *S. arcticum*$_{IIL\text{-}9}$ males were observed. Two triploid males ($X_{IIL\text{-}st}Y_{IIL\text{-}3,\ 23,\ 24}Y_{IIL\text{-}3,\ 23,\ 24}$ and $X_{IIL\text{-}st}Y_{IIL\text{-}3,\ 23}Y_{IIL\text{-}3,\ 23}$) and a triploid female ($X_{IIL\text{-}st}X_{IIL\text{-}st}X_{IIL\text{-}st}$) were also observed.

[c]In 2008 two of the males were IIL-3 *i/i* homozygotes. A $X_{IIL\text{-}st}X_{IIL\text{-}st}Y_{IIL\text{-}3,\ 23,\ 24}$ triploid male was also observed.

[d]All data for 2009 are based on a single collection on 1 May 2009. Four *S. arcticum*$_{IIL\text{-}9}$ males, one *S. arcticum*$_{IIL\text{-}19}$ male, one *S. arcticum*$_{IIL\text{-}20}$ male, and one IIL-st/st male were also observed.

$P < 0.001$). However, for genotypes having sample sizes greater than 60 (IIL-3, 23 and IIL-3, 23, 24) there was no significant difference across years (Fig. 4). The number of individuals of each genotype was significantly different for each year ($P < 0.001$ for 2007, 2008, and 2009; Fig. 5); for example, IIL-3, 23; IIL-3, 23, 24; and IIL-22, 24 types were most abundant while IIL-3, 24 and IIL-22, 23, 24 types were exceedingly rare.

Assessment of Reproductive Isolation

Based on the genotype frequencies of the IS-1 autosomal polymorphism (Fig. 6, a–c) the IIL-3 and IIL-22 types were in genetic equilibrium (2007: $\chi^2 = 0.97$, df = 2, $0.7 > P > 0.6$; 2008: $\chi^2 = 0.002$, df = 2, $P = 1$; 2009: $\chi^2 = 0.89$, df = 2, $0.8 > P > 0.7$; all 3 years: $\chi^2 = 0.891$, df = 2, $0.8 > P > 0.7$; Table 3).

DISCUSSION

A Variant of a Widespread Sibling

The sibling species *S. arcticum* (IIL-3) was initially described on cytogenetic grounds by Shields and Procunier (1982) based on material from the Canadian provinces of British Columbia and Alberta. Adler et al. (2004) subsequently described IIL-3 as *S. arcticum* sensu stricto and reported its geographic distribution (p. 815) from central Alberta south to southern British Columbia and from central Idaho to northwestern Wyoming and western Montana. *Simulium arcticum* sensu stricto is the most widespread and abundant of 17 taxa of the *S. arcticum* complex from 55 sites in western Montana (Shields unpublished data). Yet the IIL-3, 23, 24 variant described here has been found abundantly only at the Clearwater River along with only two individuals (among 272 ♂♂) at the nearby Blackfoot River. Given the exceedingly limited distribution of IIL-3, 23, 24 and the fact that only nine of the 903 male types studied here were classic IIL-3 (neither IIL-23, IIL-24 nor both), it may be that *S. arcticum* IIL-3, 23, 24 is derived from a IIL-3 ancestor and has evolved at the Clearwater River or at some location nearby.

A Cytotype New to Science

Like the IIL-3, 23, 24 type, the IIL-22, 23, 24 cytotype has been found only at the Clearwater River and at the nearby Blackfoot River (two individuals among 272 ♂♂). This extremely

TABLE 3. Test of conformity to Hardy-Weinberg equilibrium for the *Simulium arcticum* IS-1 autosomal inversion among IIL-3 and IIL-22 types at the Clearwater River.

Date		Structural types st/st	st/i	i/i	χ^2	P (df = 2)
2007	observed	197	287	88	0.971	$0.7 > P > 0.6$
	expected	202.5	275.7	93.8		
2008	observed	152	288	135	0.002	1
	expected	152.3	287.5	135		
2009	observed	222	347	119	0.830	$0.8 > P > 0.7$
	expected	223.6	337.3	127.3		
Total	observed	571	922	342	0.891	$0.8 > P > 0.7$
	expected	576.2	904.3	355.3		

limited geographic distribution also argues for the origin of IIL-22 types at or near the Clearwater River.

Cytogenetic evidence suggests that the IIL-22 inversion itself may be derived from a IIL-3 ancestor. The distal break point for the two inversions appears to be identical (Fig. 1), while the proximal break point for the two inversions differs by only one major band (Fig. 1). Both taxa possess b chromosomes of similar morphology in the male germ line. Similarity of inversion break points, sharing of the IIL-23 and IIL-24 inversions, near-identical equilibrium frequencies for the IS-1 autosomal polymorphism, and possession of similar b chromosomes all argue for a very close relationship between IIL-3 and IIL-22 types at the Clearwater River.

Fidelity to Natal Site

Descriptions of geographic distributions of taxa of black flies assume taxon stability at a site from year to year (Rothfels and Featherston 1981, Shields et al. 2007a, Shields et al. 2007b). Until now, this assumption has never been rigorously tested. Based on the three years of observations at the Clearwater River, this assumption is supported. The Geographic Distribution/Taxon Age hypothesis of Shields et al. (2007b) is also an extension of this assumption. That is, taxa with widespread distributions are hypothesized to be evolutionarily older than those with limited geographic distributions. It is reassuring to know that taxon diversity is similar from year to year at any collection site rather than being random.

Species assemblages among physically similar streams in small geographic areas appear to be unpredictable (McCreadie et al. 1997). Whether radiolabeled individuals return to their natal sites is unclear (Hunter and Jain 2000). Until this study, year-to-year cytogenetic analysis of larvae based on large sample sizes from the same site was nonexistent (Shields and Procunier 1982, Adler et al. 2004). The present cytogenetic analysis of more than 1825 individuals from the Clearwater River is unparalleled in detail and indicates remarkable similarity for all three years, not only of types present but also their frequencies and linkage groups. While we cannot assume that the same females that emerge at the Clearwater are depositing their eggs there after mating, our data suggest that the genetic contribution for each year at the Clearwater is remarkably similar. Similarities in the frequencies of the eight Y chromosome genotypes from 2007 to 2009 suggest that each Y chromosome is being transmitted and inherited in a similar way from year to year. Though meiosis is chiasmate in the *S. arcticum* complex (Shields and Procunier 1982), it appears that there is little to no recombination between X and Y chromosomes of males.

Assessment of Reproductive Status of Taxa

As stated above, the Geographic Distribution/Taxon Age hypothesis (Shields et al. 2007b) posits that taxa with large geographic distributions will be reproductively isolated in sympatry, while those with limited distributions will not. Our observations at the Clearwater River suggest that IIL-3 and IIL-22 types are not reproductively isolated. Thus, we accept our original hypothesis that the two taxa would not be reproductively isolated. This constitutes the fourth positive test of the Geographic Distribution/Taxon Age hypothesis. Similarly, the *arcticum* siblings *S. arcticum* s. s. and *S. apricarium* (IIL-7) were reproductively isolated at Little Prickly Pear Creek, the sibling *S. negativum* (IL-3•4) and the cytotype IIL-9

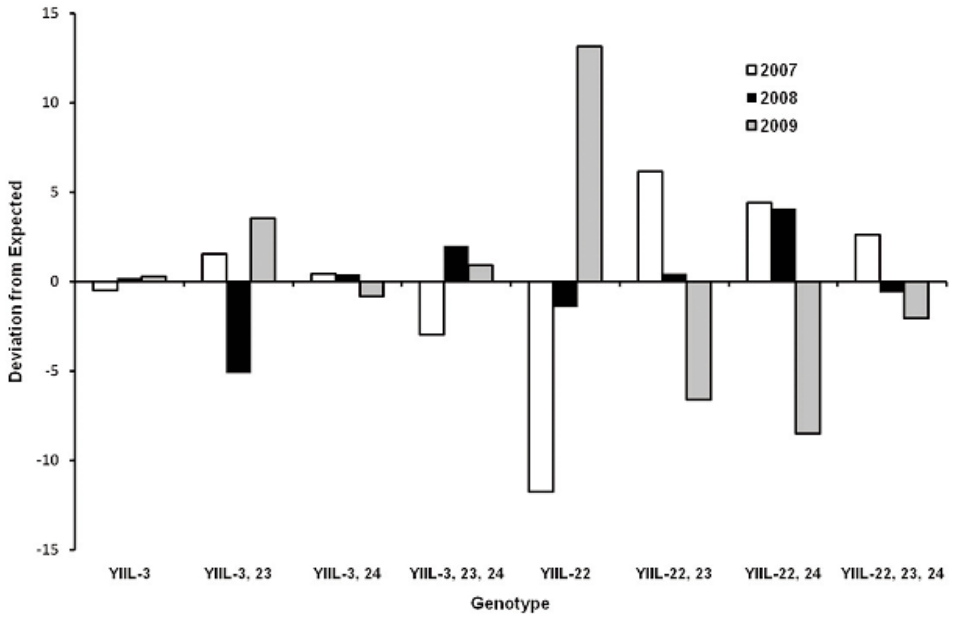

Fig. 4. Deviations from expected values testing for differences in genotypic frequencies across years. As a rule of thumb, deviations from expected values of <5 are not considered significant. Some genotypes (e.g., YIIL-22; YIIL-22, 23; and YILL-22, 24) are significantly different between years ($\chi^2 = 50.9$, df = 14, $P < 0.001$). However, for genotypes with yearly sample sizes >60, there is no significant difference across years.

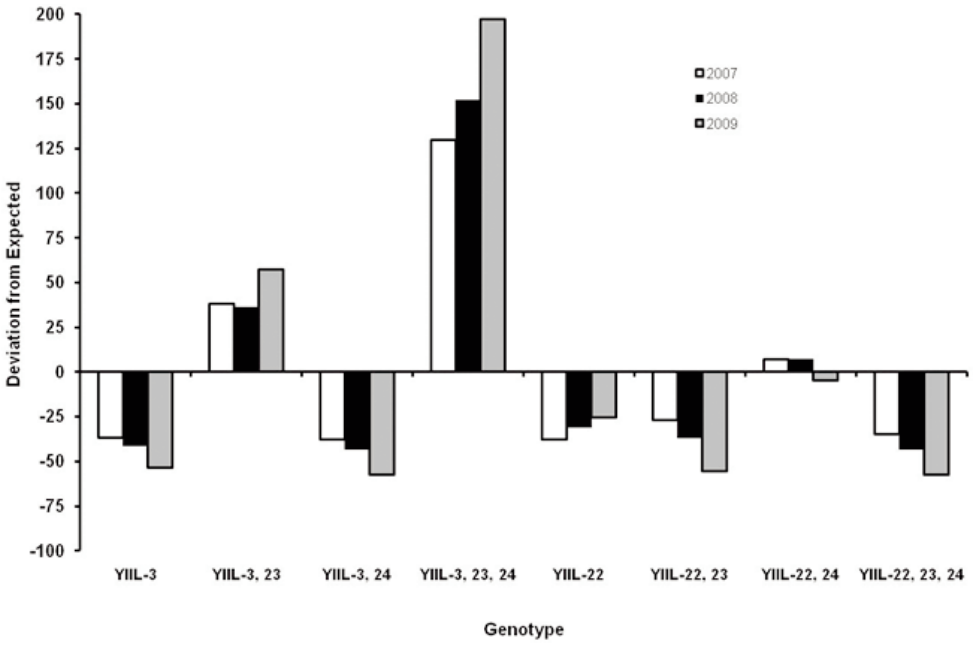

Fig. 5. Deviations from expected values testing for differences in the number of individuals between each genotype for each year. The number of individuals is significantly different between genotypes for each year ($P < 0.001$ for 2007, 2008, and 2009).

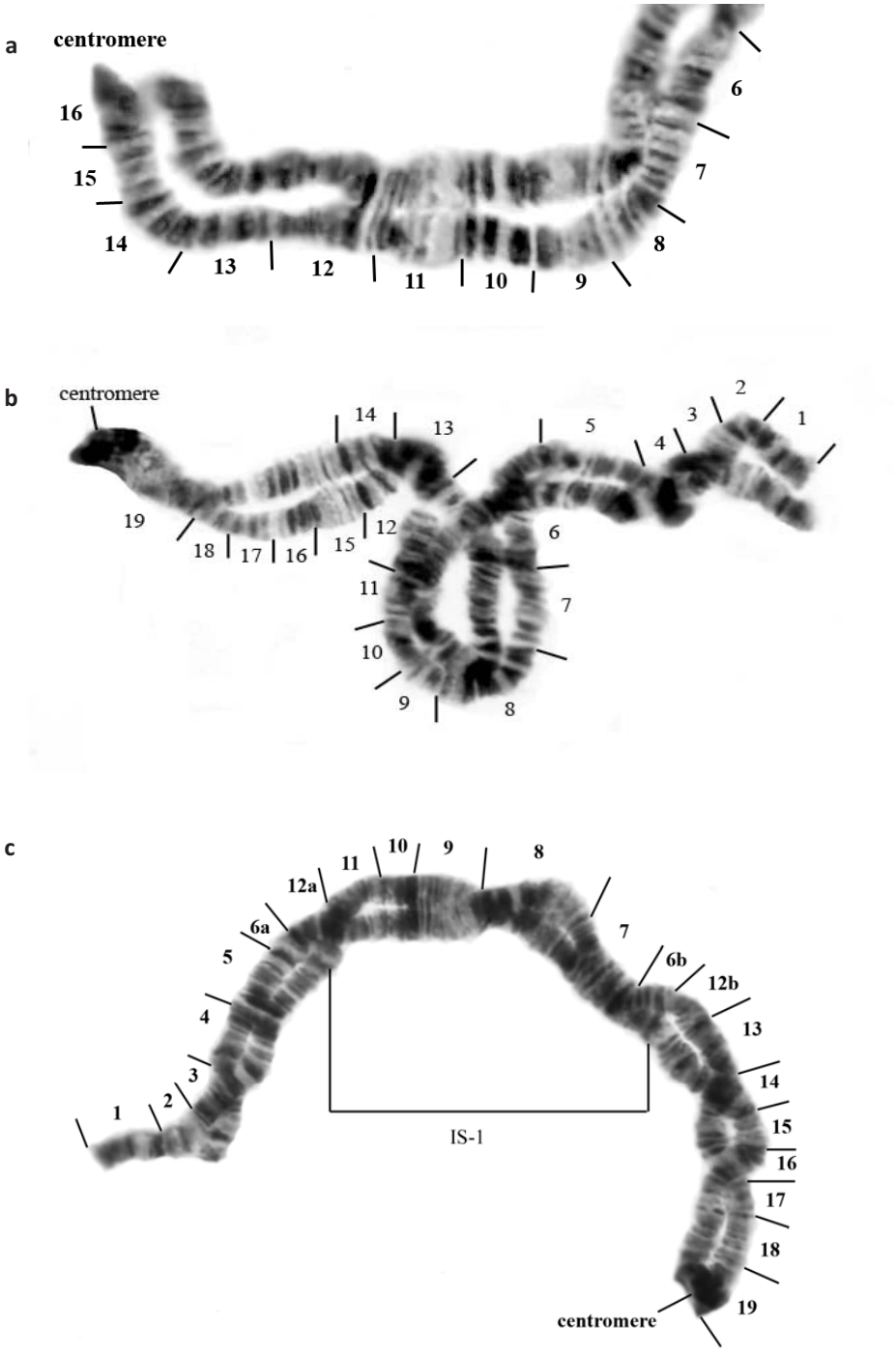

Fig. 6. The short arms of chromosome I of the *Simulium arcticum* complex: **a,** the standard, noninverted sequence for the IS-1 inversion. The centromere is to the left of the figure, and the section numbering corresponds to the standard map for IS in the *S. arcticum* complex (Shields and Procunier 1982); **b,** the IS-1 inversion heterozygote (section numbers correspond to the standard, noninverted sequence); **c,** the inverted homozygote of the IS-1 inversion. Note that distal and proximal break points for the inversion occur at the 6a–12a and 6b–12b section boundaries, respectively.

were reproductively isolated at the Blackfoot River, and the 2 cytotypes IIL-9 and IIL-19 were not reproductively isolated at Rock Creek (Shields et al. 2007b). Thus, in 4 comparative tests, we have never observed 2 reproductively isolated cytotypes in sympatry. Alternatively, all taxa having large geographic distributions have been reproductively isolated from one another when we have studied them in sympatry.

Sex-Associated Inversions and Y-Linked Autosomal Polymorphisms

Linkage to the Y chromosome for the IIL-3, IIL-22, IIL-23, and IIL-24 inversions was nearly complete with only one XIIL-22 YIIL-3, 23, 24 male and two XIIL-3 YIIL-3, 23, 24 males being observed. The Y chromosomes in the IIL-3 and IIL-22 types were most abundantly either IIL-3, 23 or IIL-3, 24, or both (99.2%) or IIL-22, 23 or IIL-22, 24 or both (79.3%). Moreover, we did not observe inverted homozygotes for either the IIL-23 or IIL-24 polymorphisms at the Clearwater River; nor did these inversions occur in females. That these inversions may be atypical may be more than idle speculation, because in our analyses of more than 12,000 larvae since 2000, only at Wise River did we commonly find more than one sex-linked inversion on any Y chromosome. However, McCreadie et al. (1995) found Y chromosomes with multiple inversions in *S. conundrum* on the Avalon Peninsula, Newfoundland, as did Rothfels and Featherston (1981) in *S. vittatum* across Canada. Nonetheless, we interpret the associations of IIL-23 and IIL-24 Y-linked inversions with IIL-3 and IIL-22 types at the Clearwater River to suggest that these unique inversions may be in a preliminary stage of association with previously existing Y chromosomes.

Inversions in Sex Chromosomes and Autosomes

Rothfels (1989) first observed that a sex-linked inversion in one sibling might be an autosomal inversion in another sibling. A case in point occurs in our studies. The IS-1 inversion is clearly autosomal in *S. arcticum* larvae at the Clearwater River since it occurs in roughly equal frequencies in males and females. However, within the *S. negativum* (IL-3•4) population at the nearby Blackfoot River, 81% of males were heterozygotes for the IS-1 inversion (Shields et al. 2007b). Thus, there may be 2 Y chromosomes, IL-3•4 and IL-3•4 + IS-1, in the *S. negativum* population at the Blackfoot River. This situation and the one at the Clearwater River where IIL-23 and IIL-24 inversions are associated with the Y chromosome in frequencies higher than expected, may be cases of coadaptive complexes that become reproductively isolated over time (Rothfels 1989).

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