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THE SIGNIFICANCE OF SEX-LINKED CHROMOSOMAL INVERSIONS IN THE SPECIATION PROCESS OF THE *SIMULIUM ARCTICUM* COMPLEX OF BLACK FLIES (DIPTERA: SIMULIIDAE)

Gerald F. Shields

ABSTRACT.—This study summarizes cytogenetic variation, particularly sex-linked chromosomal inversions which define taxa of the *Simulium arcticum* complex (Diptera: Simuliidae) in western Montana and portions of northern Idaho, Washington, and Oregon. Variation in polytene chromosomes was determined for nearly 15,000 larvae from 234 collections taken from 58 freshwater sites. The previously described siblings, *S. apricarium, S. arcticum* sensu stricto, *S. brevicercum,* and *S. saxosum,* were most numerous, while all cytotypes, with the exception of IIL-19, were found in low frequency. Additionaly, 6 new cytotypes in low frequency are described. Evidence suggests that the Y chromosome carries the testis-determining gene, and in almost all taxa of *S. arcticum,* complex paracentric inversions characterize these types. Distributions of (1) *S. brevicercum* and *S. arcticum* s. s., (2) *S. arcticum* s. s. and *S. arcticum* IIL-18, (3) *S. brevicercum* and *S. arcticum* IIL-18, and (4) *S. arcticum* IIL-9 and *S. arcticum* IIL-19 have highly significant positive geographic associations, while those of (1) *S. apricarium* and *S. brevicercum* and (2) *S. apricarium* and *S. saxosum* have highly significant negative geographic associations. The *S. arcticum* cytotypes IIS-12, IIL-17, IIL-21, IIL-22, IIL-38, IIL-51, IIL-68, IIL-73•74, and IIL-79 occur only at 2 or fewer locations. Polyploids (0.0007), pericentric inversions (0.00007) and chromosomal translocations (0.00007) are exceedingly rare. These observations and our recent DNA comparisons of chromosomally distinct types lead me to elaborate on a previously suggested model for chromosome evolution in black flies. In this model, locally distributed cytotypes may become more common with time, while widespread cytospecies may eventually become morphologically differentiated types. Contrary to the current understanding that chromosome variation may not play a significant role in the speciation process of most animals, this study suggests that chromosomal variation, at least in black flies, plays a significant role in speciation.

RESUMEN.—Este estudio resume la variación citogenética, en particular las inversiones de los cromosomas sexuales que definen a los taxa del complejo *Simulium arcticum* (Diptera: Simuliidae) en el oeste de Montana y partes del norte de Idaho, en el estado de Washington y en Oregon. Se determinó la variación en los cromosomas politénicos de aproximadamente 15,000 larvas a partir de 234 muestras que se tomaron en 58 sitios de agua dulce. Las especies hermanas descritas previamente: *S. apricarium, S. arcticum* sensu stricto, *S. brevicercum,* y *S. saxosum,* fueron las más numerosas, mientras que todos los citotipos con la excepción de IIL-19 se encontraron en frecuencia baja. Además, se describen seis nuevos citotipos de frecuencia baja. La evidencia sugiere que el cromosoma Y conlleva el gen determinante de los testículos y en casi todos los taxa de *S. arcticum* las inversiones paracéntricas caracterizan a estos tipos. Las distribuciones de (1) *S. brevicercum* y *S arcticum* s. s., de (2) *S. arcticum* s. s. y *S. arcticum* IIL-18, de (3) *S. brevicercum* y *S. arcticum* IIL-18, y de (4) *S. arcticum* IIL-9 y *S. arcticum* IIL-19 tienen asociaciones geográficas positivas altamente significativas, mientras que las distribuciones de (1) *S. apricarium* y *S. brevicercum* y de (2) *S. apricarium* y *S. saxosum* tienen asociaciones geográficas negativas altamente significativas. Los citotipos de *S. arcticum:* IIS-12, IIL-17, IIL-21, IIL-22, IIL-38, IIL-51, IIL-68, IIL-73•74 e IIL-79 se encuentran en dos o menos localidades. Los poliploides (0.0007), las inversiones pericéntricas (0.00007) y las translocaciones cromosómicas (0.00007) son extremamente raros. Estas observaciones y nuestras comparaciones recientes de ADN de tipos cromosómicamente diferentes, me llevaron a trabajar sobre un modelo sugerido previamente para la evolución de cromosomas en moscas negras en las cuales los citotipos distribuidos localmente pueden volverse más comunes con el tiempo, mientras que las citoespecies de amplia distribución pueden convertirse eventualmente en tipos morfológicamente distintos. Al contrario de la noción actual de la variación en los cromosomas puede no desempeñar un papel fundamental en el proceso de especiación de la mayoría de los animales, este estudio sugiere que la variación cromosómica, al menos en las moscas negras, desempeña un papel fundamental en la especiación.

Black flies (Diptera: Simuliidae) are important to science because, in many cases, the single morphospecies of classical taxonomy reveals itself as any number of cytologically differentiable sibling species when larval polytene chromosomes are analyzed (Rothfels 1956). In fact, the presence of reproductively isolated sibling species among presumed single morphospecies of simuliids, and their subsequent taxonomic description as valid biological species,

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TABLE 1. Taxa of the *Simulium arcticum* complex.

Taxon	Status	Reference
S. apricarium IIL-7	Species	Adler et al. (2004)
S. arcticum IIL-1	Species (formally unnamed)	Shields and Procunier (1982), Adler et al. (2004)
S. arcticum IIS-4	Species (formally unnamed)	Procunier (1984), Adler et al. (2004)
S. arcticum IIL-6	Cytotype	Shields (unpublished)
S. arcticum IIL-9	Cytotype	Shields et al. (2007b)
S. arcticum IIL-10	Cytotype	Shields et al. (2007b)
S. arcticum IIL-12	Cytotype	Adler et al. (2004)
S. arcticum IIL-13	Cytotype	Adler et al. (2004)
S. arcticum IIL-14	Cytotype	Adler et al. (2004)
S. arcticum IIL-15	Cytotype	Conflitti et al. (2010)
S. arcticum IIL-16	Cytotype	Adler et al. (2004)
S. arcticum IIL-17	Cytotype	Adler et al. (2004)
S. arcticum IIL-18	Cytotype	Shields et al. (2007a)
S. arcticum IIL-19	Cytotype	Shields et al. (2007b)
S. arcticum IIL-21	Cytotype	Conflitti et al. (2010)
S. arcticum IIL-22	Cytotype	Shields et al. (2009)
S. arcticum IIL-57•58	Cytotype	Conflitti et al. (2010)
S. arcticum IIL-68	Cytotype	Conflitti et al. (2010)
S. arcticum IIL-73•74	Cytotype	Conflitti et al. (2010)
S. arcticum s. s. IIL-3	Species	Shields and Procunier (1982), Adler et al. (2004)
S. brevicercum IIL-standard	Species	Shields and Procunier (1982), Adler et al. (2004)
S. chromatinum IIL-11	Species	Adler et al. (2004)
S. negativum IL-3•4	Species	Shields and Procunier (1982), Adler et al. (2004)
S. saxosum IIL-2	Species	Shields and Procunier (1982), Adler et al. (2004)
S. vampirum IIL-8, IIS-10•11	Species	Adler et al. (2004)

would have gone unrecognized had it not been for the initial cytogenetic analyses (Rothfels 1979). Generally, sex-linked paracentric inversions in males initially characterize cytologically differentiating taxa (Rothfels 1979). This pattern has been observed in numerous species complexes of black flies including *Prosimulium hirtipes* (Rothfels 1956), *Simulium pictipes* (Bedo 1975), *S. venustum/S. verecundum* (Rothfels et al. 1978), *S. vittatum* (Rothfels and Featherston 1981), *S. arcticum* (Shields and 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).

Nine sibling species and 16 cytotypes have been described within the *S. arcticum* complex (Shields and Procunier 1982, Procunier 1984, Adler et al. 2004, Shields et al. 2007a, 2007b, Conflitti et al. 2010; Table 1). The present study summarizes detailed cytogenetic analyses of nearly 15,000 larvae of the *Simulium arcticum* complex taken from 234 collections at 58 sites in Montana (Fig. 1), northern Idaho, Washington State, and Oregon (Fig. 2). I report frequencies of 20 taxa of the *Simulium arcticum* complex, distributions of these taxa, linkage of chromosomal inversions to X and Y chromosomes, and very rare chromosomal variation, including frequencies of pericentric inversions, chromosomal translocations, and numbers of polyploids. The present study approximates in scope the extensive studies of *S. vittatum* (Rothfels and Dunbar 1953, Rothfels and Featherston 1981) and of *S. damnosum* (Dunbar 1966, 1969, Vajime and Dunbar 1975). This study supports original observations by Rothfels (1989) which suggested that a continuum from cytotypes to cytospecies (taxa that have unique Y chromosomes and are reproductively isolated from each other) to morphospecies (taxa that have unique Y chromosomes and that can be identified on morphological grounds) may be occurring in black flies. Some of the data included in this broad comparative study have been reported in studies of correlates of genetic and environmental variation (Shields et al. 2007a) and assessments of the reproductive status of taxa in sympatry (Shields et al. 2007b, 2009, Shields and Kratochvil 2011). In the present study, I hypothesized that siblings would occur in high frequencies and have broader geographic distributions than cytotypes. I also hypothesized that linkage to the Y chromosome of various paracentric inversions in siblings and cytotypes would essentially be complete.

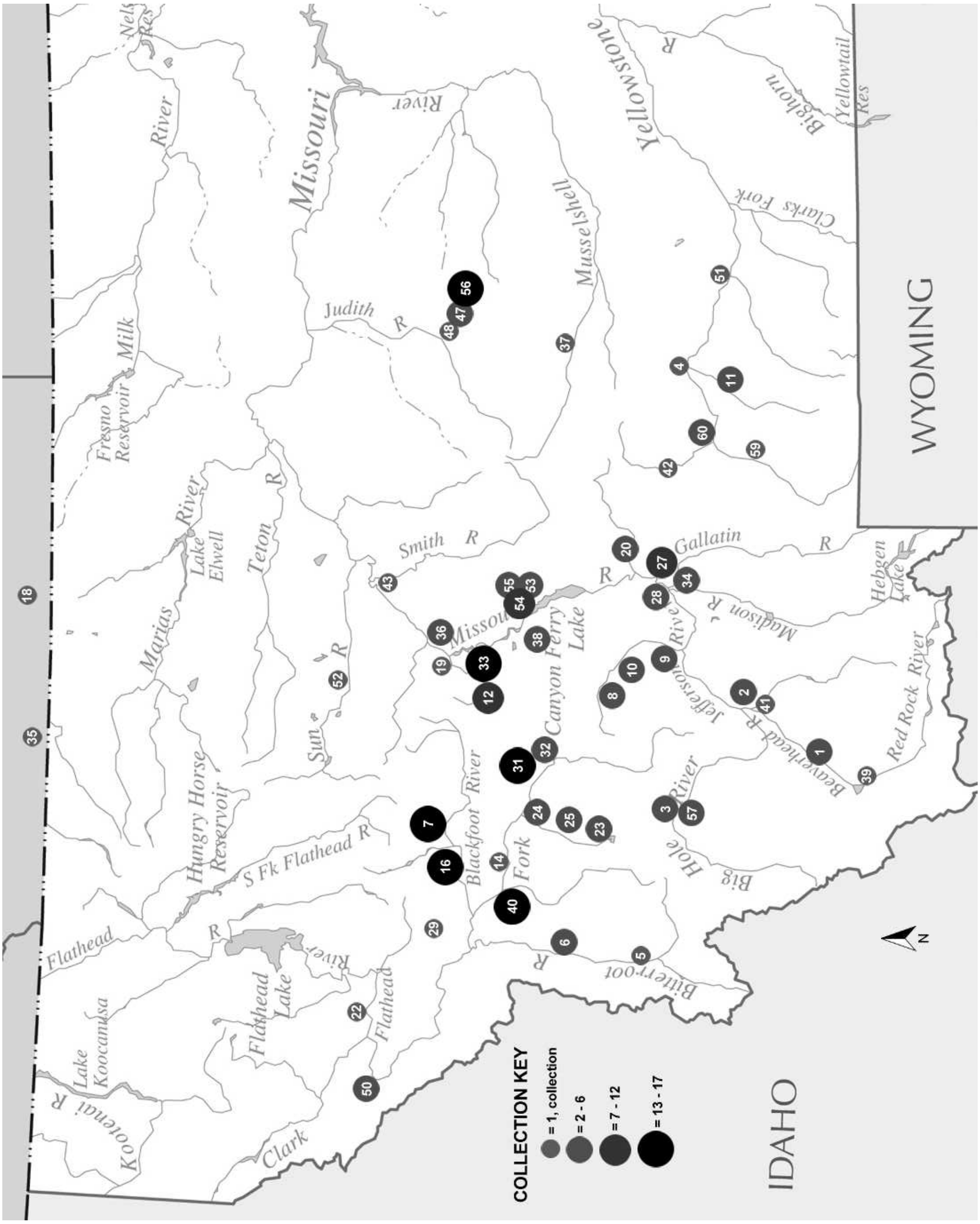

Fig. 1. Locations of collections made in the state of Montana. The size of the circles corresponds to the number of collections made at that site (see collection key). The number in each circle corresponds to the site number listed in Appendix 2.

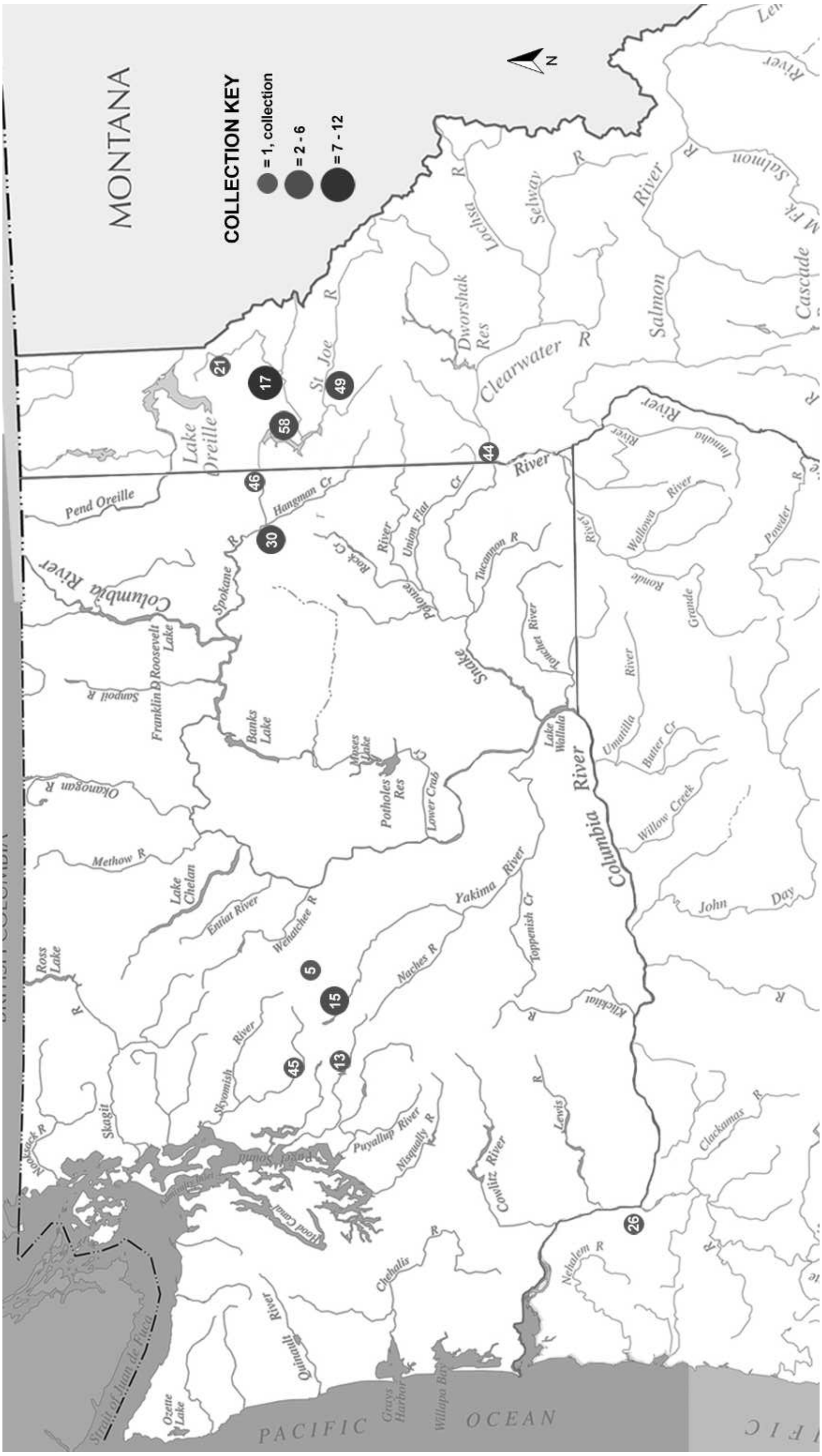

Fig. 2. Locations of collections made in Idaho, Washington, and Oregon. The size of the circles corresponds to the number of collections made at that site (see collection key). The number in each circle corresponds to the site number listed in Appendix 2.

TABLE 2. Frequencies of the 20 taxa of the *Simulium arcticum* complex observed in this study. Siblings are bolded.

Taxon	Number of males	Proportion of total	Area of distribution (km^2)
***S. arcticum* s. s. IIL-3**	**2703**	**0.361**	**103,492**
***S. apricarium* IIL-7**	**930**	**0.124**	**72,570**
***S. brevicercum* IIL-st/st**	**776**	**0.104**	**257,806**
S. arcticum IIL-9	576	0.077	40,858
***S. saxosum* IIL-2**	**553**	**0.074**	**122,495**
S. arcticum IIL-19	412	0.055	1260
S. arcticum IIL-10	296	0.040	32,476
S. arcticum IIL-22	260	0.035	1187
S. arcticum IIL-18	172	0.023	23,648
***S. negativum* IL-3•4**	**167**	**0.022**	**19,827**
S. arcticum IIL-73•74	159	0.021	6
S. arcticum IIL-68	106	0.014	12
S. arcticum IIL-21	85	0.011	one site
S. arcticum IIL-15	80	0.011	30,834
S. arcticum IIL-17	79	0.011	one site
S. arcticum IIL-79	47	0.006	one site
S. arcticum IIL-38	27	0.004	one site
S. arcticum IIL-13	22	0.003	6652
S. arcticum IIS-12	17	0.002	one site
S. arcticum IIL-51	16	0.002	one site

METHODS

Methods of larval collection, fixation, morphological identification, staining of polytene chromosomes and gonads, and chromosome analyses have been presented elsewhere (Rothfels and Dunbar 1953, Shields and Procunier 1982, Currie 1986, Adler et al. 2004). I recorded the following for each sibling species and cytotype: collection locations (58 freshwater sites), dates of collection, water temperatures, GPS coordinates, and numbers of each taxon collected at that site and date (Appendixes 1, 2). Although some sites were re-collected for various reasons (e.g., Clearwater River in 2007, 2008 and 2009), such as for determinations of annual continuity of taxa (Shields et al. 2009), we generally sampled all streams and drainages in the larger study area. I used contingency analysis and the chi-square (χ^2) test for geographic associations between taxa. Sizes of the geographic ranges were calculated using the minimum convex polygon method and ArcGIS 10.0 software. Although contingency tests assume nearly equal sample sizes, it is essentially impossible to obtain equal sample sizes of larvae. The number of larvae at any site at any time is influenced by many biological and environmental factors beyond our control. Taxa having fewer than 10 individuals were excluded from these analyses since small sample sizes could erroneously affect conclusions.

RESULTS

Frequencies and Geographic Distributions

I observed 5 sibling species and 15 cytotypes of the *S. arcticum* complex in this study (Table 2). Of the cytotypes, 3 are new to science (Figs. 3, 4). I did not observe *S. arcticum* IIL-1; *S. vampirum* IIL-8, IIS-10•11; *S. chromatinum* IIL-11; or *S. arcticum* IIS-4. *Simulium arcticum* s. s. IIL-3 comprised more than one third of males observed (Table 2). Siblings comprised 4 of the 5 most numerous taxa, and only *S. negativum* IL-3•4 was observed in intermediate frequencies (Table 2). Thirteen of the 15 cytotypes had medium to lower frequencies (<5%, Table 2). Moreover, the geographic areas of distributions of the 4 most numerous siblings were correspondingly among the largest of the data set (>72,000 m^2), while the remaining cytotypes had distributions smaller than 32,500 m^2, and 6 of these were observed at only a single site (Table 2).

Linkage of Taxon-Specific Paracentric Inversions to the Y Chromosome

I analyzed 7483 male larvae of the *S. arcticum* complex, which included 17 taxa (Table 3). Ten of these taxa had complete linkage of their taxon-specific inversions to the Y chromosome, while 6 of the 7 remaining taxa had very high linkage to the Y (range of proportion of linkage 0.967–0.997). One female was a IIL-13

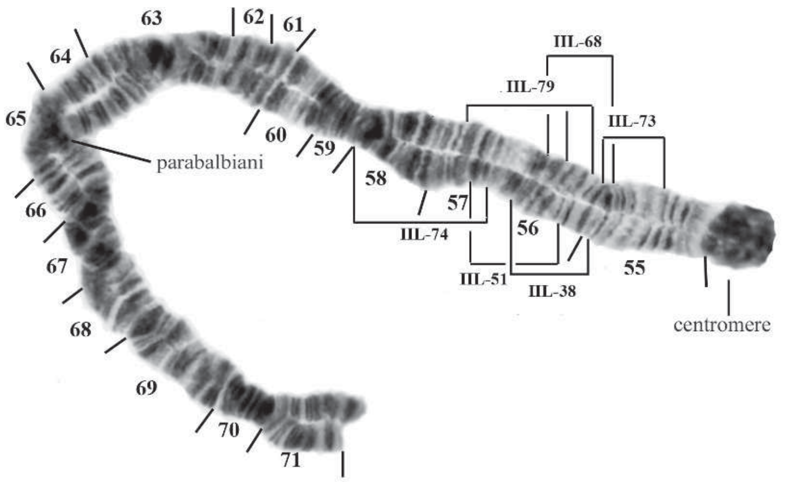

Fig. 3. Paracentric inversion breakpoints associated with the new cytotypes discovered on the IIL chromosome in this study. Larger numbers delineate sections of the long arm of chromosome II. The IIL-73•74 cytotype has 2 separate paracentric inversions associated with its Y chromosome. Inversions IIL-51, IIL-68, and IIL-73•74 were described as cytotypes in Conflitti et al. (2010), but limits of these inversions were not indicated in that publication.

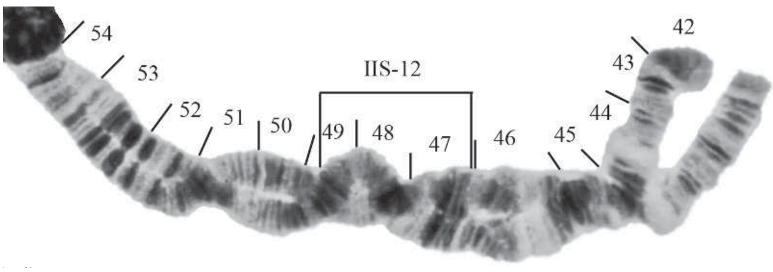

Fig. 4. The IIS chromosome arm. Larger numbers delineate sections of the short arm of chromosome II. The taxon specific IIS-12 inversion is shown in brackets.

TABLE 3. Linkage to the Y chromosome of taxon-specific inversions in the *Similium arcticum* complex.

Taxon	Sites where present	Larvae analyzed	Extent of Y-linkage
S. arcticum s. s. IIL-3	29	2703	0.997
S. arcticum IIL-9	12	468	0.981
S. arcticum IIL-10	9	296	1.000
S. arcticum IIL-13	3	30	0.667
S. arcticum IIL-15	12	80	1.000
S. arcticum IIL-17	1	81	0.975
S. arcticum IIL-18	12	183	0.967
S. arcticum IIL-19	5	500	0.994
S. arcticum IIL-21	1	86	1.000
S. arcticum IIL-22	3	261	0.996
S. arcticum IIL-38	1	27	1.000
S. arcticum IIL-51	1	16	1.000
S. arcticum IIL-68	3	106	1.000
S. arcticum IIL-73•74	2	159	1.000
S. arcticum IIL-79	1	47	1.000
S. arcticum IIS-12	1	17	1.000
S. arcticum IL-3•4	5	167	1.000

TABLE 4. Distribution of the IIL-2 inversion among females and males of this study.

Females		Males
$X_{IIL\text{-}i}$ $X_{IIL\text{-}i}$	$X_{IIL\text{-}i}$ $X_{IIL\text{-}st}$	$X_{IIL\text{-}i}$ $Y_{IIL\text{-}st}$
196	11	156

homozygote, 8 females were IIL-13 heterozygotes, and 22 males were IIL-13 heterozygotes. More study is needed to determine if this inversion is sex-linked.

Simulium saxosum and *Simulium apricarium*

Females of *Simulium saxosum* tended to be IIL-2 inversion homozygotes, while males tended to be IIL-2 heterozygotes (Shields and Procunier 1982, Adler et al. 2004). Slightly more than 5.0% of *S. saxosum* females were heterozygotes for the IIL-2 inversion (Table 4).

TABLE 5. Distribution of the IIL-7 inversion among female and male *Similium apricarium* ($G = 60.63262$, df = 2, $P \leq 0.001$).

Sex	st/st	st/i	i/i	Total
♀♀	141[a]	326[b]	238[c]	705
♂♂	71[d]	433[e]	422[f]	926
Total	212	759	660	1631
CALCULATED OBSERVED GENOTYPES				
♀♀	60.76034	–2.07127	–43.13	
♂♂	–37.4761	2.082868	50.15042	
EXPECTED GENOTYPES				
♀♀	91.63703	328.0779	285.2851	
♂♂	120.363	430.9221	374.7149	

[a]Fewer st/st ♀♀ than expected.
[b]Far more st/i ♀♀ than expected.
[c]About the number of i/i ♀♀ expected.
[d]Far fewer st/st ♂♂ than expected.
[e]Somewhat more st/i ♂♂ than expected.
[f]Somewhat more i/i ♂♂ than expected.

TABLE 6. Incidence of B chromosomes in taxa of the *Similium arcticum* complex.

Taxon	Males analyzed	Types and numbers of B chromosomes	Proportion B's per taxon
S. brevicercum	776	one acrocentric two acrocentrics	0.005
S. saxosum	553	none	—
S. arcticum s. s.	2703	one acrocentric two acrocentrics three acrocentrics four acrocentrics	0.008
S. apricarium	930	none	—
S. arcticum IIL-9	576	one acrocentric two acrocentrics three acrocentrics four acrocentrics	0.092
S. arcticum IIL-10	296	one acrocentric two acrocentrics four acrocentrics	0.024
S. arcticum IIL-13	22	two acrocentrics	0.091
S. arcticum IIL-15	80	two acrocentrics	0.025
S. arcticum IIL-17	79	none	—
S. arcticum IIL-18	171	none	—
S. arcticum IIL-19	412	one acrocentric two acrocentrics	0.005
S. arcticum IIL-21	85	one acrocentric two acrocentrics	0.082
S. arcticum IIL-22	260	one acrocentric	0.038
S. arcticum IIL-38	27	none	—
S. arcticum IIL-51	16	none	—
S. arcticum IIL-68	101	none	—
S. arcticum IIL-73•74	159	none	—
S. arcticum IIL-79	47	none	—
S. arcticum IIS-12	17	none	—

Simulium apricarium is characterized by the IIL-7 inversion and fixation of the IIS-11 autosomal inversion. IIL-7 occurs in all classes (st/st, st/i, and i/i) of both sexes (Adler et al. 2004, Shields et al. 2007a, 2007b). I analyzed more than 1600 *S. apricarium* in this study and found a significant difference ($P < 0.001$) in the distribution of genotypes and sex (Table 5). Far fewer st/i and i/i females and far fewer st/st and i/i males than expected were observed (Table 5).

Incidence of B Chromosomes

All B chromosomes observed in this study were acrocentric (centromere closest to one end of the chromosome; Table 6). *Simulium saxosum, S. apricarium, S. arcticum* IIS-12, IIL-17, IIL-18, IIL-38, IIL-51, IIL-68, IIL-73•74, and IIL-79 had no B chromosomes. *Simulium arcticum* s. s. and *S. arcticum* IIL-9 had males that possessed 1, 2, 3, and 4 B chromosomes. The single sample of *S. arcticum* IIL-9 taken on 5 April 2009 from the Spokane River, Washington, had an unusually high frequency of B chromosomes in males (39/61 or 0.64%).

Tests of Geographic Association Between Taxa

The siblings *S. brevicercum* and *S. arcticum* s. s. had highly significant geographic associations with each other, as well as with the cytotype *S. arcticum* IIL-18 (Table 7). The cytotypes IIL-9 and IIL-19 also had a highly significant geographic association. The sibling *S. apricarium* had highly significant negative geographic associations with the siblings *S. brevicercum* and *S. saxosum* (Table 7).

Extremely Rare Exceptions to Homozygotic Standard Females and Heterozygotic Males for Taxon-Specific Inversions

Inverted homozygotic females and males and double inverted females and males were extremely rare (Table 8).

Frequencies of Pericentric Inversions, Translocations, and Polyploids within the *S. arcticum* Complex

Among 14,781 larvae, I observed a single pericentric inversion (proportion = 0.00007) and a single translocation (proportion = 0.00007). Among all larvae, 8 triploids were found in males (proportion = 0.0005) and 2 were found in females (proportion = 0.0001; Table 9).

DISCUSSION

I hypothesized that when a large geographic sample was obtained, previously described siblings (Shields and Procunier 1982, Adler et al. 2004) would occur in higher frequencies and have larger geographic distributions than cytotypes, and the findings presented here support that hypothesis. The 5 siblings observed here accounted for 69% of all males analyzed. These observations correspond to those of Adler et al. (2004:814–822), who observed large geographic distributions for 8

TABLE 7. Tests of association between taxa regarding geographic distribution. Bold type indicates highly significant positive geographic associations; italic type indicates highly significant negative geographic associations.

	S. brevicercum	*S. saxosum*	*S. arcticum*	*S. apricarium*	IIL-9	IIL-10	IIL-15	IIL-18	IIL-19	IIL-22
S. sax	0.002									
S. arc	**<0.001**	0.166								
S. apr	*<0.001*	*<0.001*	0.437							
IIL-9	0.102	0.879	0.401	0.519						
IIL-10	0.889	0.264	0.426	0.528	0.026					
IIL-15	0.536	0.746	0.081	0.006	0.128	0.075				
IIL-18	**<0.001**	0.403	**<0.001**	0.049	0.159	0.033	0.621			
IIL-19	0.941	0.056	0.867	0.571	**<0.001**	0.029	0.327	0.046		
IIL-22	0.024	0.721	0.003	0.370	0.088	0.143	0.252	0.181	0.491	
IIL-68	0.009	0.198	0.081	0.778	0.123	0.291	0.252	0.173	0.219	0.408

TABLE 8. Frequencies of very rare sex-linked paracentric inversions within the *Similium arcticum* complex. This analysis does not include populations of *Simulium saxosum* and *S. apricarium* since those taxa have different distributions of X and Y linkage.

Inverted types	Variation	Frequency across all taxa
Inverted homozygotic females	IIL-13 i/i (*n* = 1)	1 in 7298 = 0.0001
Inverted homozygotic males	IIL-3 i/i (*n* = 4)	8 in 7483 = 0.001
	IIL-9 i/i (*n* = 4)	
Double inverted females	IIL-13st/i – IIL-46 st/i	3 in 7298 = 0.0003
	IIL-2 st/i – IIL-21st/ i	
Double inverted males	IIL-3 st/i – IIL-7 st/i (*n* = 4)	9 in 7483 = 0.001
	IIL-st/st – IIL-3 st/i•IIL-9 st/i	
	IIL-3 st/i – IL 3•4 st/i	
	IIL-2 st/i – IIL-3 st/i	
	IIL-2 st/i – IIL-21 st/i	
	IIL-19 st/i – IL-3•4 st/i	

TABLE 9. Frequencies of pericentric inversions, translocations, and polyploids within the *Similium arcticum* complex.

Variant type	Detail	Proportion
Pericentric inversion*	IIS-IIL inversion (*n* = 1)	0.00006
Translocation**	IIS-IIL translocation (*n* =1)	0.00006
Polyploids	Triploids, males (*n* = 8)	0.0005
	Triploids, females (*n* = 2)	0.0001

*The pericentric inversion occurred in a heterozygotic IIL-st/IIL-7i male of *S. apricarium*. The inverted segment includes sections 52, 53, and 54 of chromosome IIS and sections 55, and 56A (breakpoint just distal to the "jagged") of chromosome IIL.

**The translocation occurred in a IIL-7i/IIL7i male of *S. apricarium*. The translocated segment included the 2 "heavy" bands at the interface of sections 52 and 53 of chromosome IIS, which were translocated into the interface of sections 56 and 57 of chromosome IIL.

of 9 cytospecies of the *S. arcticum* complex. In my study, the sibling *S. negativum* IL-3•4 had an intermediate frequency of males and a small geographic distribution, but these findings may relate to the sibling's later emergence in summer in our study area when we tended to collect larvae less frequently. Even though there were 15 cytotypes described here, they accounted for less than a third of males. Correspondingly, all of the 15 cytotypes observed had intermediate to very small geographic distributions, and of these, 6 were found at only one site. Though the reproductive statuses of all taxa of the *S. arcticum* complex have not been determined, 4 independent studies (Shields et al. 2007a, 2007b, 2009) showed that siblings in sympatry are reproductively isolated while cytotypes in sympatry are not. I thus conclude that (1) siblings may be more abundant and may have larger geographic distributions than do cytotypes and (2) siblings in sympatry are reproductively isolated while cytotypes are not.

I also hypothesized that taxon-specific inversions within the *S. arcticum* complex would be completely linked to chromosomes determining sex. Most of the types observed here had extremely tight linkage to their respective Y chromosomes. Ten of the types have complete linkage to Y, while the remaining 6 have an average linkage of 0.985. These findings strongly suggest that chromosomal inversions related to sex determination in the *S. arcticum* complex possibly play an early and significant role in the speciation process. Though Coyne and Orr (2004:265) may be correct when they state, "*It is far from clear if chromosomal speciation is common in animals generally; indeed, we know of no compelling evidence for chromosomal speciation in animals,*" this may not be the case in black flies. As stated earlier, Y-linked inversions are common in complexes of black flies, and based on our assessments of molecular divergence within the *S. arcticum* complex (Conflitti et al. 2010, 2012), these inversions may occur early in the speciation process. Thus, based on our cytogenetic analysis of the largest sample ever reported for a complex of black flies, we expand on a model for chromosome-based speciation in black flies that was initially proposed by Rothfels (1989; Fig. 5). If this chromosome-based model is relevant, we should possibly observe a continuum of taxa whose earliest members (cytotypes) can be distinguished only by unique inversion linkage to the Y chromosome, and whose later members are either intermediate in the speciation process or are distinctive morphospecies.

As mentioned earlier, *S. apricarium* and *S. saxosum* are exceptions to the "male Y-linkage"

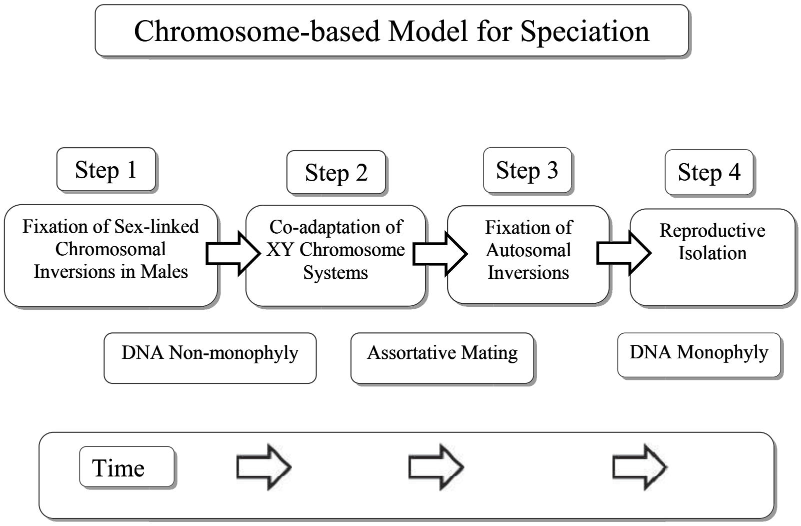

Fig. 5. Chromosome-based model for speciation in the *Simulium arcticum* complex of black flies. The 15 cytotypes described here (Table 2) support step 1 of this model. Step 2 and the designation of assortative mating in the model are supported by the presence of a remnant population of *S. arcticum* s. s. and *S. saxosum* at the Coeur d'Alene River, Idaho (Shields and Kratochvil 2011). Step 3 is supported by fixation of the IIS-11 autosomal inversion among *S. arcticum* s. s. and *S. apricarium* at Little Prickly Pear Creek (Shields et al. 2007b). Step 4 is supported by temporal reproductive isolation of *S. negativum* and *S. arcticum* IIL-9 at the Blackfoot River and by sympatric reproductive isolation of *S. arcticum* s. s. and *S. apricarium* at Little Prickly Pear Creek (Shields et al. 2007b) Designations of DNA non-monophyly and monophyly are based on Conflitti et al. (2010, 2012).

paradigm. The taxon-specific IIL-7 inversion in *S. apricarium* occurs in all genotypic categories (st/st, st/i, and i/i) of both females and males. The tendency for both females and males to possess IIL-7 heterozygotes as shown here may represent a novel and transitional form of sex-chromosome evolution. Sex in *S. saxosum* is determined by the inheritance of the X chromosome (the large majority of females being $X_{IIL\text{-}i}$ $X_{IIL\text{-}i}$, while all males are $X_{IIL\text{-}i}$ $Y_{IIL\text{-}st}$). As with the situation in *S. apricarium,* sex-linkage to the X in *S. saxosum* may represent a transitional state.

I am able to morphologically separate larvae of *Simulium negativum* from other members of the *S. arcticum* complex based on the negative head patterns of females (Adler et al. 2004) and the overall light and fragile appearance of larvae. Correspondingly, unlike all other members of the *Simulium arcticum* complex, sex in *S. negativum* is based on inversions in the long arm of chromosome I (Shields and Procunier 1982, Adler et al. 2004). This difference indicates that *Simulium negativum* may (1) be the most divergent member of the complex, (2) be a validly described morphospecies, and (3) be near the temporal apex of a chromosome-based speciation model (Fig. 5; see below regarding DNA evidence).

Our earlier studies support such a contention. We observed that (1) 2 siblings, *S. arcticum* s. s. and *S. apricarium,* were reproductively isolated in sympatry; (2) a sibling, *S. negativum,* and a cytotype, *S. arcticum* IIL-9, were temporally reproductively isolated; and (3) 2 cytotypes, *S. arcticum* IIL-9 and IIL-19, were not reproductively isolated in sympatry (Shields et al., 2007b). Moreover, a sibling, *S. arcticum* s. s., and a cytotype, *S. arcticum* IIL-22, were not reproductively isolated in sympatry (Shields et al. 2009). Such combinations of reproductive status support a continuum model of chromosomal speciation.

Rothfels (1989) proposed that speciating taxa having unique Y-linked chromosomal inversions within a black fly complex might undergo initial mating trials. These mating trials eventually lead to coadaptation of polymorphic sex chromosomes in pairs, followed by reinforcement through assortative mating and slight selective advantage. In such a case, we might expect to find geographically parapatric taxa that still share combinations of sex chromosomes in areas of overlap. Indeed, we have found such a case in which the westerly distributed *S. saxosum* and the easterly distributed *S. arcticum* s. s. still share combinational sex chromosomes, interpreted as the "remnants of mating trials," in a small area of overlap at the Coeur d'Alene River in northern Idaho (Shields and Kratochvil 2011).

We know that the majority of taxa within the *S. arcticum* complex appear relatively young in an evolutionary sense because they are not monophyletic in DNA sequence trees based on comparisons of mitochondrial and nuclear genes (Conflitti et al. 2010, 2012). Correspondingly, we might expect to observe morphologically and chromosomally distinct species at the far end of the speciation continuum model. As stated above, *S. negativum* is morphologically distinct (negative head patterns in females; Adler et al. 2004) and chromosomally distinct; males are IL-3•4 heterozygotes (Shields and Procunier 1982). Correspondingly, *S. negativum* is molecularly monophyletic to the remainder of taxa of the *S. arcticum* complex (Conflitti et al. 2010, 2012). This attribute also corresponds to other biological features of *S. negativum.* As taxa of the *S. arcticum* complex are speciating through time, we might expect to observe environmental correlates with cytogenetic diversity. Indeed, the distributions of some taxa appear to be influenced by elevation, date of collection, and water temperature. My observations based on this large sample size support our earlier observation that *S. arcticum* IIL-18 occurs at high elevations and is possibly influenced by cooler water temperatures in spring (Shields et al. 2007a). However, our earlier observation (Shields et al. 2007a) that *S. apricarium* is restricted to low elevations and that both *S. brevicercum* and *S. arcticum* s. s. are distributed randomly with respect to elevation is not supported by this more extensive analysis. Adler et al. (2004) named the IIL-7 cytospecies *S. apricarium,* which literally means "of the open." *Simulium apricarium* females possibly preferentially deposit their eggs in open (broader) streams rather than being influenced by elevation. Why female black flies oviposit at specific sites (Hunter and Jain 2000, Adler et al. 2004) deserves additional study.

Our data suggest that some taxa of the *S. arcticum* complex have highly significant positive associations with respect to geographic distribution, while other taxa do not. Both of the negative associations with respect to distribution involve only siblings, while 3 of the 4 positive associations involve both siblings and cytotypes. None of these taxa is monophyletic in DNA sequence trees (Conflitti et al. 2010, 2012), yet *S. negativum* is. More detailed study of *S. negativum* from both the cytogenetic and environmental perspectives appears warranted.

This study summarizes an analysis of a large sample of larvae of the *S. arcticum* complex across a broad geographic area. Inversions linked to sex chromosomes are omnipresent.

Frequencies and distributions of siblings are larger than those of cytotypes. Our previous studies indicate that siblings tend to be reproductively isolated when sympatric, while cytotypes are not. Our molecular studies indicate that the complex may be young in an evolutionary sense but that *Simulium negativum* is molecularly monophyletic with respect to other members of the complex. While my analysis is extensive, it covers roughly one twentieth of the distribution of the *S. arcticum* complex in western North America. Similar studies north and south of the northern Rocky Mountains are clearly needed.

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APPENDIX 1. Frequencies of taxa in the *Simulium arcticum* complex of black flies (Diptera: Simuliidae). See footnotes (p. 84)

			S. brev.	*S. sax.*			*S. arc.* s. s.			*S. apr.*						*S. arc.*		
			IIL-st/st	IIL-2			IIL-3			IIL-7						IIL-9		
Location	Date[a]	Temp.[b]	X_0Y_0	X_2X_2	X_0X_2	X_2Y_0	X_0X_3	X_0Y_3	X_3Y_3	X_0X_0	X_0X_7	X_7X_7	X_0Y_0	X_0Y_7	X_7Y_7	X_0X_9	X_0Y_9	X_9Y_9
Beaverhead River, Dillon	6/1/03	5								3	3	6	0	2	3			
Beaverhead River, Dillon	3/20/04	6								8	17	19	2	24	16			
Big Hole R., Twin Bridges	3/20/04	6								1	7	3	0	6	10			
Big Hole R., Twin Bridges	4/1/004	8								12	18	9	0	22	14			
Big Hole River, Wise River	4/15/04	4																
Big Hole River, Wise River	3/20/07	4								3	1	7	0	9	3			
Big Timber Cr.	4/6/03	7								4	3	2	0	8	4			
Bitterroot River, Darby	3/17/07	6														0	13	0
Bitterroot River Hamilton	3/28/04	9	1													0	3	0
Bitterroot River Hamilton	3/17/07	7														1	8	0
Blackfoot River Russel Gates	4/16/01	8					0	1	0							0	5	0
Blackfoot River Russel Gates	3/30/03	8					0	5	0							0	25	0
Blackfoot River Russel Gates	4/20/03	9					0	2	0	0	0	0	0	0	1	0	2	0
Blackfoot River Russel Gates	5/18/03	11					0	1	0									
Blackfoot River Russel Gates	7/12/03	12														1	3	0
Blackfoot River Russel Gates	3/28/04	4	1				0	9	0	0	0	1	0	0	1	0	50	1
Blackfoot River Russel Gates	4/2/04	6	1				0	6	0	0	1	1	0	1	1	0	1	0
Blackfoot River Russel Gates	4/1/05	7					0	10	0	0	0	0	0	2	0	1	7	0
Blackfoot River Russel Gates	4/14/06	7					0	2	0									
Blackfoot River Russel Gates	5/31/06	10																
Blackfoot River Russel Gates	6/15/06	12																
Blackfoot River Russel Gates	7/10/06	16	5													0	42	2
Blackfoot River Russel Gates	4/11/11	5					0	1	0							0	1	1
Boulder River Bison Creek,	4/15/04	2	6				0	11	0									
Boulder River Bison Creek,	4/29/04	3	17				0	18	0									
Boulder River Bison Creek,	8/15/04	15					0	1	0	0	0	0	0	0	1			
Boulder River, Cardwell	4/15/04	7								5	3	3	1	4	3			
Boulder River, Cardwell	4/29/04	8								13	11	11	14	15	6			
Boulder River, Cardwell	6/28/04	15								5	9	6	0	7	4			
Boulder River High Ore	4/1/04	4					0	19	1									
Boulder River High Ore	4/29/04	4	8	0	0	2	0	24	0									
Boulder River High Ore	6/8/04	10	1				0	13	0									
Boulder River, Sweet Grass Co.	3/11/03	4	21															
Boulder River, Sweet Grass Co.	4/6/03	6	17															
Boulder River, Sweet Grass Co.	6/23/04	11																
Canyon Creek	4/23/02	6	1				0	9	0									
Canyon Creek	5/13/02	5	2				0	7	0									
Canyon Creek	7/22/02	11					0	1	0									
Canyon Creek	4/16/03	6	2				0	17	0									
Canyon Creek	5/13/03	6	14				0	19	0									
Canyon Creek	7/9/03	10					0	2	0									
Canyon Creek	7/29/03	13	1				0	10	0									
Cedar River	3/11/05	11		18	0	2												
Clark Fork R. Bearmouth	3/18/07	4								0	5	5	1	2	1	0	1	0
Cle Elum River	5/10/03	9		33	0	7												
Cle Elum River	5/9/06	10		12	0	4												
Cle Elum River	3/24/08	5		112	4	104												
Cle Elum River	3/16/09	2		5	0	14												
Clearwater River	6/11/00	8																
Clearwater River	4/16/01	4					0	4	0									
Clearwater River	2/28/03	1																
Clearwater River	4/20/03	5					0	40	0									
Clearwater River	5/18/03	10					0	6	0									
Clearwater River	3/14/06	4														0	3	0
Clearwater River	5/8/06	11					0	24	0									
Clearwater River	4/21/07	6					0	249	0	0	0	0	0	1	0	0	3	0
Clearwater River	4/17/08	6					2	52	2									
Clearwater River	4/27/08	6					0	168	0									

for full taxon names and Appendix 2 (p. 86) for location attributes.

S. arc.																									*S. neg.*
IIL-10	IIL-13			IIL-15	IIL-17		IIL-18		IIL-19		IIL-21		IIL-22		IIL-38	IIL-51	IIL-68	IIL-73·74			IIL-79			IIS-12	IL-3·4
X_0Y_{10}	$X_{13}X_{13}$	X_0X_{13}	X_0Y_{13}	X_0Y_{15}	X_0X_{17}	X_0Y_{17}	X_0X_{18}	X_0Y_{18}	X_0X_{19}	X_0Y_{19}	X_2Y_{21}	X_0Y_{21}	X_0X_{22}	X_0Y_{22}	X_0Y_{38}	X_0Y_{51}	X_0Y_{68}	X_0Y_{73}	X_0Y_{74}	$X_0Y_{73 \bullet 74}$	X_0Y_{79}	X_2Y_{79}	$X_0Y_{3 \bullet 79}$	$X_0Y_{IIS\text{-}12}$	$X_0Y_{IL\text{-}3 \bullet 4}$
				1																					
																		0	0	1					
																		0	0	1					
				1					0	5															
									0	16															
									0	8															
									0	1															
									0	13															1
									0	2			0	1											
																									13
									0	2															
													0	1											
									0	7															
																									46
																									8
									0	3															
							0	1																	
				1																					
				2			0	1																	
				3																					
				2																					
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																								5	
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							0	1																	
							2	3																	
							0	1																	
	0	0	4																						
													0	4											
									0	4															
													0	22											
									0	2															
													0	4											
	0	0	1										0	63											1
													1	6											
													0	37											

APPENDIX 1. Continued.

			S. brev.	*S. sax.*			*S. arc.* s. s.			*S. apr.*						*S. arc.*		
			IIL-st/st	IIL-2			IIL-3			IIL-7						IIL-9		
Location	Date[a]	Temp.[b]	X_0Y_0	X_2X_2	X_0X_2	X_2Y_0	X_0X_3	X_0Y_3	X_3Y_3	X_0X_0	X_0X_7	X_7X_7	X_0Y_0	X_0Y_7	X_7Y_7	X_0X_9	X_0Y_9	X_9Y_9
Clearwater River	5/3/08	8					0	60	0									
Clearwater River	5/1/09	5					0	374	0							0	4	0
Clearwater River	4/23/10	7					0	18	0									
Coeur d'Alene River, Kingston	4/11/04	6		0	1	0	0	3	0									
Coeur d'Alene River, Kingston	3/14/05	4	1	1	3	2	0	2	0									
Coeur d'Alene River, Kingston	4/8/05	6	2	2	5	1	0	1	0									
Coeur d'Alene River, Kingston	3/15/09	2	8	2	8	6	0	14	0							0	2	0
Coeur d'Alene River, Kingston	4/3/09	3	3	2	4	7	0	4	0									
Coeur d'Alene River, Kingston	4/5/09	5	6	7	15	5	0	5	0									
Coeur d'Alene River, Kingston	3/11/10	3	18	5	19	9	0	15	0							0	1	0
Coeur d'Alene River, Kingston	3/13/10	4	12	4	11	20	0	9	0									
Coeur d'Alene River, Kingston	3/25/11	4	3	3	3	3	0	20	0									
Coeur d'Alene River, Kingston	7/23/11	14	1	0	4	4	0	1	0							0	11	0
Crowsnest Creek, Alberta	5/12/11	7	3				0	3	0									
Dearborn River	3/30/04	14	2				0	8	0	1	2	1	0	3	1	0	2	0
DeepCreek	4/6/03	6					0	4	0	0	0	0	0	0	1			
DeepCreek	2/28/04	2	1															
DeepCreek	3/20/04	2	10	0	1	0	1	11	1	0	1	0	0	0	1			
DeepCreek	4/1/04	6	4				0	13	0									
DeepCreek	5/16/04	6	6	0	1	0	0	6	0	0	0	0	0	0	1			
Flat Creek, Idaho	7/23/11	19	3	0	4	0										0	36	0
Flathead River, Perma	3/28/04	4	2													0	5	0
Flint Creek Campground	5/2/04	10	18				0	13	0									
Flint Creek Campground	6/8/04	10	5				0	3	0									
Flint Creek Campground	6/28/04	11	2															
Flint Creek Campground	8/15/04	13	1				0	2	0									
Flint Creek Hall	4/6/04	5	12				0	2	0									
Flint Creek Hall	5/2/04	10	7				0	4	0	0	5	1	0	2	3			
Flint Creek Hall	7/19/04	16	1				0	2	0	0	0	3	0	6	6			
Flint Creek Philipsburg	5/2/04	14	15				0	10	0	0	0	0	0	1	0			
Flint Creek Philipsburg	6/28/04	12	9				0	4	0	0	0	0	0	3	0			
Gales Creek, Oregon	1/21/03	4		6	0	12												
Gallatin River	4/10/02	10								3	3	10	2	7	4			
Gallatin River	2/15/03	4								0	0	1	1	4	4			
Gallatin River	3/11/03	3								3	10	7	3	16	20			
Gallatin River	4/6/03	9								4	2	3	0	7	1			
Gallatin River	4/16/03	11								0	0	0	0	3	1			
Gallatin River	8/17/03	15								1	1	0	1	4	3			
Gallatin River	3/8/04	4								1	1	3	1	4	5			
Gallatin River	3/20/07	8								1	1	4	1	8	6			
Gallatin River	3/21/10	5								10	22	9	6	21	21			
Jefferson River	3/8/04	6								1	3	0	1	4	6			
Jefferson River	3/20/04	7								3	18	20	5	23	35			
Jefferson River	5/16/04	10								1	1	0	0	1	2			
Jocko River Arlee	3/28/04	6	9				0	26	0									
Latah Creek Spokane County	5/17/03	14	2															
Latah Creek Spokane County	4/11/04	10																
Latah Creek Spokane County	3/13/05	9	6	0	0	1												
Latah Creek Spokane County	4/14/06	12	1															
Latah Creek Spokane County	3/12/10	5	2															
Little Blackfoot River, Elliston	3/30/03	6	17				0	11	0									
Little Blackfoot River, Elliston	4/17/03	5	10				0	3	0									
Little Blackfoot River, Elliston	4/28/03	7	5															
Little Blackfoot River, Elliston	5/13/03	5	2				0	1	0									
Little Blackfoot River, Elliston	6/5/03	4	2															
Little Blackfoot River, Elliston	6/29/03	8	3				0	2	0									
Little Blackfoot River, Elliston	7/9/03	10					0	1	0									
Little Blackfoot River, Elliston	8/5/03	8	2				0	2	0									

S. arc.																									*S. neg.*
IIL-10	IIL-13			IIL-15	IIL-17		IIL-18		IIL-19		IIL-21		IIL-22		IIL-38	IIL-51	IIL-68	IIL-73·74			IIL-79			IIS-12	IL-3·4
X_0Y_{10}	$X_{13}X_{13}$	X_0X_{13}	X_0Y_{13}	X_0Y_{15}	X_0X_{17}	X_0Y_{17}	X_0X_{18}	X_0Y_{18}	X_0X_{19}	X_0Y_{19}	X_2Y_{21}	X_0Y_{21}	X_0X_{22}	X_0Y_{22}	X_0Y_{38}	X_0Y_{51}	X_0Y_{68}	X_0Y_{73}	X_0Y_{74}	$X_0Y_{73•74}$	X_0Y_{79}	X_2Y_{79}	$X_0Y_{3•79}$	X_0Y_{IIS-12}	$X_0Y_{IL-3•4}$
													0	29											
									0	1			0	87											
													0	5											
7				2																					
1				1																					
1				1																					
							0	7																	
2							0	1																	
2				1			0	1																	
							0	3																	
							0	1																	
				12																					
				6																					
				3																					
	1	8	2	1																					
											1	27													
											0	18													
											0	31													
											0	2													
											0	7													
							0	4																	
							0	20																	
							0	1																	
							0	1																	
							0	1																	

APPENDIX 1. Continued.

			S. brev.	*S. sax.*			*S. arc.* s. s.			*S. apr.*						*S. arc.*		
			IIL-st/st	IIL-2			IIL-3			IIL-7						IIL-9		
Location	Date[a]	Temp.[b]	X_0Y_0	X_2X_2	X_0X_2	X_2Y_0	X_0X_3	X_0Y_3	X_3Y_3	X_0X_0	X_0X_7	X_7X_7	X_0Y_0	X_0Y_7	X_7Y_7	X_0X_9	X_0Y_9	X_9Y_9
Little Blackfoot River, Elliston	4/27/04	8	7				0	2	0									
Little Blackfoot River, Elliston	5/7/04	8	9				0	4	0									
Little Blackfoot River, Elliston	7/19/04	12	11				0	22	0							0	1	0
Little Blackfoot River, Elliston	4/7/05	7	41				0	11	0									
Little Blackfoot River, Elliston	5/12/05	7	2															
Little Blackfoot River, Elliston	4/9/06	5	5				0	3	0									
Little Blackfoot River, Elliston	4/19/09	6	17				0	16	0									
Little Blackfoot River, Elliston	4/4/11	5	204				1	114	0									
Little Blackfoot River, Elliston	4/10/11	5																
Little Blackfoot River, Garrison	4/27/04	9	4				0	2	0	0	5	9	0	10	9			
Little Blackfoot River, Garrison	6/8/04	7								0	13	11	0	3	12			
Little Blackfoot River, Garrison	6/28/04	11					0	1	0	1	8	10	1	3	11			
Little Blackfoot River, Garrison	7/19/04	11								2	2	1	2	2	3			
Little Prickly Pear Cr.	3/15/02	5					0	22	0	2	1	0	0	5	2			
Little Prickly Pear Cr.	4/4/02	7					0	26	0	0	2	0	0	0	0			
Little Prickly Pear Cr.	4/18/02	10					0	29	0									
Little Prickly Pear Cr.	4/30/02	10					0	4	0									
Little Prickly Pear Cr.	3/16/03	6					0	7	0	0	1	0	0	2	3			
Little Prickly Pear Cr.	4/10/03	9					0	24	0	0	3	1	0	7	2			
Little Prickly Pear Cr.	5/16/03	13					0	1	0	0	0	0	0	1	0			
Little Prickly Pear Cr.	3/31/05	8					0	119	0	0	5	1	0	7	7			
Little Prickly Pear Cr.	5/6/05	13					0	151	0	1	7	3	0	15	11			
Little Prickly Pear Cr.	5/26/05	10								0	7	0	0	11	0			
Little Prickly Pear Cr.	3/30/06	8					0	316	0	0	1	0	0	1	0			
Little Prickly Pear Cr.	4/1/06	9					0	10	0	0	1	0	0	0	0			
Little Prickly Pear Cr.	3/22/07	8					0	13	0									
Little Prickly Pear Cr.	3/30/07	6					0	9	0	0	0	0	0	1	0			
Little Prickly Pear Cr.	3/28/09	5					0	12	0									
Little Prickly Pear Cr.	4/15/09	5					0	24	0	0	1	0	0	0	0			
Madison River, Three Forks	4/10/02	6					0	2	0	1	10	5	1	3	4			
Madison River, Three Forks	3/11/03	3								1	1	1	0	1	5			
Madison River, Three Forks	4/8/07	9	2							0	0	2	0	0	0			
McCleese Lake Inflow	6/12/11		4															
Missouri R. Craig	3/22/07	8								9	21	5	5	45	15			
Missouri R. Craig	4/6/09	11								4	6	0	1	3	6			
Missouri R. Craig	3/28/10	8								4	5	1	1	9	11			
Musselshell R., Harlowton	4/6/03	6								3	2	0	0	4	1			
Prickly Pear Creek	3/23/04	5	5				0	64	0	0	0	0	0	1	1			
Prickly Pear Creek	4/30/04	12					0	11	0									
Red Rock River	4/1/05	8																
Rock Creek, Missoula County	4/7/01	4	1													0	3	0
Rock Creek, Missoula County	4/12/01	5														0	2	0
Rock Creek, Missoula County	3/5/02	3														0	9	0
Rock Creek, Missoula County	3/12/02	3														0	44	0
Rock Creek, Missoula County	3/17/02	4																
Rock Creek, Missoula County	4/1/02	6	1													2	11	0
Rock Creek, Missoula County	7/24/02	13								0	1	0	0	1	1			
Rock Creek, Missoula County	3/30/03	4					0	2	0	0	0	1	0	0	0	0	5	0
Rock Creek, Missoula County	3/14/05	3	2				0	2	0	0	1	0	0	1	14	1	46	0
Rock Creek, Missoula County	4/1/05	8	1				0	2	0	0	3	1	0	2	2	0	5	0
Rock Creek, Missoula County	3/14/06	2	5				0	10	0	0	1	2	0	1	3	0	106	0
Rock Creek, Missoula County	3/17/07	4														0	14	0
Rock Creek, Missoula County	4/2/07	5	10				0	13	0	0	0	1	0	0	0	0	3	0
Rock Creek, Missoula County	3/24/08	7	2													0	9	0
Rock Creek, Missoula County	4/14/09	5	1				0	7	0									
Rock Creek, Missoula County	3/11/10	2	1				0	1	0							0	25	0
Ruby River	3/20/04	5								0	0	3	2	2	5			
Shields River	5/16/04									4	3	1	5	2	3			

S. arc.																									*S. neg.*
IIL-10	IIL-13			IIL-15	IIL-17		IIL-18		IIL-19		IIL-21		IIL-22		IIL-38	IIL-51	IIL-68	IIL-73·74			IIL-79			IIS-12	IL-3·4
X_0Y_{10}	$X_{13}X_{13}$	X_0X_{13}	X_0Y_{13}	X_0Y_{15}	X_0X_{17}	X_0Y_{17}	X_0X_{18}	X_0Y_{18}	X_0X_{19}	X_0Y_{19}	X_2Y_{21}	X_0Y_{21}	X_0X_{22}	X_0Y_{22}	X_0Y_{38}	X_0Y_{51}	X_0Y_{68}	X_0Y_{73}	X_0Y_{74}	$X_0Y_{73•74}$	X_0Y_{79}	X_2Y_{79}	$X_0Y_{3•79}$	X_0Y_{IIS-12}	$X_0Y_{IL-3•4}$
1							0	2																	
1							0	2																	
3							0	1																	
7							0	9																	
							0	3																	
							0	7																	
100							2	93																	
															27	16									
				10																					
				8																					
1				11																					
				3																					
	0	0	5																						
					0	3			0	3															
					0	4			0	4															
					0	6			0	6															
	0	0	6						0	48															
					0	48																			
					1	11			0	11															
									0	4															
	0	0	4		1	4			1	93															
	0	0	5		0	3			0	5															
									1	115															
									0	5															
									0	5															
									0	13															
									0	36															

APPENDIX 1. Continued.

			S. brev.	*S. sax.*			*S. arc.* s. s.			*S. apr.*						*S. arc.*		
			IIL-st/st	IIL-2			IIL-3			IIL-7						IIL-9		
Location	Date	Temp.[a]	X_0Y_0	X_2X_2	X_0X_2	X_2Y_0	X_0X_3	X_0Y_3	X_3Y_3	X_0X_0	X_0X_7	X_7X_7	X_0Y_0	X_0Y_7	X_7Y_7	X_0X_9	X_0Y_9	X_9Y_9
Smith River	3/23/03	4								0	2	0	2	4	2			
Snake River, Lewiston, Idaho	3/24/08	3	9															
Snoqualmie River, Middle Fork	3/9/05	5		10	0	10												
Spokane River	4/5/09	8					0	1	0							0	61	0
Spring Creek, Bar 19	7/4/03	10								3	0	0	0	2	2			
Spring Creek, Bar 19	2/27/04	4								3	7	1	0	10	2			
Spring Creek, Bar 19	7/16/05	10								4	2	0	0	1	0			
Spring Creek, North Fork	5/21/05	7																
St. Joe River, St. Maries, Idaho	3/18/11	3	2	0	2	5	0	7	0									
St. Joe River, St. Maries, Idaho	3/26/11	5	8	1	13	8	0	38	0									
St. Regis River	3/28/04	6	1				0	12	0									
St. Regis River	4/11/04	7	11	0	1	0	0	35	0									
Stillwater River	8/17/03	10								1	1	0	0	3	1			
Sun River	6/18/06	10																
Trout Creek Six Mile	4/3/05	10	8															
Trout Creek Six Mile	5/5/05	11	6				0	1	0									
Trout Creek Six Mile	5/26/05	11	1															
Trout Creek Six Mile	7/14/05	10	9				0	4	0									
Trout Creek Six Mile	8/4/05	12	1				0	3	0									
Trout Creek Mouth, York Canyon	4/3/05	8	21				0	7	0	0	0	0	0	1	0			
Trout Creek Mouth, York Canyon	5/26/05	11	10				0	1	0									
Trout Creek Mouth, York Canyon	6/16/05	12					0	2	0	1	2	1	0	11	3			
Trout Creek Mouth, York Canyon	6/22/05	12								6	22	15	1	20	27			
Trout Creek Mouth, York Canyon	7/7/05	13	1							0	3	1	0	4	5			
Trout Creek Mouth, York Canyon	8/4/05	13								3	2	1	1	7	5			
Trout Creek Mouth, York Canyon	2/28/06	4					0	3	0	0	0	0	0	1	0			
Trout Creek Mouth, York Canyon	5/23/06	11	21				0	6	0	0	0	0	0	0	3			
Trout Creek, Vigilante Camp.	7/14/05	10																
Trout Creek, Vigilante Camp.	8/4/05	11	1				0	1	0									
Upper Spring Cr.	2/17/02	9																
Upper Spring Cr.	4/27/02	8																
Upper Spring Cr.	1/26/03	11																
Upper Spring Cr.	4/26/03	10																
Upper Spring Cr.	8/2/03	13																
Upper Spring Cr.	10/5/03	14																
Upper Spring Cr.	2/7/04	11																
Upper Spring Cr.	2/27/04	11																
Upper Spring Cr.	4/24/04	6																
Upper Spring Cr.	1/22/05	3																
Upper Spring Cr.	5/21/05	8																
Upper Spring Cr.	7/16/05	10																
Upper Spring Cr.	3/25/06	9																
Upper Spring Cr.	7/6/07	9																
Upper Spring Cr.	3/10/09	1																
Upper Spring Cr.	2/26/10	5																
Wise River, Beaverhead Co.	4/12/03	5																
Wise River, Beaverhead Co.	3/20/04	4																
Wise River, Beaverhead Co.	4/15/04	5					0	9	0									
Wise River, Beaverhead Co.	4/22/06	5	1							0	0	0	0	1	0			
Wise River, Beaverhead Co.	4/23/07	5					0	68	0									
Wise River, Beaverhead Co.	6/21/07	6								0	0	0	0	1	0			
Wolf Lodge Creek, Idaho	3/19/11	4	2	1	1	2	0	2	0									
Wolf Lodge Creek, Idaho	3/25/11	5		0	0	1												
Yellowstone R. Chico	4/8/07	6	3				0	17	0	0	0	0	0	0	1			
Yellowstone River, Livingston	6/10/01	8																
Yellowstone River, Livingston	3/11/03	4								0	1	4	0	6	15			
Yellowstone River, Livingston	4/6/03	5					0	10	0	0	1	0	0	1	1			
Yellowstone River, Livingston	5/16/04	6								0	8	4	0	4	7			

S. arc.																									*S. neg.*
IIL-10	IIL-13			IIL-15	IIL-17		IIL-18		IIL-19		IIL-21		IIL-22		IIL-38	IIL-51	IIL-68	IIL-73·74			IIL-79			IIS-12	IL-3·4
X_0Y_{10}	$X_{13}X_{13}$	X_0X_{13}	X_0Y_{13}	X_0Y_{15}	X_0X_{17}	X_0Y_{17}	X_0X_{18}	X_0Y_{18}	X_0X_{19}	X_0Y_{19}	X_2Y_{21}	X_0Y_{21}	X_0X_{22}	X_0Y_{22}	X_0Y_{38}	X_0Y_{51}	X_0Y_{68}	X_0Y_{73}	X_0Y_{74}	$X_0Y_{73 \cdot 74}$	X_0Y_{79}	X_2Y_{79}	$X_0Y_{3 \cdot 79}$	$X_0Y_{IIS\text{-}12}$	$X_0Y_{IL\text{-}3 \cdot 4}$
6																									
8																									
6																									
							1	0													13	1	1		
							1	5													25	7	0		
																									5
																	17								
							0	1									7								
							0	1									3								
																	11								
																	4								
4																	19								
																	4								
																	4								
2							0	1									2								
1																	33								
																	1								
																	1								
1																									
4																									
25																									
8																									
4																									
3																									
2																									
8																									
25																									
11																									
1																									
6																									
11																									
14																									
8																									
11																									
																		5	8	95					
																		0	2	11					
																		2	0	2					
							0	2										0	0	5					
																		1	3	17					
																		1	1	4					
													0	1											
1				1																					
																									85
				1																					
				4																					

APPENDIX 1. Continued.

			S. brev.	*S. sax.*			*S. arc.* s. s.			*S. apr.*						*S. arc.*		
			IIL-st/st	IIL-2			IIL-3			IIL-7						IIL-9		
Location	Date[a]	Temp.[b]	X_0Y_0	X_2X_2	X_0X_2	X_2Y_0	X_0X_3	X_0Y_3	X_3Y_3	X_0X_0	X_0X_7	X_7X_7	X_0Y_0	X_0Y_7	X_7Y_7	X_0X_9	X_0Y_9	X_9Y_9
Yellowstone River, Livingston	7/16/04	9					0	6	0	0	7	4	0	5	6			
Yellowstone River, Livingston	4/8/07	5					0	10	0									

[a]Format for dates: mm/dd/yy. Years range from 2001 to 2011.
[b]Water temperature (°C)

Taxon names:
S. brev. *Simulium brevicercum*
S. sax. *Simulium saxosum*
S. arc. s. s. *Simulium arcticum sensu stricto*
S. apr. *Simulium apricarium*
S. arc. *Simulium arcticum*
S. neg. *Simulium negativum*

S. arc.																											*S. neg.*
IIL-10	IIL-13			IIL-15	IIL-17		IIL-18		IIL-19		IIL-21		IIL-22		IIL-38	IIL-51	IIL-68	IIL-73·74			IIL-79			IIS-12	IL-3·4
X_0Y_{10}	$X_{13}X_{13}$	X_0X_{13}	X_0Y_{13}	X_0Y_{15}	X_0X_{17}	X_0Y_{17}	X_0X_{18}	X_0Y_{18}	X_0X_{19}	X_0Y_{19}	X_2Y_{21}	X_0Y_{21}	X_0X_{22}	X_0Y_{22}	X_0Y_{38}	X_0Y_{51}	X_0Y_{68}	X_0Y_{73}	X_0Y_{74}	$X_0Y_{73\bullet 74}$	X_0Y_{79}	X_2Y_{79}	$X_0Y_{3\bullet 79}$	$X_0Y_{IIS\text{-}12}$	$X_0Y_{IL\text{-}3\bullet 4}$
				5																					

APPENDIX 2. Attributes of 58 freshwater sites in Montana, Idaho, Washington, and Oregon where black fly larvae were collected for cytogenetic analysis.

Location	Site[a]	Latitude (N)	Longitude (W)	Elevation[b]
Beaverhead River, Dillon	1	45°14′05.80″	112°38′23.98″	1560
Big Hole R., Twin Bridges	2	45°38′35.37″	112°18′39.38″	1389
Big Timber Cr.	3	45°51′27.01″	109°56′17.15″	1236.6
Bitterroot River, Darby	4	45°59′45.06″	114°10′11.96″	1197.9
Blackfoot River Russel Gates	5	47°01′25.22″	113°18′42.93″	1175
Boulder River Bison Creek,	6	46°09′55.68″	112°22′05.42″	1901
Boulder River, Cardwell	7	45°52′14.92″	111°56′33.74″	1307.6
Boulder River High Ore	8	46°15′35.52″	112°11′24.39″	1551.1
Boulder River, Sweet Grass Co.	9	45°43′23.29″	109°59′48.90″	1355.7
Canyon Creek	10	46°50′26.52″	112°16′57.24″	1337.2
Cedar River	11	47°23′07.62″	122°02′59.61″	132
Clark Fork R. Bearmouth	12	46°41′52.28″	113°25′03.25″	1143.9
Cle Elum River	13	47°11′06.91″	121°00′17.57″	599.2
Clearwater River	14	47°00′01.77″	113°22′56.64″	1163.1
Coeur d' Alene River, Kingston	15	47°33′11.81″	116°16′05.05″	659.9
Crowsnest Creek, Alberta	16	51°15′12.66″	117°02′31.16″	
Dearborn River	17	47°11′54.73″	112°05′41.16″	1160.4
DeepCreek	18	46°21′16.13″	26°04′94″	1231.70
Flat Creek, Idaho	19	47°44′52.35″	116°00′51.30″	776.6
Flathead River, Perma	20	47°21′15.09″	114°36′31.40″	757.7
Flint Creek Campground	21	46°14′15.75″	113°18′28.19″	1691
Flint Creek Hall	22	46°31′44.68″	113°13′26.20″	1339.6
Flint Creek Philipsburg	23	46°20′18.94″	113°19′14.40″	1560
Gales Creek, Oregon	24	45°58′7″	123°21′5″	88.1
Gallatin River	25	45°49′28.56″	111°16′19.02″	1314
Jefferson River	26	45°54′38.40″	111°32′57.03″	1237.5
Jocko River Arlee	27	47°10′38.80″	114°05′58.48″	913.8
Latah Creek Spokane County	28	47°36′26.03″	117°24′42.40″	556.6
Little Blackfoot River, Elliston	29	46°33′23.00″	112°24′29.01″	1551.4
Little Blackfoot River, Garrison	30	46°31′08.95″	112°47′36.75″	1330.8
Little Prickly Pear Cr.	31	46°58′18.89″	112°05′00.19″	1110.4
Madison River, Three Forks	32	45°54′07.08″	111°33′06″	1239.9
McCleese Lake Inflow	33	52°23′17.84″	122°17′09.13″	
Missouri R. Craig	34	47°04′26.66″	111°57′44.98″	1055.8
Musselshell R., Harlowton	35	46°25′44.83″	109°50′28.13″	1277.1
Prickly Pear Creek	36	46°32′37.41″	111°55′27.95″	1226.50
Red Rock River	37	44°54′58.79″	112°49′24.41″	1722.4
Rock Creek, Missoula County	38	46°42′32.08″	113°40′24.48″	1089.4
Ruby River	39	45°29′28.81″	112°14′51.98″	1495.9
Shields River	40	45°55′38.04″	110°37′26.79″	1496
Smith River	41	47°22′46.66″	111°26′34.25″	1020.5
Snake River, Lewiston, Idaho	42	46°20′32.60″	117°03′02.35″	228.9
Snoqualmie River, Middle Fork	43	47°30′54.44″	121°42′34.19″	1245.1
Spokane River	44	47°41′55.01″	117°02′46.46″	620.6
Spring Creek, Bar 19	45	47°04′45.29″	109°25′45.22″	1194.2
Spring Creek, North Fork	46	47°01′20.14″	109°21′34.80″	1245.4
St. Joe River, St. Maries, Idaho	47	47°19′29.56″	116°26′29.86″	661.7
St. Regis River	48	47°17′48.93″	115°08′15.37″	816.9
Stillwater River	49	45°31′53.95″	109°26′09.26″	1307.9
Sun River	50	47°36′51.81″	112°28′23.54″	1208.8
Trout Creek Six Mile	51	46°44′02.19″	111°43′18.35″	1241.8
Trout Creek Mouth, York Canyon	52	46°42′38.26″	111°48′05.13″	1117.4
Trout Creek, Vigilante Camp.	53	46°46′01.55″	111°39′01.33″	1355.4
Upper Spring Cr.	54	47°00′18.33″	109°20′45.39″	1265.8
Wise River, Beaverhead Co.	55	45°47′28.60″	112°57′06.61″	1724.90
Wolf Lodge Creek, Idaho	56	47°37′44.95″	116°37′27.31″	664.5
Yellowstone R. Chico	57	45°21′41.36″	110°43′47.58″	1499.30
Yellowstone River, Livingston	58	45°39′32″	110°33′49″	1372

[a]Site numbers correspond to the numbered circles in Figures 1 and 2.
[b]Meters