

The *Simulium arcticum* Complex: Environmental Effects on Distribution of Taxa at Trout Creek and Reproductive Status of Taxa at the Blackfoot River

Submitted in partial fulfillment of the requirements for graduation with honors from the Department of Natural Sciences at Carroll College, Helena, Montana

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Abstract

This study had two major objectives: 1) To determine if elevation correlated with sibling distribution within the *Simulium arcticum* complex at Trout Creek, Lewis and Clark County, Montana, a previously unstudied drainage, and 2) to determine the reproductive status of siblings and cytotypes at the Blackfoot River, Missoula County, Montana, as a test of the Shields Geographic Distribution/Taxon Age Continuum Hypothesis. In both studies I used cytogenetic analysis to: compare the banding patterns of polytene chromosomes of salivary glands of larvae of the *S. arcticum* complex, identify larvae to sibling and cytotype, and score autosomal polymorphisms that could be used to determine reproductive status.

For the elevational study, frequencies of siblings at three different elevations along Trout Creek, Lewis and Clark County, Montana, were compared. Based on previous collections in different drainages, it was hypothesized that *Simulium apricarium* would be found predominately at lower elevations while *Simulium arcticum sensu stricto* would be found at higher elevations. Data collected in the present study supported this hypothesis with the caveat that *S. arcticum s. s.* was quite rare in the drainage. A new cytotype, *S. arcticum* IIL-68, was also discovered at Trout Creek.

For the study of reproductive status, the Blackfoot River at Russell Gates Campground, Missoula County, Montana, was chosen. Previous analysis of this site showed the presence of a sufficient number of siblings and cytotypes, as well as numerous autosomal polymorphisms within the *S. arcticum* complex so that a determination of reproductive status could be made. The Shields Geographic Distribution/Taxon Age Continuum Hypothesis suggests that if a cytotype and a sibling

occurred in sympatry, they should be reproductively isolated from one another. The data supported this hypothesis, as analysis of autosomal polymorphisms within the *S. negativum* and *S. arcticum* IIL-9 populations suggest that these populations are both temporally and genetically reproductively isolated. Additionally, the data suggest that two Y-chromosomes, IL-3.4 and IL-3.4 + IS-1, exist within the *S. negativum* population at the Blackfoot River.

Introduction

This thesis summarizes research on two separate but related projects concerning: 1) cytogenetic correlates with environmental factors that might influence distribution of siblings (2005) and 2) an assessment of reproductive status between siblings and cytotypes (2006).

An understanding of specific cytogenetic terms as they relate to Simuliids is initially necessary. The terms: autosomal polymorphism, sex-linked inversion, cytotype, cytospecies and sibling species all have specific, yet related meanings and are defined here for clarity of understanding of the following sections.

Autosomal polymorphism- this term refers specifically to a chromosomal segment that has been reversed 180° in orientation (an inversion) and that can occur in all three genotypic categories: as a standard homozygote (st/st), as a heterozygote (st/inv.) and as an inverted homozygote (inv./inv.), and in roughly equal frequencies in males and females.

Sex-linked inversion- a chromosomal inversion that is associated with the sex of the individual. For example, if in a sample of 1000 individuals, 500 are st/st females and 500 are st/inv. males, then the inversion is said to be sex-linked to males.

Cytotype- a group of black flies having a unique sex-linked inversion but for which information on the reproductive status of the type is as of yet unknown.

Cytospecies - a cytotype that is reproductively isolated from other such types.

Sibling species - a cytospecies for which unique features of biology have been documented and that has been formally described as a good biological species.

Environmental Factors Associated with Distribution of Siblings: Trout Creek

The presence of population specific inversions suggests that environmental factors may affect distribution of taxa (Rothfels and Featherston 1981). Many studies have attempted to determine whether or not environmental factors can be correlated with sibling distribution and diversity among *Simuliids* other than those within the *Simulium arcticum* complex (McCreadie *et al.*, 1997; McCreadie *et al.*, 2005). Within *S. arcticum*, a strong correlation has been found between sibling distribution and elevation. Specifically, analysis of 12 collection sites within four separate drainages in western Montana has shown that certain siblings are found at lower elevations while other siblings are found at higher elevations (Shields *et al.*, 2007). Thus, the first objective of this research was to further test the hypothesis that environmental factors, in this case elevation, influence the distribution of siblings of the *S. arcticum* complex.

Trout Creek, a previously unstudied drainage, in Lewis and Clark County, Montana, with a significant elevational gradient, was chosen as an additional site at which to test the presumptive correlation between genetic variation and elevation. I hypothesized that if *S. apricarium* and *S. arcticum s. s.* occurred in Trout Creek, the former would be found primarily at lower elevation sites while the latter would be found primarily at higher elevation sites.

Assessment of Reproductive Status of Siblings and Cytotypes at the Blackfoot River

Within the *Simuliids*, reproductively isolated sibling species can be identified by cytogenetic analysis of their polytene chromosomes (Rothfels 1956). Specifically, their identification is accomplished by “the presence of fixed rearrangements that separate them from their known relatives,” as well as by the “highly individualistic spectra of

floating rearrangements” (Rothfels, 1956). Additional characteristics, such as the presence of supernumerary, or B chromosomes, centromere dimorphisms, stage of meiosis in males, number of generations and their timing, larval head patterns, body color, pupal characteristics, and whether or not they over-winter as eggs or larvae are also used to characterize siblings within this complex (Shields and Procnier, 1982; Procnier and Shemanchuk, 1983; Adler *et al.*, 2004).

Such analyses have revealed that closely related siblings often occur in sympatry, suggesting that chromosomal rearrangements, especially those involving the sex chromosomes, may play a major role in the speciation process in the *S. arcticum* complex (Shields and Procnier, 1982; Rothfels, 1989). Similar situations, where an inversion involving the sex chromosome appears to be the basis for reproductive isolation, also occur in other *Simuliids* (for example in: *S. venustum/verecundum*, Rothfels *et al.*, 1978; *S. vittatum*, Rothfels and Featherston, 1981; *Prosimulium onychodactylum*, Newman, 1983; *Eusimulium aureum*, Leonhardt and Feraday, 1989; *Gymnopais* (sp.), Rothfels, 1989; *S. pugetense*, Allison and Shields, 1989; and *S. nigricoxum*, Shields, 1990).

A model suggested by Rothfels (1989) argues that some inversions involving sex chromosomes offer a reproductive advantage where certain combinations of rearranged X and Y chromosomes “may be favored reproductively.” Numerous trials may be necessary before an XY combination occurs that allows establishment of the new type, and because the new type may be characterized by “altered biological parameters,” it may be better suited to a different habitat, or it may be able to continue in the same habitat as its progenitor (Rothfels, 1989). This is supported by collections from a single site that are characterized by the presence of individuals with inversions that are completely sex-

linked, partially sex-linked, or autosomally polymorphic, suggesting different stages in the speciation process (Shields *et al.*, in review).

In accordance with Rothfels' model (1989), Shields (2006) has suggested a Geographic Distribution/Taxon Age Continuum Hypothesis (GD/TAC), stating that reproductively isolated siblings may represent ancient types, while cytotypes may possibly be more recently derived, with population specific polymorphisms representing even more recent occurrences in the diversification process.

In order to further test the GD/TAC hypothesis, I analyzed the black fly population of *S. arcticum* at the Russell Gates Campground on the Blackfoot River, Missoula County, Montana. Previous analyses of this site have shown that three siblings (*S. negativum*, *S. arcticum sensu stricto* (*s. s.*), and *S. apricarium*), as well as two cytotypes (IIL-9 and IIL-19) occur there (Shields, unpub.). Moreover, several autosomal polymorphisms appeared to be in high enough frequency that they could be used to monitor reproductive status through determinations of equilibrium frequencies. Thus, I hypothesized that combinations of siblings should be more reproductively isolated from one another than siblings and cytotypes or combinations of cytotypes at the Russell Gates site on the Blackfoot River.

These two studies, 1) investigating possible environmental influences on sibling distributions and 2) status of reproduction are logical extensions of the initial work that documented extensive cytogenetic diversity within the *S. arcticum* complex in the relatively confined geographic area of western Montana (Shields, unpub.). Both are being published (Shields *et al.*, 2007; Shields *et al.*, in review).

Materials and Methods

Trout Creek

As a test of the correlation between sibling distribution and elevation, Trout Creek, in Lewis and Clark County, Montana, was sampled at three different locations along an elevational gradient. These sites were: the mouth of Trout Creek as it enters the Missouri River ("Mile 0", 1193 m) as the low elevation site, an intermediate site ("Mile 6", 1349 m), and Vigilante Campground at the end of York Canyon Road (1424 m) as the high elevation site. Collections were made approximately every three weeks throughout the spring and summer of 2005 from April 3 to August 8. To verify the accuracy of our designation between the IIL-10 sibling and the IIL-68 cytotype that presumably differ by only one band, additional collections were made on March 22 and May 23 of 2006 and analyzed (Fig. 1). Larvae were sampled from all accessible substrates including rocks and trailing vegetation. Stream temperature was recorded on each collection date to the nearest degree centigrade. Larvae were fixed in fresh Carnoy's at the site, placed on ice until our return to the laboratory, sorted to morphospecies (Currie, 1986), stained in Feulgen (Rothfels and Dunbar, 1953) and their chromosomes subsequently scored with reference to standard chromosome maps (Figs. 1 and 2; Shields and Procunier, 1982). The percentage of *S. apricarium* and *S. arcticum s. s.* collected at each site was then compared.

Blackfoot River

The Blackfoot River at the Russell Gates Campground, Missoula County, Montana, was sampled five times at approximately three-week intervals throughout the

summer of 2006. Three collections: May 26, June 15, and July 10 yielded sufficient larvae for analysis. Collections on March 28, 2004 and April 1, 2005 by G. F. Shields were included for completeness of analysis. Previous cursory analysis of the presence of siblings, cytotypes and autosomal polymorphisms (Shields, unpub.) suggested that analysis of reproductive status of taxa at the Blackfoot River was feasible. Collection protocols, laboratory techniques, and analyses of chromosomes were identical to those used for larvae from Trout Creek. All larvae were scored for sex-linked and autosomal polymorphisms as well as for centromere band dimorphisms (Shields and Procunier, 1982) and equilibrium frequencies were determined when appropriate.

Results

Trout Creek

My first objective was to describe the diversity among cytospecies and cytotypes of the *Simulium arcticum* complex at three elevations of Trout Creek and to determine if an elevational correlation to sibling presence existed. Accordingly, larvae were collected at all three collection sites along Trout Creek (Tables 1 and 2).

Mile 0: *S. arcticum* larvae were collected in abundance at this site on all collection dates, except on May 5, 2005, when only pupae were present (Table 1). The siblings *S. brevicercum*, *S. apricarium*, and *S. arcticum s. s.* were collected at this site as well as two cytotypes, IIL-10 and IIL-68, the latter being newly discovered. The percentages of males within the sample were: *S. apricarium*, 37.5%, *S. arcticum* IIL-68, 26.67%, *S. brevicercum* 24.6%, *S. arcticum s. s.* 9.0%, and *S. arcticum* IIL-10, 2.1%. With the exception of one larva, all larvae of the *S. arcticum* complex collected after 6/22/2005 were *S. apricarium*. Additionally, *S. canonicolum* and *S. canadense* were also collected at this site (Table 2).

Mile 6: *S. arcticum* was collected on five of the six collection dates (Table 3). The siblings, *S. brevicercum*, and *S. arcticum s. s.* were found at this site, as well as the cytotypes: IIL-68, IIL-30, IIL-47 and IIL-18. The percentages of males were: cytotype IIL-68, 51.2%; *S. brevicercum*, 30.5%; *S. arcticum s. s.*, 9.8%; cytotype IIL-30, 3.7%; cytotype IIL- 47, 2.4%, and cytotype IIL-18, 2.4%. No larvae of *S. apricarium* were found at this site, suggesting that this sibling may be restricted to lower elevations. Additionally, *S. canonicolum* and *S. canadense* were also collected at this site (Table 2).

Vigilante Campground: Six collections were made at this site throughout the summer, however, only six larvae of the *S. arcticum* complex were observed (two on 7/14/2005, and four on 8/4/2005). Four of the six larvae were male and of the four males observed, one was *S. brevicercum*, one was *S. arcticum s. s.*, and two were the IIL-68 cytotypic. *S. canonicolum*, *S. canadense*, and *Prosimulium exigens* were collected in abundance at this site (Table 2). A comparison of the frequencies of each sibling at each collection site can be found in Figure 3.

Additionally, a centromere dimorphism occurred within the *S. arcticum s. s.* and *S. arcticum* IIL-68 populations. Specifically, males within these populations had one heavy centromere band and one thin centromere band in chromosome II (Table 4). The thin centromeric band appeared to be completely linked to the Y chromosome in these populations.

Blackfoot River

My second objective was to determine the reproductive status of the three siblings (*S. negativum*, *S. arcticum s. s.*, and *S. apricarium*), and the two cytotypes (IIL-9 and IIL-19) by analyzing the autosomal polymorphisms of these siblings and cytotypes at the Blackfoot River.

Collections made in the early spring were characterized by the presence of the cytotypic, *S. arcticum* IIL-9, followed by the appearance of *S. negativum* in May and June, followed by a collection in July in which only IIL-9 appeared in abundance. This suggests that the *S. negativum* sibling and the IIL-9 cytotypic are temporally isolated and that there are two generations of *S. arcticum* IIL-9.

The autosomal polymorphisms IIS-10 and IIIL-4 were present in such frequencies that they could be used to test whether or not the populations were reproductively isolated. The IIS-10 polymorphism is fixed for the standard homozygote (st/st) in the early *S. arcticum* IIL-9 population and only one heterozygote for the IIS-10 inversion was found in the late IIL-9 population. In contrast, almost all of the *S. negativum* larvae collected were inversion homozygotes for the IIS-10 inversion (i/i), suggesting that this population is fixed for the inversion homozygote (Table 6). In addition to IIL-9 and *S. negativum*, eleven IIL-19 males were also present in the 3/30/03 sample. All individuals were standard for IIS-10 (Table 6). This indicates that, like the early population of *S. arcticum* IIL-9, these IIL-19 individuals are temporally and genetically reproductively isolated from *S. negativum*. Heterozygosity for the IIIL-4 inversion appears at a low frequency in the early IIL-9 population (9.7%), is found in high numbers in the *S. negativum* population (40% on 5/23/2006 and 37.1 % on 6/15/2006), but is not present in the late IIL-9 population (Table 6). This also suggests reproductive isolation.

Additionally, the IS-1 polymorphism was found in both populations, however, in significantly different frequencies. In the early IIL-9 population, the percentage of IS-1 heterozygotes was 19.1%, and in the late population, it was 13.9%. Chi-square analysis suggests that these populations are in equilibrium with respect to the IS-1 autosomal polymorphism. In contrast, IS-1 heterozygotic males of the *S. negativum* collection were found in high frequency (81%; Table 7). This suggests that the *S. negativum* population has two different Y-chromosomes, IL-3.4 and IL-3.4 + IS-1, the latter having male determining genes at two different loci.

In the 5/23/2006 collection, six females with the IL-3.4 inversion were collected and two such females were collected on 6/15/2006. In addition, one IL-3.4 male that was heterozygotic for the IIL-3 inversion, and two IL-3.4 males that were heterozygotic for the IIL-9 inversion were found in the 3/28/2004 collection. These larvae are assumed not to be *S. negativum* as they are chromocentric and had positive head patterns, whereas the *S. negativum* population at the Blackfoot River is characterized by achromocentricity and the presence of negative head patterns in females. Therefore, I conclude that the IL-3.4 inversion in these individuals is an autosomal polymorphism.

Table 1: Summary of Sex Chromosome Diversity Within the *S. arcticum* Complex at Trout Creek, Mile 0, York Canyon, Lewis and Clark County, Montana.

Date	Water Temp. (°C)	Females				Males							Total
		X ₀ X ₀	IIL-7	IIL-7	IIL-7	X ₀ Y ₀	X ₀ Y ₃	X ₀ Y ₆₈	X ₀ Y ₁₀	IIL-7	IIL-7	IIL-7	
3/22/2006	?	6	0	0	0	2	8	4	2	0	1	1	24
4/3/2005	8	32	0	0	0	21	5	19	2	0	1	0	80
5/5/2005	8	only pupae including those of <i>S. arcticum</i>											
5/23/2006	?	-	-	-	-	25	6	33	1	0	0	3	68
5/26/2005	11	14	0	0	0	10	1	4	0	0	0	0	29
6/16/2005	10	2	1	2	1	0	2	4	0	0	11	3	26
6/22/2005	13	6	6	22	15	0	0	0	0	1	20	27	97
7/7/2005	13	0	0	3	1	1*	0	0	0	0	4	5	14
8/4/2005	8	0	3	2	1	0	0	0	0	1	7	5	19
Total	-	60	10	29	18	59	22	64	5	2	44	44	357

The percentage of *S. apricarium* males = 37.5, cytotype IIL-68 (males) = 26.67, *S. brevicercum* males = 24.6, *S. arcticum s. s.* males = 9.2, and cytotype IIL-10 (males) = 2.1. With the exception of one larva, all larvae of the *S. arcticum* complex collected after 6/22/2005 were *S. apricarium*.

Table 2: Species Richness of Black Flies at All Three Sites on Trout Creek.

Collection Date	Species Present		
	Mile 0	Mile 6	Vigilante Campground
5/5/2005		<i>S. arcticum</i> , <i>S. canonicolum</i>	<i>S. canonicolum</i>
5/26/2005	<i>S. arcticum</i>	<i>S. arcticum</i> , <i>S. canonicolum</i>	<i>P. exigens</i> , <i>S. canonicolum</i>
6/16/2005	<i>S. arcticum</i> , <i>S. canonicolum</i>	<i>S. arcticum</i> , <i>S. canonicolum</i>	<i>P. exigens</i> , <i>S. canadense</i> , <i>S. canonicolum</i>
6/22/2005	<i>S. arcticum</i> , <i>S. canadense</i> , <i>S. canonicolum</i>		
7/14/2005		<i>S. arcticum</i> , <i>S. canadense</i> , <i>S. canonicolum</i>	<i>S. arcticum</i> , <i>S. canadense</i> , <i>S. canonicolum</i>
8/4/2005	<i>S. arcticum</i> , <i>S. canadense</i> , <i>S. canonicolum</i>	<i>S. arcticum</i> , <i>S. canadense</i> , <i>S. canonicolum</i>	<i>S. canonicolum</i> , <i>S. canadense</i> , <i>S. arcticum</i>

Table 3: Sex-chromosome Diversity Within the *S. arcticum* Complex at Trout Creek, Mile 6.0.

Date	Water Temperature (°C)	Females	Males						Total	
		X ₀ X ₀	X ₀ Y ₀	X ₀ Y ₆₈	X ₀ Y ₃	X ₀ Y ₁₈	X ₀ Y ₄₇	X ₀ Y ₃₀		
4/3/2005	10	20	8	17	0	0	0	0	45	
5/5/2005	11	13	6	7	1	1	0	0	28	
5/26/2005	11	15	1	3	0	1	0	0	20	
6/16/2005	11	only larvae of <i>S. canonicolum</i> present								
7/14/2005	10	21	9	11	4	0	0	0	45	
8/4/2005	12	8	1	4	3	0	2	3	21	
Total	-	77	25	42	8	2	2	3	159	

Percentage of males: cytotype IIL-68 = 51.2, *S. brevicercum* = 30.5, *S. arcticum s. s.* = 9.8, cytotype IIL-30 = 3.7, cytotype IIL-47 = 2.4, cytotype IIL-18 = 2.4.

No larvae of *S. apricarium* have been found at this site.

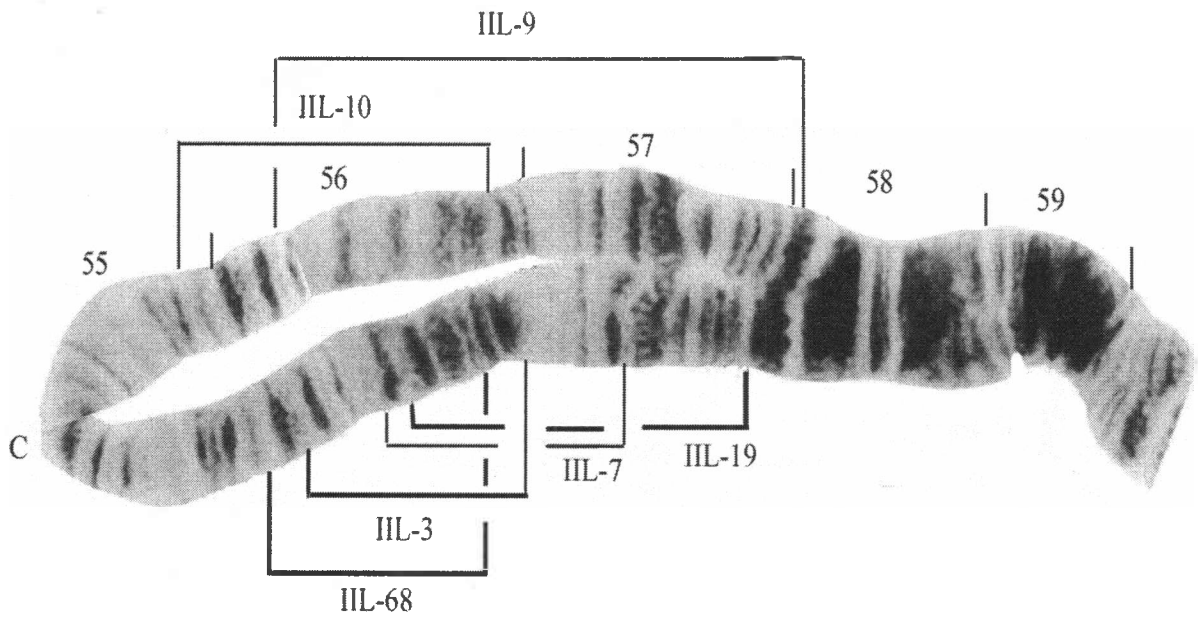


Figure 1: Sex-linked Inversions at the Base of the Long Arm of Chromosome II Observed in this Study.

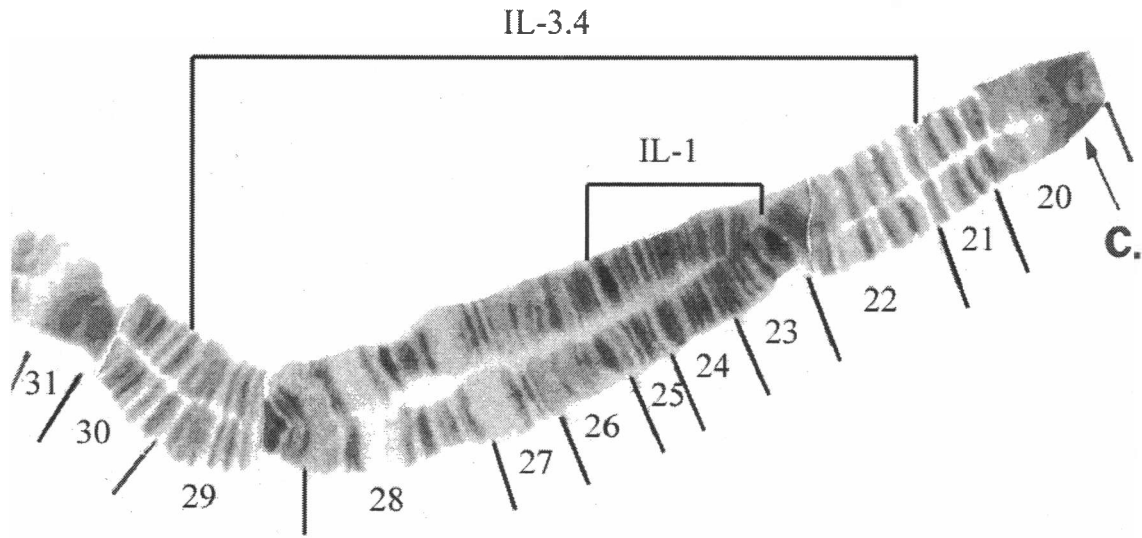


Figure 2: Sex-linked and Autosomal Inversions at the Base of the Long Arm of Chromosome I Observed in this Study.

Table 4: Distribution of Y Chromosomes and the Centromeric Band Dimorphism Within the *S. arcticum* population at Trout Creek, Mile 0, 5/23/06.

Sibling/Cytotype	Centromere Dimorphism	
	CE/CE*	CE/CT*
<i>S. brevicercum</i>	24	1
<i>S. arcticum s. s.</i> **	0	6
<i>S. arcticum</i> IIL-68**	0	34

* CE/CE denotes a centromere with both bands enhanced, CE/CT denotes a centromere with one band enhanced and the other thin.

** The thin centromeric band appears to be completely linked to the Y chromosome in the *S. arcticum s. s.* and *S. arcticum* IIL-68 populations.

Table 5: Summary of *S. arcticum* Siblings and Cytotypes Found at the Blackfoot River at Russell Gates Campground, Missoula County, Montana.

Date	Water Temp. (C)	Females		Males					Total	
		X ₀ X ₀	X ₀ X _{III-3,4}	X ₀ Y ₀	X ₀ Y _{III-3,4}	X ₀ Y _{III-3,4+IS-1}	X ₀ Y _{III-9}	X _{III-9} Y _{III-9}		
3/13/2006	-	no larvae present								
3/28/2004	-	31	0	1	0	0	27	1	60	
3/30/2003*	-	54	0	0	0	0	27	0	81	
4/1/2005*	-	17	0	0	0	0	10	0	27	
5/9/2006*	-	many small larvae								
5/18/2003	-	7	0	0	6	6	0	0	19	
5/23/2006	10	71	6	0	8	60	0	0	145	
6/15/2006	11	25	2	0	2	6	0	0	35	
7/10/2006	16	54	0	5	0	0	42	2	103	
Total	-	259	8	6	16	72	106	3	470	

*Data for these collection dates from Shields, unpub.

**Eleven IIL-19 males were also present in the 3/30/03 sample.

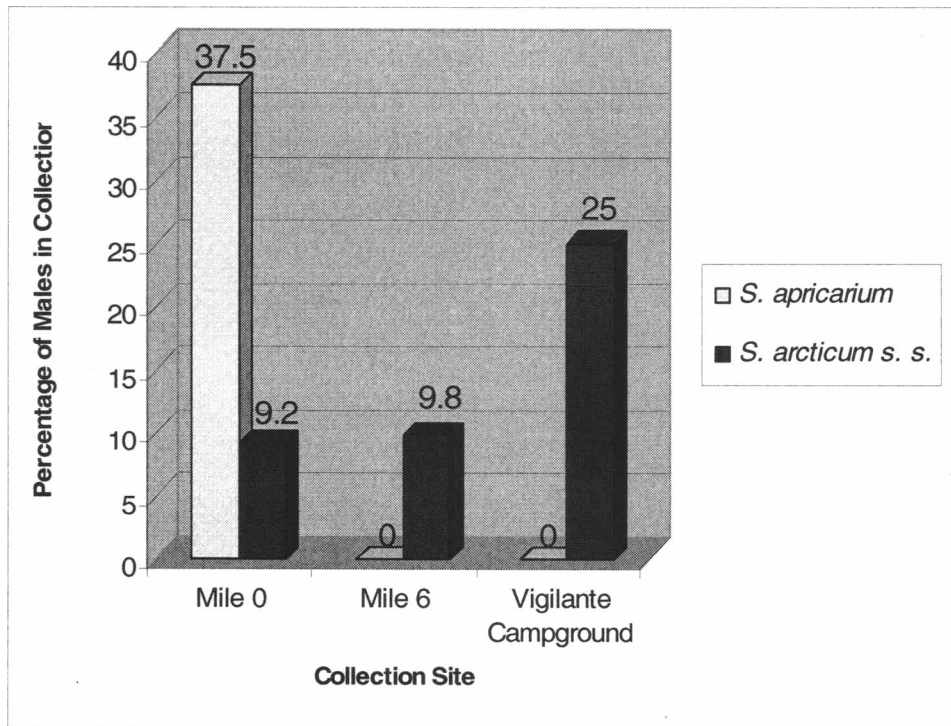


Figure 3: Comparison of Percentage of Male *S. arcticum s. s.* and *S. apricarium* at Each Elevation of the Trout Creek Drainage.

*Only six larvae of the *S. arcticum* complex were observed at the Vigilante Campground site. Of the four males observed, one was *S. brevicercum*, one was *S. arcticum s. s.* and two were cytotype IIL-68.

Table 6: Distribution of IIS-10 and IIL-4 Autosomal Polymorphisms Among the *Simulium arcticum*, IIL-9 and *S. negativum*, IL-3.4 Populations at the Blackfoot River.

Date	IIS-10			IIL-4
	st/st	st/i	i/i	% st/i
3/13/2006	no larvae present			
	<i>S. arcticum</i> IIL-9			
3/28/2004	60	0	0	9.7
3/30/2003	81	0	0	not scored
4/1/2005	10	0	0	0
	<i>S. negativum</i>			
5/9/2006	small larvae, too immature to score			
5/23/2006	3	0	145	40
6/15/2006	1	0	34	37.1
	<i>S. arcticum</i> IIL-9			
7/10/2006	102	1	0	0

Table 7: Distribution of the IS-1 Inversion Among Sexes and Populations of the *S. arcticum* Complex at the Blackfoot River.

Date	Females			Males			Chi Square Value
	st/st	st/i	i/i	st/st	st/i	i/i	
	<i>S. arcticum</i> III-9						
3/28/2004	25	6	0	30	7	0	$X^2 = 1.06$ d.f. = 2, $0.60 < p < 0.70$
3/30/2003	54	7	0	27	2	0	
4/1/2005	12	4	0	12	0	0	
Total	91	17	0	69	9	0	
	<i>S. negativum</i>, II-3.4						
5/18/2003	14	2	0	5	7	0	$X^2 = 12.6$ d.f. = 2, $0.01 < p < 0.02$
5/23/2006	68	6	0	9	59	0	
6/15/2006	27	2	0	2	6	0	
Total	109	10	0	16	7	0	
	<i>S. arcticum</i> III-9						
7/10/2006	51	4	0	42	11	0	$X^2 = 0.61$ d.f. = 2, $0.70 < p < 0.80$

Discussion

Trout Creek

One objective of this research was to further test the hypothesis that *S. arcticum s. s.* occurs at predominantly high elevations and *S. apricarium* occurs at lower elevations (Shields *et al.*, 2007).

Total Species Richness at Trout Creek

Though not a specific objective of this research, species richness of black flies was also determined in the drainage. Overall, compared to collections of fly larvae at other drainages, the species richness at Trout Creek was high. At the mouth, *S. arcticum* was collected in abundance as well as *S. canonicolum*, and *S. canadense*. Collections at the Mile 6 site consisted of *S. arcticum*, *S. canonicolum* and *S. canadense*. At both sites, *S. arcticum* and *S. canonicolum* were collected throughout the spring and summer, with *S. canadense* appearing in the June 22, 2006 collection at Mile 0 and the July 14, 2005 collection at Mile 6. At the high elevation site, only six larvae of *S. arcticum* were collected, even after extensive sampling throughout the summer. The majority of collections at Vigilante Campground consisted of *S. canonicolum*, *Prosimulium exigens* and *S. canadense*. It can be concluded that the species richness of black flies at Trout Creek is high and possibly varies elevationally and temporally.

Species Richness and Diversity Within the S. arcticum Complex

There was considerable diversity within the *Simulium arcticum* complex at Trout Creek. *S. arcticum s. s.*, *S. apricarium* and *S. brevicercum* siblings, as well as the cytotypes, IIL-68 and IIL-10 were present at Mile 0. At Mile 6, the *S. arcticum s. s.* and *S. brevicercum* siblings were present, as well as the cytotypes IIL-68, IIL-18, IIL-47 and

IIL-30. Of only six *S. arcticum* larvae collected in 2006 at Vigilante Campground, four were males: one *S. brevicercum*, one *S. arcticum* s. s. and two *S. arcticum* IIL-68.

Numerous other larvae having unique inversions in chromosome IIL were scored as well; their frequencies were low.

The second objective of this research was to determine whether or not elevational gradients could be correlated with the distribution of members of the *S. arcticum* complex at Trout Creek.

Effects of Elevation on Distribution of Taxa Within the S. arcticum Complex

It has been proposed that elevation may play a role in determining the distribution of taxa within the *Simulium arcticum* complex (Shields *et al.*, 2007). The results obtained in this study support this contention. *S. apricarium* was the second most abundant taxon of the *S. arcticum* complex in the Trout Creek Drainage (27.6% of ♂♂). It occurred only at the mouth site (low elevation; 37.5% of ♂♂) and with the exception of one larva, only after 5/26/05. *S. apricarium* was never collected at any of the higher elevation sites. *S. arcticum* s. s. was rare in the drainage (9.5% of ♂♂), yet it occurred at all three sites. However, analysis of the percentage of *S. arcticum* s. s. collected at each site tends to support the conclusion that this sibling is more prevalent at higher elevations. Thus, with the caveat that *S. arcticum* s. s. was somewhat rare in the drainage, I accept my hypothesis that *S. apricarium* would occur at low elevations and that *S. arcticum* s. s. would occur at higher elevations.

Additionally, Shields *et al.* (2007) observed that an elevational correlation with the distribution of *S. arcticum* IIL-18 is more apparent than any elevational correlation that could be seen with *S. arcticum* s. s. While *S. arcticum* IIL-18 was found in the Trout

Creek drainage, it was rare (2.4% of ♂♂ at Mile 6). However, in accordance with Shields' observation that *S. arcticum* IIL-18 occurs at higher elevations, this cytotype occurred only at the Mile 6 site.

Finally, *S. arcticum* IIL-68, a newly described cytotype, was found at all three sites and was the most prevalent taxon of the *S. arcticum* complex in the drainage (33.1% of ♂♂). This cytotype has not been found among nearly 600 other collections in Western Montana (Shields, unpub.). Among 102 individuals having the IIL-68 inversion in this study, all were male. Unfortunately, analysis of the reproductive status of this cytotype could not be determined since the frequency of autosomal polymorphisms, by which adherence to equilibrium could be tested, was low in this cytotype. Nonetheless, the unique presence of *S. arcticum* IIL-68 at Trout Creek supports the Geographic Distribution/Taxon Age Hypothesis of Shields (2006).

Interestingly, a centromere dimorphism exists that appears to be correlated with inversions within the *S. arcticum s. s.* and *S. arcticum* IIL-68 populations. The centromeres of chromosome II of males in these two populations were characterized by one thin band and one heavy band. In all cases observed, the thin centromere band was linked to the chromosome having the sex-linked inversion specific to each taxon. This appears to be a characteristic of most taxa within the *S. arcticum* complex (Shields and Procunier, 1982). The function of this centromeric dimorphism, if indeed there is one, and its association with sex, is completely unknown.

Blackfoot River

During the second year of this study I investigated the reproductive status of various taxa of the *S. arcticum* complex at locations where they occur in sympatry. Since

significant diversity has been discovered within the complex (Shields *et al.*, 2007) it is important to determine how this diversity arises. One approach is to determine how cytotypes arise and whether they are reproductively isolated from other taxa of the complex when they occur together in space and time. Therefore, the second major objective of this study was to utilize analysis of autosomal polymorphisms common to sympatric taxa to determine the reproductive status of three cytospecies: *S. negativum*, *S. arcticum s. s.*, and *S. apricarium*, and two cytotypes (IIL-9 and IIL-19) known to be present at the Blackfoot River site. The data suggest an early and late presence (two generations) of the cytotype, *S. arcticum* IIL-9, separated by a midsummer presence of the cytospecies, *S. negativum*. These observations suggest temporal and reproductive isolation for these populations at the Blackfoot River and support the Geographic Distribution/Taxon Age Continuum hypothesis of Shields (2006). The reproductive isolation between *S. arcticum s. s.*, *S. apricarium*, and two cytospecies at Little Prickly Pear Creek (Clausen, 2007) and the lack of reproductive isolation between two cytotypes, IIL-9 and IIL-19, at Rock Creek (Pickens, 2007) also support the Geographic Distribution/Taxon Age Continuum hypothesis.

Eighty-one percent of males within the *S. negativum* population were IS-1 heterozygotes, suggesting the presence of two Y chromosomes, IL-3.4 and IL-3.4 + IS-1 at the Blackfoot River. On the contrary, IS-1 is autosomal in the IIL-9 populations, as it is in most other populations of *S. arcticum* including IIL-9 and IIL-19 populations at nearby Rock Creek (Shields, unpub; Pickens, 2007). These observations not only support the contention that *S. negativum* and *S. arcticum* IIL-9 are reproductively isolated, but

they also may be representative of Rothfels' (1979) claim that "one sibling's sex-linked inversion may be another sibling's autosomal polymorphism."

Similarly, the IIS-5 inversion is fixed for the standard homozygote (st/st) in the both *S. arcticum* IIL-9 populations. In contrast, almost all of the *S. negativum* larvae analyzed were IIS-5 inversion homozygotes (i/i). This further supports the contention that the taxa are reproductively isolated.

Finally, heterozygosity for the IIIIL-4 inversion appeared at a low frequency in the early IIL-9 population (9.7%) and was found in high numbers in the *S. negativum* population (40% on 5/23/2006 and 37.1 % on 6/15/2006), yet it is not present in the late IIL-9 population. This also suggests reproductive isolation.

Additionally, *S. arcticum* IIL- 9 and IIL-19 are also found only at the Clearwater River and at the Clearwater and Bitterroot Rivers, respectively (Shields *et al.*, unpub.). In contrast, *S. negativum* has a much wider distribution from Alaska south to northern New Mexico (Shields and Procunier, 1982; Adler *et al.*, 2004; Shields, unpub.). The wider range of *S. negativum* may suggest that it is much older in evolutionary time than are either *S. arcticum* IIL-9 or IIL-19.

The *S. negativum* population at the Blackfoot River is similar to the *S. negativum* population found in collections throughout Alaska as both populations appear to be fixed as inversion homozygotes for IIS-5. However, the IS-1 inversion and the IIIIL-4 inversions do not occur in *S. negativum* populations in Alaska (Shields and Procunier, 1982). Additionally, in the Alaska populations, IL-3 and IIS-7 occurred as floating inversions (Shields and Procunier, 1982). IL-3 and IIS-7 were not found in the Blackfoot

River population. This suggests possible divergence from the ancestral *S. negativum* type.

Finally, the presence of two Y chromosomes in the *S. negativum* population at the Blackfoot River is of interest. The presence of male-determining loci, presumably at regions on different arms of the same chromosome is rare within *Simuliids*. Additional detailed studies such as this one, as well as verification of the actual sex-determining loci via *in situ* hybridization of molecular markers, may eventually elucidate the pattern of sex-determination in these genetically variable flies.

Conclusions

At Trout Creek, it appears that *S. apricarium* occurs at lower elevations. Although the data regarding the presence of *S. arcticum* s .s. were inconclusive due to the rarity of this sibling within the drainage, it can be concluded that elevation appears to be correlated with spatial separation within the *S. arcticum* complex. Additionally, a new cytotype, IIL-68 was found in this drainage.

The temporal and genetic separation of *S. arcticum* IIL-9 and *S. negativum* at the Blackfoot River suggests reproductive isolation and support the Geographic Distribution/Taxon Age Continuum hypothesis of Shields (2006), because one aspect of this hypothesis is that siblings and cytotypes should be reproductively isolated from one another. This reproductive isolation, as well as the very broad distribution of *S. negativum* and the limited distribution of *S. arcticum* IIL-9 suggest that the sibling may indeed be evolutionarily older than the cytotype.

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