Reproductive Status of Cytoforms in a Black Fly Complex in Montana

Gerald F. Shields, Department of Natural Sciences, Carroll College, 1601 Benton Ave, Helena, MT 59625
Judith A. Pickens, Department of Natural Sciences, Carroll College, 1601 Benton Ave, Helena, MT 59625
Gregory M. Clausen, Department of Natural Sciences, Carroll College, 1601 Benton Ave, Helena, MT 59625
Lindee M. Strizich, Department of Natural Sciences, Carroll College, 1601 Benton Ave, Helena, MT 59625

Abstract

We studied the reproductive status of cytospecies and cytotypes of the *Simulium arcticum* complex at four sites in Montana by comparing banding sequences of polytene chromosomes of the larval salivary glands to 1) identify cytospecies and cytotypes, 2) determine genotypes and frequencies of autosomal polymorphisms, and 3) assess the degree of reproductive isolation of taxa. We hypothesized that taxa within the complex that have large geographic distributions would be reproductively isolated in sympatry, while those that have very limited geographic distributions would not. Data from four separate collection sites support our hypothesis and possibly suggest a model for divergence within the group.

Key words: black flies, reproductive status, siblings, cytospecies, cytotypes, autosomal polymorphisms, hybrids

Introduction

In black flies (*Diptera:Simuliidae*) the morphospecies of classical taxonomy often reveals itself as any number of sibling species when polytene chromosomes of larval salivary glands are analyzed (Rothfels 1956). Taxa are described primarily on the basis of fixed-inversion sequences and by the presence of sex-linked chromosomal rearrangements and other features of their biology including the extent of shared autosomal polymorphisms, presence or absence of supernumerary or B chromosomes, the most advanced developmental stage of meiosis, and numbers of generations/year. We describe populations as cytotypes if they are cytologically distinct, whereas we designate sibling species status (cytospecies) if these cytologically defined taxa are reproductively isolated from other such forms in sympatry.

Shields and Procunier (1982) described five siblings of the *Simulium arcticum* complex in Alaska and western Canada (*S. arcticum* st, *S. arcticum* IL-3·4, *S. arcticum* IIL-1, S. *arcticum* IIL-2, and *S. arcticum* IIL-3). The former two and the latter two have now been designated *S. brevicercum*, *S. negativum*, *S. saxosum*, and *S. arcticum* sensu stricto, respectively (Adler et al. 2004). Two additional siblings, *S. arcticum* IIL8·9/IIS-10·11 *(S. vampirum*, Adler et al., 2004) and *S. arcticum* IIS-4, have been described from the Athabasca River drainage of Alberta, Canada, by Procunier and Shemanchuk (1983) and by Procunier (1984), respectively. Finally, two other siblings, *S. apricarium* and *S. chromatinum*, have been recognized (Adler et al. 2004).

We have recently documented the presence of *S. brevicercum*, *S. negativum*, *S. arcticum* sensu stricto and *S. apricarium* in Montana along with eleven cytotypes of the complex (Shields, unpub.). Distribution of siblings in the *S. arcticum* complex is associated with elevation in Montana (Shields et al. 2006). The considerable diversity within a single morphospecies and the abundance of larvae here provide opportunity to investigate the extent of reproductive isolation of taxa in sympatry, the focus of this research. Specifically, tests of the extent of reproductive isolation in sympatry can be conducted because taxa can be differentiated by the presence of unique

sex-linked chromosomal rearrangements and individuals of each type can be scored for autosomal polymorphisms that can then be subjected to genetic equilibrium analyses. While fixation of alternative inversion sequences between two taxa in sympatry may suggest reproductive isolation, sharing of identical polymorphisms does not necessarily indicate random breeding. Black flies (Rothfels 1978), including cytospecies of the *S. arcticum* complex, share autosomal polymorphisms that are retained in respective populations after reproductive isolation has occurred (Shields and Procunier, 1982; Adler et al. 2004).

Cytospecies of the *arcticum* complex are broadly distributed across western North America (Adler et al. 2004), whereas 11 new cytotypes in Montana have restricted distributions, some of which occur only at a single site within a drainage (Shields, unpub.). Patterns of geographic distribution suggest that cytospecies may be evolutionarily old and genetically divergent from other members of the complex while cytotypes may be evolutionarily young and still in the process of divergence from other types with which they are sympatric. The combination of this diversity at the level of chromosomes and knowledge of the geographic distributions of the various types suggests the possibility of defining the extent of reproductive isolation for each taxon and may allow insight into the process of speciation. As Jerry Coyne and Allan Orr (2004:69) emphasize in their recent book, *Speciation, "How can we tell which isolating barriers actually caused speciation instead of having evolved after speciation was complete? Comparative analysis of taxa at different stages of evolutionary divergence, ranging from populations through full species, may show which barriers persist throughout this transition."* In the present study, we determined distribution and frequency of autosomal polymorphisms among taxa of the *S. arcticum* complex in sympatry to estimate extent of reproductive isolation. We hypothesized that taxa with broad geographic distributions would be reproductively isolated in sympatry while those with limited distributions would not.

Materials and Methods

Assessment of Reproductive Status

Tests of reproductive status of cytological entities can be conducted in a number of ways. Taxa that develop at different times (temporal isolation) cannot produce hybrid progeny. However, reproductive status of two cytologically distinct taxa in both sympatry and synchrony can be determined by analysis of the distribution and frequency of autosomal polymorphisms and other cytological criteria. For example, presence of alternative homozygotes, without heterozygotes, suggests reproductive isolation. Floating autosomal polymorphisms among types can be tested for adherence to equilibrium frequencies and thus indicate reproductive status.

Chromosome Nomenclature

Sex chromosomes in black flies are often associated with chromosomal rearrangements. Thus, for most cytospecies and cytotypes within the *S. arcticum* complex males are heterozygous for unique, sex-linked inversions whereas females generally possess the standard chromosome sequence. Consequently, we designated the sex chromosomes of females as X_0X_0 to indicate that they possess the standard (non-inverted) chromosome sequence for corresponding sex-linked inversions in males. Males are characterized as having one chromosome, the X with the standard sequence, X_0, and a second chromosome Y with a paracentric inversion. Sex-linked inversions are numbered according to the sequence of their discovery. For example, *S. arcticum* IIL-1 males are designated $X_0\ Y_{IIL\text{-}1}$, and those in *S arcticum* s. s. as $X_0\ Y_{IIL\text{-}3}$. Sex-chromosomes in *S. apricarium* are polymorphic for all classes; thus, females can be X_0X_0, $X_0\ X_{IIL\text{-}7}$, or $X_{IIL\text{-}7}\ X_{IIL\text{-}7}$ whereas males can be X_0Y_0, $X_0\ Y_{IIL\text{-}7}$, or $X_{IIL\text{-}7}\ Y_{IIL\text{-}7}$. The subscript indicates the specific inversion characterizing each sibling or cytotype.

Selection of Sites

Presence of autosomal polymorphisms in sufficient frequencies allows statistical analyses

at sites where two or more siblings or cytotypes are present. Our previous observations (Shields, unpub.) suggested that study of the extent of reproductive isolation among cytological entities of the *S. arcticum* complex was possible at four sites (Fig. 1, Table 1).

Sample Collection and Analysis

At each collection site we sampled larvae regardless of species richness. Larvae were removed from various substrata and fixed in cold Carnoy's fixative. Upon return to the laboratory we sorted larvae of the *S. arcticum* complex to morphospecies (Currie 1986) and selected penultimate and ultimate instar larvae for analysis because they possessed polytene chromosomes of the highest quality. We used the Feulgen method of Rothfels and Dunbar (1953) to stain polytene chromosomes and gonads and used standard chromosome maps of the *S. arcticum* complex (Shields and Procunier 1982) to differentiate cytospecies and cytotypes and to determine frequencies of autosomal polymorphisms. Hardy-Weinberg equilibrium statistics were calculated where cytospecies and cytotypes were present in geographic sympatry.

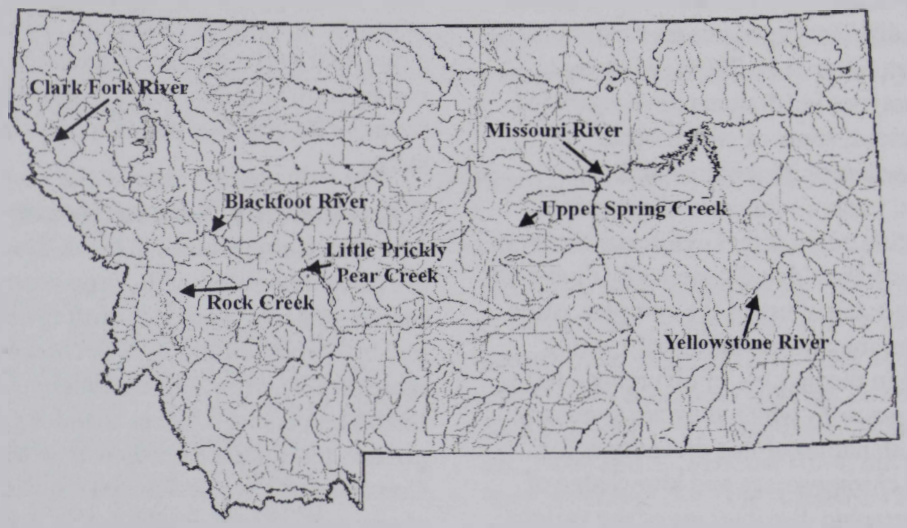

Figure 1. Map of Montana showing the three major drainage basins (Clark Fork, Missouri, and Yellowstone rivers), along with most other drainages, and the specific locations of collection sites studied here.

Table 1. Sites studied, taxa analyzed in detail, collection schemes, and autosomal polymorphisms used to determine reproductive status of various siblings and cytotypes of the *S. arcticum* complex in western Montana.

Location	Taxa Present	Collections Analyzed in Detail	Autosomal Polymophisms
Blackfoot River	*S. negativum* and *S. arcticum* IIL-9	Eight dates, four summers	IS-1, IL-3.4, IIS-10·11, IIIL-4
Upper Spring Cr.	*S. arcticum* IIL-10	Five years	None
Little Prickly Pear Creek	*S. arcticum* s. s. and *S. apricarium*	Four summers	IIS- 10·11, IIL-20
Rock Creek	*S. arcticum* IIL-9 and IIL-19	Single date (3/14/06)	IS-1, IL-1, IL-3.4

Description of Sites

Blackfoot River.— This site was chosen for study because previous analysis indicated the abundance of at least one sibling, *S. negativum* (IL-3·4, Fig. 2), and one cytotype, *S. arcticum* IIL-9 (Fig. 3). Other taxa of the complex were present but in low numbers. Also, certain autosomal polymorphisms appeared to be in sufficient abundance to perform equilibrium analysis. We sampled this site at three-week intervals from mid-March to mid-July in parts of four years.

Upper Spring Creek.— This site appeared to be unique among 40 sites analyzed (Shields, unpub.) in that only one cytotype was present year-round. This site has been sampled for five years, with the composite sample representing each month of the year.

Little Prickly Pear Creek.— Previous analyses indicated presence of two cytospecies, *S. arcticum* s. s. (IIL-3) and *S. apricarium* (IIL-7). The latter was said to be reproductively isolated from other known taxa of the complex (Adler et al., 2004), though study on the scale intended here (> 1000 larvae) had not yet been conducted. We used autosomal polymorphisms IIS-10·11 (Fig. 4) and IIL-20 to estimate reproductive status at this site.

Rock Creek.—Previous analyses indicated the presence of at least two cytotypes, *S. arcticum* IIL-9 and *S. arcticum*

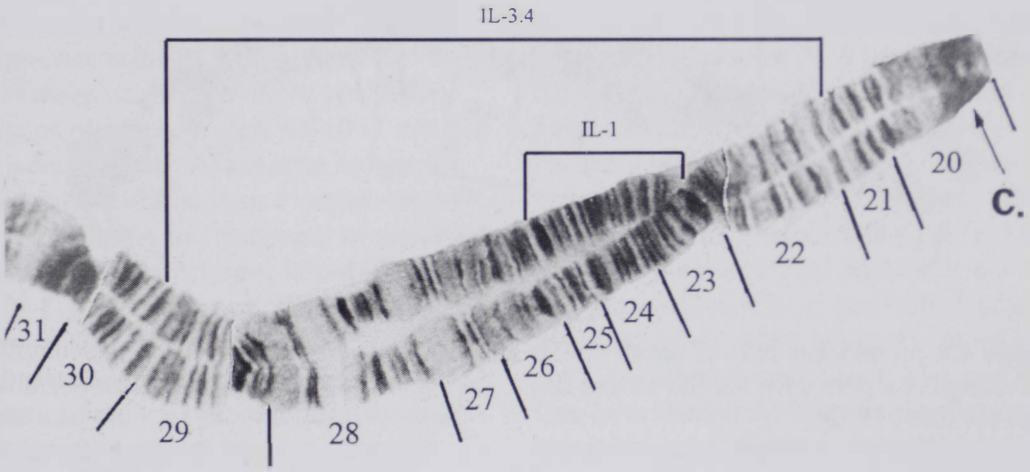

Figure 2. Chromosome map of a portion of the long arm of chromosome I of the *S. arcticum* complex (modified from figure 4 of Shields and Procunier, 1982). Brackets indicate breakpoints of the IL-3.4 and IL-1 inversions. Numbers below the chromosome indicate sections of the chromosome, as is the case in Figs. 2-6.

Figure 3. Chromosome map of a portion of the long arm of chromosome II of the *S. arcticum* complex (modified from figure 5 of Adler et al., 2004). Brackets indicate breakpoints of the IIL-3, IIL-7, IIL-9, IIL-10 and IIL19 sex-linked inversions and the IIL-20 autosomal inversion.

IIL-19, with chromosomes of excellent quality so that we could determine all three genotypes of the autosomal polymorphisms IS-1 (Fig. 5) and IL-1. Our investigation of the reproductive status of *S. arcticum* IIL-9 and IIL-19 was based on analysis of larvae collected only on 14 Mar 2006.

Results and Discussion

Blackfoot River

Our analyses of seven collections from the Blackfoot River are shown in Table 2. Because of high water at the Blackfoot in late March and April 2006; we were unable to determine presence of the *S. arcticum* complex but rather used samples collected at comparable dates in 2003 and 2004. The data suggested 1) an early presence of the cytotype *S. arcticum* IIL-9, 2) presence of the cytospecies *S. negativum* (*S. arcticum* IL-3·4) in May and June, and 3) a return of IIL-9 in July. We suggest temporal reproductive isolation because neither taxon appeared in the presence of the other though overlap of adults may have occurred. Genetic data suggested

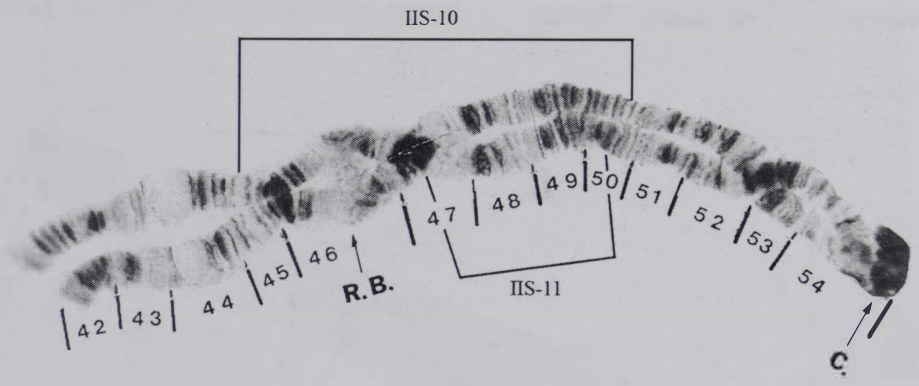

Figure 4. Chromosome map of the short arm of chromosome II of the *S. arcticum* complex indicating breakpoints for the IIS-10 and IIS-11 inversions (modified from figure 5 of Shields and Procunier, 1982).

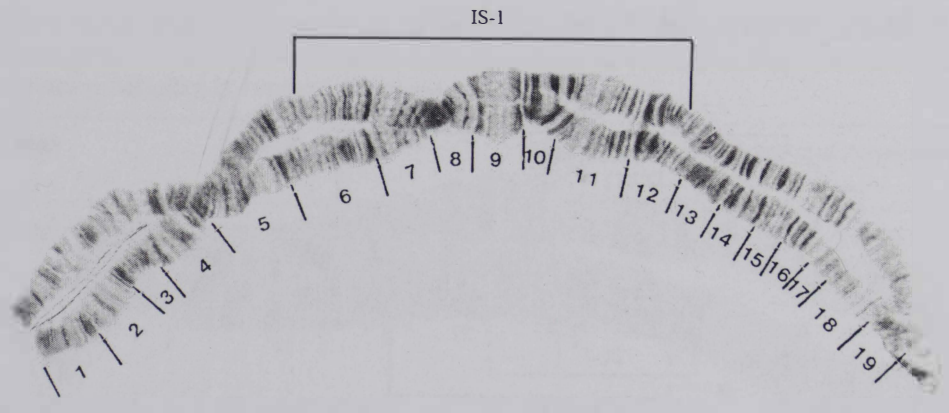

Figure 5. Chromosome map of the short arm of chromosome I of the *S. arcticum* complex (modified from figure 3 of Shields and Procunier, 1982). Brackets indicate breakpoints of the IS-1 inversion.

that if hybrids had been formed when and if the taxa were synchronous, they were formed in low numbers. Specifically, the entire population of IIL-9 was fixed for the standard homozygote of the autosomal inversion IIS-10·11 in late March (Table 3). Alternatively, 125 of 129 individuals of S. negativum collected in May and June were inverted homozygotes for IIS-10·11, which suggested near total reproductive isolation. A similar pattern occurred for the transition from *S. negativum* in June to *S. arcticum* IIL-9 in July. Although 100 percent of males after March and prior to July were *S. negativum*, no *negativum* larvae occurred in July (Table 2), and among 103 larvae analyzed from July 10, none were IIS-10·11 inverted homozygotes (Table 3). Therefore, we observed little evidence for hybridization between *S. arcticum* IIL-9 and *S. negativum* at the Blackfoot River. Eleven IIL-19 males occurred in the 30 Mar 2003 collection of which all were IIS-10·11 st/st that also indicated potential temporal and genetic reproductive isolation from *S. negativum*.

Distribution and frequency of two other autosomal polymorphisms, IIIL-4 (Fig. 6) and IS-1, were of interest. IIIL-4 occurs in low frequency (% heterozygosity) in the IIL-9 population in March and increased dramatically to ~ 33 percent heterozygosity in the negativum population in May and June, but was not present in the July population of *arcticum* IIL-9 (Table 3). This suggested additional support for reproductive isolation between *S. negativum* and the second generation of *S. arcticum* IIL-9 in July.

Early and late populations of IIL-9 had similar distributions of the IS-1 inversion, with 19.1 and 13.9 percent heterozygosities, respectively, and were in equilibrium (Table 4). However, within the *S. negativum* population, 81 percent of males were heterozygous for the IS-1 inversion (Table 4). Thus, there may be two Y chromosomes, IL-3·4 and IL-3·4 + IS-1, in the *S. negativum* population at the Blackfoot River.

Two IL-3.4 males occurred in the 28 March 2004 collection, whereas three male and two female larvae were IL-3·4 in the 10 Jul 2006 collection. One of these male larvae was a IIL-3 heterozygote, and two other males were IIL-9 heterozygotes. All these larvae were chromocentric and possessed positive head patterns, unlike the achromocentric *S. negativum* that has negative head patterns in its females (Adler et al. 2004). This suggested that the IL-3.4 inversion is autosomal in the March and July populations of *S. arcticum* IIL-9 at the Blackfoot River, while being sex-linked in the *S. negativum* population in May and June. One individual in the March collection and two in the July collection were IIL-9 inverted homozygous males (Table 2.). One male IIL-3/IIL-7 heterozygote was also observed.

Table 2. Cytological diversity of the *Simulium arcticum* complex at the Blackfoot River, Russell Gates Campground, Missoula County, Montana.

	Females		Males				
	X_0X_0	$X_0X_{IL\text{-}3.4}$	X_0Y_0	$X_0Y_{IL\text{-}3.4}$	$X_0Y_{IL\text{-}3.4+IS\text{-}1}$	$X_0Y_{IIL\text{-}9}$	$X_{IIL\text{-}9}Y_{IL\text{-}9}$
Date							
3/13/06	no larvae present						
3/28/04	31	0	1	0	0	27	1
3/30/03	54	0	0	0	0	27	0
4/1/05	17	0	0	0	0	10	0
5/9/06	many small larvae						
5/18/03	7	0	0	6	6	0	0
5/23/06	71	6	0	8	60	0	0
6/15/06	25	2	0	2	6	0	0
7/10/06	54	0	5	0	0	42	2
Total	259	8	6	16	72	106	3

Table 3. Distribution of autosomal polymorphisms among members of the *Simulium arcticum* complex at the Blackfoot River, Russell Gates Campground, Missoula County, Montana.

Date	Autosomal Polymorphisms			
3/13/06	no larvae present			
	S. arcticum IIL-9			
	st/st	IIS-10.11 st/i	i/i	IIIL-4 % st/i
3/28/04	60	0	0	9.7
3/30/03	81	0	0	not scored
4/1/05	10	0	0	0
	S. negativum			
	st/st	IIS-10.11 st/i	i/i	IIIL-4 % st/i
5/9/06	many small larvae			
5/23/06	3	0	145	40.0
6/15/06	1	0	34	37.1
	S. arcticum IIL-9			
	st/st	IIS-10.11 st/i	i/i	IIIL-4 % st/i
7/10/06	102	1	0	0
Total	247	1	125	

Figure 6. Chromosome map of the long arm of chromosome III of the *S. arcticum* complex (modified from figure 8 of Shields and Procunier, 1982). Brackets indicate breakpoints for the IIIL-4 autosomal inversion.

Table 4. Distribution of the IS-1 inversion among sexes and populations of the *S. arcticum* complex at the Blackfoot River, Russell Gates Campground, Missoula County, Montana and Chi-square Statistics Based on Equilibrium Frequencies of Genotypes.

	S. arcticum IIL-9						
	♀♀			♂♂			
	st/st	st/i	i/i	st/st	st/i	i/i	
3/28/04	25	6	0	30	7	0	
3/30/03	54	7	0	27	2	0	$X^2 = 1.06$,
4/1/05	12	4	0	12	0	0	$d.f._2$, $0.60 < p < 0.70$
	S. negativum						
5/18/03	109	10	0	16	72	0	$X^2 = 12.6$,
5/23/06							$d.f._2$, $0.01 < p < 0.02$
6/15/06							
	S. arcticum IIL-9						
7/10/06	51	4	0	42	11	0	$X^2 = 0.61$,
							$d.f._2$, $0.70 < p < 0.80$

We did not observe inversion homozygotes for IS-1. The eight females heterozygous for the IS-1 inversion may represent sex-exceptional individuals. On the contrary, IS-1 was apparently autosomal in the IIL-9 populations because in both early and late populations the distributions occurred as expected: March, $X^2 = 0.904$, d f.2, $0.70 < P < 0.80$; July, $X^2 = 0.648$, d f.2, $0.70 < P < 0.80$. Although we observed no IS-1 homozygous inverted individuals for the IIL-9 cytotype in our samples, expected probabilities indicated that there should have been at least one in each population.

The single IIS-10·11 heterozygote in the 10 July population of *S. negativum* is of interest. No males of *S. negativum* occurred in our samples from 10 July. Since this IIL-10·11 heterozygote was also heterozygous for IIL-9, it might have been a hybrid between a IIL-9 male and a *S. negativum* female. However, tight pairing of the homologs of this individual suggested that it was not a hybrid. Alaskan populations of *S. negativum* (Shields and Procunier 1982), as well as populations at the Gallatin, Sun and Yellowstone rivers in Montana, were not polymorphic for either IS-1 or for IIIL-4 (Shields, unpub.). Thus, these polymorphisms may be newly derived in the populations at the Blackfoot River.

Eleven IIL-19 st/i males occurred in the 30 March 2003 sample at the Blackfoot River of which all were IIS-10·11 st/st homozygotes, suggesting that they were reproductively isolated from *S. negativum*. Presence of two IIL-3/IIL-7 heterozygotes is of interest because only one male *S. arcticum* s. s. larva (IIL-3) and no *S. apricarium* (IIL-7) larvae were found among nearly 400 larvae analyzed from this site. These IIL-3/IIL-7 heterozygotes were also heterozygotic for a large inversion near the end of the IIL-arm. Presence of two *S. arcticum* s. s./*S. apricarium* heterozygotes was difficult to explain because the apparent parental types were either rare or nonexistent. This may be a case of very rare polymorphisms. Whereas IIIL-4 may be shared by local populations of *S. negativum* and *S. arcticum* IIL-9, our data suggested that the later population of IIL-9 may be derived from the early IIL-9 population.

Upper Spring Creek

Our analyses of larvae from Upper Spring Creek are shown in Table 5. Y-linkage of the IIL-10 inversion appears complete since all males and no females analyzed possess this inversion. Some IIL-10 larvae at this site possess as many as four B chromosomes in

their germ lines. All of these characteristics, particularly IIL-10's unique presence as larvae year-round, suggested that *S. arcticum* IIL-10 at Upper Spring Creek was reproductively isolated and may be a good biological species. The IIL-10 cytotype spends winter as larvae,

Table 5. Temporal Distribution of the *S. arcticum* IIL-10 Cytospecies at Upper Spring Creek, Fergus County, Montana.

Date	Females X_0 X_0	Males X_0 Y_{10}
1/22/05	4	11
1/26/03	11	25
2/7/04	3	2
2/17/02	3	1
2/27/04	12	8
3/25/06	5	10
4/24/04	37	25
5/21/05	8	1
7/16/05	1	6
10/5/03	9	3
Total	102	92

whereas many other taxa of black flies spend winter as eggs (Adler et al. 2004). Possibly as a consequence of this early maturation, male IIL-10 larvae at Upper Spring Creek possess mature sperm in February (Shields, unpub.). Documentation of species status for IIL-10 might require collection of pupae that could be reared to adults.

The unique presence of the IIL-10 cytotype at Upper Spring Creek may have been due to characteristics of the drainage. The creek originates from Big Spring, a natural freshwater spring with a relatively constant flow rate year-round. Moreover, water temperature at Upper Spring Creek was nearly constant yearlong having a range of temperatures of only 10 to 13 °C (Shields, unpub.). This restricted range in temperature at Upper Spring Creek occurs when other non-spring fed drainages nearby experience temperatures from 0 °C in December to 24 °C in August. Relatively constant flow rate and water temperatures at Upper Spring Creek possibly provide a unique environment for IIL-10 *arcticum*.

Little Prickly Pear Creek

We have monitored the presence, density, and number of potential hybrids of *S. arcticum* s. s. and *S. apricarium* at this site since 2002. Most populations of *S. arcticum* s. s. in allopatry are fixed for the standard form of the IIS-10·11 sequence, whereas most pure populations of *S. apricarium* are fixed for the inverted homozygote (Adler et al. 2004). Potential hybrids between the two taxa therefore, might be indicated by the presence of IIS-10·11 heterozygotes. Thirteen of the 1254 larvae analyzed (0.01 %) at this site were IIS- 10·11 heterozygotes (Table 6) and these may suggest rare hybridizations between *S. arcticum* s. s. and *S. apricarium*. However, this number could be misleading. Both IIS-10·11, and especially IIL-7, are polymorphic in some populations, especially westward in the distribution (Adler et al. 2004). This could explain six of the IIS- 10·11 st/i larvae that were IIL-st/st. The remaining seven IIS-10·11 heterozygotes may be evidence for hybridization between the taxa or they may be ancestral relics, i.e., IIL-3 types in *S. apricarium* or IIL-7 and IIS-11 types in *S. arcticum* s. s. Only one individual among 1254 larvae analyzed was a IIL-3i/IIL-7i type and this could be explained by hybridization between a *S. arcticum* s. s. male and a *S. apricarium* female. This male was IIS- 10·11 st/st, and had tight pairing of the homologues, suggesting that IIS-10·11 may be polymorphic in this population, as mentioned above.

Approximately 10 percent of *S. arcticum* s. s. larvae at Little Prickly Pear Creek were heterozygotes for the IIL-20 autosomal inversion during 2005 and 2006, and we have never seen this inversion among *S. apricarium* larvae (Shields, unpub.). We have also found the IIL-20 inversion in the nearby drainages of the Boulder River, Little Blackfoot River and Canyon Creek, but in frequencies < 1.0 percent (Shields, unpub.). Therefore, IIL-20 possibly arose at or near Little Prickly Pear Creek, and some unknown factor maintains its relatively high heterozygosity there. Taken as a whole, data from Little Prickly Pear Creek did not argue for hybridization between *S. arcticum s.* s. and S. *apricarium*. Little Prickly Pear Creek has the

Table 6. Temporal Distribution of *S. arcticum* s. s. and *S. apricarium* and Frequency of IIS-11 Heterozygotes at Little Prickly Pear Creek, Lewis and Clark County., Montana.

Date	S. arcticum s. s. ♀♀	% = 90.4 ♂♂	S. apricarium ♀♀	% = 9.6 ♂♂	IIS-11 st/i	
3/15/02	1	22	3	7	0	
3/16/03	3	7	1	15	0	
3/30/06	131	323	4	5	5	
3/31/05	109	116	8	20	3	
4/1/06	3	10	1	0	0	
4/4/02	3	26	2	0	0	
4/10/03	30	24	4	9	0	
4/18/02	17	29	2	2	0	
4/30/02	6	4	0	0	0	
5/6/05	53	63	5	14	1	
5/6/06	65	76	0	0	1	
5/26/05	5	0	8	11	3	
Total	432	701	38	83	13	n = 1254

highest density of larvae among our collection sites with an estimated 50 larvae/cm^2.

Rock Creek

Among males at Rock Creek, *S. arcticum* IIL-19 (55.4 %) and *S. arcticum* IIL-9 (37.9 %) predominated (Table 7). Five X_0Y_0 males, ten male *S. arcticum* s. s., and four male and four female *S. apricarium* also occurred in the sample. Heterozygosities of the autosomal inversions IL-1 (22.0 %) and IS-1 (15.2 %) were sufficiently high among *S. arcticum* IIL-19 and IIL-9, respectively (Table 8), so we subjected these data to tests of random mating. IL-1 was in equilibrium among the IIL-9 and IIL-19 populations as was the IS-1 inversion (Table 8). Among 534 larvae analyzed from the 14 Mar 2006 collection from Rock Creek, 13 were IIS-11 heterozygotes (three st/st females, eight IIL-9 males and two IIL-19 males). No larvae had both the IIL-7 inversion, which is polymorphic in *S. apricarium*, and any of the other sex-linked inversions characteristic of the other taxa of the arcticum complex (i. e., IIL-3, IIL-9 and IIL19) at Rock Creek. Consequently, *S. arcticum* IIL-9 and IIL-19 form a single cytospecies/cytotype at Rock Creek in March. Twelve of 534 individuals analyzed from Rock Creek were IL-3.4 heterozygotes. Five were st/st females, five were IIL-19 males, one was a st/st male and the twelfth was a IIL-9 male. IL-3.4 is the sex-linked inversion in *S. negativum* (Shields and Procunier 1982, Adler et al. 2004), but it was apparently an autosomal polymorphism among members of the *S. arcticum* complex at Rock Creek. This may be an example of the phenomenon of "one sibling's sex-linked inversion being another sibling's autosomal polymorphism" (Rothfels 1979).

Table 7. Distribution of Sex Chromosomes of the *S. arcticum* Complex at Rock Creek, Missoula County, Montana.*

	Females				Males						
	S. apricarium					*S. apricarium*					
Date	X_0X_0	X_0X_0	X_0X_7	X_7X_7	X_0Y_0	X_0Y_3	X_0Y_0	X_0Y_7	X_7Y_7	X_0Y_9	X_0Y_{19}
3/14/06	243	1	1	2	5	10	0	1	3	106	155

* Seven other larvae of the *S. arcticum* complex, each having newly discovered inversions in the long arm of chromosome II, were also observed. Since their frequencies were low, we did not include them in these analyses.

Table 8. Genotypic distributions of the IL-1 and IS-1 autosomal polymorphisms among *S. arcticum* IIL-9 and *S. arcticum* IIL-19 at Rock Creek on 14 March 2006 and Chi-square analysis suggesting random mating of the two cytotypes.

Cytotype	Autosomal	Polymophism -	IL-1	
	st/st	st/i	i/i	
IIL-9	66	35	5	$X^2 = 0.004$, d. f.$_2$, $0.90 < p < 1.0$
IIL-19	86	60	10	
Cytotype	Autosomal	Polymorphism-	IS-1	
	st/st	st/i	i/i	
IIL-9	92	14	0	$X^2 = 1.446$, d. f.$_2$, $0.50 < p < 0.60$
IIL-19	127	28	0	

Of the 13 males heterozygotic for IIS-14, 10 were IIL-9 st/i, two were IIL-st/st, and one was IIL-19 st/i., suggesting an additional rare sex chromosome in the 14 March 2006 population at Rock Creek. We hesitate to speculate on the reproductive status of other members of the *S. arcticum* complex at Rock Creek (*S. arcticum* s. s., *S. apricarium* and possibly *S. brevicercum*) because each occurred in low frequency. Finally, no data suggested reproductive isolation for the IIL-9 and IIL-19 cytotypes at Rock Creek.

Comparisons Among Sites

IIL-9 and IIL-19 and *S. negativum*.— Our results from the Blackfoot River and Rock Creek revealed several trends. *S. arcticum* IIL-9 and IIL-19 were present at both sites in March. *S. negativum* was present at the Blackfoot River in mid-summer and individuals having the IL-3.4 inversion were present in the 13 March 2006 sample from Rock Creek although that inversion appeared autosomal. Despite extensive sampling in western Montana, we found *S. arcticum* IIL-19 only at two other sites (Bitterroot River on 28 March 2004 and at the Clearwater River on 28 February 2003 and 14 March 2006, suggesting that IIL-19 has a very restricted geographic distribution and by inference, it may be evolutionarily young.

S. arcticum IIL-9 was also present at the Clearwater River on 14 March 2006, which is only 7 km from the Blackfoot River. Limited geographic distribution, early presence in spring, and sympatric presence at some sites for these two cytotypes also suggested a close relationship. The three sites in question were close geographically (< 84 km.), and each flows into the Clark Fork River that drains northwestern Montana. Sampling of additional drainages flowing into the Clark Fork River may prove informative regarding the relationships of IIL-9 and IIL-19. Finally, we never observed *S. negativum* in early spring that suggested it has adapted to mid-summer development that may be temperature dependent.

IIL-10.— S. arcticum IIL-10 was the only cytotype at Upper Spring Creek year round. We found males with the IIL-10 inversion at five widely scattered sites in our study area but always in very low numbers ($n \leq 7$) on any date. Based on ~ 30 collections at Upper Spring Creek, we have never found abundant larvae of IIL-10. We have found it in small clusters of larvae at the tips of twigs in the swiftest flowing waters. Whether low densities of IIL-10 at Upper Spring Creek and its presence in low numbers elsewhere were correlated remains unknown.

General Summary.—We monitored four collection sites for sex-linked and autosomal inversions among members of the *S. arcticum* complex in western Montana to gain insight into their reproductive status. We found little evidence for random mating between *S. negativum* and *S. arcticum* IIL-9 at the Blackfoot River or between *S arcticum* s. s. and *S. apricarium* at Little Prickly Pear Creek. *S. arcticum* IIL-10 is the only member of the *S. arcticum* complex at Upper Spring

Creek. Study of sites near Upper Spring Creek may result in a better understanding of the reproductive status of this cytotype. The cytotypes, *S. arcticum* IIL-9 and IIL-19 appear to be randomly mating at Rock Creek.

Our observations are an initial step in understanding relationships in this complex. Our current research includes 1) study of environmental factors that might influence dispersal of cytotypes, 2) study of the extent of reproductive isolation among taxa within the complex and 3) molecular analyses of larvae individually identified to cytospecies and cytotype to test hypotheses about the ages and relationships of members of the *S. arcticum* complex.

Acknowledgments

The M. J. Murdock Charitable Trust (MJMCT grants #'s 2003196 and 2005233) provided stipends for students and support for supplies and travel to collection sites. The National Geographic Society (NGS grant #7212-02) provided support for travel and equipment. The Department of Natural Sciences at Carroll College provided space, equipment and supplies. We especially thank Dr. Peter Adler, Department of Entomology, Clemson University, for his review of an earlier draft of this manuscript, help with identification of larvae and chromosomes and for his continued interest, encouragement and support of our work.

Literature Cited

Adler, P. H., D. C. Currie, and D. M. Wood. 2004. The Black Flies (Simuliidae) of North America. Comstock, Cornell University Press, Ithaca, N Y. 941 pp.

Coyne, J. A., and H. A. Orr. 2004. *Speciation*. Sinauer Associates, Inc. Sunderland, MA. 545 pp.

Currie, D. C. 1986. An annotated list of and keys to the immature black flies of Alberta (Diptera: Simuliidae). Memoirs of the Entomological Society of Canada 134:1-90.

Procunier, W. S. 1984. Cytological identification of pest species of the *Simulium arcticum* complex present in the Athabasca River and associated tributaries. Alberta Research Council Farming for the Future Final Technical Report. N. 82-101. Agriculture Canada Research Station, Lethbridge, Alberta, Canada 44 pp.

Procunier, W. S., and J.A. Shemanchuk. 1983. Identification of sibling species of black flies in Alberta using polytene chromosome analysis. Pp. 33-36. in I. J. L. Sears and T. G. Atkinson eds. Research Highlights – 1982. Agriculture Canada Research Station, Lethbridge, Alberta. 124 pp.

Rothfels, K. H. 1956. Black flies: siblings, sex and species grouping. Journal of Heredity 47:113-122.

Rothfels, K. H. 1979. Cytotaxonomy of black flies (Simuliidae). Annual Review of Entomology 24:507-539.

Rothfels, K. H., and R. W. Dunbar. 1953. The salivary gland chromosomes of the black fly *Simulium vittatum* Zett. Canadian Journal of Zoology 31:226-241.

Shields, G.F., and W.S. Procunier. 1982. A cytological description of sibling species of *Simulium (Gnus) arcticum* (Diptera; Simuliidae). Polar Biology 1:181-192.

Shields, G. F., G. M. Clausen, C. S. Marchion, T. L. Michel, K. C. Styren, C. N. Riggin, T. D. Santoro, and L. M. Strizich. 2006. The effect of elevation on the distribution of sibling species in the black fly, *Simulium arcticum* complex (Diptera: Simuliidae). Western North American Naturalist 67: 39-45.

Received 6 October 2006
Accepted 30 May 2007