

**Reproductive Status of Cytotypes within the *Simulium arcticum* Complex at Rock
Creek, Missoula Co., MT 3/14/06**

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Submitted in partial fulfillment of the requirements for graduation with honors to the
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Acknowledgements

I would like to extend my sincerest thanks to Dr. Gerald F. Shields, without whom I would not have been able to complete this research. Throughout our two years of research Dr. Shields donated an abundance of time and knowledge and instilled within me an enthusiasm for the subject matter. He also provided transportation to the NABFA meeting in 2006, which gave me invaluable experience in scientific presentation and allowed me to absorb knowledge from the many varied studies of Simuliidae. In the thesis writing process he continued to lend to me his expertise and patience without which I would not have completed this endeavor. Through his constant guidance I truly gained a mentor.

I would also like to thank the M.J. Murdock Charitable Trust for their grants (#2003196, #2005233), which provided for equipment, transportation, and a stipend salary. Their further donations allowed me to attend the Murdock symposium in the 2005-2006 and 2006-2007 years. Both occasions provided experience in the scientific community; both events enabled me to gain knowledge from my peers.

Other organizations which are worthy of thanks include Carroll College and the National Geographic Society. Both institutions provided equipment for the completion of this research. Carroll College also provided space and other expenses. Finally, I would like to thank Greg Clausen, Lindee Strizich, Katie Styrene, and Dr. Shields for providing aid in collection and analysis of the material.

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Abstract

This two year study was an in depth cytogenetic analysis of the IIL-9 and IIL-19 cytotypes of the *Simulium arcticum* complex from a March 14, 2006 collection at Rock Creek, Missoula Co., MT to assess reproductive status. In this research, conventional methods of collection, morphological classification, and chromosomal preparation and analysis were used. The reproductive status of the two cytotypes was examined using the autosomal polymorphisms IS-1 (Figure 2) and IL-1 (Figure 3), which occurred in sufficient abundance to estimate equilibrium frequencies within the 3/14/06 sample. I hypothesized that these two divergent cytotypes, IIL-9 and IIL-19 (Figure 1), would exhibit a lesser degree of reproductive isolation than previously established cytospecies.

Frequencies of the IS-1 and IL-1 autosomal polymorphisms were calculated for the IIL-9 and IIL-19 cytotypes and were compared to the equilibrium values using a Chi square analysis. The statistical analysis of the material revealed the IIL-9 and IIL-19 cytotypes to be in equilibrium at Rock Creek on 3/14/06. This would suggest that these two cytotypes are indeed not reproductively isolated from one another based on random sharing of autosomal inversions. The limited geographic distribution of the IIL-9 and IIL-19 cytotypes suggests that they may be evolutionarily young. This information coupled with the fact that they are in equilibrium at Rock Creek supports the *S. arcticum* Geographic Distribution/Taxon Age Continuum of Shields (2006).

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Introduction

The cytogenetic method has gained validity in the analysis of black flies (Diptera: Simuliidae) by allowing for the identification of sibling species, which were once regarded by classical taxonomy as a single morphospecies (Rothfels, 1956). This differentiation of siblings was made through the linkage of chromosomal rearrangements to sex (Rothfels, 1956). Other methods of describing taxa can be found in the observation of fixed-inversions, associated autosomal polymorphisms, meiotic stage, presence or absence of B chromosomes and other distinctive features of the biology of the organisms (e.g. overwintering as eggs or larvae, Rothfels, 1956). Moreover, once a sibling species differentiates from the ancestral species through cytogenetic means, single or numerous morphological variants may also be found (Rothfels, 1956).

Cytogenetic analysis also provides the opportunity to observe the process of reproductive isolation in a controlled setting since radiation within the complex appears to form a continuum from full siblings (cytospecies) to apparently recently divergent cytotypes whose relationships can be determined at the chromosome level. Thus, cytogenetic analysis of black flies allows for the opportunity to view speciation at a transitory level; a need that Jerry Coyne and Allan Orr emphasized in their recently published book Speciation (2004) as a means to gain better understanding of the speciation process.

The *Simulium arcticum* complex provides a model of ample variation to examine speciation. Shields and Procunier (1982) described four species of the *S. arcticum* complex in Alaska and observed the IIL-3 cytospecies in British Columbia and Alberta, Canada. In addition to these five-recorded taxa, four other sibling species in the *S.*

arcticum complex emerged through further research. Two of these siblings were chronicled in the Athabasca River drainage of Alberta, Canada: *S. arcticum* IIL-8.9/IIS-10.11 (Procunier and Shemanchuk, 1983) and *S. arcticum* IIS-4 (Procunier, 1984). Adler *et al.* (2004) described three other sibling species of the complex: *S. apricarium*, *S. chromatinum*, and *S. vampirum*.

Reproductive Status of Siblings and Cytotypes at Rock Creek (3/14/06)

The Shields' lab (2007) at Carroll College has discovered five siblings and 16 cytotypes of the *S. arcticum* complex in Montana. Previous analyses of taxa of the *S. arcticum* complex at Rock Creek (Missoula Co., MT) have revealed the presence of three described siblings, *S. brevicercum*, *S. arcticum sensu stricto*, and *S. apricarium*, and at least four cytotypes, IIL-9, IIL-17, IIL-13 and IIL-19. Based on previous analyses (Shields, unpub.) the cytotypes IIL-9 and IIL-19 were in sufficient numbers to warrant analyses of reproductive status. Autosomal inversions at Rock Creek included IL-1, IS-1, IIL-1, IIS-11, IIS-14, IIS-12, and IL-3,4. The IL-1 and IS-1 inversions were potentially abundant enough to allow for equilibrium analysis.

The primary focus of this research was to investigate the reproductive status of cytotypes of taxa of the *S. arcticum* complex at Rock Creek. From the *S. arcticum* Geographic Distribution/Taxon Age Continuum Hypothesis (Shields, 2006) it can be inferred that full sibling species express a greater degree of reproductive isolation and have a broader distribution than cytotypes. Due to the broader distribution, this hypothesis asserts that cytospecies are evolutionarily older than cytotypes. When applied to the Rock Creek site, the Geographic Distribution/Taxon Age Continuum Hypothesis would suggest that cytospecies such as *S. brevicercum*, *S. arcticum s. s.*, and *S.*

apricarium may be reproductively isolated from one another. Conversely, cytotypes having limited distributions are probably young in evolutionary age and may not exhibit reproductive isolation (Shields, per comm.). I thus hypothesized that IIL-9 and IIL-19 would be in genetic equilibrium at Rock Creek for the 3/14/06 collection.

Methods and Materials

Choice of Site

The site at Rock Creek, two miles south of U. S. Interstate Highway 90 on Montana Highway 348 was chosen for study because of the analysis of eight previous collections. These collections indicated the presence of: abundant larvae, two prevalent cytotypes, IIL-9 and IIL-19, of the *S. arcticum* complex, two abundant autosomal polymorphisms, and polytene chromosomes of excellent quality (Shields, unpub; Pickens, unpub.).

Collecting Samples

The larvae were collected through random sampling of rocks and leafy vegetation, and fixed in Carnoy's, a preservative of a 3:1 ratio of ethanol and glacial acetic acid. During this collection water temperature and elevation were recorded. The samples were kept on wet ice in the field to maintain chromosomal quality. Carnoy's fixative was changed until it appeared clear in a collection vial (usually after four changes).

Sample Identification

Currie (1986) was used to identify the larvae through analysis of the head patterns, sub-labial clefts, and counts of the filaments of the pupal respiratory organ. *S. arcticum* with white histoblasts were selected preferentially to ensure quality of chromosome analysis. Larvae of *S. arcticum* that were immature or had black histoblasts, as well as larvae that were not *S. arcticum* were saved for possible future study.

Staining

Larvae with white histoblasts were prepared for staining by creating an incision from the sub-labial cleft to the posterior proleg to expose the salivary glands and gonads. The larvae were then submerged in tap water for 20 minutes and blotted onto filter paper to remove silk secretions that might interfere with staining of the chromosomes. The larvae were then hydrolyzed at 64°C in .001M HCl for nine minutes. Directly after hydrolysis, the .001M HCl was removed and Feulgen stain was added to the vial. The vial was then placed in the dark for one hour. The Feulgen stain was then removed and sulfur water was added for a ten minute time period. After the sulfur water was removed, tap water was used to rinse the stained larvae. The larvae were submerged in water and placed in the refrigerator until the polytene chromosomes were prepared.

Slide Preparation

The body walls containing the salivary glands and the gonads were removed from the stained larvae and placed in a drop of 50% glacial acetic acid on a fresh slide. The salivary glands and gonads were then dissected out. The salivary glands were teased apart with dissecting needles to ensure proper spreading of chromosomes. A cover slip was placed over the salivary glands and gonads, and slight pressure was exerted on the preparation. Finally, a drop of acetocarmine was used to seal the preparation. These slides were then analyzed directly after preparation to ensure integrity of the chromosomes. The slides of males were made permanent by the process described in Adler *et al.* (2004).

Chromosomal Analysis

The standard maps of *S. arcticum* (Shields and Procnier, 1982) were used to assess the banding sequence in the IIL, IIS, IL, IS, and IIIL arms. The analysis of the

chromosomes included recording of the different inversions and centromere dimorphisms present in each individual. The sex, the most advanced meiotic stage of males, and the presence of B chromosomes was also noted.

Data Analysis

The chromosomal polymorphisms were recorded (Table 1) and the data were analyzed using a chi-square statistical test for significance (Table 2). In this analysis, Hardy-Weinberg equilibrium frequencies were calculated for the IL-1 and IS-1 inversions in the homozygous for standard (st/st), heterozygous for the inversion (st/i), and homozygous for the inversion (i/i) states based on their occurrence in the IIL-9 and IIL-19 cytotypes. These expected equilibrium values were then compared to the observed values, and the variance from expectation was assessed with a chi-square test (Hartl and Jones, 1998).

RESULTS

The major objective of this research was to determine the reproductive status of the *S. arcticum* cytotypes, IIL-9 and IIL-19, from an abundant collection at Rock Creek on 3/14/06. The autosomal polymorphisms, IS-1 and IL-1 were used to determine equilibrium frequencies for both populations (Table 2). Since IIL-9 and IIL-19 are newly discovered cytotypes with limited distributions, I hypothesized that they would not be reproductively isolated. If this hypothesis proved to be true, it would support the Shields (2006) *S. arcticum* Geographic Distribution/ Taxon Age Continuum Hypothesis.

The 3/14/06 Sample at Rock Creek

As predicted from previous analysis of the site, the 3/14/06 sample at Rock Creek included many penultimate instar larvae of the *S. arcticum* complex. Specifically, 527 larvae of the *S. arcticum* complex were analyzed at the chromosome level (Table 1). Moreover, 53.1% of these were male, 55.4% (n = 155) of which were *S. arcticum* IIL-19 and 37.9% (n = 106) were IIL-9 (Table 1). Five other males were IIL-st/st, 10 were *S. arcticum s. s.*, and four were *S. apricarium*. Because these latter taxa occurred in such low frequencies their reproductive status could not be assessed accurately; they were thus not considered further in this analysis.

Although not a major objective of this research, species richness was also assessed in the 3/14/06 sample at Rock Creek. Within the entire sample there were 106 *S. vittatum* and 27 *S. canadense*; both were immature.

Reproductive Status of the IIL-9 and IIL-19 Cytotypes

Heterozygosities of IS-1 and IL-1 (15.2 and 22.0 %, respectively) were sufficiently frequent within the sample to allow for tests of equilibrium (Table 2). The distribution of

genotypes for the IL-1 autosomal inversion in the population of IIL-9 and IIL-19 *S. arcticum* at Rock Creek on 3/14/06 strongly suggests that the cytotypes are in equilibrium ($X^2 = 0.004$, d.f.₂, $0.90 < p < 1.0$; Table 2). Similarly, the distribution of genotypes for the IS-1 autosomal inversion suggests equilibrium ($X^2 = 1.446$, d. f.₂, $0.50 < p < 0.60$; Table 2).

Additional Observations

Other notable autosomal polymorphisms were also present within the sample. The IIS-11 inversion was found in 17 individuals. The presence of this autosomal inversion may suggest the presence of *S. apricarium* population (Adler *et al*, 2004). Of these 17 individuals, heterozygotes for the IIS-11 inversion were observed in three IIL-st/st females, eight IIL-9 st/i males, and two IIL-19 st/i males. The remaining four individuals with the IIS-11 inversion were homozygotes for the inversion and were also homozygous for the IIL-7 inversion, suggesting that only these individuals were *S. apricarium*.

Another distinct autosomal polymorphism observed at Rock Creek was IL-3.4. This inversion has previously been observed to be sex linked in *S. negativum* in populations in Alaska (Shields and Procunier, 1982) and Montana (Strizich, 2007). At Rock Creek the IL-3.4 inversion was observed in 12 individuals. Five of these individuals were females having standard sequences in their IIL arms. The remaining seven individuals with the IL-3.4 inversion were male with one being IIL-st/st, one IIL-9 st/i, and five IIL-19 st/i. Homozygosity for the IL-3.4 inversion was not scored.

A new inversion, IIS-14, was observed in 13 males of the sample. Of these 13 males, there were ten IIL-9, one IIL-31, one IIL19, and one st/st male. However, a sample size of 13 males within a population of 527 is a low frequency. Moreover,

homozygosity for the IIS-14 inversion was not scored. Other autosomal polymorphisms occurred in low frequency, and thus, held little pertinence in analysis of the material.

Table 1: Distribution of Taxa of the *S. arcticum* complex at Rock Creek 3/14/06

Females			Males						Total
X ₀ X ₀	X ₀ X ₇	X ₇ X ₇	X ₀ Y ₀	X ₀ Y ₃	X ₀ Y ₇	X ₇ Y ₇	X ₀ Y ₉	X ₀ Y ₁₉	
244	1	2	5	10	1	3	106	155	527

*X and Y nomenclature are indicative of the suggested sex chromosome variation extant in males and females while the numbers associated with the letters represent inversions (Figure 1). The number 0 indicates the standard sequence.

Table 2: Genotypic Distributions of IL-1 and IS-1 Autosomal Polymorphisms Among the IIL-9 and IIL-19 Cytotypes at Rock Creek. (Calculations provided by Dr. Shields)

Cytotype	Autosomal Polymorphism- IL-1		
	st/st	st/i	i/i
IIL-9	66	35	5
IIL-19	86	60	10

* $X^2 = 0.004$, d. f. = 2, $0.90 < p < 1.0$.

Cytotype	Autosomal Polymorphism- IS-1		
	st/st	st/i	i/i
IIL-9	92	14	0
IIL-19	127	28	0

* $X^2 = 1.446$, d. f. = 2, $0.50 < p < 0.60$.

* IIL-9 and IIL19 are in equilibrium at Rock Creek on 3/14/06.

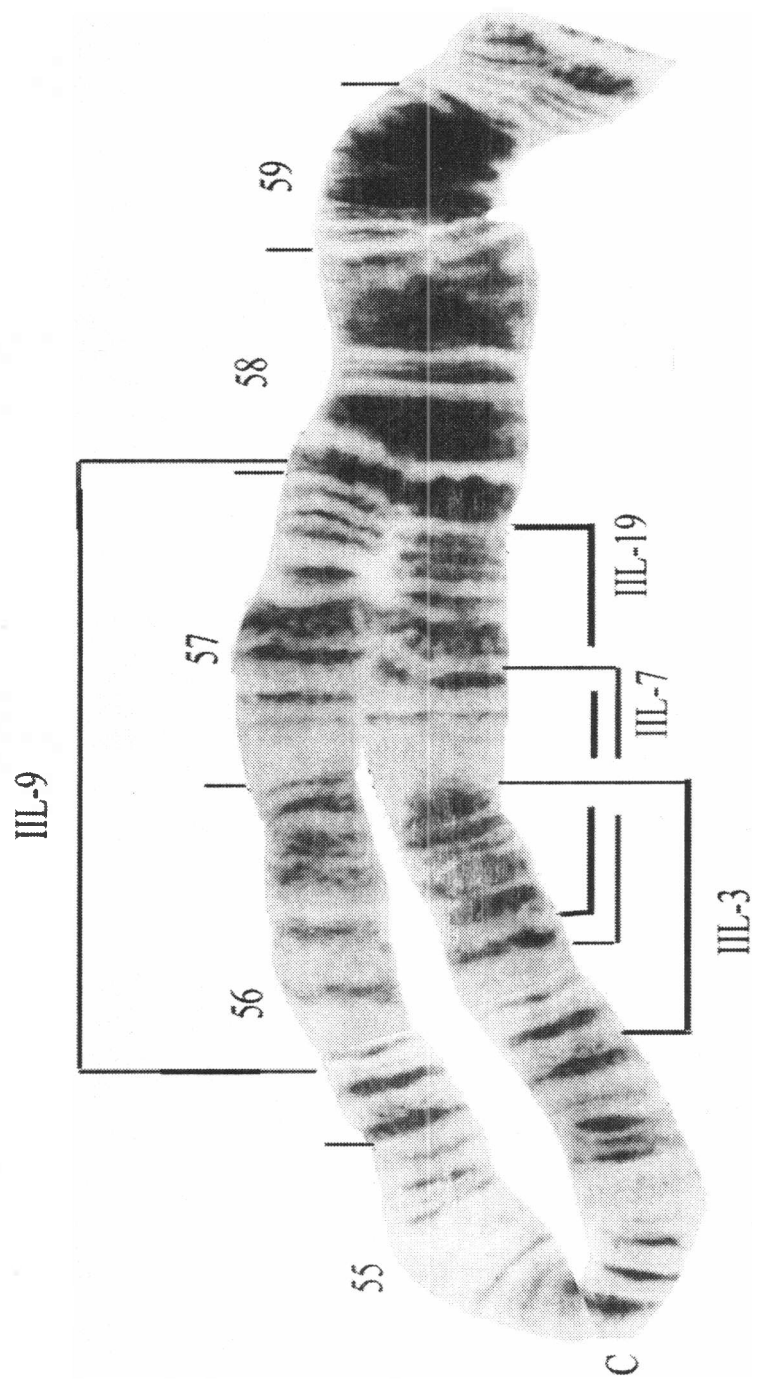


Figure 1: Chromosomal map of the standard sequence for *S. arcticum* IIL arm with IIL-3, IIL-7, IIL-19, and IIL-9 inversions in brackets.

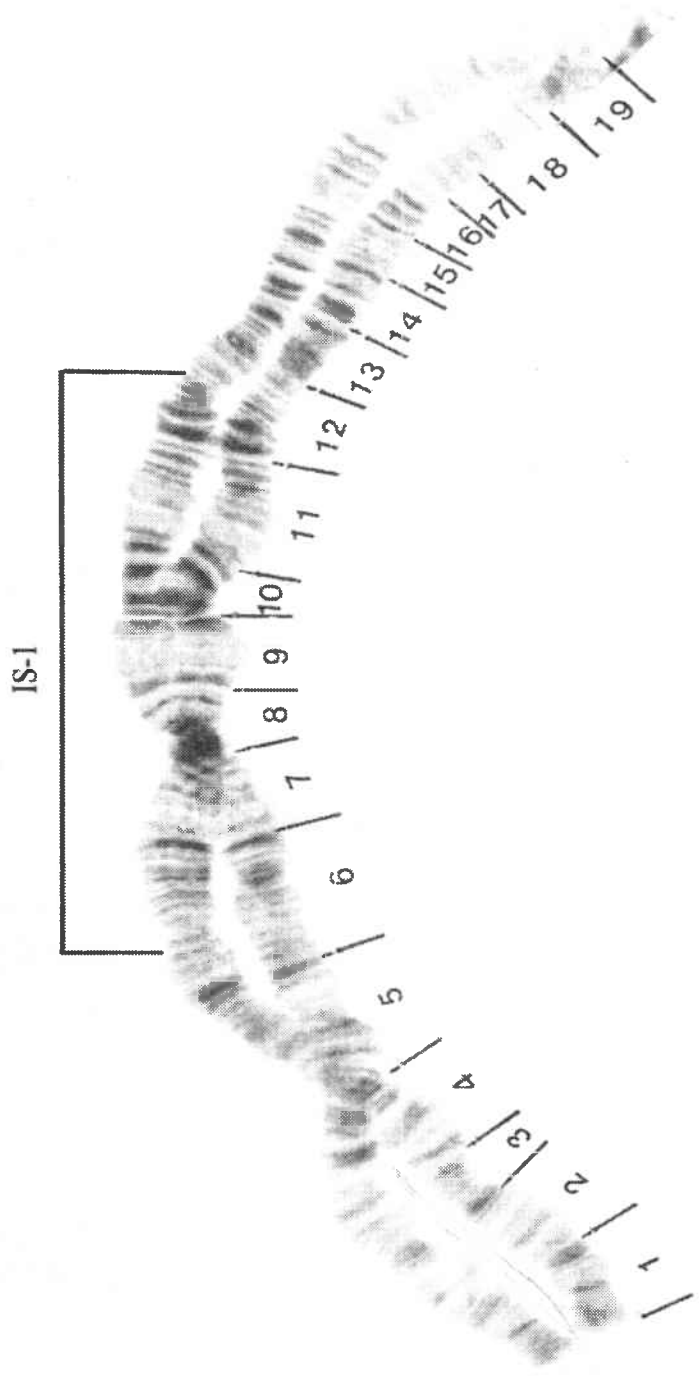


Figure 2: Chromosomal map of standard sequence for *S. arcticum* IS arm with IS-1 inversion in brackets.

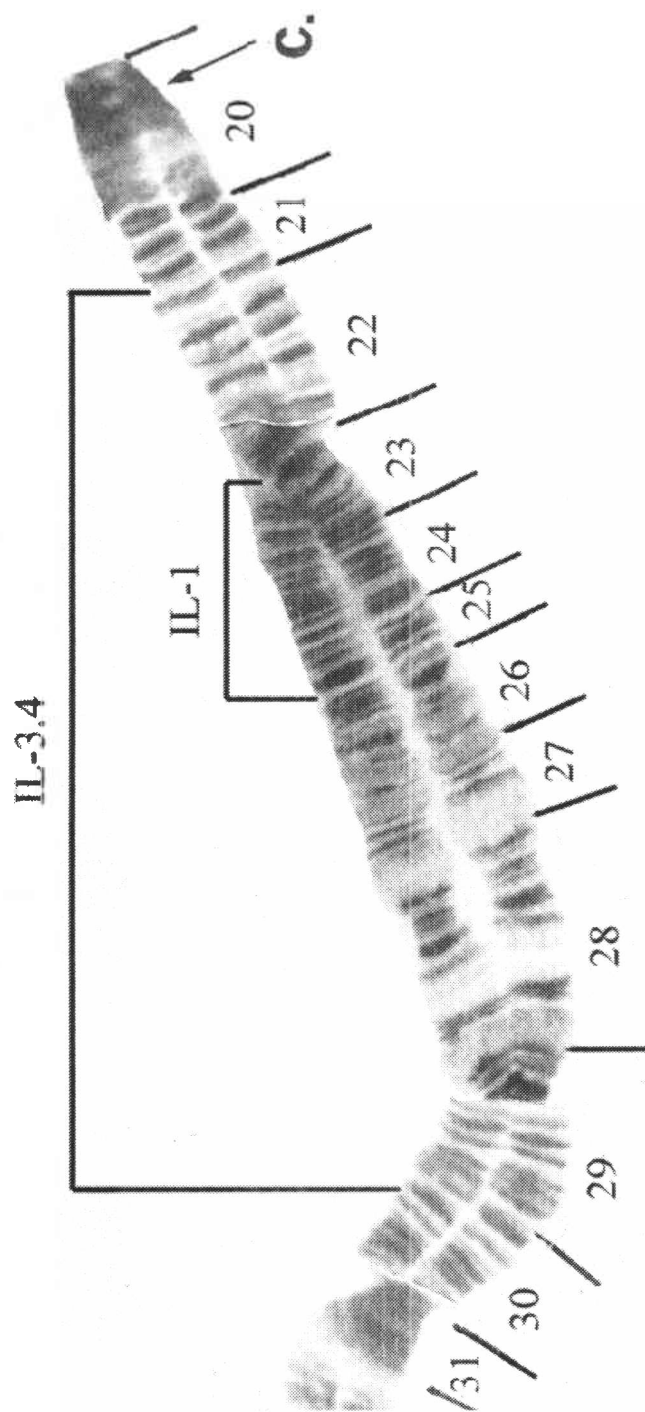


Figure 3: Chromosomal map of standard sequence for *S. arcticum* IL arm with IL-1 and IL-3.4 inversions in brackets

Discussion

Reproductive Status

The *S. brevicercum*, *S. arcticum s. s.*, and *S. apricarium* sibling species in the 3/14/06 Rock Creek sample did not occur in high frequency. In total there were five *S. brevicercum* males, ten *S. arcticum s. s.* male heterozygotes, and seven *S. apricarium* individuals (Table 1). These low frequencies of the siblings do not allow for decisive interpretation of reproductive status.

The autosomal polymorphisms IL-1 and IS-1 were used to elucidate the reproductive status of the IIL-19 and IIL-9 cytotypes. The calculated frequencies of these autosomal polymorphisms were compared to the calculated equilibrium using a chi-square analysis. The chi square values were relatively low and suggest that the IIL-19 and IIL-9 cytotypes are in equilibrium (Table 2). The observance of no homozygotes for the IS-1 inversion is not unusual because its expected value was 1.68.

IIL-9 and IIL-19 have limited distributions, because they are found only in Montana at Rock Creek and the Clearwater, Blackfoot and Bitterroot rivers (Shields, unpub.). Therefore, it is reasonable to assume that these two cytotypes may have originated within this drainage system. A limited distribution and the fact that the IIL-19 and IIL-9 cytotypes are in equilibrium would both be signs that these two cytotypes may be evolutionarily young. If this is true these data support the Shields' "Geographic Distribution/Taxon-Age Continuum Hypothesis." Further support for this hypothesis has been seen in the research of Strizich (2007) and Clausen (2007). Clausen (2007) assessed the reproductive status of *S. arcticum s. s.* and *S. apricarium*, two cytospecies of the *S. arcticum* complex that exhibit wide distribution which would suggest an earlier

evolutionary divergence. Monitoring the occurrence of these two cytospecies at the Little Prickly Pear Creek confirmed that they are good species, although there may be some evidence of hybridization. This was observed in the presence of a fixed IIS-11 inversion associated with *S. apricarium* (Adler *et al.* 2004). If the two cytospecies were hybridizing at this site one would expect to see the occurrence of heterozygotes for the IIS-11 inversion, and 13 heterozygotes for the IIS-11 inversion were observed in a population size of 1254 (Clausen, 2007). Some of these presumptive “hybrids” may be evidence of ancestral polymorphisms. Moreover, Strizich (2007) observed reproductive isolation of the *S. negativum* cytospecies and *S. arcticum* IIL-9 cytotype at the Blackfoot River drainage due to temporal isolation and genetic isolation due to the occurrence of the IS-1 inversion associated with *S. negativum*. However, the genetic isolation is not due to the IS-1 inversion.

Rothfels (1989) suggested a sympatric mode of speciation in black flies and outlined characteristics of such a population to be “1) The frequent sympatric or widely overlapping distribution of the most closely related species, 2) frequent massive sharing of chromosomal polymorphism between related species, 3) frequent and often exclusive involvement of changes in the sex-chromosome system, and 4) that such species should eventually differ in their biology and perhaps present day distribution.” Presumably, the IIL-9 and IIL-19 cytotypes have not reached reproductive isolation at Rock Creek. They do, however, exhibit overlapping distribution, an abundance of autosomal polymorphisms, and differentiation of their sex chromosomes. Previous studies such as Rothfels and Featherston (1981) and Newman (1983) observed black fly sibling species that lived in sympatry and yet exhibited reproductive isolation. Therefore, the IIL-9 and

IIL-19 cytotypes at Rock Creek may perhaps serve as a model for species occurring in sympatry at a transitory level because the heterozygous inversions present in their sex-chromosomes allows for the possibility of genetic differentiation (Newman, 1983).

Additional Observations

The IL-3.4 inversion is sex-linked in *S. negativum* populations in Alaska (Shields and Procnier, 1982) and other drainages in Montana (Shields, unpub.). However, at Rock Creek it appears to be an autosomal inversion based on its presence in both males and females. Alternatively, the IS-1 inversion is sex-linked at the nearby Blackfoot River (Strizich, 2007). These observations may be examples of what Rothfels (1979) identified as “one sibling’s sex-linked inversion being another sibling’s autosomal polymorphism.”

There were 13 IIS-14 heterozygous male larvae observed in the 3/14/06 sample at Rock Creek. IIS-14 has never been observed among any of the other approximately 10,000 larvae studied by the Shields laboratory (pers. comm.). Possibly, these larvae constitute an emerging new cytotype, however, additional analysis must be done to determine if there is sex-linkage before the cytotypic status of these new individuals can be determined. Such specifically detailed observations may be possible only when large sample sizes are studied in great detail, as has been the case here.

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