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Defining the Cytotype and Persistence Concepts in Black Flies (Diptera: Simuliidae)

Honors Thesis

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Helena, Montana

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April 1, 2012

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Acknowledgements:

The M. J. Murdock Charitable Trust (grant # 2003196 to G.F. Shields) supported my research and made the observations prior to 2011 possible. I thank the Department of Natural Science at Carroll College for providing research space, supplies and equipment. I also thank the "insect genetics group" at Carroll for interest and encouragement. My research was supported by a gift to Carroll College from Gerald and Patricia Shields.

Abstract:

Y-linked chromosomal inversions promote reproductive isolation in black flies. Time to fixation to total sex-linkage varies among incipient species. Persistence from year-to-year of chromosome types at any location is largely unstudied. Therefore, I analyzed the polytene chromosomes of a large sample (n = 800) of black fly larvae of the *Simulium arcticum* complex from the Little Blackfoot River (LBR) in western Montana to determine: 1) the extent of linkage of inversions to the Y chromosome and 2) persistence of taxa from year-to-year and compared these data to those of previous analyses, which were based on smaller sample sizes. I hypothesized that the large sample in 2011 would support previous observations. Thirteen Y chromosome types were described in 2011, whereas only nine types had been described previously. This study suggests that the IIL-10, IIL-18, IIL-38, and IIL-51 inversions are Y-linked at the LBR and that they chromosomally define cytotypes (taxa diverging from a common ancestor), which deserve more detailed study. Moreover, persistence was observed since all previous inversions, with the exception of IIL-30, IIL-35, and IIL-84 were found in 2011.

Introduction:

Variation in Y-linked chromosomal inversions among black flies may give rise to evolutionary divergence species (Rothfels, 1956). Males display these heterozygotic Y-linked inversions early in evolutionary divergence (Rothfels, 1979; Rothfels, 1989). In fact, black flies described morphologically as single biological species are often subdivided further into a large number of sibling species once their cytogenetics are investigated (Rothfels, 1979; Shields and Procunier, 1982; Adler *et al*., 2004). Moreover, this chromosomal diversification is common among many complexes of black flies

(Rothfels, 1989; Adler *et al*., 2004). Described chromosomal variation within males precedes fixation of chromosomal inversions within the *S. arcticum* complex, suggesting that cytotypes precede sibling species evolutionarily (Shields and Procunier, 1982). To date, nine sibling species and an additional twenty putative siblings, or cytotypes (Table 1), have been described within the *Simulium arcticum* complex based on chromosomal inversions in males (Shields and Procunier, 1982; Adler *et al*., 2004; Shields *et al*., 2007a; Shields *et al*., 2007b).

Table 1
Members of the *Simulium arcticum* species complex.

Taxon[a]	Status	Reference
Simulium apricarium (IIL-7)	Species	Adler et al. (2004)
S. arcticum IIL-1	Species (formally unnamed)	Adler et al. (2004) and Shields and Procunier (1982)
S. arcticum IIS-4	Species (formally unnamed)	Adler et al. (2004) and Procunier (1984)
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	Shields (unpublished)
S. arcticum IIL-14	Cytotype	Adler et al. (2004)
S. arcticum IIL-15	Cytotype	Shields (unpublished)
S. arcticum IIL-16	Cytotype	Adler et al. (2004)
S. arcticum IIL-17	Cytotype	Shields (unpublished)
S. arcticum IIL-18	Cytotype	Shields et al. (2007a)
S. arcticum IIL-19	Cytotype	Shields et al. (2007b)
S. arcticum IIL-21	Cytotype	Shields (unpublished)
S. arcticum IIL-22	Cytotype	Shields et al. (2009)
S. arcticum IIL-49,50,51,52	Cytotype	Shields (unpublished)
S. arcticum IIS-49,52	Cytotype	Shields (unpublished)
S. arcticum IIL-55	Cytotype	Shields (unpublished)
S. arcticum IIL-57,58	Cytotype	Adler et al. (2004)
S. arcticum IIL-68	Cytotype	Shields (unpublished)
S. arcticum IIL-73,74	Cytotype	Shields (unpublished)
S. arcticum IIL-84	Cytotype	Shields (unpublished)
S. arcticum IIL-85	Cytotype	Shields (unpublished)
S. arcticum sensu stricto (IIL-3)	Species	Adler et al. (2004) and Shields and Procunier (1982)
S. brevicercum (IIL-standard)	Species	Adler et al. (2004) and Shields and Procunier (1982)
S. chromatinum (IIL-11)	Species	Adler et al. (2004)
S. negativum (IL-3,4)	Species	Adler et al. (2004) and Shields and Procunier (1982)
S. saxosum (IIL-2)	Species	Adler et al. (2004) and Shields and Procunier (1982)
S. vampirum (IIL-8; IIS-10,11)	Species	Adler et al. (2004)

[a] Names include unique chromosomal inversions defining taxa: I = first chromosome, II = second chromosome, L = long arm, S = short arm.

Table 1. Members of the *Simulium arcticum* complex (Conflitti *et al.*, 2010).

At issue regarding cytogenetic analysis and the implications of the significance of each of these stages in the diversification process is not only the distinction between sex-linked inversions and those that are autosomal within a given population but also whether sex-linked inversions that characterize cytotypes remain constant, or nearly so, from year

to year. In reality, as new chromosomal variation is discovered and described it is assigned to either sex-linked or autosomal categories depending on the extent of the linkage. However, sample size affects these determinations and incorrect identifications and interpretations regarding the classification to sibling, cytotype and autosomal inversion categories could be made when sample sizes are small.

Shields *et al*., (2009) observed the consistent presence of identical cytoforms as well as frequencies of autosomal polymorphisms in a three-year study at the Clearwater River, Montana. However, the Shields *et al*., (2009) study is the only analysis addressing presence and frequencies of types year after year. Thus, additional study of types and frequencies is necessary to determine the validity and application of initial cytogenetic interpretations.

I chose to study the LBR at Elliston, Montana for the following important reasons: 1) from earlier observations, at least two siblings and potentially four cytotypes of the *S. arcticum* complex were present in spring populations (Shields *et al*., 2007a); 2) large numbers of penultimate larvae were known to be present in the first and second weeks of April (Shields et al., 2007a, and unpublished data) so that larger collections could be made for this study and, 3) the health of larvae at this time was known to be good and thus the necessity of obtaining chromosomes of high quality was assured (Shields *et al*., 2007a, and unpublished data).

The present research: 1) attempts to determine whether putative cytoforms initially described from small sample sizes at the LBR (Shields *et al*., 2007a and unpublished data) remain completely sex-linked or nearly so when large sample sizes are analyzed; and 2) determines whether species richness and cytogenetic diversity remain similar from

year-to-year. Based on the previous observations by Shields *et al*., (2009) at the Clearwater River, I hypothesized that taxon diversity (number and types of siblings and cytotypes) and cytogenetic variation observed in the large sample of 2011 would essentially be similar to those of the earlier, smaller collections. The objective of this study was to use conventional methods of cytogenetic analysis to obtain a large sample size to test the aforementioned hypotheses.

Methods and Materials:

Collection:

I sampled the LBR near Elliston, Montana (46° 33’ 20’’ N, 112° 24’ 30’’ W) on April 4th 2011 to ensure an abundance of material for analysis. A second collection was made by G. F. Shields at the same location on April 10th 2011. All larvae were fixed immediately in 45 ml. vials containing a fresh solution of Carnoy’s fixative (3:1 ratio of 100% ethanol to glacial acetic acid). Vials were filled to ¼ volume with larvae and the fixative was changed until it became colorless (usually four changes). Larvae were stored on ice until our return to the laboratory at which time they were stored at -20° C. Larvae of the *Simulium arcticum* complex were selected by observing their head patterns, sub-labial clefts, and the morphology of gill filaments (page 79, Currie, 1986). They were further selected for appropriate maturity indictaed by the presence of white penultimate histoblasts.

Slide Preparation:

Each larva was opened vertically down the abdomen to expose the salivary glands and gonads. Larvae were placed in cold tap water for 10 mins. and each was blotted dry on filter paper to remove excess silk which could inhibit staining. Entire carcasses with

attached salivary glands and gonads were stained via the Feulgen method (Rothfels and Dunbar 1953). Both salivary glands and gonads were placed on a clean microscope slide in a drop of 50% glacial acetic, covered with a cover slip and gently squashed. Chromosomes were then analyzed for variation, especially inversions in the long arm of chromosome II, using the standard chromosome maps for the genus *Simulium* (Shields and Procunier 1982, Adler *et. al*., 2004). The latest stage of meiosis in males, presence or absence of supernumerary or B chromosomes, centromere dimorphism (either enhanced Ce or thin, Ct) and the polymorphism for the IS-1 chromosomal inversion were also determined.

Larvae were cytotyped into various categories based on the banding sequences of the long arm of chromosome II (Figure 1). These data were then compared to previous cytogenetic observations from this site at comparable time periods (Shields *et al*. 2007a and Shields, unpublished data).

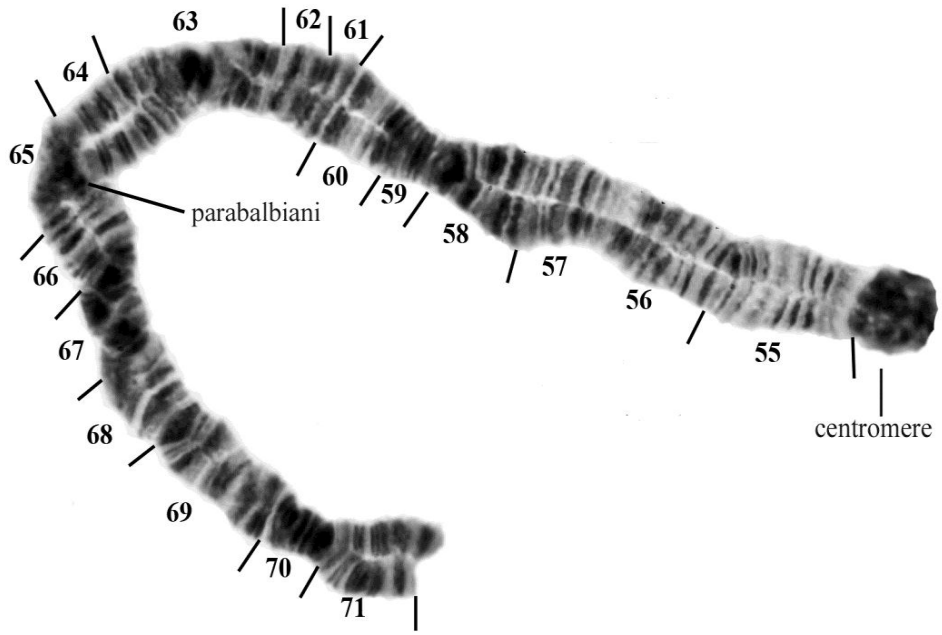

Fig. 1. The long arm of chromosome II of *S. arcticum.* Numbers refer to identified sections of the chromosome. The entire genome is numbered from 1 to 100, beginning with the short arm of chromosome I and ending with the long arm of chromosome III.

Results:

The first objective of this study was to determine if there were changes in the putative cytoforms described at the LBR in previous years, which were based on smaller sample sizes (Shields et al., 2007a and unpublished data), as compared to a larger 2011 sample size. For comparative purposes, I used observations from previous collections (3/30/03, 4/7/05, 4/9/06, 4/17/03 and 4/19/09; Shields *et al.*, 2007a). The total sample for all five collections was N = 313 larvae, while the average number per collection was 62.6 larvae. Cytoforms previously described at the LBR were IIL-3, IIL-10, II-18, IIL-30, IIL-35, IIL-51, and IIL-84. Of these inversions, IIL-3, IIL-10, IIL-18, and IIL-51 were also found in the larger sample (Table 3).

The second objective was to determine if the richness of variation of cytotypes remained similar to previous samples. Variation increased with the increased sample size. With the larger sample size, I found additional types in females and males, sometimes with varying frequencies (Figure 3). Nine X-linked inversions were discovered in the 2011 collection (Table 2). None had previously been observed. The IIL-84 inversion was observed previously, but not in 2011. The majority of female larvae were X_0X_0 in both analyses. Eleven X chromosome types were observed in 2011; but only three had been observed in previous analyses (Table 2).

Table 2. Diversity of X chromosomes of females of the *Simulium arcticum* complex at the LBR for two time periods.

Date	X_0X_0	X_0X_2	X_0X_3	X_0X_{18}	X_0X_{20}	X_0X_{31}	X_0X_{44}	X_0X_{65}
previous	198 (0.990)	0	1 (0.005)	0	0	0	0	0

2011	206 (0.916)	1 (0.004)	1 (0.004)	2 (0.009)	5 (0.022)	3 (0.013)	1 (0.004)	1 (0.004)

Date	X_0 X_{66}	X_0 X_{84}	X_0 X_{103}	X_0 X_{105}
previous	0	1 (0.005)	0	0
2011	1 (0.004)	0	2 (0.009)	2 (0.009)

Thirteen types of Y-linked inversions were described in 2011 (Table 3), whereas only nine types had been described previously. IIL-84 was observed previously, but not in 2011. The frequency of IIL-10 types increased ten-fold in 2011; IIL-20 was present in 2011 but absent in previous studies and IIL-30 was present in previous studies but absent in 2011. Moreover, IIL-38 and IIL-51, which had not previously been described, were found to be completely Y-linked at the LBR (Figure 2). The Y-linkage for lower frequency inversions found in the 2011 sample is uncertain (Table 4). One example of this is IIL-31, where the proportion of females to males is 3:2. If the inversion was completely Y-linked, that value would be expected to be 1.0.

Table 3. Diversity of Y chromosomes of males of the *Simulium arcticum* complex at the LBR for two time periods.

Date	X_0Y_0	X_0Y_2	X_0Y_3	X_0Y_{10}	X_0 Y_{18}	X_0 Y_{20}	X_0 Y_{30}	X_0 Y_{31}
previous	115 (0.452)	0	53 (0.209)	9 (0.035)	48 (0.189)	0	12 (0.047)	3 (0.012)
2011	204 (0.354)	1 (0.002)	114 (0.198)	100 (0.173)	93 (0.161)	12 (0.021)	0	2 (0.003)

Date	X_0Y_{35}	X_0Y_{38}	X_0Y_{40}	X_0 Y_{41}	X_0 Y_{51}	X_0 Y_{84}	X_0 Y_{102}	X_0 Y_{103}
previous	4 (0.016)	0	0	0	2 (0.008)	8 (0.031)	0	0
2011	0	27 (0.047)	2 (0.003)	2 (0.003)	16 (0.028)	0	1 (0.002)	1 (0.002)

Date	$X_0 Y_{104}$	$X_0 Y_{105}$
previous	0	0
2011	1 (0.002)	1 (0.002)

Table 4. Linkage analysis of taxa of the *S. arcticum* complex at the LBR, Montana.

Inversion	Number of Females	Number of Males	Proportion on Y	Y-linked?
IIL-2	**1**	**1**	**0.50**	**?**
IIL-3	**1**	**114**	**0.991**	**+**
IIL-10	**0**	**100**	**1.0**	**+**
IIL-18	**2**	**93**	**0.979**	**+**
IIL-20	**5**	**12**	**0.710**	**?**
IIL-31	**3**	**2**	**0.666**	**?**
IIL-38	**0**	**27**	**1.0**	**+**
IIL-40	**0**	**2**	**1.0**	**?**
IIL-41	**0**	**2**	**1.0**	**?**
IIL-44	**1**	**0**	**0**	**?**
IIL-51	**0**	**16**	**1.0**	**+**
IIL-65	**1**	**0**	**0**	**?**
IIL-66	**1**	**0**	**0**	**?**
IIL-102	**0**	**1**	**1.0**	**?**
IIL-103	**2**	**1**	**0.333**	**?**
IIL-104	**0**	**1**	**1.0**	**?**
IIL-105	**2**	**1**	**0.333**	**?**

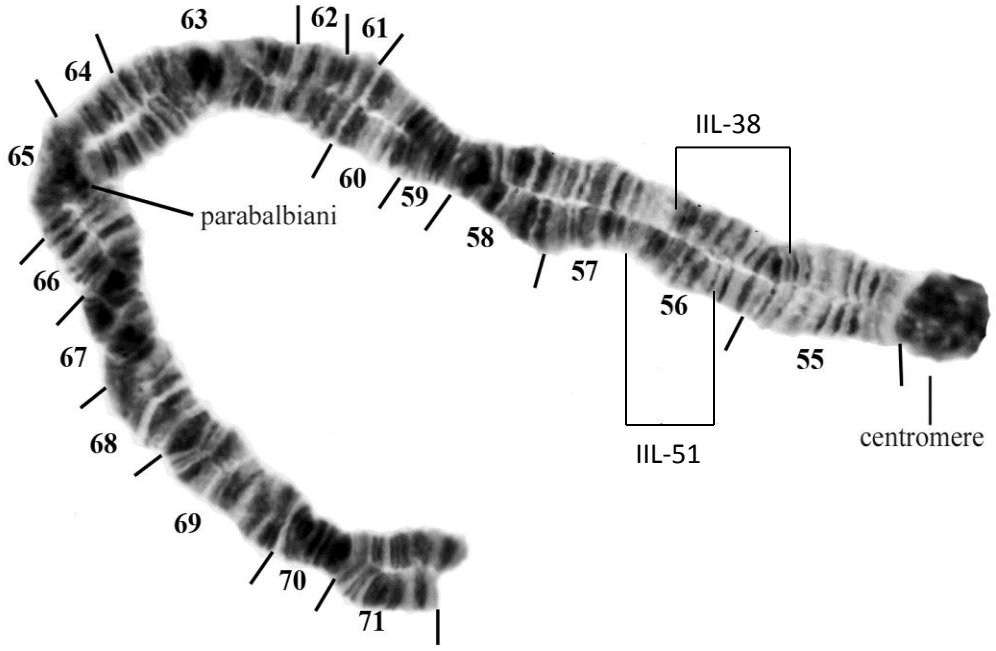

Figure 2. Newly described Y-linked inversions at the LBR. Brackets indicate limits of inversions.

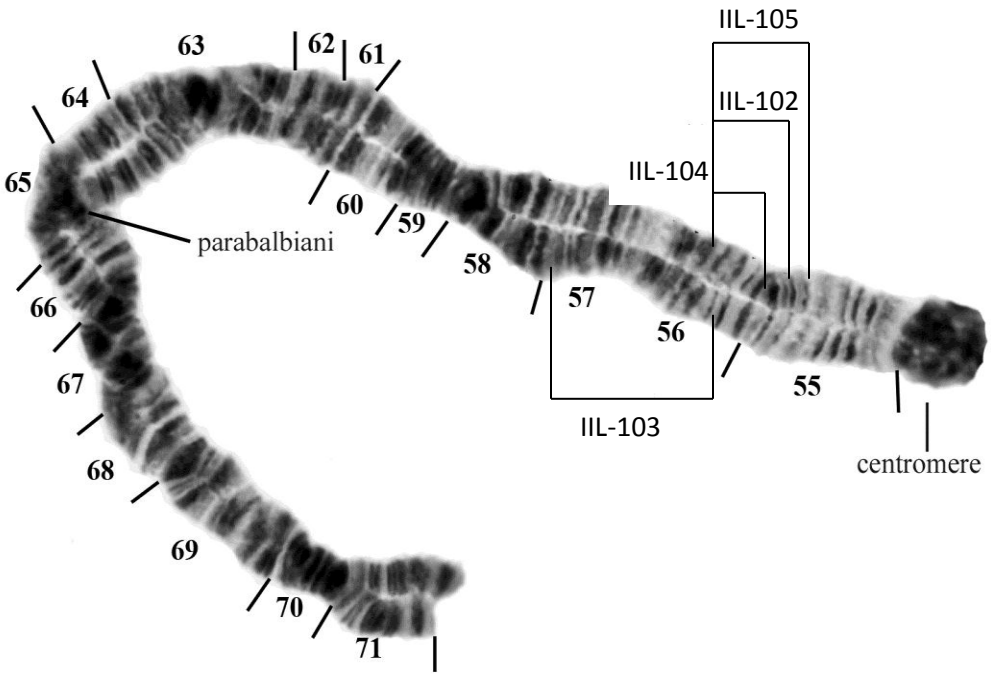

Figure 3. Newly described inversions at the LBR. Brackets indicate limits of inversions.

Discussion:

Nine new inversions on the X chromosome were discovered in 2011. This suggests that, according to Rothfels' Chromosome-based Speciation Model (1989), in some cases linkage to the Y is not totally complete. The variety in females suggests that complete Y-linkage may have not occurred yet. Taxon defining inversions previously studied were also observed in 2011. For males, IIL-30, IIL-35, and IIL-84 were the only inversions previously seen that were not seen in my sample. The frequencies of these inversions during previous years were relatively low. Four Y-linked inversions that had not been previously described were also found. This may mean that changes are still occurring in the population from year to year. New inversions are occurring in addition to previously described cytoforms.

The similarity of the frequencies of inversions at the same site from year to year supports the conclusions from the studies at the Clearwater River (Shields *et al*., 2009). Shields *et al.* (2009) found that the same cytotypes occurred in three consecutive years. I compared data from three consecutive years, and analyzed IIL-3 and IIL-22 inversions in conjunction with other inversions found on the other arm of IIL. The number of males analyzed in each year was similar and showed a frequency gap, in the case of $X_0Y_{\mathrm{IIL}-3,23,24}$, as low as 0.9%. This is extremely similar for the large samples of N=568 to N=686 larvae per year. My sample also showed similar frequencies from year-to-year, even though the 2011 sample was almost thirteen times larger than those of previous individual years. There were some changes when the larger sample was studied because I did not find all previously described cytoforms, when I had expected to find the previous cytotypes and additional, less frequent, types.

My study differs from the Clearwater River in the frequency of IIL-10. In 2011, the frequency of IIL-10 was 10 times that of previous studies. If my collections were made at the peak of the IIL-10 season, the frequency of that inversion would be at its highest value. Comparing this to the previous years, which combined five different studies, a previous one year peak similar to the 2011 peak may have been masked.

The IIL-20 inversion, which is known to be autosomal at other locations (Shields et al., 2007b; 2010) was not observed in previous studies at the LBR. The ratio of five females to twelve males further suggests that IIL-20 is autosomal at the LBR, as previously suggested by Shields *et al.* (2007a). Previous studies at Little Prickley Pear Creek in 2005 and 2006 described IIL-20 as autosomal. One interesting aspect of that study was that IIL-20 was found in simultaneously with other inversions on the opposite IIL-20 arm. My study found that only five out of the seventeen IIL-20 inversions observed were in conjunction with other inversions such as IIL-3 and IIL-18. This further suggests that IIL-20 is autosomal.

The status of Y-linkage is well known for common inversions at the LBR, but is unknown for some lower frequency inversions. The IIL-3, IIL-10, and IIL-18 male to female ratios from my 2011 study confirm previous analyses (Shields and Procunier, 1982 and Adler *et al.*, 2004) that those inversions are sex-linked. Moreover, the IIL-51 inversion, presumed to be Y-linked at the LBR in previous studies was completely Y-linked in 2011. According to Rothfels Chromosome-based Model of Speciation, IIL-51 is a fixed sex-linked inversion in males, and therefore, may be undergoing mating trials and may be considered a cytotype.

This study suggests that: 1) the IIL-10, IIL-18, IIL-38, and IIL-51 inversions are Y-linked at the LBR, 2) they chromosomally define cytotypes (taxa diverging from a common ancestor), and 3) their cytogenetics and general biology deserve additional study.

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