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Testing the Cytotype Concept in Black Flies (Diptera: Simuliidae)

Honors Thesis

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Abstract

Paracentric inversions have been linked to maleness in 17 taxa of the *Simulium arcticum* complex (Diptera: Simuliidae). Linkage to maleness not only defines taxa but also suggests that the sex determining gene or genes lie within the inversion or close to it. Determination of whether an inversion is sex-linked or alternatively is autosomal essentially depends on the analysis of many individuals. This research may thus determine whether linkage is absolute or nearly so. Previous research on nearly 15,000 larvae from 234 sites indicated that inversion linkage to maleness was nearly complete for specific inversions. These include the inversions IIL-3 s. s., IIL-9, IIL-10, IIL-15, IIL-17, IIL-18, IIL-19, IIL-21, IIL-22, IIL-38, IIL-51, IIL-68, IIL-73•74, IIL-79, IIS-12, and IL-3•4. IIL-15 has been observed in 80 male larvae and has not been observed in any females at 12 sites throughout western Montana. The present study tested the linkage of the IIL-15 inversion to maleness on the Y chromosome of larvae in the *Simulium arcticum* complex found at Prickly Pear Creek, Jefferson County, Montana. This site had already presented a decent sample of the IIL-15 inversion, with 14 male larvae. Inversion variation in the IIL arm was determined for 201 larvae. Four males had the IIL-15 inversion while none of the 80 females had it. Thus, to date, all larvae of the *S. arcticum* complex having the IIL-15 inversion have been males, suggesting that this rearrangement is Y-linked. The previously described sibling species, *S. brevicercum* and *S. arcticum* sensu stricto were observed most commonly in my sample, while eight new inversions were found. Of these, seven were discovered in males and one of them was discovered in a female, suggesting that some of these inversions may be autosomal.

Introduction

The study of polytene chromosomes in larval black flies (Diptera: Simuliidae) has demonstrated that a single morphospecies can be differentiated into multiple sibling species by comparisons of unique banding patterns (Rothfels, 1956). Previous analyses of the *Simulium arcticum* complex have led to the discovery of widespread chromosomal variation that has been suggested to play a significant role in the speciation process of black flies (Shields, 2013). A correlation exists in black flies between sex determination and paracentric chromosomal inversions (Rothfels, 1989). This pattern has been observed in numerous species complexes of black flies including: *Prosimulium hirtipes* (Rothfels, 1956), *P. heldon onychodactylus* (Newman, 1983), *Eusimulium pugetnese* (Allison and Shields, 1989), *E. aureum* (Leonhardt and Feraday, 1989), *Simulium pictipes* (Bedo, 1975), *S. venustum/verecundum* (Rothfels *et al.*, 1978), *S. vittatum* (Rothfels and Featherston, 1981), *S. arcticum* (Shields and Procunier, 1982; Shields, 2013), and *S. tuberosum* (McCreadie *et al.*, 1995). The complete Y-linkage of ten types of inversions and an almost complete Y-linkage of an additional six types of inversions of taxa within the *S. arcticum* complex strongly suggest that some chromosomal inversions relate to sex determination in black flies (Shields, 2013). Molecular studies of inversion specific individual larvae suggest that inversions occur early in the speciation process (Conflitti *et al.*, 2010, 2014).

In black flies, a sex chromosome is traditionally known as a chromosomal pair in which one homolog is heterozygous in one sex (often times the male) and homozygous in females (Rothfels, 1956). Rothfels (1979) suggested that it is on these sex chromosomes that most of the differentiation between closely related species most frequently and

initially occurs. This diverse differentiation is most commonly found as the linkage of a paracentric chromosomal inversion mutation to the X chromosome, the Y chromosome, or both. These can also be simple, including a small number of bands positioned close together, or complex, including a large number of bands spanning a large portion of the chromosomal arm (Rothfels, 1979). Inversions may also overlap, such as in *S. negativum* IL-3•4 (Shields and Procunier, 1982).

Paracentric inversions may also be autosomal, i.e. occurring in each of the three genotypes, standard/standard (st/st), standard/inverted (st/i), inverted/inverted (i/i), as well as in both sexes. Since the sex-determining gene or genes have not been identified nor located on a specific chromosome in black flies, it is necessary to rely on the association between the presence of the specific inversion and the sex of the individual (Shields, 2013) to determine sex linkage. Consequently, assignment of an inversion as sex-linked depends on collection and analysis of numerous larvae, which may not always be possible. In other words, larvae with specific paracentric inversions must be correlated with either maleness or femaleness in order to be classified as being sex-linked. Shields (2013) identified 17 taxa within the *Simulium arcticum* complex whose unique paracentric inversions were linked to maleness or nearly so. For example, the IIL-3 sensu stricto inversion (species-specific for *S. arcticum* s. s.) was associated with maleness in 2,703 of 2,711 larvae analyzed (Shields, 2013). Similarly, the IIL-10 inversion was found in all 296 males analyzed (Shields, 2013). Thus, cytogenetic determinations of whether paracentric inversions are associated with maleness depend on analysis of large sample sizes. However, some larvae having unique paracentric inversions associated with maleness may be extremely rare and may occur only at one

site, such as *S. arcticum* IIL-17, a rare inversion found only at Rock Creek, Missoula County, Montana (Shields, 2013).

Cytotypes (populations that have a unique inversion linked to the Y chromosome but the reproductive statuses have not yet been determined) of black flies are usually morphologically indistinguishable in all stages of the life-cycle, including larvae, pupae and adults (Adler *et al.,* 2004). Often times, the only notable differences in some of these most closely related species and cytotypes are the inversions in the banding patterns of the sex chromosomes (Rothfels, 1956, Rothfels, 1979). Currently within the *S. arcticum* complex, there are nine sibling species and 16 cytotypes (Shields and Procunier, 1982; Adler *et al.,* 2004; Shields, 2013). It has been suggested that Y-linked inversions differentiate taxa of *S. arcticum* through sympatric speciation (Shields, 2013), but some of these cytotypes are rare and found only at one location.

The IIL-15 mutation in *S. arcticum* has been found at 12 sites including, the Big Hole River near Twin Bridges, the Bitterroot River in Darby, the Boulder River in Bison Creek, the Boulder River High Ore, the Deep Creek, the Flint Creek Hall, the Gallatin River, the Jocko River Arlee, the Madison River near Three Forks, the Prickly Pear Creek, the Yellowstone River Chico, and the Yellowstone River near Livingston (Table 1). A total of 80 individual larvae have been analyzed with the IIL-15 inversion mutation, all of which were heterozygotic males (Shields, 2013). Prickly Pear Creek and the Gallatin River had the largest numbers of *S. arcticum* lavae with the IIL-15 inversion (Table 1). Fourteen individuals were found in the two samples collected from Prickly Pear Creek, and 21 individuals were found in three different sample collections from the Gallatin River (Table 1). Prickly Pear Creek also appeared to have a large diversity

including *S. brevicercum* (IIL-standard), *S. arcticum s. s.*, *S. apricarium* IIL-7, and *S. arcticum* IIL-10 (Shields, 2013).

Table 1: Distribution of *S. arcticum* IIL-15 males (Shields, 2013).

Location	Total IIL-15
Big Hole River, Twin Bridges	1
Bitterroot River, Darby	1
Boulder River Bison Creek	1
Boulder River High Ore	7
Deep Creek	4
Flint Creek Hall	1
Gallatin River	21
Jacob River Arlee	1
Madison River, Three Forks	18
Prickly Pear Creek	14
Yellowstone River, Chico	1
Yellowstone River, Livingston	10

The present study tests whether the IIL-15 paracentric inversion is linked to maleness at Prickly Pear Creek, Jefferson County, Montana. Previous studies indicate that all IIL-15 larvae at Prickly Pear Creek were males, and no females were found (Shields, 2013). Based on these previous analyses, it is hypothesized that with analysis of a larger sample from Prickly Pear Creek, all IIL-15 inversion types will be male.

Materials and Methods

Black fly larvae were collected from Prickly Pear Creek using standard procedures (Shields and Procunier, 1982). Both GPS coordinates and date of collection were recorded. Identification to species complex was performed through dissection and

identification of the histoblast, as well as identification of head and cleft patterns (Currie, 1986). Staining of the polytene chromosomes and gonads was standard (Rothfels and Dunbar, 1953). Both salivary glands were dissected from the larvae and placed on slides for chromosome analysis. Gonads from the larvae were also dissected and placed on the slides for an on slide identification of sex for each individual. Chromosome analysis and interpretation were performed using previously described maps (Shields and Procunier, 1982), and nomenclature (Shields and Procunier, 1982, Adler *et al*., 2004). For each larva, sex was recorded and the IIL chromosome arm was particularly analyzed to interpret if inversions were present. All prepared slides were then placed in a -80°C freezer for storage.

Results

The objective of this research at Prickly Pear Creek was to study the linkage of the IIL-15 inversion to maleness on the Y-chromosome. Results are shown in Table 2. Of 201 larvae analyzed, four males were observed with this inversion, one of which was in combination with the new inversion IIL-12 found on the same chromosome arm. Although few individuals with the specific IIL-15 inversion were found, a great deal of chromosome diversity was observed at Prickly Pear Creek (Table 2). Note that 72 female larvae were found to be homozygous standard (st/st), and 27 male larvae were identified as *S. bervicercum*. These individuals are not included in Table 2.

Table 2: Types of inversions identified, the combination of inversions, sexes of the larvae, and the proportion of linkage to the Y chromosome.

Inversion	Number of Males	Number of Females	Proportion on Y
IIL-3	68	0	1.0
IIL-3/IIL-20	3	0	1.0
IIL-3,45	1	0	1.0
IIL-3/IIL-66	1	0	1.0
IIL-3/IIL-108	1	0	1.0
IIL-12	1	0	1.0
IIL-15	3	0	1.0
IIL-15,112	1	0	1.0
IIL-18	1	1	0.5
IIL-20	1	5	0.167
IIL-20/IIL-56	1	0	1.0
IIL-35	1	0	1.0
IIL-40	1	0	1.0
IIL-41	1	0	1.0
IIL-45	0	1	0.0
IIL-76	1	0	1.0
IIL-107	0	1	0.0
IIL-109	1	0	1.0
IIL-111	1	0	1.0
IIL-113	2	0	1.0
IIL-114	2	0	1.0
IIL-115	2	0	1.0

The majorities of male larvae (68 individuals) had the IIL-3 inversion (st/i) and were therefore identified as *S. arcticum s. s.* Another four males had the IIL-3 inversion, yet also another inversion on either the same IIL chromosomal arm or the complementary IIL chromosomal arm. Also, 12 larvae contained new inversions (IIL-107, IIL-108, IIL-109, IIL-111, IIL-112, IIL-113, IIL-114, and IIL-115) that have not previously been observed (Fig. 1).

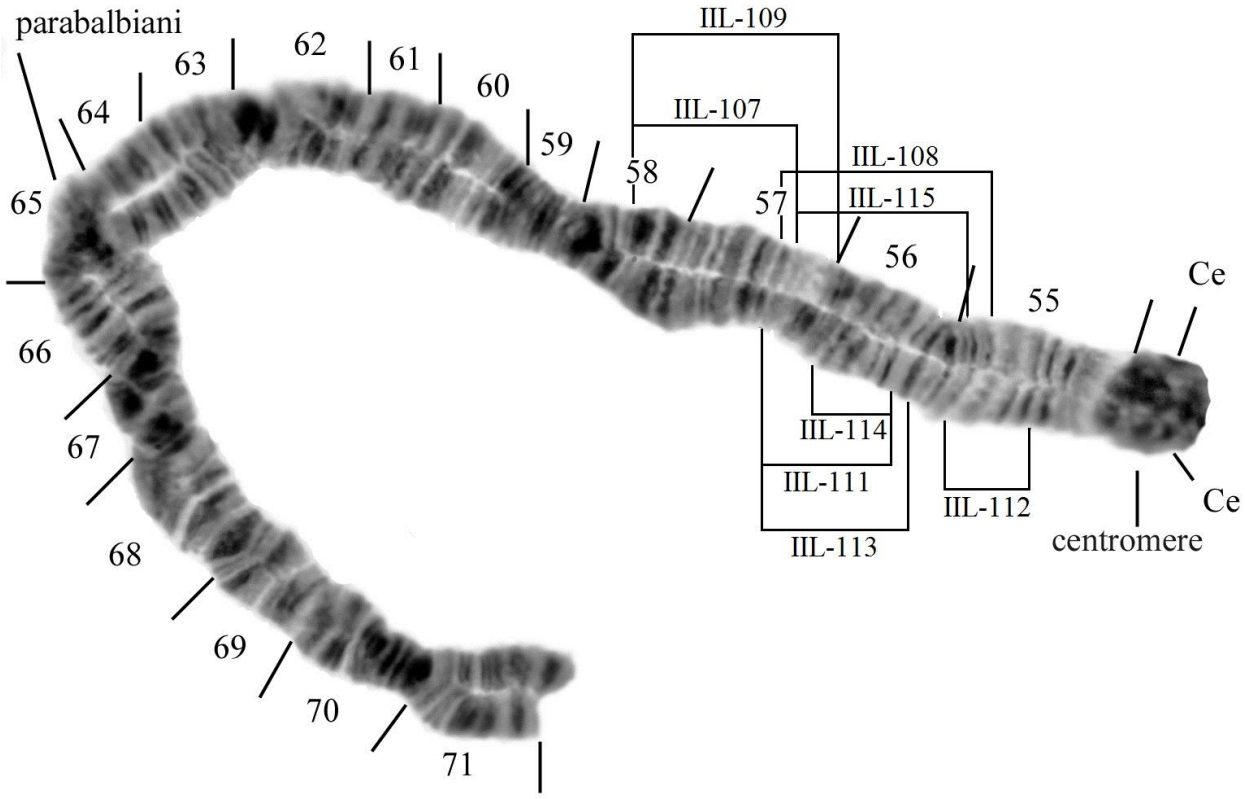

Figure 1: Describes mapping for each new inversion on the IIL chromosome arm

One male had the inversion IIL-12 (st/i) at Prickly Pear Creek. This is a rare inversion that has never before been observed outside the state of Oregon (Adler *et al.*, 2004).

Discussion

This study focused on obtaining a large sample size of IIL-15 individuals from Prickly Pear Creek as well as determining if the inversion was linked to maleness on the Y chromosome. It was hypothesized that this inversion would be solely found in males, thus supporting the suggestion of Shields (2013) that IIL-15 is in fact linked to maleness. The evidence in this study supports this hypothesis. Four individuals were observed with

the IIL-15 inversion, thus I have no evidence to suggest that IIL-15 is not linked to maleness on the Y chromosome. Though a small number of larvae with the IIL-15 inversion were observed, the data do show that IIL-15 is solely found in males.

For this analysis, I used the previous research described in Table 1 by Shields (2013). The site with the highest numbers of IIL-15 is the Gallatin River with a total of 21 larvae (Shields, 2013). However, these larvae were observed over a series of eight years in nine different collections. Of these nine collections at the Gallatin River, *S. arcticum* with the IIL-15 inversion were observed in three males. Because of this fairly rare occurrence, the Gallatin River may not be an ideal collection site for future study of IIL-15. However, the Gallatin River has shown us that collections during the second week of April (around the same time we collected the larvae in this study) offer the largest numbers of the IIL-15 inversion. The site with the second highest frequency of IIL-15 is the Madison River near Three Forks with a total of 18 individuals (Shields, 2013). Contrary to the larvae found at the Gallatin River, these individuals were observed in two of three different collections taken at this site. The two collections containing IIL-15 individuals both occurred during the second week of April, while the collection lacking IIL-15 individuals took place at the beginning of March. This site supported findings at the Gallatin River suggesting that the second week of April was prime time for finding IIL-15 individuals.

Prickly Pear Creek offered slightly different results. Two collections were taken from this site, one at the end of March (3/23/04) and the second at the end of April (4/30/04). Together, these sites yielded 14 larvae, a fairly significant number for two collections. However, the first collection yielded 11 larvae while the collection towards

the end of April only yielded three. This suggests that at this particular site, earlier collections, perhaps towards the end of March, may yield higher numbers of larva with the IIL-15 inversion than collections that take place in April. If collections were to be performed in April, it may be beneficial to collect during the second week of April at Madison River, Three Forks.

One-hundred eight larvae had inversions on the IIL chromosome arm, and 22 of these were unique. Individuals with multiple inversions can either have an inversion on both the X and Y chromosome arms, or multiple inversions on a single arm. This adds to previous evidence suggesting that a single species or cytotype can have multiple sex chromosome rearrangements, as well as undifferentiated sex chromosomes (Procunier 1982, McCreadie *et al.,* 1995).

This conclusion is further supported by the findings on IL-3•4 in the *S. arcticum* complex (Shields and Procunier, 1982). The IL-3•4 (st/i) sibling differs from other *arcticum* sibling species by containing sex determination on the IL-arm, and is recognized based on the presence of the IL-3•4 inversion (Shields and Procunier, 1982). Females remain undifferentiated and have the same banding patterns on the IL arm as those female individuals found to be standard (Shields and Procunier, 1982). Also, 62 larvae collected from Coeur d'Alene River, Shoshone County, Idaho were observed as IIL-2/IIL-3 (i/i) combinational types (Shields and Kratochvil, 2011). This finding suggested that the population of flies collected at this site may be a putative ancestor to present day *S. saxosum* and *S. arcticum* s. s. (Shields and Kratochvil, 2011, Conflitti *et al.,* 2014).

Of these 22 inversions observed, eight were new inversions that have not yet been previously described in the literature. Seven of these new inversions (IIL-108, IIL-109, IIL-111, IIL-112, IIL-113, IIL-114, and IIL-115) were observed in males. This suggests that these inversions may have linkage to the Y chromosome. IIL-107 was a new inversion analyzed in a female suggesting that the inversion is either autosomal or that fixation of this inversion to the sex chromosome has not yet occurred. Sex-related rearrangements have the potential to be partially linked, sex exceptions, ancestral relicts or a result of crossing-over (Rothfels *et al.*, 1978). What may also be presumed to be a single species with multiple sex chromosomes could actually be a combination of two or more species, each with a unique sex-chromosome sequence (Adler *et al.*, 2004). These eight new inversions could possibly be additional formations of separate incipient species or simply partially linked sex inversions although additional study of these inversions is suggested in order to make these specific conclusions.

Adding to the diversity of Prickly Pear Creek was a male larva found with the IIL-12 inversion. This is a rare inversion that has previously been described as linked to maleness on the Y chromosome and as a cytotype that has only been observed in coastal regions throughout Oregon (Adler *et al.*, 2004). This finding may suggest that this inversion allows for survival in more mountainous regions, rather than previously living in a solely coastal environment. Further study of this site would be indicative of whether other additional individuals with the IIL-12 inversion are present.

Of the total 121 male larvae analyzed in this study, 68 males were identified with the IIL-3 s. s. inversion. This was overwhelmingly the most prevalent inversion observed at this site, and it supports previous findings (Shields, 2013). Within the two collections

at Prickly Pear Creek performed in this study, a total of 75 male larvae were identified as IIL-3 sensu stricto. Of all the collections performed throughout western Montana, northern Idaho, and eastern Washington, a total of 2703 larvae were identified as IIL-3 s. s., making up 0.361 percent of the total number of male larvae analyzed (Shields, 2013). Also, after an investigation of reproductive status and formal biology, *S. arcticum* IIL-3 sensu stricto was determined to be a separate morphospecies that has complete linkage to maleness on the Y chromosome (Shields and Procunier, 1982; Adler *et al.*, 2004). Previous analysis has determined the extent of linkage of IIL-3 s. s. to the Y chromosome to be 0.997 (Shields, 2013). The data in the present study support these conclusions with an extent of linkage to the Y chromosome of 1.000.

Included in the 201 larvae analyzed, I also observed one male and one female larvae with the IIL-18 inversion, yielding a 0.5 linkage to the Y chromosome. Conversely, in previous studies, a proportion of linkage for this inversion has been suggested to be 0.967, a significantly greater extent of Y-linkage (Shields, 2013). These differences are most likely the result of significant differences in sample size. IIL-18 has been found at 12 sites including Boulder River Bison Creek, Boulder River High Ore, Canyon Creek, Flint Creek Campground, Flint Creek Hall, Flint Creek Philipsburg, Little Blackfoot River, Elliston, St. Joe River, St. Maries, Idaho, Trout Creek Six Mile, Trout Creek Mouth, York Canyon, and Wise River, Beaverhead Co. (Shields, 2013). From these sites, 183 larvae with the IIL-18 inversion were observed, and 172 were males (Shields, 2013). Prickly Pear Creek has had no previous history of yielding this particular inversion, yet as mentioned before, the diversity at Prickly Pear Creek is large and continually increasing. Collecting from sites previously known to yield high

numbers of the IIL-18 inversion may give us more data to obtain a more accurate extent of Y-linkage.

With the presence of four male larvae with the IIL-15 inversion, I can accept the hypothesis that IIL-15 is linked to maleness on the Y chromosome. However, additional study is needed to make more accurate conclusions. Additional study is also suggested for the presence of sex linkage in *S. arcticum* IIL-107, IIL-108, IIL-109, IIL-111, IIL-112, IIL-113, IIL-114, and IIL-115, as well as further presence of IIL-12 at Prickly Pear Creek.

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