

**A Longitudinal Analysis of the Distributions of and An Assessment of the
Reproductive Status of Two Siblings of the *S. arcticum* Complex at Little Prickly
Pear Creek**

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ABSTRACT

Shields (2006) has proposed the *S. arcticum* Geographic Distribution/Taxon-Age hypothesis. Accordingly, cytospecies with broad, geographic distributions may be evolutionarily old and would be expected to be more reproductively isolated in sympatry than presumably younger cytotypes that have more restricted distributions. Given that no large-scale reproductive isolation test had been performed between *S. arcticum s. s.* and *S. apricarium*, and since Little Prickly Pear Creek (LPPC) offered the opportunity for such studies, one was performed there. Accordingly, I hypothesized that the two cytospecies, *S. arcticum s. s.* and *S. apricarium* would be reproductively isolated at LPPC.

Little Prickly Pear Creek was the single exception among the five drainages previously studied upon which the *S. arcticum s. s.* high/ *S. apricarium* low hypothesis was based. The site was unique in that the ratio of the two taxa was reversed from the previous observations at the other four drainages, i.e. *S. arcticum s. s.* predominated at the original low elevation site at LPPC. I therefore studied this drainage at four equally spaced locations to determine if some other abiotic factor, such as water temperature, might influence the distribution of these cytospecies. Since colder water is usually found at higher elevations and its temperature increases as it moves to lower elevations, I hypothesized that as I sampled down stream from the original site the frequency of *S. apricarium* would increase. I used conventional methods of collection and cytogenetic analysis to study 1108 larvae of the *Simulium arcticum* complex at four sites including the original one. First, I found that *S. apricarium* did not increase in frequency as I sampled down stream. In fact, *S. apricarium* was only dominant in one of 15 collections.

Therefore, I reject my first hypothesis. In addition, I found little evidence for hybridization between the two cytospecies. This observation supports the Geographic Distribution/Taxon-Age hypothesis of Shields (2006).

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INTRODUCTION

Environmental Effects on the Distribution of Siblings

Currently, there are roughly 260 known species of black flies distributed throughout North America (Adler *et al.*, 2004). Mountainous regions are more species rich than geographical regions with little elevational relief (Adler *et al.*, 2004). Thus, with western Montana being a mountainous region, could elevation be a factor affecting the distribution of black flies? Shields *et al.* (2007) found a correlation between elevation and distribution of sibling species within four drainages in Montana in which the *Simulium arcticum* complex occurs. In the present study, Little Prickly Pear Creek (LPPC) was chosen because it was the single exception to the *S. arcticum sensu stricto* high/ *S. apricarium* low hypothesis (Shields *et al.*, 2007). However, no studies had been done to determine if this species distribution was due to any other abiotic factors such as water temperature. Therefore, I hypothesized that this species distribution may be the result of differences in water temperatures between upstream and downstream sites. For example, water should warm as it flows downstream. Could the distribution of the two species be caused by differences in temperature rather than elevation? The upper and lowermost sites at LPPC differ in elevation by only 86 meters which made LPPC a suitable location for testing a temperature correlation hypothesis. Consequently, I hypothesized that *Simulium arcticum s. s.*, previously found at predominately higher elevations (Shields *et al.*, 2007) would be the dominant sibling species at the original site of LPPC, where the water was anticipated to be cooler (Shields *et al.*, 2007). In contrast, *Simulium apricarium* was expected to increase in frequency as I sampled downstream to warmer waters.

Assessment of Reproductive Status of Siblings in Sympatry

Shared autosomal polymorphisms can be used to determine equilibrium frequencies among taxa in sympatry. Adler *et al.* (2004) suggested that *Simulium arcticum s. s.* and *Simulium apricarium* might be reproductively isolated in sympatry. The distribution of *S. arcticum s. s.* is limited to the northwest (Montana, Idaho, Alberta, and British Colombia) while *Simulium apricarium* is distributed in the southwest (Montana, southern Idaho, California, Utah, New Mexico, Arizona, Colorado, Wyoming, and Nebraska; Adler *et al.*, 2004). However, no reproductive isolation test had been conducted for these taxa at rare locations where their distributions overlap and where it is possible to analyze numerous individuals. Therefore, I hypothesized that the two taxa would be reproductively isolated at LPPC. In previous cursory studies the two taxa were fixed for alternative forms of the IIS-10·11 autosomal polymorphism (Riggin, 2004). That is, *S. arcticum s. s.* possessed the standard genotype for IIS-10·11 (st/st) while *S. apricarium* possessed the inverted homozygote (i/i). Thus, reproductive status between these two species could be determined by the presence or absence of heterozygotes with respect to the autosomal polymorphism IIS-10·11.

MATERIALS AND METHODS

Environmental Effects on Distribution of Siblings

Selection of Study Sites

The original site studied by Shields *et al.* 2007 was located 2 miles north of exit 219 off U.S. Interstate 15 on LPPC. This collection site was the single exception among 14 other sites within five drainages (each drainage had 3 sites) whose purpose it was to study any correlation of sibling distribution with elevation (Shields *et al.*, 2007). In testing my hypothesis, I sampled at the original site and at three other equally spaced sites, concluding at the mouth of LPPC as it enters the Missouri River near Wolf Creek.

Collections were made at three-week intervals beginning in late-March and continuing until the quality of polytene chromosomes was such that analysis became impossible (usually in late May) presumably due to inferior health of larvae. With the elevation decreasing by only 86 meters from the first to the last site, it was reasoned that water temperature should increase as I moved along the elevational gradient and thus, the frequency of *S. apricarium* should increase.

Sample Collection

The temperature at each site was recorded to the nearest tenth of a degree Celsius, and freshly collected larvae were placed into fresh, cold Carnoy's fixative, which was then changed frequently until the solution become clear (Shields and Procnier 1982). On March 31, 2005, it was noted at the mouth site, that unlike the other three upstream sites, larvae occurred on both rocks and trailing vegetation. It was reasoned that perhaps some component of the egg cases might allow attachment to different types of substrata

by each sibling. Thus, larvae from the mouth site were analyzed separately based on their attachment to either rocks or trailing vegetation.

Larvae were sorted to morphospieces (Currie, 1986) in the laboratory. Larvae of the *S. arcticum* complex were then stained in Feulgen (Rothfels and Dunbar, 1953). To determine the taxonomic status of each larva (either *S. arcticum s. s.* or *S. apricarium*), I used the chromosome maps of Shields and Procunier (1982).

Reproductive Status

Most *Simulium arcticum s. s.* and *Simulium apricarium* are alternatively fixed homozygotes (st/st vs. i/i), for the autosomal inversion, II-S 10·11 (Riggin, 2004). *Simulium apricarium* sibling status is partially determined based on the presence II-S 10·11 being double inverted. Thus, to determine if *Simulium arcticum s. s.* and *Simulium apricarium* were reproductively isolated in sympatry, I scored all three genotypes for each individual for the IIS-10·11 autosomal inversion.

RESULTS

Distribution Patterns

The first objective of this study was to determine if the frequency of *S. apricarium* would increase as I sampled down stream from the original site to the mouth of the Little Prickly Pear (LPPC). Table 1 shows the ratio of *S. arcticum s. s.* to *S. apricarium* for each site by date for both the 2005 and 2006 collecting seasons. *S. apricarium* was never found to be the dominant sibling species except on May 26, 2005

at the mouth site. Notably, there was also a decrease in frequency of *S. apricarium* from 14.9% in 2005 to 1.7% in 2006.

Surprisingly, the water temperature did not vary greatly between sites or within the sites for the two collecting years (2005 8-12° C, 2006 6-9.5° C), which may have influenced the results of this study. Furthermore, despite the higher temperatures at the mouth site when compared to the original site, larvae there were less mature than those of the three previous sites, on March 31, 2005 and again on March 30, 2006.

For each site there was no significant increase in *S. apricarium* between the two sample dates in the same year and the trends appeared to be the same for both 2005 and 2006. Also, contrary to expectation, *S. apricarium* did not increase in frequency as samples were made downstream from the original site.

In addition, there seems to be no preference for egg deposition for either *S. arcticum s. s.* or *S. apricarium* either on rocks or trailing vegetation. An exception occurred on May 26, 2005, in which *S. apricarium* seemed to preferentially adhere to trailing vegetation. However, the number of larvae analyzed for the site was very small, thus the significance of this observation is uncertain.

Reproductive Isolation

The second objective of this research was to determine if *S. arcticum s. s.* and *S. apricarium* were reproductively isolated at LPPC. With the exception of a small number of larvae possessing new paracentric inversions in chromosome IIL, *S. arcticum s. s.* and *S. apricarium* were the only sibling species present at LPPC during the collecting period. This made the assessment of reproductive status easier; which would

have been more difficult if there were more taxa present. Thirteen of the nearly 1000 larvae analyzed (1.4%) were heterozygotes for the autosomal inversion IIS-10-11, which may suggest rare hybrids between *S. arcticum s. s.* and *S. apricarium* (Table 2). Sixty-seven larvae possessed the IIL-20 autosomal inversion in various combinations (Table 3). Notably, there were 11 male larvae in which IIL-20 and IIL-3 inversions overlapped.

The data also suggest that the two sibling species may be reproductively isolated since IIL-20 was not found in any *S. apricarium* individuals, but was commonly found in many *S. arcticum s. s.* individuals and often superimposed on the IIL-3 inversion (Table 3).

Table 1. Sex Chromosome Diversity in the *S. arcticum* complex at four sites along Little Prickly Pear Creek, Lewis and Clark County, Montana, summers of 2005 and 2006.

| | Females | | Males | | Proportion |
|--------------------------|---------|-------|------------|-------|--|
| | St/St | IIL-7 | IIL-3 St/i | IIL-7 | |
| Date & Temp | | | | | <i>S. arcticum</i> s.s./ <i>S. apricarium</i> |
| Original Site | | | | | |
| 3/31/05, 8°C | 12 | 1 | 21 | 5 | 0.85 : 0.15 |
| 5/06/05, 13°C | 11 | 1 | 23 | 9 | 0.78 : 0.22 |
| 3/30/06, 6°C | 22 | 0 | 67 | 1 | 0.99 : 0.01 |
| 5/06/06, 8°C | 21 | 0 | 22 | 0 | 1 : 0 |
| Kilometer 4.0 | | | | | |
| 3/31/05, 10°C | 34 | 3 | 42 | 4 | 0.92 : 0.08 |
| 5/06/05, 13°C | 9 | 2 | 6 | 1 | 0.83 : 0.17 |
| 3/30/06, 6°C | 19 | 2 | 65 | 1 | 0.96 : 0.04 |
| Kilometer 9.8 | | | | | |
| 3/31/05, 12°C | 23 | 1 | 18 | 2 | 0.94 : 0.06 |
| 5/06/05, 13°C | 22 | 0 | 32 | 4 | 0.94 : 0.06 |
| 3/30/06, 6°C | 32 | 1 | 51 | 3 | 0.96 : 0.04 |
| Mouth, Kilometer 17.9 | | | | | |
| Rock | | | | | |
| 3/31/05, 12°C | 10 | 1 | 23 | 3 | 0.90 : 0.10 |
| 5/06/05, 13°C | 0 | 0 | 10 | 2 | 0.80 : 0.20 |
| 3/30/06, 9°C | 11 | 1 | 75 | 1 | 0.98 : 0.02 |
| 5/06/06, 9°C | 19 | 0 | 27 | 0 | 1 : 0 |
| Branch | | | | | |
| 3/31/05, 12°C | 6 | 0 | 2 | 2 | 0.80 : 0.20 |
| 5/06/05, 13°C | 5 | 1 | 7 | 2 | 0.80 : 0.20 |
| 5/26/05, 13°C | 4 | 7 | 0 | 11 | 0.18 : 0.82 |
| 3/30/06, 9°C | 28 | 0 | 60 | 0 | 1 : 0 |
| 5/06/06, 9°C | 18 | 0 | 27 | 0 | 1 : 0 |
| Total | 306 | 21 | 578 | 51 | |

Table 2. Assessment of Reproductive Status of *S. arcticum s. s.* and *S. apricarium* for the summers of 2005 and 2006 at LPPC.

| Date | <i>S. arcticum s. s.</i> | | <i>S. apricarium</i> | | IIS-11 st/i |
|---------|--------------------------|-------|----------------------|-------|-------------|
| | Females | Males | Females | Males | |
| 3/31/05 | 85 | 106 | 6 | 16 | 5 |
| 3/30/06 | 112 | 318 | 4 | 6 | 3 |
| | | | | | |
| 5/06/05 | 47 | 78 | 4 | 18 | 1 |
| 5/06/06 | 58 | 76 | 0 | 0 | 1 |
| | | | | | |
| 5/26/05 | 4 | 0 | 7 | 11 | 3 |
| | | | | | |
| Total | 306 | 578 | 21 | 51 | 13 |

N=956

Table 3. Association of the IIL-20 autosomal inversion with sex chromosomes of *S. arcticum s. s.* at Little Prickly Pear Creek (summer 2006).

| Females | Males | | | |
|-----------------|-----------------------|-------------------|-----------------|------------------------|
| X_0X_{IIL-20} | $X_{IIL-20}Y_{IIL-3}$ | $X_0Y_{IIL-3:20}$ | $X_{IIL-20}Y_0$ | $X_{IIL-20}Y_{IIL-41}$ |
| 23 | 29 | 11 | 3 | 1 |

DISCUSSION

Effect of Elevation on the Distribution of Cytospecies

My original hypothesis was that the frequency of *S. apricarium* would increase as I sampled down stream from the original site to the mouth of LPPC. This hypothesis was based on previous research by Shields *et al.*, (2007) which suggested that *S. apricarium* and *S. arcticum s. s.* occurred at low and high elevations, respectively, more often than expected by chance alone. Based on the analysis of nearly 1,000 larvae from multiple collections at the four LPPC sites, there is no support for this hypothesis. In 14 of the 15 collections, *S. arcticum s. s.* was undoubtedly the dominant cytospecies either, alone or at its lowest ratio with *S. apricarium* (78:22). The single exception to this pattern occurred on May 26, 2005 at the mouth site, where *S. apricarium* was the dominant cytospecies in the ratio of 18:82. However, this reversal does not seem temperature dependent, since only 20 days earlier the same site, along with the other three, had the same temperature, 13° C, but *S. arcticum s. s.* was the dominant cytospecies there. The reversal may be due to random sampling error caused by the harsh collecting conditions on that day. Therefore, I reject my original hypothesis.

Surprisingly, the water temperature did not vary significantly between sites for the two years of this study. A possible explanation for this lack of variance in temperature could be that the elevational decrease between the sites was not significant enough to create a noticeable difference in water temperature. Despite the temperature differences between the original site and the mouth site, on March 31, 2005 and again on March 30, 2006, the larvae were less mature at the mouth site than at the other three upstream sites, which was contrary to my expectation. Although not studied in this investigation, a

reasonable explanation may be stream velocity. The presence of a beaver dam at the original site may have caused water to move faster through the collection site by forcing it to flow through a narrow opening, thereby increasing maturation of larvae via enhanced feeding. Moreover, LPPC flows through a deep canyon that is generally oriented north and south and thus is in the shadow of the sun most of the summer. Correspondingly, the portion of LPPC that I studied may remain cooler than other similar drainages and thus inhibit females of *S. apricarium* from depositing eggs there.

A secondary hypothesis was that eggs and larvae might have a preference for attachment to either rocks or trailing vegetation at the mouth site. Based on analysis of 363 larvae from 2005 and 2006, there seems to be no preference for egg deposition on either rocks or vegetation by either *S. arcticum s. s.* or *S. apricarium*. Similar observations have been made for members of the *arcticum* complex at Rock Creek, Missoula County by Pickens (2007).

When Peter Adler named IIL-7, he gave it the name *S. apricarium*, which means “of the open,” because it was found in open spaces. This observation could have had an effect on my results, since three of the four sample sites were located in a deep canyon. The female *S. apricarium* could have preferentially selected against these locations for egg deposition because they were not in an open area, thereby decreasing the frequency of *S. apricarium* in the drainage.

Assessment of Reproductive Status of Cytospecies at LPPC

According to the Geographic Continuum/Taxon-Age Hypothesis of Shields (2006), cytospecies with broad, geographic distributions may be evolutionarily old and

would be expected to be more reproductively isolated in sympatry than presumably younger cytotypes that have more restricted distributions. Therefore, my second hypothesis was that the cytospecies, *S. arcticum s. s.* and *S. apricarium* would be reproductively isolated at LPPC. Based on analysis of nearly 1,000 larvae from 15 collections from 2005 and 2006, only 13 potential hybrids were observed. The fact that IIS-10-11 and particularly IIL-7 are polymorphic in some California populations (Adler, pers. comm.) could explain six of the potential hybrids. The remaining seven heterozygotes could be real evidence of hybridization or, alternatively they could be ancestral types that were left from diverging progenitors. One of these seven larvae was a IIL-3/IIL-7 heterozygote suggesting a real hybrid. However, this individual was standard for the IIS-10-11 autosomal inversion, which argues against this contention.

Since IIL-20 was found only in *S. arcticum s. s.* it is likely that this autosomal polymorphism is a recent derivative within the *S. arcticum s. s.* taxon. If the IIL-20 inversion had been ancestral and if the two taxa were hybridizing, then some IIL-20 individuals would have been seen among *S. apricarium* larvae. Therefore, taking all observations into account, I accept my second hypothesis that the two taxa are essentially reproductively isolated at LPPC. Thus, the data suggest support for the Geographic Continuum/Taxon-Age Hypothesis of Shields (2006).

Similar studies by Shields *et al.* (2007) and Shields and Procnier (1982) exhibit comparable results to my reproductive status study. Shields *et al.* (2007) used similar methods to investigate the reproductive status of members of the of *S. arcticum* complex at both the Blackfoot River and Rock Creek. The results of the first study showed that at the Blackfoot River *S. arcticum* IIL-9 and *S. negativum* are the dominant taxa present and

that they are reproductively isolated. In addition to being located in sympatry, they are temporally isolated. Conversely, at Rock Creek, the two cytotypes, *S. arcticum* IIL-9 and IIL-19 are dominant, but have autosomal polymorphisms in equilibrium, suggesting that they are not reproductively isolated. The results of these studies again suggest support for the Geographic Continuum/Taxon-Age Hypothesis of Shields (2006).

In the Shields and Procunier (1982) study, five sibling species were cytologically described within the *S. arcticum* complex: IIL- standard, IIL-1, IIL-2, IIL-3, and IL-3.4. The results of the study showed the different species to be temporally and sympatrically isolated, with respect to one another. The results of both Shields and Procunier (1982) and Shields *et al.* (2007) lend support to the hypothesis that different sibling species within the *S. arcticum* complex can be reproductively isolated either temporally or sympatrically, as I found with *S. arcticum s. s.* and *S. apricarium*, while cytotypes may not be.

Finally, the IIL-20 autosomal inversion was found at a significantly higher frequency than at any other surrounding sites (Shields, pers. comm.). A possible explanation for this could be that IIL-20 originated at LPPC and has some unknown advantage at the site. Among the IIL-20 inversion types, eleven were overlapped with the IIL-3 inversion in males. Such autosomal/sex-linked interactions of overlapping inversions in the base of chromosome IIL in the *S. arcticum* complex are extremely rare, being found only in one or two other cases, such as in IIL-22.23.24 *S. arcticum* at the Clearwater River (Shields, pers. comm.), but apparently are not selected against.

We assume that in the ancestral state the male sex-determining gene or genes lie near the base of chromosome IIL. The first sex-linked inversion either included the sex-

locus or the breakpoints of the inversion were near it. Autosomal inversions such as IIL-20 could interfere with sex determination, particularly when they overlapped with sex-linked inversions, as they do here with IIL-3. Apparently, in this case sex determination remains stable.

A post hoc observation revealed that the distribution frequencies for the two sibling species were 0.93 and 0.07. An ideal distribution frequency of 0.5 for both sibling species would have provided the best possible scenario for testing a distribution hypothesis because it helps minimize the possibility of random sampling error.

Therefore, can a fair assessment of sibling distribution be given with my frequencies? Possibly, however perhaps it should be considered a precursor study until a more ideal drainage is found.

Major Conclusions:

- 1) The frequency of *S. apricarium* did not increase as samples were made down stream for the original site. In addition, there seems to be no preference for egg deposition on either rocks or vegetation by either species, at least at the mouth site.
- 2) As expected, *S. arcticum s. s.* and *S. apricarium* are reproductively isolated from each other in the drainage.

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