Trends in Entomology
Vol. 12, 2016

Original Communication

Determining causal factors of diversity in the *Simulium arcticum* complex of black flies (Diptera: Simuliidae)

Gerald F. Shields*

Department of Life and Environmental Sciences, Carroll College, 1601 N. Benton, Ave., Helena, Montana 59625-0002, USA.

ABSTRACT

This research attempts to identify causal factors responsible for divergence within the black fly *Simulium arcticum* complex. Earlier analysis at the landscape level suggested that sex-chromosomes of larvae of the *S. arcticum* complex were more similar to those of larvae in the same river corridor even when sites in different corridors were closer in Euclidian distance. However, this was not always the case; some sites in the same corridors had larvae with very different sex chromosomes. To investigate these phenomena at the local level, sex chromosomes of larvae of *S. arcticum* at new sites were analyzed and compared to those of previously analyzed sites. Twelve of the 14 comparisons in the present study had larvae with either different sex chromosomes or larvae that had different frequencies of sex chromosomes, or both. These observations suggest that while there may be a river corridor effect, gravid female black flies may also be choosing oviposition sites that are ecologically and physically appropriate for survival of their offspring. Additional studies of ecological and physical parameters at the microhabitat level are encouraged.

KEYWORDS: black flies, polytene chromosomes, sex-linked inversions, distributions, river corridors

INTRODUCTION

Descriptions of genetic, ecological and physical factors that promote divergence within and among species are not well documented [1]. In fact, the large majority of descriptions of factors that promote diversity in populations are only hypothesized and may occur after the fact when reproductive isolation is already complete [1]. These authors stress the importance of opportunities to describe these processes based on actual diverging taxa in a continuum from initial divergence to full reproductive isolation. Black flies (Diptera: Simuliidae) present an opportunity to potentially identify and describe such processes since most 'species' of black flies originally defined on morphological grounds as single biological entities are in reality complexes of sibling species based on analysis of their larval polytene chromosomes [2, 3, 4]. At least eleven species complexes of black flies have been described [5] and these would not have been recognized had it not been for the original cytogenetic analyses [6, 4]. Accordingly, in most cases the first hint of genetic diversity occurs when putative sex determining genes diverge and become associated with paracentric chromosomal inversions, mostly in male black flies [4].

The *Simulium arcticum* Malloch complex of black flies is one of the most diverse groups world-wide, second only to the *S. damnosum* complex in Africa. Nine sibling species of the *S. arcticum* complex are characterized by fixed, sex-linked chromosomal inversions [5] while 22 additional cytotypes have also been described [7]. Cytotypes possess unique sex-linked chromosomal inversions but tests of their reproductive status are forthcoming. Accordingly, a chromosome model of speciation

*Email id: gshields@carroll.edu

in the *S. arcticum* complex has been suggested that hypothesizes through time a continuum of divergence from cytotypes to full reproductively isolated sibling species [7]. This chromosome model of speciation has been supported by molecular analyses (comparisons of mitochondrial and nuclear DNAs) [8, 9, 10, 11]. This complex of variously diverging taxa provides an opportunity to possibly identify factors that may be important as causative determinants of divergence. Elevation seems to be important with regard to the geographic distribution of some siblings and cytotypes [12]. All siblings and some cytotypes are found in unique habitats [13]. Moreover, larvae within the complex that occur within the same river corridor are generally more similar chromosomally than are larvae in different river corridors even when the latter are closer in Euclidian distance [14, 15]. Also, larvae are chromosomally most similar in streams of equivalent size, especially if those streams are < 25 km distant [14].

These analyses have led to the formulation of the "river corridor" hypothesis which in its simplest form suggests that larvae are more similar chromosomally if they occur in the same river corridor, possibly implying not only that females might return to their natal sites to oviposit [14, 15] but also an increase in geographic distribution from a founding population. While this hypothesis may be supported at a broad landscape scale [14], there are exceptions to this hypothesis at the microhabitat level. For example, chromosome similarity occurs within some river corridors but not in others [14]. These 'exceptions' to the river corridor hypothesis provided the opportunity to determine if there is a scale effect involved with oviposition decisions by gravid female black flies from landscape (entire river corridors) to microhabitat (local) levels. The focus of the present research is to test the river corridor hypothesis at an ever more localized level, that of the microhabitat [16]. To this end new sites were sampled and new populations of *S. arcticum* were compared to the sex chromosome diversity of other populations previously analyzed within the same river corridors. It was hypothesized that larvae within the same river corridors would have similar chromosomes.

MATERIALS AND METHODS

Collection of larvae

Sites in six different river corridors were chosen for study so that the chromosome characteristics of *S. arcticum* larvae from them could be compared to previously analyzed larvae from other sites in the same river corridors (Table 1; Figure 1). Taxa of the *S. arcticum* complex are multivoltine, have more than one generation, [5]. Accordingly, specimens were collected from late February through early April so as to obtain only first generation larvae to correspond with previously analyzed samples. All sites had to be visited at least twice to obtain penultimate instar larvae since they have chromosomes of the best morphologies [14]. Larvae were collected from submergent rocks, twigs and trailing vegetation in several areas of the swiftest portions of each stream. Larvae were immediately fixed in freshly mixed 3:1 100% ethyl alcohol: glacial acetic acid at ice temperature (5° C). Collection vials were filled with larvae only to one third volume and the fixative was changed until it remained clear (usually four changes).

Analysis of *S. arcticum* larvae

In the laboratory *S. arcticum* larvae were sorted based on the morphology of their head patterns, postgenal clefts and gill filaments of the histoblasts [17]. Larvae were opened ventrally with fine dissection needles, placed in tap water for ½ hour, and blotted onto filter paper to remove silk. They were then hydrolyzed for exactly nine minutes in a pre-warmed vial of 1N HCl at 64 °C and stained in Feulgen in the dark for one hour [18]. Salivary glands and gonads were removed and placed into one drop of 50% glacial acetic acid and squashed under a cover slip. The chromosome maps of Shields and Procunier [19] were used to analyze polytene chromosomes. A macrogenomic investigation (analysis of all chromosomes and rearrangements) [15] was not performed but rather all populations of *S. arcticum* were characterized according to their sex-chromosomes [7]. A G-test for goodness of fit [20] was completed to determine if the sex chromosomes at each of the sampled sites were significantly different from those of other site(s) in the same river corridor. Results of sex-chromosome comparisons are presented in alphabetical order for each river corridor.

Table 1. Information on collection sites.

River corridor	Collection site	GPS	Distance from upstream site	Collection date(s)	Elevation in meters
Blackfoot River	Russell Gates	47°01'26.40" N, 113°18'34.64" W.		Multiple	1180
	Sunset Hill Rd.	46°56'41.21" N, 113°22'31.20" W.	7 km.	3/8	1153
	Roundup	46°56' 45.43" N, 113°25'48.07" W.	9 km.	2/20, 3/8, 3/9	1131
Clearwater River	Clearwater Campground	47°00'03.66" N, 113°22' 51.43" W.		Multiple	1165
	Mouth of the Clearwater R.	46° 57'54.47" N, 113°22' 36.10" W.	4 km.	3/9	1155
Dearborn River	Highway 287	47°11'56.50" N, 112°05'27.28" W.		3/30	1159
	Mouth of the Dearborn River	47°07'46.53" N, 111°54'39.45" W.	16 km.	3/27	1072
Lt. Blackfoot River	Elliston, Mt.	46 33'23.19" N, 112 24'20.70" W.		3/30, 4/4, 4/7, 4/9	1552
	Garrison Mt.	46 30'59.03" N, 112 47'29.20" W.	33 km.	4/27	1331
Missouri River	Townsend, Mt.	46°20'10.50" N, 111°31'47.16" W.		3/12, 3/27	1165
	Craig, Mt.	47°04'27.27"N, 111°57'40.63"W.	100 km.	3/22, 3/28, 4/6	1053
Rock Creek	Sawmill	46°37'24.54 N, 113°39'05.56 W		3/10	1162
	Spring Creek	46°39'56.32" N, 113°40'02.12" W.	5 km.	4/10	1155
	Original Site	46° 41'25.00" N, 113°40'10.00" W.	5 km.	3/11, 3/12, 3/14 (2)	1085

RESULTS

Blackfoot River corridor

Nearly ¼ of male larvae are *S. arcticum* s. s. (*S. arcticum* IIL-3) at the upstream site (Russell Gates Campground) of this corridor (Table 2a). *S. arcticum* s. s. does not occur at either of the two downstream sites (Tables 2b and c). Alternatively, *S. arcticum* IIL-9 and IIL-19 make up 75.2, 100 and 97.2% of *S. arcticum* larvae at these three sites, respectively (Tables 2a, b, and c). *S. apricarium* was present at 2.8% at the Roundup site.

The Sunset Hill Road site is highly significantly different from the upstream Russell Gates Campground site, $G = 59.04$, d. f. = 5, $P < 0.001$. Likewise, the Roundup site is highly significantly different from the Russell Gates site, $G = 56.93$, d. f. = 5, $P < 0.001$. However, the Sunset Hill Road and Roundup sites are not significantly different, $G = 7.80$, d. f. = 6, $P = 0.253$.

Clearwater River corridor

S. arcticum s. s. male larvae dominate at the Clearwater Campground, and *S. arcticum* IIL-22

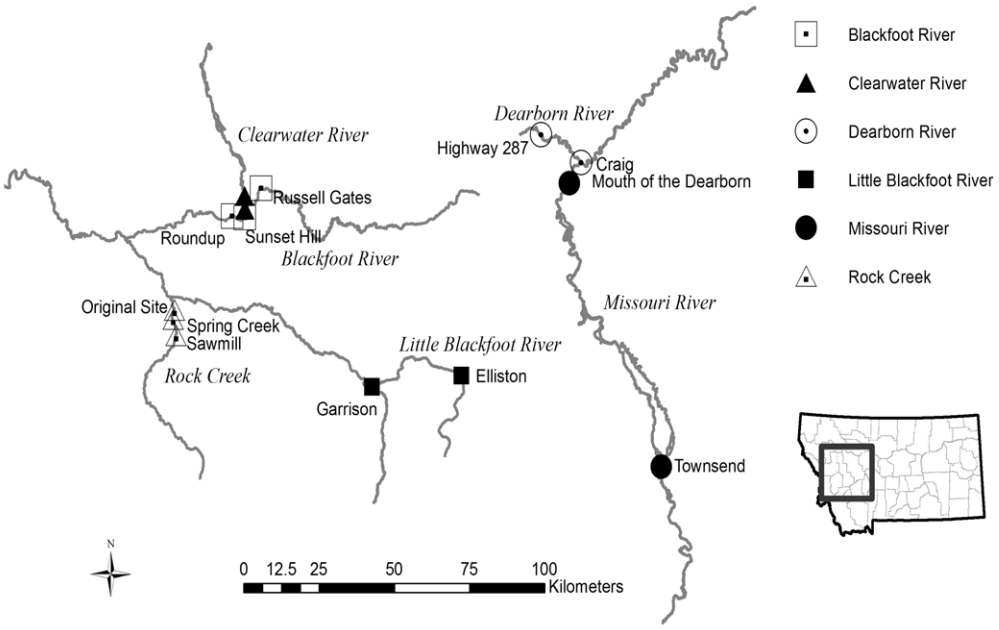

Figure 1. Locations of sites compared in this study.

Table 2a. Russel Gates Campground, Blackfoot River, Missoula, Co., Montana. N = 448.*

Date	**X_0 X_0**	**X_0 Y_3**	**X_7 Y_7**	**X_0 Y_9**	**X_0 Y_{19}**
3/28, 29, 30 4/1, 4/2, 4/11	274	39	4	101	30
% of total	61.2	8.7	0.9	22.5	6.7
% of males		22.4	2.3	58.0	17.2

Table 2b. Sunset Hill Road, Missoula County, Montana. N = 77.

Date	**X_0 X_0**	**X_0 X_9**	**X_0 X_{50}**	**X_0 Y_9**	**X_0 Y_{19}**
3/8/2015	23	1	1	31	21
% of total	29.9	1.3	1.3	40.3	27.2
% of males				59.6	40.4

Table 2c. Roundup, Missoula County, Montana. N = 66.

Date	**X_0 X_0**	**X_0 X_0**	**X_7 Y_7**	**X_0 Y_9**	**X_0 Y_{19}**
2/20, 3/8/, 3/9	27	2	1	22	14
% of total	40.9	3.0	1.5	33.3	21.2
% of males			2.7	59.4	37.8

*X_0 refers to the undifferentiated state of the X chromosome. Y_7, Y_9 and Y_{19} refer to the respective chromosome inversions that characterize those larvae.

is also relatively abundant there (Table 3a). Neither of these taxa occur at the mouth of the Clearwater which is only 4 km. downstream (Table 3b). The mouth of the Clearwater is dominated by *S. arcticum* IIL-9 and IIL-19 males, while only 5% of males are IIL-19 at the upstream Campground site (Table 3a). The Clearwater Campground site is highly significantly different from the downstream Mouth site, G = 316.94, d. f. = 9, P = 0.001.

Dearborn River corridor

It is possible to identify all females and males of *S. apricarium*, (*S. arcticum*, IIL-7) since all genotypes, IIL-st/st, IIL-st/7, and IIL-7/7 occur in both sexes. These larvae are also achromocentric and fixed for the autosomal inversion, IIS-11. With the exception of a single IIL-9 individual, the Mouth of the Dearborn River is a pure *S. apricarium* site (Table 4b). *S. apricarium* is also present at the

Table 3a. Clearwater River, Clearwater Campground, Missoula, Co., Montana. N = 2195.

Date	$X_0 X_0$	$X_0 Y_3$	$X_0 Y_9$	$X_0 Y_{13}$	$X_0 Y_{19}$	$X_0 Y_{22}$
multiple	841	1055	10	5	7	277
% of total	38.3	48.1	0.5	0.2	0.3	12.6
% of males		77.9	0.7	0.4	0.5	20.4

Table 3b. Mouth of the Clearwater River, Missoula County, Montana. N = 60.

Date	$X_0 X_0$	$X_0 X_{IL\text{-}3.4}$	$X_0 Y_0$	$X_0 Y_7$	$X_7 Y_7$	$X_0 Y_9$	$X_0 Y_{19}$
3/9/2016	18	1	2	2	1	31	5
% of total	30.0	1.7	3.3	3.3	1.7	51.7	8.3
% of males			4.9	4.9	2.4	75.6	12.2

Table 4a. Dearborn River, Highway 287, Lewis and Clark Co., Montana. N = 24.

	***S. apricarium* females**				***S. apricarium* males**				
Date	$X_0 X_0$	$X_0 X_0$	$X_0 X_7$	$X_7 X_7$	$X_0 Y_0$	$X_0 Y_0$	$X_0 Y_7$	$X_7 Y_7$	$X_0 Y_9$
3/30/2004	4	1	2	1	8	0	3	1	2
% of total	16.7	4.1	8.3	4.1	33.3	0	12.5	4.1	8.3
% of sex	50.0	12.5	25.0	12.5	57.1	0	21.4	7.1	14.3

Table 4b. Mouth of the Dearborn River, Lewis and Clark Co., Montana. N = 50.

	***S. apricarium* females**			***S. apricarium* males**			
Date	$X_0 X_0$	$X_0 X_7$	$X_7 X_7$	$X_0 Y_0$	$X_0 Y_7$	$X_7 Y_7$	$X_0 Y_9$
3/27/16	2	10	1	0	23	13	1
% of total	4.0	20.0	2.0	0	46.0	26.0	2.0
% by sex	15.4	76.9	7.7	0	60.7	35.7	3.6

upstream site but that site is dominated by *S. brevicercum* (Table 4a). The Dearborn, Highway 287 site is highly significantly different from the Mouth of the Dearborn site, G = 37.79, d. f. = 7, P = 0.001.

Little Blackfoot River corridor

The Elliston (upstream site) is one of the most diverse sites studied [7] having at least four types of male larvae (*S. brevicercum*, *S. arcticum* s. s., *S. arcticum* IIL-10 and *S. arcticum* IIL-18; Table 5a). On the contrary, the downstream, Garrison site, is essentially a *S. apricarium* site, constituting 85.5% of all larvae there while none of those types occurs upstream (Tables 5b and 5a). The upstream Little Blackfoot site at Elliston is highly significantly different from the down steam, Garrison site, G = 401.69, d. f. = 10, P < 0.001.

Missouri River corridor

Both Missouri River sites are pure *S. apricarium* sites (Tables 6a and 6b).

Rock Creek corridor

No larvae were present at the Sawmill, Rock Creek site on March 10, 2016. On the same day hundreds of *S. arcticum* larvae were present at the Rock Creek, original site. Moreover, all larvae at Spring Creek, a tributary of Rock Creek, on April 10 were *S. vittatum*. *S. vittatum* has never been observed at the original site at Rock Creek, which is essentially a *S. arcticum* IIL-9 and IIL-19 site (93%; data not shown).

Table 5a. Little Blackfoot River, Elliston, Montana. N = 896.

Date	**$X_0 X_0$**	**$X_0 Y_0$**	**$X_0 Y_3$**	**$X_0 Y_{10}$**	**$X_0 Y_{18}$**
multiple	274	267	139	107	109
% of total	30.6	29.8	15.5	11.9	12.2
% of males		42.9	22.3	17.2	17.5

Table 5b. Little Blackfoot River, Garrison, Montana. N = 124.

	***S. apricarium* females**				***S. apricarium* males**				
Date	**$X_0 X_0$**	**$X_0 X_0$**	**$X_0 X_7$**	**$X_7 X_7$**	**$X_0 Y_0$**	**$X_0 Y_3$**	**$X_0 Y_0$**	**$X_0 Y_7$**	**$X_7 Y_7$**
multiple	11	1	26	30	4	3	1	16	32
% of total	8.9	0.8	21.0	24.2	3.2	2.4	0.8	12.9	25.8
% by sex	16.2	1.5	38.2	44.1	7.1	5.4	1.8	28.6	57.1

Table 6a. Missouri River, Townsend, Montana. N = 64.

	***S. apricarium* females**			***S. apricarium* males**		
Date	**Xo Xo**	**Xo X_7**	**$X_7 X_7$**	**Xo Yo**	**Xo Y_7**	**$X_7 Y_7$**
3/12/2016	4	7	3	4	10	12*
3/27/2016	4	7	1	2	6	4
Total	8	14	4	6	16	16
% by sex	30.8	53.8	15.4	15.8	42.1	42.1
% of total	12.5	21.9	6.3	9.4	25.0	25.0

*One male was a triploid, IIL-7 i/i/i.

Table 6b. Missouri River, Craig, Montana. N = 151.

	***S. apricarium* females**			***S. apricarium* males**		
Date	**Xo Xo**	**Xo X_7**	**X_7 X_7**	**Xo Yo**	**Xo Y_7**	**X_7 Y_7**
3/22/2007	9	21	5	5	45	15
3/28/2010	4	5	1	1	9	11
4/6/2009	4	6	0	1	3	6
Total	17	32	6	7	57	32
% by sex	30.9	58.2	10.9	7.3	59.3	33.3
% of total	11.3	21.2	4.0	4.6	37.7	21.2

DISCUSSION

The objective of this research was to compare the morphologies of sex-chromosomes of larvae of the *S. arcticum* complex at sites within six river corridors to determine similarities or differences within each corridor. If sites within each corridor had similar chromosome morphologies, then both a river corridor effect (pattern) and a gravid female choice of sites (process affecting the pattern) might be supported. If sites within the same drainage had larvae with different sex-chromosome morphologies no corridor effect might be supported. Only two of the 14 sites (Townsend and Craig on the Missouri River and Sunset Hill Road and Roundup on the Blackfoot River) had no significant differences in sex-chromosome morphologies between upstream and downstream sites. Both sites on the Missouri River were pure *S. apricarium* sites and these were the only sites that exclusively had identical taxa present. Taxa at the Sunset Hill Road and Roundup sites on the Blackfoot River had similar chromosome morphologies. All other corridors had either different chromosomes present at sites or the relative proportions of each of those chromosome types differed significantly. This general observation suggests that although a river corridor effect may be present for the *S. arcticum* complex at the landscape level (Shields and Hokit, 2016), it is probably not present at the microhabitat level, and therefore the original hypothesis that the same river corridors would have larvae with similar sex-chromosomes is rejected.

Observations on the Missouri and Clearwater rivers are simultaneously interesting and unpredicted. Sites in these corridors were the most distant (Missouri, 100 km.) and the nearest (Clearwater, 4 km.), yet the former had an identical taxon present (*S. apricarium*) while the latter had taxa of the *S. arcticum* complex present that arguably could not be more different. This leads one to suggest that at the local level, river corridor and Euclidian distance may not be an important factor determining divergence. Sites on the Missouri River are not only separated by a considerable distance (100 km.) but also by a 40 km. long lake and by two man-made dams (Hauser and Holter). Possibly, blockage of water by dams may not influence oviposition sites by female flies. Also, ecological differences (not studied here) at the two Clearwater River sites might support a model where gravid female black flies are choosing sites based on the appropriateness of offspring survival (process) rather than factors involving Euclidian distance or river corridor effects (pattern).

That gravid female black flies may be using ecological cues to determine where to lay eggs is supported by observations on the stability of community structure [21] and detailed analysis of taxa over a three-year period at the Clearwater River Campground [22]. In the former study which was based on 39 comparisons of nearly 8,500 larvae from 19 sites, chromosome diversity did not change from-year-to-year [21]. In a more detailed site study at the Clearwater Campground during 2007, 2008 and 2009 in which 1825 larvae were analyzed, the year-to-year percentage of types

varied by less than three percent in all categories and in all years [22]. These observations suggest that females are using environmental cues to return to an appropriate site each year to oviposit [15].

Small sample sizes of analyzed larvae may affect conclusions about divergence [7]. However, if one compares sex-chromosome diversity of the *S. arcticum* complex at the eight sites for which at least 500 larvae have been analyzed over multiple springs, the genotypes at each site remain essentially the same from year-to-year [7]. This possibly supports the fact that gravid females are choosing environmentally appropriate sites in which to lay their eggs.

Studies that investigate physical and ecological determinants that influence decisions of oviposition sites by female black flies at both regional and local levels are needed [5]. The present study suggests that more detailed research at the microhabitat level may reveal those factors that determine choice of oviposition sites by female black flies.

At a macroscale species richness is high in montane regions but decreases as physical relief declines [5]. Stream width and depth, flow rates, stream types, vegetation and other unknown features may influence choice of oviposition sites by females [23, 24]. Accordingly, future studies should investigate these ecological and physical variables at locations for which abundant cytogenetic data are already available.

ACKNOWLEDGEMENTS

G. F. S. is funded through the James J. Manion Fund of Carroll College as Endowed Chairman of Biological Sciences. At Carroll College, Grant Hokit gave statistical advice and helped with the figure. Dr. Peter Adler, Clemson University, commented on an earlier draft of this manuscript and has supported this research from the beginning.

CONFLICT OF INTEREST STATEMENT

The author declares no conflict of interest.

REFERENCES

1. Coyne, J. and Orr, A. 2004, Speciation. Sinauer Associates Inc., Sunderland, Massachusetts.
2. Rothfels, K. 1956, J. Heredity, 47, 113.
3. Rothfels, K., Feraday, R. and Kaneps, A. 1978, Can. J. Zool., 56, 1110.
4. Rothfels, K. 1989, Genome, 32, 500.
5. Adler, P., Currie, D. and Wood, M. 2004, The Black Flies (Simuliidae) of North America. Comstock. Cornell University Press, Ithaca, NY. 941 pp.
6. Rothfels, K. 1979, Ann. Rev. Entom., 24, 507.
7. Shields, G. F. 2013, Mono. W. N. Amer. Nat., 6, 64.
8. Conflitti, I., Kratochvil, M., Spironello, M., Shields, G. and Currie, D. 2010, Mol. Phy. Evol., 57, 245.
9. Conflitti, I., Shields, G., Murphy, R. and Currie, D. 2012, Can. Entom., 144, 323.
10. Conflitti, I., Shields, G., Murphy, R. and Currie, D. 2014, Mol. Phy. Evol., 78, 43.
11. Conflitti, I., Shields, G., Murphy, R. and Currie, D. 2015b, Biol. J. Linn. Soc., 115, 13.
12. Shields, G., Clausen, G., Marchion, C., Michel, T., Styren, K., Riggin, C., Sontaro, T. and Strizich, L. 2007a, W. N. Amer. Nat., 67, 39.
13. Conflitti, I., Shields, G., Murphy, R. and Currie, D. 2015a, J. Evol. Biol., 28, 1625.
14. Shields, G. and Hokit, G. 2016, Fresh. Sci., 35, 1023.
15. Adler, P., Kudelova, T., Kudela, M., Seitz, G. and Igniatovic-Cupina, A. 2016, PLoS One, 11(1), e0147673. doi:10.1371/journal.pone.0147673.
16. Adler, P. and McCreadie, J. 2009, Medical and Veterinary Entomology, 2nd Ed., Editors Mullen and Durden.
17. Currie, D. 1986, Mem. Ent. Soc. Can., 134, 1.
18. Rothfels, K. and Dunbar, R. 1953, Can. J. Zool., 31, 226.
19. Shields, G. and Procunier, W. 1982, Polar Biol., 1, 181.
20. McDonald, J. H. 2014, Handbook of Biological Statistics (3rd), Sparky House Publishing.
21. Shields, G. 2015, Annals. Entom. Soc. Amer., 108, 487.
22. Shields, G., Christiaens, B., van Leuven, M. and Hartman, A. 2010, W. N. Amer. Nat., 69, 511.
23. McCready, J. and Colbo, M. 1991b, Can J. Zool., 69, 2651.
24. McCready, J. and Adler, P. 1998, Oecologia, 114, 79.