Phenotypic and genetic variation within the *Cricotopus sylvestris* species-group (Diptera, Chironomidae), across a Nearctic - Palaearctic gradient

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Intraspecific variation sometimes obscures species boundaries and makes identification of certain Chironomidae difficult. This is true for many species in the genus *Cricotopus*. We used DNA barcode data and multivariate statistical analyses to investigate which phenotypic characters in populations of the *Cricotopus sylvestris* species-group are useful for species identification. Specimens collected across a broad latitudinal range from the Southwest United States through subarctic Canada to northern Norway formed nine distinct barcode clusters. Body size of adult flies decreased by 51% from the northern to southernmost populations. Meristic characters in wings and legs were strongly related to overall body size, and related morphometric ratios were not species specific. Antennal ratio increased significantly with body size, thus limiting its value in species delimitation. Non-metric ordinations of setal and coloration pattern data were characteristic for most species in the *sylvestris*-group. DNA barcode data worked well in separating morphologically different populations, except for the case of *C. (I.) sylvestris* and *C. (I.) trifasciatus*, which were distinguished by ordination of color pattern, but not by barcoding data. These two species appeared closely related, and we conclude that sequence data from neutral nuclear markers will be necessary to determine if these are genetically distinct species, or whether there is merely a high level of environmental plasticity in pigmentation within this geographically widespread barcode cluster.

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INTRODUCTION

The genus *Cricotopus* van der Wulp, 1874 is one of the largest in the Orthocladiinae, containing five subgenera, with species distributed across the globe (Cranston et al. 1989). Aquatic macrophytes and algae provide typical habitat for *Cricotopus* larvae: species of the genus are known to inhabit a wide range of water bodies, from pristine streams and brooks to eutrophic ponds and brackish estuaries (Hirvenoja 1973; Boesel 1983). Certain species may become so abundant in eutrophic waters that adult swarms can reach nuisance proportions (Spies 2000; Hirabayashi et al. 2004). Species-level identification of adult *Cricotopus* is difficult because high levels of intraspecific variation lead to overlap in the values of morphological traits between species. This is particularly true for many mensural data; coloration and the pattern of setae on thorax and the abdominal tergites are therefore important for identification of *Cricotopus* species (Hirvenoja 1973). Within a species, however, pigmentation varies seasonally and geographically (LeSage & Harrison 1980; Boesel 1983; Oliver & Dillon 1988) and can potentially obscure otherwise good differences

between species.

Hirvenoja (1973) revised the genus *Cricotopus* for the western Palaearctic and his revision has become the main reference for further work on this genus. Within the subgenus *Isocladius* (Kieffer, 1909) he placed twelve species in the *sylvestris* species-group. Six of the Palaearctic *sylvestris* gr. species are also reported from the Nearctic (Poole & Gentili 1998). Although several *Cricotopus* species are regarded as Holarctic (Hirvenoja 1973), there could be some bias in identification of Nearctic species, because a translation (Simpson et al. 1983) of Hirvenoja's (1973) keys are used in North America. Further taxonomic challenges in North American *Cricotopus* are a number of undescribed taxa (own observation) and the fact that certain populations seem particularly difficult to identify. For instance, Nearctic populations of *C. (I.) sylvestris* Fabricius, 1794 and *C. (I.) trifasciatus* Meigen, 1813 have proved difficult to distinguish, and there have been conflicting reports as to which species is more abundant in North America (LeSage & Harrison 1980, Boesel 1983). There has been debate over which Nearctic populations warrant species status: e.g., *C. (I.) lebetis* Sublette, 1964 was ultimately recognized as a Nearctic relative of *C. (I.) tricinctus* Meigen, 1818 (Epler et al. 2000), whereas *C. (I.) remus* Sublette, 1964 has been synonymized with *C. (I.) trifasciatus* (Boesel 1983). *Cricotopus. (I.) flavibasis* Malloch, 1915 and *C. (I.) myriophylli* Oliver, 1984, represent additional *sylvestris* gr. species, unique to the Nearctic (Boesel 1983; Oliver 1984).

A combination of traits is used to distinguish adult males of the *sylvestris* species-group from the six other groups within the subgenus *Isocladius*. The superior volsella is always rounded and hump-shaped; the median setae form a single longitudinal row on the abdominal tergites, while the anterior transverse row of tergal setae is absent. On the tarsi, small pulvilli are visible, and light markings are usually present on the legs and abdomen. Within the *sylvestris*-group Hirvenoja (1973) used the size and abundance of tergal setae, the number of sensillae chaetica, and various morphometric ratios to distinguish species. While color pattern is the main criterion for identification of half of the species in this group, Hirvenoja also mentioned variation from light to dark-pigmented forms of C. *(I.) sylvestris*, C. *(I.) trifasciatus*, C. *(I.) speciosus* Goetghebuer, 1921 and C. *(I.) tricinctus*. In the extreme case, discrimination of dark forms of *C. (I.) sylvestris* from *C. (I.) glacialis* Edwards, 1922 may only be possible when adult females and pupal exuviae can be associated with adult males (Hirvenoja 1973).

Phenotypic plasticity refers to the ability of environmental conditions to alter the expression of the genotype of an organism. Both temperature regime and food quality control size and growth rate of chironomids: larvae from colder habitats mature at larger size, on a given diet (Gresens 1997). Chironomid taxonomy has accounted for body size variation by expressing measurements of antenna, leg and wing structures in terms of morphometric ratios. Nevertheless, plasticity in body size raises the issue of allometry, wherein the relative proportions of body parts change with body size. Allometry leads to differences in shape among large and small individuals of the same species, such that the values of morphometric ratios themselves change with body size (McKie & Cranston 2005). Allometric responses of morphometric ratios and shape of the hypopygium have been reported for several species of chironomids (Kobayashi 1998; McKie & Cranston 2005) and plasticity of pigmentation in response to temperature has also been demonstrated experimentally for several species of *Conchapelopia* Fittkau, 1957 (Kobayashi and Hayashi 2001).

Our study focused on adult male specimens of the *Cricotopus sylvestris* species group, where the problems posed by allometry and phenotypic plasticity have not been addressed. Our goals were to determine how well species identifications based on phenotype corresponded to genetically defined populations and to identify which traits were most effective in distinguishing species within the *C. Sylvestris*-group. Our analysis emphasized multivariate analyses of traits that are not expressed as morphometric ratios: i.e., color pattern and distribution of thoracic and tergal setae. Given the potential for observing phenotypic plasticity in populations spanning a wide range of latitude and environmental conditions, it was crucial to have genetic data to define populations independent of phenotype. Thus we combined quantitative analyses of phenotype with DNA sequence data from the cytochrome c oxidase subunit 1 (COI) gene, (i.e., "DNA barcodes") from specimens collected from sites across a broad geographic range, including the United States, Canada, Iceland and northern Norway. We sought to answer the following questions: 1) To what extent does allometry bias the values of measurement ratios across populations? 2) How useful are leg and wing ratios for distinguishing species within the *C. sylvestris*-group? 3) How well do multivariate analyses of setae and pigmentation data correspond to genetically distinct populations? 4) Which particular setal and pigment variables are most useful in distinguishing species of the *C. sylvestris*-group?

METHODS

Nucleotide sequence data was obtained for 64 specimens, including one *C. Cricotopus cylindraceous* gr. specimen which served as the outgroup. Tissue samples for barcoding consisted of 1-2 legs per specimen, preserved in 96% ethanol. Gene sequence data was provided by the Canadian Centre for DNA Barcoding, University of Guelph, according to their standard procedures for extraction, PCR and sequencing. Partial COI sequences and metadata for specimens are available through the Barcode of Life Datasystems (Ratnasingham & Hebert 2007). A taxon identification tree was constructed using MEGA 5 software (Tamura et al. 2011) employing the Neighbor-Joining method and 500 bootstrap replicates. Pairwise distances within the final COI dataset, consisting of 658 codon positions, were computed using the Kimura 2-parameter substitution model

(Kimura 1980) in MEGA 5.

Phenotypic measurements were made on thirty eight cleared and slide mounted specimens. We calculated several of the morphometric ratios reported by Hirvenoja (1973). The length of femur, tibia and each tarsal segment (i.e., t_1,…t_5) were measured for fore-, mid- and hindleg. These lengths were used to calculate the following "leg ratios":

BV = (femur + tibia + t_1) / (t_2 + t_3 + t_4 + t_5)
LR = (t_1/tibia)
SV = (femur + tibia) / t_1

The antennal ratio was calculated as:

AR = (apical flagellomere) / (combined length of basal flagellomeres).

McKie and Cranston (2005) reported wing length to be an accurate indicator of total body size in chironomids. Wing length was measured from arculus to wingtip. Two wing ratios (Hirvenoja 1973) were then calculated:

VR_C = (distance between R_{2+3} and R_{4+5}) / (distance from tip of R_1 to tip of R_{4+5})

VR_{CU} = (distance from arculus to FCu) / (distance from arculus to RM).

Three additional measurements were made to quantify wing shape: the distance from FCu to RM, the distance from FCu to the tip of R4+5, and the distance from FCu to the furthest extent of the anal lobe.

The following groups of thoracic setae were quantified: dorsocentrals, antepronotals, acrostichals, prealars and scutellars. The pattern of setation of abdominal tergites 3 and 4 are important for discrimination of *sylvestris* gr. species. The number of medial and lateral setae on these tergites was recorded and the diameter of the medial setae relative to the laterals was classified as 0= medial and lateral of similar diameter, or 0.5 = medials slightly wider than laterals, 1 = medial setae clearly wider than laterals. The number of sensilla chaetica, appearing at 400 times magnification as a row of small depressed hooklets along the edge of t_1 of the hindleg, was recorded.

We devised a numerical scoring system to quantify patterns of pigmentation on the scutum, abdominal tergites and legs. This approach required a modest amount of simplification but it allowed us to capture major patterns and subject them to statistical analysis. Thoracic pigmentation was assigned several scores, on a scale from 0 (pale yellow exoskeleton, no pigmentation), to 5 (black, very dark pigmentation). Thoracic pigment scores were recorded for the anterior and posterior scutum, the humeral area, scutellum and postnotum. Areas that were darkened by uncleared musculature were avoided in the scoring process. Leg pigmentation scores included 0 (presence of a light ring contrasting sharply with brown color of segment), 1 (a pale brown ring on a darker brown ground), and 2 (no ring, segment uniformly pigmented). The femur, tibia and all tarsomeres of fore-, mid- and hind-leg received separate scores. Quantification of tergal pigmentation was most complex: the distribution of dark pigment was scored separately for abdominal tergites 1- 8, plus the anterior and posterior part of the hypopygium. In most cases, tergites exhibited a more-or-less transverse band of pigment; therefore, the average locations of the anterior and posterior edges of this pigment band were recorded as the percent of distance back from the anterior of the tergite. For example, a brown pigment band extending from the anterior of a tergite to its midpoint would receive a score of 0 for its anterior margin and a score of 50 for its posterior margin The presence of different pigment patterns not resembling transverse bands was recorded as a separate variable, with a code number (0 to 10) for a particular pattern, e.g., 4 = two posterolateral spots, whereas 7 = one medial triangular spot. Thus each abdominal tergite received 3 pattern-variables.

Multivariate statistics were initially used to distinguish variation in leg and wing measurements that were simply due to body size from independent variation in leg or wing shape, which might prove species-specific. Principal components analysis (PCA) was judged to be applicable given the distribution of variables; PCAs based on correlation matrices were applied separately to the leg and wing datasets. Significant principal component axes (PCs), which summarized independent aspects of morphological variation, were then subject to linear regression on wing length (i.e., surrogate for body size). Morphometric ratios were also regressed on wing length to detect any persistent allometry. Linear regressions were run on both raw data and on log-transformed data; these gave similar results. Although allometric relationships of body size and shape are typically nonlinear, the range of body size, although substantial in this study, was not large enough to detect the nonlinear component of the size-shape relationships. Regression diagnostics including the distribution of residuals from the regression model and the proportion of variance explained, R^2, showed that it was more accurate to report the regressions on raw data. Regressions were performed using Microsoft Excel® spreadsheet software.

Both the setae and pigment datasets contained large numbers of variables with highly non-normal distributions. Variables that did not actually vary among specimens were removed from analysis. Setae and pigment datasets were analyzed separately because the difference in magnitude of their variables would have biased the results. Non-metric multidimensional scaling (NMDS) was used to determine which groups of variables (i.e., ordination axes) best distinguished genetically distinct populations. All multivariate statistical procedures were run using PC-ORD v. 5 software (McCune & Mefford 2006).

RESULTS

The taxon identification tree revealed nine genetically distinct populations, i.e., "barcode clusters" (Figure 1). Three barcode clusters closely matched Hirvenoja's (1973) descriptions of *C. (I.) ornatus* (Meigen, 1818), *C. (I.) tricinctus* and *C. (I.) glacialis*, whereas the largest cluster contained diverse populations of both *C. (I.) sylvestris* and *C. (I.) trifasciatus*. The remaining five clusters were given provisional designations because they differed noticeably from Hirvenoja's descriptions, based largely on material collected in Finland. Two of these populations were from Finnmark, Norway: *Cricotopus (I.)* sp. 3, which resembled *C. (I.) pilitarsis*, and *C. (I.)* sp. 5 which did not clearly resemble any described species. The three Canadian populations included *C. (I.)* sp. 4, in the *sylvestris*-group, plus *C. (I.)* sp. 6 and *C. (I.)* sp. 7, which were both close to *C. (I.) ornatus*, although they differed from each other in the length of setae and intensity of pigmentation.

Given the range of body size represented, allometry should have been observed if present. Wing length (i.e., body size) decreased by 51% from the largest (2.94 mm) to smallest individual (1.44 mm). Multivariate analyses of leg and wing measurements were unable to detect any trends in shape independent of size. The PCA on wing dimensions (not including wing length) returned only one significant PC axis which accounted for 85% of total variation in wing dimensions. A linear regression of these PC scores on wing length was significant (R^2 = 97%, $p < 0.0001$), thus virtually all variation in wing measurements across populations was due to body size. Similarly, a PCA of leg segment length gave one significant PC axis explaining 92% of total variation, which was also closely linked to body size (R^2 = 85%, p = 0.0001). No systematic variation in leg segmentation independent of body size was detected.

Four of the six morphometric ratios examined were isometric. None of the regressions of leg ratios (BV_1, LR_1 and SV_1) and wing ratio VR_C on wing length were significant: p-values ranged from 0.716 to 0.270, with R^2 ranging from 0.5% to 4% of variation explained by body size. Although leg and wing ratios did not vary systematically with body size, their values overlapped broadly for most barcode groups, as typified by BV_1 (Figure 2). Only antennal ratio showed clear evidence of being allometric (R^2 = 62%, $p < 0.0001$); AR increased in value with body size across populations (Figure 2). Too few individuals were available for each of the barcode clusters to determine whether the same allometric relation existed within each population. Wing ratio VR_{CU} showed a weak but statistically significant relation to size ($p = 0.002$, R^2 = 27%), however this was influenced by one individual with aberrant wing venation, so the regression was not considered strong evidence for allometry (Figure 2).

The ability of thoracic and abdominal setae to distinguish barcode clusters was examined via NMDS ordination; the best solution, based on a Euclidean distance matrix, had three axes. Axes 1 and 2 together explained 88% of variation in the data, and clearly separated *C. (I.) ornatus*, plus C. *(I.)* sp. 3, *C. (I.)* sp. 6 and *C. (I.)* sp. 7 (Figure 3) from the remaining barcode clusters. Correlations of the original setae variables with axis scores (Table 1) indicate that Axis 1 contrasted the numbers of tergal, dorsocentral and scutellar setae vs. the number of acrosticals and the robustness of scutellar and medial tergal setae. The number of sensilla chaetica was also correlated with Axis 1 scores. Thus *C. (I.) ornatus* and *C. (I.)* sp. 3 (nr. *pilitarsis*) exhibited high scores on Axis 1 (Figure 3) because they had more lateral tergal setae, but narrower scutellar and medial tergal setae (ca. 2.5 µm diameter) compared to *Cricotopus (I.) sylvestris* and related barcode clusters (medial setae ca. 5 µm). Axis 2 contrasted of the numbers of lateral setae on tergites 3 and 4, with the robustness of the medial setae on these tergites and the scutellum. The number of sensilla chaetica and prealar setae also contributed to higher scores on Axis 2. Therefore *C. (I.) sylvestris* and related populations are located in the upper left corner of Figure 3.

Pigmentation patterns were summarized by a NMDS ordination, based on a Sørensen distance matrix. The best solution had 6 significant axes but only 2 were retained, due to their effectiveness in summarizing color variation (e.g., final STRESS = 10). When ordination results were associated with barcode clusters three distinct phenotypes were revealed within the largest barcode group, which according to Hirvenoja (1973) corresponded to *C. (I.) sylvestris* and *C. (I.) trifasciatus* small form (Figure 4). Within this largest barcode cluster, a third dark-pigmented Canadian population of *C. (I.) sylvestris* largely intermingled with *C. (I.) ornatus* and three other barcode clusters. *Crictopus (I.) glacialis* formed a tight cluster distinct from *C. (I.) sylvestris*, but which completely overlapped with that of *C. (I.)* sp. 3. Correlations of the original pigment variables with the ordination scores (Table 2) indicated high scores on the first axis reflected the width and form of pigment bands on tergites 1,4 and 7, white gonocoxites and light bands on tarsal segments 2 and 3 of the midleg. Lower scores on Axis 1 reflected darker pigment on the scutellum and location of the pigment band on tergite 2. Given our scoring system, individuals with darker pigment on these leg segments as well as darker pigmentation of the scutellum, and posterior scutum and humeral area (e.g. *C. (I.) ornatus* and *C. (I.) glacialis*) had higher scores on Axis 1 (Figure 4). Axis 2 generally emphasized the brightness of light rings on the legs, especially on the femur, tibiae and first 3 tarsal segments of the mid and hind legs, as well as brightness of the gonocoxites and posterior scutum. The extent of pigmentation of the abdominal tergites was represented by both axes, whereas presence of a medial pigment spot on tergite 4 (typical of *C. (I.) sylvestris*) was uniquely represented by axis 2. In summary, individuals in the upper left corner of Figure 4 (e.g., *C. (I.) trifasciatus*, *C. (I.) tricinctus*) have brightly banded legs, strongly contrasting pigment patterns on the thorax, and a light-colored scutellum. Individuals with low scores on both axes had darker legs with

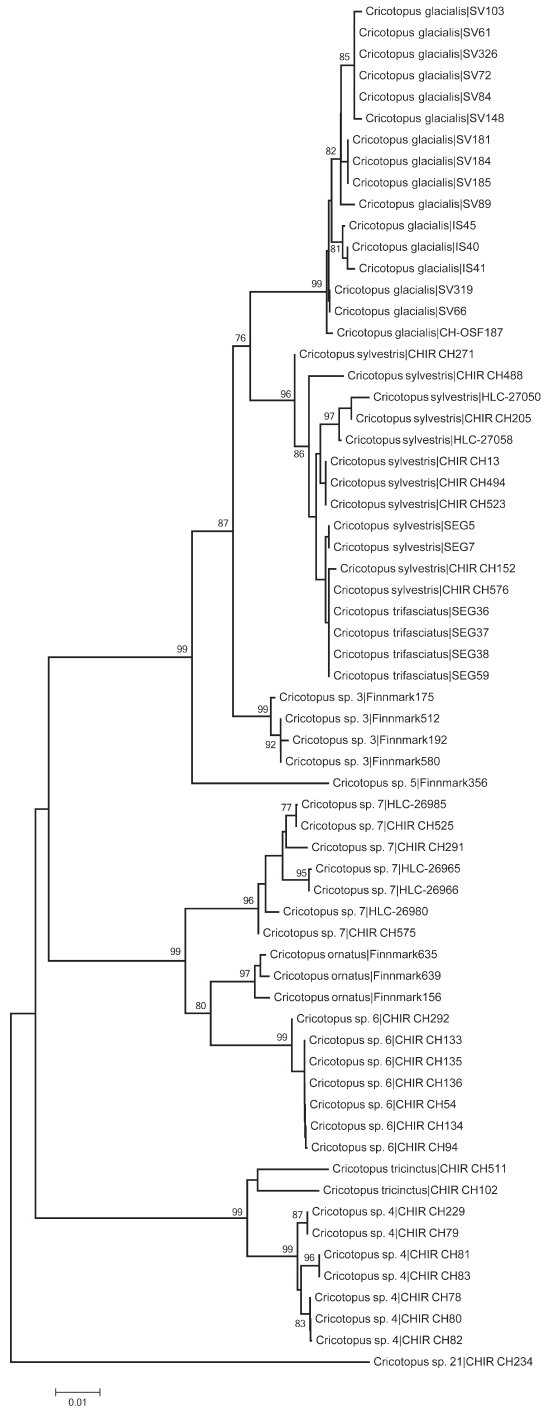

Figure 1. Taxon identification tree: a neighbor-joining tree with bootstrap support based on 1000 random replications. The COI dataset included 658 positions. Distances were computed using the Kimura 2-parameter method. Individual specimen codes indicate location of collection site: "SV"= Svalbard, Norway; "IS" = Iceland; "OSF"= Oslofjord, Norway; "CHIR CH" and "HLC" = Churchill, Manitoba, Canada; "SEG5" - 7 = Nevada, USA; "SEG59"= Minnesota, USA; "SEG36" – 38 = Maryland, USA; "Finnmark"= northern Norway.

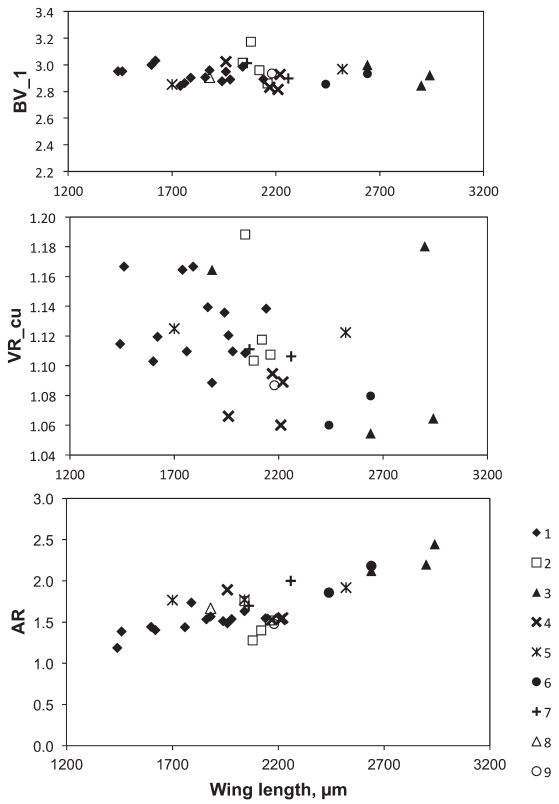

Figure 2. Morphometric ratios for foreleg (BV_1), wing venation (V_{CU}), and antennae (AR) plotted against size (wing length). BV_1 was isometric, but both AR and V_{CU} showed evidence of allometric relations with size.

Table 1. Pearson correlations of the original setae variables with NMDS ordination scores. Correlations (R^2) between the original data and scores in reduced dimensions: axis 1 $R^2 = 0.516$, axis 2 $R^2 = 0.359$, axis 3 $R^2 = 0.113$. Final "Stress" = 4.48.

Axis	1	2	3
sensilla chaetica	***.531***	***.622***	***.523***
dorsocentral setae	***.677***	.108	***.661***
prealar setae	.278	***.496***	.312
scutellar setae	***.549***	-.024	***.393***
acrostichal setae	***-.419***	-.132	***.626***
antepronotal setae	***.397***	-.220	.341
lateral setae-tergite 3	***.823***	***-.708***	***.358***
medial setae-tergite3	***.396***	.213	***.635***
lateral setae-tergite 4	***.801***	***-.713***	.361
medial setae tergite 4	***.464***	-.047	.605
robustness-scutellar	***-.507***	***.604***	-.155
robustness-medial	***-.608***	***.664***	-.189

Table 2. Pearson correlations of the pigmentation variables with NMDS ordination scores. Only variables with correlations greater than 0.300 are listed. Subscripts refer to fore (1), mid (2) or hind (3) leg. "T#" refers to a specific abdominal tergite; "T#a" refers to the location of the anterior edge and "T#p" to the posterior edge of a pigment band. Correlations (R^2) between the original data and scores in reduced dimensions: axis 1 $R^2 = 0.649$, axis 2 $R^2 = 0.298$. "Stress" = 10.75.

Axis	1	2
Contrast of leg rings	.343	***-.411***
$Femur_1$	.328	***-.700***
$Tibia_1$	***.360***	-.325
$Femur_2$	.215	***-.593***
$Tibia_2$	.184	***-.377***
1^{st} $tarsomere_2$	.219	***-.455***
2^{nd} $tarsomere_2$	***.515***	***-.669***
3^{rd} $tarsomere_2$	***.475***	***-.495***
$Femur_3$	.279	***-.593***
$Tibia_3$	.137	***-.401***
1^{st} $tarsomere_3$	.112	***-.385***
2^{nd} $tarsomere_3$	.156	***-.470***
3^{rd} $tarsomere_3$	.198	***.328***
T1p	***.532***	***-.899***
T2a	***-.509***	***.891***
T3a	.088	***.552***
T4a	***.735***	-.147
T4p	***.886***	***-.706***
T4 (pattern)	-.236	-.318
T5a	.268	***.401***
T6p	-.331	.267
T7p	***.387***	-.299
T7	***.350***	-.135
T8p	-.280	***.378***
Gonocoxites	***.476***	***-.441***
Posterior scutum	-.003	***-.430***
Scutellum	***-.532***	-.023

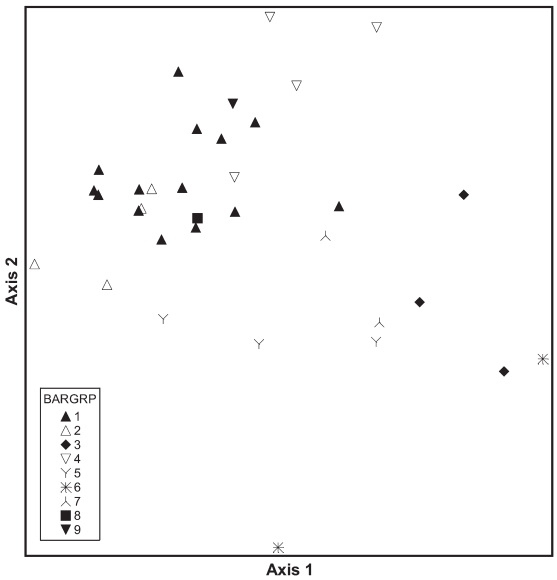

Figure 3. NMDS axis scores for an ordination of counts of thoracic and abdominal seta. Barcode groups as numbered in legend: 1 = *sylvestris* gr. spp.; 2 = *C. (I.) glacialis*; 3 = *C. (I.)* sp. 3; 4 = *C. (I.)* sp. 4; 5 = *C. (I.) sp. 7*; 6 = *C. (I.) ornatus*; 7 = *C. (I.)* sp. 6; 8 = *C. (I.)* sp. 5; 9 = *C. (I.) tricinctus*.

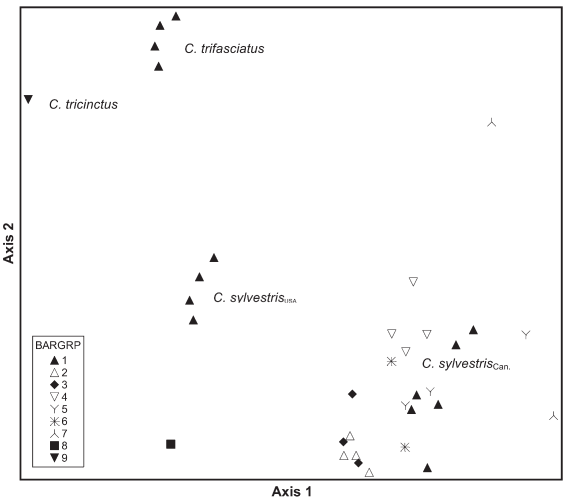

Figure 4. NMDS axis scores for an ordination of pigment pattern on legs, thorax and abdominal tergites. Barcode groups are coded as in Figure 3.

less obvious rings, and more extensive tergal pigment bands. The "typical" form of *C. (I.) sylvestris*, with a triangular or round pigment patch on tergite 4 had intermediate scores on Axis 1.

Although neither setae nor pigmentation alone were sufficient to distinguish all nine barcode clusters within the *C. (I.) sylvestris*-group, these two datasets did provide complimentary information. To further characterize unidentified barcode clusters we sought additional morphological features whose shape was more difficult to quantify: the inferior volsella and the gonostylus (Figure 5). The inferior volsella of *C.* (*I.*) nr. *pilitarsis* is relatively broad and blunt, whereas in *C. (I.) ornatus* it is more slender and parallel-sided. In contrast, the inferior volsellae of *C. (I.) sylvestris* and *C. (I.) trifasciatus* were distinctly triangular. Although the appearance of the gonostylus may be biased by its angle of presentation in slide-mounted specimens, we found consistent differences in shape between populations. Canadian *C. (I.)* sp. 4 had an elongate, "cucumber-shaped" gonostylus, whereas gonostyli of *C. (I.) ornatus* and *C. (I.) sylvestris* were clearly tapered at the base. Gonostyli of *C.*

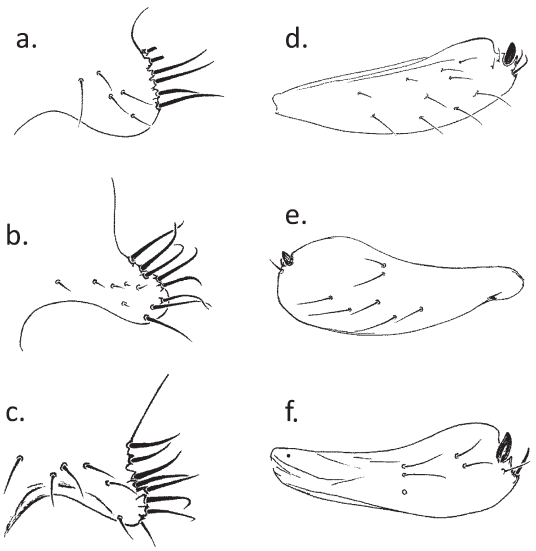

Figure 5. Variation in structure of the male hypopygium. Inferior volsella of: a. *C. (I.) sp. 3*; b. *C. (I.) ornatus*; c. *C. (I.) trifasciatus.* Gonostylus of: d. *C. (I.) sp.* 4; e. *C. (I.) ornatus*; f. *C. (I.) sylvestris.*

(I.) sylvestris and *C. (I.) trifasciatus* were distinctly curved and generally more slender than that of *C. (I.) ornatus*, which was quite broad distally (Figure 5).

DISCUSSION

The extremes observed for size/wing length generally reflected latitude: the largest specimens were collected from Finnmark (latitude 70°N), whereas the smallest specimens were reared during summer in Maryland (latitude 39°N). It was not possible to distinguish latitudinal and seasonal effects on body size, because the majority of specimens were collected between the months of June and August. Antennal ratio significantly increased with size, showing allometric changes in the length of the terminal flagellomere relative to the basal antennal segments. Clear evidence of allometry in both the AR and the BV leg ratio has also been reported in two wide-ranging Australian chironomid species (McKie & Cranston 2005). We found weak evidence for allometry in the wing ratio VR_{CU}, which appeared to decrease with size, but a larger sample size is needed to decide whether or not an outlier does represent the normal range of variation. Morphometric analysis of wing venation in *Drosophila lummei* Hackman, 1972 did find variation in wing shape consistent with genetic differences in geographically distant populations (Haas & Tolley 1998). However, our ordination of wing dimensions failed to show any variation that was not simply due to size, and suggests that wing ratios may not be informative in the *sylvestris*-group. For the *sylvestris*-group species studied, leg ratios and the wing ratio, VR_C, were free of allometric effects, but their values varied widely within and between species, with no evidence of size-independent pattern. Oliver and Dillon (1988) similarly concluded that leg ratios were not useful for distinguishing *Cricotopus* species from the Canadian arctic. Boesel (1983) found both AR and leg ratios highly variable among *Cricotopus* in the eastern United States. Unfortunately it is impossible to generalize about the occurrence of allometry as it appears to affect only certain morphometric ratios, on a species and gender specific basis (Kobayashi 1998; McKie & Cranston 2005). Within the *sylvestris*-group, ratios may have some use in distinguishing particular pairs of species, but should be treated with caution until more thorough sampling of geographical variation of populations has been achieved.

The ordination of setae was particularly effective in separating a group consisting of *C. (I.) ornatus*, *C. (I.)* sp. 3, *C. (I.)* sp. 6 and *C. (I.)* sp. 7 from the remaining populations (Figure 3). The number of lateral tergal setae played an important role as noted by Oliver & Dillon (1988) who found them useful in distinguishing *C. (I.) sylvestris* from *C. (I.) ornatus*. The ordination also emphasized the robustness of scutellar setae and median tergal setae, traits which Hirvenoja (1973) used to separate *C. (I.) ornatus* and *C. (I.) relucens* from the remaining *sylvestris*-group species. The first ordination axis strongly emphasized the number of sensilla chaeticae and dorsocentral setae. Hirvenoja (1973) considered the number of tarsal sensilla chaetica to be very important in separating particular pairs of species, whereas the dorsocentrals merit further comparison across a wider range of populations to confirm their utility.

The ordination of color pattern was most successful in distinguishing populations of *C. (I.) sylvestris*, *C. (I.) glacialis*, *C. (I.) trifasciatus* and *C. (I.) tricinctus*, which commonly pose the greatest difficulty in identification of adults (LeSage & Harrison 1980; Boesel 1983; Oliver & Dillon 1988). Ironically, *C. (I.) glacialis,* from Svalbard, and *C. (I.)* sp. 3, from Finnmark, clustered tightly, but were separated from the "dark-form" *C. (I.) sylvestris* population (from Canada). Oliver and Dillon (1988) concluded that although *C. (I.) glacialis* appeared similar to dark *C. (I.) sylvestris* from the Canadian arctic, *C. (I.) glacialis* was restricted to the Palaearctic. The remaining Canadian barcode clusters plus *C. (I.) ornatus* from Finnmark grouped together in the ordination, reflecting the trend for arctic populations to be more darkly pigmented than their temperate zone relatives (Oliver & Dillon 1988). The analyses of pigmentation and setae thus yielded complementary information for distinguishing barcode clusters.

Given the complex and subtle variation in pigmentation of legs, thorax and abdomen in many *Cricotopus* species, the ability to visualize trends in color patterns is valuable. Numerical quantification of color patterns followed by ordination analysis appears to be a useful tool for comparative studies of *Cricotopus* populations. Methods of coding pigment variables can be modified to capture different types of markings, or more precise banding patterns, as required. However, the next step in phenotypic analysis would be to expand the

dataset to include greater latitudinal and seasonal sampling of populations to capture a wider range of within-species variation in pigmentation. If color patterns are indeed uniquely associated with particular species, the discontinuities observed in the ordination should remain, but if environmental plasticity dominates, one would expect to see continuous gradations of color pattern among barcode clusters.

Our inability to resolve *C. (I.) sylvestris* and *C. (I.) trifasciatus* using COI sequence data suggests that either the analyzed specimens represent one highly plastic species, or two evolutionarily young species that are have not yet had the time to diverge genetically, or two species that have hybridized one or more times resulting in mitochondrial introgression. Cytogenetic evidence indicates that hybridization within the *sylvestris* species-group also contributes to the high degree of variation in the group. Compared to sympatric populations of *C. (I.) ornatus* and *C. (I.) tricinctus*, *C. (I.) sylvestris* had a lower number of chromosomes, which showed a high degree of polymorphism, and were characterized by incomplete pairing of homologues. This evidence suggests that speciation of *C. (I.) sylvestris* involved hybridization and has occurred relatively recently (Michailova 1976, 1980). Mitochondrial genes such as COI, which are maternally inherited, would not detect the effects of hybridization; instead, sequence data from neutral nuclear genes are necessary to test the hypothesis that *C. (I.) sylvestris* and *C. (I.) trifasciatus* constitute a single species.

Finding genetic markers that can accurately distinguish very closely related and evolutionarily young species is a challenge. Barcoding data is widely used in delineation of animal species, and a pairwise sequence divergence of 2% has been found to characterize the great majority of interspecific comparisons across many genera (Hebert et al. 2003). However, COI sequence data were considerably less successful in distinguishing species of Diptera within a dataset composed of a large number of closely related species (Meier et al. 2006). The need for multiple genetic markers is further emphasized by Carew et al. (2011) in their study of a taxonomically difficult complex of *Procladius* Skuse, 1889 species: COI data resolved only half of the six genetically distinct clusters that were detected by Cytb and CAD sequence data. Each of these genes provided unique information for these species. Ekrem et al. (2010) also found the nuclear marker CAD superior to COI in reconstruction of genus-level relations within tribe Tanytarsini.

The difficulty in distinguishing intra- and interspecific variation in *Cricotopus* has been well documented, and is especially great in the *sylvestris*-group (Hirvenoja 1973; Boesel 1983; Oliver & Dillon 1988). The ability to connect phenotypic and genotypic variation should improve our ability to delimit species across a broad geographic range. To this end, we defined a numerical method which was successful in summarizing complex patterns of pigmentation and which facilitated comparison of coloration among individuals and populations. A similar analysis of the distribution of setae complemented the ability of pigmentation data to distinguish populations. Mapping the identity of barcode clusters onto the results of ordinations of phenotypic data showed the distinctiveness of many described species. It also showed various degrees of intergradation of traits between Palaearctic species (e.g., *C. (I.) ornatus*) and similar Nearctic populations. Barcode data was not always able to distinguish some phenotypically and geographically distinct populations, e.g. *C. (I.) sylvestris* and *C. (I.) trifasciatus*. Given the evidence for divergence of geographically distant populations and for hybridization within sympatric species, sequence data from nuclear genes, such as CAD, are also required to define closely related *sylvestris*-group species. Our study of populations across a Palaearctic-Nearctic gradient is an initial step in formally addressing geographic variation of these species, and it underscores the impact of under-sampling (Oliver & Dillon 1988, Meier et al. 2006) on the perceived "gap" (phenotypic or genetic) between species. More comprehensive sampling both within and among species across their geographical ranges is needed to improve our ability to "draw the line" between species with confidence.

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Appendix. List of specimens used in molecular analyses. Associated larval and pupal exuviae are abbreviated "Lex" and "Pex", respectively.

BOLD Sample ID	Species	Identifier	Life stage	Country	Province	Region
CH-OSF187	Cricotopus glacialis	Elisabeth Stur	Male adult	Norway	Oslo	Oslo
IS40	Cricotopus glacialis	Elisabeth Stur	Larva	Iceland		Geysir
IS41	Cricotopus glacialis	Elisabeth Stur	Larva	Iceland		Geysir
IS45	Cricotopus glacialis	Elisabeth Stur	Female adult	Iceland		Geysir
SV103	Cricotopus glacialis	Elisabeth Stur	Male adult	Norway	Svalbard	Adventdalen
SV148	Cricotopus glacialis	Elisabeth Stur	Male adult	Norway	Svalbard	Adventdalen
SV181	Cricotopus glacialis	Elisabeth Stur	Male adult	Norway	Svalbard	Nordenskioldland
SV184	Cricotopus glacialis	Elisabeth Stur	Male adult	Norway	Svalbard	Nordenskioldland
SV185	Cricotopus glacialis	Elisabeth Stur	Male adult	Norway	Svalbard	Nordenskioldland
SV319	Cricotopus glacialis	Elisabeth Stur	Male adult	Norway	Svalbard	Nordenskioldland
SV326	Cricotopus glacialis	Elisabeth Stur	Larva	Norway	Svalbard	Nordenskioldland
SV61	Cricotopus glacialis	Elisabeth Stur	Female adult	Norway	Svalbard	Adventdalen
SV66	Cricotopus glacialis	Elisabeth Stur	Female adult	Norway	Svalbard	Adventdalen
SV72	Cricotopus glacialis	Elisabeth Stur	Male adult	Norway	Svalbard	Adventdalen
SV84	Cricotopus glacialis	Elisabeth Stur	Female adult	Norway	Svalbard	Adventdalen
SV89	Cricotopus glacialis	Elisabeth Stur	Female adult	Norway	Svalbard	Adventdalen
Finnmark156	Cricotopus ornatus	Elisabeth Stur	Male adult	Norway	Finnmark	Vardo
Finnmark635	Cricotopus ornatus	Elisabeth Stur	Male adult	Norway	Finnmark	Sor-Varanger
Finnmark639	Cricotopus ornatus	Elisabeth Stur	Male adult	Norway	Finnmark	Sor-Varanger
Finnmark175	Cricotopus sp. 3	Elisabeth Stur	Male adult	Norway	Finnmark	Kautokeino
Finnmark192	Cricotopus sp. 3	Elisabeth Stur	Male adult	Norway	Finnmark	Kautokeino
Finnmark512	Cricotopus sp. 3	Elisabeth Stur	Male adult	Norway	Finnmark	Kautokeino
Finnmark580	Cricotopus sp. 3	Elisabeth Stur	Male adult	Norway	Finnmark	Lebesby
Finnmark356	Cricotopus sp. 5	Elisabeth Stur	Male adult	Norway	Finnmark	Porsanger
CHIR_CH229	Cricotopus sp. 4	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
CHIR_CH78	Cricotopus sp. 4	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
CHIR_CH79	Cricotopus sp. 4	Elisabeth Stur	Female adult	Canada	Manitoba	Churchill
CHIR_CH80	Cricotopus sp. 4	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
CHIR_CH81	Cricotopus sp. 4	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
CHIR_CH82	Cricotopus sp. 4	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
CHIR_CH83	Cricotopus sp. 4	Elisabeth Stur	Female adult	Canada	Manitoba	Churchill
CHIR_CH133	Cricotopus sp. 6	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
CHIR_CH134	Cricotopus sp. 6	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
CHIR_CH135	Cricotopus sp. 6	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
CHIR_CH136	Cricotopus sp. 6	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
CHIR_CH292	Cricotopus sp. 6	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
CHIR_CH54	Cricotopus sp. 6	Elisabeth Stur	Male pupa, Lex	Canada	Manitoba	Churchill
CHIR_CH94	Cricotopus sp. 6	Elisabeth Stur	Female pupa, Lex	Canada	Manitoba	Churchill
CHIR_CH291	Cricotopus sp. 7	Elisabeth Stur	Larva	Canada	Manitoba	Churchill
CHIR_CH525	Cricotopus sp. 7	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
CHIR_CH575	Cricotopus sp. 7	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
HLC-26965	Cricotopus sp. 7	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill

Locality	Latitude	Longitude	Elev	Collection Date	Collectors
Blankvann, Karussputten	60.013901	10.662700	360	19-Jul-2010	L.O. Hansen & M. Steinert
Stream from hot springs	64.311996	-20.301001	123	01-Oct-2006	T. Ekrem & E. Stur
Stream from hot springs	64.311996	-20.301001	123	01-Oct-2006	T. Ekrem & E. Stur
Stream from hot springs	64.311996	-20.301001	123	01-Oct-2006	T. Ekrem & E. Stur
at Gruvedalen	78.224998	15.650000	50	11-Jul-2005	M. Skjøstad
at Todalen	78.160004	15.830000	100	07-Jul-2005	M. Skjøstad
Griegfjella, Griegbekken	78.009003	13.662000	20	07-Aug-2008	T. Ekrem & K. Hårsaker
Griegfjella, Griegbekken	78.009003	13.662000	20	07-Aug-2008	T. Ekrem & K. Hårsaker
Griegfjella, Griegbekken	78.009003	13.662000	20	07-Aug-2008	T. Ekrem & K. Hårsaker
Griegfjella, Griegbekken	78.009003	13.662000	20	07-Aug-2008	T. Ekrem & K. Hårsaker
Griegfjella, Griegbekken	78.009003	13.662000	20	07-Aug-2008	T. Ekrem & K. Hårsaker
at Gruvedalen	78.224998	15.650000	50	07-Jul-2005	M. Skjøstad
at Gruvedalen	78.224998	15.650000	50	07-Jul-2005	M. Skjøstad
at Gruvedalen	78.224998	15.650000	50	07-Jul-2005	M. Skjøstad
at Gruvedalen	78.224998	15.650000	50	09-Jul-2005	M. Skjøstad
at Gruvedalen	78.224998	15.650000	50	09-Jul-2005	M. Skjøstad
Indre Kiberg, rockpools	70.269402	30.945400	3	29-Jul-2010	T. Ekrem
Grense Jakobselv, rockpools	69.791603	30.795799	7	01-Aug-2010	T. Ekrem
Grense Jakobselv, rockpools	69.791603	30.795799	7	01-Aug-2010	T. Ekrem
Kautokeinoelva, near Masi	69.448196	23.757601	275	24-Jul-2010	T. Ekrem
Kautokeinoelva, near Masi	69.448196	23.757601	275	31-Aug-2010	A. Anderson
Lahpoluoppal, at Nahpoljohka river	69.210297	23.761999	320	16-Aug-2010	T. Ekrem & E. Stur
near Njallavarri, at lake	70.452103	27.010099	62	28-Jul-2010	T. Ekrem
near Gaggavann, at fen	69.823700	25.200899	106	16-Jun-2010	T. Ekrem & E. Stur
23 km E Churchill, Ramsay Creek	58.730999	-93.779999	13	15-Aug-2006	T.Ekrem & E.Stur
22 km E Churchill, CNSC, pond at road	58.737000	-93.819000	11	12-Aug-2006	E.Stur & T.Ekrem
22 km E Churchill, CNSC, pond at road	58.737000	-93.819000	11	12-Aug-2006	E.Stur & T.Ekrem
22 km E Churchill, CNSC, pond at road	58.737000	-93.819000	11	12-Aug-2006	E.Stur & T.Ekrem
22 km E Churchill, CNSC, pond at road	58.737000	-93.819000	11	12-Aug-2006	E.Stur & T.Ekrem
22 km E Churchill, CNSC, pond at road	58.737000	-93.819000	11	12-Aug-2006	E.Stur & T.Ekrem
22 km E Churchill, CNSC, pond at road	58.737000	-93.819000	11	12-Aug-2006	E.Stur & T.Ekrem
16 km E Churchill, Bird Cove, Rock Bluff B	58.771999	-93.843002	5	11-Aug-2006	E.Stur & T.Ekrem
16 km E Churchill, Bird Cove, Rock Bluff B	58.771999	-93.843002	5	11-Aug-2006	E.Stur & T.Ekrem
16 km E Churchill, Bird Cove, Rock Bluff B	58.771999	-93.843002	5	11-Aug-2006	E.Stur & T.Ekrem
16 km E Churchill, Bird Cove, Rock Bluff B	58.771999	-93.843002	5	11-Aug-2006	E.Stur & T.Ekrem
16 km E Churchill, Bird Cove, Rock Bluff C	58.765999	-93.867996	5	16-Jul-2007	T.Ekrem & E.Stur
16 km E Churchill, Bird Cove, Rock Bluff C	58.765999	-93.867996	3	12-Aug-2006	T.Ekrem & E.Stur
16 km E Churchill, Bird Cove, Rock Bluff C	58.765999	-93.867996	3	12-Aug-2006	T.Ekrem
16 km E Churchill, Bird Cove, Rock Bluff C	58.765999	-93.867996	5	16-Jul-2007	T.Ekrem & E.Stur
16 km E Churchill, Bird Cove, Rock Bluff B	58.771000	-93.852997	3	22-Jul-2007	E.Stur
2 km NW Churchill, Churchill Harbour	58.778999	-94.195000	2	25-Jul-2007	P.D.N. Hebert
Town of Churchill, 111 Hearne St., backyard	58.769001	-94.160004		11-Aug-2007	J.Lankshear & J.McGowan

Continued on next page.

Appendix. Continued.

BOLD Sample ID	Species	Identifier	Life stage	Country	Province	Region
HLC-26966	Cricotopus sp. 7	Elisabeth Stur	Female adult	Canada	Manitoba	Churchill
HLC-26980	Cricotopus sp. 7	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
HLC-26985	Cricotopus sp. 7	Elisabeth Stur	Female adult	Canada	Manitoba	Churchill
CHIR_CH234	Cricotopus sp. 21	Susan E. Gresens	Male adult	Canada	Manitoba	Churchill
CHIR_CH13	Cricotopus sylvestris	Susan E. Gresens	Male adult	Canada	Manitoba	Churchill
CHIR_CH152	Cricotopus sylvestris	Susan E. Gresens	Male adult	Canada	Manitoba	Churchill
CHIR_CH205	Cricotopus sylvestris	Susan E. Gresens	Male adult	Canada	Manitoba	Churchill
CHIR_CH271	Cricotopus sylvestris	Susan E. Gresens	Male adult	Canada	Manitoba	Churchill
CHIR_CH488	Cricotopus sylvestris	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
CHIR_CH494	Cricotopus sylvestris	Susan E. Gresens	Male adult	Canada	Manitoba	Churchill
CHIR_CH523	Cricotopus sylvestris	Susan E. Gresens	Male adult	Canada	Manitoba	Churchill
CHIR_CH576	Cricotopus sylvestris	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
HLC-27050	Cricotopus sylvestris	Susan E. Gresens	Male adult	Canada	Manitoba	Churchill
HLC-27058	Cricotopus sylvestris	Elisabeth Stur	Male adult	Canada	Manitoba	Churchill
SEG36	Cricotopus trifasciatus	Susan E. Gresens	Male adult	United States	Maryland	Baltimore Co.
SEG37	Cricotopus trifasciatus	Susan E. Gresens	Male adult	United States	Maryland	Baltimore Co.
SEG38	Cricotopus trifasciatus	Susan E. Gresens	Male adult, Pex	United States	Maryland	Baltimore Co.
SEG5	Cricotopus sylvestris	Susan E. Gresens	Male adult	United States	Nevada	Clark County
SEG59	Cricotopus trifasciatus	Susan E. Gresens	Male adult, Pex, Lex	United States	Minnesota	Ramsey Co.
SEG7	Cricotopus sylvestris	Susan E. Gresens	Male adult, Pex	United States	Nevada	Clark County
CHIR_CH102	Cricotopus tricinctus	Susan E. Gresens	Female adult	Canada	Manitoba	Churchill
CHIR_CH511	Cricotopus tricinctus	Susan E. Gresens	Male adult	Canada	Manitoba	Churchill

Locality	Latitude	Longitude	Elev	Collection Date	Collectors
Town of Churchill, 111 Hearne St., backyard	58.769001	-94.160004		11-Aug-2007	J.Lankshear & J.McGowan
Town of Churchill, 111 Hearne St., backyard	58.769001	-94.160004		11-Aug-2007	J.Lankshear & J.McGowan
Town of Churchill, 111 Hearne St., backyard	58.769001	-94.160004		11-Aug-2007	J.Lankshear & J.McGowan
23 km E Churchill, Ramsay Creek	58.730999	-93.779999	13	15-Aug-2006	T.Ekrem & E.Stur
23 km E Churchill, Ramsay Creek	58.730999	-93.779999	13	15-Aug-2006	T.Ekrem & E.Stur
26 km SE Churchill, Twin Lakes burn site	58.618000	-93.806999	33	14-Aug-2006	T.Ekrem & E.Stur
23 km E Churchill, Ramsay Creek	58.730999	-93.779999	13	15-Aug-2006	T.Ekrem & E.Stur
23 km E Churchill, Ramsay Creek	58.730999	-93.779999	13	15-Aug-2006	T.Ekrem & E.Stur
Town of Churchill, 111 Hearne St., backyard	58.769001	-94.160004	8	21-Jul-2007	J.McGowan
2 km NW Churchill, Cape Merry	58.786999	-94.197998	3	25-Jul-2007	E.Stur & T.Ekrem
16 km E Churchill, Bird Cove, Rock Bluff B	58.771000	-93.852997	3	22-Jul-2007	E.Stur
2 km NW Churchill, Churchill Harbour	58.778999	-94.195000	2	25-Jul-2007	P.D.N. Hebert
13 km E Churchill, Eastern Creek	58.754002	-93.948997		20-Aug-2006	T.Ekrem
13 km E Churchill, Eastern Creek	58.754002	-93.948997		20-Aug-2006	T.Ekrem
Towson University, Lily Pond	39.395000	-76.605003	127	22-Jul-2010	Susan Gresens
Towson University, Lily Pond	39.395000	-76.605003	127	22-Jul-2010	Susan Gresens
Towson University, Lily Pond	39.395000	-76.605003	127	22-Jul-2010	Susan Gresens
Lake Meade, marina	36.029999	-114.776001	366	20-Mar-2010	Mark Wolfire
Shoreland, Lake Owasso	45.029999	-93.129997	270	20-Oct-2010	Susan Gresens
Lake Meade, marina	36.029999	-114.776001	367	20-Mar-2011	Mark Wolfire
26 km SE Churchill, Twin Lakes fen	58.632000	-93.786003	22	15-Aug-2006	E.Stur & T.Ekrem
11 km S Churchill, Churchill River weir	58.675999	-94.167999	1	25-Jul-2007	T.Ekrem