A "complex" problem: delimiting sibling species boundaries in black flies (Diptera: Simuliidae)

Ida M. Conflitti, Gerald F. Shields, Douglas C. Currie[1]

Abstract—Rapid and recent lineage radiations pose challenges to systematists. Using members of the highly diverse *Simulium arcticum* Malloch complex, we tested whether the cytochrome *c* oxidase subunit I (COI) barcoding gene can differentiate black fly sibling species. Members of the *S. arcticum* complex were monophyletic in relation to two morphospecies and two cryptic species of the *Simulium malyschevi* Dorogostaisky *et al.* and *Simulium noelleri* Friederichs species-groups, respectively. Of five *S. arcticum* sibling species analysed, only *Simulium negativum* Adler *et al.* was monophyletic. No other members of the complex could be distinguished using COI barcodes. The inability to resolve *S. arcticum* sibling species resulted because (1) haplotypes were shared between species and (2) the distribution of interspecific genetic distances completely overlapped the range of variation within species. Potential sources of incongruence between barcode data and species boundaries include imperfect taxonomy, inadequate genetic information, incomplete lineage sorting, and/or introgressive hybridization. We ruled out imperfect taxonomy because chromosomal, ecological, and distributional evidence support the validity of *S. arcticum* sibling species. Therefore, current nomenclature should be maintained pending further study. We conclude that one or more of the latter three sources of incongruence could be responsible for the lack of reciprocal monophyly among species of the *S. arcticum* complex.

Résumé—Les radiations rapides et récentes des lignées évolutives posent des défis aux systématiciens. En utilisant des membres du très divers complexe d'espèces *Simulium arcticum* Malloch, nous avons testé si le gène code-barre de la sous-unité cytochrome *c* oxydase I (COI) peut différencier entre espèces jumelles de mouches noires. Les membres du complexe *S. arcticum* sont monophylétiques par rapport à deux espèces morphologiques du groupe *Simulium malyschevi* Dorogostaisky *et al.* et deux espèces cryptiques de *Simulium noelleri* Friederichs. Parmi les cinq espèces analysées au sein du complexe *S. arcticum*, seul *Simulium negativum* Adler *et al.* est monophylétique. Aucun des autres membres du complexe ne peut être distingué par le code-barre COI. L'incapacité de différencier entre espèces jumelles du complexe *S. arcticum* provient (1) d'un partage d'haplotypes entre espèces et (2) du chevauchement entre les distributions des distances génétiques inter- et intraspécifiques. Les sources potentielles d'incongruité entre les données des codes-barres et la circonscription des espèces peuvent provenir d'une taxonomie imparfaite, d'une insuffisance d'information génétique, d'une séparation incomplète des lignées, et/ou d'introgression par hybridation. Nous écartons la possibilité d'une taxonomie imparfaite parce que des preuves chromosomiques, écologiques et géographiques soutiennent la validité des espèces reconnues au sein du complexe *S. arcticum.* Par conséquent, la nomenclature actuelle doit être maintenue en attendant une étude plus approfondie. Nous concluons qu'une ou plusieurs des trois autres sources d'incongruité pourrait être responsable de l'absence de monophylie réciproque entre espèces du complexe *S. arcticum.*

Introduction

Accurate species diagnosis is central to biological research. Delimiting species based on morphological criteria is often complicated by phenotypic plasticity, genetic variability, cryptic taxa, and peculiarities of different life stages or genders (Hebert *et al*. 2003a, 2003b). To overcome these challenges, Hebert *et al*. (2003a, 2003b) proposed a standardized genetic identification system for animal species. DNA barcoding uses sequence diversity in the mitochondrial gene,

Received 25 January 2011. Accepted 26 April 2011.

I.M. Conflitti, D.C. Currie,[1] Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario M5S 3B2, Canada; and Department of Natural History, Royal Ontario Museum, Toronto, Ontario M5S 2C6, Canada
G.F. Shields, Department of Natural Sciences, Carroll College, Helena, Montana 59601, United States of America

[1]Corresponding author. Department of Natural History, Royal Ontario Museum, 100 Queen's Park, Toronto, Ontario M5S 2C6, Canada (e-mail: dc.currie@utoronto.ca).
doi:10.4039/tce.2012.29

 © 2012 Entomological Society of Canada

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press

cytochrome *c* oxidase subunit I (COI), to create genetic profiles for taxonomically verified specimens. Reference libraries of barcode sequences serve as species identifiers for individuals of unknown origin. For this system to succeed at discriminating taxa, COI divergence within species cannot exceed genetic distance between species (Hebert *et al*. 2003a, 2003b). By focusing on rapidly evolving mitochondrial DNA (mtDNA) in general (and COI in particular) the diversity requirement is typically met for closely allied species, provided the taxa in question have persisted for a sufficient period of time (Hebert *et al*. 2003a, 2003b). Hence, DNA barcoding has demonstrated success in several animal groups (*e.g*., spiders, Barrett and Hebert 2005; mayflies, Ball *et al*. 2005; beetles, Monaghan *et al*. 2005; fish, Ward *et al*. 2005; amphibians, Smith *et al*. 2008; birds, Kerr *et al*. 2007; among others). However, when reproductive isolation between species is recent, taxonomic diagnosis based on molecular criteria becomes more difficult; factors such as inadequate genetic signal, incomplete lineage sorting, and introgressive hybridization tend to obscure species boundaries (Funk and Omland 2003). Consequently, DNA barcoding may be less informative in cases of recent lineage radiations.

Black flies (Diptera: Simuliidae) provide an opportunity to test COI's ability to distinguish recently diverged species. In general, simuliids are taxonomically difficult due to their small size and structural homogeneity. Morphologically indistinguishable sibling species are also common throughout the family; cytological analyses of larval polytene chromosomes often reveal the presence of chromosomally distinct sibling species within morphologically defined nominal species. When this is the case, band-by-band examination of chromosomes provides the only means to accurately identify species. As such, chromosomal rearrangements have become accepted taxonomic and phylogenetic indicators in black flies (Rothfels 1979, 1981, 1989; Adler *et al*. 2004). Various lines of evidence suggest sibling species complexes are the product of recent chromosome-based speciation (Rothfels 1989).

Few studies have attempted to diagnose black fly sibling species using COI barcodes. Some efforts were met with limited success. For example, using the barcoding fragment, Ilmonen *et al*. (2009) distinguished *Simulium juxtacrenobium* Bass and Brockhouse, a chromosomally and morphologically distinct species, from *Simulium crenobium* (Knoz) and *Simulium vernum s.s* Macquart. However, *S. crenobium* and *S. vernum s.s.* did not have distinct COI profiles, even though species status was confirmed based on breeding habitat and chromosomal data (Ilmonen *et al*. 2009). Likewise, a 920 base-pair (bp) region of the COI gene could not discriminate between *Simulium dussertorum* Craig and *Simulium oviceps* Edwards, two morphologically similar members of the *S. oviceps* group (Joy *et al*. 2007). These taxa belong to a large radiation of simuliids within the Society Islands, French Polynesia (Joy *et al*. 2007).

Other barcoding studies of black fly sibling species have proven fruitful. In Britain, *Simulium reptans* (L.) consists of two morphologically distinct larval types that lack identifiable variation as adults and pupae. Whether these entities are distinct or variants of a single species has been debated (reviewed in Day *et al*. 2008). DNA barcoding delineated the larval morphotypes into two highly supported monophyletic clades. Moreover, genetic distance was 10-fold greater between, than within each cluster. Because this level of genetic differentiation is consistent with that found within and between species in other taxa, as well as with that established by the DNA barcoding consortium for delimiting species (Hebert *et al*. 2004), a re-evaluation of the status of *S. reptans* variants was proposed (Day *et al*. 2008). Interestingly, COI barcodes have also been used to successfully distinguish geographically localized populations of single black fly species (*Prosimulium neomacropyga* Peterson in the Rocky Mountains, United States [Finn *et al*. 2006]; *Simulium tani* Takaoka and Davies in Thailand [Pramual *et al*. 2005]).

Finally, in a comprehensive study, Rivera and Currie (2009) showed that barcoding was not only effective in discriminating among recognized Nearctic black fly species, but also confirmed cryptic diversity, as previously revealed by cytological analyses. These authors evaluated 58 morphologically distinct species, as well as five known and three suspected sibling species complexes (known complexes analysed: *Helodon onychodactylus* Dyar and Shannon complex, *Stegopterna mutata* (Malloch) complex, *Simulium arcticum* Malloch complex, *Simulium tuberosum* (Lundstrom) complex, and *Simulium venustum* Say

© 2012 Entomological Society of Canada

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press

complex; suspected complexes analysed: *Simulium craigi* Adler and Currie, *Simulium quebecense* Twinn, and *Simulium murmanum* Enderlein). In virtually all cases, genetic distances within morphospecies were comparatively lower than that found within-species complexes, indicating the presence of cryptic diversity. Moreover, the neighbour-joining (NJ) tree depicted deep divergences within complexes, suggesting that more than one species was sampled. Given that individuals belonging to species complexes were not cytologically analysed, Rivera and Currie (2009) could not resolve whether divergent clades within complexes corresponded to cytologically recognized sibling species. Therefore, the performance of DNA barcoding for accurately identifying black fly sibling species remains unclear.

Here, we build on Rivera and Currie (2009) by examining the extent to which barcoding captures species boundaries and allows identification of black fly sibling species. We focus on the *S. arcticum* complex – the second largest sibling species assemblage known for black flies (Adler and Crosskey 2010) and the most diverse Nearctic complex studied by Rivera and Currie (2009). The *S. arcticum* complex contains nine reproductively isolated sibling species (*i.e.*, "cytospecies") and an additional 21 chromosomally distinct types that may or may not represent valid species (*i.e.*, "cytotypes" Adler *et al.* 2004; Shields *et al.* 2007a, 2007b, 2009, unpublished data; Adler and Crosskey 2010).

These taxa are distributed throughout the Nearctic region, primarily in the Western Cordillera, from Alaska to Arizona (Adler *et al.* 2004). Although the *S. arcticum* complex occurs widely in western North America, a large portion of its biodiversity is concentrated in the Rocky Mountains of Montana, United States (Adler *et al.* 2004; Shields *et al.* 2007a, 2007b, 2009). Within this geographic region, larvae of at least four sibling species and 14 cytotypes are known to coexist (Adler *et al.* 2004; Shields *et al.* 2007a, 2007b, 2009, unpublished data; Adler and Crosskey 2010).

The following four lines of evidence collectively demonstrate that *S. arcticum* taxa are the product of a rapid and recent lineage radiation: (1) lack of morphological differentiation among members of the complex, (2) the nature of the chromosome rearrangements defining *S. arcticum* cytospecies and cytotypes (reviewed in Rothfels 1989), (3) recent phylogenetic analyses (Conflitti *et al.* 2010), and (4) high taxonomic diversity concentrated in Montana, United States. Given that Montana is generally not considered a biodiversity hotspot, one can speculate that *S. arcticum* populations in the Rocky Mountains have experienced rapid chromosome-based speciation as a result of recent glacial cycles.

We previously investigated phylogenetic relationships among 26 *S. arcticum* individuals representing four species (*S. apricarium* Adler *et al.*, *S. arcticum s.s.*, *Simulium negativum* Adler *et al.*, and *Simulium saxosum* Adler *et al.*) and two cytotypes (IIL-10 and IIL-73,74) (Conflitti *et al.* 2010). Maximum parsimony and Bayesian analyses based on 12S, COII, *cyt b*, and ITS-1 gene sequences failed to resolve species-level monophyly of any taxon except *S. negativum*.

In that preliminary study, larvae were not cytologically analysed. Instead, individuals were sampled from presumed taxon-pure localities in Montana and Alaska, United States. Therefore, it is possible that non-monophyly resulted from imperfect taxonomy, because collection sites may not have remained pure at the time of sampling. This general approach was taken to avoid challenges presented by molecular studies of structurally homogeneous taxa distinguished only via chromosome banding pattern analysis. Specifically, genetic studies require ethanol-fixed larvae, while cytogenetic identifications can only be made using individuals preserved in Carnoy's solution, a fixative known to degrade DNA (Post *et al.* 1993; Koch *et al.* 1998). Although Pramual *et al.* (2005) recently reported success in obtaining genetic data from Carnoy's fixed material, our concerns regarding efficient DNA extraction and amplification, as well as replicability of results, precluded the use of their methods in our preliminary study (Conflitti *et al.* 2010).

To eliminate erroneous taxonomy as the cause of non-monophyly, we performed a second analysis based on cytologically verified taxa collected from Montana and adjacent states (Conflitti *et al.* 2010). We used the COI barcoding fragment along with COII gene sequences to investigate phylogenetic relationships among 42 individuals representing 13 taxa within the *S. arcticum* species complex. Again, individuals did not form monophyletic lineages that matched their cytotaxonomic designations. Overall, we could not confidently reject

© 2012 Entomological Society of Canada

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press

inadequate phylogenetic information, incomplete lineage sorting, and/or introgressive hybridization as the cause of non-monophyly of *S. arcticum* taxa (Conflitti *et al*. 2010).

As in Conflitti *et al*. (2010), our study focused on cytologically verified *S. arcticum* individuals from Montana and surrounding regions. However, only formally described (*i.e*., nominal) sibling species were included in our analysis. Monophyly of these taxa may have been obscured in previous studies by the inclusion of potentially non-reproductively isolated forms (*i.e*., cytotypes). We also extended our analysis by including more individuals per species sampled over a broader geographic range. This ensured that molecular diversity within each species was adequately represented. As products of a recent lineage radiation, members of the *S. arcticum* complex provide a challenging test of the efficacy of DNA barcoding in resolving species boundaries.

Materials and methods

Taxon sampling and chromosome analysis

We sampled 70 individuals representing five described species within the *S. arcticum* complex (Table 1). Where possible, we included multiple individuals per species (range = 2–30, mean = 14) from various localities in Montana and surrounding states (Idaho and Washington). Sequence data for *S. negativum* was extracted from GenBank. All remaining *S. arcticum* individuals were newly collected, identified, and sequenced for our study. Collected larvae were identified to the *S. arcticum* complex according to Currie (1986). Heads and thoraces were removed and retained in 95% ethanol for molecular analysis. Abdomens were preserved in Carnoy's fixative (1 part glacial acetic acid, 3 parts 95% ethyl alcohol) for cytogenetic identification; fixative was changed until it remained clear. Then, abdomens were stained with Feulgen (Rothfels and Dunbar 1953) to enable visualization of polytene chromosomes and gonads. Chromosome maps for the *S. arcticum* species complex (Shields and Procunier 1982; Adler *et al*. 2004) were used to identify larvae to chromosome type. Although the *S. arcticum* complex was the focus of this study, we included exemplars of two closely related, morphologically and chromosomally distinct species (*Simulium decimatum* Dorogostaisky *et al.* and *S. murmanum*) for comparison. *Simulium decorum* Walker and *Simulium noelleri* Friederichs were chosen as the outgroup based on previous morphological and molecular evidence (Adler *et al*. 2004; Rivera and Currie 2009). Sequences for *S. decimatum* and *S. murmanum*, as well as outgroup taxa, were extracted from GenBank (Table 1).

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from larval heads and thoraces using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, Missouri). Standard DNA barcoding primers (LCO1490 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HCO2198 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′; developed by Folmer *et al*. 1994) were used to target a 658 bp region of the COI gene. Each 25 μL polymerase chain reaction (PCR) consisted of 2.5 μL Fisher brand buffer (Fisher Scientific, Waltham, Massachusetts), 1.5 μL MgCl, 0.8 μL dNTP, 1 μL of each primer, 0.1 μL Fisher brand *Taq* DNA polymerase (Fisher Scientific), and 1 μL template DNA. PCR cycling parameters were as follows: 1 min at 96°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C; and finally 7 min at 72°C. Amplification products were visualized on 1.5% agarose gels stained with ethidium bromide. Ten microlitres sequencing reactions contained 2 μL 5× sequencing buffer (Applied Biosystems, Carlsbad, California), 1 μL either forward or reverse primer, 0.5 μL BigDye Terminator (Applied Biosystems), and either 2 or 4 μL purified DNA. Products were bi-directionally sequenced on an ABI 3730 DNA Analyser (Applied Biosystems). Table 1 contains GenBank accession numbers for each sequence obtained.

Data analysis

COI sequences were aligned manually using BioEdit Sequence Alignment Editor v7.0.9.0 (Hall 1999). The majority of sequences were 610 bp in length; longer barcodes were trimmed to this position. The two *S. negativum* sequences acquired from GenBank were only 587 bp in length. To fit the start position of our dataset, five bases were trimmed from the 5′-region of these sequences. After trimming, 28 bases (identified as "N") were added to the 3′-end of the sequences and treated as missing data in all

© 2012 Entomological Society of Canada

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press

Table 1. List of *Simulium* taxon samples with corresponding collection information and GenBank accession numbers.

Species	Collection locality (abbreviation)	No. of specimens	Collection date	GenBank no.	Reference
Outgroup					
Simulium decorum	Manitoba, Duck Mountain Provincial Park vicinity (MB-DM)	1	05-15-2005	FJ524677	Rivera and Currie (2009)
	Manitoba, Neepawa vicinity (MB-NP)	1	05-14-2005	FJ524678	Rivera and Currie (2009)
	Manitoba, Trembowla vicinity (MB-TM)	1	05-16-2005	FJ524676	Rivera and Currie (2009)
	Ontario, Algonquin Provincial Park vicinity (ON-AQ)	5	08-19-2005	FJ524670–FJ524674	Rivera and Currie (2009)
	Wyoming, Townsend Creek (WY-TS)	1	07-03-2006	FJ524675	Rivera and Currie (2009)
Simulium noelleri	Nunavut, Airplane Lake vicinity (NU-AP)	2	07-15-2003	FJ524746; FJ524748	Rivera and Currie (2009)
	Nunavut, Arviat vicinity (NU-AV)	5	07-09-2003	FJ524740; FJ524742; FJ524744; FJ524745; FJ524747	Rivera and Currie (2009)
	Nunavut, Rankin Inlet vicinity (NU-RI)	6	07-18-2003; 07-20-2003; 07-21-2003	FJ524739; FJ524741; FJ524743; FJ524749–FJ524751	Rivera and Currie (2009)
Simulium malyschevi species group					
Simulium decimatum	Nunavut, Arviat vicinity (NU-AV)	4	07-09-2003; 07-10-2003	FJ524663; FJ524664; FJ524666; FJ524667	Rivera and Currie (2009)
	Nunavut, McConnell River (NU-MC)	3	07-11-2003	FJ524665; FJ524668; FJ524669	Rivera and Currie (2009)
Simulium murmanum	Manitoba, Duck Mountain Provincial Park vicinity (MB-DM)	2	05-15-2005; 05-16-2005	FJ524733; FJ524734	Rivera and Currie (2009)
	Ontario, Algonquin Provincial Park vicinity (ON-AQ)	1	08-16-2005	FJ524735	Rivera and Currie (2009)
Simulium arcticum species complex					
Simulium apricarium	Montana, Little Prickly Pear Creek (MT-LP)	14	04-06-2009; 04-15-2009; 03-28-2010	JF815152–JF815165	

© 2012 Entomological Society of Canada

Table 1. *Continued*

Species	Collection locality (abbreviation)	No. of specimens	Collection date	GenBank no.	Reference
Simulium arcticum sensu stricto	Idaho, Coeur d'Alene River (ID-CA)	7	04-03-2009; 03-11-2010; 03-13-2010	JF815105–JF815111	
	Montana, Clearwater River (MT-CW)	16	05-01-2009	JF815128–JF815143	
	Montana, Little Prickly Pear Creek (MT-LP)	7	03-28-2009; 04-15-2009	JF815166–JF815172	
Simulium brevicercum	Montana, Little Blackfoot River (MT-LB)	8	04-18-2009	JF815144–JF815151	
Simulium negativum	Montana, Blackfoot River (MT-BF)	2	06-09-2009	HM210497; HM210498	Conflitti *et al.* (2010)
Simulium saxosum	Idaho, Coeur d'Alene River (ID-CA)	6	03-11-2010; 03-13-2010	JF815112–JF815117	
	Washington, Cle Elum River (WA-CE)	10	03-16-2009	JF815118–JF815127	

analyses. Note that, because no variable regions were lost, sequence trimming had no effect on subsequent analyses. Genetic variation was assessed using PAUP* v4.0b10 (Swofford 2003). Pair-wise sequence divergence within and between taxa was calculated using the Kimura 2-parameter (K2P) model. This model provides the best estimate of divergence when genetic distances are low, as in recently diverged taxa (Nei and Kumar 2000). A NJ tree of K2P distances was created to provide a graphic representation of species divergence. Clade support was estimated using 10,000 bootstrap replicates (faststep search; Felsenstein 1985). Summary statistics of intraspecific and interspecific distances (*i.e.*, minimum, maximum, and mean), as well as histograms were produced using Microsoft Excel 2007.

Many COI sequences differed by few substitutions. In some cases, sequences were shared among species. As such, a network representation of genetic distances might help to determine relationships. A statistical parsimony network (Templeton *et al*. 1992) was constructed using TCS v1.21 (Clement *et al*. 2000) to investigate haplotype relationships within the *S. arcticum* species complex.

Conventional barcoding terminology was used to discuss species status. Species were defined based on two main criteria: (1) reciprocal monophyly among COI barcodes and (2) interspecific divergence values greater than a standard threshold of 10× the mean intraspecific variation for the group under study (Hebert *et al*. 2004). Applying a strict threshold as the basis for defining species may not be appropriate for certain taxa. In particular, this approach tends to overlook species with short evolutionary histories (Hebert *et al*. 2004), such as members of the *S. arcticum* complex. Therefore, we used the threshold value simply as a tool for making comparisons, as well as for testing barcoding's ability to differentiate black fly sibling species.

Results

We obtained 68 new barcode sequences, 610 bp in length, for four nominal species within the *S. arcticum* species complex. Variability within the complex was calculated based on five sibling species; sequences for the fifth species, *S. negativum*, were acquired from a previous study (Table 1). No insertions, deletions, or stop

© 2012 Entomological Society of Canada

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press

codons were found indicating that all sequences constituted functional mitochondrial products (Funk and Omland 2003). Consistent with results from other insect studies, our mtDNA dataset showed strong A + T bias in base composition (A: 27.0%, T: 37.1%, G: 16.9%, C: 19.0%; A + T =64.1%; Clary and Wolstenholme 1985; Nardi *et al*. 2001).

Figure 1 presents the NJ tree based on K2P distances, along with bootstrap support (BSS). Significant support was obtained for monophyly of the *S. arcticum* complex (BSS = 97%). However, within the complex only *S. negativum* was monophyletic (BSS = 100%). No other sibling species could be distinguished using the barcoding gene. The *S. arcticum* specimens analysed, excluding *S. negativum*, cluster into two weakly supported groups, with each species and locality represented at least once in both subclades. The majority of nodes associated with the *S. arcticum* complex lack BSS. Morphologically and chromosomally distinct species (*S. decimatum* and *S. murmanum*), as well as outgroup taxa, were monophyletic and highly supported (BSS = 100% in all cases).

The results for the *S. arcticum* complex are not surprising given that identical sequences were shared within, as well as between, species. The haplotype network illustrates this point (Fig. 2). Fifty-seven COI haplotypes were found among the 70 *S. arcticum* individuals examined. These are clustered into two subgroups that reflect the division between *S. negativum* and the remaining members of the complex, as displayed in the NJ tree. Most barcode haplotypes were present only in a single individual. Although four haplotypes were shared by individuals within species, three were shared between species. Cases of interspecific haplotype sharing included the following sibling species: *S. arcticum s.s.* and *S. saxosum*; and *S. arcticum s.s.*, *Simulium brevicercum* Knowlton and Rowe, and *S. saxosum*. The network approach supports incongruence between barcode data and species boundaries of *S. arcticum* taxa.

Within the *S. arcticum* complex, intraspecific K2P distances ranged from 0.00%–3.20% with a mean of 1.21% (Fig. 3A). The highest divergence value was found in *S. saxosum*; the lowest was present in all *S. arcticum* species. Similar interspecific distances were obtained (0.00%–5.43%; mean = 1.66; Fig. 3B). The slightly greater range of interspecific distances, compared with intraspecific values resulted from the inclusion of *S. negativum* in divergence calculations. Specifically, distances between *S. negativum* and all other members of the complex ranged from 3.54%–5.43%, whereas interspecific values among the latter were 0.00%–3.38%. Therefore, excluding *S. negativum*, the distribution of COI variability within and between *S. arcticum* sibling species completely overlapped one another. Average intraspecific distance within the *S. arcticum* complex was greater than that of all other species analysed, the exception being *S. murmanum*, a suspected species complex (Adler *et al*. 2004) (*S. decorum* = 0.46%, *S. noelleri* = 0.19%, *S. decimatum* = 0.36%, *S. murmanum* = 3.52%, *S. arcticum* complex = 1.21%). In contrast, mean interspecific divergence within the complex was significantly lower than that observed for other species comparisons (*S. decorum* = 11.94%, *S. noelleri* = 10.60%, *S. decimatum* = 10.88%, *S. murmanum* = 10.77%, *S. arcticum* complex = 1.66%). Overall, the *S. arcticum* complex showed interspecific distances that are comparable to within, rather than among species diversity of other taxa.

In terms of a benchmark value for defining species, a 10× interspecific divergence threshold was calculated as 10.60% (average intraspecific distance across all taxa = 1.06%). This value may be inflated because of high within-species variation for *S. murmanum*, as well as for *S. arcticum* taxa. Nevertheless, all members of our broadly defined outgroup (*S. decorum*, *S. noelleri*, *S. decimatum*, and *S. murmanum*) had average interspecific divergence values equal to or greater than the cut-off criteria. In contrast, no member of the *S. arcticum* complex met this traditional barcode threshold for delimiting species.

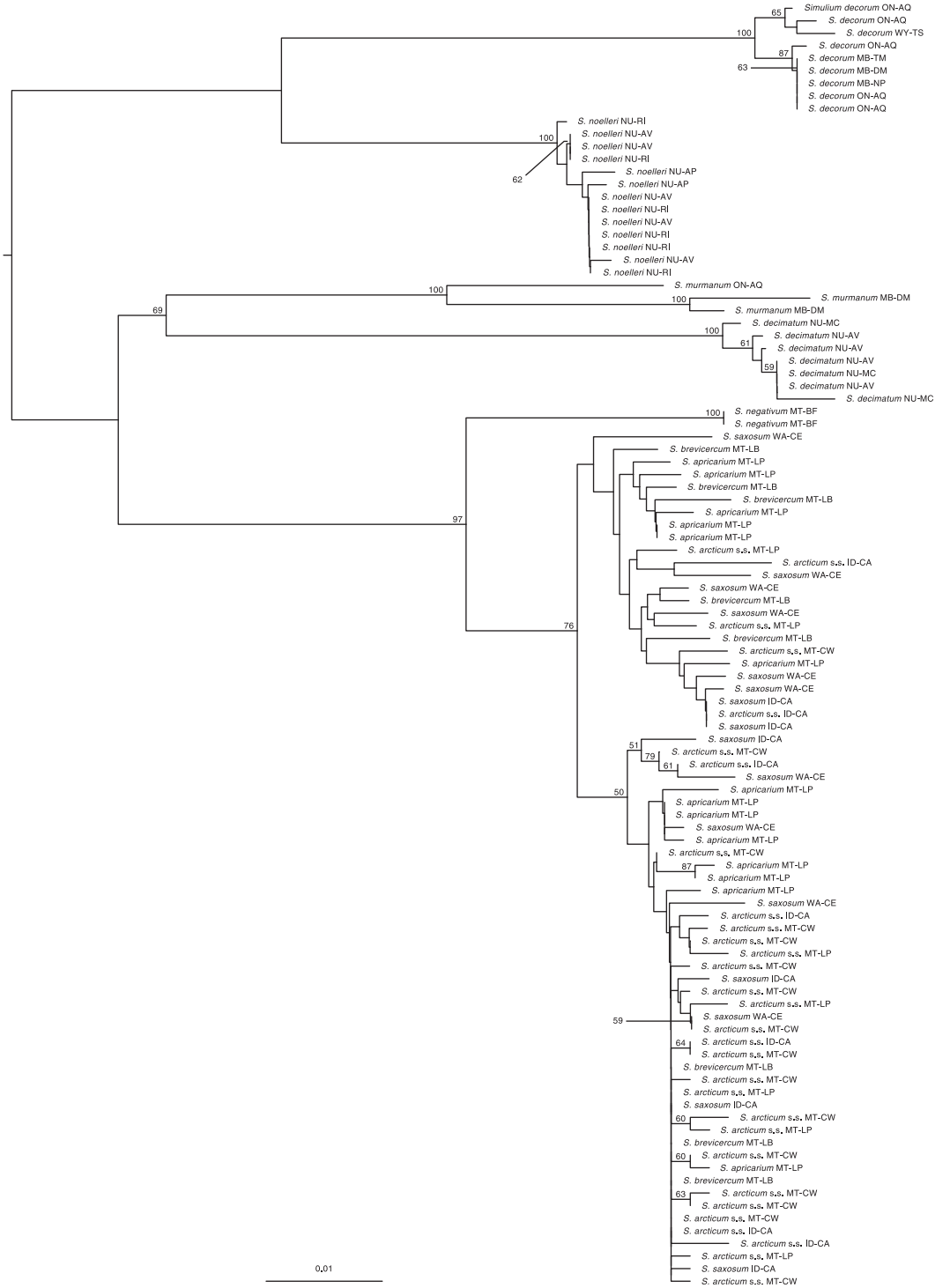
Discussion

We assessed the ability of barcoding to differentiate members of a recent black fly lineage radiation. The *S. arcticum* species complex was monophyletic in relation to the outgroups, two closely related members of the *Simulium malyschevi* species group (*S. decimatum* and *S. murmanum*) and two more distantly related members of the *S. noelleri* group (*S. decorum* and *S. noelleri*; Fig. 1). Of five morphologically indistinguishable

© 2012 Entomological Society of Canada

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press

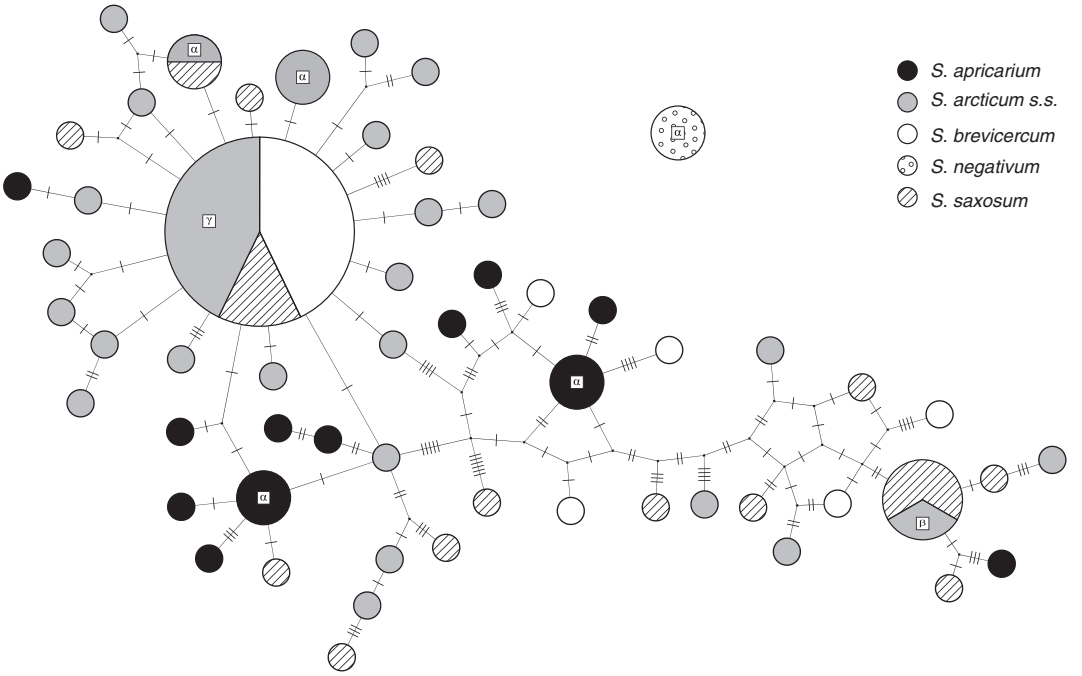
Fig. 1. Neighbour-joining tree for *Simulium* taxa generated using K2P distances. Analysis is based on 102 cytochrome *c* oxidase subunit I sequences, 610 bp in length. Numbers above the nodes indicate bootstrap support (≥50%).



© 2012 Entomological Society of Canada

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press

Fig. 2. Parsimony network showing 95% probability linkages among 57 cytochrome *c* oxidase subunit I haplotypes obtained from 70 individuals representing five *Simulium arcticum* sibling species. Ticks across network connections represent mutational changes. The area of each circle is proportional to the number of individuals containing the haplotype. Haplotypes marked by α, β, and γ, are present in two, three, and seven individuals, respectively. All remaining haplotypes are found in only a single individual.



but chromosomally distinct *S. arcticum* sibling species, only *S. negativum* was recovered with robust molecular support. No other members of the complex could be distinguished using COI barcodes. Our results are consistent with and significantly augment the main findings of Conflitti *et al*. (2010). In our study we built on Conflitti *et al*. (2010) by sampling more extensively geographically and numerically from individual locations (*i.e*., over four times as many individuals per species on average) to ensure adequate representation of genetic variation within each sibling species.

DNA barcoding will accurately recognize species when (1) sequences are not shared between sister taxa and (2) intraspecific genetic variation does not exceed interspecific divergence (Hebert *et al*. 2004). Failures in species identification are proportional to the amount of overlap between the distribution of intra- and interspecific distances (Meyer and Paulay 2005). In our study, three *S. arcticum* sibling species (*S. arcticum s.s.*, *S. brevicercum*, and *S. saxosum*) shared identical barcode haplotypes (Fig. 2). Moreover, the distribution of interspecific genetic distances completely overlapped the range of variation within species (Fig. 3). Consequently, the COI gene could not differentiate most members of the *S. arcticum* complex.

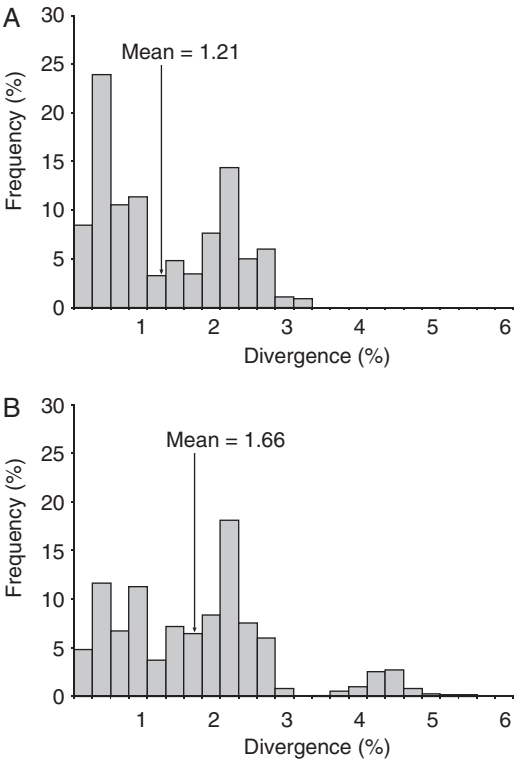
Diagnosis based on molecular criteria is particularly difficult when species are separated by a short history of reproductive isolation because various processes can result in shared DNA haplotypes and lack of reciprocal monophyly. Funk and Omland (2003) recognized five sources of mtDNA failure in detecting species boundaries: gene paralogy, imperfect taxonomy, inadequate genetic information, incomplete lineage sorting, and introgressive hybridization. Conflitti *et al*. (2010) thoroughly evaluated these factors in relation to the *S. arcticum* species complex and each process will be mentioned only briefly here.

The presence of undetected paralogous gene sequences is not likely the cause of sibling species

© 2012 Entomological Society of Canada

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press

Fig. 3. Frequency distribution of intraspecific (A) and interspecific (B) K2P distances for five members of the *Simulium arcticum* species complex.



non-monophyly. No insertions, deletions, or stop codons were encountered in our dataset indicating that all sequences constitute functional mitochondrial products (Funk and Omland 2003). However, lack of COI divergence may indicate that the majority of *S. arcticum* complex members represent a single randomly breeding species. Although experimental mating crosses provide the most direct method of establishing reproductive status, difficulties in rearing black flies in a laboratory setting generally preclude these types of studies (*i.e.*, black flies typically require fast flowing water during larval and pupal stages, as well as blood meals and mating swarms for adults). Instead, evidence for isolation among black fly sibling species is provided by cytogenetic analysis of natural populations. In general, reproductively isolated cytospecies maintain their unique chromosomal rearrangements in sympatry with other closely related taxa (Rothfels 1989; Adler *et al*. 2010). Specifically, the distribution of chromosomal variation is in Hardy–Weinberg disequilibrium between isolated populations. In this way, cytogenetic studies provide significant evidence of reproductive status. *Simulium arcticum* sibling species differ considerably in their cytological features, most having distinct sex-linked chromosomal rearrangements. Sex chromosomes in the complex range from undifferentiated (*S. brevicercum*) to containing inversions that are either X-linked in females (*S. saxosum*) or Y-linked in males (*S. apricarium*, *S. arcticum s.s.*, and *S. negativum*; Adler *et al*. 2004). Moreover, cytogenetic studies have shown that sympatric *S. arcticum* sibling species maintain their unique sex-linked and autosomal inversions (Shields and Procunier 1982; Adler *et al*. 2004; Shields *et al*. 2007b). Other lines of evidence, such as geographic distribution and ecology, further support species status of *S. arcticum* taxa (Shields and Procunier 1982; Adler *et al*. 2004; Shields *et al*. 2007a, 2007b). Finally, all *S. arcticum* individuals analysed by us were cytologically verified ruling out the possibility of incorrect identification of morphologically indistinguishable taxa. Overall, imperfect taxonomy is unlikely for the sibling species we analysed. Therefore, although DNA barcoding failed to distinguish most taxa in the *S. arcticum* complex, it is premature to modify current (2012) taxonomy based solely on one short gene fragment.

Another explanation for lack of reciprocal monophyly is inadequate genetic information. Single gene analyses may not provide sufficient variation to resolve recently diverged taxa (Moritz and Cicero 2004). The weak support for the majority of bifurcations within the *S. arcticum* complex in our NJ tree (BSS $<$ 50%; Fig. 1) supports this view. Incomplete lineage sorting may also be responsible for shared haplotypes among, and lack of reciprocal monophyly between, *S. arcticum* sibling species. Establishment of reciprocal monophyly is a function of time since isolation. During early stages of divergence, when lineage sorting is incomplete, ancestral polymorphisms are shared between species (Funk and Omland 2003). This appears to be the case for members of the *S. arcticum* complex. Most notably, haplotype γ (Fig. 2) was shared between sibling species sampled from distant geographic regions (*S. arcticum s.s.* from Coeur d'Alene River, Idaho and Clearwater River and Little

© 2012 Entomological Society of Canada

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press

Prickly Pear Creek, Montana; *S. brevicercum* from Little Blackfoot River, Montana; and *S. saxosum* from Coeur d'Alene River, Idaho). Another possible example of shared ancestral polymorphism is identical COI sequences among *S. arcticum s.s.* and *S. saxosum* from Clearwater River, Montana, and Cle Elum River, Washington, respectively (haplotype α, Fig. 2). Both cases suggest that lineage sorting is incomplete in the *S. arcticum* complex. Given that adult black flies can travel long distances (*e.g.*, *S. vampirum* Adler *et al.*, a member of the *S. arcticum* complex; Fredeen 1969; Charnetski and Haufe 1981), it may also suggest that gene flow is occurring between sibling species. Members of black fly species complexes have been known to hybridize naturally and under experimental conditions (reviewed in Adler *et al.* 2010). Recurrent hybridization could account for the patterns we observed. Gene flow among multiple species can strongly affect mitochondrial tree topologies, making it difficult to infer routes of genetic exchange between species (Funk and Omland 2003). However, hybridization often can be detected when individuals possess haplotypes of sympatric lineages (Funk and Omland 2003). A possible example involves *S. arcticum s.s.* and *S. saxosum* from Coeur d'Alene River, Idaho (haplotype β, Fig. 2). Future studies based on microsatellite data can test whether ongoing gene flow is occurring at this locality.

Lack of species-specific genetic variation is not unique to the *S. arcticum* complex. In general, diagnostic molecular variation is difficult to obtain for black fly sibling species originally discovered through cytotaxonomy (reviewed in Adler *et al.* 2010). Limited success in finding sibling species-specific genetic differences contrasts with the scenario for black fly morphospecies: morphologically defined black fly species are typically represented by a single cluster of closely related DNA sequences, distinct from those of all other species. As in other studies, the morphospecies we analysed (*i.e.*, outgroup species *S. decimatum* and *S. murmanum*) were each monophyletic and strongly supported (Fig. 1). With this in mind, the genetic cohesion of individuals representing *S. negativum* can be explained. *Simulium negativum* was strongly supported in the tree (Fig. 1) and distinct from the main haplotype network (Fig. 2), indicating that this species diverged relatively early from the remaining members of the complex. This early split is consistent with morphological and cytological evidence (Shields and Procunier 1982; Adler *et al.* 2004). *Simulium negativum* is among the only *S. arcticum* taxa to have accumulated morphological differences. Although *S. negativum* adults and pupae are not easily distinguished from other sibling species, larvae can usually be recognized by the negative head-spot pattern on females, as well as by the unpigmented suboesophageal ganglion in both sexes (Adler *et al.* 2004). Cytogenetically, *S. negativum* differs from other members of the complex in that male sex determination is based on the IL, rather than the IIL chromosome arm (Shields and Procunier 1982). Alternatively, monophyly of *S. negativum* relative to other *S. arcticum* taxa may be an artefact of sample size (only two individuals were analysed). More extensive sampling will be required to test monophyly of this sibling species adequately.

Our results support the view that eastern and western populations of *S. murmanum* represent separate species (Adler *et al.* 2004). We observed a marked (4.62%) divergence between eastern (Algonquin Park, Ontario) and western (Duck Mountain Park, Manitoba) populations of *S. murmanum* (Fig. 1). This pattern was also observed by Rivera and Currie (2009), based on a larger sample size ($n = 15$) than ours, and by Adler *et al.* (2004), based on chromosomal differences between eastern (Labrador, Quebec, Ontario) and western (Alaska, Yukon, Alberta) populations. Possibly *Simulium corbis* Twinn, currently (2012) considered a junior synonym of *S. murmanum*, will be revalidated and applied to the eastern population.

Outgroup taxa of the *S. noelleri* group (*S. decorum* and *S. noelleri*) are morphologically indistinguishable but were recognized with significant support by barcoding (Fig. 1). Similar to members of the *S. arcticum* complex, identification of *S. decorum* and *S. noelleri* relies on chromosomal data (Adler *et al.* 2004). In contrast, however, evidence suggests that lineage sorting is complete and hybridization limited between these species. Differences in speciation rate and geographic separation could be responsible for reciprocal monophyly among *S. noelleri* and *S. decorum*, and the lack of it between species of the *S. arcticum* complex. The *S. noelleri* species

© 2012 Entomological Society of Canada

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press

group consists of nine nominal species distributed throughout the Holarctic Region. However, only two species, *S. decorum* and *S. noelleri*, are currently recognized within the Nearctic Region (Adler *et al*. 2004). In contrast, the highly diverse *S. arcticum* complex is restricted to North America, with most of its members localized to the Rocky Mountains of Montana (Adler *et al*. 2004; Shields *et al*. 2007a, 2007b, 2009). We speculate that most members of the complex diverged recently, as a result of isolation in inter-mountain valleys caused by mountain glaciers south of the continental ice sheets during the Wisconsinan glacial maximum. Subsequent rapid radiation of *S. arcticum* sibling species over a relatively short geological time span would likely result in iterative episodes of reproductive isolation before complete sorting of ancestral polymorphisms.

Wisconsinan-aged glacial cycles may also explain the origin of the sister-pair *S. noelleri* and *S. decorum*. *Simulium noelleri* was probably restricted to Beringia (central Alaska and Yukon) during the glacial maximum, whereas *S. decorum* probably survived south of the continental ice sheet (Currie 1997). This assumption is supported by the present-day distribution of the two species in the Nearctic Region: *S. noelleri* at high latitudes from Alaska to Hudson Bay, *S. decorum* from coast-to-coast as far south as Florida, with sympatry occurring only in northwestern North America (Alaska, Yukon, Northwest Territories). The larger geographic separation and slower speciation rate relative to members of the *S. arcticum* complex perhaps explains why lineage sorting is complete between *S. noelleri* and *S. decorum*.

Mechanisms that limit hybridization between sympatric *S. noelleri* and *S. decorum* would also promote reciprocal monophyly. One possibility is that differences in mating behaviour prevent gene flow between *S. noelleri* and *S. decorum*. Mating among *S. decorum* individuals takes place in ground-level aggregations at emergence and oviposition sites (Davies and Peterson 1956). Although *S. noelleri* mating is unreported, small groups of males, possibly representing mating swarms, have been observed hovering under trees (Edwards 1920). In contrast, mating behaviour evidently does not differ among members of the *S. arcticum* complex and ample opportunity for hybridization exists, especially in Montana where many *S. arcticum* sibling species have overlapping distributions.

Our study supports the view that COI barcodes effectively discriminate among black fly morphospecies (Rivera and Currie 2009). We also have demonstrated that barcoding can be useful for diagnosing cryptic black fly species. However, COI's ability to recognize morphologically indistinguishable taxa depends on the history of the species being considered. In particular, DNA barcoding could not differentiate members of a recent lineage radiation. However, barcoding can flag exceptional species assemblages, such as the *S. arcticum* complex, that are worthy of further study. In cases where species boundaries are blurred by incomplete lineage sorting and/or introgressive hybridization, supplemental analyses of rapidly evolving nuclear (microsatellites and introns) and mitochondrial (control region) loci will be required. As recommended by Ilmonen *et al*. (2009) and Adler and Huang (2011), an integrated approach incorporating multiple sources of evidence (*i.e*., morphology, ecology, cytology, and multi-locus molecular datasets) will be crucial for understanding recent radiations of black flies.

Acknowledgements

The *Manual of Nearctic Diptera* is arguably the most important contribution to the systematics and taxonomy of flies during the 20th century. The coordinators of the Manual (Frank McAlpine, Bob Peterson, Guy Shewell, Herb Teskey, Dick Vockeroth, and Monty Wood), together with some of the most influential dipterologists of the time, established a foundation that stimulated and accelerated subsequent research on the Diptera. We are particularly indebted to Monty Wood, whose outstanding contributions to the study of black flies continue to inspire new generations of "simuliidologists". This research was supported, in part, through funding to the Canadian Barcode of Life Network from Genome Canada (through the Ontario Genomics Institute), Natural Sciences and Engineering Research Council of Canada (NSERC), and other sponsors listed at www.BOLNET.ca. Additional funding was provided by an NSERC Discovery Grant to DCC, an NSERC Canada Graduate Scholarship to IMC, and grants from

© 2012 Entomological Society of Canada

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press

the M.J. Murdock Charitable Trust (#2003196 and #2005233) to GFS. Laboratory costs at the Royal Ontario Museum were supported, in part, from an NSERC Discovery Grant to Robert W. Murphy. Comments from Peter H. Adler greatly improved an earlier version of this manuscript. We thank D.C. Winter for assistance with Microsoft Excel scripts and Jean-Marc Moncalvo for providing the French translation of the abstract.

References

Adler, P.H., Cheke, R.A., and Post, R.J. 2010. Evolution, epidemiology, and population genetics of black flies (Diptera: Simuliidae). Infection Genetics and Evolution, **7**: 846–865.

Adler, P.H., and Crosskey, R.W. 2010. World blackflies (Diptera: Simuliidae): a comprehensive revision of the taxonomic and geographical inventory (2010). Clemson University, South Carolina [online]. Available from http://entweb.clemson.edu/biomia/pdfs/ [accessed 24 January 2011].

Adler, P.H., Currie, D.C., and Wood, D.M. 2004. The black flies (Simuliidae) of North America. Cornell University Press, New York.

Adler, P.H., and Huang, Y. 2011. Integrated systematics of the Simuliidae (Diptera): evolutionary relationships of the little-known Palearctic black fly *Simulium acrotrichum*. The Canadian Entomologist, **143**: 612–628.

Ball, S.L., Hebert, P.D.N., Burian, S.K., and Webb, J.M. 2005. Biological identifications of mayflies (Ephemeroptera) using DNA barcodes. Journal of the North American Benthological Society, **24**: 508–524.

Barrett, R.D.H., and Hebert, P.D.N. 2005. Identifying spiders through DNA barcodes. Canadian Journal of Zoology, **83**: 481–491.

Charnetski, W.A., and Haufe, W.O. 1981. Control of *S. arcticum* Malloch in Northern Alberta, Canada. *In* Blackflies: the future for biological methods in integrated control. *Edited by* M. Laird. Academic Press, New York. pp. 117–132.

Clary, D.O., and Wolstenholme, D.R. 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. Journal of Molecular Evolution, **22**: 252–271.

Clement, M., Posada, D., and Crandall, K. 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology, **9**: 1657–1660.

Conflitti, I.M., Kratochvil, M.J., Spironello, M., Shields, G.F., and Currie, D.C. 2010. Good species behaving badly: non-monophyly of black fly sibling species in the *Simulium arcticum* complex (Diptera: Simuliidae). Molecular Phylogenetics and Evolution, **57**: 245–257.

Currie, D.C. 1986. An annotated list of and keys to the immature black flies of Alberta (Diptera: Simuliidae). Memoirs of the Entomological Society of Canada, **134**: 1–90.

Currie, D.C. 1997. Black flies (Diptera: Simuliidae) of the Yukon, with reference to the black fly fauna of northwestern North America. *In*Insects of the Yukon. *Edited by* H.V. Danks and J.A. Downes. Biological Survey of Canada (Terrestrial Arthropods), Ottawa, Ontario. pp. 563–614.

Davies, D.M., and Peterson, B.V. 1956. Observations on the mating, feeding, ovarian development, and oviposition of adult black flies (Simuliidae, Diptera). Canadian Journal of Zoology, **34**: 615–655.

Day, J.C., Goodall, T.I., and Post, R.J. 2008. Confirmation of the species status of the blackfly *Simulium galeratum* in Britain using molecular taxonomy. Medical and Veterinary Entomology, **22**: 55–61.

Edwards, F.W. 1920. On the British species of *Simulium* – II. The early stages; with corrections and additions to Part I. Bulletin of Entomological Research, **11**: 211–246.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution, **39**: 783–791.

Finn, D.S., Theobald, D.M., Black, W.C., and Poff, N.L. 2006. Spatial population genetic structure and limited dispersal in a Rocky Mountain alpine stream insect. Molecular Ecology, **15**: 3553–3566.

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, **3**: 294–299.

Fredeen, F.J.H. 1969. Outbreaks of the black fly *Simulium arcticum* Malloch in Alberta. Quaestiones Entomologicae, **5**: 341–372.

Funk, D.J., and Omland, K.E. 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. Annual Review of Ecology, Evolution, and Systematics, **34**: 397–423.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, **41**: 95–98.

Hebert, P.D.N., Cywinska, A., Ball, S.L., and deWaard, J.R. 2003a. Biological identifications through DNA barcodes. Proceedings of the Royal Society B: Biological Sciences, **270**: 313–321.

Hebert, P.D.N., Ratnasingham, S., and deWaard, J.R. 2003b. Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society B: Biological Sciences, **270**: S96–S99.

Hebert, P.D.N., Stoeckle, M.Y., Zemlak, T.S., and Francis, C.M. 2004. Identification of birds through DNA barcodes. PLoS Biology, **2**: 1657–1663.

Ilmonen, J., Adler, P.H., Malmqvist, B., and Cywinska, A. 2009. The *Simulium vernum* group (Diptera: Simuliidae) in Europe: multiple character

© 2012 Entomological Society of Canada

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press

sets for assessing species status. Zoological Journal of the Linnean Society, **156**: 847–863.

Joy, D.A., Craig, D.A., and Conn, J.E. 2007. Genetic variation tracks ecological segregation in Pacific island black flies. Heredity, **99**: 452–459.

Kerr, K.C.R., Stoeckle, M.Y., Dove, C.J., Weigt, L.A., Francis, C.M., and Hebert, P.D.N. 2007. Comprehensive DNA barcode coverage of North American birds. Molecular Ecology Notes, **7**: 535–543.

Koch, D.A., Duncan, G.A., Parsons, T.J., Pruess, K.P., and Powers, T.O. 1998. Effect of preservation methods, parasites, and gut content of black flies (Diptera: Simuliidae) on polymerase chain reaction products. Journal of Medical Entomology, **35**: 314–318.

Meyer, C.P., and Paulay, G. 2005. DNA barcoding: error rates based on comprehensive sampling. PLoS Biology, **12**: 2229–2238.

Monaghan, M.T., Balke, M., Gregory, T.R., and Vogler, A.P. 2005. DNA-based species delineation in tropical beetles using mitochondrial and nuclear markers. Philosophical Transactions of the Royal Society B: Biological Sciences, **360**: 1925–1933.

Moritz, C., and Cicero, C. 2004. DNA barcoding: promise and pitfalls. PLoS Biology, **10**: 1529–1531.

Nardi, F., Carapelli, A., Fanciulli, P.P., Dallai, R., and Frati, F. 2001. The complete mitochondrial DNA sequence of the basal Hexapod *Tetrodontophora bielanensis*: evidence for heteroplasmy and tRNA translocations. Molecular Biology and Evolution, **18**: 1293–1304.

Nei, M., and Kumar, S. 2000. Molecular evolution and phylogenetics. Oxford University Press, New York.

Post, R.J., Flook, P.K., and Millest, A.L. 1993. Methods for the preservation of insects for DNA studies. Biochemical Systematics and Ecology, **21**: 85–92.

Pramual, P., Kuvangkadilok, C., Baimai, V., and Walton, C. 2005. Phylogeography of the black fly *Simulium tani* (Diptera: Simuliidae) from Thailand as inferred from mtDNA sequences. Molecular Ecology, **14**: 3989–4001.

Rivera, J., and Currie, D.C. 2009. Identification of Nearctic black flies using DNA barcodes (Diptera: Simuliidae). Molecular Ecology Resources, **9**: 224–236.

Rothfels, K.H. 1979. Cytotaxonomy of black flies (Simuliidae). Annual Review of Entomology, **24**: 507–537.

Rothfels, K.H. 1981. Cytological approaches to the study of black fly systematics and evolution. *In* Application of genetics and cytology in insect systematics and evolution. *Edited by* M.W. Stock. Forest, Wildlife, and Range Experiment Station, University of Idaho, Moscow, Idaho. pp. 67–83.

Rothfels, K.H. 1989. Speciation in black flies. Genome, **32**: 500–509.

Rothfels, K.H., and Dunbar, R.W. 1953. The salivary gland chromosomes of the black fly *Simulium vittatum* Zett. Canadian Journal of Zoology, **31**: 226–241.

Shields, G.F., Christiaens, B.A., Van Leuven, M.L., and Hartman, A.L. 2009. Reproductive status and continuity of taxa of the *Simulium arcticum* complex (Diptera: Simuliidae) at the Clearwater River, Montana (2007, 2008, and 2009). Western North American Naturalist, **69**: 511–520.

Shields, G.F., Clausen, G.M., Marchion, C.S., Michel, T.L., Styren, K.C., Riggin, C.N., *et al* 2007a. The effect of elevation on the distribution of sibling species in the *Simulium arcticum* complex (Diptera: Simuliidae). Western North American Naturalist, **67**: 39–45.

Shields, G.F., Pickens, J.A., Clausen, G.M., and Strizich, L.M. 2007b. Reproductive status of cytoforms in a black fly complex in Montana. Intermountain Journal of Science, **13**: 32–43.

Shields, G.F., and Procunier, W.S. 1982. A cytological description of sibling species of *Simulium* (*Gnus*) *arcticum* (Diptera: Simuliidae). Polar Biology, **1**: 181–192.

Smith, M.A., Poyarkov, N.A., and Hebert, P.D.N. 2008. CO1 DNA barcoding amphibians: take the chance, meet the challenge. Molecular Ecology Notes, **8**: 235–246.

Swofford, D.L. 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods) version 4. Sinauer Associates, Sunderland, Massachusetts.

Templeton, A.R., Crandall, K.A., and Sing, C.F. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics, **132**: 619–633.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., and Hebert, P.D.N. 2005. DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society B: Biological Sciences, **360**: 1847–1857.

© 2012 Entomological Society of Canada

https://doi.org/10.4039/tce.2012.29 Published online by Cambridge University Press