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Ephemeroptera, Plecoptera, and Trichoptera fauna of Churchill (Manitoba, Canada): insights into biodiversity patterns from DNA barcoding

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Abstract. The insect orders Ephemeroptera, Plecoptera, and Trichoptera (EPT) are particularly important for freshwater ecological and biomonitoring studies, but difficulties in their identification to species level impede research. DNA barcoding provides a solution to this problem by linking newly collected specimens to a reference library of authoritatively identified specimens. Here, we consider the ways in which patterns of intraspecific and interspecific genetic divergences in the barcode region can provide rapid insights into the taxonomic identity, morphological features, and geographical distributions of species. Our study led to a >5× increase in the EPT fauna, including 68 caddisfly, 37 mayfly, and 7 stonefly species, recorded from Churchill. DNA barcoding also aided detection of rare taxa, allowed identification of otherwise unidentifiable life stages, revealed several potentially new species of caddisflies and mayflies, and suggested the presence of cryptic species. The new insights into this fauna and the strong congruence between morphological and molecular characters affirm the utility of DNA barcoding for rapid characterization of the diversity of EPT faunas. We also explore the phenology and habitat preferences of Churchill's trichopterans and demonstrate that comprehensive sampling is important for documenting biodiversity through DNA barcoding.

Key words: aquatic insects, COI, biodiversity inventory, species boundaries, species checklist, phenology, habitat preference.

Community ecology studies on Arctic aquatic insects have been limited despite their value for understanding the impacts of climate change (Quinlan et al. 2005, Wrona et al. 2006a, b), for monitoring water quality (Bowman et al. 2009, Rosenberg and Resh 1993), and for improving knowledge of biodiversity in the Arctic. Among other practical difficulties, such as funding and availability of expertise, species identification using morphological characters presents a serious impediment to community analysis and is one of the reasons why so few studies have been conducted. This impediment arises because description of most aquatic insect species has been based on the morphologically variant life stage, typically adult males (with some exceptions, e.g., in some mayflies). As a result, the identification of females and immature stages often is not possible (although mayfly larvae are often more easily identified than adults). Nevertheless, females typically contribute at least ½ of the adult population, and immature forms are typically the predominant stage in the water.

Characterizing an unknown fauna is always challenging, even in areas with only moderate diversity, because qualified taxonomists are often unavailable, especially for less charismatic taxa that comprise the bulk of biodiversity. Moreover, routine identification of large numbers of specimens is not the primary goal for academic taxonomists (Holzenthal et al. 2010) and diverts them from finding and describing new species, writing identification keys, building electronic resources, and reconstructing phylogenies. Investment in the

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training and employment of additional nonacademic taxonomists is much needed, and deoxyribonucleic acid (DNA) barcoding—the use of a short standardized mitochondrial cytochrome c oxidase subunit I (COI) gene region for animal species discrimination (Hebert et al. 2003)—shows much promise as an alternate route for the rapid analysis of biodiversity. This method simply requires access to sequencing technology and a DNA library against which to compare sequences.

Both global and local studies are underway to establish DNA reference libraries and to document biodiversity using DNA barcodes. Global DNA barcode reference libraries—barcode sequences linked to voucher specimens identified by qualified taxonomists and housed in permanent repositories—are being built rapidly for selected eukaryote groups, including some key aquatic insect orders, e.g., Ephemeroptera (mayflies), Plecoptera (stoneflies), Trichoptera (caddisflies) (EPT), and Odonata (dragonflies and damselflies). A complementary approach, termed *barcoding biotas*, is being used to survey all eukaryote species at particular sites. Such endeavors contribute to the global libraries of the target taxonomic groups, and provide a better overall understanding of biodiversity in focal ecosystems. One low-Arctic Canadian site (Churchill, Manitoba) has been chosen as a testing ground for documenting all multicellular life via DNA barcoding. The barcode reference library for EPT groups at Churchill (Zhou et al. 2009) represents the 1st order-level installment of this large-scale initiative. Zhou et al. (2009) investigated the overall genetic divergence patterns in COI sequences and demonstrated the utility of DNA barcode clusters for constructing species accumulation curves as a complementary approach to documenting unknown local diversity. Species boundaries and life-history traits of the Churchill EPTs are discussed in detail in the present work.

Churchill is an ideal location for an intensive study of the EPT fauna because of its modest taxonomic diversity, complex biogeographical linkages reflecting its location at the transition zone between the boreal and tundra biomes, and its wide variety of habitats. The drainage system at Churchill is characterized by abundant lentic water bodies (ponds, lakes, and temporary pools), small streams, and one major river, the Churchill River. Tundra ponds and lakes are the predominant freshwater habitats by area. Most of these habitats form isolated pools, but some are linked by fast, shallow creeks. The Churchill River is a large, slow-flowing river with a width of 1 km where it empties into Hudson Bay.

During our previous work on Churchill's EPT fauna, DNA barcodes greatly facilitated sorting of morphospecies, differentiation of closely related species, discovery of potentially new species, and revelation of cryptic species. The present paper examines genetic divergence patterns in greater detail and explores how DNA barcodes perform in discriminating among EPT species compared to using morphological species boundaries. In addition, an updated checklist for Churchill EPT species is provided. During the course of this work, clear differences among species in phenology and habitat preferences were observed. Because caddisflies, the most species-rich order among EPTs at Churchill, were collected intensively for 5 seasons, the temporal and spatial distributions of the Churchill trichopterans were analyzed. Last, the feasibility of conducting rapid biotic surveys—initially using DNA barcodes alone—is discussed. Several critical factors that might affect the efficiency of efforts to barcode entire biotas are summarized.

Methods

Specimen collection, identification, and sequencing

We first compiled prior records for the EPT fauna at Churchill to generate a draft checklist. This list was subsequently enlarged through collection programs led by researchers at the Biodiversity Institute of Ontario and by collaborators from 2002 to 2007 (except 2003, when none were made). The timing of expeditions in different years was varied to help ensure seasonal coverage. Adult samples were collected using ultraviolet (UV) light traps, sweep and aerial nets, Malaise traps, and pitfall traps. Larval samples were collected with a kicknet and by handpicking. Sampling efforts were structured to maximize microhabitat diversity. Adult specimens were pinned or preserved in 95% ethanol, and all larval samples were kept in 95% ethanol. Trichoptera specimens were deposited in the Biodiversity Institute of Ontario, University of Guelph, at the University of Manitoba, and in the University of Minnesota Insect Collection. Ephemeroptera and Plecoptera specimens were deposited in the Biodiversity Institute of Ontario.

EPT specimens were identified with current morphological keys by 3 authors of this paper (XZ, LMJ, and RED). Representative specimens of certain caddisfly species also were examined by R. Blahnik, J. Morse, and D. Ruiter, and burrowing mayfly identifications were confirmed by W. P. McCafferty. Morphological identifications were independent of molecular analysis. When morphological identification was impossible (e.g., some larvae, females, and damaged specimens), DNA barcodes were used to

associate these specimens with identified representatives of the taxon in question.

Sequences analyzed in our study have been published in a companion paper (Zhou et al. 2009). COI barcodes were acquired at the Canadian Centre for DNA Barcoding at the University of Guelph. Standard barcoding protocols for DNA extraction (Ivanova et al. 2006), polymerase chain reaction (PCR) amplification, and sequencing (deWaard et al. 2008, Hajibabaei et al. 2005) were followed. Detailed DNA analysis protocols and a new reverse primer designed for recovering a shorter fragment (325 base pairs [bp] on the 5′ end) of the barcode region for EPTs are provided in Zhou et al. (2009).

Barcode sequences and associated trace files and voucher information (taxonomy, image, collection information) are accessible in the Barcode of Life Data System (BOLD) (Ratnasingham and Hebert 2007), within the projects 'Ephemeroptera of Churchill', 'Plecoptera of Churchill', and 'Trichoptera of Churchill 2002/2004/2005/2006/2007'. COI sequences are also available in GenBank under accession numbers GU113533–GU115809. Detailed voucher information for all analyzed EPT specimens also is provided in supplementary table S1 in the companion paper by Zhou et al. (2009).

Genetic data analysis

COI sequences for each order were aligned in MEGA 4.0 (Tamura et al. 2007) with the integrated ClustalX method with default parameters, and alignments were verified using amino acid translation. Unique haplotypes were recognized using analytical tools available at the DNA Barcoding Tools website (www.ibarcode.org; Singer and Hajibabaei 2009) and imported into MEGA for tree construction using the Neighbor-Joining method (Saitou and Nei 1987) with pairwise deletion of missing sites and Kimura-2-Parameter (K2P) distances (Kimura 1980). Bootstrap values were obtained using 1000 replicates.

Genetic distances were calculated using the Nearest Neighbor Summary option in BOLD with K2P distances for all sequences >420 bp to examine intraspecific vs interspecific divergence patterns. Maximum intraspecific divergences and minimum distances to nearest neighbors were grouped into a series of categories: 0–1%, 1–2%, 2–3%, etc. The frequencies of each of these categories were plotted for each of the 3 orders.

Species discovery in caddisflies: temporal and spatial pattern analyses

All caddisfly samples collected from 2002 to 2007 were used to examine the extent of temporal overlap in species occurrence. Pooled across years, the emergence season was divided into 3 nearly equal periods (P1–P3, each consisting of 18–20 d). Overlap in species composition between these 3 periods was displayed with a Venn diagram.

All 2007 Trichoptera samples were collected within a 1-mo period and with detailed habitat information and, thus, were used in an analysis of habitat use. Three habitat types were designated for the Churchill area: tundra lentic environments (collected adjacent to ponds or lakes), tundra creeks (collected adjacent to fast-moving creeks), and river (collected next to the Churchill River). The division of Churchill's aquatic habitats is not based on their surface areas or water volumes, but rather on their physical attributes, which are most important in determining the distribution of caddisfly larvae (e.g., Wiggins 1996). Overlap in species composition also was displayed with a Venn diagram.

Caddisflies were most intensively collected in 2006 and 2007 (84% of total caddisfly specimens), and the collecting dates in these 2 y were complementary (5–26 August 2006 and 30 June–27 July 2007). Thus, the 2006 and 2007 samples were used to build species accumulation curves to explore the trends in species occurrence across the season. Accumulated species number, counting all individuals, was plotted against collection dates (sampling bias uncorrected). To eliminate potential bias caused by heterogeneity in species abundance or collecting/processing effort, the accumulated species number divided by the total number of individuals collected on each collection date also was included (sampling bias corrected) in the same figure.

Results

Tracing species boundaries in Churchill EPTs with DNA barcodes

A total of 2277 COI sequences were collected from 2436 individuals, including 68 caddisfly, 37 mayfly, and 7 stonefly species (morphologically distinguishable taxa confirmed by DNA barcodes, summarized in Table 1; but see later discussion regarding 2 *Nemoura* species). COI was effective for separating all species of EPTs at Churchill. Members of each morphological species formed a monophyletic cluster in the COI Neighbor-Joining tree. Furthermore, all morphospecies with multiple representatives had bootstrap values >99%, except the caddisfly *Limnephilus partitus* Walker (92%) (Figs 1–3). A few morphological species showed relatively high intraspecific divergence (Table 1) with multiple haplotype groups represented in such taxa. Those morphospecies with intraspecific divergence

Table 1. Summary of Ephemeroptera, Plecoptera, and Trichoptera at Churchill, Mantoba. * = not collected in this study, Δ = new record in Manitoba, # = new record for Canada, seq = sequence, intra = intraspecific divergence, max = maximum, NN = nearest neighbor, dist = distribution, NA = North America, H-N= Holarctic or Northern Transcontinental, N/A = statistics not available (single sequence), — = not collected in this study.

Species	No. seq	Mean Intra	Max Intra	Nearest neighbor	Distance to NN	Processing ID of NN	Dist	Literature
Trichoptera								
Apataniidae								
* *Apatania crymophila* McLachlan	—	—	—	—	—	—	H-N	Ross 1938
Apatania stigmatella (Zetterstedt)	2	0.80	0.80	*Apatania zonella*	10.47	CUCAD446-07	H-N	Lehmkuhl and Kerst 1979
Δ *Apatania zonella* (Zetterstedt)	1	N/A	N/A	*Apatania stigmatella*	10.47	CUCAD538-07	H-N	
Brachycentridae								
Brachycentrus americanus (Banks)	7	0.00	0.00	*Brachycentrus fuliginosus*	13.02	CUCAD742-07	H-N	
Brachycentrus fuliginosus (Walker)	1	N/A	N/A	*Brachycentrus americanus*	13.02	CUCAD711-07	NA	
Glossosomatidae								
Glossosoma intermedium (Klapalek)	13	0.51	1.54	*Glossosoma velonum*	11.47	CUCAD736-07	H-N	
Δ *Glossosoma velonum* Ross	7	0.74	1.43	*Glossosoma intermedium*	11.47	CUCAD657-07	NA	
Protoptila tenebrosa (Walker)	33	0.18	0.64	*Agrypnia macdunnoughi*	25.79	CUCAD544-07	NA	
Hydropsychidae								
Arctopsyche ladogensis (Kolenati)	12	0.00	0.00	*Ceratopsyche alternans*	21.82	LCHIP116-07	H-N	
Ceratopsyche alhedra (Ross)	1	N/A	N/A	*Ceratopsyche bronta*	13.54	CUCAD075-07	NA	
Ceratopsyche alternans (Walker)	101	0.44	1.44	*Ceratopsyche alhedra*	15.92	CUCAD187-07	NA	Flannagan and Flannagan 1982, Lehmkuhl and Kerst 1979, as *Hydropsyche recurvata* (Banks)
Ceratopsyche bronta (Ross)	1	N/A	N/A	*Ceratopsyche vexa*	11.76	CUCAD073-07	NA	Flannagan and Flannagan 1982
Ceratopsyche vexa (Ross)	1	N/A	N/A	*Ceratopsyche bronta*	11.76	CUCAD075-07	NA	Denning 1943
* *Cheumatopsyche analis* (Banks)	—	—	—	—	—	—	NA	Lehmkuhl and Kerst 1979, as *C. pettiti* Banks
Cheumatopsyche campyla Ross	13	2.38	5.25	*Ceratopsyche alhedra*	21.33	CUCAD187-07	NA	
Hydroptilidae								
Hydroptila consimilis Morton	17	0.85	2.70	*Hydroptila spatulata*	20.12	CUCAD779-08	NA	
Hydroptila spatulata Morton	1	N/A	N/A	*Hydroptila consimilis*	20.12	CUCAD793-08	NA	
Oxyethira sp. CHU1	1	—	—	—	—	—		

Table 1. Continued.

	Species	No. seq	Mean Intra	Max Intra	Nearest neighbor	Distance to NN	Processing ID of NN	Dist	Literature
	Leptostomatidae								
	Lepidostoma togatum (Hagen)	29	1.05	2.66	*Asynarchus montanus*	17.96	COC092-05	NA	
	Leptoceridae								
	Ceraclea annulicornis (Stephens)	32	0.01	0.19	*Ceraclea excisa*	4.32	CUCAD727-07	H-N	Lehmkuhl and Kerst 1979
	Ceraclea arielles (Denning)	24	0.05	0.48	*Ceraclea resurgens*	15.24	CUCAD568-07	NA	
Δ	*Ceraclea excisa* (Morton)	11	0.08	0.30	*Ceraclea annulicornis*	4.32	CUCAD174-07	H-N	
	Ceraclea resurgens (Walker)	1	N/A	N/A	*Ceraclea arielles*	15.24	CUCAD407-07	NA	
	Mystacides interjectus (Banks)	11	1.06	2.23	*Mystacides sepulchralis*	10.73	CUCAD677-07	NA	Yamamoto and Wiggins 1964
	Mystacides sepulchralis (Walker)	1	N/A	N/A	*Mystacides interjectus*	10.73	CUCAD028-07	NA	
	Oecetis cf. *inconspicua* (Walker) CHU1	1	N/A	N/A	*Oecetis* cf. *inconspicua* CHU2	12.46	CUCAD729-07	NA	
	Oecetis cf. *inconspicua* (Walker) CHU2	20	0.79	1.86	*Oecetis* cf. *inconspicua* CHU1	12.46	DSTRI588-07	NA	
	Oecetis cf. *ochracea* (Curtis) CHU1	2	0.30	0.30	*Oecetis* cf. *ochracea* CHU2	7.45	CUCAD403-07	H-N	
	Oecetis cf. *ochracea* (Curtis) CHU2	3	0.20	0.30	*Oecetis* cf. *ochracea* CHU1	7.45	CUCAD161-07	H-N	
	Triaenodes reuteri McLachlan	3	0.10	0.15	*Oecetis* cf. *inconspicua* CHU2	17.00	CUCAD729-07	H-N	Lehmkuhl and Kerst 1979, as *T. griseus* Banks
	Limnephilidae								
	Anabolia bimaculata (Walker)	9	0.20	0.96	*Asynarchus montanus*	12.66	DSTRI534-07	NA	
Δ	*Arctopora pulchella* (Banks)	9	0.33	1.43	*Limnephilus hageni*	10.38	COC088-05	NA	
*	*Asynarchus iteratus* McLachlan	—	—	—	—	—	—	H-N	Lehmkuhl and Kerst 1979
Δ	*Asynarchus lapponicus* (Zetterstedt)	2	0.66	0.66	*Asynarchus montanus*	9.34	DSTRI362-07	H-N	
	Asynarchus montanus (Banks)	173	0.85	3.92	*Asynarchus lapponicus*	9.34	CUCAD402-07	NA	
Δ	*Asynarchus mutatus* (Hagen)	77	0.05	0.46	*Asynarchus montanus*	11.27	DSTRI362-07	NA	

Table 1. Continued.

Species		No. seq	Mean Intra	Max Intra	Nearest neighbor	Distance to NN	Processing ID of NN	Dist	Literature
	Asynarchus rossi (Leonard & Leonard)	35	0.00	0.00	*Anabolia bimaculata*	14.68	DSTRI376-07	NA	
	Grammotaulius interrogationis (Zetterstedt)	119	0.63	2.83	*Limnephilus picturatus*	9.92	DSTRI580-07	NA	
Δ	*Lenarchus fautini* (Denning)	4	1.01	2.01	*Arctopora pulchella*	11.22	COC034-05	NA	
	Limnephilus canadensis Banks	1	N/A	N/A	*Limnephilus perpusillus*	7.06	MHTRI011-06	NA	
	Limnephilus externus Hagen	66	0.09	0.62	*Limnephilus nigriceps*	9.66	DSTRI512-07	H-N	
	Limnephilus femoralis Kirby	94	0.29	1.20	*Arctopora pulchella*	11.58	COC034-05	H-N	Fischer 1968
Δ	*Limnephilus fischeri* Ruiter	13	0.13	0.31	*Limnephilus hageni*	3.46	GUCAD147-08	NA	
Δ	*Limnephilus hageni* Banks	122	0.35	1.43	*Limnephilus fischeri*	3.46	DSCNI047-07	NA	
	Limnephilus infernalis (Banks)	17	0.00	0.00	*Limnephilus externus*	9.69	DSTRI414-07	NA	
	Limnephilus kennicotti Banks	14	0.13	0.32	*Limnephilus sericeus*	9.51	CUCAD289-07	NA	
	Limnephilus moestus Banks	3	0.10	0.15	*Limnephilus canadensis*	11.42	CUCAD186-07	NA	
	Limnephilus nigriceps (Zetterstedt)	5	0.33	0.83	*Limnephilus picturatus*	5.75	DSTRI582-07	H-N	
	Limnephilus partitus Walker	12	0.89	1.72	*Limnephilus sansoni*	4.09	DSTRI471-07	NA	
	Limnephilus perpusillus Walker	2	0.31	0.31	*Limnephilus canadensis*	7.06	CUCAD186-07	NA	Flannagan and Flannagan 1982
	Limnephilus picturatus McLachlan	6	0.29	0.62	*Limnephilus nigriceps*	5.75	DSTRI512-07	H-N	
	Limnephilus rhombicus (Linnaeus)	1	N/A	N/A	*Limnephilus externus*	10.44	DSTRI075-06	H-N	
Δ	*Limnephilus sansoni* Banks	87	0.37	4.01	*Limnephilus partitus*	4.09	DSTRI604-07	NA	
	Limnephilus sericeus (Say)	3	0.10	0.15	*Limnephilus kennicotti*	9.51	DSCNI046-07	H-N	
*	*Nemotaulius hostilis* (Hagen)	—	—	—	—	—	—	NA	Flannagan and Flannagan 1982, Flint 1960
	Onocosmoecus unicolor (Banks)	1	N/A	N/A	*Asynarchus mutatus*	17.73	COC010-05	H-N	
	Philarctus bergrothi McLachlan	6	0.00	0.00	*Limnephilus kennicotti*	14.05	DSTRI563-07	H-N	Lehmkuhl and Kerst 1979, as *P. quaeris* (Milne)

Table 1. Continued.

Species	No. seq	Mean Intra	Max Intra	Nearest neighbor	Distance to NN	Processing ID of NN	Dist	Literature
Molannidae								
Molanna flavicornis Banks	45	0.02	0.36	*Agrypnia obsoleta*	21.74	CUCAD379-07	H-N	Lehmkuhl and Kerst 1979
Philopotamidae								
Chimarra socia Hagen	1	N/A	N/A	*Hydroptila consimilis*	25.76	CUCAD796-08	NA	
Phryganeidae								
Agrypnia colorata Hagen	71	0.07	0.92	*Agrypnia improba*	13.36	COC041-05	H-N	
Agrypnia deflata (Milne)	15	0.05	0.15	*Agrypnia obsoleta*	3.45	CUCAD379-07	NA	
Agrypnia improba (Hagen)	2	0.00	0.00	*Agrypnia straminea*	8.57	CUCAD524-07	NA	
Agrypnia macdunnoughi (Milne)	14	0.42	1.54	*Agrypnia straminea*	10.70	CUCAD145-07	NA	
Δ *Agrypnia obsoleta* (Hagen)	1	N/A	N/A	*Agrypnia deflata*	3.45	COC040-05	H-N	
* *Agrypnia pagetana* Curtis	—	—	—	—	—	—	H-N	Wiggins 1960
Agrypnia straminea Hagen	32	0.01	0.18	*Agrypnia improba*	8.57	COC041-05	NA	Lehmkuhl and Kerst 1979
Banksiola crotchi Banks	21	0.44	1.07	*Agrypnia macdunnoughi*	11.76	CUCAD572-07	NA	
Phryganea cinerea Walker	9	0.16	0.30	*Banksiola crotchi*	15.23	CUCAD355-07	NA	
Ptilostomis semifasciata (Say)	6	0.15	0.46	*Banksiola crotchi*	12.60	CUCAD100-07	NA	
Polycentropodidae								
Neureclipsis crepuscularis (Walker)	3	0.00	0.00	*Polycentropus aureolus*	19.92	DSTRI586-07	NA	
Polycentropus aureolus (Banks)	4	0.00	0.00	*Psychomyia flavida*	19.90	CUCAD325-07	NA	
Psychomyiidae								
Psychomyia flavida Hagen	4	0.23	0.46	*Polycentropus aureolus*	19.90	DSTRI586-07	H-N	Lehmkuhl and Kerst 1979
Rhyacophilidae								
Rhyacophila angelita Banks	41	0.01	0.17	*Agrypnia improba*	22.54	COC041-05	NA	
Average for Trichoptera		0.34	0.98		12.21			
Ephemeroptera								
Baetidae								
Acentrella turbida (McDunnough) D	1	N/A	N/A	*Acentrella turbida* E	19.36	EPCHU531-07		
Acentrella turbida (McDunnough) E	2	0.15	0.15	*Plauditus* sp. CHU1	18.89	EPCHU506-07		

Table 1. Continued.

	Species	No. seq	Mean Intra	Max Intra	Nearest neighbor	Distance to NN	Processing ID of NN	Dist	Literature
	Acerpenna pygmaea (Hagen)	7	1.23	1.93	*Procloeon fragile*	20.25	EPCHU141-07	NA	Harper and Harper 1981
	Acerpenna sp. CHU1	9	0.00	0.00	Baetidae sp. CHU1	2.77	EPCHU340-07		
	Baetidae sp. CHU1	11	0.59	1.12	*Acerpenna* sp. CHU1	2.77	EPCHU374-07		
	Baetis brunneicolor McDunnough	59	0.20	1.86	*Baetis hudsonicus*	13.03	EPCHU086-07	NA	Harper and Harper 1981, McClure 1943, Morihara and McCafferty 1979
	Baetis bundyae Lehmkuhl	4	0.15	0.30	*Baetis hudsonicus*	4.24	EPCHU086-07	NA	Harper and Harper 1981
	Baetis hudsonicus Ide	3	0.31	0.46	*Baetis bundyae*	4.24	EPCHU319-07	NA	Ide 1937, McClure 1943, Morihara and McCafferty 1979
	Baetis phoebus McDunnough[a]	176	0.01	0.78	*Procloeon mendax*	20.55	EPCHU037-07	NA	Morihara and McCafferty 1979, as *B. flavistriga* McDunnough
	Plauditus cf. *dubius* (Walsh)	25	0.99	2.80	*Acentrella turbida* E	20.36	EPCHU531-07		Harper and Harper 1981
	Plauditus sp. CHU1	2	0.00	0.00	*Acentrella turbida* E	18.89	EPCHU531-07		
	Plauditus sp. CHU2	7	0.15	0.30	*Plauditus* sp. CHU1	20.23	EPCHU506-07		
*	*Procloeon inanum* (McDunnough)	—	—	—	—	—	—	NA	Harper and Harper 1981
Δ	*Procloeon fragile* (McDunnough)	5	0.12	0.30	*Procloeon mendax*	17.89	EPCHU031-07	NA	
Δ	*Procloeon mendax* (Walsh)	11	0.08	0.31	*Procloeon fragile*	17.89	EPCHU275-07	NA	
	Baetiscidae								
	Baetisca laurentina McDunnough	9	0.03	0.16	*Metretopus borealis*	20.23	EPCHU557-07	NA	
	Ephemerellidae								
Δ	*Dannella lita* (Burks)	3	0.00	0.00	*Dannella simplex*	12.79	EPCHU001-07	NA	
	Dannella simplex (McDunnough)	4	0.10	0.15	*Dannella lita*	12.79	EPCHU545-07	NA	Allen and Edmunds 1961
Δ	*Ephemerella needhami* McDunnough	16	0.06	0.30	*Ephemera simulans*	20.24	EPCHU244-07	NA	
	Eurylophella bicolor (Clemens)	1	N/A	N/A	*Dannella lita*	23.42	EPCHU545-07	NA	
	Ephemeridae								
	Ephemera simulans Walker	22	0.87	2.17	*Paraleptophlebia praepedita*	16.76	EPCHU229-07	NA	
	Heptageniidae								

Table 1. Continued.

Species		No. seq	Mean Intra	Max Intra	Nearest neighbor	Distance to NN	Processing ID of NN	Dist	Literature
Δ	*Ecdyonurus criddlei* (McDunnough)	4	0.00	0.00	Heptageniidae XZ sp. CHU1	16.71	EPCHU218-07	NA	
	Heptagenia pulla (Clemens)	27	0.11	0.76	Heptageniidae XZ sp. CHU1	16.53	EPCHU218-07	NA	
	Heptageniidae sp. CHU1	1	N/A	N/A	*Heptagenia pulla*	16.53	EPCHU540-07		
	Heptageniidae sp. CHU2	1	N/A	N/A	*Rhithrogena manifesta*	10.65	EPCHU283-07		
	Leucrocuta jewetti (Allen)	10	0.58	1.26	*Ecdyonurus criddlei*	16.76	EPCHU541-07	NA	
	Maccaffertium terminatum (Walsh)	8	0.47	1.11	*Leucrocuta jewetti*	20.01	EPCHU553-07	NA	
*	*Maccaffertium vicarium* (Walker)	—	—	—	—	—	—	NA	Bednarik and McCafferty 1979
*	*Rhithrogena jejuna* Eaton	—	—	—	—	—	—	NA	Ide 1954, Jacobus and McCafferty 2001
Δ	*Rhithrogena manifesta* Eaton	5	0.00	0.00	Heptageniid XZ sp. CHU2	10.65	EPCHU495-07	NA	
Leptohyphidae									
	Tricorythodes mosegus Alba-Tercedor & Flannagan	1	N/A	N/A	*Ephemera simulans*	24.48	EPCHU241-07	NA	
Leptophlebiidae									
	Leptophlebia cupida (Say)	3	0.10	0.15	*Paraleptophlebia praepedita*	17.83	EPCHU229-07	NA	
Δ#	*Paraleptophlebia aquilina* Harper & Harper	3	0.31	0.46	*Leptophlebia cupida*	21.08	EPCHU021-07	NA	
	Paraleptophlebia debilis (Walker)	4	0.00	0.00	*Paraleptophlebia* sp. CHU1	5.58	EPCHU179-07	NA	
	Paraleptophlebia praepedita (Eaton)	55	0.19	0.97	*Metretopus borealis*	16.15	EPCHU557-07	NA	Harper and Harper 1981
	Paraleptophlebia sp. CHU1	4	0.00	0.00	*Paraleptophlebia debilis*	5.58	MHCOL145-07		
Metretopodidae									
	Metretopus borealis Eaton	6	0.10	0.15	*Siphlonurus phyllis*	14.41	EPCHU055-07	H-N	Edmunds 1957
Siphlonuridae									
	Parameletus chelifer Bengtsson	1	N/A	N/A	*Paraleptophlebia praepedita*	19.02	EPCHU229-07	H-N	
*	*Parameletus midas* McDunnough	—	—	—	—	—	—	NA	Harper and Harper 1981
	Siphlonurus alternatus (Say)	49	0.08	0.77	*Siphlonurus phyllis*	7.18	EPCHU085-07	H-N	
	Siphlonurus phyllis McDunnough	5	0.71	1.38	*Siphlonurus alternatus*	7.18	EPCHU105-07	NA	

Table 1. Continued.

Species	No. seq	Mean Intra	Max Intra	Nearest neighbor	Distance to NN	Processing ID of NN	Dist	Literature
Average for Ephemeroptera		0.25	0.65		14.97			
Plecoptera								
Capniidae								
* *Capnia nearctica* Banks	—	—	—	—	—	—	H-N	Ricker 1964
* *Capnia vernalis* Newport	—	—	—	—	—	—	H-N	Ricker 1938
Chloroperlidae								
Hastaperla brevis (Banks)	13	0.07	0.30	*Haploperla orpha*	10.89	PLCHU217-08	NA	Frison 1942
Haploperla orpha (Frison)	3	0.20	0.30	*Haploperla brevis*	10.89	PLCHU206-08	NA	
Nemouridae								
Amphinemura linda (Ricker)	180	0.34	2.54	*Nemoura arctica*	17.68	PLCHU218-08	H-N	Ricker 1952
Nemoura arctica Esben-Peterson	4	0.00	0.00	*Nemoura* sp. CHU1	6.05	PLCHU222-08	H-N	Ricker 1952
Nemoura sp. CHU1	3	0.22	0.35	*Nemoura arctica*	6.05	PLCHU218-08		
Nemoura sp. CHU2	1	N/A	N/A	*Nemoura arctica*	8.79	PLCHU218-08		
* *Shipsa rotunda* (Claassen)	—	—	—	—	—	—	H-N	Ricker 1944
Perlidae								
* *Acroneuria carolinensis* (Banks)	—	—	—	—	—	—	NA	Stark and Gaufin 1976
* *Acroneuria lycorias* (Newman)	—	—	—	—	—	—	NA	Ricker 1964
* *Claassenia sabulosa* (Banks)	—	—	—	—	—	—	H-N	Ricker 1964
Perlodidae								
* *Diura bicaudata* (Linnaeus)	—	—	—	—	—	—	H-N	Ricker 1964
* *Isogenoides frontalis* (Newman)	—	—	—	—	—	—	NA	Frison 1942
* *Isoperla decolorata* (Walker)	—	—	—	—	—	—	H-N	Ricker 1944
Isoperla sp.	9	1.30	3.13	*Haploperla brevis*	20.57	PLCHU206-08		
* *Isoperla marlynia* Needham and Claassen	—	—	—	—	—	—	NA	Frison 1942
* *Isoperla transmarina* (Newman)	—	—	—	—	—	—	NA	Frison 1942
Pteronarcyidae								
* *Pteronarcys dorsata* (Say)	—	—	—	—	—	—	NA	McClure 1943, as *T. shelfordi* Frison
Average for Plecoptera		0.35	1.10		11.56			

[a] *Baetis phoebus* McDunnough is being reinstated from synonymy with *B. flavistriga* McDunnough by LMJ, XZ, and PDNH, unpublished data

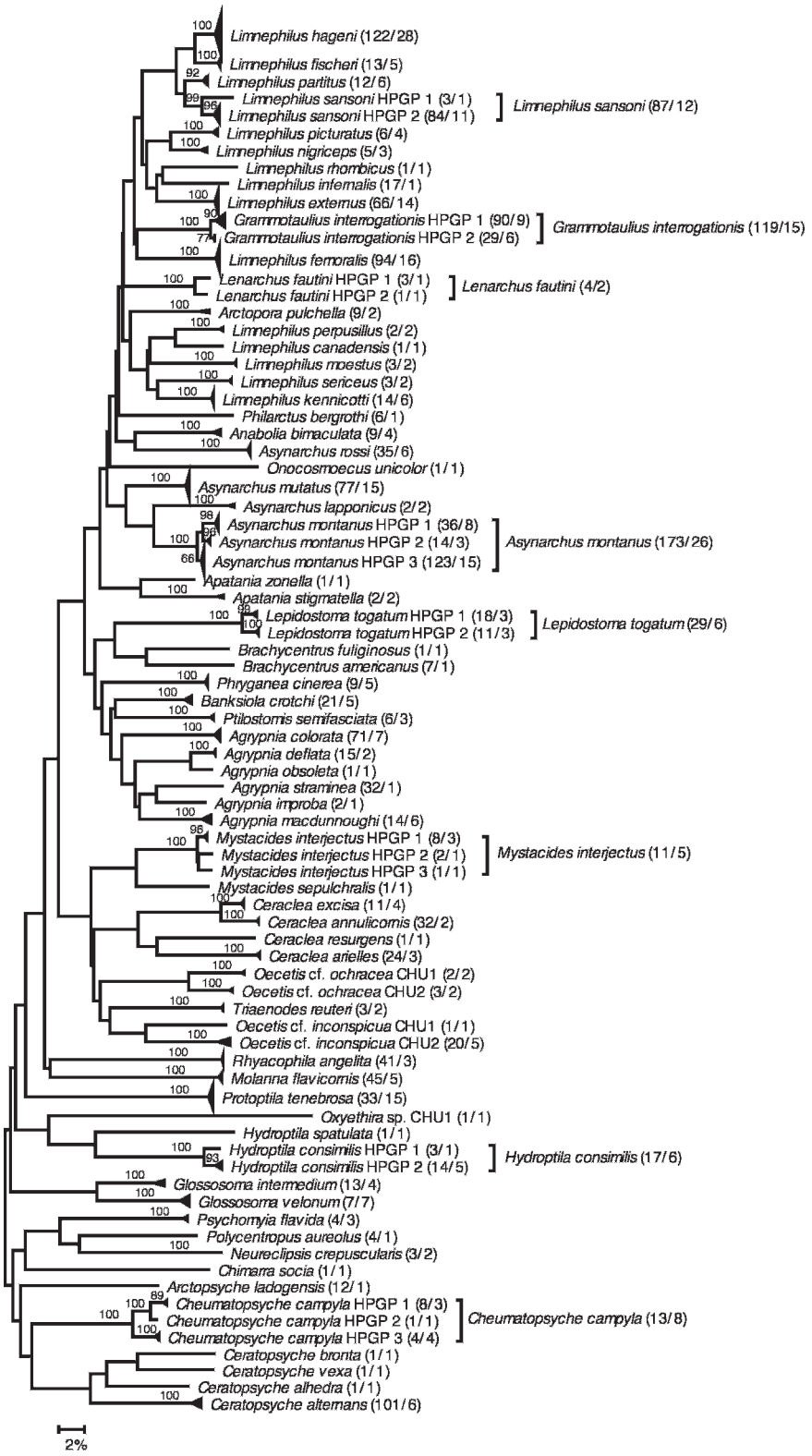

FIG. 1. Neighbor-Joining tree of unique cytochrome c oxidase subunit I (COI) haplotypes of Trichoptera from Churchill. A total of 1500 COI sequences representing 68 species of Trichoptera are represented. Haplotypes of the same morphospecies are collapsed into triangles whose height represents the number of distinct haplotypes and width represents the extent of intraspecific divergence. Species showing relatively large intraspecific divergences (>2%) are highlighted in brackets to the right, and haplotype groups (HPGP) within these species are shown on the tree. Numbers in parentheses represent the total number of sequences and the number of haplotypes for each species or haplotype group. Bootstrap values are provided for all morphospecies and for each distinct haplotype group with multiple representatives. Haplogroups represented by multiple individuals with the same haplotype possess a bootstrap value of 100%, e.g., *Limnephilus infernalis*.

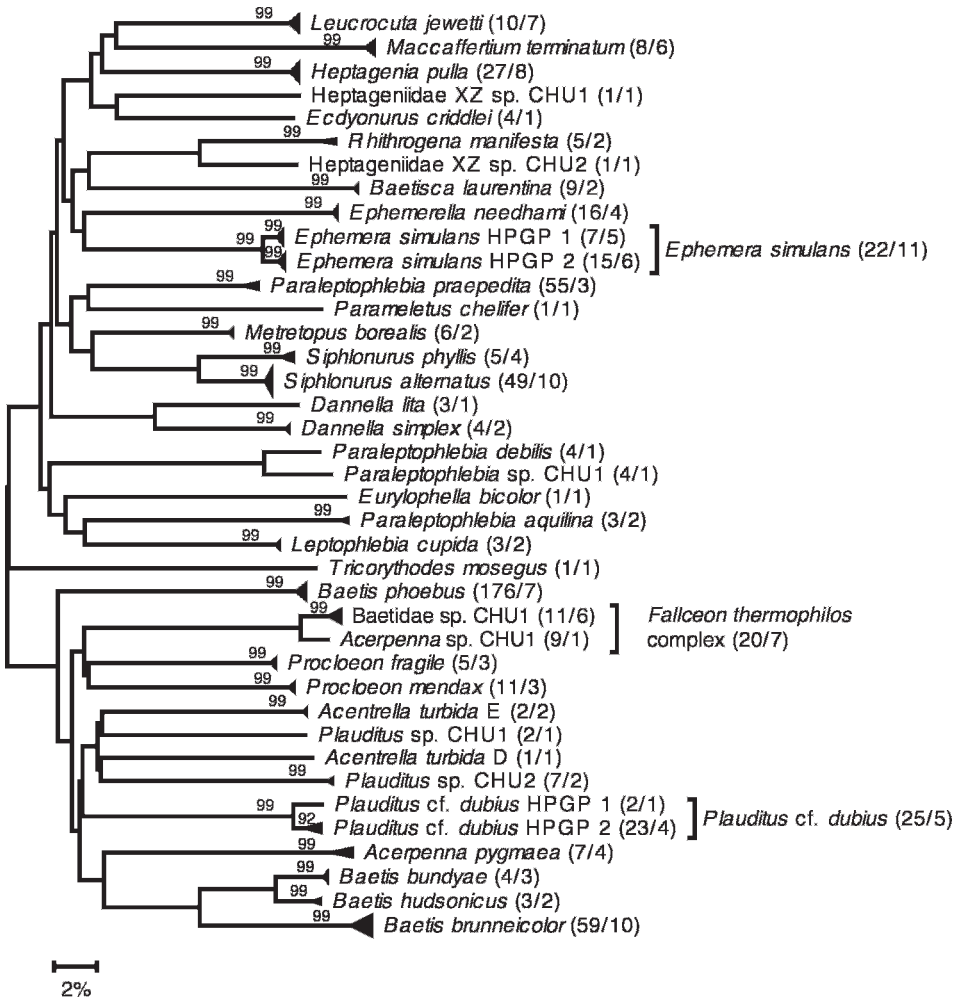

FIG. 2. Neighbor-Joining tree of unique cytochrome c oxidase subunit I (COI) haplotypes of Ephemeroptera from Churchill. A total of 564 COI sequences representing 36 species of Ephemeroptera represented. Figure annotations are the same as those in Fig. 1.

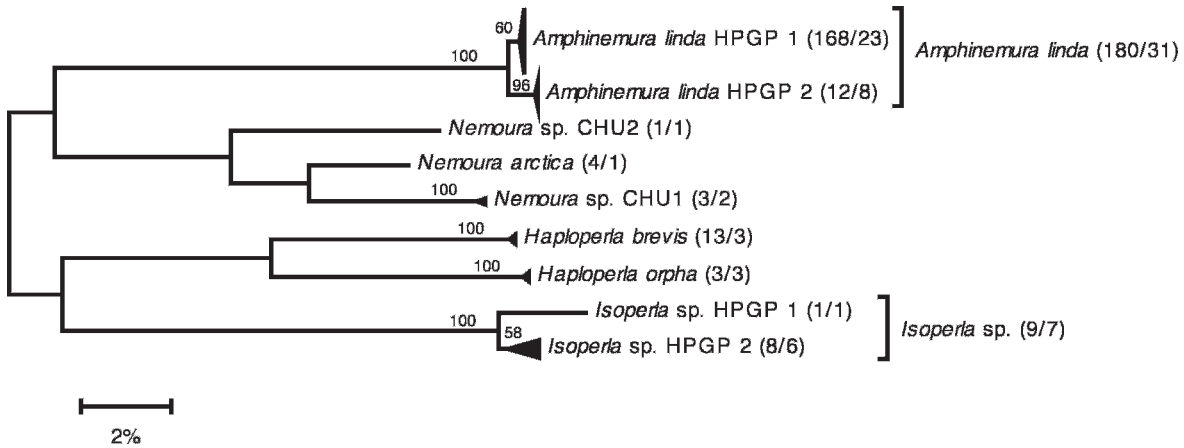

FIG. 3. Neighbor-Joining tree of unique cytochrome c oxidase subunit I (COI) haplotypes of Plecoptera from Churchill. A total of 213 COI sequences representing 7 species of Plecoptera are represented. Figure annotations are the same as those in Fig. 1.

values >2% are shown in brackets in Figs 1–3 (haplogroups within these species are noted as HPGPs). This threshold was used because past studies have shown that intraspecific divergences rarely exceed this value in sympatry (Hebert et al. 2003, 2004, Ball et al. 2005, Hogg et al. 2009). Most cases of large intraspecific divergence reflect cryptic diversity (Williams et al. 2006, Smith et al. 2007, Decaëns and Rougerie 2008, Ståhls and Savolainen 2008, Vaglia et al. 2008, Basquin and Rougerie 2009, Hausmann et al. 2009, Hebert et al. 2009). Of course, exceptions have been observed (Wiemers and Fiedler 2007, Alexander et al. 2009). Therefore, cases of >2% intraspecific divergence are highlighted in our study to emphasize taxa that deserve further attention for potential taxonomic revision (see discussion of morphological characters below), but species names based on morphology are used for all lineages, even those with deep genetic divergence.

For the most part, interspecific distances of Churchill's EPTs did not overlap with intraspecific divergences. In fact, average distance to nearest neighboring species (NN distance) was greater than maximum intraspecific divergences by a factor of ≥10× in each order (Table 1). NN distances were greater than average intraspecific divergences by ≥30×. Genetic patterns were similar across the 3 orders. Mean intraspecific divergences for species within each order ranged from 0.25 to 0.35%, whereas the averages of the maximum intraspecific divergences ranged from 0.65 to 1.10% and NN distances ranged from 11.6 to 14.97%.

The few cases of overlap between intraspecific and interspecific distance values reflected large intraspecific divergences in 3 caddisfly and 2 mayfly species combined with shallow distances between 4 caddisfly and 1 mayfly species pairs (Fig. 4, noted in panels A and B). At least some of these cases probably reflected the presence of cryptic species or a species complex with a recent diversification history (e.g., *Limnephilus sansoni* Banks, *Cheumatopsyche campyla* Ross; see Discussion). In addition, morphological identification of some samples was uncertain (e.g., *Isoperla* sp.; see Discussion), but the relevant samples were temporarily treated as the same morphospecies.

Extended checklists and distribution patterns for Churchill's EPT fauna

Few published studies exist for EPT species at Churchill, and the available records are scattered. McClure (1943) recorded 2 mayfly, 1 stonefly, and several unidentified caddisfly species from this region. For stoneflies, most Churchill records came from the taxonomic treatments, such as Frison (1942), Ricker (1952), and Stark and Gaufin (1976). A study of Churchill caddisflies, motivated by an attempt to solve identification difficulties encountered in an ecological study (Lehmkuhl and Kerst 1979), provided an annotated checklist of 10 species collected from 1971 to 1979. Species records of Churchill EPTs from previous studies are compiled in Table 1 with notes on the original references. Most historical records were extracted from a faunistic review of the EPTs of Manitoba by Flannagan and Flannagan (1982).

Most of the EPT species collected in our study were new records to Churchill, and 18 were new to Manitoba (Table 1), including 1 new record for Canada. Lehmkuhl and Kerst (1979) reported that 7 of the 10 caddisfly species collected at Churchill in their study were endemic to North America. In our study, of the 66 caddisfly species that have been assigned to a nominal species or species complex, 44 are Nearctic, whereas 22 are Holarctic. Three of the identified mayflies (*Metretopus borealis* Eaton, *Parameletus chelifer* Bengtsson, *Siphlonurus alternatus* (Say)) also have Holarctic distributions, but the rest are Nearctic (Table 1). Our collections and literature records suggest that 19 stonefly species occur at Churchill, including those defined by temporary names (*Nemoura* sp. CHU1, *Nemoura* sp. CHU2) and the unidentified *Isoperla* (Table 1). Of the 16 named stonefly species, 3 have Holarctic, circumpolar distributions: *Capnia nearctica* Banks, *Nemoura arctica* Esben-Petersen, and *Diura bicaudata* (Linneaus). Several other species have northern transcontinental distributions: *Capnia vernalis* Newport, *Amphinemura linda* (Ricker), *Shipsa rotunda* (Claassen), *Isoperla decolorata* (Walker), and *Claassenia sabulosa* (Banks), and the rest have eastern Nearctic distributions with Churchill as the western limit of their range.

Most of the caddisfly species collected at Churchill have a transcontinental distribution in North America. Eleven species are new for Manitoba (Table 1), among which *Glossosoma velonum* Ross, *Lenarchus fautini* (Denning), *Limnephilus sansoni*, and *Agrypnia obsoleta* (Hagen) represent the easternmost records in North America. *Asynarchus rossi* (Leonard and Leonard) is regarded as a rare species in North America, with a locally abundant disjunct population in Minnesota (Houghton and Holzenthal 2003). This species was collected in Churchill only during 9–26 August 2006, but it was common (n = 46). Five caddisfly species previously recorded from Churchill were not detected during our study (Table 1, marked with *).

Both mayfly larvae and adults were included in our DNA analysis, enabling the association of unknown

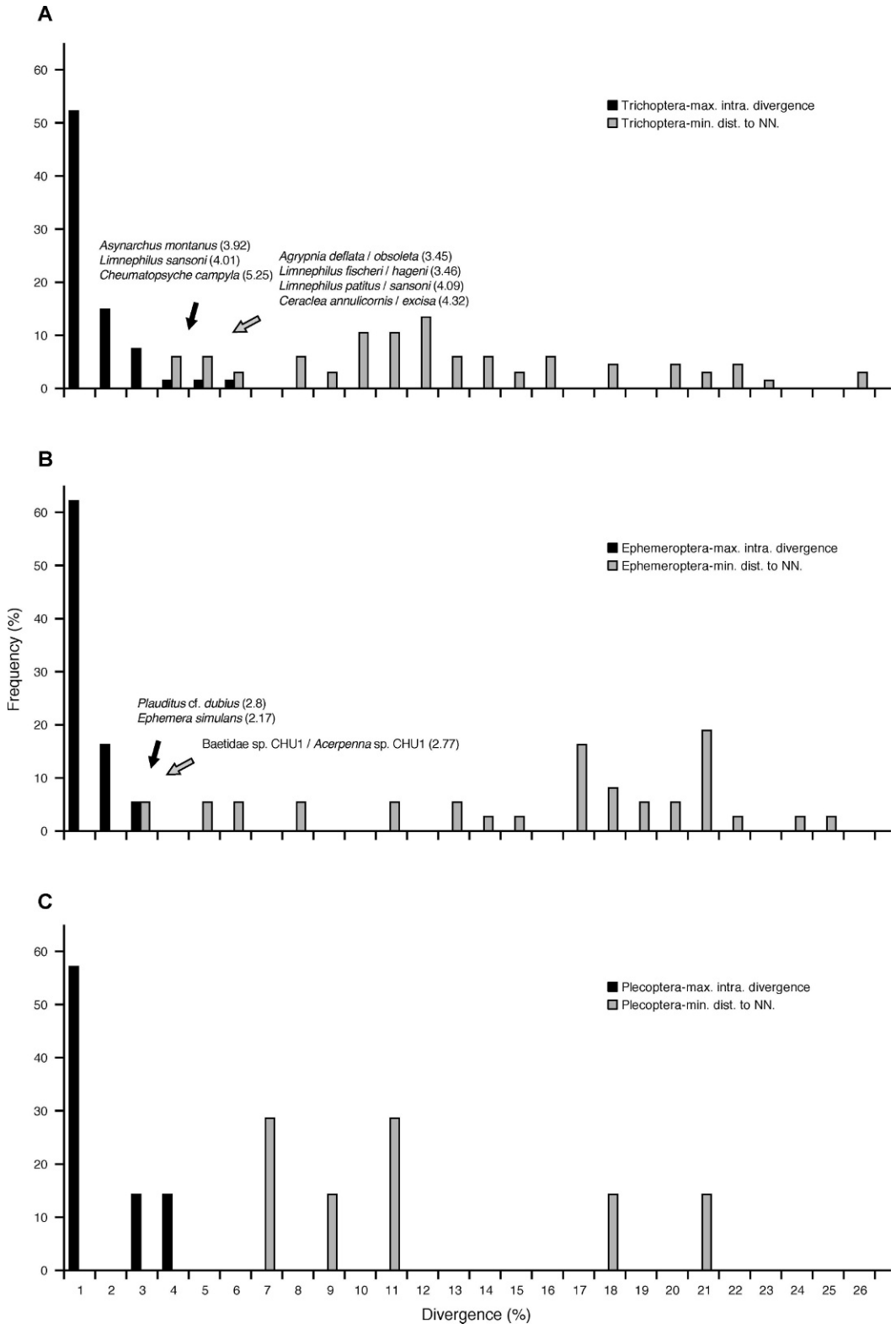

FIG. 4. Intraspecific vs interspecific cytochrome *c* oxidase subunit I (COI) distances in Trichoptera (A), Ephemeroptera (B), and Plecoptera (C) at Churchill. Frequencies of the maximum intraspecific divergence and minimum distance to nearest neighboring species are plotted for the 3 orders. Identities of the species and nearest neighboring species pairs appearing in the overlapping portion are provided with their divergence values. Arrows indicate cases of overlap between intra- and interspecific distance values. Black and grey arrows in panels A and B indicate species showing large intraspecific divergences and species pairs possessing shallow interspecific distances, respectively. Max. intra. divergence = maximum intraspecific divergence, min. dist. to NN = minimum distance to nearest neighbor.

life stages and confirmation of current associations. About ⅓ of the mayfly species in our study were represented by both adults and larvae: *Baetis brunneicolor* McDunnough, *Baetis bundyae* Lehmkuhl, *Baetis phoebus* McDunnough, *Baetisca laurentina* McDunnough, *Dannella lita* (Burks), *Ecdyonurus criddlei* (McDunnough), *Ephemera simulans* Walker, *Heptagenia pulla* (Clemens), *Leptophlebia cupida* (Say), *Procloeon fragile* (McDunnough), and *Siphlonurus alternatus* (Say). The larva of *P. fragile* has not been described, but it was associated with adults via barcodes in our study. However, additional material is needed before their formal description because our specimens were damaged. One mayfly species, *B. phoebus*, is being reinstated from synonymy with *B. flavistriga* McDunnough based on the present DNA barcode data and concordant morphological evidence from a review of eastern Nearctic species of the genus *Baetis* Leach (LMJ, XZ, and PDNH, unpublished manuscript). Eight mayfly species are reported from Manitoba for the first time, including 1 new record for Canada (Table 1). Among these, the distribution range of *Paraleptophlebia aquilina* Harper & Harper, a boreal species previously known only from Oregon (Harper et al. 1995, Harper and Harper 1986, Parsons et al. 1991), is significantly extended eastward. This species is very similar to the eastern US species *Paraleptophlebia assimilis* (Banks), which might be considered more likely to occur in Manitoba, but the 2 species are differentiated easily by size and color (Harper and Harper 1986). The occurrence of *E. criddlei* at Churchill represents a significant northeastern range extension from the western US and Alberta, Canada (McDunnough 1927). However, some identifications of *Nixe* and *Ecdyonurus* species are problematic in central Canada (Webb and McCafferty 2008). We failed to recollect 4 mayfly species previously recorded from Churchill (Table 1, marked with *), but 2 of these species might be represented in our DNA library by haplotypes that can be identified only to the family Heptageniidae at the moment.

We collected only 5 stonefly species that could be identified to species with morphological traits, and 2 additional genetically distinct Nemourinae (probably *Nemoura*) were each represented by only a larva and females, respectively. The low number of stonefly species reflected our use of collecting techniques that were inefficient for sampling stonefly larvae and that our sampling began too late to collect early-emerging species. For example, 2 early-spring-emerging stoneflies, *Capnia nearctica* and *Capnia vernalis*, occur at Churchill but emerge while snow is still on the ground. Larval exuviae of several large species (Perlidae and Perlodidae of Table 1) were observed during our collections, so we know that these species also occur although we failed to collect them.

Temporal and spatial distributions of Churchill caddisflies

The overall shapes and trends of the bias-corrected (number of accumulated species/specimen) and bias-uncorrected (number of accumulated species) species accumulation curves (Fig. 5) were very similar, a result suggesting that the temporal distributions of the detected species are independent of abundances. The shallow slope for the initial sampling dates reflects the combined effect of undersampling (caused by limited collection activity in the early season) and the lower species richness at this time. The subsequent steep slope suggests that this later period is the most critical period for sampling species diversity in Trichoptera.

Emergence of adult Churchill caddisfly species was bimodal with many species emerging around the summer solstice, followed by a lull in activity, then a resumption of species emerging in late summer (Fig. 5). Only 14 species (21%) were collected throughout the entire season (Fig. 6A), and nearly ½ (31/68) were collected within only 1 of the 3 periods. Among caddisfly species detected in 2 periods, adjacent time periods had the greatest overlap in species composition as expected, whereas only 1 species was shared between the earliest and latest time periods.

Of the 68 species of adult caddisflies, 42 were collected from lentic habitats, 29 from the Churchill River, and 8 from small streams (Fig. 6B). Only 4 species (*Agrypnia colorata* Hagen, *Asynarchus montanus* (Banks), *Ceraclea excisa* (Morton), and *Mystacides interjectus* (Banks)) were detected in all 3 habitats, and these cases might reflect adult dispersal because the larvae of these genera are mostly lentic. Overlap in species was greatest between the Churchill River and lentic habitats, and no species were shared only between creeks and the Churchill River. Only 2 medium- to large-sized caddisflies, *Asynarchus mutatus* (Hagen) and *Phryganea cinerea* Walker, were shared between creeks and lentic habitats. They were each represented by a single specimen collected in the creeks, probably because of adult flight. Once larval habitat use is investigated, we expect a substantial decrease in overlap between habitats.

Discussion

We investigated the species diversity of Ephemeroptera, Plecoptera, and Trichoptera (EPTs) at a site in the Canadian subarctic. Patterns of intraspecific and interspecific divergence in DNA barcodes were

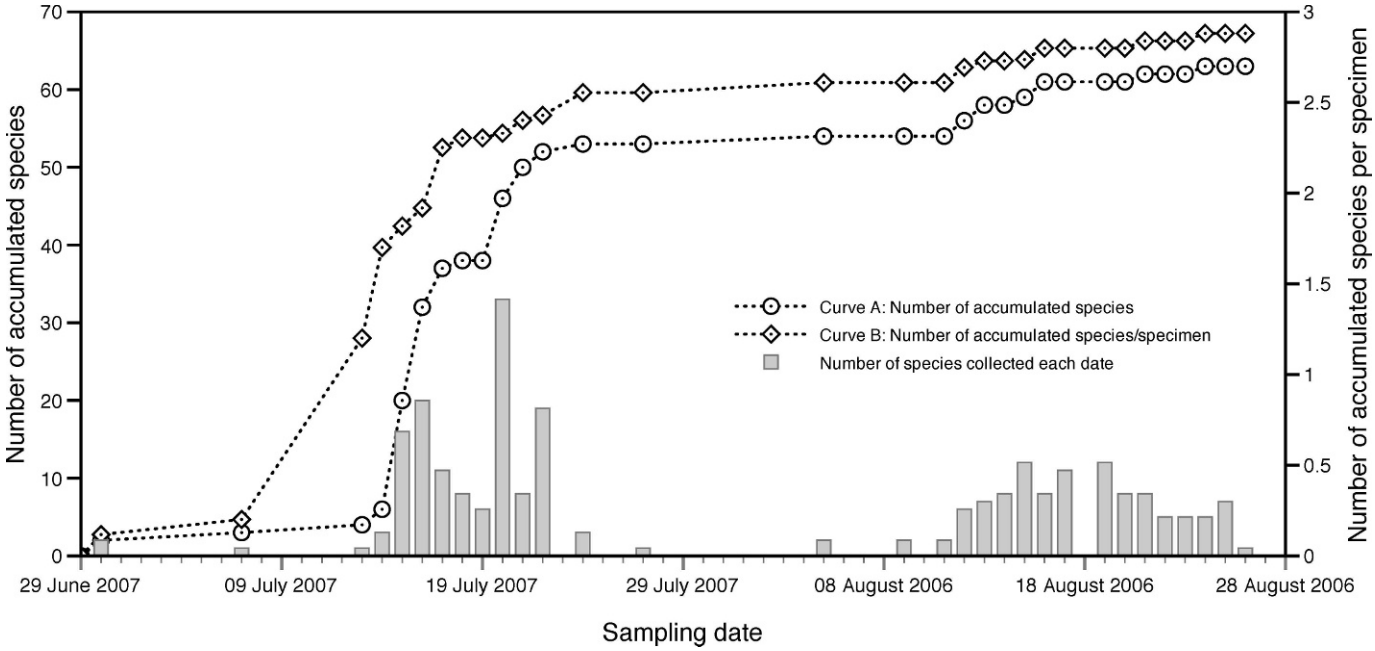

FIG. 5. Temporal distribution of Trichoptera species richness in samples collected in 2006 and 2007. The accumulated number of morphospecies (curve A, represented by circles) is plotted against sampling dates during 5 to 26 August 2006 and 30 June–27 July 2007 to explore the trends in species occurrence in caddisflies at Churchill. The accumulated number of morphospecies divided by the number of samples collected on each day also is included as a correction for potential sampling intensity or abundance bias (curve B).

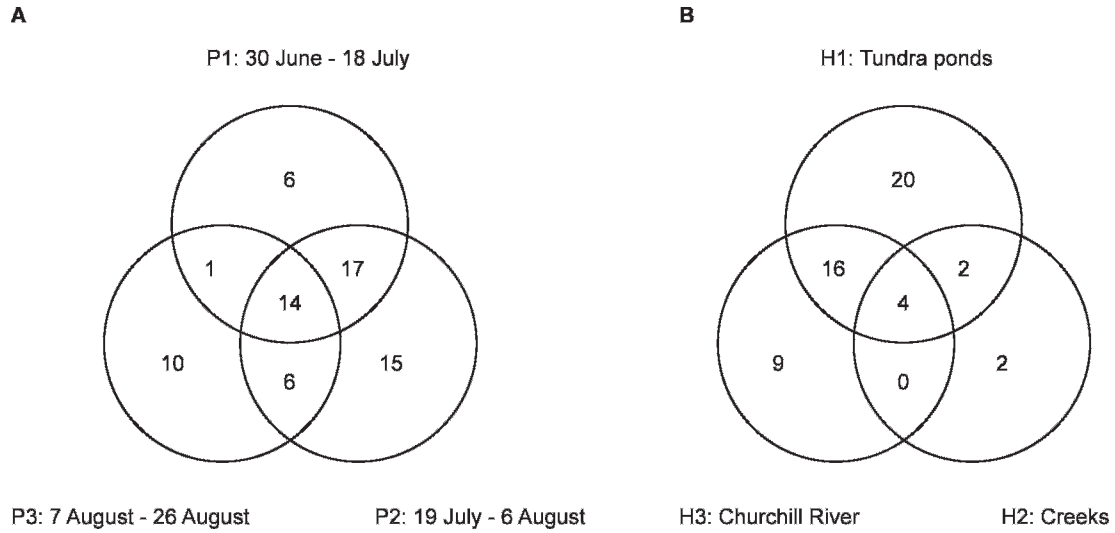

FIG. 6. Temporal overlap and habitat usage in Churchill Trichoptera. A.—Temporal overlap in Trichoptera (2002–2007). Collection dates are divided into 3 periods (P1–P3), each consisting of 18–20 d. The number of species collected during each period is noted in the corresponding area. For example, 14 species were collected throughout P1 to P3, whereas 10 species were collected only during P3. B.—Habitat preference in Trichoptera (2007). Aquatic habitats at Churchill are categorized into 3 types: tundra ponds, tundra creeks, and the Churchill River. The number of species collected in each habitat is noted in the corresponding area. For example, 4 species were collected from all habitats, whereas 9 species were collected only from the Churchill River.

compared with the current taxonomic status and morphological characteristics of species encountered. This combined approach was successful for characterizing this fauna, and resulted in an expanded understanding of the extent of species diversity and distribution, especially among caddisflies.

The barcode- and morphology-based approaches to species recognition were largely concordant. Members of most valid species showed low intraspecific divergences (<2%), much higher interspecific differences, and high bootstrap values for terminal nodes in the Neighbor-Joining tree. The usual discontinuity between maximum intraspecific divergences and distances to the nearest neighboring species indicates the presence of a *barcode gap*, and supports the use of barcode-based identification systems for future ecological and monitoring studies at Churchill. Moreover, in cases where large intraspecific divergences were observed, morphospecies always formed monophyletic groups so these cases do not represent a problem for a barcode-based identification system built for this site. The DNA barcode results also were instrumental for complementing morphological data, so that rare species were revealed and species pairs with subtle morphological differences were separated.

The balance of this discussion is divided into 3 sections. The 1st considers the new insights gained about the diversity of EPT species at Churchill through DNA barcodes. The 2nd describes how DNA barcoding can aid biodiversity surveys across time, space, and researchers. Last, we discuss how lessons from the present study can improve the efficiency of efforts to build comprehensive barcode libraries for all animal species at Churchill and other sites.

DNA barcoding provides finer taxonomic resolution in biotic surveys

DNA barcoding is crucial in detecting rare taxa and species pairs with subtle diagnosis.—Three closely related Hydropsychidae (Trichoptera) species, *Ceratopsyche alhedra* (Ross), *Ceratopsyche bronta* (Ross), and *Ceratopsyche vexa* (Ross), were each represented in our collections by a single female whose presence was detected during the barcode analysis of numerous specimens of *Ceratopsyche alternans* because of their distinct COI sequences. Morphological examination by an independent specialist (R. Blahnik, University of Minnesota) had revealed only the presence of *C. bronta* and *C. vexa*. The third female of *Ceratopsyche* remained unidentifiable via morphology, but its COI sequence was very similar to specimens of *C. alhedra* from sites in Ontario, Minnesota, and Wisconsin (XZ, unpublished data) and was deeply divergent from other congeneric taxa (10.2% sequence divergence from its nearest neighboring species, *Ceratopsyche sparna* (Ross); XZ, unpublished data). Because of the similarity between female hydropsychids and the subtle diagnostic characters (genitalia structures that require dissection and clearing), these locally rare female hydropsychids would have been overlooked in routine morphological sorting. Such oversight probably is a common error in surveys where 1 dominant species co-occurs with several closely related species in much lower abundance. However, this biodiversity heterogeneity can be detected through large-scale DNA barcoding efforts.

DNA barcodes also revealed an unexpected range extension of a caddisfly species by differentiating members of a morphologically similar species pair. A female *Agrypnia* (BOLD Sample ID: 07PROBE-01729) originally identified as *Agrypnia deflata* (Milne), was re-examined when it showed 3.45% sequence divergence from its nearest neighboring *A. deflata* specimen from Churchill. This re-inspection revealed its close affinity to *A. obsoleta*, a species previously thought restricted to northern and central Europe, Asia, and northwestern North America. Although very similar, the females of these sister species can be differentiated by the shape of segment X. The female in question possesses lighter forewings, more highly developed lateral lobes of tergum X, and a deep terminal notch on the median lobe of tergum X, characters used for recognition of *A. obsoleta* (Wiggins 1998). In addition, its COI barcode formed a monophyletic lineage with Mongolian *A. obsoleta* samples, with a within-group divergence of 1.3% and a number of synapomorphic nucleotide variations separating this group from *A. deflata* (XZ, unpublished data). Because this species pair is very similar in morphology, Wiggins (1998) questioned the validity of certain North American records of *A. obsoleta*, especially those southeast of the northern Yukon, and concluded that this species was restricted to Beringia. In contrast, the new record for this species at Churchill indicates a much larger expansion from Eurasia. A thorough investigation of the dispersal history of these taxa is beyond the scope of this paper, but more extensive geographic coverage of COI sequences, perhaps together with other gene markers, should provide detailed insights. Moreover, the larvae of North American *A. deflata* have not been positively associated. The barcode reference library for Trichoptera will assist with the association of larval samples collected from a geographical range of populations and might provide additional larval characters to distinguish *A. deflata* and *A. obsoleta*.

Two members of the *Oecetis inconspicua* (Walker) complex also were differentiated via morphology and DNA barcodes. Considerable variation in the shape of the aedeagus and the inferior appendages on the X^{th} abdominal segment were observed among the Churchill specimens, but most individuals followed descriptions and illustrations of the species (Ross 1944). This species complex requires revision (Floyd 1995, Ross 1944), so most Churchill *O. inconspicua* samples were assigned to a provisional identification, *O.* cf. *inconspicua* CHU2. However, the COI sequence of 1 specimen (BOLD Sample ID: 06-PROBE-0862) was 12.54% different from its nearest neighboring *O.* cf. *inconspicua* CHU2 specimen. Morphological examination revealed that the spine inside its aedeagus was antisymetric to that of typical *O. inconspicua*, a character that had not been observed previously in this group (J. Morse, Clemson University, personal communication). Because this specimen was otherwise indistinguishable from *O.* cf. *inconspicua* CHU2, it probably is an unknown species that would have been overlooked in routine morphological sorting. This single specimen of *O.* cf. *inconspicua* CHU1 awaits the collection of additional specimens before it is formally described.

The mayflies *Baetis hudsonicus* Ide and *B. bundyae* that we examined were very similar morphologically, with primary differences being the relative length of caudal filaments. *Baetis bundyae* is widespread in northern and western North America, but *B. hudsonicus* has a distribution restricted to the far north (Giberson et al. 2007, McCafferty and Randolph 1998). DNA barcode analyses at Churchill provide evidence for maintaining the validity of these sibling species and refute past suggestions of their synonymy (Morihara and McCafferty 1979). Furthermore, *B. bundyae* was considered as a subspecies of *B. macani* Lehmkuhl until McCafferty (1994) reinstated it to full species status based on anecdotal evidence from Europe, where the 2 species might co-occur. Further genetic analyses of all eastern Nearctic *Baetis* species, including *B. hudsonicus* and *B. bundyae*, and published European *Baetis vernus* Curtis-group species, including *B. macani* (from Ståhls and Savolainen 2008 and Williams et al. 2006), are being conducted by authors of this paper (LMJ, XZ, and PDNH, unpublished data). The preliminary results indicate that our Churchill *B. bundyae* are nested within 1 of the cryptic European *B. macani* haplotype groups that is confined to lotic habitats and possesses narrow gills and invisible tracheae. This haplotype group was proposed by Ståhls and Savolainen (2008) as true *B. bundyae* distributed in Finland. Thus, our findings at Churchill confirm their results.

Two barcode haplogroups of *Acentrella turbida* (McDunnough) (noted as *A. turbida* D and E in our paper) were collected at Churchill, but 5 lineages of this species have been detected thus far from North America (XZ and LMJ, unpublished data). Unlike the adults, the *Acentrella* larvae associated with these species (LMJ and XZ, unpublished data) differ in body coloration, setation, and leg morphology. However, names cannot be assigned to these species until detailed investigation of related species in *Acentrella* and other genera is complete (Lugo-Ortiz and McCafferty 1998, McCafferty et al. 2005). Unfortunately, our attempts to recover DNA barcodes from dry, pinned specimens contemporaneous with types in this genus (>50 y old) were not successful, a result suggesting that the recovery of full-length barcodes from mayfly types is not feasible at this time.

Similar observations were made in other taxonomic groups, such as *Oecetis* cf. *ochracea* (Curtis) CHU1 and CHU2, *Plauditus* sp. CHU1 and CHU2, and *Fallceon thermophilos* (McDunnough) complex (see following discussion). In each case, cryptic morphotypes were first detected and differentiated by DNA barcodes and then confirmed by additional morphological scrutiny.

DNA barcoding sheds light on species delimitation in a mayfly complex with highly variable morphology.— Hindwings have been very important to species- and genus-level identifications of mayfly adults from the family Baetidae in middle North America (Traver 1935, Waltz and Burian 2008). However, recent findings have complicated long-held notions of generic boundaries (McCafferty et al. 2008) because some genera previously identified by hindwing characteristics demonstrate considerable variation that overlaps with other genera.

Some specimens that we examined revealed further complications. A group of small minnow mayfly specimens was very difficult to identify because of high variability in hindwing morphology, in contrast to overall morphological similarity including size and coloration. These specimens are collectively referenced in this paper as the ''*Fallceon thermophilos* complex'' (Fig. 2) because the presence of a long 3^{rd} vein in the hindwing is characteristic of that species (McCafferty et al. 2008). DNA barcoding separated these difficult specimens into 2 haplotype groups, in which sequence variability was concordant with observed morphological divergence, a result that allowed identification of morphological characteristics that separated members of the 2 clusters.

Specimens in Cluster I lacked sequence diversity (9 individuals with an identical barcode sequence) and

demonstrated low variation in hindwing morphology. Members of this cluster possess a very long, marginal intercalary vein on the hindwing that resembles the longitudinal vein parallel to the 2nd longitudinal vein, but this vein does not extend to the base of the wing. The overall shape of the wing is reminiscent of the genus *Acerpenna*, although it does not have the same undulate upper margin. Thus, this cluster is recognized in this study as *Acerpenna* sp. CHU1.

In contrast, Cluster II is much more diverse in its hindwing morphology, to the extent that individuals might be regarded as members of different genera. These specimens also have what appears to be either a long intercalary vein or a 3rd longitudinal vein. In some specimens, this vein runs parallel to the 2nd longitudinal vein and nearly reaches the basal part of the wing. These specimens were the basis for the initial *F. thermophilos* identification of the 2 clusters that was made without knowledge of barcode results. Among these specimens, the hindwing vein in question matches narrative descriptions of *F. thermophilos* but is longer than the vein figured by Traver (1935). In other Cluster II specimens, the vein is shorter and angles slightly towards the 2nd vein. These specimens are generally similar to Cluster I, but the shape of the upper margin of the wing is more convexly undulate. In yet other Cluster II specimens, the 3rd vein is angled toward the 2nd vein and connects to it. When this happens, the 2nd vein is bent slightly toward the upper margin of the wing, and creates a distinctive fork that is consistent with the present concept of the genus *Diphetor* in North America, which contains a single polytypic species, *Diphetor hageni* (Eaton) (Meyer and McCafferty 2001). However, the hindwings of our specimens appeared to be slightly more narrow and elongate than in *Diphetor*. In addition, the barcode sequences of the Churchill samples are very different from *D. hageni* specimens from Indiana, Florida, and Pennsylvania (with a distance of 19.5% to each group's nearest neighboring member), which themselves form a monophyletic group with deep within-group divergence (with a mean intraspecific divergence of 5.1%; XZ, unpublished data). Because of these taxonomic uncertainties, members of Cluster II have simply been treated as Baetidae sp. CHU1 (Fig. 2).

These observations demonstrate the utility of DNA barcode data as a precursory means of specimen sorting, especially for cases where traditional diagnostic characteristics are highly variable or poorly characterized. Our findings shed light on difficulties associated with proper identification of adults in the family Baetidae and, therefore, bring into question some historical taxonomic work, especially those involving single, few, or only a small series of specimens. We emphasize the need for more extensive study of series of adult specimens of both sexes, ideally associated with larvae.

DNA barcoding suggests potential cryptic species.—In a few cases, distinct COI haplotype clusters were present among individuals assigned to a single species under current species hypotheses. In these cases, no consistent diagnostic morphological characters were found to separate members within each group, but studies have not extended to an examination of larval morphology or habitat selection.

Two caddisfly species, *Limnephilus sansoni* and *Cheumatopsyche campyla*, each showed deep intraspecific divergences (maximum 4.01% and 5.25%, respectively), with this variation falling into 2 or 3 distinct COI clusters, respectively. We did not find consistent morphological traits that would differentiate adult members of these distinct COI haplogroups. This result is not surprising because taxonomic ambiguities are known in species of *Limnephilus* and *Cheumatopsyche*. Similar intraspecific divergence patterns at barcode loci also were observed in *C. campyla* collected from a much broader geographic region in eastern North America, where the groupings of COI haplotypes were not correlated to geography (XZ, unpublished data). The high mitochondrial divergences within specimens from single localities, combined with close affinity of the major COI haplotypes across broad geographic areas, suggests that *C. campyla* might include cryptic species, several of which often occur in sympatry. However, when several closely related *Cheumatopsyche* species were included in the analysis, the Churchill *C. campyla* haplotypes seemed to intermix with at least 3 eastern Nearctic *Cheumatopsyche* species—*C. speciosa* (Banks), *C. ela* Denning, and *C. pasella* Ross (XZ, J. L. Robinson, C. J. Geraci, C. R. Parker, O. S. Flint, D. Etnier, D. Ruiter, REDW, LMJ, and PDNH, unpublished data). Such results suggest that this species complex might have undergone recent speciation and is subject to incomplete lineage sorting and gene introgression, which also has been reported in other caddisfly species (Pauls et al. 2009, Waringer et al. 2007). The taxonomic uncertainty in the *C. campyla* complex was indeed reflected in our Churchill material, where an independent specialist named the same set of *Cheumatopsyche* specimens as *C.* nr. *ela*. Nevertheless, when considering Churchill material alone, DNA barcoding can precisely assign query samples (e.g., larvae) to the same haplotype groups, despite their taxonomic ambiguity.

No *Limnephilus sansoni* from other regions are currently available to us, but the deep divergence

between the 2 haplogroups (HPGP1 and HPGP2; Fig. 1) at Churchill matches interspecific distances between valid *Limnephilus* species at this locality, e.g., *L. hageni* and *Limnephilus fischeri* Ruiter (see Table 1). Furthermore, both haplogroups of *L. sansoni* are genetically close to some Norwegian specimens of *Limnephilus femoratus* (Zetterstedt) (XZ, unpublished data), a species also known from the Kuril Islands in the Western Pacific (Minakawa et al. 2004). The similarity in COI sequences between Churchill *L. sansoni* and Norwegian *L. femoratus*, and the disjunct records of the latter species, suggest the need for further study to clarify their status. Even though high intraspecific divergence (e.g., bracketed terminals in Figs 1–3) does not necessarily suggest cryptic speciation, such observations draw attention to the potential need for taxonomic revision.

One *Isoperla* stonefly species could not be identified to species level because the most important diagnostic character, the aedeagus, was not extruded in the field. Thus, members of this cluster of samples were simply treated as *Isoperla* sp. in this paper. One female specimen (07PROBE-02689) had a mean divergence of 2.85% from other members of the cluster and contributed to the large maximum intraspecific divergence within the group (3.13%). This specimen cannot be differentiated from the others by color. Given that diagnosing female *Isoperla* is difficult, the collection of fresh, male specimens with extruded aedeagi is needed to determine the identity of this and other *Isoperla*.

DNA barcoding enables consistency among biodiversity surveys

DNA barcoding has the potential to act as a powerful quality assurance tool for biodiversity surveys by ensuring consistency in taxonomic assignments through time and across space. Taxon concepts change over time (e.g., see Table 1, regarding EPT synonyms in previous studies) and the application of names can vary among specialists (e.g., *Cheumatopsyche campyla* samples examined in this study) and over time for the same specialist as species concepts change. It is time-consuming and sometimes impractical to incorporate all relevant information about synonymies when interpreting a series of biotic surveys—taxonomic expertise, keys used, and life stages collected vary tremendously across studies. Moreover, barcoding increases the value of biotic surveys conducted by nonspecialists. For example, none of the caddisflies collected at Churchill in an early faunistic study (McClure 1943) was identified even to family level because of the limited taxonomic expertise of the researcher. Such records contribute little to our understanding of the Trichoptera assemblage. By contrast, future studies of Churchill's EPTs will benefit from the present DNA barcode library even if taxonomic assignments are revised over time.

DNA barcode results also can aid the comparison of biodiversity surveys at different locations. In addition to differences in taxonomic concepts across biogeographic regions and a lack of access to comparative or type material, geographical variability in morphology can make it difficult to achieve consistent species-level assignments. DNA barcodes, in combination with morphological and ecological traits, are invaluable for overcoming this problem.

Last, even when species identities remain unknown, barcoding provides a useful interim system for documenting biodiversity. The linking of DNA barcodes to formal species names in biosurveillance is certainly desirable, but is not always possible in the short term if species are undescribed or poorly known. However, DNA barcodes enable comparisons of provisional taxonomic entities collected in various surveys even when specific identification is impossible. For example, several EPT samples (females, larvae, or subimagos) encountered in our study could be identified only to genus or family level because their COI sequences did not match any available reference barcodes. However, the lack of a specific identification did not prevent their detection and registration. The fact that they were readily differentiated by barcodes from related taxa further suggests that these unknowns will gain a species identification as the barcode reference library expands. Linnaean names can be provided to the identifiable specimens acquired in future works and associated with samples collected in the current study via the DNA barcode library. Of course, progress towards achieving a comprehensive DNA barcode reference library of the North American EPT groups also might eventually link currently unknown COI haplotypes to described species.

Critical factors for conducting comprehensive biodiversity surveys: time and space

Several factors might lead to more comprehensive regional biotic surveys. The 2 most critical factors are temporal and habitat coverage. In addition to improvements to our understanding of species diversity through barcoding efforts, the dramatic extension of the Churchill caddisfly and mayfly checklists (and discovery of new species and records for Canada) are obvious benefits of our extensive collecting efforts over time and space for that region.

Our samples covered most of the emergence season for the fauna under investigation. Programmed life cycles characterized by long winter diapause and condensed summer development are one of the typical adaptations of aquatic insects for life in cold climates (reviewed in Danks 2007). Therefore, most adult insects emerge within a short time window in the subarctic. This pattern was reported in the study of biotic communities of the Churchill area (McClure 1943) and was also observed for the caddisflies in our study. The time frame with the highest arthropod diversity in the former study (~July 12) coincided with the peak emergence period in our caddisfly records (July 16–23). However, even within such a short active period, different Churchill caddisfly species showed distinct adult flight patterns. Only 19 of 68 caddisfly species flew for >4 wk. Most species (44) were encountered only over <2 wk, some late in the season (e.g., *Anabolia bimaculata* (Walker), *Asynarchus rossi*, *Limnephilus externus* Hagen, *Limnephilus infernalis* (Banks), *Limnephilus nigriceps* (Zetterstedt), *Limnephilus sansoni*, *Rhyacophila angelita* Banks, and others). Broad coverage of the emergence season is critical for understanding the biodiversity composition in any biosurveillance study.

Broad habitat coverage is also important for comprehensive species surveys. Strong habitat associations were observed even in Churchill caddisfly adults, whose mobility allowed for travel across habitat types. For example, ~½ of the caddisfly species were collected only from 1 of the 3 habitat categories. A clear message from our study is that, as with traditional surveys, sampling in a variety of habitat types should be done in efforts to barcode biota.

Many cold-climate aquatic insects have life histories that extend for ≥2 y (Danks 2007), and in such cases, adults might be present in alternate years. Such cases are known for certain Lepidoptera species at Churchill, and our records revealed many caddisflies that were collected in only 1 y. However, a longer sampling program is necessary to test if this pattern was a chance observation or an important characteristic of caddisfly population dynamics in the region.

This contribution has substantially extended the species checklists for Trichoptera and Ephemeroptera at Churchill. However, despite 5 y of collecting, we failed to encounter several species recorded in previous studies, especially for Plecoptera. Moreover species accumulation curves (Zhou et al. 2009) indicate that species richness has not reached an asymptote in any of the 3 groups. This situation is typical of most biodiversity surveys, but rare species, or those with multiyear life cycles, can be incorporated into the reference barcode library over time.

Conclusion

DNA barcoding of the Ephemeroptera, Plecoptera, and Trichoptera at Churchill has proven effective in identifying species, detecting rare taxa, suggesting potential cryptic species, and in documenting α diversity. Our existing knowledge of these 3 groups of relatively well-studied freshwater insects helps interpretation of the barcode data and leads to suggestions for improving biosurveillance techniques and efforts to barcode entire biotas. The value of barcoding EPTs will increase when the DNA library is used to associate all life stages of a species, to improve the comparability of parallel biosurvey results, and to increase the efficiency and coverage of the biological monitoring of freshwater systems.

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