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Eight new provincial species records of mayflies (Ephemeroptera) from one Arctic watershed river in British Columbia

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Abstract.—We repeatedly sampled 8 sites on the Crooked River in British Columbia's Arctic watershed for adult and nymph mayflies (Ephemeroptera) over the course of 2 years. Using taxonomic keys and DNA barcoding, we report 8 new species records for the province: 5 members of the family Baetidae (*Acerpenna pygmaea*, *Baetis phoebus*, *Baetis vernus*, *Iswaeon anoka*, and *Procloeon pennulatum*), 1 Heptageniidae (*Leucrocuta hebe*), 1 Leptohyphidae (*Tricorythodes mosegus*), and 1 Siphlonuridae (*Siphlonurus alternatus*). Three of these, *Acerpenna*, *Iswaeon*, and *Leucrocuta*, are also new genus records for the province. In total, we detected 40 species in 8 families as indicated by clustering into BINs (Barcode Index Numbers), by morphological keys, and by matches in the Barcode of Life Database. One of those species, *Ameletus vernalis*, is of conservation concern. Our analysis indicated that a number of other specimens may represent new species or genus records for British Columbia. In addition, this unique and anthropogenically impacted river may contain cryptic species of *Baetis tricaudatus* (Baetidae), *Leptophlebia nebulosa* (Leptophlebiidae), and *Paraleptophlebia debilis* (Leptophlebiidae).

Resumen.—Muestreamos en repetidas ocasiones ninfas y adultos de moscas efímeras (Ephemeroptera) en ocho sitios del río Crooked en la cuenca ártica de British Columbia, durante dos años. Usando claves taxonómicas y códigos de barras de ADN, reportamos ocho nuevas especies en la provincia. Dichas especies se componen de: (1) cinco miembros de la familia Baetidae (*Acerpenna pygmaea, Baetis phoebus, Baetis vernus, Iswaeon anoka* y *Procloeon pennulatum*), (2) un miembro de la familia Heptageniidae (*Leucrocuta hebe*), (3) un miembro de la familia Leptohyphidae (*Tricorythodes mosegus*) y (4) un miembro de la familia una Siphlonuridae (*Siphlonurus alternatus*). Además, tres de ellas (*Acerpenna, Iswaeon* y *Leucrocuta*) son nuevos registros de géneros en la provincia. Usando las técnicas de agrupamiento del Índice del Código de Barras (BIN, por sus siglas en inglés), claves morfológicas y coincidencias encontradas en las bases de datos de los códigos de barras, en total, detectamos 40 especies de ocho familias. Una de esas especies, *Ameletus vernalis*, se encuentra en peligro de extinción. Nuestro análisis indicó que cierta cantidad de otros ejemplares podrían representar registros de nuevas especies o géneros en British Columbia. A si mismo, es posible que este río de características únicas y antropogénicamente afectado albergue especies crípticas de *Baetis tricaudatus* (Baetidae), *Leptophlebia nebulosa* (Leptophlebiidae) y *Paraleptophlebia debilis* (Leptophlebiidae).

Anthropogenic climate change is impacting global biodiversity, especially in lotic ecosystems (Meyer et al. 1999, Parmesan 2006, Bowman et al. 2010). The Ephemeroptera (mayflies) comprise one of the major invertebrate orders in streams and rivers. Many mayflies require cool, well-oxygenated water, and many are sensitive to pollutants (Bauernfeind and Moog 2000, Richardson and Kiffney 2000). As a result, managers frequently use metrics based at least in part on mayfly biodiversity as an ecological indicator of water quality (Lenat and Barbour 1994, Bauernfeind and Moog 2000).

Morphology-based identification of mayflies can be challenging, as detailed keys and expertise developed over years are often needed for definitive identification, immature larvae are not identifiable in keys, and expertise may be limited to particular groups. In addition, keys to species level are not available for some genera (Kenner et al. 2001). The use of DNA barcoding and DNA sequence databases of verified specimens has facilitated identification of

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mayflies to species level (Ball et al. 2005, Ratnasingham and Hebert 2007). The alignment of a 648-bp fragment of the cytochrome c oxidase I (COI) gene sequence has been successfully used in delineating species (Hebert et al. 2003). COI sequence analyses also revealed that DNA sequence variation of the COI gene seldom exceeds 2% within a species (Hebert et al. 2003), and for mayflies a 2% threshold has been shown to be a good standard (Webb et al. 2012). COI sequences often reveal clear divergence and much greater biodiversity than expected within existing species (Blaxter et al. 2004). Such divergences may suggest presumptive species that are then organized into operational taxonomic units (OTU). The Barcode Index Number (BIN) system takes into account existing taxonomy (to either family, genus, or species level) and uses established algorithms to generate BINs that facilitate the identification of putative OTUs or species (Ratnasingham and Hebert 2013).

In British Columbia there are currently 94 known species, but these records reflect collections mainly limited to the southern and coastal regions of the province (Wigle and Thommasen 1990, McCafferty et al. 1994, Zloty 1996, McCafferty and Randolph 1998, Jacobus and McCafferty 2001, Scudder 2007, McCafferty et al. 2012). With the exception of an identification of a mayfly from the Liard Hot Springs in northeastern British Columbia (Kenner et al. 2001), no work has been published in the central and northern interior British Columbia.

Our objective was to explore the mayfly diversity of the Crooked River because of the river's initially observable insect abundance and seeming diversity, because of its unique characteristics, and because it may provide an indication of the diversity of the surrounding, minimally explored area. Our work has revealed that this one system contains a large proportion of British Columbia's 94 known species of mayflies. In addition, we report 8 new species records for the province, which include 3 genera previously not observed in British Columbia. We also report a number of other records that may represent new species/genera or cryptic species.

Study Area

The Crooked River is a small system on the very southern edge of the Arctic watershed in the central interior of British Columbia (Fig. 1). It flows northward out of Summit Lake, and its water eventually enters the Williston Reservoir and later flows to the Arctic Ocean via the Peace River and then the Mackenzie River. It varies from wide lake-like areas to meandering portions to fast-flowing riffles and straight runs. The river is partially fed by several springs that help to moderate seasonal temperature changes throughout its length. The headwaters are also situated near an extinct volcano, Teapot Mountain, which may provide mineral nutrient input to the river. The Crooked River is susceptible to regular spring flooding beyond its banks, which functions to provide further allochthonous input. The river is home to substantial populations of fish, and casual observations reveal that it supports massive populations of a variety of aquatic invertebrates along with copious streamside and instream vegetation.

While a provincial park offers protection to a small portion, the river is otherwise highly impacted by anthropogenic activities and mainly unprotected. A major highway and a rail line closely parallel much of the river. Proposed oil pipelines, ongoing logging, and related road and bridge building pose ongoing and cumulative impacts. The Crooked River also receives a great deal of recreational attention, including camping, angling, hunting, and off-road vehicle use; it is easily accessed by roads and is only about a 1-h drive from Prince George, British Columbia (population ~80,000). To our knowledge, the northern portions of British Columbia's vast Interior Plateau have not been historically well surveyed for insect biodiversity in any way even slightly proportional to their geographic extent.

METHODS

We sampled 8 sites along the Crooked River on a near-weekly basis during the springs and summers of 2014 and 2015, starting in early May of each year and ending in late August. In locations where permission was required (i.e., Crooked River Provincial Park), we collected under the British Columbia Ministry of Environment Park Use Permit #107171. We used kicknets and hand sampling to collect nymphs (2014), and hand sampling (2014 and 2015), sweep nets (2015), and Malaise traps (2015) to collect adults. Collection sites

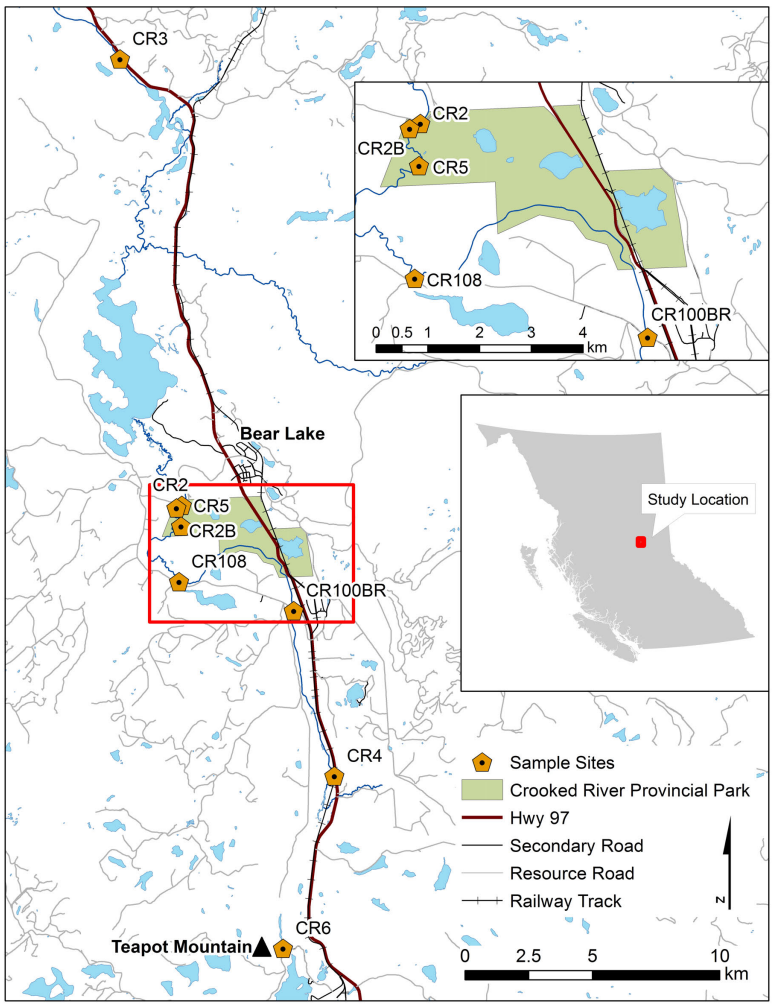

Fig. 1. Map of the Crooked River in British Columbia, Canada, showing main features in the sampling area. Sample sites are CR2 (54.484° N, 122.721° W), CR2B (54.484° N, 122.721° W), CR3 (54.643° N, 122.743° W), CR4 (54.388° N, 122.633° W), CR5 (54.478° N, 122.719° W), CR6 (54.328° N, 122.669° W), CR100BR (54.446° N, 122.653° W), and CR108 (54.458° N, 122.722° W).

were located and designated as follows (Fig. 1): CR2 (54.484° N, 122.721° W), CR2B (54.484° N, 122.721° W), CR3 (54.643° N, 122.743° W), CR4 (54.388° N, 122.633° W), CR5 (54.478° N, 122.719° W), CR6 (54.328° N, 122.669° W), CR100BR (54.446° N, 122.653° W), and CR108 (54.458° N, 122.722° W). Sampling for nymphs was carried out at all 8 sites along with some limited adult sampling. Intensive adult sampling with sweep nets (half-hour total effort per site per visit) and Malaise traps was carried out at CR2B, CR3, CR4, and CR108 in 2015.

Over the course of the study, we completed 109 sampling events and collected a total of 7212 mayflies. Captured insects were immediately placed in 80% ethanol and transported to the lab, where they were stored in a freezer until they could be processed. Samples were first sorted to morphospecies, and then at least to genus for nymphs (Needham et al. 1935, Clifford 1991, Merritt and Cummins 1996, Needham 1996) and to family or sometimes genus level for adults (Hafele and Hughes 2004). Following sorting, 201 specimens were sent to the Canadian Centre for DNA Barcoding, and we received back 197 successful barcode (cytochrome oxidase I) sequences >300 base pairs in length. Sequenced specimens

and their associated sequence data were vouchered at the Biodiversity Institute of Ontario and the Barcode of Life Database (BOLD) (Ratnasingham and Hebert 2007). These are publicly available as data set https://dx.doi.org/10.5883/DS-CREPH. The barcode sequences are also available at GenBank (accession numbers MH916886 through MH917082). Barcode Index Numbers (BINs) were assigned automatically at BOLD in 38 of 40 cases. Where species names diverged between BOLD and Mayfly Central (https://www.entm.purdue.edu/mayfly/na-species-list.php [accessed 28 June 2017]), we used the current nomenclature listed at Mayfly Central. This only affected *Neoleptophlebia heteronea* and *Neoleptophlebia memorialis*, which were listed at BOLD as *Paraleptophlebia heteronea* and *Paraleptophlebia memorialis.* Tiunova and Kluge (2016) transferred these species from *Paraleptophlebia* to *Neoleptophlebia.*

The known ranges for the species that we identified from the Crooked River were determined using several databases and region-specific identification guides. Online databases of known mayfly ranges or within-province accounts were NatureServe (NatureServe 2017), E-fauna BC (Klinkenberg 2017), and Mayfly Central, as well as published species accounts for mayflies in British Columbia (Wigle and Thommasen 1990, McCafferty et al. 1994, Needham 1996, Zloty 1996, McCafferty and Randolph 1998, Jacobus and McCafferty 2001, Scudder 2007, and McCafferty et al. 2012). The ranges of those species that were determined from sequencing to be in the Crooked River were examined, and species not known to be present in British Columbia according to any of the databases that we checked were considered new records for the province. DNA sequences were aligned in BOLD via MUSCLE to generate neighbor-joining trees (Edgar 2004) using the Kimura 2-parameter model (Kimura 1980). Trees were visualized with FigTree v.1.4.3 (http://beast.bio.ed.ac.uk/figtree [accessed 28 June 2017]).

RESULTS AND DISCUSSION

New Mayfly Species for British Columbia

The Crooked River contains at least 40 species of mayflies in 9 families (Table 1). Eight of the records in 4 families are new records for British Columbia. These are *Acerpenna pygmaea* (Hagen, 1861) (Baetidae); *Baetis phoebus* McDunnough, 1923 (Baetidae); *Baetis vernus* Curtis, 1834 (Baetidae); *Iswaeon anoka* (Daggy, 1945) (Baetidae); *Procloeon pennulatum* (Eaton, 1870) (Baetidae); *Leucrocuta hebe* (McDunnough, 1924) (Heptageniidae); *Tricorythodes mosegus (*Alba-Tercedor & Flannagan, 1995) (Leptohyphidae); and *Siphlonurus alternatus* (Say, 1824) (Siphlonuridae). *Acerpenna pygmaea* (and a second *Acerpenna* species), *Iswaeon anoka*, and *Leucrocuta hebe* are each also the first species records in their respective genera for the province.

Our analyses align with the concepts (1) that BINs generally correspond to discrete species among many insect and other arthropod taxa (Hebert et al. 2003, Smith et al. 2017); (2) that Ephemeroptera specimens with >2% divergence are usually discrete species (Zhou et al. 2009, Webb et al. 2012, Cordero et al. 2016); and (3) that the North American mayfly assemblage has been substantially and accurately surveyed (Zhou et al. 2009, 2010, Webb et al. 2012, Cordero et al. 2016).

BINs Reveal Potential New Species

A number of sequenced specimens clustered in existing BINs but were not identifiable to the species level by that method. These included

1 *Ameletus* sp. (BOLD:ABA4299),
1 *Acerpenna* sp. (BOLD:AAC3979),
1 *Callibaetis* sp. (BOLD:ACI3026),
1 *Procloeon* sp. (BOLD:AAG5056),
1 Baetidae (BOLD:ADA1160),
1 *Ephemerella* sp. (BOLD:ACL4202),
2 *Cinygmula* spp. (BOLD:ADA2747 and BOLD:ABA3456),
1 Heptageniidae (BOLD:ADA2851), and
1 *Siphlonurus* sp. (BOLD:AAF3899).

For BOLD:ABA4299, identified in our analysis as *Ameletus* sp., the nearest neighbor (8.3%) is BOLD:ACK1998, identified as *Ameletus doddsianus*. For BOLD:AAC3979, identified in our analysis as *Acerpenna* sp., the nearest neighbor (2.55%) is BOLD:AAC4626, identified as *Acerpenna* sp. For BOLD:ACI3026, identified in our analysis as *Callibaetis* sp., the nearest neighbor (3.36%) is BOLD:AAC7440, identified as *Callibaetis ferrugineus*. For BOLD:AAG5056, identified in our analysis as *Procloeon* sp., the nearest

Table 1. Specimens of Ephemeroptera collected along the Crooked River, British Columbia, in 2014 and 2015 that were DNA barcoded (cytochrome oxidase I [COI]), along with their barcode-assigned identifications[a].

Specimen ID	BIN	Family	ID	Collection sites[b]	Notes
E55-CR1 and 1 other	BOLD:ABA4299	Ameletidae	*Ameletus* sp.	CR108	
E51-CR1 and 2 others	BOLD:AAF2991	Ameletidae	*Ameletus vernalis*	CR3, CR108	NatureServe N3 (US), N2 (CAN)[c]
E-178-CR2BS and 3 others	BOLD:AAC3979	Baetidae	*Acerpenna* sp.	CR2B, CR3, CR108	
E-143-CR3S and 4 others	BOLD:AAB7866	Baetidae	*Acerpenna pygmaea*	CR3	New BC genus/species record
E77-CR100 and 6 others	BOLD:AAA2041	Baetidae	*Baetis phoebus*	CR4, CR100, CR108	New BC species record
E11-CR5 and 4 others	BOLD:AAJ9779	Baetidae	*Baetis tricaudatus*	CR5	See Fig. 3
E-140-CR4S and 15 others	BOLD:AAL5544	Baetidae	*Baetis tricaudatus*	CR3, CR4, CR5, CR100, CR108	See Fig. 3
E18-CR6	BOLD:AAB1424	Baetidae	*Baetis vernus*	CR6	New BC species record
E95-CR2B and 2 others	BOLD:AAE0585	Baetidae	*Callibaetis ferrugineus*	CR2B, CR100	
E33-CR3 and 11 others	BOLD:ACI3026	Baetidae	*Callibaetis* sp.	CR6	
E54-CR1 and 6 others	BOLD:AAC2232	Baetidae	*Diphetor hageni*	CR2B, CR4, CR100, CR108	
E79-CR2B and 7 others	BOLD:ABY7648	Baetidae	*Iswaeon anoka*	CR2B, CR3	New BC genus/species record
E-180-CR4S	BOLD:ADA1160	Baetidae	none given	CR4	
E72-CR4	NO BIN	Baetidae	none given	CR4	
E38-CR2B	BOLD:AAC9431	Baetidae	*Procloeon pennulatum*	CR2B	New BC species record
E203.CR6	BOLD:AAG5056	Baetidae	*Procloeon* sp.	CR6	
E24-CR6	BOLD:AAA7515	Caenidae	*Caenis youngi*	CR6	
E-192-CR4S_rerun and 2 others	BOLD:AAZ4020	Ephemerellidae	*Attenella margarita*	CR2B, CR4, CR108	
E14-CR2B and 5 others	NO BIN	Ephemerellidae	*Drunella* sp.	CR2B, CR4	
E1-CR4 and 5 others	BOLD:AAL1912	Ephemerellidae	*Drunella grandis*	CR2, CR4, CR108	
E67-CR2B and 18 others	BOLD:AAZ1958	Ephemerellidae	*Ephemerella dorothea infrequens*	CR2B, CR3, CR4, CR6, CR100, CR108	

TABLE 1. Continued.

Specimen ID	BIN	Family	ID	Collection sites[b]	Notes
E40-CR2 and 4 others	BOLD:ACL4202	Ephemerellidae	*Ephemerella* sp.	CR2, CR4, CR108	
E78-CR100 and 6 others	BOLD:AAL0644	Ephemerellidae	*Ephemerella tibialis*	CR2B, CR4, CR100, CR108	
E-159-CR2BS and 2 others	BOLD:AAA7231	Ephemeridae	*Ephemera simulans*	CR2B, CR3	
E90-CR1 and 2 others	BOLD:ABA3456	Heptageniidae	*Cinygmula* sp.	CR4, CR108	See Fig. 2
E56-CR2 and 7 others	BOLD:ADA2747	Heptageniidae	*Cinygmula* sp.	CR2, CR4, CR5, CR108	See Fig. 2
E16-CR100	BOLD:AAX7171	Heptageniidae	*Epeorus albertae*	CR100	
E26-CR3 and 1 other	BOLD:AAC9881	Heptageniidae	*Leucrocuta hebe*	CR3	New BC genus/species record
E-158-CR3S and 2 others	BOLD:AAC3145	Heptageniidae	*Maccaffertium terminatum*	CR3	
E41-CR1 and 8 others	BOLD:ADA2851	Heptageniidae	none given	CR2, CR108	
E25-CR6_rerun and 3 others	BOLD:ADA2500	Leptohyphidae	*Tricorythodes mosegus*	CR3, CR6	New BC record
E82-CR100 and 12 others	BOLD:AAA7017	Leptophlebiidae	*Leptophlebia nebulosa*	CR2, CR2B, CR3, CR4, CR100, CR108	See Fig. 4
E61-CR2	BOLD:AAD3328	Leptophlebiidae	*Leptophlebia nebulosa*	CR2	See Fig. 4
E8-CR4 and 9 others	BOLD:AAZ2457	Leptophlebiidae	*Neoleptophlebia heteronea*	CR2B, CR4, CR108	
E91-CR2	BOLD:AAZ4066	Leptophlebiidae	*Neoleptophlebia memorialis*	CR2	
E36-CR1 and 3 others	BOLD:ABW1850	Leptophlebiidae	*Paraleptophlebia bicornuta*	CR4, CR100, CR108	
E31-CR6 and 3 others	BOLD:AAE2997	Leptophlebiidae	*Paraleptophlebia debilis*	CR2B, CR4, CR6, CR108	See Fig. 5
E-127-CR1UM	BOLD:ADA2951	Leptophlebiidae	*Paraleptophlebia debilis*	CR108	See Fig. 5
E30-CR4 and 1 other	BOLD:AAA4673	Siphlonuridae	*Siphlonurus alternatus*	CR4, CR108	New BC species record
E93-CR100 and 3 others	BOLD:AAF3899	Siphlonuridae	*Siphlonurus* sp.	CR3, CR100, CR108	

[a]Specimens are vouchered at the University of Guelph, Centre for Biodiversity Genomics, and sequence data are publicly available as indicated.
[b]Geographic coordinates of the collection sites are as follows: CR2: 54.484° N, 122.721° W; CR2B: 54.484° N, 122.721° W; CR3: 54.643° N, 122.743° W; CR4: 54.388° N, 122.633° W; CR5: 54.478° N, 122.719° W; CR6: 54.328° N, 122.669° W; CR100BR: 54.446° N, 122.653° W; and CR108: 54.458° N, 122.722° W.
[c]The conservation-related note is via NatureServe (2017).

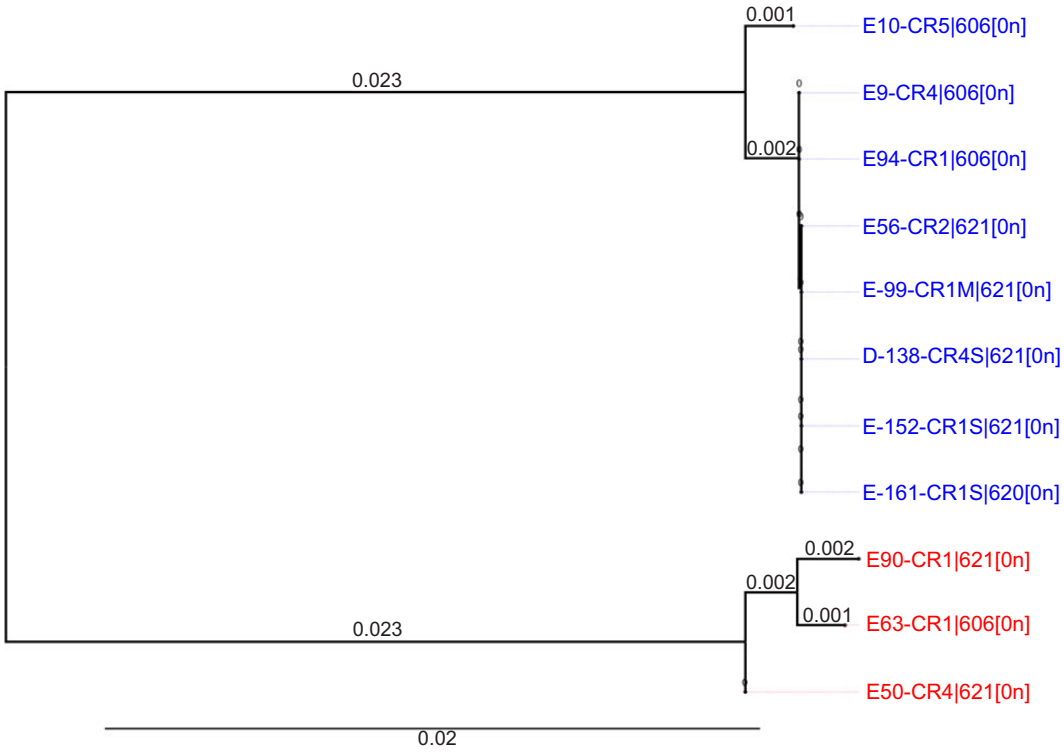

Fig. 2. Tree of specimens identified as *Cinygmula* spp., with a scale bar showing a 2% divergence. Specimens in blue were in BOLD:ADA2747, while specimens in red were in BOLD:ABA3456. The specimen names are followed by the length of the sequences and the number of ambiguous bases in brackets.

neighbor (2.23%) is BOLD:ADB5062, identified as a *Procloeon* sp. For BOLD:ADA1160, identified in our analysis only to family level, Baetidae, the nearest neighbor (10.84%) is BOLD:ABW0366, identified as *Anafroptilum* sp. For BOLD:ACL4202, identified in our analysis as *Ephemerella* sp., the nearest neighbor (5.4%) is BOLD:AAL0646, identified as an *Ephemerella* sp. For BOLD:ABA3456, identified in our analysis as *Cinygmula* sp., the nearest neighbor (4.48%) is BOLD:ADA2747, identified as a *Cinygmula* sp. For BOLD:ADA2747, identified in our analysis as *Cinygmula* sp., the nearest neighbor (4.48%) is BOLD:ABA3456, identified as a *Cinygmula* sp. For BOLD:ADA2851, identified in our analysis only to family level, Heptageniidae, the nearest neighbor (6.86%) is BOLD:ACS9812, identified as *Ecdyonurus simplicioides*. For BOLD:AAF3899, identified in our analysis as *Siphlonurus* sp., the nearest neighbor (3.00%) is BOLD:AAZ1962, identified as *Siphlonurus occidentalis*.

In 2 cases no BIN was assigned. In one case, a specimen identified as *Drunella flavilinea* (Ephemerellidae)—possibly due to somewhat poor sequence data for those specimens. In another case, a Baetidae specimen had no current close match in the BOLD database and so either is simply uncollected in that context or represents a previously undescribed species. In the case of the *Cinygmula* spp. listed above, the 11 specimens cluster into 2 separate BINs (BOLD:ADA2747 and BOLD:ABA3456) that are >4% divergent (Fig. 2), indicating that they are most likely separate species. There is an additional challenge with *Cinygmula*, as definitive identification of *Cinygmula* nymphs to species level is nearly impossible. In the case of *Cinygmula*, a strong case can be made for the use of OTUs to recognize separate species.

In most cases, these specimens were not the first to be collected in their respective BINs, although in no case were previous collections extensive; often ours were the majority of specimens in the BIN. In the case of the

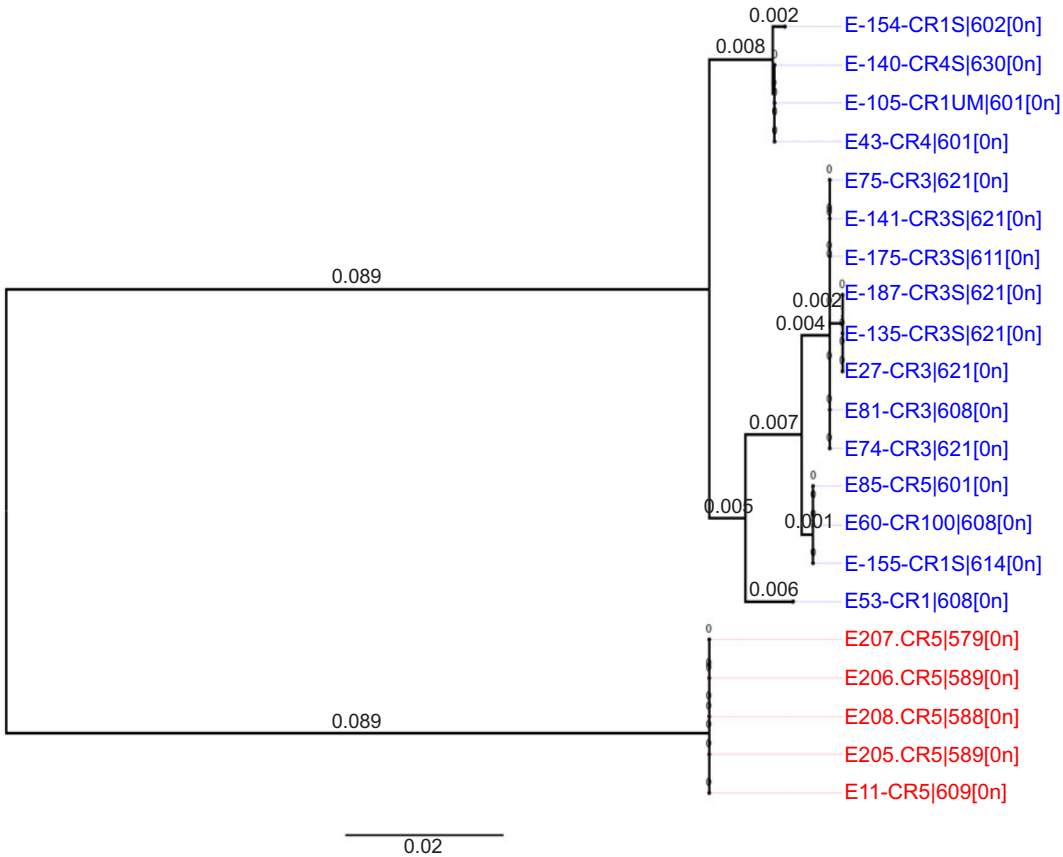

Fig. 3. Tree of specimens identified as *Baetis tricaudatus*, with a scale bar showing a 2% divergence. Specimens in blue were in BOLD:AAL5544, while specimens in red were in BOLD:AAJ9779. The specimen names are followed by the length of the sequences and the number of ambiguous bases in brackets.

unidentified Heptageniidae (BOLD:ADA2851), our specimens are the first recorded in the BOLD database. In most other cases, public specimens in the same BINs are from British Columbia, Alberta, Yukon, or Montana; although in some cases associated BIN members were from Saskatchewan, northern Manitoba, or southern Ontario.

There are a number of potential explanations for our classification based on these BINs. There is the possibility that some of these specimens are indeed undescribed species. However, it is also possible that some are Holoarctic species known from other parts of the Arctic but are yet to be identified in British Columbia (Kjærstad et al. 2012, Cordero et al. 2016). In addition, the BOLD database is not complete, and not all of the mayfly species known to British Columbia have DNA barcodes. For example, at the time of this writing, there were still 4 *Ameletus* spp. in British Columbia without DNA barcodes in BOLD. The same is true for 2 species of *Procloeon*, 1 species of *Anafroptilum*, 1 species of *Ephemerella*, 5 species of *Cinygmula*, and 2 species of *Siphlonurus*.

In 3 cases, we sequenced multiple specimens that matched to one species in BOLD but were in fact in separate BINs. Tree-based analyses indicate potential cryptic species in all 3 cases. *Baetis tricaudatus* (Baetidae) is represented by 2 BINs (BOLD:AAJ9779 and BOLD:AAL5544) that are >17% divergent (Fig. 3). *Leptophlebia nebulosa* (Leptophlebiidae) is represented by 2 BINs (BOLD: AAA7017 and BOLD:AAD3328) that are >3% divergent (Fig. 4). *Paraleptophlebia debilis* (Leptophlebiidae) is represented by 2 BINs (BOLD: AAE2997 and BOLD:ADA9584) that are >2% divergent (Fig. 5), although the

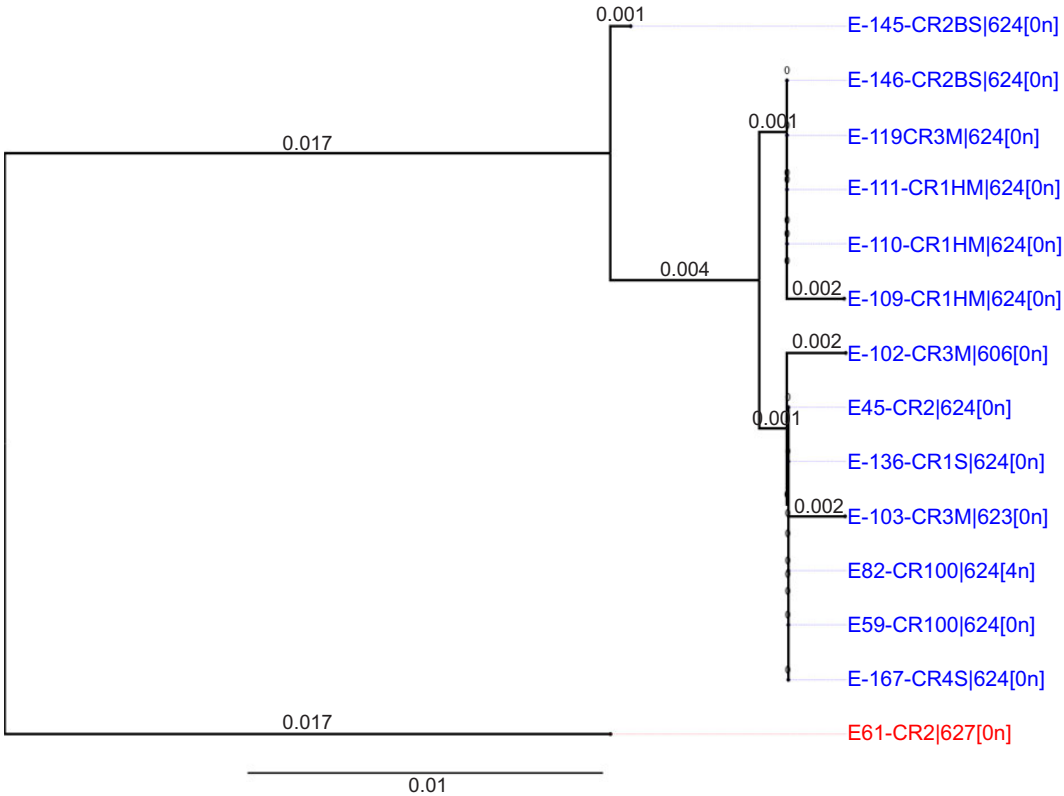

Fig. 4. Tree of specimens identified as *Leptophlebia nebulosa*, with a scale bar showing a 1% divergence. Specimens in blue were in BOLD:AAA7017, while specimens in red were in BOLD:AAD3328. The specimen names are followed by the length of the sequences and the number of ambiguous bases in brackets.

single specimen in BOLD:ADA9584 contains 3 bases that were ambiguous and are called "N" in its sequence, so the distance calculation may not be reliable.

We collected one species that is of conservation concern. *Ameletus vernalis* (Ameletidae), collected at both CR3 and CR108, is listed with a U.S. national status of N3 (vulnerable) and a Canadian national status of N2 (imperiled) by NatureServe (NatureServe 2017). The presence of a species of potential conservation concern highlights the need for ongoing monitoring and expanded protection of the Crooked River.

Mayfly Diversity of the Crooked River

Using morphological keys and DNA barcoding, we detected a considerable diversity of species within the Ephemeroptera on the Crooked River, suggesting a highly productive and currently healthy system with a diversity of habitat that is beneficial for mayflies and likely for other invertebrate taxa also. Our work has extended the range distribution for 8 mayfly species and 3 genera to the west slope of the Canadian Rocky Mountains and, in some cases, into the Arctic watershed. We have also described a number of specimens that may also be new records for this region or perhaps previously undescribed species, highlighting the need for biodiversity exploration in central and northern British Columbia. Such information may be transferable to other rivers and streams in the southern end of the Arctic watershed, although some caution is required before extrapolating species ranges to nearby systems due to the unique characteristics that make the Crooked River particularly amenable to a diversity of species. This work represents the first baseline database of mayfly species for the Crooked River and perhaps other parts of this substantially unexplored region. A baseline database is vital for ongoing monitoring due to the unique nature of this system and the ongoing and increasing anthropogenic pressures along its entire length.

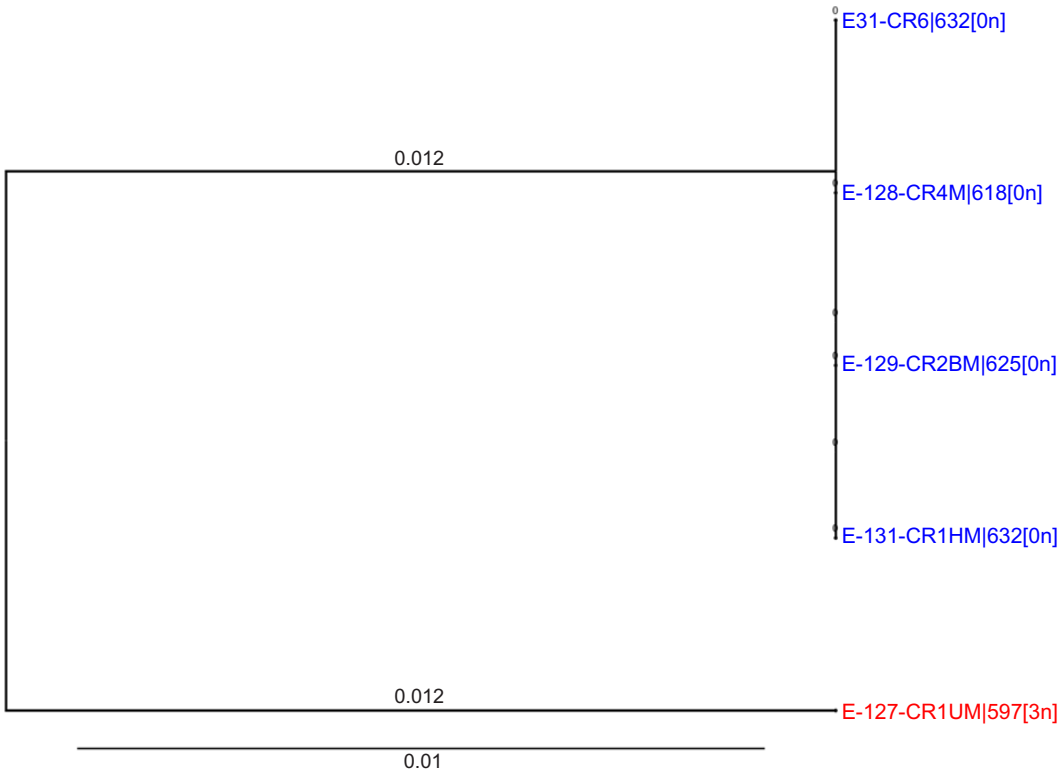

Fig. 5. Tree of specimens identified as *Paraleptophlebia debilis*, with a scale bar showing a 1% divergence. Specimens in blue were in BOLD:AAE2997, while specimens in red were in BOLD:ADA2951. The specimen names are followed by the length of the sequences and the number of ambiguous bases in brackets.

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