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Biological identifications of mayflies (Ephemeroptera) using DNA barcodes

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Abstract. We tested the efficacy of DNA barcodes in identifying mayfly species primarily from the northeastern United States and central Canada. We sequenced a 630-base-pair segment of the mitochondrial gene, cytochrome c oxidase 1 (COI), from 1 individual of each of 80 species to create a reference sequence profile. We used these reference sequences to identify 70 additional specimens representing 32 of the species that were in the profile. DNA barcodes correctly identified 69 of the 70 test specimens. The sole exception was an individual identified morphologically as *Maccaffertium modestum* that showed deep genetic divergence from other *M. modestum* specimens. Mean sequence divergence within species was 1%, whereas mean divergence among congeneric species was an order of magnitude greater (18%). We conclude that DNA barcoding can provide a powerful tool for mayfly species identification.

Key words: aquatic invertebrates, mayfly, species identification, mtDNA, COI.

Accurate identification of species is fundamental to both basic and applied aquatic research. Studies of community structure, food-web dynamics, biodiversity, and biomonitoring depend critically on the accuracy of species discrimination and identification. Despite an increasing demand for taxonomic expertise in the aquatic sciences, the number of taxonomists continues to dwindle (New 1996, Stribling et al. 2003). Furthermore, morphology-based identification of many aquatic invertebrate species is particularly challenging. Taxonomic keys often exist only for a certain life stage or gender. Thus, assigning species names to specimens is often impossible in studies where immatures make up a large proportion of the sample (as in biomonitoring). Years of experience also may be required before investigators can effectively use the taxonomic keys that are available. Last, phenotypic variation in taxonomically important traits can pose significant challenges during species identification. Such variation can lead to misidentification of specimens (Kondratieff and Voshell 1984, Wolf and Mort 1986, Burian 2001), regardless of whether the variation is environmentally or genetically based. In other cases, different species exhibit extremely limited morphological variation despite reproductive isolation and large genetic divergence between them; such cryptic species can be detected only through genetic analysis (Funk et al. 1988, Hogg et al. 1998, Witt and Hebert 2000).

A DNA-based species identification system (DNA barcoding, Hebert et al. 2003a) offers a promising supplemental technique for the identification of taxa for which morphology-based identifications are problematic, for associating different life stages, and for enabling those investigators lacking the morphology-based skills to identify taxa reliably. DNA barcodes consist of short DNA sequences that function as unique species identifiers. Species in a variety of animal groups have been discriminated reliably using an ~650-base-pair fragment of the mitochondrial gene, cytochrome c oxidase 1 (COI) (Hebert et al. 2003a, 2004a, b, Hogg and Hebert

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2004). For example, Hebert et al. (2003b) showed that 13,000 closely related species pairs from a range of animal phyla regularly showed deep genetic divergences, enabling reliable species identifications. Thus, DNA barcoding holds the potential to serve as a species identification system for all animal life, an approach that will be particularly valuable when morphology-based identifications are difficult or access to taxonomic expertise is limited.

We tested the efficacy of DNA barcodes for the identification of mayflies collected primarily from the northeastern United States and central Canada. We targeted mayflies because of their importance in aquatic research, particularly in biomonitoring studies where samples regularly consist of immatures. Moreover, the identification of many mayfly species through morphology remains problematic (Kondratieff and Voshell 1984, McCafferty and Pereira 1984, Waltz et al. 1996, 1998, Baumgardner and McCafferty 2000, Jacobus and McCafferty 2000, 2002, Burian 2001) despite the importance of mayflies in aquatic research. The specific aim of our study was to test the ability of COI to correctly identify mayfly species by comparing their sequence similarity to a pool of reference sequences from individuals of known taxonomic identity.

Methods

Sequences

We sequenced COI from 150 mayfly specimens, including representatives from 80 species (~15% of the North American species), 30 genera (~30% of the North American genera) and 12 of the 21 families known from North America. We obtained most of our samples from the northeastern United States and central Canada, but we sequenced additional specimens from geographic locations across North America and a single leptohyphid specimen that was collected in Costa Rica, Central America. We focused on species that are relatively common and for which taxonomic keys are available.

All specimens used in our study had been preserved in 75 to 95% ethanol for periods ranging from 1 to 20 y. When whole nymphs or adults were obtained, we removed a single leg or a very small tissue piece from the thorax or leg and preserved the remainder of the specimen. This approach provided ample DNA for sequencing, but also ensured the preservation of a voucher specimen (housed at the University of Guelph) for further morphological examination. We extracted total DNA in 50 μL of Proteinase K for 24 to 36 h at 55°C. We used the primer pair LC01490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HC02198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) to amplify a 658-base-pair fragment of COI (Folmer et al. 1994). DNA was amplified using the following PCR reaction: 5 μL of 10× PCR buffer pH 8.3 (10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% NP-40), 38.8 μL of distilled water, 200 mM of each dNTP, 1 unit of Taq polymerase, 0.3 μM of each primer, and 1 to 3 μL of DNA template. The polymerase chain reaction (PCR) thermal regime consisted of 1 cycle of 94°C for 1 min, 5 cycles of 94°C for 5 min, 45°C for 1.5 min, and 72°C for 1.5 min. We followed this cycle with 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and a final cycle of 72°C for 5 min. We visualized all PCR products on 1% agarose gels using ethidium bromide. We gel-purified PCR products using the Qiaex II kit (Qiagen, Mississauga, Ontario) and sequenced them on an ABI 377 automated sequencer (Applied Biosystems, Foster City, California) using the Big Dye v.3 dye termination kit.

We aligned sequences using Sequence Editor 1.9 (Applied Biosystems). Alignment was straightforward because of the absence of indels. We pruned the sequences to 630 base pairs before further analysis. We have provided a list of mayfly taxa, their locations of collection, and GenBank accession numbers in Appendix 1.

COI profiles

We tested the ability of COI to identify species correctly by creating a COI profile or reference data set based upon a single sequence from each of the 80 mayfly species. We created a profile neighbour-joining (NJ) tree of Kimura-2-parameter (K2P) distances, using MEGA2.1 (available from: www.megasoftware.net). The K2P model provides the best metric when genetic distances are low (Nei and Kumar 2000). We used a simple NJ algorithm because the goal of barcoding is to provide species identification based on sequence similarity rather than to reconstruct deeper phylogenetic relationships accurately. Furthermore, NJ provides the necessary speed of analysis for the large data sets that are typical

of DNA barcoding studies. We used a jumping bristletail (*Machiloides* sp.) sequence obtained from GenBank as the outgroup.

Test taxa

We tested the ability of the COI profile to generate correct species identifications for 70 test specimens representing 32 of the 80 species included in the profile by determining the sequence congruence between the 80 profile specimens and the 70 test specimens. These test specimens were mock unknowns because their species identifications were known from prior morphological examination by 2 of us (SKB and JMW) who are taxonomists of North American mayflies. We used these mock unknowns to assess the congruence between morphological and COI-based species identification. Whenever possible, we obtained our test specimens from a population different from that of the profile specimen. When >1 test specimen was available for a species, we tried to use specimens from geographically distant populations (i.e., different states/provinces, eastern vs western North America) to provide a greater sample of the variation in COI throughout the species' ranges. However, in ~13% of the cases, we had only one test specimen, and it was from the same population as the profile specimen. We considered identifications based on the COI profile successful if the test sequences, when added one at a time to the NJ profile tree, grouped most closely with their conspecific in the profile. We calculated identification success rate as the % of test specimens that grouped correctly with their conspecific.

Data analysis

We calculated intraspecific sequence divergences based on K2P distances for all species for which we had sequences from ≥2 individuals. For each species, we calculated all pairwise divergences and plotted them as a frequency histogram. We omitted *Stenacron interpunctatum* from this calculation because it represents a putative species complex (see Discussion). Similarly, we omitted a single *Maccaffertium modestum* specimen because its morphological identification was ambiguous. We calculated interspecific K2P divergences between congeneric species pairs for all genera represented by ≥2 species. When we had sequences for several individuals of a species, we used one randomly chosen individual to represent that species. We calculated K2P divergences for all congeneric species pairs and plotted them as a frequency histogram. We calculated mean intra- and interspecific K2P divergences as the overall mean of all pairwise comparisons within each species and the mean of all pairwise comparisons within each genus, respectively.

Results

Profile sequences

Eleven of the 12 families in our NJ profile were represented by >1 species. Nine of these families formed cohesive groups in the NJ profile (Fig. 1). The 2 exceptions were the largest North American families, Heptageniidae and Ephemerellidae. The Heptageniidae formed 3 small subgroups, with each group including species of 1 or 2 genera. The Ephemerellidae also formed 3 subgroups, each representing a different genus. The genera, *Dannella* and *Attenella*, for which only a single profile sequence was represented, grouped with the genus *Eurylophella*. In no case did any of these heptageniid or ephemerellid species infiltrate another family grouping. Furthermore, genera that were represented by ≥2 species formed cohesive groups. The single exception was *Baetis flavistriga*, which occurred on a long branch as the most basal of the Baetidae. Thus, all but 1 specimen grouped correctly with their appropriate family and genus.

Test sequences

Ninety-nine percent (69 of 70) of the test specimens were identified correctly to species by the COI profile (Appendix 2). The sole exception was one individual identified through morphological analysis as *M. modestum*. This individual did group with members of its genus, but showed deep divergences from the profile specimens of both *M. modestum* and *M. mediopunctatum* (13.7% and 12.2%, respectively).

Nucleotide diversity

Mean intra- and interspecific (congeneric) divergences differed by more than an order of

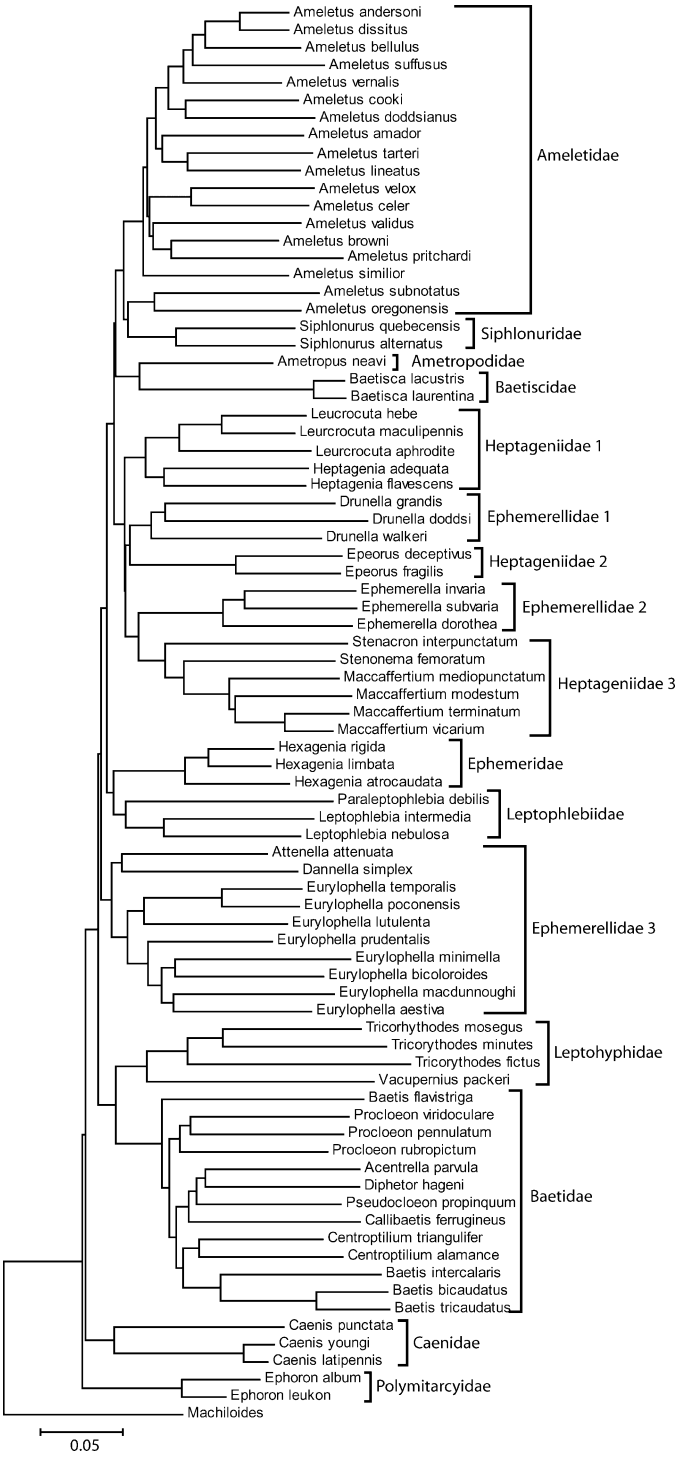

FIG. 1. Neighbor-joining profile of 80 mayfly species from 12 North American families.

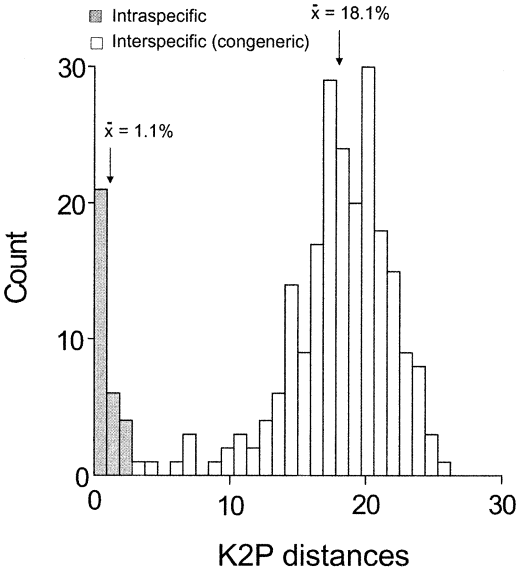

FIG. 2. Histogram of intra- and interspecific (congeneric) genetic Kimura-2-parameter (K2P) divergences.

magnitude (Fig. 2). The sole overlap in the distribution of mean intra- and interspecific divergences consisted of 2 individuals of the obligate parthenogen, *Centroptilum triangulifer*, from geographically distant populations (Maine and Pennsylvania). These individuals showed a pairwise distance of 6.6%, much larger than all other intraspecific pairwise distances. When these 2 *C. triangulifer* individuals were omitted from the data set, the distribution of mean intra- and interspecific divergences did not overlap (Fig. 2). However, when individual congeneric species pairs were examined, 2 pairs (*Baetisca laurentina*/*B. lacustris*, divergence = 3.8% and *Caenis latipennis*/*C. youngi*, divergence = 3.3%) showed divergences similar to the upper limit of intraspecific distances observed among individuals of *Maccaffertium vicarium*. Mean intraspecific divergences among *M. vicarium* individuals was 2.2%; however, the maximum divergence observed between pairs of individuals was 3.4%. Mean nucleotide divergence among the 19 genera for which we had ≥2 species ranged from a low of 3.8% in the *Baetisca* to a high of 22.8% in the genus *Tricorythodes* (Table 1).

Discussion

Efficacy of COI-based identification

Our results indicate that a COI-based identification system should be extremely effective in

TABLE 1. Mean and range of interspecific Kimura-2-parameter nucleotide divergences for species in 19 mayfly genera belonging to 11 different families.

			% divergence	
Family	Genus	No. of species	Mean	Range
Ameletidae	*Ameletus* Eaton	18	18.3	6.5–24.8
Baetidae	*Baetis* Leach	4	20.2	8.5–24.6
	Callibaetis Eaton	2	19.9	–
	Centroptilum Eaton	2	17.8	–
	Procloeon Bengtsson	3	19.0	18.8–19.2
Baetiscidae	*Baetisca* Walsh	2	3.8	–
Caenidae	*Caenis* Stephens	3	14.1	3.3–19.7
Ephemerellidae	*Drunella* Needham	3	22.4	22.4–22.5
	Ephemerella Walsh	3	15.1	13.7–16.2
	Eurylophella Teinsuu	7	20.1	9.5–24.2
Ephemeridae	*Hexagenia* Walsh	3	10.2	7.1–12.4
Heptageniidae	*Epeorus* Eaton	2	12.9	–
	Heptagenia Walsh	2	17.1	–
	Leucrocuta Flowers	3	13.3	9.6–16.0
	Maccaffertium Bednarik	4	10.9	7.2–15.2
Leptohyphidae	*Tricorythodes* Ulmer	3	22.8	18.3–25.8
Leptophlebiidae	*Leptophelbia* Westwood	2	17.8	–
Polymytarcidae	*Ephoron* Williamson	2	7.3	–
Siphlonuridae	*Siphlonurus* Eaton	2	14.2	–

identifying mayfly species from the northeastern United States and central Canada. We successfully identified all but 1 of the 70 test specimens using the COI profile. The only ambiguous identification involved a single specimen that was identified morphologically as *M. modestum* but showed deep divergence from specimens of both *M. modestum* and *M. mediopunctatum* (13.7 and 12.2%, respectively; Appendix 2). These high sequence divergences suggest that this specimen may either represent an unknown cryptic species that has been confused morphologically with *M. modestum* or a species not present in the profile data set. One of us (SKB) found that the specimen (a late instar larva) was generally consistent with the known morphological concept of *M. modestum*, but this species exhibits significant phenotypic variation, particularly in abdominal color patterns (Bednarik and McCafferty 1979). Existence of a cryptic species complex within *M. modestum* has also been suggested based on differences in adult swarming behaviour (D. Funk, Stroud Research Center, personal communication).

Evidence for additional cryptic species was encountered in 2 other cases, but these lineages formed sister taxa. *Maccaffertium vicarium* specimens clustered into 2 distinct groups, showing 3.4% sequence divergence, the upper limit of the intraspecific divergences we observed. Three of these 5 specimens were collected from different locations in Maine, but only 2 of them grouped together. The 3rd specimen clustered most closely with 2 individuals from Ontario. Similarly, we observed 3 distinct groups among 9 specimens of *Stenacron interpunctatum*, a taxon that has long been suspected to be a species complex, despite the failure to identify consistent morphological characters enabling their discrimination (Spieth 1947, Lewis 1974). Sequence divergences between these 3 groups were large (10–17%, far greater than any other intraspecific divergences we observed) but were consistent with the sequence divergences typically observed between other congeneric species pairs. Hence, our data provide the first genetic evidence for the existence of a *S. interpunctatum* species complex. Moreover, these large genetic divergences did not correlate with geographic distance. Specimens from the Androscoggin River (AR) population in Maine fell into 2 genetically distinct groups.

COI was extremely successful in identifying species, and it revealed phylogeographic population structure in several cases. Specimens from geographically proximate populations generally clustered more closely with each other than they did with specimens from other geographically distant populations. For example, *B. laurentina* from 2 populations in Maine showed greater similarity to each other than to individuals from a population from Saskatchewan. Similar patterns were seen with *Ephemerella subvaria*, *Eurylophella prudentalis*, *Ameletus cooki*, and *Ameletus andersoni*.

Deeper taxonomic groups

The COI data also recovered deeper taxonomic groups; congeneric taxa formed cohesive groups. A single exception was *B. flavistriga*, which failed to group with its 3 congeners and occurred on a long branch basal to the other baetids. Baetid mayflies are known for their uncertain taxonomy and a number of species have been transferred recently to different genera (McCafferty et al. 1994, Waltz and McCafferty 1997, Lugo-Ortiz and McCafferty 1998, McCafferty and Waltz 1990, Wiersema 2000). Thus, our data suggest that the taxonomic position of *B. flavistriga* should be re-examined. COI sequences usually grouped confamilial species, and 9 of the 11 families with multiple representatives formed cohesive groups. The Heptageniidae and Ephemerellidae, 2 of the largest families, did not form cohesive groups, probably because of undersampling of the total taxon diversity in these families (Hebert et al. 2003a). That none of the Heptageniidae and Ephemerellidae sequences infiltrated any other family groupings supports our hypothesis that taxon undersampling is responsible for the lack of cohesiveness. Homoplasy also is possible, but it is unlikely that very divergent taxa would converge on an identical or near-identical 630-base-pair barcode. Lack of cohesion of species in large insect orders also has been observed by Hebert et al. (2003a), who found that COI sequences of Coleoptera, the largest insect order, formed distinct subgroups that separated taxa according to suborder.

The NJ tree based on all 150 sequences (profile and test sequences, Appendix 2) showed minor topological shifts from the NJ tree containing only the 80 profile sequences. These changes did not affect the success of species identifica-

tion because the shifts involved deeper nodes. For example, the Ameletidae in the profile tree formed a single, cohesive group, but they formed 2 groups in the total tree. Nevertheless, no ameletid sequence infiltrated any other group; conspecifics always grouped together, indicating that these minor differences in topology did not affect the ability of COI to correctly identify species. We also stress that the purpose of DNA barcoding is not to recover deeper phylogenetic relationships, so the cohesion (or lack thereof) of groups above the species level has no bearing on the success of COI-based species identifications.

Nucleotide divergences at the intraspecific level were an order of magnitude lower than those among congeneric species. A similar result was observed for Lepidoptera (Hebert et al. 2003a) and Collembola (Hogg and Hebert 2004), but the magnitude of divergences was larger in mayflies (mean intra- and interspecific distances of 1% and 18%, respectively) than in lepidopterans (0.25 and 6.8%, respectively). These differences may be a consequence of the higher mean G+C content (40%) in mayflies compared to lepidopterans (31%) (PDNH, unpublished data). Furthermore, some mayfly species recently have been transferred to different genera (McCafferty and Waltz 1990, Waltz and McCafferty 1997, Wang and McCafferty 2004). Consequently, the placement of some highly divergent species pairs in a single genus may have contributed to the high levels of sequence divergence seen among mayfly congeners. Mean intra- and interspecific nucleotide divergences differed by an order of magnitude and did not overlap, but the distributions of individual intra- and interspecific divergences overlapped slightly. The highest intraspecific divergences (6.6%) involved specimens of the obligate asexual species *C. triangulifer* from Pennsylvania and Maine (Gibbs 1977). When *C. triangulifer* was excluded, the upper limit of intraspecific divergences was 3.4%, comparable to the lower limit of interspecific divergences observed for 2 congeneric species pairs, *B. laurentina*/*B. lacustris* and *C. latipennis*/*C. youngi*, which differed by 3.8% and 3.3%, respectively. These cases of low interspecific divergence may reflect that these are young species pairs. However, low interspecific divergence also could be a consequence of lower substitution rates in these taxa.

Effects of geographic separation on COI divergence

Levels of sequence divergence among geographically distant mayfly populations were not high enough to complicate species identification by blurring species boundaries. In fact, genetic divergences were only slightly higher for species for which specimens were from populations separated by >2000 km (e.g., *Maccaffertium terminatum*, *M. vicarium*, and *B. laurentina*) than for species for which specimens came from the same locations. Moreover, these genetic distances were well within the range of intraspecific distances in our complete data set. Further study is needed to more fully evaluate the extent of COI diversity among populations of very widely distributed species, but we stress that the purpose of our study was to evaluate the potential of a COI-based identification system for mayflies, beginning with sampling focused primarily at a regional scale (northeastern United States and central Canada).

We did not exhaustively survey COI diversity across the entire geographic range of any species. Furthermore, we used only a single representative of each species in the COI profile because this approach provided the most stringent test of the ability of DNA barcodes to identify species. However, studies are now in progress to evaluate fully the efficacy of a COI-based identification system for species with extremely large geographic distributions (e.g., on the order of 10^2–10^4 km). We do not expect such analyses to lead to taxonomic confusion. Rather we anticipate that these analyses will enable accurate species identifications and (potentially) correct identification of the region from which the specimen was obtained. Given that we obtained successful species identifications of all but one test specimen, based on only a single representative of each species in the COI profile, we are confident that a DNA databank, containing multiple reference sequences of each species, will be equally able to provide successful species identification. Moreover, the goal of DNA reference databanks will be to include sequences of specimens collected from throughout each species' geographic range and to encompass the range of COI diversity found in each species.

Potential limitations of COI-based DNA barcoding

DNA barcoding using COI will be unable to provide accurate species identification in some

cases. 1) COI is a mitochondrial gene, and mitochondrial genes typically are inherited maternally in animals. F1 hybrids would be indistinguishable from their maternal parent, but nuclear genes could be used to confirm hybrid status where hybridization is suspected. However, given the relative rarity of natural hybrids between animal species, COI should provide a reliable species identification system for most species. 2) Very young species pairs might be difficult to identify using a COI-based system. This problem may be particularly noticeable if the species have ancestrally polymorphic mitochondrial haplotypes that do not sort according to subsequent speciation events (Funk and Omland 2003). 3) Identifications using DNA barcodes (like identifications using morphology) will not work successfully for all species. However, the deep genetic divergences between most congeneric taxa suggest that such misidentifications will be relatively infrequent among the Ephemeroptera, and other studies have confirmed that this conclusion is probably general in the animal kingdom (Hebert et al. 2003b, Hogg and Hebert 2004). 4) The goal of profile-sequence databases is to include as much taxonomic coverage as possible. However, species identifications will not be possible if the specimen for which identification is sought is not represented in the profile-sequence database. In such cases, the COI profile should provide the next-highest level of identification (e.g., genus, subfamily, or family).

Practicality of DNA barcoding in aquatic research

The high success with which COI correctly identified mayfly species is consistent with studies in other invertebrates (Hebert et al. 2003a, b, Hogg and Hebert 2004) and vertebrates (Hebert et al. 2004b). A molecular identification system, using small subunit RNA, was effective at classifying nematodes into molecular operational taxonomic units (Floyd et al. 2002). A similar approach (based on molecular diagnostic tools) has been used for the identification of forensically important Diptera (Sperling et al. 1994, Wells and Sperling 2001), forest pest species (Sperling and Hickey 1994), human hookworm (Zhan et al. 2001), and the malaria vector *Anopheles minimus* (Van Bortel et al. 2000). Our study focused on the identification of mayflies, but we are confident that DNA barcoding will be successful in species identification of other aquatic invertebrates. Aquatic taxa such as daphniid and rotifer species, which exhibit marked phenotypic plasticity, have been distinguished reliably using COI (Derry et al. 2003, Adamowicz et al. 2004). Given the success of the COI profile in identifying mayflies and other insect taxa (Lepidoptera: Hebert et al. 2003a, Collembola: Hogg and Hebert 2004), the potential for successful identification of many other aquatic insect taxa using COI is extremely high.

Taxonomic expertise is currently limited, and morphological identification is often fraught with difficulties (e.g., identifcation of eggs and early instar larvae, damaged specimens, or fragments of specimens). Thus, a DNA-based identification system would have significant benefits for aquatic research. In particular, a DNA-based system could provide an important tool for species identification in biomonitoring. The need for species-level identification in biomonitoring is contentious (see Bailey et al. 2001, Lenat and Resh 2001), but DNA barcoding could provide the option of species-level identification when taxonomic discrimination at the species level is warranted. It could also ensure uniform quality of taxonomic results in studies where the quality of taxonomic data might be compromised by the inability to identify early instars, damaged specimens, or fragments of specimens (Stribling et al. 2003). Moreover, the increased taxonomic resolution delivered by DNA barcoding would provide more sensitive measures of the magnitudes and types of environmental impacts (Lenat and Resh 2001).

Inclusion of DNA barcoding in biomonitoring studies is not unrealistic, given the increasing accessibility and affordability of DNA sequencing. DNA sequencing service facilities are becoming more common at universities and, thus, can provide access to sequencing for researchers who do not have in-house sequencing capability. Furthermore, DNA extraction, PCR, and sequencing can be delivered for $<$\$5 (US currency) from high through-put sequencing facilities. These costs will continue to drop, making routine use of DNA sequence-based identification tools possible. Initially, strategies that focus on characterization of specimens that cannot be identified through morphological examination can make DNA barcoding a feasible option. However, automation of DNA-based taxon identification for entire samples collected in biomonitoring studies could be delivered using DNA

microarrays. Development of these tools is currently underway (SLB, Bio-protection Centre, Lincoln University).

In summary, DNA barcoding can provide a powerful supplement to the traditional morphological approach to species identification. In some cases (e.g., aquatic biomonitoring), DNA barcoding systems (i.e., microarrays) may be developed to automate taxon identification as a means to provide rapid, efficient, and consistently accurate identifications. However, we stress that DNA barcoding is not meant to replace traditional taxonomic approaches. In fact, DNA barcoding cannot be accomplished without the involvement and expertise of taxonomists who can identify specimens from which reference sequences are obtained and who can deal with taxonomic issues resulting from the discovery of provisional species based on significant genetic divergences.

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APPENDIX 1. Species identifications, location of collection, and Genbank accession numbers for profile and test speciments.

Family	Species	Location of collection	Location abbreviation	Accession number
Profile				
Ameletidae	*Ameletus amador* Mayo	Dry Creek, California	DC, CA	AY3267863
	A. andersoni Zloty	Holter Gulch Creek, Washington	HGC, WA	AY3267827
	A. bellulus Zloty	Ford Creek, Alberta	FC, AB	AY3267829
	A. browni McDunnough	Glibert Brook, Vermont	GB, VT	AY3267795
	A. celer McDunnough	Jumping Pound Creek, Alberta	JPC, AB	AY3267867
	A. cooki McDunnough	Prairie Creek, Alberta	PC, AB	AY3267831
	A. dissitus Eaton	Santa Clara, California	SC, CA	AY3267866
	A. doddsianus Zloty	South Boulder Creek, Colorado	SBC, CO	AY3267864
	A. lineatus Traver	Thorncrag Brook, Maine	TB, ME	AY3267830
	A. oregonensis McDunnough	Jumping Pound Creek, Alberta	JPC, AB	AY3267835
	A. pritchardi Zloty	Elbow River, Alberta	ER, AB	AY3267865
	A. similior McDunnough	Ford Creek, Alberta	FC, AB	AY3267837
	A. subnotatus Eaton	Clearwater, Alberta	CW, AB	AY3267834
	A. suffussus McDunnough	Elbow River, Alberta	ER, AB	AY3267836
	A. tarteri Burrows	Hills Creek, West Virginia	HC, WV	AY3267828
	A. validus McDunnough	Prairie Creek, Alberta	PC, AB	AY3267826
	A. velox Dodds	Jumping Pound Creek, Alberta	JPC, AB	AY3267832
	A. vernalis McDunnough	Bow River, Alberta	BR, AB	AY3267833
Ametropodidae	*Ametropus neavei* McDunnough	S. Saskatchewan River, Saskatchewan	SSR, SK	AY3267868
Baetidae	*Acentrella parvula* (McDunnough)	N. Saskatchewan River, Saskatchewan	NSR, SK	AY3267796
	Baetis bicaudatus Dodds	East River, Colorado	ER, CO	AY3267797
	B. flavistriga McDunnough	Hwy 9 Creek, Saskatchewan	H9C, SK	AY3267860
	B. intercalaris McDunnough	Lutteral Creek, Ontario	LC, ON	AY3267799
	B. tricaudatus Dodds	Lutteral Creek, Ontario	LC, ON	AY3267798
	Callibaetis ferrugineus (Walsh)	Lutteral Creek, Ontario	LC, ON	AY3267804
	Centroptilium alamance (Traver)	Chillisquaque Creek, Pennsylvania	CC, PA	AY3267806
	C. triangulifer (McDunnough)	Tributary of Dead River, Maine	TDR, ME	AY3267805
	Diphetor hageni (Eaton)	Low Creek, Saskatchewan	LC, SK	AY3267800
	Procloeon pennulatum (Eaton)	McVey Creek, Saskatchewan	MC, SK	AY3267803
	P. rubropictum (McDunnough)	McVey Creek, Saskatchewan	MC, SK	AY3267800
	P. viridoculare (Berner)	N. Branch Dead River, Maine	NBDR, ME	AY3267801
	Pseudocloeon propinquum (Walsh)	S. Saskatchewan River, Saskatchewan	SSR, SK	AY3267859

APPENDIX 1. Continued.

Family	Species	Location of collection	Location abbreviation	Accession number
Baetiscidae	*Baetisca lacustris* McDunnough	S. Saskatchewan River, Saskatchewan	SSR, SK	AY3267807
	B. laurentina McDunnough	Torch River, Saskatchewan	TR, SK	AY3267808
Caenidae	*Caenis latipennis* Banks	Grand River, Ontario	GR, ON	AY3267916
	C. punctata McDunnough	Cedar Creek, Missouri	CC, MO	AY3267825
	C. youngi Roemhild	Lily Pond, Saskatchewan	LP, SK	AY3267824
Ephemerellidae	*Attanella attenuata* (McDunnough)	Delaware River, Pennsylvania	DR, PA	AY3267809
	Dannella simplex (McDunnough)	White Clay Creek, Pennsylvania	WCC, PA	AY3267810
	Drunella doddsi (Needham)	South Boulder Creek, Colorado	SBC, CO	AY3267909
	D. grandis (Eaton)	Colorado	CO	AY3267812
	D. walkeri (Eaton)	Nezinscott River, Maine	NR, ME	AY3267811
	Ephemerella dorothea (Needham)	West Virginia	WV	AY3267813
	E. invaria (Walker)	Little Androscoggin River, Maine	LAR, ME	AY3267814
	E. subvaria McDunnough	Lutteral Creek, Ontario	LC, ON	AY3267815
	Eurylophella aestiva (McDunnough)	White Clay Creek, Pennsylvania	WCC, PA	AY3267862
	E. bicoloroides (McDunnough)	Wyalusing Creek, Pennsylvania	WYC, PA	AY3267840
	E. lutulenta (Clemens)	Emerald Lake, Vermont	EL, VT	AY3267839
	E. macdunnoughi Funk	Meshoppen Creek, Pennsylvania	MC, PA	AY3267841
	E. minimella (McDunnough)	N. Branch Dead River, Maine	NBDR, ME	AY3267838
	E. poconoensis Funk	Lake Lacawac, Pennsylvania	LL, PA	AY3267861
	E. prudentalis (McDunnough)	Famys Branch, Delaware	FB, DE	AY3267842
	E. temporalis (McDunnough)	Moosehead Lake, Maine	ML, ME	AY3267843
Ephemeridae	*Hexagenia atrocaudata* McDunnough	Luteral Creek, Ontario	LC, ON	AY3267819
	H. limbata (Serville)	Detroit River, Ontario	DR, ON	AY3267901
	H. rigida McDunnough	St. Lawrence River, Ontario	SLR, ON	AY3267817
Heptageniidae	*Epeorus deceptivus* (McDunnough)	East River, Colorado	ER, CO	AY3267820
	E. fragilis (Morgan)	Bruce Trail Creek, Ontario	BTC, ON	AY3267821
	Heptagenia adaequata McDunnough	S. Saskatchewan River, Saskatchewan	SSR, SK	AY3267816
	H. flavescens (Walsh)	S. Saskatchewan River, Saskatchewan	SSK, SK	AY3267915
	Leucrocuta aphrodite (McDunnough)	Nezinscott River, Maine	NR, ME	AY3267822
	L. hebe (McDunnough)	Unnamed creek, Saskatchewan	UC, SK	AY3267823
	L. maculipennis (McDunnough)	S. Saskatchewan River, Saskatchewan	SSR, SK	AY3267900

APPENDIX 1. Continued.

Family	Species	Location of collection	Location abbreviation	Accession number
	Maccaffertium mediopunctatum (McDunnough)	Lutteral Creek, Ontario	LC, ON	AY3267852
	M. modestum (Banks)	Androscoggin River, Maine	AR, ME	AY3267851
	M. terminatum (Walsh)	S. Saskatchewan River, Saskatchewan	SSR, SK	AY3267853
	M. vicarium (Walker)	Unnamed creek, Saskatchewan	UC, SK	AY3267854
	Stenacron interpunctatum (Say)	Androscoggin River, Maine	AR, ME	AY3267850
	Stenonema femoratum (Say)	Sabattus Pond, Maine	SP, ME	AY3267855
Leptohyphidae	*Tricorythodes fictus* Traver	Llano River, Texas	LR, TX	AY3267903
	T. Minutus Traver	Sandy Creek, Texas	SC, TX	AY3267848
	T. mosegus Alba-Tercedor & Flannagan	Lutteral Creek, Ontario	LC, ON	AY3267847
	Vacupernius packeri (Allen)	Rio Caracol, Costa Rica	RC, CR	AY3267846
Leptophlebiidae	*Leptophlebia intermedia* (Traver)	Moose Pond, Maine	MP, ME	AY3267856
	L. nebulosa (Walker)	Little Androscoggin River, Maine	LAR, ME	AY3267857
	Paraleptophlebia debilis (Walker)	Unnamed creek, Saskatchewan	UC, SK	AY3267849
Polymitarcyidae	*Ephoron album* (Say)	S. Saskatchewan River, Saskatchewan	SSR, SK	AY3267858
	E. leukon Williamson	Grand River, Ontario	GR, ON	AY3267896
Siphlonuridae	*Siphlonurus alternatus* (Say)	N. Branch Dead River, Maine	NBDR, ME	AY3267845
	S. quebecensis (Provancher)	Nezinscott River, Maine	NR, ME	AY3267844
Test				
Ameletidae	*A. andersoni*	Corvallis, Oregon	CO, OR	AY3267911
	A. andersoni	Willow Creek, Oregon	WC, OR	AY3267926
	A. andersoni	Willow Creek, Oregon	WC, OR	AY3267935
	A. bellulus	Ford Creek, Alberta	FC, AB	AY3267929
	A. browni	Gilbert Brook, Vermont	GB, VT	AY3267927
	A. cooki	Prairie Creek, Alberta	PC, AB	AY3267918
	A. cooki	Calgary, Alberta	CG, AB	AY3267917
	A. lineatus	Unnamed Creek, Ontario	UC, ON	AY3267934
	A. lineatus	Bruce Trail Creek, Ontario	BTC, ON	AY3267940
	A. lineatus	Country Rd. 22 Creek, Ontario	CR22C, ON	AY3267920
	A. lineatus	White Clay Creek, Pennsylvania	WCC, PA	AY3267933
	A. oregonensis	Jumping Pound Creek, Alberta	JPC, AB	AY3267928
	A. oregonensis	Jumping Pound Creek, Alberta	JPC, AB	AY3267936
	A. oregonensis	Jumping Pound Creek, Alberta	JPC, AB	AY3267938
	A. pritchardi	Elbow River, Alberta	ER, AB	AY3267941
	A. similior	Ford Creek, Alberta	FC, AB	AY3267939
	A. suffussus	Ford Creek, Alberta	FC, AB	AY3267930

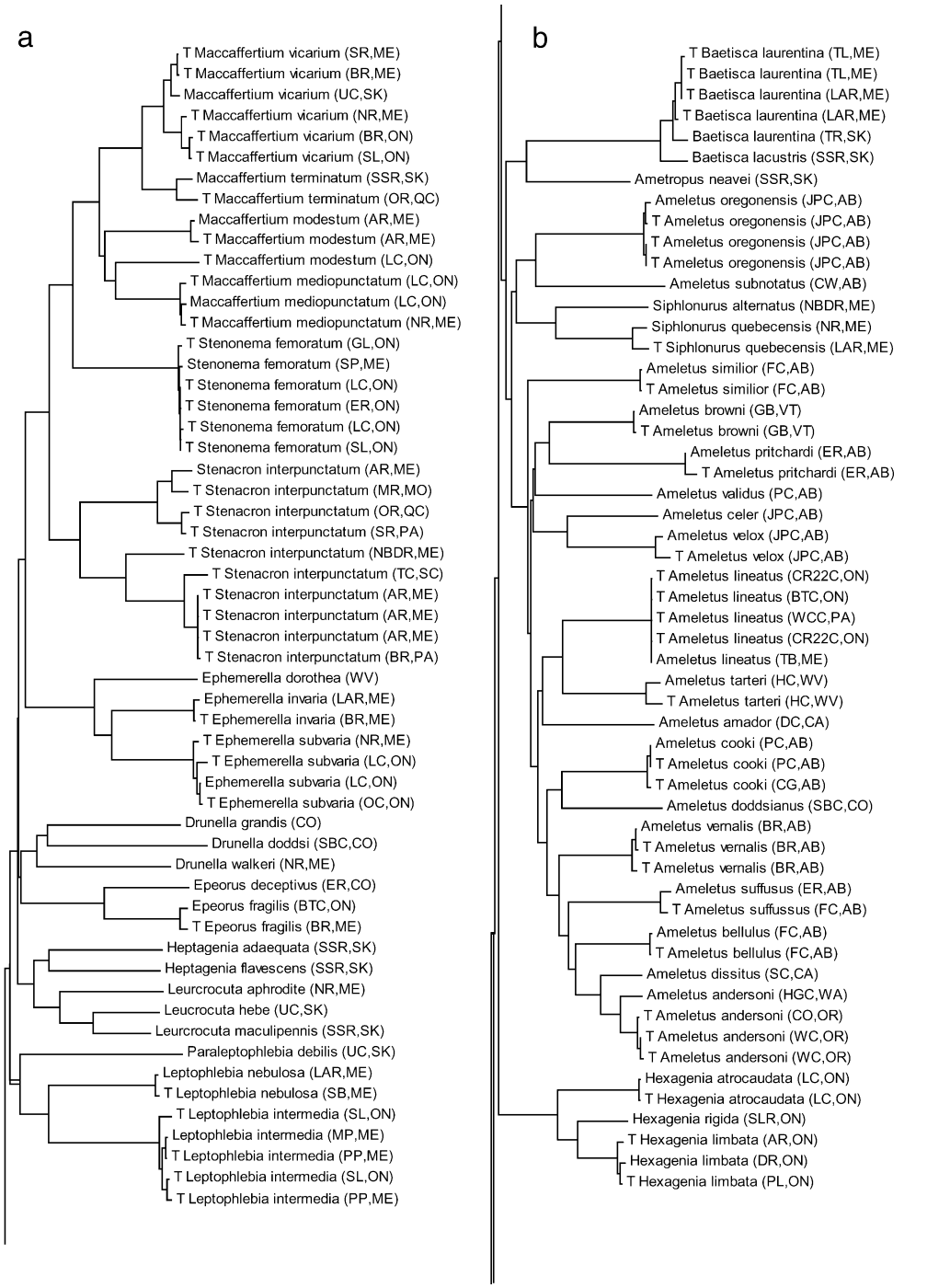
APPENDIX 1. Continued.

Family	Species	Location of collection	Location abbreviation	Accession number
	A. tarteri	Hills Creek, West Virginia	HC, WV	AY3267932
	A. velox	Jumping Pound Creek, Alberta	JPC, AB	AY3267944
	A. vernalis	Bow River, Alberta	BR, AB	AY3267919
	A. vernalis	Bow River, Alberta	BR, AB	AY3267931
Baetidae	*B. tricaudatus*	Eramosa River, Ontario	ER, ON	AY3267899
	C. triangulifer	Chillsquaque Creek, Pennsylvania	CC, PA	AY3267895
Baetiscidae	*B. laurentina*	Thompson Lake, Maine	TL, ME	AY3267906
	B. laurentina	Thompson Lake, Maine	TL, ME	AY3267937
	B. laurentina	Little Androscoggin River, Maine	LAR, ME	AY3267904
	B. laurentina	Little Androscoggin River, Maine	LAR, ME	AY3267905
Ephemerellidae	*D. simplex*	White Clay Creek, Pennsylvania	WCC, PA	AY3267908
	E. invaria	Bear River, Maine	BR, ME	AY3267910
	E. subvaria	Lutteral Creek, Ontario	LC, ON	AY3267912
	E. subvaria	Ospringe Creek, Ontario	OC, ON	AY3267913
	E. subvaria	Nezinscott River, Maine	NR, ME	AY3267914
	E. bicoloroides	Wyalusing Creek, Pennsylvania	WYC, PA	AY3267924
	E. prudentalis	N. Branch Dead River, Maine	NBDR, ME	AY3267922
	E. prudentalis	Famys Branch, Delaware	FB, DE	AY3267925
	E. prudentalis	Androscoggin River, Maine	AR, ME	AY3267921
Ephemeridae	*H. atrocaudata*	Lutteral Creek, Ontario	LC, ON	AY3267902
	H. limbata	Puslinch, Ontario	PL, ON	AY3267897
	H. limbata	Ausable River, Ontario	AR, ON	AY3267818
Heptageniidae	*E. fragilis*	Bear River, Maine	BR, ME	AY3267907
	M. mediopunctatum	Lutteral Creek, Ontario	LC, ON	AY3267879
	M. mediopunctatum	Nezinscott River, Maine	NR, ME	AY3267880
	M. modestum	Androscoggin River, Maine	AR, ME	AY3267877
	M. modestum	Lutteral Creek, Ontario	LC, ON	AY3267878
	M. terminatum	Ottawa River, Quebec	OR, QC	AY3267881
	M. vicarium	Sunday River, Maine	SR, ME	AY3267884
	M. vicarium	Bear River, Maine	BR, ME	AY3267885
	M. vicarium	Nezinscott River, Maine	NR, ME	AY3267883
	M. vicarium	Beaver River, Ontario	BR, ON	AY3267882
	M. vicarium	Sasajewan Lake, Ontario	SL, ON	AY3267887
	S. interpunctatum	Mississippi River, Missouri	MR, MO	AY3267872
	S. interpunctatum	Ottawa River, Quebec	OR, QC	AY3267869
	S. interpunctatum	Susquehanna River, Pennsylvania	SR, PA	AY3267870
	S. interpunctatum	N. Branch Dead River, Maine	NBDR, ME	AY3267874
	S. interpunctatum	Turkey Creek, South Carolina	TC, SC	AY3267876

APPENDIX 1. Continued.

Family	Species	Location of collection	Location abbreviation	Accession number
	S. interpunctatum	Androscoggin River, Maine	AR, ME	AY3267871
	S. interpunctatum	Androscoggin River, Maine	AR, ME	AY3267873
	S. interpunctatum	Androscoggin River, Maine	AR, ME	AY3267875
	S. interpunctatum	Birch Run, Pennsylvania	BR, PA	AY3267942
	S. femoratum	Guelph Lake, Ontario	GL, ON	AY3267891
	S. femoratum	Lutteral Creek, Ontario	LC, ON	AY3267886
	S. femoratum	Lutteral Creek, Ontario	LC, ON	AY3267889
	S. femoratum	Eramosa River, Ontario	ER, ON	AY3267888
	S. femoratum	Sasajewan Lake, Ontario	SL, ON	AY3267890
Leptophlebiidae	*L. intermedia*	Sasajewan Lake, Ontario	SL, ON	AY3267892
	L. intermedia	Sasajewan Lake, Ontario	SL, ON	AY3267898
	L. intermedia	Pleasant Pond, Maine	PP, ME	AY3267893
	L. intermedia	Pleasant Pond, Maine	PP, ME	AY3267943
	L. intermedia	Sabattus Pond, Maine	SP, ME	AY3267894
Siphlonuridae	*S. quebecensis*	Little Androscoggin River, Maine	LAR, ME	AY3267923

APPENDIX 2. Neighbor-joining tree of profile (n = 80) and test (n = 70) sequences. Test sequences are preceded by a T. Location abbreviations are in parentheses. Locations and abbreviations are as in Appendix 1.



APPENDIX 2. Continued.