Barcoding Ephemeroptera, Plecoptera, and Trichoptera of North America

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INTRODUCTION

The three insect orders Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies), or "EPT", are aquatic macroinvertebrates commonly encountered in various freshwater habitats. Because of their great diversity and abundance, they are widely used as bioindicators of water quality. However, the difficulties in species-level identification, especially of immature stages, have hindered such activity. The DNA barcoding initiative provides an alternative route in species identification with a number of advantages that complement the conventional morphological approach. For example, DNA barcoding can improve resolution on taxonomic identification by discriminating cryptic species (Hajibabaei et al. 2006, Hebert et al. 2004) and enable confident association of larval and adult stages (Zhou et al. *in press*, Pfenninger et al. 2007).

Churchill Project

Approximately 1,000 caddisfly specimens, including at least 28 species, were collected in Churchill, Manitoba, Canada, from 2004 to 2006. A number of species are represented by many individuals. Churchill caddisflies, including a number of closely related limnephilid species (Limnephilidae), provide a good opportunity to test the ability of COI in delimiting species boundaries in Trichoptera, especially within highly diversified genera.

Impacts of Formalin Exposure on DNA Quality

At the launching stage of the EPT barcoding project, one of the bottlenecks that has hindered progress in establishing the COI barcode reference library is the limited resource of specimens. Although EPT specimens are frequently collected in previous and on-going biomonitoring programs, they have been routinely exposed to formalin solution for a period of time in order to preserve morphological characters. To test the impacts of formalin exposure on subsequent DNA analyses, we sequenced 187 individuals from 4 common freshwater macroinvertebrate species that were preserved in formalin for various periods of time.

METHODS

Churchill caddisflies were captured in Malaise traps and light traps from 2004 to 2006 in collection trips conducted by the University of Guelph and the University of Manitoba. All specimens were initially preserved in 95% ethanol, most of which were later pinned and preserved as dry specimens. The majority of caddisfly specimens were barcoded as part of the Polar Research Observatories for Biodiversity and the Environment (PROBE) project. When possible, caddisflies were identified to species level based on adult morphology.

Four cultured macroinvertebrate species, *Hexagenia limbata*, *Tubifex tubifex*, *Hyalella azteca*, and *Chironomus riparius* were used as model organisms to test the impacts of formalin exposure on DNA analyses. Specimens were initially preserved in 1:3 solution of 10% buffered neutral formalin and water, and then transferred to 70% ethanol solution in individual tubes. Five individuals of each taxon were exposed for various time periods of 2, 5, 10, 15, and 20 days. An independent test set was also conducted using nearly identical conditions except the specimens were preserved in formalin with the addition of sediments to emulate the inclusion of organic material in typical benthic samples in the field.

COI sequences were analyzed in BOLD and MEGA 4.0 beta using neighbor-joining method.

RESULTS

Churchill Trichoptera:

COI barcodes were collected from 616 Churchill caddisflies, including 28 species from all 3 suborders of Trichoptera (Integripalpia, Annulipalpia, and "Spicipalpia") (Table 1). Limnephilidae is clearly the most diversified and abundant group among all Churchill caddisflies. Nearly all caddisfly species collected in Churchill have a very wide geographic distribution, but the majority represent new records for the Churchill region. Genetic divergence of COI sequences was calculated in BOLD. In general, conspecifics are well clustered while the interspecific divergence among Trichoptera species, especially those from different suborders are great (Figs. 1, 2). The mean intraspecific divergence is 0.52% (0-4.02%), while the mean interspecific distance is 10.84% (3.51%-18.12%).

Three limnephilid species, *Grammotaulius interrogationis, Limnephilus hageni*, and *Asynarchus batchawanus* are represented by a great number of individuals (92, 108, and 107 respectively). Distinct haplotype clusters are recognized in *G. interrogationisi* and *A. batchawanus*, suggesting the potential of cryptic species. Similarly, haplotypes of *L. sansoni* are clustered into two distinct groups. Such potential species boundaries may be clarified using independent gene markers (such as 28S nuclear DNA) or ecological evidence (such as sub-division of microhabitats).

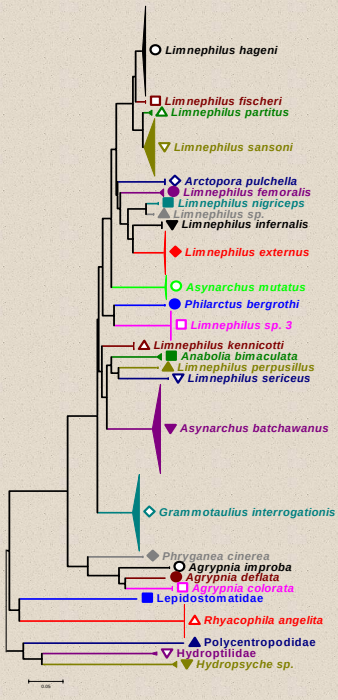

Fig. 1. COI neighbor-joining tree of Churchill Trichoptera constructed in MEGA 4.0 beta. Terminal lineages (individuals of the same species) are compressed into a solid elongated triangle, whose height is proportional to the number of conspecifics condensed, and whose width is proportional to its intraspecific divergence.

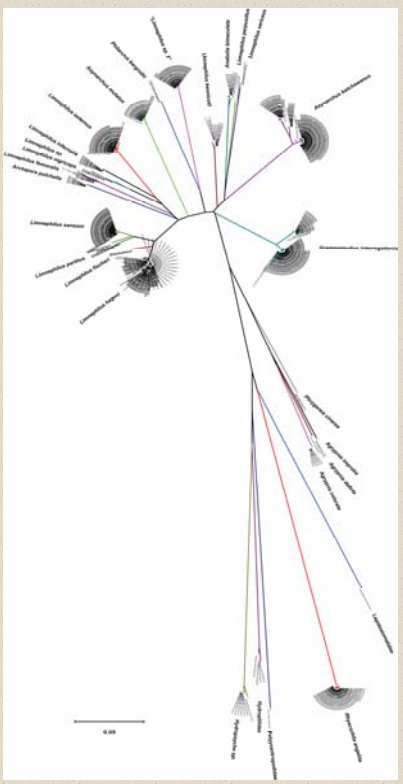

Fig. 2. COI radiation tree of Churchill Trichoptera constructed in MEGA 4.0 beta using neighor-joining. Taxon name is marked at the terminal of each haplotype cluster.

Table 1. Checklist of Churchill Trichoptera

Taxon	# of individuals
Hydropsychidae	
Hydropsyche sp.	7
Polycentropodidae	1
Hydroptilidae	4
Rhyacophilidae	
Rhyacophila angelita	40
Lepidostomatidae	1
Limnephilidae	
Anabolia bimaculata	8
Arctopora pulchella	7
Asynarchus batchawanus	107
A. mutatus	29
Grammotaulius interrogationis	92
Limnephilus externus	52
L. femoralis	9
L. hageni	108
L. infernalis	8
L. kennicotti	9
L. nigriceps	3
L. partitus	6
L. perpusillus	2
L. sansoni	69
L. sericeus	2
L. sp.	2
L. sp. 3	35
L. sublunatus	3
Philarctus bergrothi	2
Phryganeidae	
Agrypnia colorata	5
A. deflata	1
A. improba	2
Phryganea cinerea	2

Impacts of formalin exposure on DNA quality:

In a single trial, 180 out of 187 (95.7%) PCR amplifications using Folmer primers (Folmer et al. 1994) produced a product apparent on visualization gel. 170 PCR products (90.9% of the total samples) were then successfully sequenced. In general, DNA quality was high (with full length and low noise) when formalin-exposure was shorter than 15 days. Sequence quality began to decrease after 20-day exposure (although many 20-day-exposure samples did yield high-quality sequences). Meanwhile, preserving specimens with sediments did not seem to have significant impacts on DNA quality. Qualitative results, i.e. electropherograms of COI sequences collected from various species, with various formalin-exposure time periods and sediment, are shown in Fig. 3.

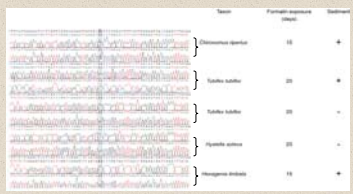

Fig. 3. Electropherograms of COI sequences collected from 4 freshwater macroinvertebrate species, with various formalin-exposure time periods and sediment.

Discussion

COI barcodes were effective in delimiting the species of caddisflies at Churchill. Although the number of taxa included in this analysis is limited, Churchill specimens provide an interesting case study, where the local fauna is relatively thoroughly sampled. A great number of individuals were sequenced for several abundant species, which has revealed some distinct clustering patterns in haplotypes of *A. batchawanus, L. sansoni*, and *G. interrogationis*. The potential of discovering the evolutionary and ecological principles that had led to the clustering of these haplotypes would have been overlooked if only a few conspecifics had been sampled.

Success in sequencing formalin preserved freshwater macroinvertebrates was surprisingly high. Furthermore, the mayfly larva, *Hexagenia limbata*, seemed to have lower success rate in PCR amplifications, which was likely a primer issue. Consequently, the overall performances of PCR and sequencing can be further improved with additional PCR efforts. The result of this pilot experiment suggests that the aquatic macroinvertebrate specimens collected by routine methods in various bioinventory and biomonitoring programs may serve as good sources for DNA barcoding without requirement of significant modifications on the existing biomonitoring protocols.

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