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DNA barcoding to confirm morphological traits and determine relative abundance of burrowing mayfly species in western Lake Erie

Curt L. Elderkin [a,b,*,1], Lynda D. Corkum [c], Claudia Bustos [c], Erica L. Cunningham [b,1], David J. Berg [d]

[a] Department of Biology, The College of New Jersey, Ewing, NJ 08628 USA
[b] Department of Zoology, Miami University, Oxford, OH 45056 USA
[c] Department of Biological Sciences, University of Windsor, Windsor, ON N9B 3P4 Canada
[d] Department of Zoology, Miami University, Hamilton, OH 45011 USA

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ABSTRACT

Burrowing mayfly species of the genus *Hexagenia* are well known indicators of environmental health in lakes and rivers. Two species, *H. limbata* and *H. rigida*, are indistinguishable as nymphs and as adult females. Our objectives were to develop a genetic technique to distinguish between the two species and identify morphological features that separate cryptic nymphs and adult females. Fifty nymphs were collected before emergence from 10 sites throughout the western basin of Lake Erie in 2004 and 2005. Using known specimens of adult aerial male *H. limbata* and *H. rigida*, we used the mitochondrial cytochrome c oxidase subunit 1 (COI) gene to identify a 16 base pair (bp) difference between species. DNA sequencing confirmed correct species identification based on differences in abdominal pigmentation patterns on adult female imagos in 19 of 20 cases; the lone exception was a female with very faint pigmentation. Pigmentation patterns between species were consistent on nymphs, subimagos and imagos of both sexes. Populations of both species are panmictic across the western basin of Lake Erie, but *H. limbata* is the numerically dominant species, representing 70 to 100% of nymphs at sites in both years. A separate lineage of *H limbata* was discovered in the samples. The ability to distinguish nymphs of the two species will aid in developing more sensitive ecosystem indicators.

Introduction

Taxonomic sufficiency in the identification of organisms is based on a number of factors. Study objectives, logistics, budget restrictions, and statistical effect-size deemed appropriate determine the accuracy of species identification to detect ecological patterns (Bailey et al., 2001; Corkum and Ciborowski, 1988; Jones, 2008). Synoptic surveys or index-based bioassessment studies often use coarse taxonomic resolution, trophic guilds, morpho-behavioural type, or size category to develop empirical relationships between biota and environmental variables (Corkum and Ciborowski, 1988). By contrast, species identification provides the most discriminatory information among samples (Lenat and Resh, 2001) and is typically required for studies in biodiversity, physiology, toxicity, and environmental impact (Rosenberg et al., 1986).

Cryptic species are indistinguishable morphologically, but are divergent with respect to other traits (Freeman and Herron, 2004) such as DNA sequences or behaviors. The Consortium for the Barcode of Life (Frézal and Leblois, 2008) has developed a standardized molecular identification system (Hebert et al., 2003) to rapidly sort among species that are morphologically indistinguishable. This DNA barcode is a short (about 650 bp) fragment of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene that uses the mouse mitochondrial genome as a reference (Hebert et al., 2003). The COI gene has been used effectively to identify many eukaryotic species, including invertebrates such as springtails (Hogg and Hebert, 2004), mayflies (Ball et al., 2005), and chironomids (Pfenninger et al., 2007; Sinclair and Gresens, 2008), where specific life history stages, damaged specimens, or cryptic species are difficult to identify using morphological traits.

Historically, burrowing mayflies of the genus *Hexagenia* were conspicuous in the benthos of western Lake Erie (Britt et al., 1973). Populations collapsed following a period of anoxia in 1953 (Britt, 1955) and remained low for about 40 years. Since 1994, populations in western Lake Erie have greatly increased in abundance (Krieger et al., 2007). As a result, mayflies have been considered to be an important taxon as a water quality indicator for lake recovery (Schloesser et al., 2009). *Hexagenia* species normally spend 1–2 y in the nymphal

* Corresponding author at: Department of Biology, The College of New Jersey, PO Box 7718, Ewing NJ 08628, USA. Tel.: +1 609 771 2819.
E-mail addresses: elderkin@tcnj.edu (C.L. Elderkin), corkum@uwindsor.ca (L.D. Corkum), bergdj@muohio.edu (D.J. Berg).
[1] Present address: Environmental Security, Marine Corps Base Camp Pendleton, Camp Pendleton, CA 92055 USA.

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stage (Corkum et al., 1997), the life history stage which cannot be identified taxonomically using morphological traits. Life history interpretation of *Hexagenia* species is difficult because populations have a protracted emergence period (Corkum et al., 1997), often exhibit multiple cohorts (Heise et al., 1987), delayed hatching of eggs (Gerlofsma, 1999), differential growth of males and females (Wright et al., 1982), and wide variability in growth rates of individuals from the same egg mass (Hanes and Ciborowski, 1992; Hunt, 1953). *Hexagenia limbata* and *H. rigida* frequently co-occur, contributing to the complexity of life history analysis (Corkum et al., 1997).

Identification of *H. limbata* and *H. rigida* has been based solely on the genitalia of the aerial male imagos (Burks, 1953) and the chorionic features of fertilized eggs (Koss, 1968). Additionally, naturalists have consistently observed a dark band along the outer margin of the hind wing in *H. limbata* adults (Corkum, personal observation). Although nymphal collections have been made throughout the western basin during the recovery of the species (Schloesser et al., 2000), the relative abundance of nymphs between species is unknown. Accordingly, researchers who study nymphs or adult females of *Hexagenia* have had to group the two species into a single genus for study (e.g., Edsall et al., 1999; Schloesser et al., 2000).

In this study, we tested the hypothesis that the DNA barcode technique can be used to discriminate the two cryptic species of *Hexagenia* from western Lake Erie and then used this technique to estimate relative abundance of nymphs collected from sites in the western basin of Lake Erie in 2004 and 2005. Finally, we identified and described morphological traits that distinguish nymphs and female imagos of these two species and confirmed these identifications using the barcode analysis.

Materials and methods

Fifty mayfly nymphs were collected using an Ekman grab sampler from each of ten sites in the western basin of Lake Erie in late May 2004 and again in 2005 before *Hexagenia* emergence in June. Ten sites across the basin with high densities of mayfly nymphs were chosen from historically sampled locations described in Appendix 1 of Schloesser et al. (2000). We retained the same designations for these sites. Lake sediment from the grab samples was washed in a 500 μm mesh sieve bucket and individuals collected until 50 nymphs were obtained from a site. Nymphs were rinsed in lake water, individually flash frozen in liquid nitrogen, and transported back to Miami University, Ohio, where they were stored at −80 °C until analysis.

In June 2004 and 2005, known specimens of male imagos (*H. limbata* and *H. rigida*) were collected after sunset at dock lights near shore from both the Detroit River at Windsor, Ontario (42°20'27" N, 82°56'56" W) and Colchester Harbour, Ontario (41° 59' N, 82° 55' W), on the north shore of western Lake Erie. Male specimens were preserved in 80% ethanol, identified to species using penial lobes (Burks, 1953), and sent to Miami University for genetic analysis. In June 2006, we collected female *Hexagenia* imagos after sunset from the same two sampling locations in Ontario. Individual female imagos (clear wings with extruded eggs) that were attracted to dock lights were collected and preserved in 80% ethanol.

Based on years of sampling, counting and measuring nymphs and adults of *Hexagenia* from Lake Erie, we noticed two types of pigmented patterns on the abdomens of the mayflies that were present among nymphs, subimagos, and imagos (Corkum & Bustos, personal observations). Using UTHSCSA Image Tool, we measured the amount of pigmented areas on the 6th (arbitrarily selected) abdominal segment of the female imagos compared to the entire surface area of the tergum. Analysis of covariance (ANCOVA) was used to test for significant differences in slopes and intercepts for pigmented areas between species. We assigned a code number and sent 20 individuals of each specimen type from the University of Windsor to Miami University. To test if these morphological patterns represented distinct species, specimens underwent DNA barcode analysis.

Total DNA was extracted from legs of individual nymphs and thoracic tissue of individual imagos using a Qiagen™ tissue extraction kit according to the manufacturer's specifications. Isolated DNA was quantified and an aliquot was diluted to 0.005 μg/μL. A 681-base-pair sequence of the CO1 gene was amplified with the polymerase chain reaction. Primers 22me (5'- GGTCAACAAATCATAAAGATATTGG-3') and 700dy (5'-TCAGGGTGACCAAAAAATCA-3'; modified from Folmer et al., 1994) were used with the following cycle repeated 35 times: 94 °C for 30 sec; 42 °C for 30 sec; 72 °C for 1.5 min with a hot start of 94 °C for 2 min. PCR products were isolated on a 2% agarose gel and DNA was extracted using a Qiagen™ gel extraction kit, according to the manufacturer's specifications. The product was then amplified again using the same primers, and forward and reverse sequences were determined using an Applied Biosystems™ Genetic Analyzer

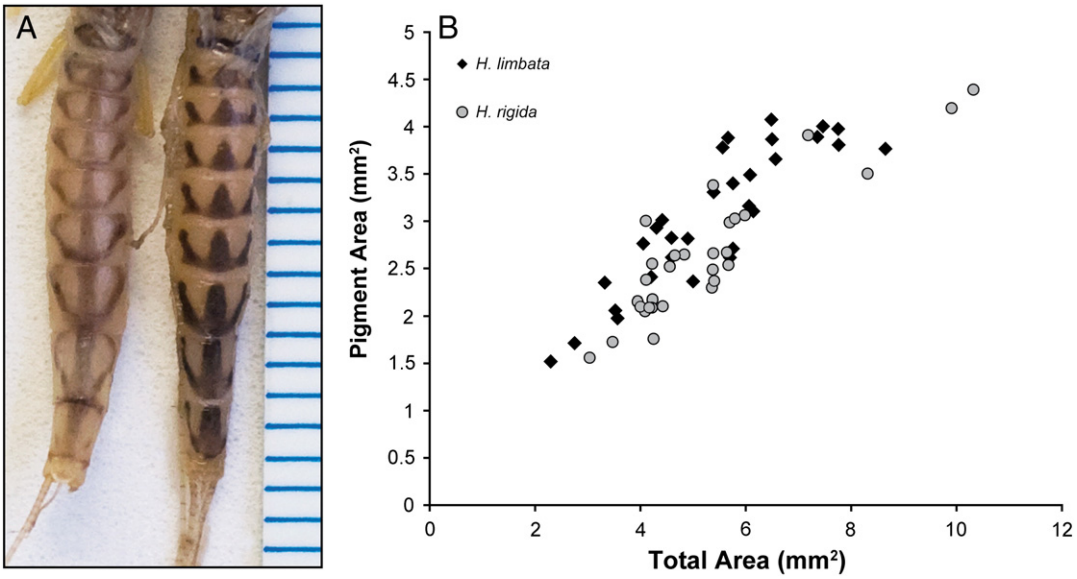

Fig. 1. Abdominal pigmentation (A) that distinguishes females of *H. limbata* (left) from *H. rigida* (right). Ruler lines (provided for scale) are 1 mm apart. (See text for description of pigmentation patterns.) The relationship between pigmented area (B) on the 6th abdominal tergum and the total area of the 6th abdominal tergum for female imagos of *H. limbata* (◆) and *H. rigida* (◯). Photograph by Peter Marval.

Table 1
Of 681 base pairs of the mitochondrial CO1 gene examined for *H. limbata* and *H. rigida*, there were 16 fixed interspecific nucleotide substitutions. The relative positions (s added) of these substitutions are listed below. A = adenine, T = thymine, C = cytosine, G = guanine.

CO1 position (base pair #)																
Species	42	81	147	375	387	486	514	537	546	558	561	564	627	633	651	654
H. limbata	A	G	C	T	T	A	T	C	C	G	T	C	T	C	C	C
H. rigida	G	A	T	C	C	T	C	T	T	A	C	A	C	A	T	T

model 3100, 3130xl, or 3730 using manufacturers specifications. Sequences were aligned in WordPad and MacClade v4.06 (Maddison and Maddison, 2003).

Sequences were analyzed in Arlequin v2.0 (Schneider et al., 2000) to examine base pair (bp) differences within and between species. Mismatch distributions, which display differences in nucleotide sequences among pairs of individuals, were used to visualize the genetic differences within and between *Hexagenia* species and determine whether fixed nucleotide differences were present. If our hypothesis that DNA Barcoding will be useful for *Hexagenia* is correct, then mismatch analysis will yield a bi-modal distribution with a "Barcoding Gap" between species (Alexander et al., 2009; Meyer and Paulay, 2005). A Neighbor-joining tree was generated for a 609 bp fragment of the COI gene with an Approximate Likihood-Ratio Test (aLRT) (Phylogeny.fr; Dereeper et al., 2008). The phylogeny was calculated using an HKY85 substitution model, which was the optimum model calculated

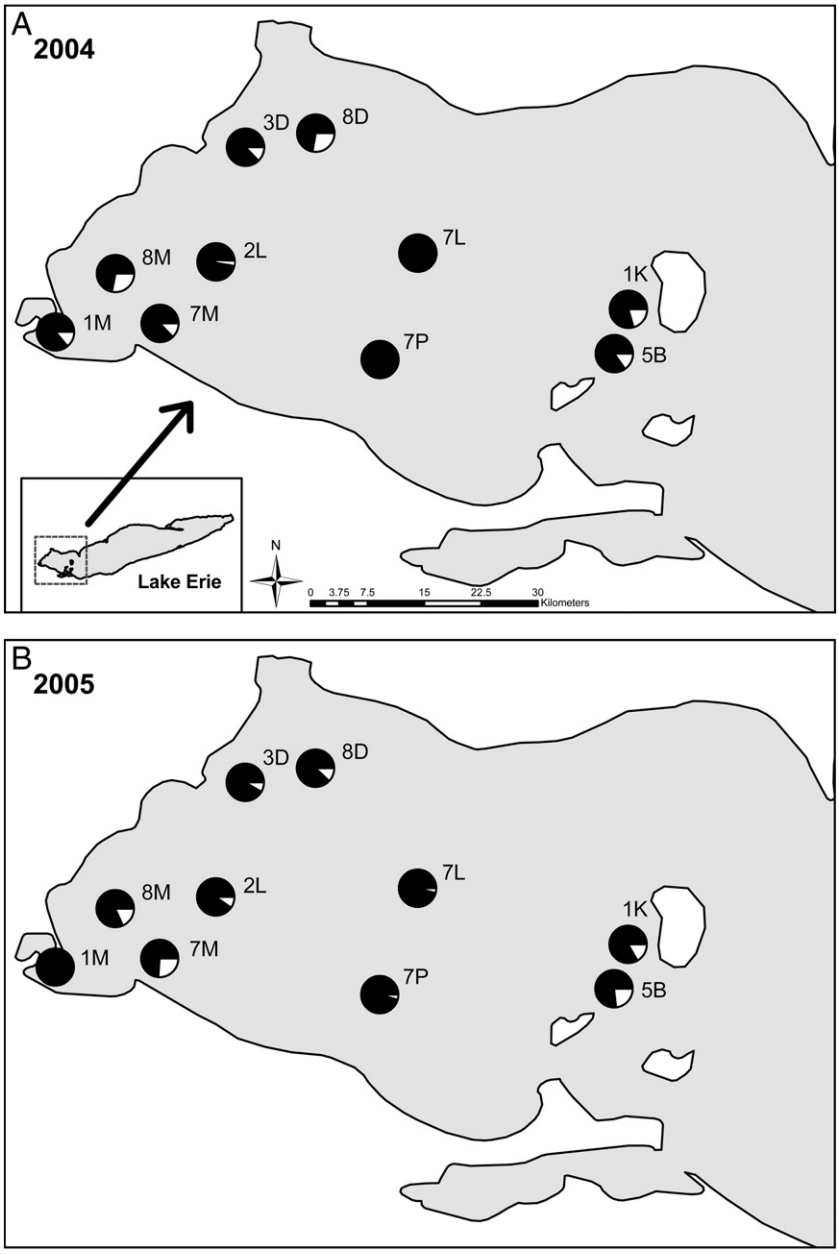

Fig. 2. Spatial distribution of *H. limbata* (dark sector) and *H. rigida* (light sector) at the 10 sampling sites in the western basin of Lake Erie in 2004 (A) and 2005 (B). Site codes are those of Appendix 1 in Schloesser et al. (2000).

using jMODELTEST (Posada, 2008). Outgroup and additional *H. ridgida*/*H. limbata* sequences were taken from Genbank and aligned by eye in MS Wordpad. Individual nymphs from each site were assigned a species identification based on the COI sequencing. We used these results to determine the relative abundances of the two species for 2004 and 2005, and compared these abundances to male emergence data from the same year (sampling described above).

Results

Abdominal pigment patterns of female imagos differed between the two species (Fig. 1). In *H. limbata,* one broad medial and two narrow lateral bands of dark pigment extend anteriorly from the transverse pigment band across the base of each tergum, giving a wine glass shape when viewed from the base. In *H. rigida*, one medial and two lateral bands of pigmentation extend the length of the tergum, merging with the transverse band, forming an overall crown pattern when viewed from above. These patterns were also present on other life stages (female subimagos, nymphs, male subimagos and imagos). Although the patterns were present on both adult male stages (subimagos and imagos), the dark background of males made the patterns more difficult to discern compared with the pigmented patterns on adult females and nymphs. There was a significant difference between intercepts for the regression lines comparing the pigmented and total areas of the 6th abdominal tergum between species (ANCOVA, $F_{1,\ 57} = 18.25$, $P < 0.001$), indicating more pigment was present on *H. limbata* than *H. rigida*. However, the slopes of the regression lines did not differ significantly between species (ANCOVA, $F_{1,56} = 0.724$, $P = 0.398$; Fig. 1).

Hexagenia limbata and *H. rigida* were distinguished based on fixed differences at 16 nucleotide positions of 681 bp that were examined (Table 1). This analysis was based on COI findings from adult male imagos that are morphologically distinguishable between species using genitalia. Of the coded female imagos that were analyzed, 19 of 20 specimens identified using morphological pigmented patterns on the abdomen were correctly identified. Pigmented markings on the single misidentified specimen were faint.

These bp differences enabled us to determine the relative abundance of nymphs for each species at the 10 sampling sites in 2004 (Fig. 2A) and 2005 (Fig. 2B). Species abundances changed between years, but there were more nymphs of *H. limbata* than *H. rigida* at every site during both years. In 2004, nymphs from sites in the middle of the western basin (7 L and 7P) were entirely *H. limbata.* In 2005, a few *H. rigida* nymphs were present at these central locations, whereas a site in the southwest corner of the lake, near the Maumee River mouth (1 M) had only *H. limbata* nymphs.

For each year, analysis of molecular variance (AMOVA) showed that 100% of the genetic variation was within sites (0% among sites, $F_{st} = 0.00$), suggesting that populations of *H. limbata* and *H. rigida* were panmictic spatially and that genetic diversity was homogenized among sites. Examination of a mismatch distribution combining both species revealed three modes centered at differences of 3 bp, 11 bp, and 44 bp (Fig.3). When mismatch distributions were created separately for each species (based on the fixed nucleotide differences), a bimodal distribution, centered at differences of 2 bp and 10 bp, was present for *H. limbata* (Fig. 4B). The mismatch distribution for *H. rigida* was unimodal, centered on 2 bp (Fig. 4A). Phylogenetic analysis shows distinct monophyletic branches for *H. rigida* and *H. limbata* respectively (Fig. 5). However *H. limbata* sequences formed two significant clades within the species, corresponding to the two modes found in the mismatch analysis for *H. limbata* (Fig. 4B).

Discussion

We distinguished the species *H. limbata* and *H. rigida* using fixed differences in the mitochondrial COI gene. The barcoding technique was successful in identifying nymphal specimens collected from the western basin that previously could not be distinguished morphologically. *Hexagenia limbata* nymphs were more abundant than *H. rigida* at all 10 sample sites throughout western Lake Erie in 2004 and 2005, representing 70 to 100% of nymphs collected. Three sites were entirely *H. limbata* for one of the two years. The prevalence of *H. limbata* as nymphs is consistent with proportions of adult male imagos collected from the northern shore of western Lake Erie (Corkum, 2010). Based on a 12-year monitoring program of male imagos (distinguished using genitalia, Burks, 1953, page 40), *H. rigida* was the dominant species in 1997, *H. limbata* and *H. rigida* were co-dominant in 2000, and *H. limbata* was dominant in 2002 (Corkum, 2010). Once *H. limbata* became the dominant species (> 90%) in 2000 to 2002 (depending on the site), it remained so. By 2004, most nymphs in the western basin of Lake Erie were *H. limbata*, although both species were present throughout.

The mismatch distributions support our hypothesis that DNA barcoding can be used to differentiate these two cryptic mayfly species in

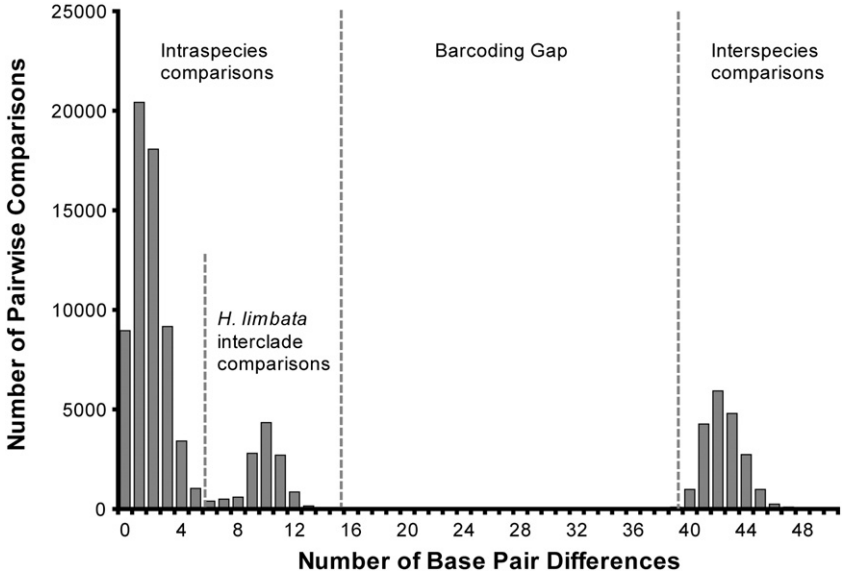

Fig. 3. Mismatch distributions displaying the number of base pair differences among all individuals. Note the tri-modal nature of the distribution with a considerable barcoding gap between pairwise comparisons of *H. limbata* and *H. ridgida.*

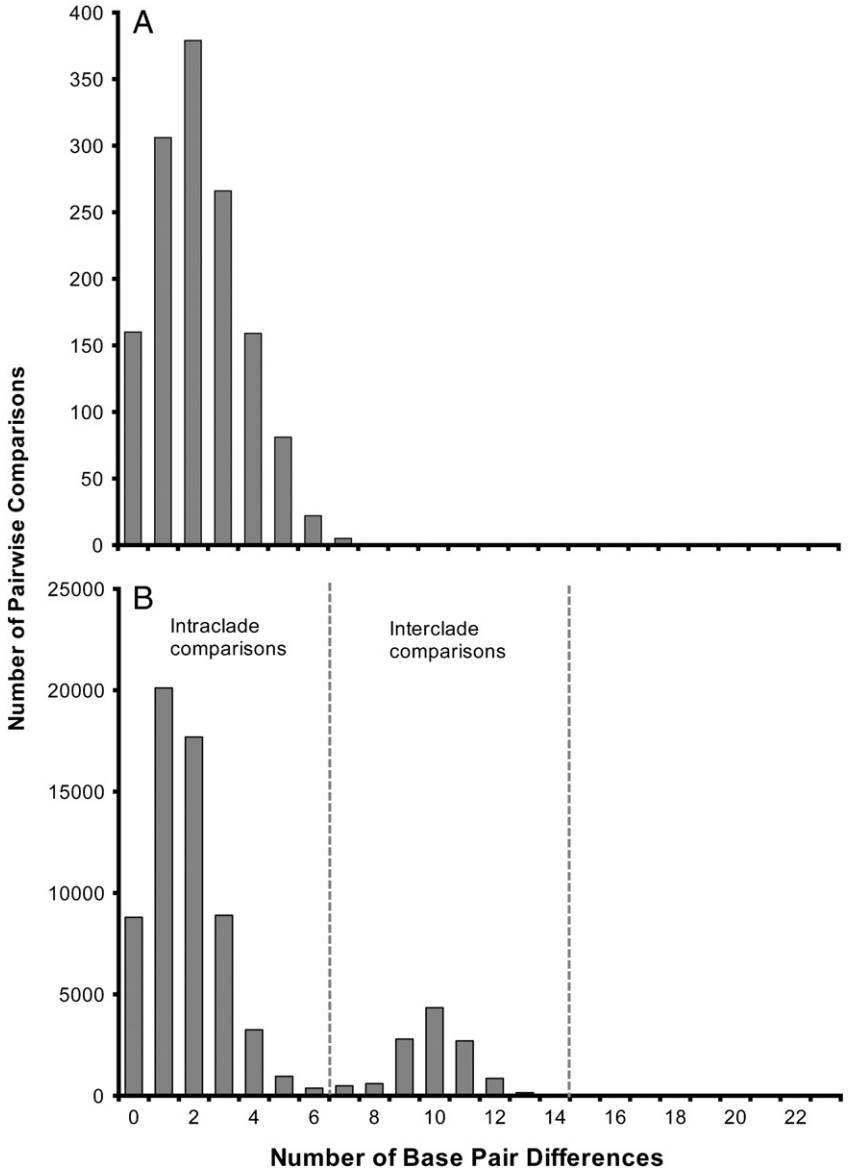

Fig. 4. Mismatch distributions displaying the number of base pair differences between *H. rigida* individuals (A) and between *H. limbata* individuals (B). Note the bi-modal plot B indicating both intra and interclade comparisons within *H. limbata* (see discussion for explanation).

western Lake Erie. DNA barcoding analysis is dependent on the assumption of a significant gap between intraspecific and interspecific DNA variation (Meyer and Paulay, 2005), especially for identifying cryptic species. Previous results of barcoding analysis have been inconclusive if investigators encountered considerable overlap between intra- and interspecific variation (Alexander et al., 2009; Meyer and Paulay, 2005). Our results confirm the existence of a significant gap between *H. limbata* and *H. rigidia* COI sequences and support our hypothesis that DNA barcoding can be used to distinguish these cryptic species. However, our results revealed the presence of two distinct intraspecific clades within *H. limbata*. Based on our phylogenetic analysis, this variation clearly represents an evolutionarily significant unit of *H. limbata* (Moritz, 1994), where there is reciprocal monophyly of the two *H. limbata* clades.

Four species of *Hexagenia* occupy waterbodies in Ontario and Ohio and overlap in their distributions, including *H. atrocaudatus*, *H. bilineata, H. limbata*, and *H. rigida* (Randolph, 2002). A previous analysis of three species of *Hexagenia* (*H. atrocaudatus, H. limbata, H. rigida*) using COI showed that *H. limbata and H. rigida* were closely related and *H. atrocaudatus* was more distant from the two (Ball et al., 2005). We found a similar evolutionary relationship among *Hexagenia* spp. in our phylogenetic analysis; however the *H. limbata* clade II has been thus far undocumented in the western basin. Possibly, the *H. limbata* clade II could be a previously synonomized species (S. Burian, personal communication). Further, considering the recent recolonization of the western basin, multiple clades in a single population could represent population admixture due to multiple overlapping dispersal events from distinct source populations.

Hexagenia rigida was the initial colonizer of western Lake Erie after the recovery of the lake from a major eutrophication disturbance (Corkum, 2010). Biotic resistance theory assumes that the incumbent limits opportunities for new arrivals (Vermeij, 1991; Ricciardi and Whoriksey, 2004). However, an analysis based on aerial adult *Hexagenia* (Corkum, 2010) and nymphs (this study) confirms that the subsequent colonizer, *H. limbata*, is now the dominant burrowing mayfly in western Lake Erie. This is consistent with relative abundances in Lake Erie prior to the collapse of mayfly populations in the mid-20th Century, when approximately 75% of burrowing mayflies were *H. limbata* (Chandler, 1963).

Our ability to distinguish the two species of *Hexagenia* will enable us to determine competitive interactions or changes in life history traits in response to environmental challenges. Although *Hexagenia*

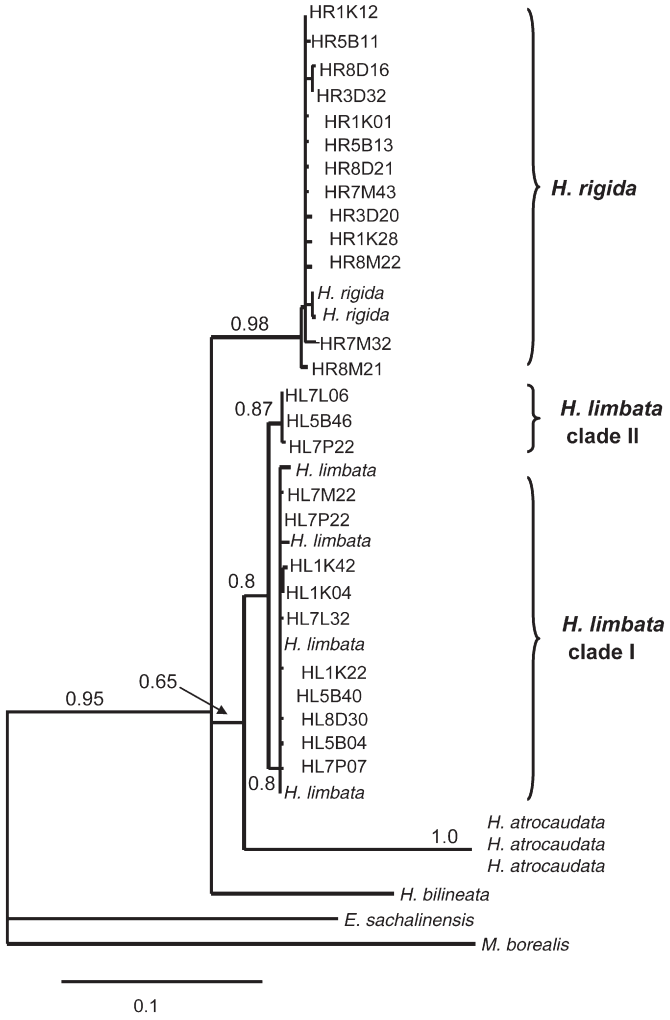

Fig. 5. Neighbor-Joining tree of *H. rigida* and *H. limbata* from the western basin of Lake Erie (abbreviated HR and HL with identification number). Numbers on branches represent the proportion of Approximate Likelihood-Ratio (aLRT) branch support. Sequences labeled genus/species were taken from previously published sequences using Genbank.

is a known indicator of elevated oxygen conditions at the sediment-water interface in the Laurentian Great Lakes (Krieger et al., 1996; Schloesser et al., 2000) and the Mississippi River Basin (Fremling, 1964a,b), understanding the population dynamics of each species will enhance their usefulness for bioassessment of lakes and rivers.

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