



# A preliminary molecular assessment of the taxonomic validity of *Hexagenia orlando* Traver, 1931 (Ephemeroptera: Ephemeridae)

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DOI: 10.31383/ga.vol4iss2pp43-49

## Abstract

In an effort to evaluate the taxonomic uncertainty of the species status of *Hexagenia orlando* Traver, 1931 (Ephemeroptera: Ephemeridae), molecular data were used for the first time to test its current classification. Mitochondrial cytochrome c oxidase subunit I (COI) haplotypes were evaluated using three types of analyses in the form of distance-based, tree-based, and model-based delimitation methods. All analyses consistently recovered *H. orlando* as a valid species. These preliminary results lend evidence reinforcing the current classification scheme and encourage renewed scrutiny of adults and nymphs to hopefully identify additional morphological characters that may serve to separate *H. orlando* and its congener *Hexagenia limbata* (Serville, 1829).

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### Received

September, 2020

### Accepted

October, 2020

### Published

December, 2020

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## Keywords

*burrowing mayflies,*  
*DNA barcoding,*  
*species delimitation,*  
*taxonomic hypothesis*

## Introduction

Understanding species boundaries and proper classification of life is at the heart of biology. Dozens of species concepts and delimitation methods have been utilized to address these questions (De Queiroz, 2007; Zachos, 2018). The several data used in defining species include classical morphology, meristics, various molecular

data, and ecology. Ideally, multiple lines of evidence should be considered when testing a species hypothesis and this is often necessary to resolve conflicting hypotheses.

Some members of the burrowing mayfly genus *Hexagenia* Walsh, 1863 (Ephemeroptera:

Ephemerae) are good candidates for additional scrutiny. The genus consists of eight nominal species i.e., *Hexagenia albivitta* (Walker, 1853), *Hexagenia atrocaudata* McDunnough, 1924, *Hexagenia bilineata* (Say, 1824), *Hexagenia callineura* Banks, 1914, *Hexagenia limbata* (Serville, 1829), *Hexagenia mexicana* Eaton, 1885, *Hexagenia orlando* Traver, 1931, *Hexagenia rigida* McDunnough, 1924 (Barber-James et al., 2013) that inhabit lotic and lentic systems throughout the New World. Of the five North American species, only two have been recorded from the state of Florida, USA: *H. limbata* and *H. orlando* (Berner and Pescador, 1988; Pescador and Richard, 2004). While *H. limbata* is a lentic and lotic species ranging across much of North America (e.g., Orfinger and Lucky, 2019), *H. orlando* appears to be restricted to sand-bottom lakes of central Florida and southern Georgia (Berner and Pescador, 1988; Pescador and Richard, 2004; McCafferty et al., 2010).

*Hexagenia orlando* has a complex taxonomic history. Described by Traver in 1931, the species has subsequently been treated variously as a subspecies of *Hexagenia munda* (itself now a junior synonym of *H. limbata*) and as a valid species (Spieth, 1941; Berner, 1950; McCafferty, 1975; Berner and Pescador, 1988; Pescador and Richard, 2004). The taxonomic uncertainty stems from morphological similarity of adults and nymphs. Currently considered a valid species, adults are separated based solely on smaller body size relative to *H. limbata*, color pattern, and its unique ecology in inhabiting sand-bottom lakes (Berner and Pescador, 1988; Pescador and Richard, 2004). The genitalia of imagoes of the two species are only marginally different and not reliable in discriminating between them (Pescador and Richard, 2004). Furthermore, nymphs of the two species are not currently separable morphologically (Pescador and Richard, 2004; McCafferty et al., 2018). Speculation has arisen that *H. orlando* may in fact be an ecophenotype of the more widespread *H. limbata* (Pescador and Richard, 2004), and it has again recently been suggested that *H. orlando* is likely synonymous with *H. limbata* (McCafferty et al., 2018).

Fortunately, modern molecular delimitation methods and available public data make it possible to reevaluate the current species hypothesis, which is the impetus of this study. DNA barcoding using a

roughly 648 bp fragment of the mitochondrial gene cytochrome c oxidase subunit I (COI) has been widely employed in both identification and testing of species boundaries in recent years, including in aquatic insects (Hebert et al., 2003; Puillandre et al., 2012; Webb et al., 2012; Cardoni et al., 2015; Tenchini et al., 2018). This method exploits the typically high intraspecific specificity and interspecific disparity of this DNA barcode to reliably identify species. The goal of this paper therefore is to use COI to reexamine the interesting case of the *H. orlando* species hypothesis in an effort to lend additional evidence to the longstanding confusion surrounding its taxonomy. To accomplish this aim, three analytical strategies are employed and compared: distance-based, tree-based, and model-based methods.

## Material and methods

### Sequence Data

Nucleotide data were mined from the Barcode of Life Data System (BOLD; Ratnasingham and Hebert, 2007; www.boldsystems.org). All available COI haplotypes for *Hexagenia orlando* (n=2) were extracted. While more exemplars of the species would be ideal and statistically more robust, two is still informative for addressing the question at hand in light of the high intraspecific consistency of the barcoding fragment of COI. Twenty-one COI sequences of *H. limbata* were haphazardly selected to encompass a variety of localities. Finally, four sequences of two *Ephemera* Linnaeus, 1758 species were extracted for use as outgroup taxa for the phylogenetic analysis. A summary of the sequences used in analyses is provided in Table 1. Nucleotide sequences were aligned using ClustalW using default settings (Larkin et al., 2007).

### Distance Method

Pairwise uncorrected p-distance, or the number of base differences per site between sequences, was calculated for all *Hexagenia* sequences using the pairwise deletion option in MEGA X (Kumar et al., 2018). 1st+2nd+3rd+noncoding codon positions were used. The resulting values were converted to percentages.

**Table 1.** Classification and accession numbers of specimens used in sequence analyses and phylogenetic reconstruction.

Species	Locality	Life Stage	BOLD ID
<i>Hexagenia orlando</i>	Florida, USA	Adult	FAMAY059-07
<i>Hexagenia orlando</i>	Florida, USA	Adult	FAMAY057-07
<i>Hexagenia limbata</i>	Ontario, CA	Adult	BBEPT249-10
<i>Hexagenia limbata</i>	Ontario, CA	Adult	BBEPT266-10
<i>Hexagenia limbata</i>	Ontario, CA	Adult	BBEPT267-10
<i>Hexagenia limbata</i>	Ontario, CA	Immature	CDINV016-07
<i>Hexagenia limbata</i>	Ontario, CA	Immature	CDINV024-07
<i>Hexagenia limbata</i>	Ontario, CA	Immature	CDINV211-07
<i>Hexagenia limbata</i>	Ontario, CA	Immature	CDINV212-07
<i>Hexagenia limbata</i>	Ontario, CA	Immature	CDINV213-07
<i>Hexagenia limbata</i>	Ontario, CA	Immature	CDINV214-07
<i>Hexagenia limbata</i>	Ontario, CA	Adult	CNPPC1841-12
<i>Hexagenia limbata</i>	Minnesota, USA	Immature	EINVA436-19
<i>Hexagenia limbata</i>	Manitoba, CA	Adult	EPCHU246-07
<i>Hexagenia limbata</i>	Manitoba, CA	Adult	EPCHU247-07
<i>Hexagenia limbata</i>	Manitoba, CA	Adult	EPCHU248-07
<i>Hexagenia limbata</i>	Manitoba, CA	Adult	EPCHU249-07
<i>Hexagenia limbata</i>	Manitoba, CA	Adult	EPCHU250-07
<i>Hexagenia limbata</i>	Manitoba, CA	Adult	EPCHU251-07
<i>Hexagenia limbata</i>	Illinois, USA	Immature	INHSE128-09
<i>Hexagenia limbata</i>	South Dakota, USA	Immature	INHSE130-09
<i>Hexagenia limbata</i>	Illinois, USA	Immature	INHSE131-09
<i>Hexagenia limbata</i>	Indiana, USA	Adult	LJMAY107-09
<i>Ephemera simulans</i>	Alberta, CA	Adult	BBEPT143-10
<i>Ephemera simulans</i>	Alberta, CA	Adult	BBEPT144-10
<i>Ephemera danica</i>	Sofiya, BG	Immature	BGMAY086-11
<i>Ephemera danica</i>	Sofiya, BG	Adult	BGMAY087-11

### Tree Methods

Model optimization was performed using the model selection feature of Topali v2 (Milne et al., 2009). A Maximum Likelihood phylogeny was constructed using the General Time Reversible (GTR) model and 1000 bootstrap replicates in MEGA X (Kumar et al., 2018). Initial trees for the heuristic search were obtained by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale and then annotated in Dendroscope 3 (Huson and Scornavacca, 2012).

### Poisson Tree Processes (PTP) Model

The outgroups were trimmed from the resulting phylogeny with the greatest log likelihood, which was then subjected to the Poisson tree processes (PTP) model to determine species boundaries (Zhang et al., 2013; code available at [www.exelixis-lab.org/software.html](http://www.exelixis-lab.org/software.html)). PTP is a single-locus species delimitation method that relies on nucleotide substitution information, implementing a model assuming gene tree branch lengths generated by two independent Poisson process classes (inter- and intraspecific substitution events; Zhang et al., 2013). This approach has been shown to generally outperform other approaches such as OUT-picking methods or the General Mixed Yule Coalescent (GMYC) model (Zhang et al., 2013; Dellicour and Flot, 2018).

### Results and Discussion

Pairwise uncorrected p-distance results are presented as percentages in Table 2. Intraspecific distance of *H. orlando* is 0.68% while mean intraspecific distance of *H. limbata* is 0.78% ( $\pm 0.01\%$ ; range 0.0%-2.28%). Interspecific distance was found to be 9.16% ( $\pm 0.005$ ; range 8.51%-10.18%).

The final tree with the highest log likelihood (-1909.81) is presented in Figure 1. In total, 23 ingroup nucleotide sequences (two of *H. orlando* and 21 of *H. limbata*) and four outgroup sequences were used. Of the *Hexagenia*, two clades were recovered with corresponding to a nominal species. Both monophylies had high statistical support.

The PTP model partition recovered two species clusters. One grouping consisted of the two *H. orlando* with the other comprised of the 21 *H. limbata*, each with low support (0.507 and 0.004, respectively).

The results were all congruent in supporting the validity of the current classification scheme. Intraspecific genetic distances were well under the 2.2% threshold typically applied to species boundaries, including in the Ephemeroptera (Ball et al., 2005; Zhou et al., 2009; Malakauskas and Zonca, 2018; Suh et al., 2019). Interspecific distances were likewise an order of magnitude higher. The gene tree supported the same conclusion with high bootstrap support, while the model-based approach produced the same classification but with lower support.

These early results add to the scant morphological evidence suggesting that *H. orlando* should indeed be considered a valid taxon. Renewed morphological study should be considered to search for consistent characters for use in separating these two species. The current study had a very explicit goal that was narrow in scope. The results of this study, while informative, should be interpreted with caution given the low sample size available for *H. orlando* sequence data.

Being the first molecular test of the validity of *H. orlando*, the data here provide additional evidence that suggests that the current taxonomy reflects biological reality. Still, the weaknesses of the data available do not provide a definitive resolution. Additional taxon sampling across the genus with more replicates and loci should be the next step in evaluating species boundaries and phylogeny of this charismatic genus of mayflies which has yet to be evaluated phylogenetically. Taking advantage of molecular data publicly available through BOLD and GenBank would be a logical first step to accomplish these goals.

## Conclusion

Single-locus species delimitation using COI is not a panacea and should not be relied upon as the only line of evidence to identify, delimit, or describe species. However, in conjunction with integrative taxonomic methods, COI is a robust tool in addressing challenging species hypotheses as the one presented here. Here, three popular analyses

were performed on mitochondrial COI data in an effort to test the validity of *H. orlando*. The results, while preliminary, support the current species status of *H. orlando*. In addition to helping resolve the taxonomic uncertainty surrounding a species of burrowing mayfly, the present effort can also serve as a case study in using multiple tests of species boundaries in order to compare results, highlight potential uncertainty, and evaluate confidence in the overall conclusion in an area of study that has endless similarly problematic cases.

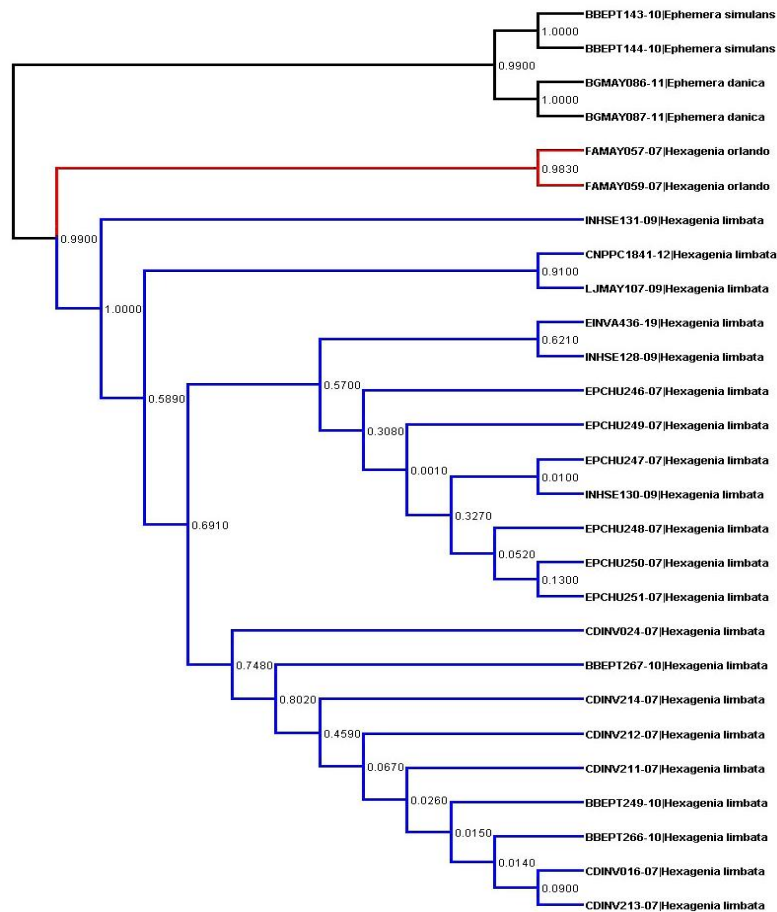
## Acknowledgement

I wish to thank Jan Peters (Florida A&M University) for her discussion on the taxonomy of *Hexagenia orlando* and David Liu for providing additional *H. orlando* for examination. Multiple reviewers provided helpful comments that helped to improve the manuscript. This work was supported by the McIntire-Stennis Program from the USDA National Institute of Food and Agriculture. The sequence data used are publicly available on BOLD and the result of other researchers' efforts to provide open access to their molecular data, for which I am thankful.

## Conflict of Interest

The author declare no conflict of interest.





**Figure 1.** A Maximum Likelihood phylogeny constructed using the barcoding segment of COI. Red branches correspond to the *Hexagenia orlando* clade while blue branches correspond to the *Hexagenia limbata* clade. Outgroup taxa are shown with black branches. Values at nodes represent bootstrap support. Branch tips display sequence ID and taxon. The tree with the highest log likelihood (-1909.81) is shown.

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