

# Phylogenetic Analysis of *Erythroneura* leafhoppers

Michael B. Roberts and Randy Hunt  
Indiana University Southeast

## Abstract:

*Erythroneura* is a genus of leafhoppers containing 54 described species specializing on a variety of trees and shrubs in the eastern United States. Species are currently separated morphologically, primarily using the structure of male genitalia. Two of these species, *E. aclys* and *E. bistrata*, are specialists on redbud trees (*Cercis canadensis*). Each species includes a range of color morphs. Mating behavior in these insects is mediated by vibrational signals. Studies of courtship behavior within and between different color morphs suggest that they are distinct species (Hunt et al., unpublished). The purpose of this project was to determine if these color morphs can be separated into distinct lineages based on mtDNA sequences. The phylogenetic analysis was based on sequences from the cytochrome oxidase I gene (about 750 bp). MEGA 6 was used to align sequences and to construct trees using the maximum likelihood method with bootstrapping to determine confidence in branches. The potato leafhopper (*Empoasca fabae*) was used as the outgroup. *E. aclys* and *E. bistrata* formed monophyletic groups, as did the *E. aclys* light form morph within the larger *E. aclys* clade. These results support the evidence for reproductive isolation in our behavioral studies and suggest that there are currently unrecognized species within *Erythroneura*. Future studies will include using sequences from the entire CO1 gene and other mitochondrial genes, as well as expanding the number of populations sampled for each species.

## Introduction

Leafhoppers (Cicadellidae) are one of the most abundant and diverse groups of insects with over 4000 described species in the United States alone (Cocroft and Rodriguez 2005). Our current understanding of leafhopper diversity is based almost entirely on the morphological species definition. There have been relatively few studies aimed at clarifying the taxonomy of closely related species based on the biological species definition (i.e. reproductive isolation) or by phylogenetic analysis. In the few groups that have been studied in depth, cryptic species are often found (Drosopoulos and Claridge 2006). This project focuses on determining the possible existence of unrecognized species within the leafhopper genus *Erythroneura*. There are 54 recognized species within this genus and many specialize on redbud trees (*Cercis canadensis*). Two of the most common species are *E. aclys* and *E. bistrata*. Each of these species is defined primarily based on characteristics of the male genitalia (Dmitriev and Dietrich 2007). Color patterns are variable within each species, although relatively discrete color morphs exist. Preliminary studies of mating signals emitted by individuals representing the different color morphs and populations of *E. aclys* and *E. bistrata* suggest that each of these species may actually form a complex of reproductively isolated species (Hunt et al., unpublished). The purpose of this pilot project was to determine if species and populations can be separated using mtDNA.

## Methods

**Taxa.** Individuals were collected, reared in the laboratory, preserved in 95% ethanol and stored at 20° C prior to DNA extraction (see Table 2).

**DNA Extraction.** DNA was extracted from a single individual from each of the species and populations. Extractions were done using a dNeasy kit™ (QIAGEN™, California).

**PCR.** mtDNA cytochrome c oxidase subunit 1(CO1) (~750 bp) was amplified using the following primers (Table 1). PCR products were purified using a UltraClean® (MO BIO Laboratories, Inc.™, California) PCR clean-up kit.

Table 1. Primers used for PCR amplification and sequencing.

Name	COI Location	Sequence
Forward	1517	5'-CWA CAA AYC ATA AGG ACA TYG GAA C-3' *
Reverse	2191	5'-CCT GGT AAA ATT AAA ATA TAA ACT TC-3' **

\*Zanhizer (unpublished, personal communication)  
\*\*Simon et al. (1994)

**Phylogenetic Analysis.** Sequences were edited and aligned using MUSCLE, which is available in the program MEGA 6 (Tamura et al. 2013). Trees were constructed using maximum parsimony and maximum likelihood procedures available in MEGA 6.

Table 2. Species and populations that were sequenced. The potato leafhopper (*Empoasca fabae*) was used as the outgroup. The vibrational attraction call emitted by males of each species is shown.

Species and Population	Male Attraction Call
<i>Erythroneura aclys</i> Light Form Lexington, KY	
<i>Erythroneura aclys</i> Dark Form Louisville, KY	
<i>Erythroneura aclys</i> Light Form Turkey Run State Park, IN	
<i>Erythroneura aclys</i> Light Form Monticello, IN	
<i>Erythroneura aclys</i> Dark Form Tippecanoe State Park, IN	
<i>Erythroneura aclys</i> Dark Form Turkey Run State Park, IN	
<i>Erythroneura bistrata</i> Red Form Louisville, KY	
<i>Erythroneura bistrata</i> Two Tone Turkey Run State Park, IN	
<i>Erythroneura bistrata</i> Two Tone New Albany	
<i>Erythroneura bistrata</i> Two Tone Cherokee National Forest, TN	
<i>Erythroneura bistrata</i> Two Tone Gatlinburg, TN	
<i>Empoasca fabae</i> Outgroup New Albany, IN	

## Results

The populations of *E. aclys* formed a monophyletic group and each was separated with high confidence based on bootstrap values (Fig. 1). The *E. bistrata* color morphs and populations also formed a monophyletic group.

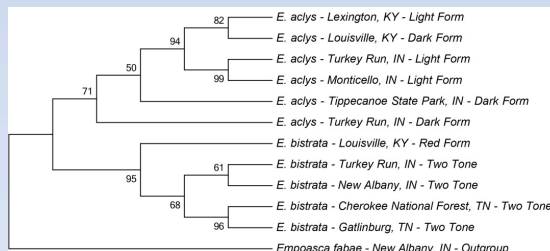


Figure 1. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura 1992). The tree with the highest log likelihood (-1623.2597) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join 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 analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 578 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

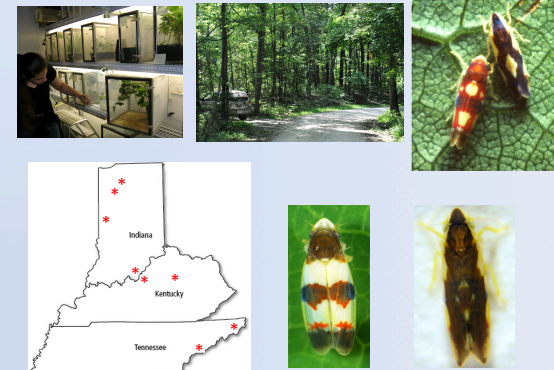


Figure 2. Collecting sites (see Table 2)

## Discussion

- Results of this pilot study show that populations and species of *Erythroneura* can be separated using mtDNA. This is especially encouraging since the analysis was based only on the partial sequences of one gene.
- The six populations of *E. aclys* form a monophyletic group and were separated with high confidence based on bootstrap values. The five populations of *E. bistrata* also formed a clear monophyletic group. Within both species closely related populations also were located within the same geographic region.
- Phylogenetic results support the hypothesis that there are additional species within the genus *Erythroneura*. In a separate study the vibrational signals emitted by males from the populations and species included in this study are distinct and detailed studies of mating behavior for some combinations of populations show that they are reproductively isolated.
- Future studies will include more species and populations collected over a broader geographic range. Such studies may provide clues about the interaction between natural and sexual selection on speciation in this diverse group of insects (see Coyne and Orr 2004, McNett and Cocroft 2008). Furthermore, a well supported phylogeny will provide a framework for understanding mating signal evolution.

## Literature Cited

- Cocroft, R.B. and Rodriguez, R.L. 2005. The behavioral ecology of insect vibrational communication. *BioScience*. 55:323-334.
- Coyne, J.A. and Orr, H.A. 2004. *Speciation*. Sinauer Associates, Inc. Sunderland, MA.
- Dmitriev, D. and Dietrich, C.H. "Review of the new world Erythroneurini (Hemiptera: Cicadellidae: Typhlocybinae) 1. Genera *Erythroneura*, *Erasmoneura*, *Rossmoneura*, and *Hymetta*." *Illinois Natural History Survey*. 2007. 38: 60-61.
- Drosopoulos, S. and Claridge, M.F. 2006. *Insect Sounds and Communication: Physiology, Behavior, Ecology, and Evolution*, CRC Press, Boca Raton, FL.
- McNett, G.D. and Cocroft, R.B. 2008. Host shifts favor vibrational signal divergence in *Enchenopa binotata* treehoppers. *Behavioral Ecology*. 19:650-656.
- Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., and Flook, P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* 87:651-701.
- Tamura K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C content biases. *Molecular Biology and Evolution* 9:678-687.
- Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* 30: 2725-2729.

## Acknowledgements

We thank the Fall 2013 Evolutionary Biology class for their contribution to this project.

