USING CYTOCHROME C OXIDASE I TO DETERMINE THE PHYLOGENETIC RELATIONSHIPS AMONG SUBSPECIES OF *CERCYONIS PEGALA* (NYMPHALIDAE: SATYRINAE)

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Abstract

The common wood-nymph, *Cercyonis pegala*, is a North American butterfly found throughout the United States, northern Mexico, and southern Canada. *Cercyonis pegala agawamensis* is a recently described subspecies that is differentiated from other subspecies of *C. pegala* based on habitat, behavior, and phenotype. A major defining characteristic is habitat, with *C. pegala agawamensis* found in salt marsh habitat, *C. pegala maritima* in coastal upland habitat, and *C. pegala nephele* in a northeastern upland habitat. The level of genetic differentiation between the subspecies is unknown; therefore, we investigated the phylogenetic relationships among *C. pegala agawamensis*, *C. pegala nephele*, and *C. pegala maritime* using the mitochondrial cytochrome c oxidase I (COI) gene. COI is a 648 base-pair region that is used as a barcoding standard to help identify the relationships between species and subspecies. Eight mtDNA haplotypes were found among 31 samples of the three subspecies. The data show that *C. pegala nephele* is genetically different from *C. pegala agawamensis* and *C. pegala maritima*. However, there is no genetic differentiation between *C. pegala maritima* and *C. pegala agawamensis*. Small phenotypic differences used to identify samples sometimes placed subspecies in atypical habitats (for example, *C. pegala maritima* in salt marsh habitat). Therefore, there does not appear to be a genetic, morphological or habitat basis to justify separate subspecies status for *C. pegala agawamensis*.

Introduction

Cercyonis pegala, commonly known as the "wood nymph," was first described by Fabricius in 1775 (Sourakov, 1995). The butterfly species lives in sunny open woodlands, wet meadows, and savannas distributed throughout the United States, northern Mexico, and southern Canada, where it feeds on nectar, tree sap, and decaying matter. Many subspecies of *Cercyonis pegala* have been identified, including two that occur in New England, *C. pegala maritima* and *C. pegala nephele*. *Cercyonis pegala agawamensis* is a new subspecies of *C. pegala* that was recently described based on phenology, flight period, seasonality, habitat, behavior, and phenotype (Arey and Grkovich, 2014). *C. pegala agawamensis* predominately inhabits coastal salt marshes and estuarine meadows along the New England coast. This subspecies feeds on nectar from flowering plants within the habitat and or nearby habitats.

There have been different views on the number of subspecies of *Cercyonis pegala* (Sourakov, 1995). Under the biological species concept, a species is a major subdivision of a genus that is composed of individuals that are able to mate with one another and produce viable offspring. A subspecies is a subdivision of a species. Subspecies designate populations from specific geographic regions that are genetically differentiated from other such populations of the same species (Merriam Webster, 2016), although the subspecies definition is the subject of considerable debate (e.g., Braby et al 2012). Arey and Grkovich (2014) hypothesized that *C. pegala agawamensis* evolved due to ecological isolation that followed the last glaciation of the Pleistocene. During the Pleistocene, glaciers retreated northward, which allowed the temperatures to warm in the Northern hemisphere. Because *C. pegala* does not inhabit densely forested areas, as the glaciers retreated and forests spread, populations of *C. pegala* may have been isolated in open

habitats, such as riverbanks, open hilltops, and along waterways. Some populations of *C. pegala* may have been isolated at the coastline and coastal marshes. Arey and Grkovich (2014) hypothesize that these ecologically and geographically isolated coastal populations formed the subspecies *C. pegala agawamensis*. Isolation should have resulted in genetic differences between *C. pegala agawamensis* and the other subspecies. Prior to this study, the level of genetic differentiation between *C. pegala agawamensis* and the other subspecies was unknown. It is important to determine if there is any genetic differentiation in order to explore the *C. pegala agawamensis* subspecies designation proposed by Arey and Grkovich (2014).

In this study, we investigated whether *C. pegala agawamensis* is genetically different from other subspecies of *C. pegala*. A mitochondrial gene, cytochrome c oxidase I (COI), was used to examine phylogenetic relationships among the subspecies. COI is a barcoding region that is used to help elucidate differences between species and subspecies (Hebert 2003). If *C. pegala agawamensis* is a subspecies that was separated from other populations of *C. pegala* during the Pleistocene, then we would expect to see numerous differences in the COI gene.

Materials and Methods

Sample Collection

Butterflies were collected in salt marshes and fields in Newbury, Salisbury, and Essex, MA using nets during the summer of 2016. Matthew Arey, the author of the paper describing *C. pegala agawamensis*, donated additional samples from Unity and Bowdoinham, ME. He also identified each of our samples to subspecies.

DNA Extraction

The QIAamp DNA Minikit was used to extract DNA from 25 mg of the butterfly abdomen. The Standard Qiagen protocol for "DNA Purification from Tissues" was followed, except the tissue was ground manually using a pestle, 10 µL of PBS was used, and samples were incubated in 56℃ water bath overnight.

PCR

The PCR Protocol for *Taq* DNA Polymerase with Standard *Taq* Buffer from New England BioLabs was used. The reactions were done using touchdown PCR at the following temperatures: 1 cycle at 94℃ for 1 min, 5 cycles at 94℃ for 1 min, 45℃ for 1.5 min, 72℃ for 1.5 min, 35 cycles at 94℃ for 1 min, 50℃ for 1.5 min, 72℃ for 1 min, final extension at 72℃ for 5 min, and hold at 8℃ (Herbert et al. 2003). The COI primers were LCO1490 and HCO2198 from Folmer et al (1994).

Gel Electrophoresis

The PCR products were electrophoresed on a 1.2% agarose gel using 1X TAE buffer. Gel Red was added to the melted agarose before it was poured. The DNA ladder was 50 ng/µL and 6X Loading Dye was used. Each well contained a total volume of 10 µL of sample with Loading Dye. Images of the gels were taken using Thermo Scientific my ECL Imager.

Data Analysis

The PCR products were sent to GeneWiz (South Plainfield, NJ) or New England BioLabs, (Ipswich, MA), for DNA sequencing. After editing the sequences and assembling the contigs using *Sequencher* (Version 4.1.4, Gene Codes Corporation), *Geneious* (Kearse et al., 2012) was used to align the sequences and construct a phylogenetic tree using neighbor joining. Bootstrap values were obtained by resampling 1,000 trees. A sequence for *Cercyonis meadii,* which is a related species from Midwestern North America, was obtained from GenBank and used as an outgroup for the phylogenetic tree. A haplotype network was constructed using PopART, (http://popart.otago.ac.nz).

Results

The mtDNA COI gene, as shown in Figures 1 and 2, was sequenced for 31 butterflies that represent a mix of the three *C. pegala* subspecies (12 *C. pegala maritima*, 15 *C. pegala agawamensis*, and 4 *C. pegala nephele*,). We identified eight mtDNA haplotypes in this set of samples (Figure 3). There are 20 base pair differences seen among the three subspecies, of which 12 are in *Cercyonis pegala nephele.* The most common haplotype is shared by 20 of the 31 samples and includes all 12 of *C. pegala maritima* specimens and 8 of the *C. pegala agawamensis* specimens. Additional haplotypes that differ by one, three, or six base pair differences are seen in several *C. pegala agawamensis* specimens, shown in Figure 3. All *C. pegala maritima* samples were collected in Essex and Newbury, MA, while *C. pegala agawamensis* were collected in Essex, Newbury, and Salisbury, MA. The *C. pegala nephele* samples were all collected in Maine. The phylogenetic tree (Figure 4) shows that there are few or no differences between various specimens of *C. pegala agawamensis* and *C. pegala maritima*, but *C.*

pegala nephele is different from these two subspecies. All the *C. pegala* specimens form a monophyletic group, distinct from *C. meadii,* with 100% bootstrap support, but there are no strongly supported monophyletic subspecies groups. The two *C. pegala nephele* groups consist of two identical sequences from each of two sampling sites.

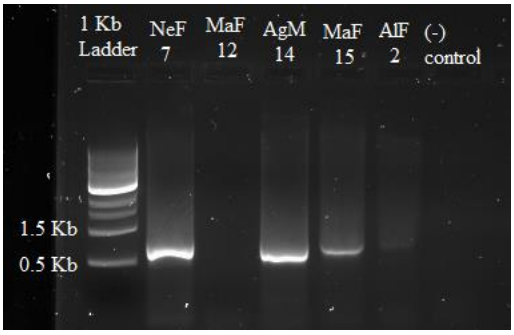

Figure 1. **COI PCR products.** This is a photograph of an agarose gel showing that the gene COI is 648 base-pairs long. In this case, three of the five samples were at the correct length and concentration to clean and sequence the products.

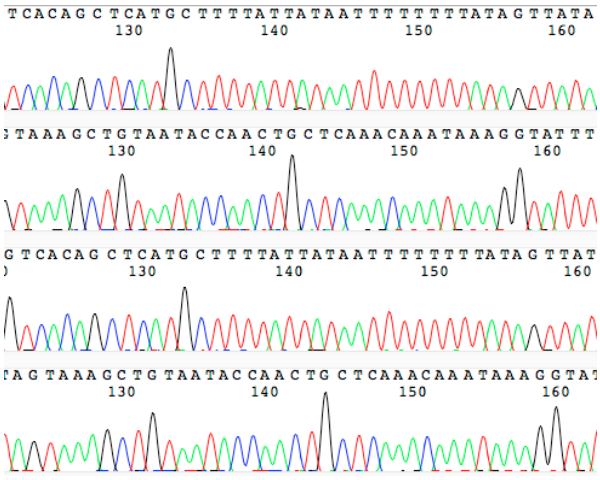

Figure 2. Chromatogram of *C. pegala nephele*, which shows DNA sequence data obtained from New England BioLabs. The raw data were edited to determine if there are any differences between the subspecies.

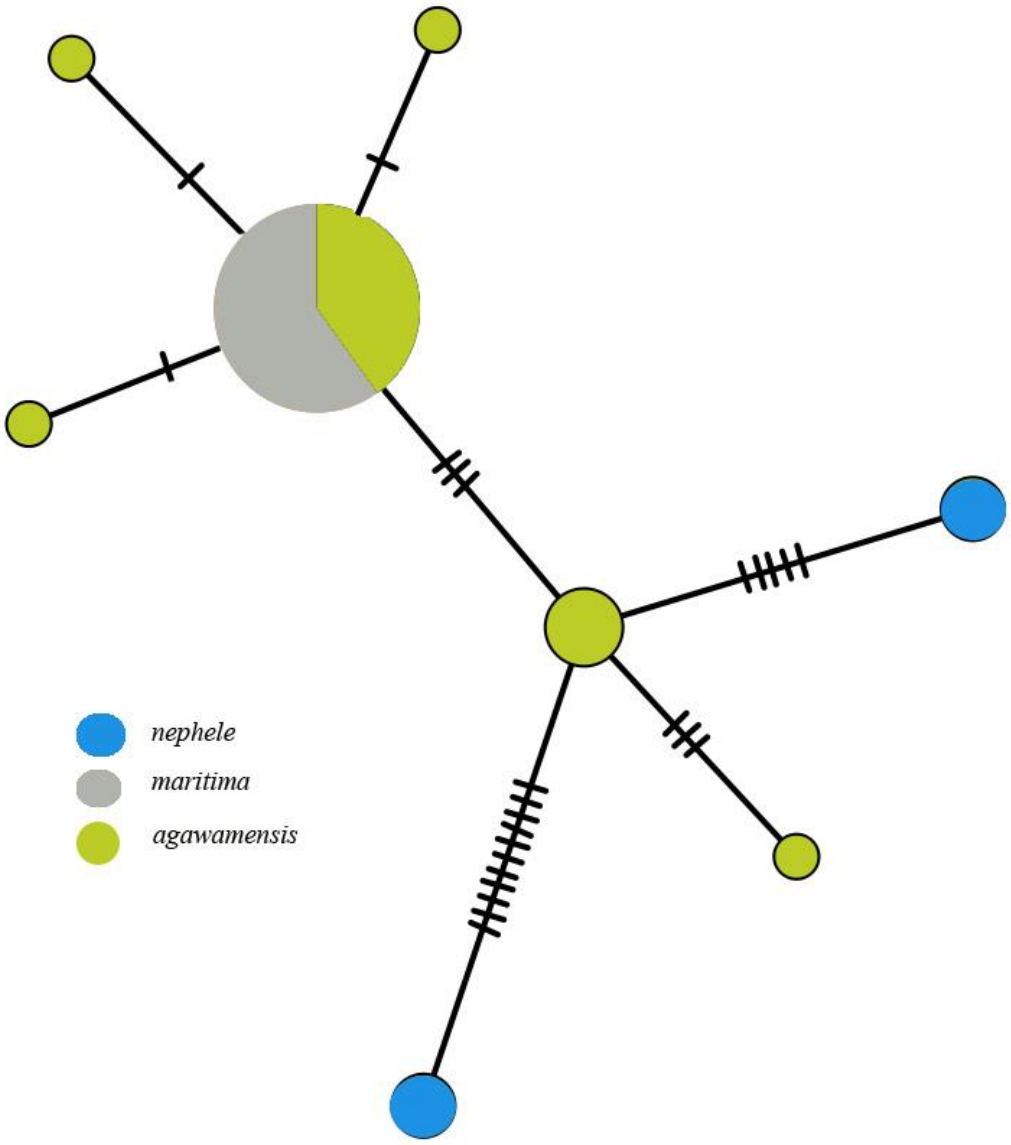

Figure 3. Haplotype network of the three subspecies, which aids in visualizing the genetic relationships between haplotypes. Hash marks represent the number of base pair differences between the haplotypes. The size of each haplotype circle represents the relative number of individuals with that particular sequence. The circles are color coded by subspecies.

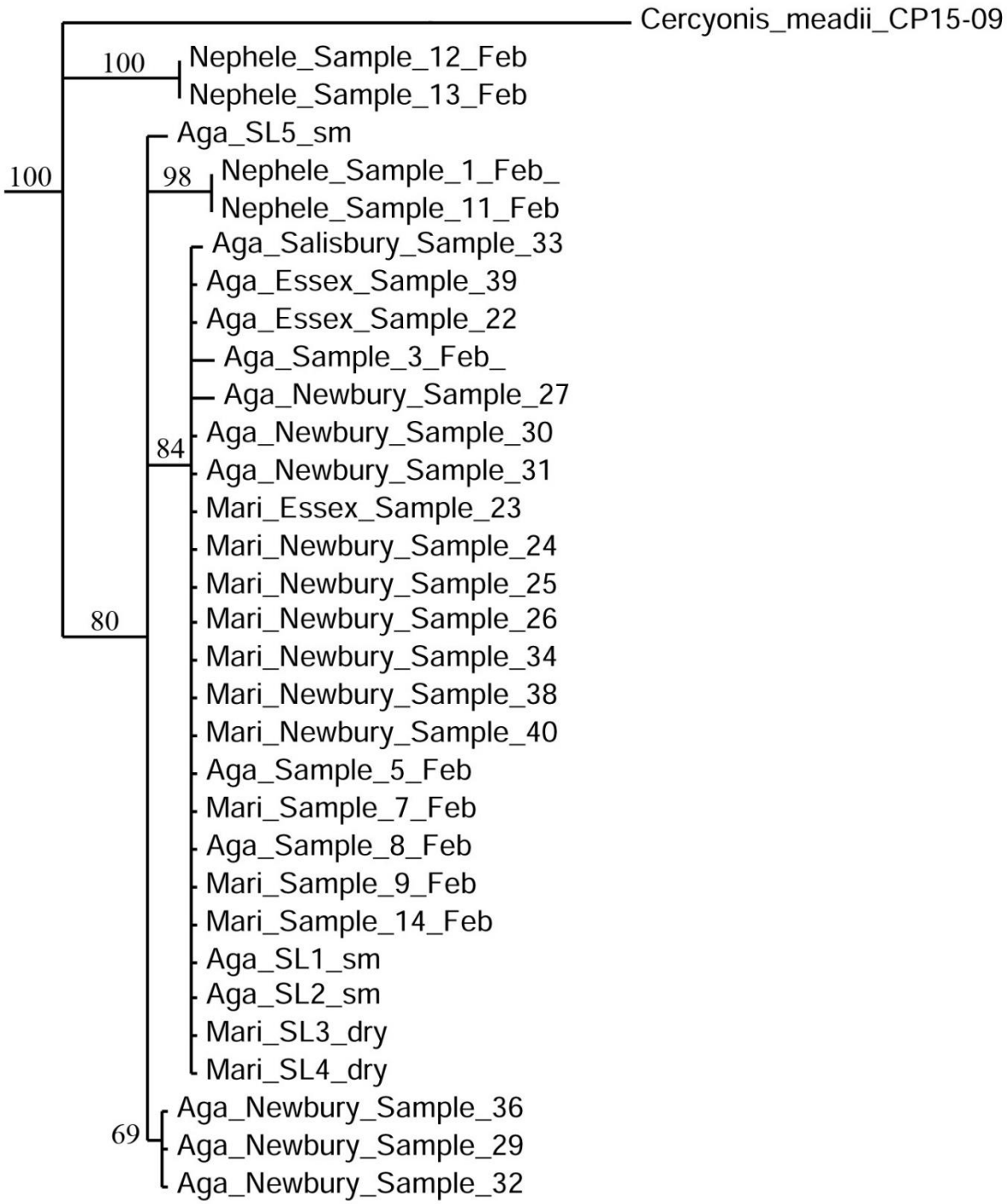

Figure 4. Phylogenetic tree of our sequence data for the three subspecies. Numbers above the branches are bootstrap support values. Branch lengths indicate genetic distance, but the branch for *C. meadii* is not drawn to scale.

Discussion

The phylogenetic relationships among three North American subspecies of *C. pegala* that occur in New England suggest that there is no genetic differentiation between *C. pegala agawamensis* and *C. pegala maritima*. The lack of genetic differences challenges that *C. pegala agawamensis* and *C. pegala maritima* are two separate subspecies. Our samples were identified by wing morphology by the scientist that described *C. pegala agawamensis* (Matthew Arey); in addition, collection habitat was noted. The majority of *C. pegala agawamensis* were collected in salt marshes. Yet, there were exceptions for both subspecies. *Cercyonis pegala maritima* was identified among samples caught in the salt marsh and *C. pegala agawamensis* was identified among samples caught in the upland meadows. Therefore, a sharp distinction by habitat for the subspecies was not corroborated by this study.

The basis for the hypothesized morphological distinctions that separate the two subspecies may in fact be natural wing pattern variation within the species. Sourakov (1995) raised *C. pegala* subspecies from Ohio and noticed that offspring had three or four eyespots, whereas another generation only produced one regular eyespot. He concluded that even large differences such as the number of eyespots on the wings may not be enough to differentiate "subspecies." Arey and Grkovich (2014) state that *C. pegala agawamensis* males and females are slightly larger than their counterparts in *C. pegala maritima*, and the forewing eyespots are more distinct in *C. pegala agawamensis*, compared to *C. pegala maritima*. Furthermore, described habitat for *Cercyonis pegala maritima* is dry open fields, while *C. pegala agawamensis* is confined to salt marshes. Our research calls into question the taxonomic significance of the small morphological

differences that do not sort by genetic haplotype or always correlate with specific habitats.

Our *C. pegala nephele* samples include two individuals each from sampling sites in Bowdoinham and Unity, Maine. The two samples from each Maine locality are identical to each other, but are genetically distinct from the other sampling location. The *C. pegala nephele* samples are also genetically distinct from *C. pegala agawamensis* and *C. pegala maritima*. All the *C. pegala nephele* are from Maine, while our *C. pegala agawamensis* and *C. pegala maritima* samples are from a small area of coastal Massachusetts, making it difficult to know if the patterns of genetic differentiation reflect geography or subspecies status. Additional samples of each subspecies from a broader geographic region are needed to distinguish between these two possibilities and will be the subject of future research.

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