Molecular Analysis of Grasshopper Populations to aid in Prairie Restoration Efforts

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Abstract

Although the Wisconsin Department of Natural Resources and non-government organizations have invested heavily in prairie restoration over the past decade, little effort had been made to evaluate whether insect species that inhabit these projects are also restored to pre-settlement diversity. To evaluate the effect of prairie restoration attempts on insect species diversity, eighty individual red-legged grasshoppers, Melanoplus femurrubrum (DeGeer), were collected from 7 populations in 3 relic and 4 restored grasslands. Molecular analyses were designed to obtain gene sequence data by polymerase chain reactions (PCR) amplification and sequencing the mitochondrial genes cytochrome oxidase subunit I (COI) and cytochrome B (cytB). Sixty eight M. femurrubrum sequences obtained for COI and fifty seven cytochrome b sequences were aligned and compared. These data may ultimately be used to improve the management of relic and restored grasslands.

Introduction

The last 150 years has proven devastating to the prairie ecosystems in the State of Wisconsin. Today, less than 0.1 percent of the original 2.1 million acres remains (Henderson & Sample, 1995). The Wisconsin Department of Natural Resources and other non-government agencies have been attempting to restore damaged lands back to their original prairie state. A prairie restoration is referred to "the purposeful assembly of plant and animal communities in order to reconstruct a stable ecosystem that is compositionally and functionally similar to that which originally existed" (Robertson, pg. 1, 2004). Unfortunately, restoring degraded lands has proven to be a difficult task. Traditionally,

success is measured by surveying plant species diversity within the reconstructed prairie (Westman, 1991) and comparing it to similar, intact, relic prairies. However, surveying plants omits mobile organisms such as insects, small mammals, and microbial species. We would like to assess the use of relatively mobile organisms such as M. femurrubrum to provide further comparison between reconstructed and relic prairies.

To further restoration efforts, grasshoppers can be used as a molecular tool to evaluate prairie authenticity. In a recent guide to the grasshoppers of Wisconsin, Kirk and Bomar (2005), demonstrate that *Melanoplus Femurrubrum* is a grasshopper that enjoys a widespread distribution (Fig.1). Studying grasshopper populations will help us to evaluate the quality of remnant prairies (Bomar, 2001; Bomar & Secrist, 2002) and the relative success or failure of the reconstruction.

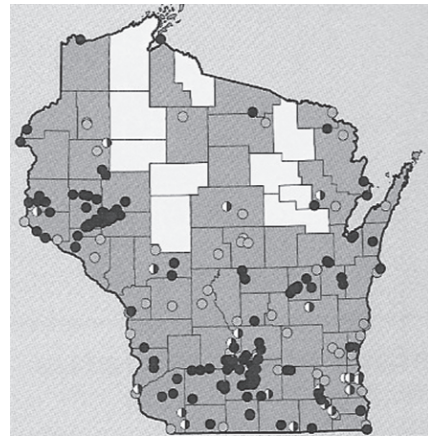

Figure 1. *M. femurrubrum* Accounts (Kirk and Bomar, 2005)

Recent literature has shown that small, isolated populations of grasshopper species can be genetically distinct (Knowles 2001 a, b; Chapco & Litzenberger 2002 a, b; Chapco, Kuperus, & Litzenberger, 1999) as well as morphologically unique (Lockwood, 1989; Chapco & Litzenberger 2002b). However, these studies focus on long time spans, such as Pleistocene glaciations (i.e. within the last 2 Myr) (Knowles & Otte, 2000; Knowles, 2001) as a source of genetic isolation of sibling species and subsequent speciation. We are interested in evaluating more recent ecological events (ca. 150 years) and their impact on genetic deviations within *M. femurrubrum*. Moreover, individuals in the field of orthopteran taxonomy have stated "…those relationships are unlikely to be resolved through the use of gross morphological characters such as those employed to date. Almost certainly it will be necessary to use molecular traits to group them properly" (Perez-Gelabert & Otte, 2000, p. 129).

In this study, we share the development of methods to obtain mitochondrial gene sequences to assess *M. femurrbrum* populations in restored and relic prairies. Since mitochondrial genes are highly conserved, show no recombination, and change slowly (Simon et al.,

1994), comparison of these sequences allow the inference of evolutionary relationships between organisms. Ultimately, these data will be utilized to construct evolutionary trees and compare diversity of grasshopper species in relic and restored prairies.

Methodology

Specimen collection.

Eighty grasshoppers were collected from restored and relic prairies previously described by Bomar (2001) and hayfields from West-Central Wisconsin. At least five males and five females were collected from seven locations (3 restored, 3 relic prairies, and 1 hayfield). Specimens were stored in -20°C after collection to preserve DNA integrity.

Muscle Dissection and DNA Extraction.

Specimens were pinned and labeled as museum specimens. One hind femur was removed from each specimen and muscle tissue rich in mitochondrial DNA was aseptically extracted from each femur. To prevent the chitinous exoskeleton from interfering with DNA polymerase during polymerase chain reaction (PCR) amplification, a forceps and scalpel were used to isolate only femoral muscle tissue. DNA was extracted from the muscle tissue using the MoBio Laboratories, Inc. UltraClean™ soil DNA isolation kit (Solana Beach, CA). DNA was concentrated 5X in a centrifugal evaporator (Speed-Vac, Thermo Savant, Waltham, MA). DNA size and quantity was estimated by 1% agarose gel electrophoresis. DNA extracted directly from the muscle tissue samples was used as a template for PCR and analyzed by gene sequencing as described below.

Mitochondrial gene amplification and cleaning for sequencing.

Custom PCR primers were synthesized by SIGMA Genosys (The Woodlands, TX). Primers for COI were previously described by Simon et al. (1994) (L2N.3014 and CI3.1718). Primers CB9 and CB10 for *cyt*B (Chapco et al., 1997, 1999) were synthesized by IDT Oligonucleotides (Coralville, IA), PCR enzymes and reagents were obtained from Amersham Biosciences (puReTaq Ready-To-Go PCR beads). Amplification conditions were modified from Chapco et al. (1999) and Knowles (2001a,b). The amplification mix included 1X TAE buffer, 10 pmol each primer, 1.0 mg/ml bovine serum albumin (Promega, Madison, WI), 1 Ìl of the DNA template, nano-pure de-ionized water (DEPC treated) (Fisher Scientific) and ~2.5 U puReTaq™ DNA Polymerase

(PCR-Ready-To-Go, Amersham Biosciences). The amplification reaction for COI consisted of an initial denaturation step of 2 min at 94ºC, followed by 12 s at 94ºC, 12 s at 58ºC, 72ºC for 55 s, and 72ºC for 7 minutes. The amplification reaction for cytB consisted of (the first ten cycles) an initial denaturation step of 2 min and 12 s at 94ºC, followed by a 0.5ºC ramp per cycle for 10 cycles of 56ºC for 12 s, and 72ºC for 55 s. After the first ten cycles the reaction consisted of 12 s at 94ºC, 61ºC for 12 s, 72ºC for 55 s, and a final extension of 72ºC for 7 minutes.

DNA Purification and Quantification.

Following amplification, the mitochondrial DNA was cleaned of excess primers, nucleotides, and DNA polymerase using the Wizard® SV Gel and PCR Clean-up System, (Promega Corp, Madison, WI). Relative abundance and approximate size of the PCR product was determined by 1% agarose gel electrophoresis. Spectrophotometry was performed at wavelengths of 260nm, 280nm, and 360nm on purified mitochondrial DNA to determine an average DNA concentration for COI. Concentrations for *cyt*B were determined through template dilutions in ethidium bromide-agarose plate flourometry (Winfrey, Rott, & Wortman, 1997).

Mitochondrial gene sequencing

Purified mitochondrial DNA amplicons were sent to UW-Madison Biotechnology Center Sequencing Facility for sequencing reactions. PCR products were sequenced in both the forward and reverse directions using BigDye Terminator v. 3.1 mix (Applied Biosystems) sequencing reactions and an Applied Biosystems 3730xl automated DNA sequencer. The forward and reverse PCR primers were used as primers for the sequencing reactions.

Sequence Analysis

To confirm primer specificity of COI and *cyt*B amplification, a Blastn search (Altschul, Gish, Miller, Myers, & Lipman, 1990) was conducted using a sequenced gene. The search compares a nucleotide query sequence against a nucleotide sequence database in the National Center for Biotechnology Information (NCBI) database and generated closely matching sequences ranked by sequence score or degree of similarity. Sequences were trimmed of non-reportable base pairs at the beginning and end of the sequences, reverse compliments were created, forward and reverse sequences were joined, complete sequences were assembled and edited of ambiguities (IUPAC, 1985), and all sequences were aligned

using a sequence alignment editor (Hall, 1998). During final sequence alignment the genus *Melanoplus* and other grasshopper COI and *cyt*B gene sequences were downloaded from Genbank (Benson et. al., 2000) and used to compare sequences from the *Melanoplus* genus and *M. femurrubrum* DNA sequences (AF228996, AF229004).

Results

By comparison to a 50 bp ladder, the length of the COI band was roughly 780 bp (Figure 2) and the length of *cyt*B band was roughly 300 bp (Figure 3), corresponding to predicted lengths of the PCR products. Following amplification, mitochondrial DNA quantified by spectrophotometry yielded concentrations of 55 ng/µl or 13 ng/bp for COI. Flourometry yielded *cyt*B concentrations of 1-2 ng/µl.

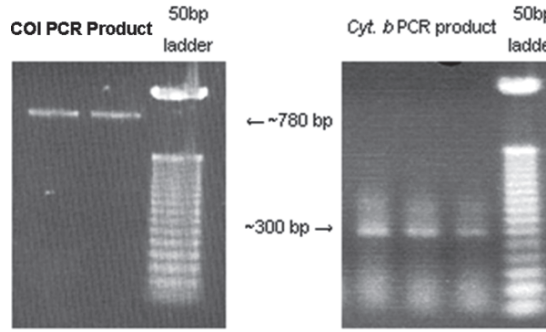

Figure 2, 3. Gel electrophoresis results for PCR amplications of mitochondial genes COI and *cyt*b aside a 50bp DNA step ladder.

Of the eighty specimens collected, sequenceable mitochondrial DNA was extracted from sixty-eight COI and fifty-seven cytB specimens. Mitochondrial DNA was sequenced as observed in the chromatogram of COI and each nucleotide was able to be identified and color-coded accordingly (Figure 4).

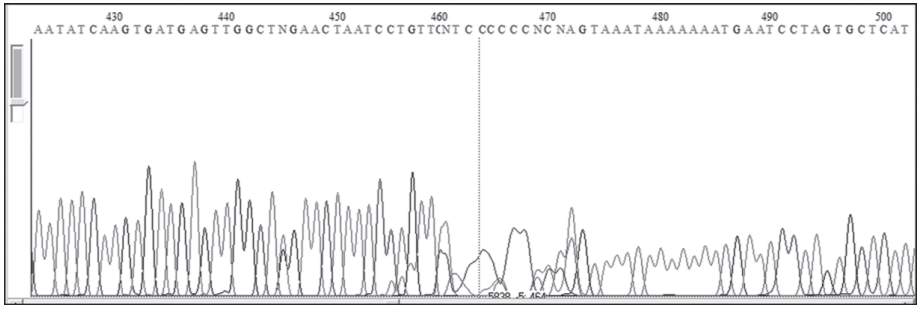

Figure 4. Chromatogram of COI sequence

Sequences were compared to database sequences during a BLASTn search. Our sequences were most similar to M. femurrubrum COI sequences in GenBank (Figure 5). During sequence analysis, DNA sequences were aligned so each sequence started at the same nucleotide allowing for further analysis (Figure 6).

Sequences producing significant alignments:		Score (Bits)	E Value
gi|11464663|gb|AF228994.1|AF228994	Melanoplus femurrubrum cyt...	1265	0.0
gi|11464657|gb|AF228991.1|AF228991	Melanoplus pinicola haplot...	1164	0.0
gi|11464659|gb|AF228992.1|AF228992	Melanoplus dawsoni cytochr...	1160	0.0
gi|11464661|gb|AF228993.1|AF228993	Melanoplus huroni cytochro...	1154	0.0
gi|11464694|gb|AF229009.1|AF229009	Melanoplus artemisiae cyto...	1116	0.0
gi|11464692|gb|AF229008.1|AF229008	Melanoplus pinicola haplot...	1114	0.0
gi|11464690|gb|AF229007.1|AF229007	Melanoplus ascensus cytoch...	1110	0.0
gi|11464667|gb|AF228996.1|AF228996	Melanoplus stupefactus hap...	1108	0.0
gi|11464681|gb|AF229003.1|AF229003	Melanoplus bivittatus cyto...	1035	0.0
gi|11464671|gb|AF228998.1|AF228998	Melanoplus femurnigrum hap...	1003	0.0
gi|11037380|gb|AF270459.1|	Melanoplus crux isolate 1025crux c...	995	0.0
gi|11464688|gb|AF229006.1|AF229006	Melanoplus lakinus haploty...	993	0.0
gi|11464665|gb|AF228995.1|AF228995	Melanoplus stupefactus hap...	993	0.0
gi|11464683|gb|AF229004.1|AF229004	Melanoplus lakinus haploty...	967	0.0
gi|11037388|gb|AF270463.1|	Melanoplus moyense isolate 1003.mo...	963	0.0
gi|11037360|gb|AF270449.1|	Melanoplus payetti isolate 1011pay...	961	0.0

Figure 5. BLASTn results of an M. femurrubrum COI amplicon

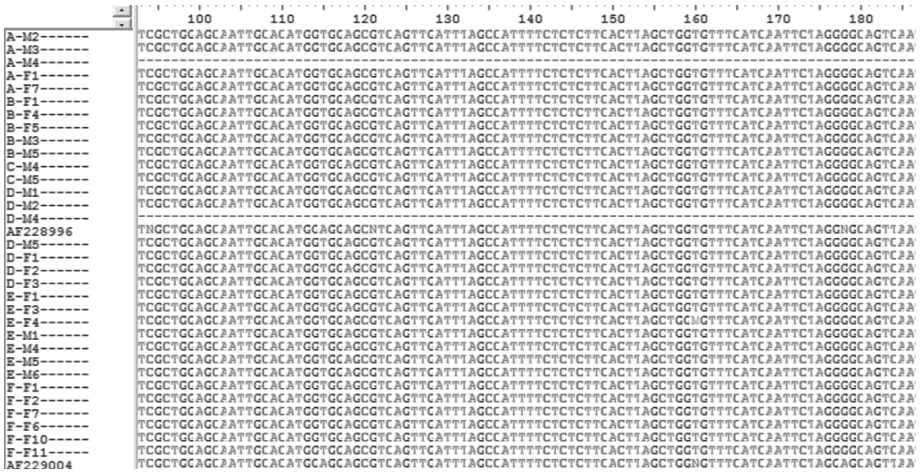

Figure 6. Sequence alignment of M. femurrubrum COI sequences

Discussion

PCR amplifications of the extracted DNA were successful (Fig. 2 and 3). The bands observed occur at the approximate base pair length expected. *Cyt*B bands are ~ 300 bp and the predicted length is ~ 258 bp. COI bands appear as 780 bp and the predicted length is 1317 bp.

The PCR amplification process proved to be a difficult task at first because chitin within the femur exoskeleton binds to DNA polymerase. This competition between the DNA and chitin was avoided by altering the DNA extraction method so no exoskeleton was used and only muscle tissue was emulsified and lysed.

After concentrating DNA through evaporation, half of the sample pool contained a significant amount of DNA for sequencing based on their bright bands in gels. Other samples had low DNA yields based on bands that were weak in intensity. To overcome this, the PCR product was re-amplified until gel bands were brighter (the brighter the band, the more products). After pooling, the COI PCR product was near the 10ng/bp needed for sequencing. Due to low quantities of PCR product for *cyt*B sequencing, all PCR amplifications were pooled and concentrated. The result was sufficient DNA for sequencing.

Overall, the sequences displayed chromatograms with very uniform peaks characterizing quality DNA and sequence reactions. After alignment of the DNA sequences, it revealed very similar sequences (Fig. 5). Sequencing also revealed some ambiguities that had to be corrected such as overlapping nucleotide peaks and others that could not be corrected (460 bp, Fig. 4). For example, when the sequencing reactions proceeded through a region rich in cytosines or guanines, the polymerase had a problem interpreting them. As a result, the peak quality in that region of the chromatogram decreased sometimes beyond analysis.

In conclusion, this research provides a molecular tool to evaluate grasshopper populations. This tool may by more beneficial when taken a step further by examining the phylogeny of *M. femurrubrum* in relic and stored prairies. These combined data may be used to improve the management of relic and restored grasslands.

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