Genetic variation in Montana populations of the rangeland grasshopper, Melanoplus sanguinipes
by Paul Joseph Parrinello

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences
Montana State University

Abstract:
Several methods were evaluated for effectiveness in revealing spatial and temporal genetic variation in three Montana populations of the rangeland grasshopper, Melanoplus sanguinipes (F.). Samples were from the annual collections of the U.S.D.A. Rangeland Insect Lab in Bozeman, MT, and taken at sites near Broadus, Havre, and Three Forks. The methods used to assess genetic variability were Restriction Fragment Length Polymorphism (RFLP) analysis of Southern blots, Random Amplification of Polymorphic DNA (RAPD), RFLP analysis of Polymerase Chain Reaction (PCR) products, and DNA sequencing. RFLP's of Southern blots and RAPD trials revealed little information. RFLP's of PCR products did not show variability in a 2500 base pair (bp) fragment with partial sequences of the cytochrome b gene and the large subunit of rRNA (16S) gene, and the complete NADH dehydrogenase subunit 1 (ND-1), tRNAleu, and tRNAser genes. Incomplete digests made results difficult to interpret in this application of RFLP techniques. DNA sequencing did not reveal significant variation between populations in an ND-1 region sequence. However, novel sequences in the ND-1 and 16S genes were revealed. These sequences were compared with homologous ND-1 and 16S sequences in 11 species of insects representing 6 different orders, and consensus trees were constructed using the Phytogeny Inference Package (PHYLIP) Programs. The trees showed high levels of genetic similarity between M sanguinipes and another Orthopteran, Locusta migratoria. The trees also supported the grouping of two Lepidopteran and two Hymenoptera species, but offered limited resolution for the Dipterans, Homopterans, and Coleopterans. These results concur with previous phylogenetic studies based on insect mitochondrial DNA (mtDNA). Although no genetic differences were uncovered in this study, a potentially useful methodology for discovering polymorphic sequences was determined. By testing more insect mtDNA PCR primers, regions that have useful polymorphisms could be amplified and subsequently sequenced.

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APPROVAL

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Paul Joseph Parrinello, Jr.

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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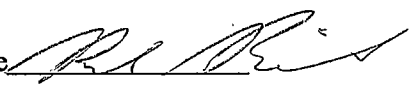
Signature 

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ABSTRACT

Several methods were evaluated for effectiveness in revealing spatial and temporal genetic variation in three Montana populations of the rangeland grasshopper, *Melanoplus sanguinipes* (F.). Samples were from the annual collections of the U.S.D.A. Rangeland Insect Lab in Bozeman, MT, and taken at sites near Broadus, Havre, and Three Forks. The methods used to assess genetic variability were Restriction Fragment Length Polymorphism (RFLP) analysis of Southern blots, Random Amplification of Polymorphic DNA (RAPD), RFLP analysis of Polymerase Chain Reaction (PCR) products, and DNA sequencing. RFLP's of Southern blots and RAPD trials revealed little information. RFLP's of PCR products did not show variability in a 2500 base pair (bp) fragment with partial sequences of the cytochrome b gene and the large subunit of rRNA (16S) gene, and the complete NADH dehydrogenase subunit 1 (ND-1), $tRNA^{leu}$, and $tRNA^{ser}$ genes. Incomplete digests made results difficult to interpret in this application of RFLP techniques. DNA sequencing did not reveal significant variation between populations in an ND-1 region sequence. However, novel sequences in the ND-1 and 16S genes were revealed. These sequences were compared with homologous ND-1 and 16S sequences in 11 species of insects representing 6 different orders, and consensus trees were constructed using the Phylogeny Inference Package (PHYLIP) Programs. The trees showed high levels of genetic similarity between *M. sanguinipes* and another Orthopteran, *Locusta migratoria*. The trees also supported the grouping of two Lepidopteran and two Hymenoptera species, but offered limited resolution for the Dipterans, Homopterans, and Coleopterans. These results concur with previous phylogenetic studies based on insect mitochondrial DNA (mtDNA). Although no genetic differences were uncovered in this study, a potentially useful methodology for discovering polymorphic sequences was determined. By testing more insect mtDNA PCR primers, regions that have useful polymorphisms could be amplified and subsequently sequenced.

INTRODUCTION

Rangeland grasshoppers (Orthoptera: Acrididae) have been studied extensively in Montana with regard to changes in community structure (Kemp, 1992a) and density dependence (Kemp and Dennis, 1993) over time. Periodic outbreaks of grasshoppers have damaged agricultural and range areas (Hewitt, 1983). The relationship between climate and outbreaks has been asserted (for example, Fielding and Brusven, 1990), but is too variable to use in forecasting (Kemp and Dennis, 1993). Also, little is known about the genetic characteristics of the species that make up these communities. Two major concerns in assessing these characteristics are genetic variability over time, and genetic variability between geographically distinct populations.

We chose to examine *Melanoplus sanguinipes* because of its important role in grasshopper communities as the greatest contributor to fluctuations in rangeland grasshopper density (Kemp, 1992b). The study began as an effort to test the hypothesis that genetic variation occurs temporally and spatially in Montana populations of this species. The molecular techniques used to show this variation were restriction analysis using Southern blots, restriction analysis of PCR products, and DNA sequencing. The two RFLP methods were generally unreliable and uninformative. Sequencing did not expose significant variation but did reveal novel 16S ribosomal RNA and ND-1 mtDNA sequences. These were compared to other available homologus insect sequences from various orders and entered in the GenBank database.

These and other molecular techniques have been implemented to reveal clear quantitative differences between closely related species, subspecies, and populations. Allozyme analyses, for example, have been used to study variation in *Drosophila* (Lewontin and Hubby, 1966), to conduct phylogenetic anaylses between and within seven species of the Coleopteran genus *Chauliognathus* (Howard and Shields, 1990), and to differentiate between populations of the weevil *Rhinocyllus conicus* (Klein and Seitz, 1994). Allozymes are proteins that vary in amino acid sequence without changes in identity or function. By electrophoretically separating several

allozymes from different individuals, a quantitative measure of genetic distance (that is ultimately reflective of DNA sequence differences) between the individuals can be attained. However, redundance in the amino acid code and other translational factors can affect the utility of this technique.

Random amplification of polymorphic DNA (RAPD) is a PCR technique that uses small (8-12 bp) arbitrary primers to produce amplified DNA fragments of various sizes. The presence or absence of a given fragment is determined by the presence or absence of a template DNA sequence complementary to two of the primers. Differences in DNA sequence between samples are evidenced by the differing band patterns that result when randomly primed samples are compared electrophoretically. This technique has been used to distinguish colonies of predatory Coccinellids (Roerdanz and Flanders, 1994) and its potential for use in systematics and population genetics studies of grasshoppers was asssessed by Chapco, et al. (1992). The technique is facile, but artifactual results from inconsistent priming are a potential problem.

Since the 1980's, the use of mitochondrial DNA (mtDNA) has become widespread in phylogenetic and taxonomic studies. The study of mtDNA has several distinct advantages over other molecular techniques. The mtDNA molecule has a finite size (around 16 kb for most species) and exhibits conservation in overall sturcture. It is commonly thought to evolve more rapidly and have a greater proportion of coding sequences compared to nuclear DNA, which has many repeated and non-coding regions (Brown, et. al., 1979), although there is recent evidence to the contrary (Lynch and Jarrell, 1993). MtDNA is generally inherited maternally and cytoplasmically, and thus is not subject to recombinations during meiosis (Avise, et. al., 1979). With a few exceptions (Harrison, et al., 1985), mtDNA is homoplasmic in each individual.

Until recently, most mtDNA studies employed restriction fragment length polymorphisms (RFLP's). These polymorphisms were revealed by hybridizing labeled mtDNA probes to digested DNA on Southern Blots (Southern, 1975). Phylogenetic resolution between and within species could be detected in many instances. Martel and Chapco (1995) used these techniques to study genetic variation and population structure of banded-wing grasshoppers (subfamily Oedipodinae) and Chapco, et. al. (1994) examined variation within and among members

of the genus *Melanoplus*. Harrison, et al., (1987) used RFLP's of mtDNA to investigate hybridization of two cricket species, *Gryllus firmus* and *G. pennsylvanicus*, in relation to geographic location and soil type. Intraspecific variation between two subspecies of honey bee (*Apis mellifera mellifera* and *A. m. carnica*) has also been revealed (Smith and Brown, 1990). Distinction between broods of 17-year cicadas was accomplished by applying RFLP techniques to a large mitochondrial PCR fragment (Simon, et. al., 1993).

In the last few years, advances in technology have allowed the sequencing of mtDNA (Sanger, et al., 1977) to become a widely used tool for phylogenetic and taxonomic studies. Generally, the template DNA is a mitochondrial PCR fragment. The DNA sequence of an individual can be directly compared to a homologous sequence from another individual or group of individuals, and sequence similarities and differences may be used to determine phylogenies. Several regions of the mitochondrial genome have been shown to have high phylogenetic utility at various taxonomic levels. Sequences in the cytochrome oxidase I (COI), cytochrome oxidase II (COII), and cytochrome oxidase III (COIII) genes are the most commonly examined. An interesting cladistic analysis of ten insect orders was conducted using 673 bp of the COII gene (Liu and Beckenbach, 1992) and phylogenetic relationships between 6 species of spruce budworm (genus *Choristoneura*) were determined using COI and COII sequences and the tRNA leucine gene that lies between them (Sperling and Hickey, 1994). Jermiin and Crozier (1994) compared the cytochrome b sequence of the ant (*Tetraponera rufoniger*) to those of *Drosophila yakuba* (Clary and Wolstenhome, 1985) and *Apis mellifera* (Crozier and Crozier, 1993). The large ribosomal RNA gene (16S) is another frequently sequenced mitochondrial region. Hymenopteran phylogeny was analyzed using 16S sequences (Derr, et al., 1992) and variation within and among blackflies (Diptera: Simuliidae) has been studied in detail by Xiong and Kocher (1991, 1993). Seven NADH dehydrogenase subunit genes are distributed throughout the mitochondrial genome; subunit one (ND-1) is frequently sequenced. Weller, et al. (1994), compared phylogenies based on ND-1 sequences to phylogenies based on nuclear sequences that code for the 28S ribosomal RNA, and created a phylogeny based on both sequences. Sequence data from 3 mitochondrial regions (COIII, ND-1, and 16S) were combined to provide an intraspecific phylogeny for

geographically and morphologically distinct subspecies of *Cicindela dorsalis* Say, a North American tiger beetle (Vogler and DeSalle, 1993).

The number of published mtDNA sequences for insect species is increasing rapidly as new PCR primers are found. Improved access to sequence databases (such as GenBank) through the Internet have made searching for homologous sequences much easier. Widely available programs such as PHYLIP (Phylogeny Inference Programs; Felsenstein, 1993) allow sequence data to be quickly converted into phylogenetic data.

From this research, it is evident that sequencing gives clearer and more useful results than the RFLP techniques, and that future efforts studying the genetics of *Melanoplus sanguinipes* should be directed at finding, amplifying, and sequencing a more variable region of the mtDNA.

MATERIALS AND METHODS

Grasshopper Samples

Melanoplus sanguinipes samples were obtained from the annual collections of the USDA Rangeland Insect Laboratory in Bozeman, MT. These collections were made at several sites throughout Montana from 1986 to 1992. To attain maximum geographical separation, grasshoppers from sites near Broadus, Havre, and Three Forks were used. The collections were maintained at -15° C, but were transferred to a -20° C freezer upon arrival. Live samples (a non-diapause strain from Colorado) were also used to determine if freezing at -15° C caused DNA degradation. Live grasshoppers were maintained for several days in a plastic tube that contained grass and sawdust, and had a removable screen on one end.

DNA extraction

Three protocols for DNA extraction were tried. The first (Davis, et al., 1980) entailed homogenizing grasshoppers either on dry ice with a mortar and pestle or by chopping finely with a razor blade, then placing the homogenate on ice in a 2.0 ml centrifuge tube. Both live and frozen grasshoppers were used. Additionally, various body parts were removed (head, abdomen, and intestinal tract) from some samples to determine effects on extraction efficiency. Six hundred and fifty µl buffer (0.1 M EDTA, 0.2 M Tris), 100 µl 10% SDS, and 20 µl proteinase K (10 mg/ml) were added to the sample tubes, which were then incubated 2 hours at 65° C, followed by 3-24 hours incubation at 37° C. Four hundred µl of 5 M potassium acetate were then added to each sample, prior to a 45 minute incubation on ice. The sample tubes were centrifuged at 14,000 RPM for 10 minutes, and 600 µl of the resulting supernatant were decanted into 1.5 ml centrifuge tubes. The tubes were filled with phenol-

chloroform-isoamyl alcohol (25:24:1) and spun 12 minutes at 14,000 RPM. The top, aqueous layer was decanted into a new tube, to which was added 1 volume phenol-chloroform-IAA. This mixture was centrifuged 2.5 minutes at 14,000 RPM, and the aqueous layer was decanted into a new tube. The DNA was precipitated by adding 1 volume isopropanol and freezing (-20° C) for 2 hours, then pelleted by spinning 15 minutes at 14,000 RPM. Pellets were rinsed by adding 30 µl 70% ethanol and centrifuging 3 minutes at 14,000 RPM. The ethanol was then discarded and any that remained was allowed to evaporate at room temperature. The dried pellet was resuspended in 150 µl double-distilled water (ddH_2O).

The second procedure (Rand and Harrison, 1989) started with homogenization in liquid nitrogen using a mortar and pestle. Whole grasshoppers and grasshoppers without intestinal tracts and heads were used. The homogenate was placed in a 1.5 ml tube and 500 µl of buffer (0.1 M Tris, 0.05 M EDTA, 0.2 M sucrose, 0.5% SDS) were added. The procedure called for the addition of 5 µl diethylpyrocarbonate, which was unavailable, so 2-mercaptoethanol was substituted. Sample tubes were incubated at 70° C for 30 minutes prior to additional homogenization with a glass pestle. The tubes were then filled with 5 M potassium acetate, and placed on ice for 1 hour. Using a glass pestle, the solid contents of the tube were compressed, and the supernatant was decanted into new tubes, which were then filled with isopropanol and frozen overnight. Samples were then spun 10 minutes at 14,000 RPM. The supernatant was decanted into new tubes and incubated 10 minutes at 37° C, with 100 µl TE buffer and 2 µl RNAase (10 µg/µl). One volume of phenol was added, then samples were briefly vortexed and spun 10 minutes at 14,000 RPM. The aqueous layer was decanted and extracted as above, first with phenol-chloroform-IAA, then with chloroform-IAA. The chloroform extraction was spun for 3 minutes at 14,000 RPM. DNA was precipitated by adding 1 volume of 95% ethanol to the supernatant and freezing for 1 hour, then pelleted by centrifugation (15 minutes at 14,000 RPM). DNA pellets were rinsed in 70% ethanol and resuspended in water as in the first protocol.

The third extraction protocol was modified from Gustincich, et al. (1991). Grasshopper leg muscles were ground in 300 µl TE buffer (pH 8.8) in 2.0 ml microcentrifuge tubes. Six hundred µl DTAB (8%

dodecyltrimethylammonium bromide, 1.5 M NaCl, 100 mM Tris-HCL [pH 8.8], 50 mM EDTA) were mixed in, and sample tubes were incubated 15 minutes at 68° C. Six hundred µl chloroform were mixed in, and tubes were centrifuged 2 minutes at 10,000 RPM. The aqueous layer was decanted, re-extracted with chloroform, and transferred to 1.5 ml centrifuge tubes containing 1.0 ml of CTAB (5% hexadecyltrimethylammonium bromide. 0.4 M NaCl) diluted 1:10 with ddH_20. The tubes were inverted gently and centrifuged 2 minutes at 10,000 RPM. The supernatant was decanted and pellets were resuspended in 300 µl 1.2 M NaCl, then precipitated in 750 µl ice-cold ethanol. The DNA was re-pelleted by centrifugation for 2 minutes at 10,000 RPM. The supernatant was decanted and pellets rinsed in 300 µl 70% ethanol. Pellets were air-dried and dissolved in 100 µl ddH_20.

DNA Purification

Several purification methods were tried in an effort to facilitate PCR amplification of more samples. The procedure for use of the Rapid Pure Miniprep Kit (Bio 101), began with the mixing of 70 µl DNA sample with 250 µl Glassmilk Spinbuffer, and the placement of the mixture in a spin filter. Centrifugation was for 1 minute at 14,000 RPM, and the filtrate was discarded. The DNA in the filter was rinsed twice by adding 250 µl of wash solution (provided) and centrifuging for 1 minute at 14,000 RPM. The DNA was then eluted into a 1.5 ml centrifuge tube with 50 µl TE buffer, and centrifuged for 30 seconds at 14,000 RPM.

DNA was also purified using a Prep-A-Gene (Bio-Rad Laboratories) Kit. One hundred µl of DNA sample were vortexed with 315 µl binding buffer (provided), and the mixture was allowed to incubate 5 minutes at room temperature. Five µl Prep-A-Gene powder were added, and the tube was spun 30 seconds at 14,000 RPM. The supernatant was discarded, and the resulting pellet was resuspended in 50 pellet volumes of binding buffer. The tube was spun again, and the pellet re-suspended in binding buffer. Fifty pellet volumes of wash buffer were added, and the sample tube was vortexed and spun 30 seconds at 14,000 RPM; this was repeated twice. One pellet volume of elution buffer was added, and the sample tube was incubated for 5 minutes at 37° C. The tube was spun 30 seconds at 14,000 RPM and the supernatant transferred to a new tube; this was done

three times. Twenty-five µl ddH_2O were added to the 6 µl extracted DNA.

Another procedure, using Chelex (Bio-Rad), was also tried. Twenty µl sample DNA and 20 µl Chelex 100 (5%) were added to a centrifuge tube and incubated with shaking at 37° C for 45 minutes. The sample tube was gently shaken by hand and placed in a heat block at 95° C for 5 minutes. It was shaken again by hand, then spun for 30 seconds at 14,000 RPM. The supernatant was transferred to a new tube.

A Sepharose (Pharmacia) column was also used for purification. The column was prepared by puncturing the bottoms of 0.5 ml and 1.5 ml centrifuge tubes with a small-gauge hypodermic needle tip, and placing the smaller tube inside the larger. Twenty-five µl of glass beads suspended in TE buffer were pipetted into the smaller tube (using a cut-off tip). A layer of 500 µl Sepharose CL-6B was pipetted in a similar manner over the glass beads. The two tubes were placed inside a glass test tube that fit into a metal sleeve in a swinging bucket rotor centrifuge, and the column was spun 5 minutes at 1800 RPM. The punctured 1.5 ml tube was replaced with a new, intact tube. Fifty µl DNA sample were added and spun through the column for 5 minutes at 1800 RPM, and the treated sample was collected in the new 1.5 ml tube.

Several methods for gel-purifying DNA were tested. The first used a polyester plug spin insert (PEPSI, Glenn and Glenn, 1994). A 1 ml pipette tip cut at the wider end was placed inside a 1.5 ml centrifuge tube, with the tapered end of the tip in the bottom of the tube. A small amount of polyester fiberfill was inserted into the tip and a slice of agarose gel containing the desired DNA fragment was placed on top of the fiber. Then 50 µl TBE was placed in the bottom of the tube, and tubes were spun 1 minute at 4000 g.

Also, methods for gel-purification of DNA using low melting point agarose were used. A procedure modified from Sambrook, et. al. (1989), began with melting the gel slice containing the fragment of interest in 2 volumes 20 mM Tris-Cl, 1 mM EDTA (pH 8.0), for 5 minutes at 65° C. An equal volume of phenol (equilibrated to pH 8.0 with 0.1 M Tris-Cl) was added, and the mixture vortexed for 20 seconds. The sample tube was spun for 10 minutes at 7000 RPM. The aqueous layer was removed, and again incubated 5 minutes at 65° C, then re-extracted with phenol. The aqueous phase was re-extracted (without heating) with phenol-chloroform-

IAA, and again with chloroform-IAA. The aqueous layer was then transferred to a new tube, and DNA was precipitated by freezing for 1-4 hours in 2 volumes ethanol and 0.2 volumes 10 M ammonium acetate. DNA was pelleted by centrifugation at 14,000 RPM for 25-30 minutes. The pellet was rinsed by spinning for 2.5 minutes at 14,000 RPM with 25 µl 70% ethanol. The ethanol was removed with a disposable glass pipette (pulled over a flame to create a narrow opening) and the pellet was suspended in 15 µl ddH_2O.

DNA from low melting point agarose gels was also recovered using a Magic Miniprep Kit (Promega). The gel slice containing the desired DNA fragment was melted in a 1.5 ml centrifuge tube at 70° C. One ml of PCR Preps Resin was added, and the tube was vortexed for 20 seconds. A Magic Mini-Column was attached by a Luer-lock to a 3 ml syringe barrel, and the column tip was placed in a vacuum manifold. The resin/DNA mixture was pipetted into the syringe barrel, then drawn into the column with the vacuum. Washing was facilitated by drawing 2 ml of 80% isopropanol through the column, then the column was dried by vacuuming for an additional two minutes. Any remaining isopropanol was removed by centrifuging the column in a new 1.5 ml tube for 20 seconds at 12,000 g. DNA was eluted out of the column with 25 µl TE buffer by spinning for 20 seconds at 12,000 g.

DNA Analysis with Restriction Fragment Length Polymorphisms

DNA Restriction

Samples with DNA concentrations greater than 0.25 µg/µl were digested in reactions using 5.0 µg of DNA, 40 U of restriction endonuclease, the appropriate buffer at a 1X concentration, and ddH_20 to 20 µl. Enzymes with 4-base and 6-base recognition sites were used. Restriction reactions were incubated overnight at 37° C. Digested samples were mixed with 3.5 µl loading dye and run on a 1% agarose gel in 1X TBE buffer. A lane of 20 µl lHindIII standard was also loaded into the gel. Electrophoresis was generally done overnight at 20 MA. Gels were stained in 1X TBE (enough to cover the gel) with 10-µl ethidium bromide (EtBr) for 30 minutes under agitation at room temperature. The gel was then photographed with UV light, and the positions of the

lHindIII bands were noted relative to a ruler placed next to the standard lane. The gel was trimmed with a sterile razor blade at the 564 bp band and near the 23,130 bp band and along the extreme top and bottom edges (to remove any upturned areas). The upper left hand corner was removed to mark the orientation of the gel. The following enzymes were used: AluI, ApaI, AvaI, BamHI, BanI, BglII, ClaI, DdeI, DpnII, DraI, EcoRI, EcoRV, HaeIII, HhaI, HindIII, HinfI, HincII, HpaI, KpnI, MspI, PstI, PvuII, RsaI, SacI, SalI, SmaI, TaqI, and XbaI.

Southern Transfer

The DNA in the trimmed gel was denatured by agitation and immersion in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was briefly rinsed in ddH_2O, then immersed and gently agitated in 0.5 M Tris, 1.5 M NaCl, pH 7.5 for 30 minutes (to reduce alkalinity), using an orbit shaker. Concurrently, a Zetabind nylon membrane was prepared for blotting as follows: the membrane was cut to the dimensions of the gel and soaked, first in ddH_2O for 5 minutes, then for 5 minutes in 20X SSC. Vertical, upward transfer was in 20X SSC, using three sheets of Whatman 3MM Chromatography filter paper cut to gel size and a stack of paper towels about 30 cm thick as a wick. Following overnight transfer, the membrane was rinsed in 2X SSC, twice for 15 minutes with gentle agitation. DNA was fixed to the membrane by baking in a vacuum oven. The membrane was placed between two sheets of filter paper and baked for 2 hours at 80° C under 20 lbs. pressure. The membrane was then soaked in a hybridization tube with 30 ml 0.1X SSC, 0.5% SDS. The tube was rotated for one hour at 65° C. The membrane was removed from the tube, blotted dry between two sheets of filter paper and stored in a sealed plastic bag at 0° C.

A more efficient procedure using ammonium acetate as a transfer agent was used for later blots. The DNA on the trimmed gel was denatured by two 15 minute soaks in 1.5 M NaCl, 1.5 M NaOH with gentle agitation, followed by two 15 minute soaks in 0.2 M NaCl, 0.1 M NaOH, also with gentle agitation. The gel was then washed twice in 1 M ammonium acetate, with gentle agitation (15 minutes per wash). The membrane was soaked for 5 minutes in ddH_2O, then for 5 minutes in 1 M ammonium acetate. Membrane transfer was as above, substituting 1 M ammonium acetate for 20X SSC. DNA was fixed to the membrane by baking for 2 hours at 80°

C.

Transformation of mt DNA Probes

Several different mtDNA probes were used in hybridizations. An 8.0 kb fragment from *Gryllus affirmus* (pGA 8.0), 6.0 kb (pDS 6.0) and 3.6 kb (pDS 3.6) fragments from *Drosophila silvestris*, and a 900 bp PCR fragment (900 RL) that could be generated from any *Drosophila* template (with included primers) were received from Dr. Rob DeSalle at the Museum of American History. Probes of *Melanoplus sanguinipes* mtDNA were sent by Dr. William Chapco from the University of Regina. They were: pDM1 (8.0 kb), pDM2 (6.8 kb), and pDM3 (1.2 kb). All probes were sent in pUC 18 plasmids. Two transformation procedures were tried, in order to insure an adequate supply of probes. A heat-shocking procedure began with preparation of competent cells using the following procedure: Two 500 ml flasks with 50 ml LB media were autoclaved. One was inoculated (using a flame-sterilized inoculation loop) with JM101 *E. coli* cells, the other with JM109 *E. coli* cells, and both were incubated overnight at 37° C with shaking at 225 RPM. (*E. coli*. strains were prepared by streaking minimal media plates with glycerol cultures and incubating at 37° C for 1-2 days.) Two dilutions (1:50 and 1:500) of each bacteria strain were prepared by adding overnight culture to new 50 ml LB media aliquots in 500 ml flasks. Cells were incubated at 37° C with shaking at 225 RPM, until the optical density at 600 nm reached 0.5-0.6 (around 3 hours for the 1:50 dilution). The cells were then transferred to ice-cold 50 ml polypropylene tubes and placed on ice for 5-10 minutes. Cells were centrifuged at 4000 RPM for 10 minutes at 4° C in a Sorvall Rotor. The resulting supernatant was discarded and drained off by inverting the tube for 1 minute, and the pellet of cells was suspended by vortexing in 10 ml ice-cold 0.1 M $CaCl_2$. Cells were stored on ice 5-10 minutes before centrifugation at 4000 RPM for 10 minutes at 4° C. The supernatant was decanted as before, and the pellet was resuspended by vortexing in 2 ml 0.1 M $CaCl_2$. Two hundred µl of competent cell suspension were aliquoted to sterile 1.5 ml centrifuge tubes. One µl of each probe DNA in pUC plasmids was added to separate cell aliquots, and the tubes were placed on ice for 30 minutes, along with an aliquot of cells with no DNA added (negative

control). The tubes were heated in a 42° C water bath for 90 seconds, then chilled on ice 1-2 minutes. Cells were allowed to recover by incubation at 37° C in 800 µl SOC media. Recovered cells were poured on LB agar plates with 35 mg/ml ampicillin. Twenty-five µl Bluo-gal and 20 µl isopropylthio-β-D-galactoside (IPTG) were spread on the plates with a sterile bent glass rod prior to plating. Agar plates were incubated overnight at 37° C. White colonies were selected and transferred to liquid LB media by dabbing with a toothpick, which was then dropped into 3 ml sterile LB media in 15 ml glass test tubes. Liquid cultures were incubated overnight at 37° C with shaking at 225 RPM. DNA minipreps (Sambrook, et. al., 1989) were started by filling 1.5 ml centrifuge tubes with the overnight liquid cultures, and spinning for 1 minute at 14,000 RPM. The media was decanted, and any that remained after drainage by inversion was removed by aspiration. The pellet was resuspended by vortexing in 100 ml of Solution I (50 mM glucose, 25 mM Tris-Cl pH 8.0, and 10 mM EDTA pH 8.0). Two hundred µl Solution II (0.2 N NaOH, 1% SDS) were added, and tubes were gently inverted several times to mix. The tubes were stored on ice 5-10 minutes prior to the lysis of cells by addition of 150 µl of ice-cold Solution III (3 M potassium acetate, 11.5% acetic acid). Tubes were inverted and stored 3-5 minutes on ice before centrifugation at 14,000 RPM at 4° C for 5 minutes. The supernatant was decanted into a new 1.5 ml centrifuge tube, and 2 volumes room-temperature 95% ethanol were added. The tubes were vortexed briefly, and the DNA was allowed to precipitate for 2 minutes at room temperature. Centrifugation at 14,000 RPM for 5 minutes at 4° C pelleted the DNA. The supernatant was removed by aspiration, then the pellet was rinsed by the addition of 1 ml 70% ethanol, and centrifugation at 14,000 RPM at 4° C for 2.5 minutes. The 70% ethanol was removed by aspiration, and the pellet was allowed to air-dry for 10 minutes. The DNA was resuspended by the addition of 50 µl TE buffer with 20 mg/ml RNAase (DNAses were removed from the RNAase by boiling for 5 minutes.)

Another transformation procedure using electroporation of competent cells was tried. An overnight culture of JM 101 *E. coli* cells was prepared and new LB media was inoculated and grown to an OD_{600} of 0.4, as above. Cells were pelleted in ice-cold 50 ml polypropylene tubes by centrifugation for 12 minutes at 4° C at 4,000 RPM in a Sorvall Rotor. The pellet was then resuspended in 6 ml sterile 20% glycerol, and spun for 12

minutes at 4° C at 4,000 RPM. This wash repeated 3 times, then the pellet was resuspended in 1 ml 20% glycerol, transferred to a 1.5 ml centrifuge tube, and spun at 4° C for 30 seconds at 10,000 RPM. The media was decanted, and the pellet resuspended in 5 volumes of 20% glycerol. The competent cells were used in electroporation transformations, or frozen at -70° C. Immediately before electroporation, 0.5 µl of probe DNA in pUC 18 plasmid were added to 30 µl competent cells. This mixture was placed on an electroporation plate, and subject to a brief 400V charge. The cells were retrieved using a pipette, by mixing them with 30 µl of the 1 ml of SOC media used for recovery (1 hour at 37° C in a shaking water bath). Cells were plated (10 µl and 100 µl) on LB agar plates with 100 mg/ml ampicillin, 25 µl Bluo-gal and 20 µl IPTG, then incubated overnight at 37° C. Colony selection and mini-preps were as above.

Radiolabeling Probes

Probes were radiolabeled using a nick-translation procedure. In a 1.5 ml centrifuge tube, the following were mixed: 5 µl dNTP's without dATP, 3 µl plasmid DNA, 3 µl [a-P^{32}]dATP, and 34 µl ddH_2O. Five µl DNA polymerase/DNAase I were mixed in, and the tube was centrifuged 5 seconds, prior to a 1 hour incubation at 15° C. Stop buffer (5 µl) was then added.

Hybridization

Nylon membranes were first prehybridized in a solution of 0.7 g SDS powder, 0.1 g BSA, 20 ml 0.5 M EDTA pH 8.0, 5.26 ml 0.5 M Na_2HPO4 pH 7.2, and 4.5 ml ddH_2 0. The 10 ml obtained were adequate for prehybridization of 100 cm^2 of membrane area. The membranes and prehybridization solution were placed in a seal-capped tube in a mini-oven, and incubated overnight at 60° C. Radiolabeled probes were boiled at 95° C for five minutes, then added to the prehybridized membranes. Incubation was overnight at 60° C in a rotating oven. Membranes were rinsed in the hybridization tubes, twice with 50 ml of 2X SSC, 0.1% SDS (15 minutes at 30° C in a rotating oven), then twice with 50 ml 0.1X SSC, 1% SDS (15 minutes at 30° C in a rotating oven). Used rinse solutions were discarded in a liquid radioactive waste jar. Membranes were blotted dry with filter paper, and

covered with plastic wrap. Autoradiography film was placed on the membrane in a light-proof film holder, and exposure was overnight at -70° C. Films were developed by immersing in developer (1 minute), stop solution (1 minute), and in fixative solution (5 minutes).

Polymerase Chain Reaction (PCR) Methods

Random Amplification of Polymorphic DNA (RAPD)

Several attempts to reveal genetic variation using random primers were made. Ten base-pair primers from a RAPD kit (Operon Technologies) were used. Twenty-five µl reactions were prepared with the following components: 16.75 µl ddH_2O, 2.5 µl 10X *Taq* buffer, 3.0 µl DNTP's, 1.5 µl 50 mM MgCl , 0.25 µl *Taq* polymerase, 1.0 µl primer, and 1.0 µl sample DNA. Primers from kit "A", numbers 4, 6, 7, 8, 10, and 12 were used in separate reactions. DNA from sample MS9 was used because it seemed to be easily digested by many enzymes, which indicated it might be a better preparation. It was diluted 1:100 with ddH_2O. A control reaction with no DNA was also used. Reactions were performed in 500 µl tubes and were overlaid with approximately 25 µl sterile mineral oil prior to thermocycling with the following parameters: profile 1: 95° C for 2 minutes, one cycle, profile 2: 94° C for 1 minute, 36° C for 1 minute, 72 ° C for 2 minutes, 45 cycles, profile 3: 72° C for 5 minutes, one cycle. RAPD reactions were also done with the annealing temperature raised from 36° C to 38° C (profile 2, second segment). Reactions were visualized on 0.7% agarose, EtBr-stained 0.5X TBE gels.

PCR with Mitochondrial Primers

Homology searches using mitochondrial sequences of sea urchin (Jacobs, et al., 1988) *Locusta migratoria* (McCracken, et al., 1987; Uhlenbusch, et al., 1987), *Apis mellifera* (Crozier and Crozier, 1993), *Drosophila yakuba* (Clary and Wolstenholme, 1985), *Xenopus laevis* (Roe, et al., 1985), mouse (Bibb, et al., 1981), and humans (Anderson, et al., 1981), revealed many possible primers. Primer sequences from homology searches and the literature are given in Table 1.

Table 1. Oligonucleotide Sequences for primers used in amplification of *Melanoplus* mtDNA

Primer	Sequence	Region
14969	5'-TTTACTACCAAATCCACC-3'	12sc rRNA
14970	5'- GGGGTATGAACCC A/G GTAGCT-3'	$tRNA^{meth}$
23944	5'-TATCATAACGAAAACGAGGTAA-3'	ND-1
23945	5'-TAGAAATGAAATGTTATTCGTTT-3'	16S RNA
25681	5'-TATGTACTACCATGAGGACA-3'	Cyt b
25682	5'-CGCCTGTTTAACAAAAACAT-3'	16S rRNA
31288	5'-CGCCTGTTTATCAAAAACAT-3'	16S rRNA
33052	5'-GTAAATAAAACTAAAAAACC-3'	COIa
34941	5'-TCAACAAAGATGTCAGTATCA-3'	COIIIb
34942	5'-AATATGGCAGATTA A/G TGCA-3'	$tRNA^{leu}$
C2-J-3696	5'-GAAATTTGTGGAGCAAATCA-3'	COII A611
37744	5'-AGCCCATGAAATCCTGTTGCGA-3'	COII
37743	5'-GAGCATCACCACTAATAGAACA-3'	COIII

The first primer set that was tried (14969 and 14970) flanked the A&T region, which has shown high levels of variability in many species (Lewis, et al., 1994). Primer 14969 was based on a conserved sequence in the 12sc rRNA, and primer 14970 was taken from a conserved region of the $tRNA^{meth}$ gene. The PCR product should have been around 2000 bp long. Oligonucleotide primers were ordered from Integrated DNA Technologies, Inc., and on arrival were dissolved in 400 µl ddH_2O. To determine the concentration of primers, OD_{260} measurements were taken, and DNA concentrations calculated by the following formula:

$[DNA] = (A_{260} \times 3x10^4 \text{ mg/l})/N \times \text{Dilution Factor} \times 330 \text{ amu}$

(N= number of bases, Dilution Factor = 200)

The concentration of primer 14969 was found to be 104 μM, while that of primer 14970 was 58 μM. The desired primer concentration in the PCR reaction was approximately 10 μM, so primers were diluted accordingly in ddH_2O.

The proper annealing temperature for the primers was calculated by the following formula:

Annealing temperature = (4 x the number of C+G bases) + (2 x the number of A+T bases)

The annealing temperature for primer 14969 was 64° C and 54° C for primer 14970. An annealing temperature of 50° C (profile 2, step 2) was used to lower reaction specificity. The following thermocycling parameters were used: profile 1: 95° C for 2 minutes, one cycle, profile 2: 94° C for 1 minute, 50° C for 1 minute, 72° C for 2.5 minutes, 40 cycles, profile 3: 72° C for 5 minutes, one cycle. The reaction mix was (per reaction): 13.25 μl ddH_2O, 2.5 μl 10X *Taq* buffer, 3.0 μl DNTP's, 1.0 μl 25 mM $MgCl_2$, 0.25 μl *Taq* polymerase, 2.0 μl each primer, and 1.0 μl sample DNA. *Drosophila melanogaster* DNA was used as a template, with ddH_20 dilutions of 1:10, 1:50, 1:100, and 1:250 in separate reactions. The reaction was also tried with the annealing temperature lowered to 40° C.

Primers designed to amplify a 2000 bp region of mtDNA were also tried. Primers 23944 and 23945 were diluted to appropriate concentrations as before. PCR was performed with the following parameters: profile 1: 95° C for 2 minutes, one cycle, profile 2: 94 ° C for 1 minute, 40 ° C for 1 minute, 72° C for 1.5 minutes, 40 cycles, profile 3: 72° C for 5 minutes, one cycle. The reaction mix was (per reaction): 16.75 μl ddH_2O, 2.5 μl 10X *Taq* buffer, 3.0 μl DNTP's, 1.0 μl 25 mM $MgCl_2$, 0.25 μl *Taq* polymerase, 1.0 μl each primer, and 1.0 μl sample DNA. Template DNA was generally diluted 1:100 with sterile ddH_2O. The reaction was then tested for optimum magnesium concentration by trying 0.5 μl increments from 0.5 μl to 2.5 μl, and for DNA concentrations by trying different dilutions of the template DNA.

The primer 25681 was designed from a sequence in the cytochrome b gene (Jermiin and Crozier, 1993).

It was paired with primer 25682, a 16S rRNA sequence. PCR to attain the desired 2500 bp fragment was performed with the following parameters: profile 1: 95° C for 1 minute, 1 cycle, profile 2: 94° C for 1 minute, 45° C for 1 minute, 72° C for 1.5 minutes, 40 cycles, profile 3: 72° C for 5 minutes, one cycle. The reaction mix was (per reaction): 16.75 µl ddH_2O, 2.5 µl 10X *Taq* buffer, 3.0 µl DNTP's, 1.0 µl 25 mM $MgCl_2$, 0.25 µl *Taq* polymerase, 1.0 µl each primer, and 1.0 µl sample DNA. Magnesium concentration was optimized as above, and several different annealing temperatures were tried. Results were improved greatly by using the following parameters: profile 1: 95° C for 2 minutes, one cycle, profile 2: 94° C for 1 minute, 45° C for 1 minute, 72° C for 1.5 minutes, 4 cycles, profile 3: 94° C for 1 minute, 47° C for 1 minute, 72° C for 1.5 minutes, 36 cycles, profile 4: 72° C for 5 minutes, one cycle. The reaction mix was (per reaction): 16.75 µl ddH_2O, 2.5 µl 10X *Taq* buffer, 3.0 µl DNTP's 1.0 µl 25 mM $MgCl_2$, 0.25 µl *Taq* polymerase, 1.0 µl each primer, and 1.0 µl sample DNA.

New combinations of these primers were subject to PCR. Primer 25682 was paired with primer 23944, primer 23945 was paired with primer 25681, primer 25681 was paired with primer 31288 (a primer similar to 25862 but with a single substitution), and primer 14970 was paired with primer 23944.

Primers with sequences in cytochrome oxidase genes were also tried. Primer 33502 is a conserved sequence in the COIa gene. It was paired with primer 14969 to produce a putative PCR product that would amplify the A&T rich region. PCR was first tried with a conventional reaction mix (16.75 µl ddH_2O, 2.5 µl 10X *Taq* buffer, 3.0 µl DNTP's, 1.0 µl 25 mM $MgCl_2$, 0.25 µl *Taq* polymerase, 1.0 µl each primer, and 1.0 µl sample DNA) and PCR parameters with longer extension times (profile 1: 95° C for 1 minute, one cycle, profile 2: 94° C for 1 minute, 42° C for 1 minute, 72° C for 3 minutes, 4 cycles, profile 3: 94° C for 1 minute, 45° C for 1 minute, 72° C for 3 minutes, 36 cycles, profile 4: 72° C for 5 minutes, one cycle. PCR with these primers was also tried in a reaction mix for "long PCR" (Cheng, et. al., 1994), which consisted of the following: 20 mM tricine (pH 8.7), 85 mM potassium acetate, 9.6% glycerol, 0.2 mM DNTP's, 0.2 µM primers, 2% DMSO, 1.1 mM magnesium acetate. The paper specified the use of rTth polymerase and Vent polymerase, which were unavailable, so 1U *Taq* polymerase was substituted. The reaction was also tried with Tris-Cl buffer (pH 8.4)

substituted for tricine. PCR parameters were: profile 1: 95° C for 1 minute, one cycle, profile 2: 94 C for 1 minute, 42° C for 1 minute, 72° C for 2 minutes, 4 cycles, profile 3: 94° C for 1 minute, 45° C for 1 minute, 72° C for 2 minutes, 36 cycles profile 4: 72° C for 5 minutes, one cycle.

Another primer based on cytochrome oxidase sequences (Simon, 1993) was tried. Primer 34941 was based on a conserved region in the COIIIb gene, and it was paired with primer 34942, a sequence in the $tRNA^{leu}$ gene (Sperling and Hickey, 1994). PCR was performed using this reaction mix: 16.75 µl ddH_2O, 2.5 µl 10X buffer, 3.0 µl DNTP's 1.0 µl 25 mM $MgCl_2$, 0.25 µl *Taq* polymerase, 1.0 µl each primer, and 1.0 µl sample DNA). Several parameter combinations were tried. The first was: profile 1: 95° C for 2 minutes, 1 cycle, profile 2: 94° C for 1 minute, 45° C for 1 minute, 72° C for 1.5 minutes, 40 cycles, profile 3: 72° C for 5 minutes, one cycle. The second set of parameters was: profile 1: 95° C for 2 minutes, 1 cycle, profile 2: 94° C for 1 minute, 43° C for 1 minute, 72° C for 1.5 minutes, 4 cycles, profile 3: 94° C for 1 minute, 47° C for 1 minute, 72° C for 1.5 minutes, 36 cycles, profile 4: 72° C for 5 minutes, one cycle. PCR was also tried with parameters similar to the four-profiled ones above, with annealing temperatures of 44° C and 48° C, and 40° C and 48° C. PCR was also attempted with a reaction mix using 4 times the usual primer concentration.

Primer 34941 was also paired with a primer sent by Chris Simon (pers. comm.), C2-J-3696, a sequence located in the COII (A611) gene. The reaction mix used was: 14.25 µl ddH_2O, 2.5 µl 10X buffer, 3.0 µl DNTP's, 0.25 µl *Taq* polymerase, 2.0 µl each primer, and 1.0 µl sample DNA). The buffer was sent with the primer and consisted of the following: 3.5 ml 1.0 M Tris-Cl pH 8.8, 100 µl BSA (100 mg/ml), 125 µl 1.0 M $MgCl_2$, and 1.275 ml ddH_2O. DNA from *Magicauda septemdecim* was used as a template in a positive control reaction, and DNA from various *Melanoplus* and *Anabres simplex* (Mormon cricket) samples were also used as prospective templates. PCR was performed with the following parameters: profile 1: 95° C for 2 minutes, 1 cycle, profile 2: 94° C for 1 minute, 50° C for 1 minute, 72° C for 1.25 minutes, 40 cycles, profile 3: 72° C for 5 minutes, one cycle. PCR was also attempted with these parameters using an annealing temperature of 48° C. The primers were also tried with a reaction mix of: 10.25 µl ddH_2O, 2.5 µl Perkin-Elmer 10X PCR buffer, 3.0 µl DNTP's, 1.0 µl

25 mM $MgCl_2$, 0.25 µl *Taq* polymerase, 1.0 µl each primer, and 5.0 µl sample DNA. The templates were conventional extractions of *Melanoplus* and *Anabres* DNA diluted 1:5, undiluted DTAB/CTAB extractions of *Melanoplus* DNA, and DTAB/CTAB extractions diluted 1:5 and 2:5. The PCR parameters were: profile 1: 95° C for 2 minutes, 1 cycle, profile 2: 94° C for 1 minute, 45° C for 1 minute, 72° C for 1.5 minutes, 4 cycles, profile 3: 94° C for 1 minute, 49° C for 1 minute, 72° C for 1.5 minutes, 36 cycles, profile 4: 72° C for 5 minutes, one cycle. PCR was also tried as above with annealing temperatures in the second and third profiles of 43° C and 45° C, respectively, using reaction volumes of 25, 50, and 100 µl.

Primers sequences from lichen grasshoppers (*Trimerotropis saxatilis*) were sent by Ann Gerber (pers. comm.). Primer 37744 and primer 37743 are also sequences from cytochrome oxidase genes. The reaction mix was: 16.75 µl ddH_2O, 2.5 µl 10X buffer, 3.0 µl DNTP's, 1.0 µl 25 mM $MgCl_2$, 0.25 µl *Taq* polymerase, 1.0 µl each primer, and 1.0 µl sample DNA. The first set of PCR parameters tried was: profile 1: 95° C for 2 minutes, 1 cycle, profile 2: 94° C for 1 minute, 55° C for 1.5 minutes, 72° C for 2.75 minutes, 38 cycles, profile 3: 72° C for 5 minutes, one cycle. PCR was also tried using the same parameters with the annealing temperature lowered to 46° C. The second set of parameters tried was: profile 1: 95° C for 2 minutes, 1 cycle, profile 2: 94° C for 1.25 minutes, 42° C for 1.5 minutes, 72° C for 2.75 minutes, 6 cycles, profile 3: 94° C for 1.25 minutes, 47° C for 1.5 minutes, 72° C for 2.75 minutes, 32 cycles, profile 4: 72° C for 5 minutes, one cycle.

PCR using the primer combination of 25682 and 23944 provided DNA amplification from the greatest number of samples. The approximate size of the PCR product was 1557 bp. The reaction mix initially used was: 16.75 µl ddH_2O, 2.5 µl 10X buffer, 3.0 µl DNTP's, 1.0 µl 25 mM$MgCl_2$, 0.25 µl *Taq* polymerase, 1.0 µl each primer, and 1.0µl sample DNA, with the following PCR parameters: profile 1: 95° C for 2 minutes, 1 cycle, profile 2: 94° C for 1 minute, 43° C for 1 minute, 72° C for 1.5 minutes, 4 cycles, profile 3: 94° C for 1 minute, 45° C for 1 minute, 72° C for 1.5 minutes, 36 cycles, profile 4: 72° C for 5 minutes, one cycle. Changing the annealing temperatures in profiles 2 and 3 to 45° C and 47° C, respectively, improved amplification. The magnesium concentration in the reaction mix was optimized to 1.75 µl 25 mM $MgCl_2$, greatly increasing the