Molecular Diagnostics of Economically Important Wireworm Species (Coleoptera: Elateridae) in the Midwestern United States

A Thesis presented to the Faculty of the Graduate School at the University of Missouri-Columbia

In Partial Fulfillment of the Requirements for the Degree

Master of Science

By

Erica Lindroth

Dr. Thomas Clark, Thesis Supervisor

August 2007

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

Molecular Diagnostics of Economically Important Wireworm Species (Coleoptera: Elateridae) in the Midwestern United States

presented by Erica Lindroth,

a candidate for the degree of master of science,

and hereby certify that, in their opinion, it is worthy of acceptance.

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Dr. Thomas Clark

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Dr. Robert Sites

_____

Dr. Brenda Beerntsen

_____

Dr. George Smith

To my husband, for his patience.

Acknowledgements

I would like to thank my advisor, Dr. Tom Clark, for his support and advice. I would like to thank my committee members, Dr. Bob Sites, Dr. Brenda Beerntsen, and Dr. George Smith. Many thanks to the myriad students, extension workers, entomology professors, and farmers who helped me obtain wireworm samples, including: Tom Kuhar, John Van Duyn, Pat Davis, Chris DiFonzo, Gerald Wilde, Gary Hein, D. Moellenbeck, Lyndon Brush, Ben Puttler, Wayne Bailey, Francis Lloyd, Lisa Meihls, Kevin Steffey, Billy Fuller, Eileen Cullen, Greg Andrews, Greg Kerr, Gary Cramer, and Leslie Loehr. I would like to thank Sandy Briesacher for her help with laboratory procedures. Finally, I would like to thank Akekawat Vitheepradit for helping me learn about phylogenetic analyses and always letting me know when there was free food.

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Introduction

Wireworms, the common name for larval click beetles (Coleoptera: Elateridae), are a serious soil-dwelling pest of many different crops all around the world (Ivashchenko 1981, Klinger 1974, Wightman 1978). Fifteen species of wireworms are known to attack corn (*Zea mays* L.) in the Midwestern United States (Riley & Keaster 1981, Ward and Keaster 1977). Damage caused by wireworms can result in substantial yield loss and may even require some stands to be replanted (Simmons, et al 1998, Smith, et al. 1981, Lefko, et al 1998). In some cases up to 35% of a crop might be lost due to wireworm damage (Apablaza, et al 1977).

Wireworms damage corn in multiple ways. Larvae first attack the seed, eating the germ and reducing the remainder to mere fragments (Kulash and Monroe 1955, Munson et al 1986). Damage of this type results in the corn failing to germinate and may necessitate replanting (Cheshire, et al. 1987). Wireworms may also bore into the underground portion of the stem or eat the roots of the plant (Simmons, et al., 1998). Damage of this type results in withering and structural instability (Munson et al 1986, Kulash and Monroe 1955).

Elaterids are a difficult group to study for many reasons. They are extremely difficult to distinguish morphologically on the species level (Riley and Keaster 1981), whether as adults or as larvae. Some species are cryptic in the larval stage. Control and sampling measures are problematic due to their subterranean habitat and are complicated by the difficulty in identifying species. Finally, their long life cycle is an impediment to

rearing and breeding studies. The purpose of this project is to identify the species of wireworms attacking corn in the Midwest using molecular diagnostics. This will increase the ease and accuracy of identification, facilitating further investigations into their control. This study was initiated with the following goals:

1. Construct a molecular phylogeny of the species of wireworms that are known pests of corn in the Midwest and develop an initial phylogenetic hypothesis concerning economically important wireworm species.
2. Use molecular diagnostics to distinguish the morphologically cryptic species of wireworms identified by T. J. Riley and A.J. Keaster.
3. Determine the genetic structure of *Melanotus depressus* (Melsheimer) populations in the Midwest. *M. depressus* is the most common wireworm encountered in this study.

Taxonomy and Distribution

Click beetles are classified within family Elateridae, which is contained within super family Elateroidea in the suborder Polyphaga within order Coleoptera. Elateridae is a large family with representatives in many places around the world. There are approximately 400 genera and 9000 species in the family, and more are discovered every year (Parker 1982). Their common name is derived from their ability to "click". A lobe on the prosternum extending back into a mesosternal depression is the mechanism that allows the beetle to click (Parker 1982). The ability to click is thought to be a mechanism to escape predation.

Adults are generally narrow-bodied with the pronotum sharply pointed at the posterior corners (Parker 1982). Adults range in size from just a few millimeters up to 4.5 centimeters in length. Larvae are elateriform, hard-bodied, and range in color from light tan to dark brown. Size varies with age as well as species; older larvae are usually larger. Where such distinctions are possible, characters on the head and ninth abdominal segment are used to distinguish larvae at the species level (Riley and Keaster 1981).

According to Riley and Keaster (1979, 1983, 1981) fifteen species in six genera of Elateridae are associated with damage to corn and other grains in the Midwest. The genera are *Agriotes*, *Melanotus*, *Aeolus*, *Conoderus*, *Hemicrepidius*, and *Limonius*. *Melanotus* is the most common genus as well as the most destructive (Riley and Keaster 1981). Ten species of Melanotus are known to be associated with corn, while just one species in each of the other genera is known to be economically important (Riley and Keaster 1979, 1981).

The most economically important species in Missouri are *Melanotus depressus* (Melsheimer), *Melanotus verberans* (LeConte), *Melanotus lanei* (Quate), and *Melanotus opacicollis* (LeConte) (Riley and Keaster 1981). *M. lanei* and *M. opacicollis* are morphologically indistinguishable in the larval stage, as are *M. dietrichi* and *M. communis*. *Agriotes mancus* and *Limonius dubitans* do not occur in Missouri, although they have been collected in Nebraska, Iowa, Illinois, Indiana, and Ohio (Riley and Keaster 1981).

Click beetles have not been observed to migrate great distances. The larvae likely do not move a great deal, although they are known to migrate vertically through the soil (Fisher, et al. 1975) and occasionally crawl across the soil surface (Keaster, et al 1975).

Females exhibit very little flight activity, indicating that infestation into adjoining areas may take quite some time (Brown and Keaster 1986).

Biology and Life Cycle

Elaterids tend to have comparatively long life cycles for insects. Adults emerge in late spring and early summer and are active for about a month, with males emerging prior to females (Brown and Keaster 1986, Doane 1961). Data from pitfall and flight traps suggest that males exhibit more flight activity than females, indicating that females do not travel far to find oviposition sites (Brown and Keaster 1986, Doane 1961).

According to Fisher et al. (1975), eggs are oviposited in late spring and early summer and hatch in early to late summer. Elaterids spend anywhere from one to several years in the larval state, depending on the species (Stone 1980, Simmons, et al 1998). During this stage feeding periods alternate with periods of dormancy (Keaster, et al 1975). Many species spend one or more winters in the larval stage, although a few species with shorter life cycles may overwinter as pupa or adults. (Fisher, et al 1975).

Habitat preferences have been more thoroughly investigated than life history characteristics, perhaps because they are less time consuming to study. Wireworms have specific moisture and temperature preferences, as documented by Campbell in 1937. They prefer soil moisture content of 9-12%, and experience fatality in soil that is desiccated or saturated (Campbell 1937, Lefko, et al 1998). Wireworms prefer a soil temperature range of about 50-75 degrees Fahrenheit (Campbell 1937, Fisher, et al 1975). They migrate vertically through the soil twice each year. The first migration occurs in spring when the population moves up into shallower soil as the surface temperature increases. In mid-summer they move back down as the soil temperature increases. The

second migration occurs in fall when the wireworms once again move upwards as temperatures begin to cool then again return to deeper soil in late October to overwinter (Fisher, et al 1975). Wireworms move to a depth of 6 inches or less when the soil temperature ranges from 55 to 75 degrees Fahrenheit and move to depths below 6 inches when the soil temperature rises above 75 degrees or falls below 55 degrees Fahrenheit (Fisher, et al 1975). Keaster, et al (1975) also noted that wireworms exhibited negative phototaxis and those exposed to excessive light experienced higher mortality.

In addition to the above preferences, wireworms appear to exhibit partiality towards different parts of a field. Riley and Keaster (1984) found that wireworms are more likely to be found in fresh manure pats and the soil underneath them than in a decomposed manure pat or soil without manure. *Melanotus* larvae and adults are also more likely to inhabit the southern side and center of a field than the western side of a field (Brown and Keaster 1986).

Certain fields are more susceptible to wireworm damage than others. Riley and Keaster (1984) suggest that crops planted in former cow pastures may be especially susceptible due to the increased numbers of wireworms under manure. Land given over to the Conservation Reserve Program (CRP) (instituted in 1985 to increase natural habitat and decrease soil erosion) may also be more likely to harbor damaging levels of wireworms when replanted (Lefko, et al 1998a, 1998b, Simmons, et al 1998). Land in the CRP is planted with native grasses and left fallow for 10 years, making it an ideal habitat for Elaterids (Lefko, et al 1998a, 1998b).

Wireworm Management

Control of wireworms is complicated by their subterranean habitat. Remedial control methods (i.e., application of pesticides after damage has begun) are ineffective at controlling soil-dwelling pests (Munson, et al 1986). The most economical means of dealing with wireworm damage may simply be to replant the crops (Lefko, et al 1998). Widespread pesticide application is not generally worthwhile economically, as wireworm damage is sporadic (Smith, et al 1981, Arnold 1981). The best means of dealing with wireworms is early detection of areas with large populations (Smith, et al 1981).

There are a variety of sampling and baiting methods available for determining wireworm population levels. Sampling is as simple as taking several soil cores from a field and counting the number of wireworms in them by hand. The process can be made more efficient with the use of equipment such as the Self-Propelled Soil Sampler, a modified Cushman Turf-Truckster (Outboard Marine Corp., Lincoln, Nebraska), developed by Smith, et al., in 1981 for monitoring the presence of soil insect pests. However, soil cores may not be the best means of wireworm detection, as a null result does not necessarily mean there is not a potentially damaging population present (Parker 1994). The use of baits is an effective means of enhancing sampling and determining the presence of wireworms before planting. Apablaza et al (1977) found that wireworms of several species (*M. depressus*, *M. verberans*, and *L. dubitans*) were most attracted to 1:1 mixtures of corn and wheat in laboratory experiments. Seal, et al. (1992) found that several other species of wireworms in the genus *Conoderus* were most attracted to mixtures of corn and wheat in field tests. Germinating seeds produce carbon dioxide, which wireworms use to locate their food (Doane et al 1975). Ward and Keaster (1977)

devised a bait trap using solar energy as well as food to attract wireworms earlier in the spring. Covering the bait with a plastic sheet to warm the soil around it was shown to be quite effective in attracting wireworms early in the year. Kirfman, et al. (1986) modified the trap by embedding the bait in vermiculite, wrapping it in fine tulle and placing it in a porous container with a lid positioned loosely over it. This design allows for a shorter sampling time because the bait seed germinates more quickly and processing the traps is also easier because vermiculite does not clump the way soil does.

A second type of trap found to be successful in attracting *Melanotus* is the pheromone trap. Keaster, et al (1987) found that the pheromone of the tufted apple bud moth could be used as an attractant to detect the presences of adult click beetles. *M. depressus* and *M. similis* were the species found to be most attracted to the pheromone. Pheromone trap surveying has been successful in British Columbia and Washington, although the pheromone composition is proprietary information (Vernon, et al 2001).

The Agricultural Guide published by the University of Missouri (Munson, et al 1986) recommends placing two baited traps per acre two to three weeks before planting. The current economic threshold for wireworms is an average of one wireworm per bait trap (Simmons 1998). Once it has been determined whether a field has an economically damaging population of wireworms, decisions can be made about control measures. A variety of methods for dealing with wireworm infestations have been evaluated.

Insecticides must be applied before or at planting. Application at planting is usually more effective and worthwhile economically (Munson, et al 1986). There are three general methods for applying insecticides during planting: in-furrow application, row-band application, and seed treatment (Munson, et al 1986, Arnold 1981). Although

some of the following chemicals are no longer in use, the insecticides Carbofuran, Chlorpyrifos, and Terbufos have been effective when applied in-furrow and the insecticides Carbofuran, Chlorpyrifos, Ethoprop, Fonofos, and Terbufos have been effective in row-band applications (Munson, et al 1986). More recently, imidacloprid has become the most common chemical used for control of soil insect pests (Albajes, et al 2003). Kulash and Monroe (1955) evaluated the efficacy of insecticides applied to corn seed, applied to the soil as a spray, and applied at planting mixed with a fertilizer. Of these three treatments a combination of the fertilizer-insecticide mixture and the soil treatment produced the largest number of healthy plants. However, they found that while the differences in methods were statistically significant, they could be attributed to chance. Arnold (1981) found that the insecticide permethrin is effective in controlling wireworm damage while the insecticide fenvalerate is only moderately effective. Chemigation is an alternative method of pesticide application that is not always as effective as other means, but costs less (Chalfant, et al 1993).

Non-chemical controls for wireworms do exist, although they vary in their utility. Flooding has been shown to reduce wireworm populations, but fields need to be flooded for four to five weeks with water temperatures around 24 degrees Celsius (Hall and Cherry 1993). This technique obviously has limited applicability for farmers not near major bodies of water or in climates with short growing seasons. Tilling may be a more effective cultural control measure. Pique et al (1998) discovered that fields in Catalonia that underwent extensive tilling did not require the application of pesticides. In a different study, however, Belcher (1989) found there was no significant difference in the presence of wireworms among fields that were tilled conventionally, minimally, or not tilled at all.

Molecular Diagnostic Techniques

There are a variety of molecular diagnostic techniques available for analyzing the genetic composition of a population. These methods can be divided into three general areas: protein electrophoresis, DNA sequencing, and analysis of DNA fragments.

There are a number of different ways of cleaving DNA and analyzing the resulting fragments. DNA fragments can vary in conformation, size, restriction endonuclease cleavage at recognition sites, and PCR priming sites. These techniques generally include visualization via the gel electrophoresis process described below and amplification using polymerase chain reaction (PCR).

PCR is an extremely useful technique that allows very small amounts of DNA to be amplified for use in sequencing and molecular diagnostics. To amplify a segment of DNA two primers must be prepared, one corresponding to the 5' end of the fragment, the other corresponding to the 3' end of the fragment. A mixture containing the DNA to be amplified, the primers, the four deoxynucleoside triphosphate building blocks, and DNA polymerase goes through a cycle of heating and cooling. Heating the mixture separates the DNA into two strands. When the mixture is cooled the polymerase builds two new strands of DNA using the denatured strands and the primers as a template. The heating and cooling cycle is repeated about 30 times over the course of a few hours, resulting in the creation of a large quantity of DNA. (Nelson and Cox 2005) PCR is an important tool that is used in many different types of molecular systematic studies. It can be used to determine genetic variation within populations (de Leon and Jones 2004) and among different species (Navarro and Weaver 2004). It is commonly used to amplify genes in

preparation for DNA sequencing (Szalanski, et al 2004), microsatellite analysis (Batley, et al 2004), and fragment analysis techniques such as RFLP (Silva, et al 2003).

Restriction endonucleases cleave DNA at specific recognition sites, resulting in restriction fragment length polymorphisms, or RFLPs. Mutations result in the deletion or insertion of these recognition sites, resulting in fragments of differing lengths (Hillis 1996). A large number of restriction endonucleases for a wide variety of highly conserved segments of DNA have been catalogued, making RFLP analysis of many different types of organisms feasible (Hillis 1996). RFLP has been shown to be very useful in identifying potentially invasive species of fruit fly in New Zealand (Armstrong 1997) and to track outbreaks of Mediterranean fruit flies in Florida (Silva, et al 2003). RFLP has been use to differentiate among mosquitoes in the genus *Anopheles* in South East Asia (Van Bortel 2000). Szalanski, et al (2003) showed that morphologically cryptic species of *Reticulitermes* termites can be identified using PCR-RFLP analysis.

Microsatellites are short, repeating segments of DNA, generally between two and ten base pairs in length, found near coding loci (Loxdale and Lushai 1998). The length of these repeating sections is highly variable, much more so than the coding loci they are near (Hillis 1996). This makes them convenient for study, as the conserved coding loci can be used as primers to amplify the microsatellites via PCR (Loxdale and Lushai 1998). After the microsatellite segments are amplified they are analyzed using gel electrophoresis. Like RFLPs, different fragment lengths are considered different alleles (Loxdale and Lushai 1998). Microsatellites are often used in molecular diagnostic studies. Llewellyn, et al (2003) used microsatellites to determine that the aphid *Sitobion*

avenae is highly migratory based on the lack of intraspecific differentiation over long distances.

Randomly amplified polymorphic DNAs, or RAPDs, create DNA fragments of differing length based on differences in PCR priming sites. Sequence differences in the recognition sites used by PCR primers will result in randomly amplified polymorphic fragments produced during PCR (Hillis 1996). They have been used in a variety of studies, such as the analysis of genetic variation in aphids (Clements 2000). The major drawback to RAPDs is that they do not differentiate between heterozygous individuals and homozygous dominant individuals because of the dominant nature of the markers (Loxdale and Lushai 1998). Amplified fragment length polymorphism, or AFLP, tests for the presence or absence of restriction fragments rather than for fragment length (Loxdale and Lushai 1998). In this technique, genomic DNA is cleaved using a common-site and a rare-site restriction endonuclease (Loxdale and Lushai 1998). Adaptor sequences are ligated onto the cleaved segments, which are then amplified via radioactive PCR (Loxdale and Lushai 1998). The resulting fragments are visualized through gel electrophoresis.

The procedure for gel electrophoresis is relatively simple. Hillis (1996) outlines the procedure. After the DNA fragments or proteins in question have been extracted they are loaded onto the gel, which is generally made from a type of sugar. The gel is placed in a well that is connected to a power supply. A buffer solution is added to protect against changes in pH that would otherwise occur when an electrical current is run through the gel. When current is run through the gel the negatively charged DNA fragments migrate to the positive end of the gel. The extent of a fragment's movement is

generally based on its size in relation to the pores in the gel. When electrophoresis is complete the fragments are stained to allow visualization. (Hillis 1996)

DNA sequencing is a powerful tool because it provides direct genetic information. There are two basic steps involved in sequencing a section of DNA. The first step is to isolate the desired fragments of DNA using a process such as PCR or cloning (Hillis 1996). Next, the isolated nucleic acids must be sequenced. This is typically done using an automated sequencer based on the Sanger sequencing method (Hillis 1996). If sequencing technology is not readily available, it is possible to send samples to universities or private companies that provide sequencing services (Szalanski, et al 2004).

DNA sequencing can provide a great deal of information on intra- and interspecific diversity. Combined with geographical data it provides a way of measuring the genetic structure of populations and gene flow among them (Hillis 1996). Caccone and Sbordoni (2001) were able to determine the molecular biogeography of Bathysciine beetles through sequencing their mitochondrial DNA. It can be a valuable tool in conservation as it enables researchers to look at the factors that affect very small populations, such as inbreeding and reduced heterozygosity (Hillis 1996). DNA sequencing has been used in the identification of blow fly species used in forensic studies (Chen 2004). Navarro and Weaver (2004) used the ITS-2 region of rDNA to investigate the molecular phylogeny of mosquitoes in the subgenus *Culex*. Tokuda , et al (2004) used the COI region of mDNA to investigate both intra- and interspecific relationships among gall midges.

Molecular population genetics and molecular systematics have a wide variety of applications in entomology. The most obvious use of genetics and molecular diagnostics

is in insect systematics, particularly in distinguishing between morphologically cryptic species. However, phylogenetics has also been used to great effect in many other areas.

There are many examples of the use of molecular markers in insect systematics. West, et al (1997) used PCR-RFLP to identify female and immature mosquitoes of the genus *Aedes*, which cannot be identified morphologically. Phylogenetic relationships among species can also be determined, as Knowles and Otte (2000) did for 25 species of montane grasshoppers in western North America. More recently, mosquitoes from Phnom Penh have been analyzed using PCR-AFLP (Paupy 2004). Rao et al (2006) used DNA sequencing to distinguish two cryptic species of Tipulidae at the molecular level. De Leon and Jones (2004) demonstrated natural variation in glassy-winged sharpshooters using DNA fingerprinting methods based on PCR. Pest species, such as *Anopheles* mosquitoes (Van Bortel 2000) or *Diabrotica* beetles (Clark et al 2001) are often the subject of molecular systematic studies, as this type of knowledge may aid in control efforts.

Phylogenetic analyses have been used extensively to study insect-plant interactions. Mardulyn, et al (1997) elucidated the evolutionary history of interactions between *Gonioctena* leaf beetles and their host plants. They used allozymes and mtDNA sequence data to determine that there is not always a correspondence between insect phylogeny and host affiliation. Downie, et al (2001) used mtDNA and RAPD analysis of nuclear DNA to determine that geography was more important than host-plant traits in producing diversification in the grape phylloxera *Daktulosphaira vitifoliae* Fitch.

Phylogenetic experiments also contribute to colonization and dispersal studies. Juan, et al (1995) sequenced mtDNA in beetles of the genus *Pimelia* that inhabit the

Canary Islands and Iberia. They compared their data to geographical and geological data to determine the order in which the islands were colonized. They found that the order of island colonization corresponded well to the order of island formation. Carew and Goodisman (2004) used PCR-RFLP to analyze populations of two mite species (*Colomerus vitis* Pagenstecher and *Calepitrimerus vitis* Nalepa) that are known grapevine pests. They found high levels of differentiation between the two species although they live very close together, indicating that their dispersal rate is very low. Scheffer and Lewis (2006) used mitochondrial sequence data to determine the phylogeography of the vegetable pest *Liriomyza sativae*.

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Chapter II: Molecular Diagnostics and Phylogenetic Analysis

Introduction

Wireworms (Coleoptera: Elateridae) are a common soil-dwelling pest of corn, *Zea mays* L., and other field crops throughout the Midwestern United States and the world. As wireworms are generalist herbivores they do not specifically target field crops, but damage can become quite severe when pasture or land in the Conservation Reserve Program (CRP) is brought into production for field crops such as corn (Riley and Keaster 1984, Lefko et al 1998a, 1998b). Feeding by wireworms can damage crops in several different ways. Larvae may attack the seed, eating the germ and reducing the remainder to mere fragments (Kulash and Monroe 1955, Munson et 1986). Damage of this type results in the corn failing to germinate and may necessitate replanting (Cheshire, et al. 1987). Withering and structural instability (Munson et al 1986, Kulash and Monroe 1955)can also result when wireworms bore into the underground portion of the stem or feed on the roots of more mature plants (Simmons, et al., 1998). In some cases up to 35% of a crop might be lost due to wireworm damage (Apablaza et al 1977).

Fifteen species in six genera are associated with damage to corn and other grains in the Midwest (Riley and Keaster 1979, 1983, 1981). The genera are *Agriotes*, *Melanotus*, *Aeolus*, *Conoderus*, *Hemicrepidius*, and *Limonius*. *Melanotus* is the most common genus as well as the most destructive (Riley and Keaster 1981). Ten species of *Melanotus* are known to be associated with corn, while just one species in each of the other genera is known to be economically important (Riley and Keaster 1979, 1981). *Agriotes mancus* and *Limonius dubitans* do not occur in Missouri, although they have been collected in Nebraska, Iowa, Illinois, Indiana, and Ohio (Riley and Keaster 1981).

The most economically important species in Missouri are *Melanotus depressus* (Melsheimer), *Melanotus verberans* (LeConte), *Melanotus lanei* (Quate), and *Melanotus opacicollis* (LeConte) (Riley and Keaster 1981).

Several economically important elaterid species belong to cryptic species complexes that are considered to be morphologically indistinguishable during the damaging larval stage. For example, the *M. lanei-M. opacicollis* and the *M. dietrichi-M. communis* species complexes are morphologically indistinguishable in the larval stage (Riley and Keaster 1981). Furthermore, wireworm species are difficult to distinguish and are often only identifiable as adults using male genitalia with no female specific characters (Smith and Enns 1977 and 1978). Consequently, species specific research on biological and ecological topics critical to the understanding and design of effective management strategies for wireworms continues to be a problematic endeavor.

Molecular systematics has enhanced our ability to elucidate economically important insect species in problematic genera lacking discrete morphological characters (Avise 1994). Many techniques have been utilized to distinguish populations, biotypes, and cryptic species using methods such as esterase markers (Krysan et al. 1989), random amplified polymorphic DNA (RAPD) (Clements 2000), amplified fragment-length polymorphism (AFLP) (Blanc 2006), microsatellites (De Barro), and gene sequencing (mitochondrial, ribosomal, and nuclear) (Rao et al 2006, Navarro and Weaver 2004, De Barro 2005). While most of these molecular methods have a significant degree of systematic utility, sequence analysis of mitochondrial genes have proven taxonomic utility in terms of identifying morphologically cryptic species or life stages (Clark et al. 2001, Austin et al. 2006) and providing insight into evolutionary and phylogeographic

questions associated with economically and medically important insect species complexes (De Barro et al. 2005; Hsieh et al. 2006; Scheffer and Lewis 2006). In general, all of these questions are currently lacking in the wireworm literature. Here we develop an initial phylogenetic hypothesis regarding economically important species of wireworms, and test the utility of the mitochondrial cytochrome oxidase subunit 1 (COI) gene in distinguishing morphologically cryptic species in the genus *Melanotus*.

Materials and Methods

Sampling and Identification

Specimens were collected from various locations in the Midwestern and Eastern U.S (Fig. 1). Samples were obtained by digging in fields with known wireworm populations and by placing traps according to the methods of Ward and Keaster (1977). Extension entomologists and farmers sent in wireworm samples from distant locations. Identifications were made using available dichotomous keys (Riley and Keaster 1981 and Riley 1983) and museum specimens previously identified by T.J. Riley. Adult and larval reference specimens were obtained from the Enns Entomology Museum at the University of Missouri. Samples were stored in 95% ethanol or -80° C.

DNA Collection and Analysis

DNA was extracted from wireworms using the QIAGEN DNeasy Tissue Kit (QIAGEN, Valencia, CA). Extraction was performed on the abdomen; the anterior and posterior sections of the larvae were preserved at -80°C as a reference. DNA extraction from adult museum specimens was performed using DNAzol (Gibco BRL/Life Technologies, Gaithersburg, MD), with the technique described by Junqueira et al (2002). Portions of the mtDNA cytochrome oxidase subunit I (COI) gene were amplified

via PCR using the primers C1-J-1718 (5' GGAGGATTTGGAAATTGATTAGTTCC 3'), C1-N-2191 (5' CCCGGTAAAATTAAAATATAACTTC 3'), C1-J-2183 (5' CAACATTTATTTTGATTTTTTGG 3'), and TL2-N-3014 (5' TCCAATGCACTAATCTGCCATATTA 3') from Simon et al (1994).

30 µl PCR reactions were performed with 10 mM dNTPs, 5 U/µl Amplitaq, 25 mM $MgCl_2$, 10X PCR buffer, 20mM sense and antisense primers (all reagents Applied Biosystems, Foster City, CA), extracted DNA, and water to volume. A PCR cycle of 60 s at 94°C, 30 s at 52°C, and 90 s at 72°C was repeated 35 times in a GeneAmp 2700 PCR System (Applied Biosystems, Foster City, CA). The PCR product was then purified using the Qiaquick PCR Purification Kit (QIAGEN, Valencia, CA). Samples were sequenced at the University of Arkansas Department of Microbiology and Immunology DNA Sequencing Core Facility (Little Rock, AR) using an ABI 377 sequencer.

Sequences were edited and aligned using BioEdit 7.0.5.2 (Hall, 1999). MacClade 4.06 (Maddison and Maddison 2003) was used to obtain proper sequence format for PAUP 4.0b10, and CLC Free Workbench 2.5.2 (www.clcbio.com) was for translations. P-distances were computed in MEGA2 (Kumar et al 2004). Tree construction and phylogenetic analyses were performed using PAUP 4.0b10 (Swofford 2003) and ModelTest 3.7 (Posada and Crandall 1998). Minimum evolution trees were constructed using the neighbor joining method. Maximum parsimony analysis was performed using a heuristic search. An unweighted maximum likelihood analysis was performed using the evolutionary model GTR+G derived from ModelTest 3.7 (Posada 2005). Bootstrap analyses of 1000 replicates were performed to assess clade support. COI sequence data

from the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (GenBank AF278549) were added as an outgroup taxon.

Results

Phylogenetic Analysis

DNA was sequenced from 318 individuals comprising 11 of the 15 species of wireworms known to be economically important in the Midwestern United States. Table 2 lists the Genbank accession numbers for the species sequenced. DNA sequencing resulted in 1343 bp of the COI gene, comprising the majority of the gene. All species in the genus *Melanotus* were successfully amplified with the primers provided by Simon et al (1994), but the primers C1-J-2183, and TL2-N-3014 were not successful in amplifying *Hemicrepidius memnonius* or *Limonius dubitans*, and the primers C1-J-1718 and C1-N-2191 were not successful in amplifying *Aeolus mellillus*.

Of the 1343 bases sequenced, 281 characters were parsimony informative. Nucleotide frequencies were biased towards A and T by 65.03%. Seven distinct haplotypes were identified within the species *Melanotus depressus*. Three different analyses were performed to determine phylogenetic relationships. Fig. 2 depicts the most parsimonious tree, Fig. 3 is a minimum evolution tree, and Fig. 4 is a maximum likelihood tree. In all cases the numbers above the branches indicate bootstrap values.

All three trees place *C. lividus* in a separate clade, and place *M. depressus* as basal to the other species within the genus. The maximum parsimony and minimum evolution analyses also depict similar relationships among the different species within *Melanotus*. *M. indistinctus*, *M. pilosus*, *M. lanei*, and *M. opacicollis* form a distinct clade, as do *M. verberans*, *M. communis*, and *M. dietrichi*. *M. similis* and *M. cribulosus* are placed in

their own clade in the first two analyses. The maximum likelihood analysis differs from the first two analyses by placing *M. cribulosus* and *M. similis* into a clade with *M. communis* and *M. dietrichi*. *M. verberans* is in the clade containing *M. indistinctus*, and *M. pilosus* forms a comb with *M. lanei* instead of forming a strict group. High bootstrap values, typically accepted as 80 or higher with values under 50 not shown, indicate strong support for a clade. The bootstrap value for the clade separating *M. depressus* from the other *Melanotus* species is 99, which indicates very strong support for this clade. Likewise, the clade containing *M. cribulosus* and *M. similis* has a bootstrap value of 100, as does the clade containing the *M. dietrichi-M. communis* species complex.

Identification of Cryptic Species

523 bp of the COI gene from the mitochondrial genome were sequenced from 16 *M. communis, M. dietrichi, M. lanei,* and *M. opacicollis* larvae. 523 bp of the COI gene were also extracted from adult museum specimens from the reference collection of T.J. Riley. Due to the difficulty of extracting DNA from very old specimens – specimens were collected in the 1970s - museum specimen sequence data was obtained from only *M. lanei* and *M. dietrichi*. Museum specimen sequence data was compared with unknown larval sequence data from the four cryptic species. Nucleotide *p*-distances were used to assess relationships between museum specimens and unknown specimens. Tables 2 and 3 depict the percentage of differences between the museum specimens sequence data and the unknown larval sequence data. Nucleotide *p*-distances were also used to construct minimum evolution trees showing the relationships between the known and unknown specimens. In Fig. 5 the museum specimen *M. dietrichi* clearly aligns into a clade with the unknown larval specimens WWU5, WWX6, and WWU3, providing evidence that

these three specimens are also *M. dietrichi* while those not in the clade are likely *M. communis*. In Fig. 6 the museum specimen *M. lanei* does not clearly align with either clade, but instead comes out basal to both. However, the clade containing WWI7, WWI6, and WWI9 is slightly closer to *M. lanei* and thus was designated as such, therefore the other clade was assumed to be *M. opacicollis*.

To test the tentative identifications made using nucleotide *p*-distances, the unknown specimens were included in the multi-species phylogenetic analyses using the full 1343 bp of the COI gene. In all three analyses the unknown species formed clades consistent with their previous identifications (Figs. 2, 3, and 4). The two *M. communis* specimens grouped together, as did the *M. lanei* and *M. opacicollis* specimens. In addition, *M. communis* consistently formed a sister group to *M. dietrichi*. The bootstrap support for these groups is very strong.

Discussion

Phylogenetic data reflects species morphology as described by Riley and Keaster (1981). For example, *M. depressus* is the only species in the genus without paired striations on the posterior segments and is also placed basally to the other species in all three trees. *M. depressus* also seemed to be the most widely distributed and commonly encountered species in this study. *M. similis* and *M. cribulosus* are depicted as sister species and also appear to be closely related morphologically. Anecdotal morphological evidence indicates that *M. indinstinctus* is closely related to *M. opacicollis* and *M. lanei* and analysis puts these species in the same clade with strong bootstrap support (Riley and Keaster 1981). The morphologically cryptic species *M. dietrichi* and *M. communis* are shown to be sister species. It was expected that *M. lanei* and *M. opacicollis* would also be

sister species because they are morphologically indistinguishable, and although they were not, they were in the same clade.

Species determination analysis showed distinct mitochondrial separation of the various species complexes. These findings provide insight into the elucidation of cryptic wireworm species and may allow future researchers to target specific environments where certain species are likely to be found. For example, wireworms have been shown to have specific soil temperature and moisture preferences (Campbell 1937). Some wireworms prefer sandy soils (Keaster and Fairchild 1960) while others prefer cow pastures (Riley and Keaster 1984). This is especially important, as adult elaterids do not specifically target field crops for oviposition (Lefko et al 1998a, 1998b). In addition, accurate identification should prove useful in instigating more effective pest control measures and more accurate investigations into the life history and ecology of these organisms.

The data presented here provides an initial view of the phylogenetic relationships among economically important wireworm species, as well as the first species identifications based on DNA analysis. Future studies should include genomic DNA, as well as a wider variety of species to elucidate relationships with wireworm pest species from other regions of North America. It might also be useful to conduct an analysis using both pest and non-pest species within the genus *Melanotus*, as this could potentially help predict whether other species have the capability of becoming pests. It would be advantageous for future studies to include recently obtained adult specimens for easier DNA extraction.

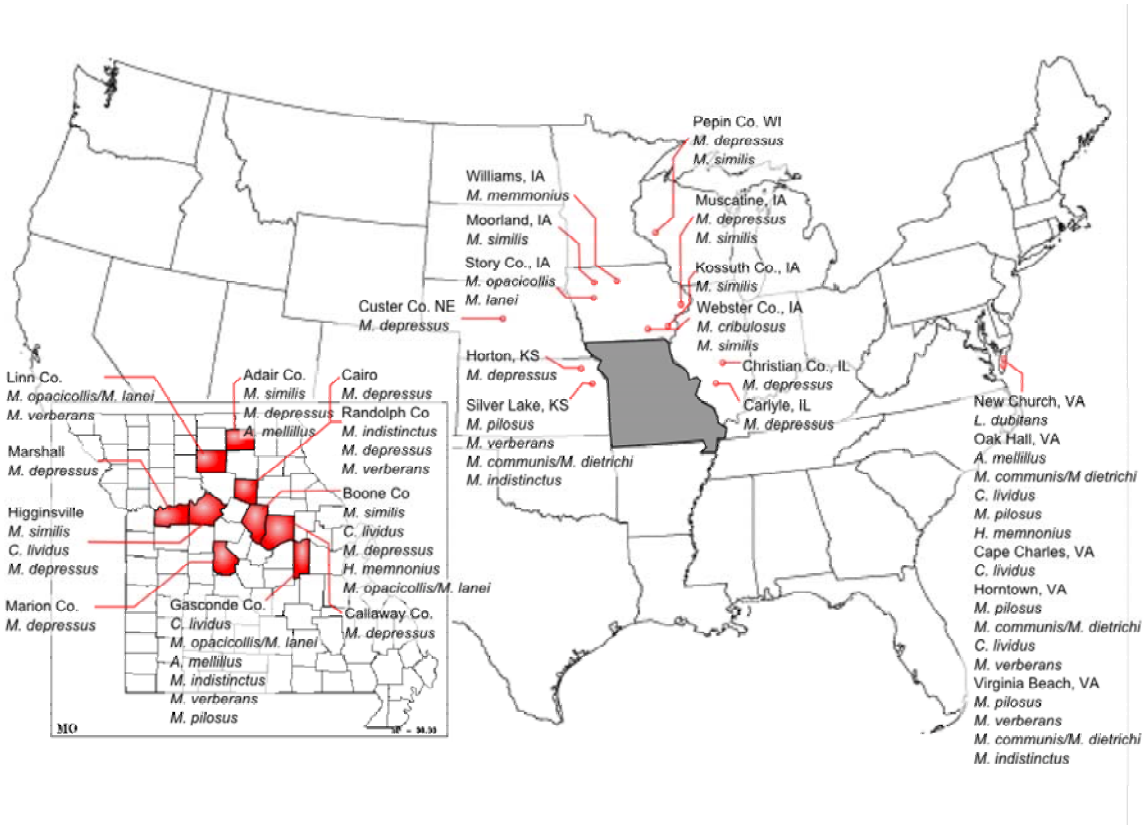

Figure 1. Collection locations across the United States and Missouri.

Table 1. Collection locations.

Location	Species Collected
Moorland, IA	*M. similis*
Story Co., IA	*M. opacicollis/M. lanei*
Muscatine, IA	*M. depressus, M. similis*
Kossuth Co., IA	*M. similis*
Williams, IA	*H. memnonius*
Webster Co., IA	*M. cribulosus, M. similis*
Pepin Co., WI	*M. depressus, M. similis*
Horton, KS	*M. depressus*
Silver Lake, KS	*M. pilosus, M. verberans, M. communis/M. dietrichi, M. indinstinctus*
Custer Co., NE	*M. depressus*
Carlyle, IL	*M. depressus*
Christian Co., IL	*M. depressus*
Boone Co., MO	*M. similis, C. lividus, M. depressus, H. memnonius, M. opacicollis/M. lanei*
Higginsville, MO	*M. similis, C. lividus, M. depressus*
Marshall, MO	*M. depressus*
Cairo, MO	*M. depressus*
Adair Co., MO	*M. similis, M. depressus, A. mellillus*
Randolph Co., MO	*M. indinstinctus, M. depressus, M. verberans*
Gasconde Co., MO	*C. lividus, M. opacicollis/M. lanei, A. mellilus, M. indistinctus, M. verberans, M. pilosus*
Marion Co., MO	*M. depressus*
Forage Research Station, MO	*M. opacicollis/M. lanei, M. verberans*
Callaway Co., MO	*M. depressus*
New Church, VA	*L. dubitans*
Oak Hall, VA	*C. lividus*
Painter, VA	*A. mellillus, M. communis/M. dietrichi, C. lividus, M. pilosus, H. memnonius*
Cape Charles, VA	*C. lividus*
Horntown, VA	*M. pilosus, M. communis/M. dietrichi, C. lividus, M. verberans*
Virginia Beach, VA	*M. pilosus, M. verberans, M. communis/M. dietrichi, M. indistinctus*

Table 2. Genbank accession numbers for the species identified and used in this study.

Wirworm Species	Genbank Accession Number
M. dietrichi	EF424473
M. communis	EF424474
M. cribulosus	EF424475
M. indistinctus	EF424476
M. lanei	EF424477
M. opacicollis	EF424478
M. pilosus	EF424479
M. similis	EF424480
M. verberans	EF424481
M. depressus	EF424482
C. lividus	EF424483

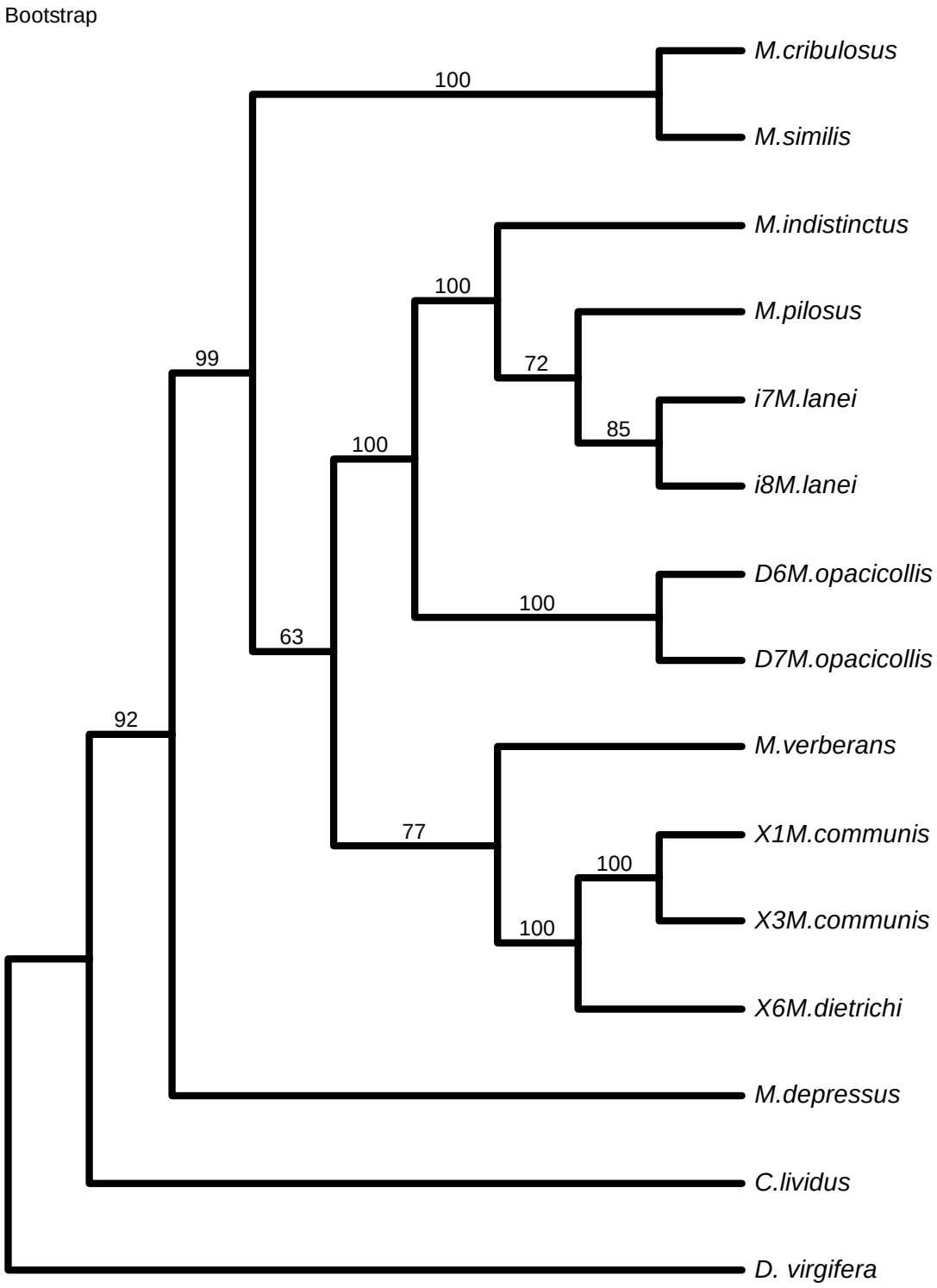

Figure 2. Maximum parsimony tree. Consensus sequences. Heuristic search (simple, TBR), 1000 rep bootstrap. Tree length = 1291, CI = 0.811, RI = 0.728, 281 parsimony-informative characters.

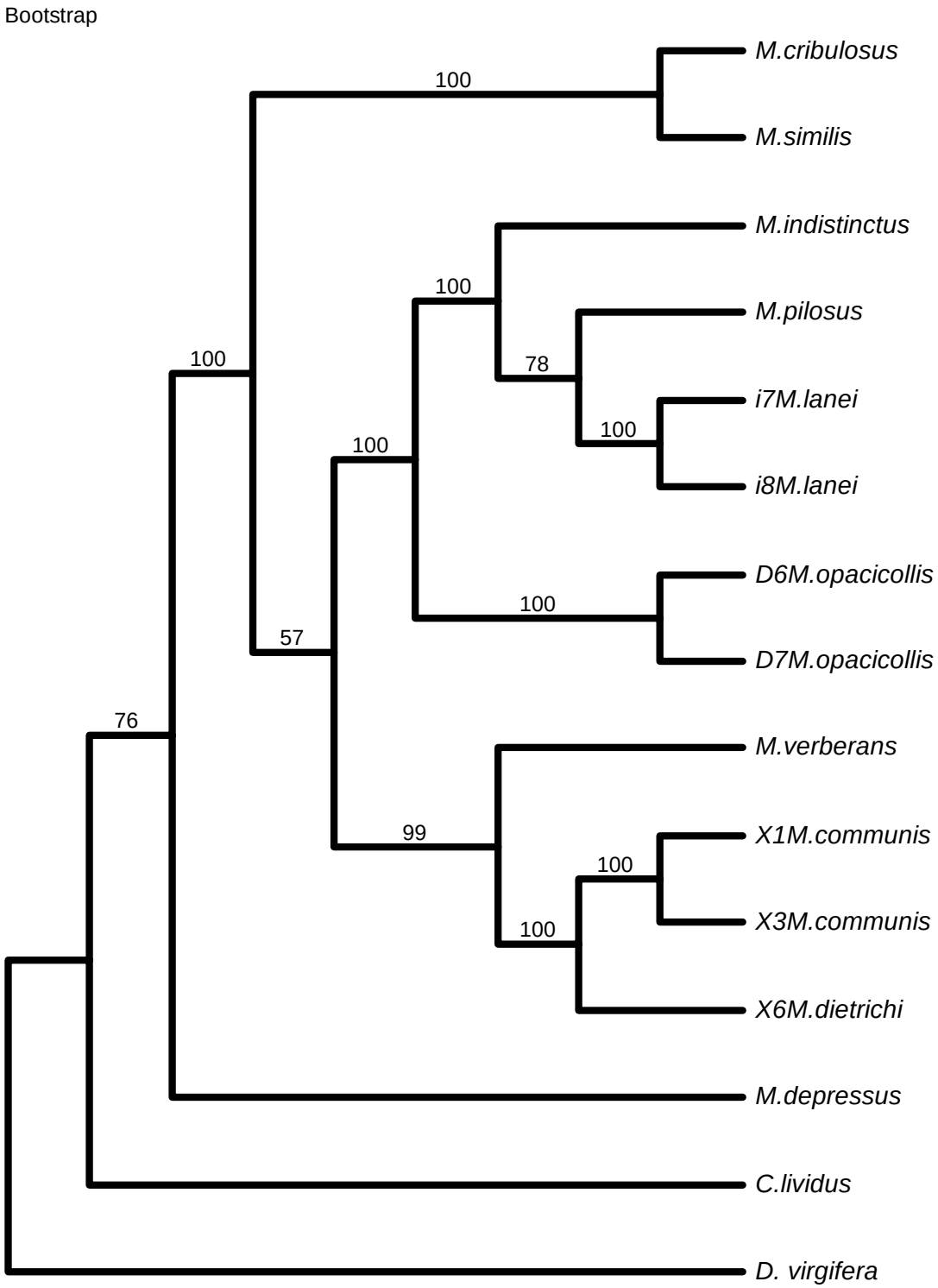

Figure 3. Minimum evolution tree. Consensus sequences. Uncorrected p-distances. neighbor joining, 1000 rep bootstrap, ME-score = 0.87258.

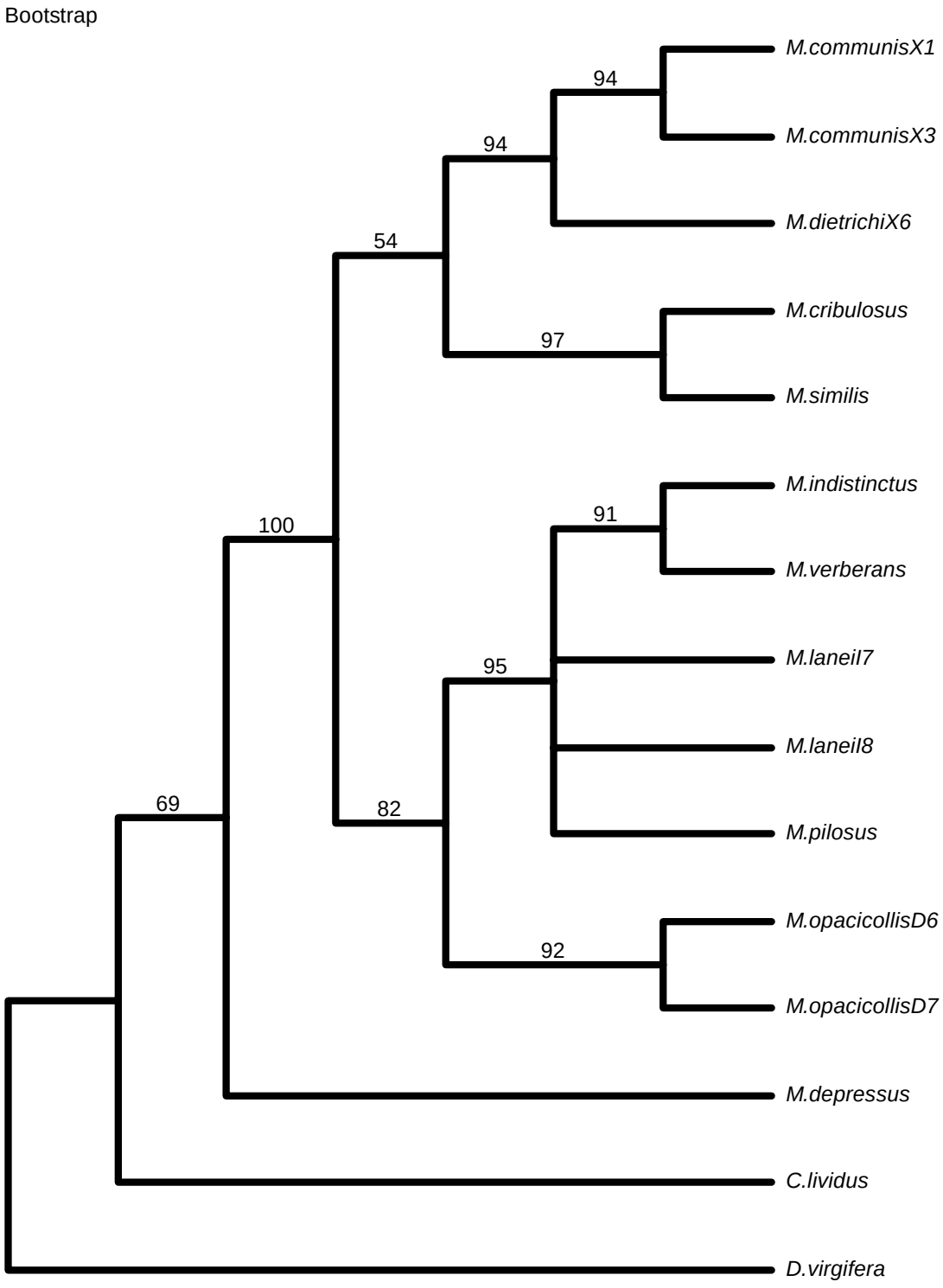

Figure 4. Maximum likelihood tree. GTR + G model, -Ln = 6204.18440. 1000 replicate bootstrap, heuristic search.

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Chapter III: *M. depressus* Population Genetics

Introduction

Wireworms are a sporadic but important pest of field crops throughout the Midwestern United States. Fifteen species of wireworms in six genera are associated with damage to corn and other field crops in the Midwest (Riley and Keaster 1979, 1983, 1981). Wireworms can damage corn in several different ways. Larvae may attack the seed, eating the germ and reducing the remainder to mere fragments (Kulash and Monroe 1955, Munson et al 1986). Damage of this type results in the corn failing to germinate and may necessitate replanting (Cheshire, et al. 1987). Wireworms may also bore into the underground portion of the stem or eat the roots of the plant (Simmons, et al., 1998). Damage of this type results in withering and structural instability (Munson et al 1986, Kulash and Monroe 1955). In some cases up to 35% of a crop might be lost due to wireworm damage (Apablaza et al 1977).

Melanotus is the most common genus as well as the most destructive (Riley and Keaster 1981). Ten species of *Melanotus* are known to be associated with corn, while just one species in each of the other five genera is known to be economically important (Riley and Keaster 1979, 1981). The most economically important species in Missouri are *Melanotus depressus* (Melsheimer), *Melanotus verberans* (LeConte), *Melanotus lanei* (Quate), and *Melanotus opacicollis* (LeConte) (Riley and Keaster 1981). *M. depressus* is also one of the most commonly encountered species in the Midwest.

Although *M. depressus* is widely distributed, click beetles have not been observed to migrate long distances (Brown and Keaster 1986). Adults emerge in late spring and early summer and are active for about a month, with males emerging prior to females

(Brown and Keaster 1986, Doane 1961). Data from pitfall and flight traps suggest that males exhibit more flight activity than females, indicating that females do not travel far to find oviposition sites (Brown and Keaster 1986, Doane 1961). Females exhibit very little flight activity, suggesting that infestation into adjoining areas may take quite some time (Brown and Keaster 1986). If the dispersal rate of *M. depressus* is low, it is reasonable to assume that there is relatively low gene flow among populations (Avise 2004).

The mitochondrial genome has recently been shown to be useful in determining the genetic structure of some insect species. Vandyke et al (2004) used the COI and COII regions of the mitochondrial genome to examine the population dynamics of the montane grasshopper, *Melanoplus alpinus* Scudder. The study clarified the evolutionary mechanisms that were active on the genus *Melanoplus* during the Pleistocene. The COI gene was also used to determine the distribution of morphologically indistinguishable biotypes of the sweetpotato whitefly *Bemisia tabaci* Gennadius (Hsieh et al 2006). This sort of study can be used to determine the frequency and time of invasion events of pests into new areas.

Understanding the population dynamics of pest populations is important for control efforts. Beyond studies that examine the biology and ecology of *M. depressus,* it is also important to study the gene flow and population structure of this economically important species. Here we attempt to elucidate the genetic structure of *M. depressus* populations in the Midwest.

Materials and Methods

Sampling and Identification

Specimens were collected from 12 locations in the Midwestern United States (Table 1). Samples were obtained by digging in fields with known wireworm populations and by placing traps according to the methods of Ward and Keaster (1977). Extension entomologists and farmers enabled us obtain samples from distant locations. Identifications were made using available dichotomous keys (Riley and Keaster 1981 and Riley 1983) and museum specimens previously identified by T.J. Riley. Adult and larval reference specimens were obtained from the Enns Entomology Museum at the University of Missouri. Samples were stored in 95% ethanol or at -80° C.

DNA Collection and Analysis

DNA was extracted from wireworms using the Quiagen DNeasy Tissue Kit (QIAGEN, Valencia, CA). Extraction was performed on the abdomen; the anterior and posterior sections of the larvae were preserved at -80°C as a reference. Portions of the mtDNA cytochrome oxidase subunit I (COI) gene were amplified via PCR using the primers C1-J-1718 (5' GGAGGATTTGGAAATTGATTAGTTCC 3'), C1-N-2191 (5' CCCGGTAAAATTAAAATATAACTTC 3'), C1-J-2183 (5' CAACATTTATTTTGATTTTTTGG 3'), and TL2-N-3014 (5' TCCAATGCACTAATCTGCCATATTA 3') from Simon et al (1994).

30 µl PCR reactions were performed with 10 mM dNTPs, 5 U/µl Amplitaq, 25 mM $MgCl_2$, 10X PCR buffer, 20mM sense and antisense primers (all reagents Applied Biosystems, Foster City, CA), extracted DNA, and water to volume. A PCR cycle of 60 s at 94°C, 30 s at 52°C, and 90 s at 72°C was repeated 35 times in a GeneAmp 2700 PCR

System (Applied Biosystems, Foster City, CA). The PCR product was then purified using the Qiaquick PCR Purification Kit (QIAGEN, Valencia, CA). Samples were sequenced at the University of Arkansas Department of Microbiology and Immunology DNA Sequencing Core Facility (Little Rock, AR) using an ABI 377 sequencer.

Sequences were edited and aligned using BioEdit 7.0.5.2 (Hall, 1999) and MacClade 4.06 (Maddison and Maddison 2005). CLC Free Workbench 2.5.2 (www.clcbio.com) was used to translate DNA sequences to protein sequences. To ascertain genetic structure, populations were grouped based on location – northern, southern, eastern, and western. Haplotype frequency and AMOVA analyses were performed using Arlequin 3.0, and significance was estimated with 1,023 permutations (Excoffier et al 2005). Tree construction and phylogenetic analyses were performed using PAUP 4.0b10 (Swofford 2003) and MEGA 3.1 (Kumar et al 2004).

Results

M. depressus specimens were collected from 12 different locations in the Midwest. DNA was extracted from the COI gene of 55 individuals and the amplicon was1343 bp in length. Nucleotide base frequencies were biased towards A and T, with A at 29.9%, T at 34.64%, C at 19.76%, and G at 15.67%. The proportion of genetic distance between haplotypes was an average of 0.01, while the difference between all haplotypes and the outgroup, *C. lividus*, was 0.16. Analysis revealed seven haplotypes within the 12 populations sampled. Haplotypes 1, 2, and 3 were the most commonly encountered haplotypes among all locations. Haplotype frequencies among all locations are detailed in Table 3. AMOVA analysis of haplotypes and locations revealed significant genetic structure. There was significant genetic variation among groups, within populations, and

among populations within groups. There was more variation within populations (71.26%) and among populations within groups (25.24%) than between groups (3.50%). The corresponding fixation indices also indicated a high level of genetic variation, with FSC = 0.26158, FST = 0.28745, and FCT = 0.03503. FSC and FST were significant ($p > .25$) while FCT was not. The lowest amount of variation was once again between groups.

Of the 1343 bp sequenced, 281 characters were parsimony informative. Parsimony analysis of genetic data from the 12 locations revealed three most parsimonious trees. The bootstrap consensus tree (Fig. 2) was constructed using parsimony analysis and differs from the strict consensus of the three trees only in that location 12 is shown branching independently instead of directly basal to the clade containing location 9. Minimum evolution analysis (Fig. 3) differs from parsimony analysis in the relationship between locations 11 and 6 and does not clarify the relationship among locations 1, 4, and 5.

Discussion

High genetic variability among sympatric populations is not unexpected, as the behavior and flight activity (Brown and Keaster 1986) indicate that there is little genetic exchange among populations of *M. depressus*. Observations of elaterid pests of other genera as adults (Doane 1961) utilized pitfall traps to collect adult wireworms, indicating little, if any, flight activity and thus very low dispersal rate. The slow dispersal rate of wireworms to new areas may also be attributed to their relatively long life cycle (Vernon et al 2001). Avise (2004) suggests that species with a low dispersal rate will have a greater amount of population genetic structure than species with high dispersal rates, and that this is especially likely in species in which the females do not disperse.

Although the natural dispersal rate based on behavioral studies appears to be very low, there are several haplotypes that are relatively wide spread. For example, deph7 is present in Missouri, Iowa, and Wisconsin. Other species with low dispersal rates and high genetic structure such as aphids have been shown to move great distances through environmental events, causing more genetic variability within populations than between populations (Serikawa et al 2007). Wireworms are known to survive in anaerobic conditions, such as flooding, for periods of several weeks (Hall and Cherry 1993). The survival period is even longer when temperatures are cool (Hall and Cherry 1993), such as during spring flooding. It is possible that wireworm populations in floodplains have been carried long distances by floods, much as aphids are carried long distances by storms. Wireworm species are also known to have been introduced to North America from Europe through the importation of the soil that occasionally accompanies crops (Vernon et al 2001). It is possible that *M. depressus* is transported across state lines in a similar manner, causing certain haplotypes to be widely distributed.

The data presented here is an initial view of *M. depressus* populations in the Midwestern United States, and is the first study of this kind on wireworm pest populations. It would be advantageous in further studies to collect from a wider variety of locations and perhaps employ additional genes or markers, such as microsatellites. Population studies have been able to elucidate insecticide-resistant biotypes in other pest species (Khasdan et al 2005), and future studies should examine whether different wireworm populations have different levels of susceptibility to common control methods such as seed treatments. Future studies should also examine whether wireworm

populations in floodplains exhibit greater diversity and gene flow than populations in upland areas.

Table 1. Locations where *M. depressus* larvae were collected.

Location	County/State	N	Date Collected	Collector
1. MU Dairy Farm	Boone Co., MO	2	June-July 2005	E. Lindroth, F. Lloyd
2. Higginsville	Lafayette Co., MO	4	May 2005	T. Clark
3. Cairo	Randolph Co., MO	8	May 2005	L. Brush
4. Carlyle,	Clinton Co., IL	8	May 2005	L. Loehr
5. Kirksville	Adair Co., MO	8	June 2005	L. Brush
6. Emerson	Marion Co., MO	9	May 2005	E. Lindroth
7. Plum City	Pepin Co., WI	1	May 2005	G. Andrews, G. Kerr
8. Marshall	Saline Co., MO	1	June 2005	T. Clark
9. Callaway	Custer Co., NE	8	June 2005	G. Hein
10. Horton	Brown Co., KS	1	May 2005	G. Wilde
11. Muscatine	Muscatine Co., IA	4	June 2004	D. Moellenbeck
12. Jacksonville	Randolph Co., MO	1	May 2005	E. Lindroth, L. Meihls

Table 2. GenBank accession numbers for *M. depressus* haplotypes.

M. depressus Haplotype	GenBank Accession Number
Deph1	EF546384
Deph2	EF546385
Deph3	EF546386
Deph4	EF546387
Deph5	EF546388
Deph6	EF546389
Deph7	EF546390

Table 3. Haplotypic frequencies within the populations at 12 locations.

	Boone Co., MO	Higginsville MO, Lafayette Co.	Cairo MO, Randolph Co.	Carlyle IL, Clinton Co.	Adair Co., MO	Marion Co., MO	Pepin Co., WI	Marshall MO, Saline Co.	Callaway Co., NE	Horton KS, Brown Co.	Muscatine IA, Muscatine Co.	Jacksonville MO, Randolph Co.
Deph1	1	0	0	0.667	0.625	0.222	0	1	0.5	1	0	0
Deph2	0	0	0.5	0.111	0	0	0	0	0.25	0	0.5	1
Deph3	0	0	0	0.111	0.25	0.556	0	0	0	0	0.25	0
Deph4	0	0	0	0	0.125	0	0	0	0.25	0	0	0
Deph5	0	0	0	0.111	0	0	0	0	0	0	0	0
Deph6	0	0	0.5	0	0	0.222	0	0	0	0	0	0
Deph7	0	1	0	0	0	0	1	0	0	0	0.25	0

Table 4. Population structure for AMOVA analysis.

AMOVA groups	Locations within group
North	7, 11
Central Missouri	1,3,12,8
East	5,6,4
West	2,9,10

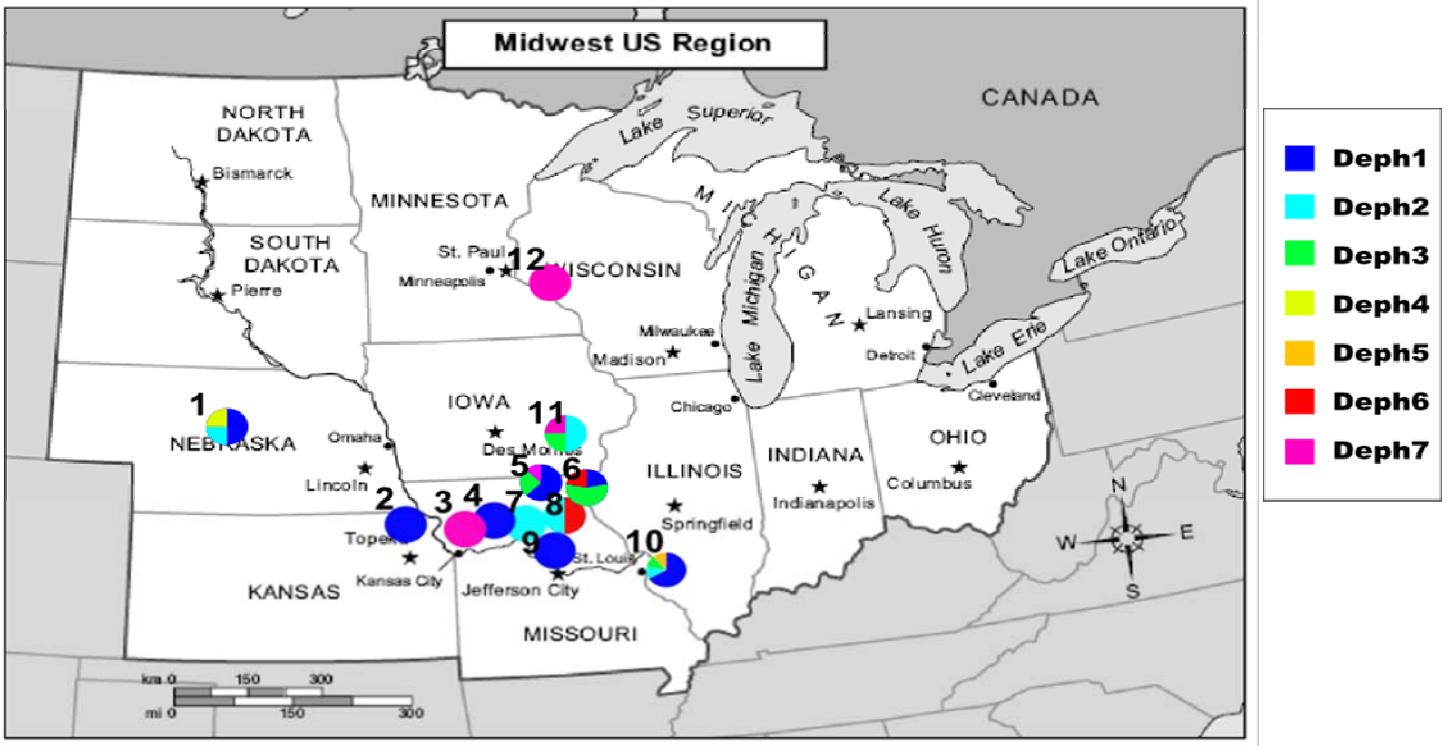

Figure 1. Haplotype map depicting haplotype frequencies across the Midwest.

Table 5. AMOVA comparing genetic differentiation among groups. Significance of $p < 0.05$, significance for fixation indices $p > .25$.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation Indices
Among groups	3	3.198	0.01139 Va	3.50	FCT = 0.03503
Among populations within groups	8	5.642	0.10366 Vb	25.24	FSC = 0.26158
Within populations	44	12.875	0.29261 Vc	71.26	FST = 0.28745
Total	55	21.714	0.41065		

Significance tests:

Vc and FST : P(rand. value < obs. value) = 0.00000
P(rand. value = obs. value) = 0.00000
P(rand. value <= obs. value) = 0.00000+-0.00000

Vb and FSC : P(rand. value > obs. value) = 0.00000
P(rand. value = obs. value) = 0.00000
P(rand. value >= obs. value) = 0.00000+-0.00000

Va and FCT : P(rand. value > obs. value) = 0.28837
P(rand. value = obs. value) = 0.00000
P(rand. value >= obs. value) = 0.28837+-0.0122

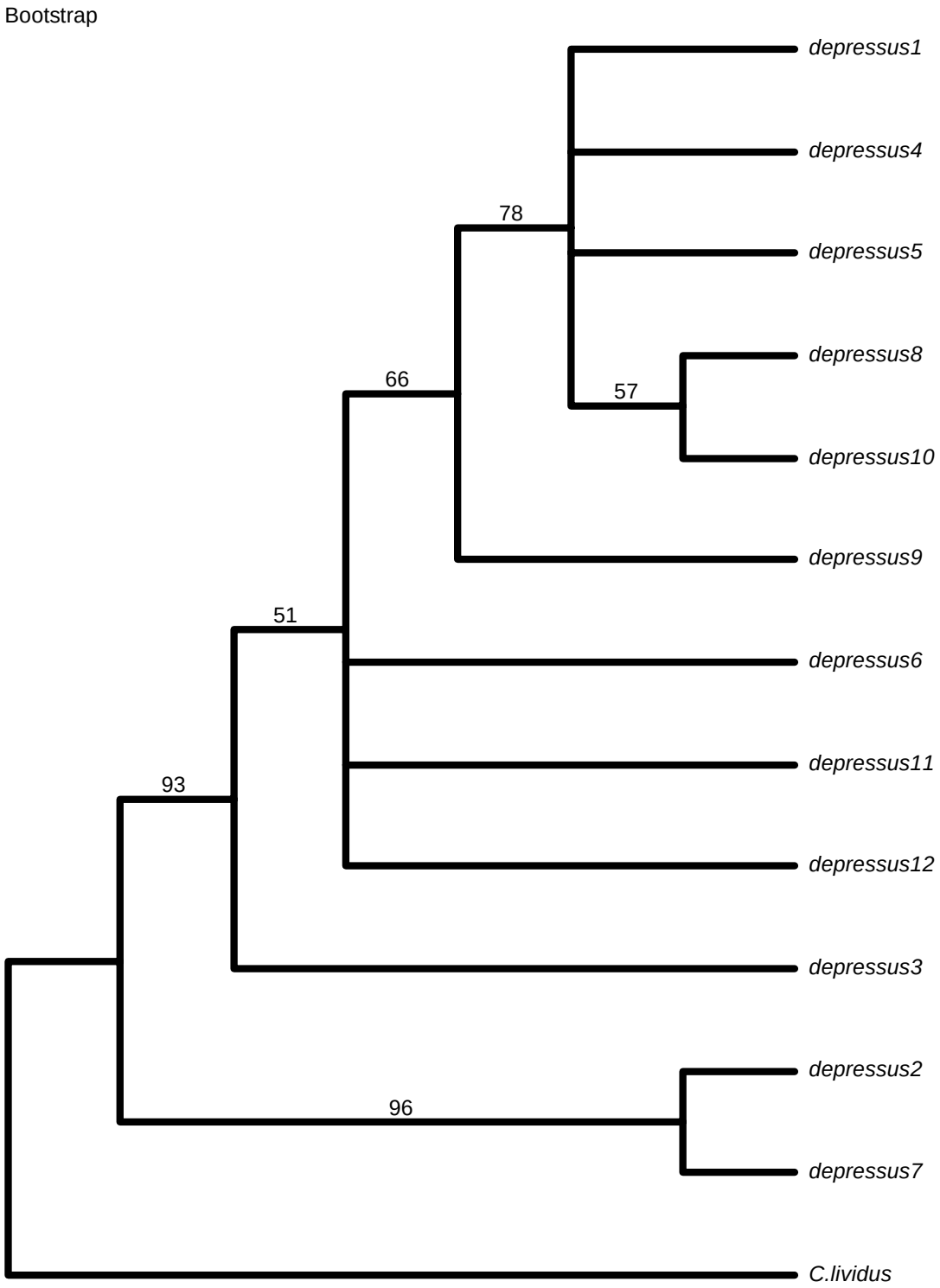

Figure 2. Maximum parsimony tree. Consensus sequences. 1000 replicate bootstrap. Tree length = 269, CI = 0.952, RI = 0.776.

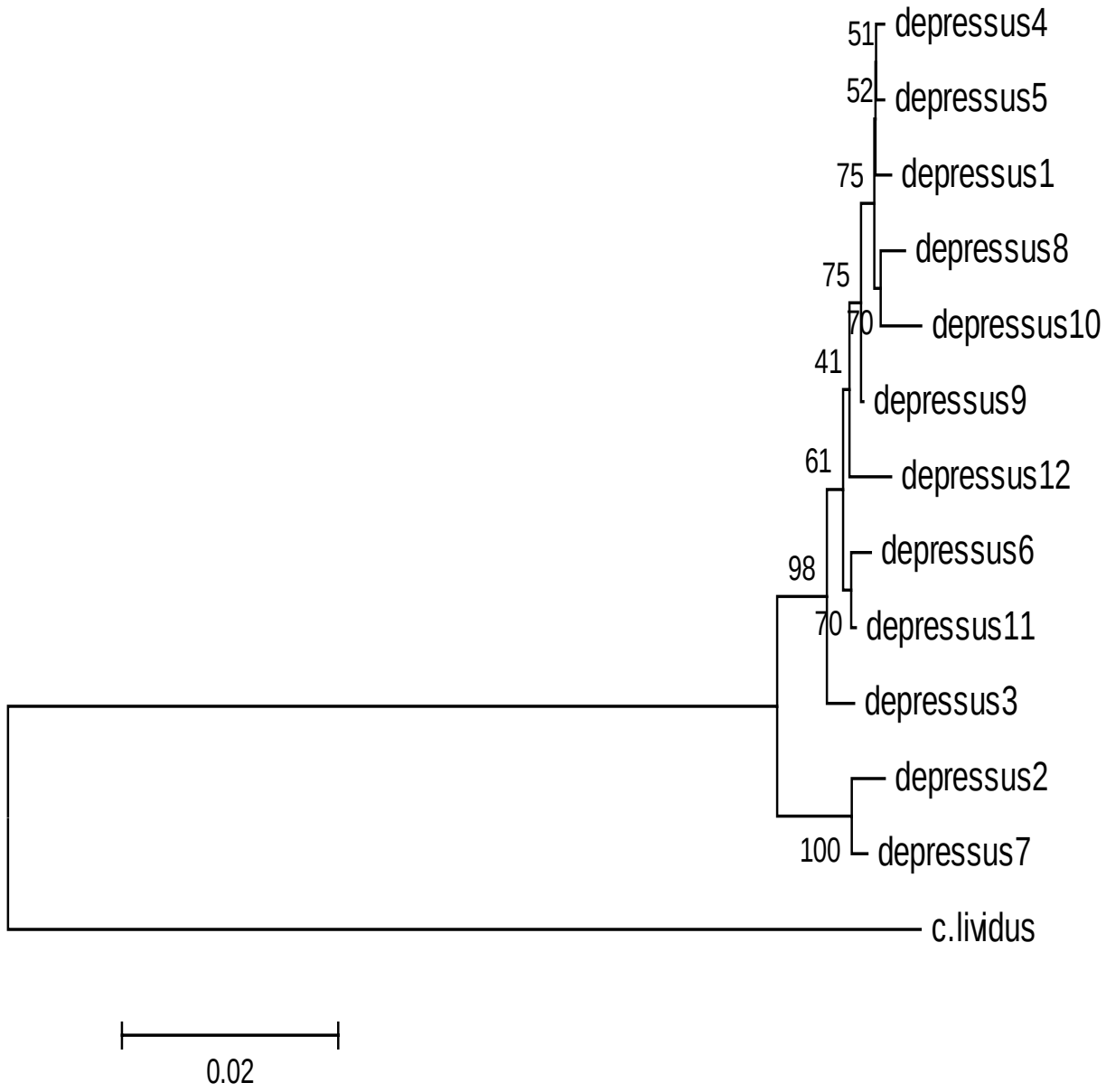

Figure 3. Minimum evolution tree. Consensus sequences. Uncorrected *p*-distances. 1000 replicate bootstrap, neighbor joining.

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