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Population genetics of Thomomydoecus minor: Ectoparasitic chewing lice on Thomomys bottae pocket gophers of the Rio Grande Valley in New Mexico

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POPULATION GENETICS OF *THOMOMYDOECUS MINOR*; ECTOPARASITIC CHEWING LICE ON *THOMOMYS BOTTAE* POCKET GOPHERS OF THE RIO GRANDE VALLEY IN NEW MEXICO

An Abstract of a Thesis

Submitted

in Partial Fulfillment

of the Requirements for the Degree

Master of Science

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ABSTRACT

Thomomydoecus minor is an ectoparasitic chewing louse that lives in intimate association with the pocket gopher *Thomomys bottae*. Chewing lice are wingless, obligate, host specific parasites that spend their entire life cycle on the fur of their host. Pocket gophers are fossorial and asocial, cut off for most of their lives even from members of their own species. Thus, the life histories of both chewing lice and pocket gophers have been predicted to limit transmission of lice from host to host, thus limiting gene flow among louse infrapopulations found on different hosts and increasing the effect of louse population bottlenecks that occur when lice colonize new host individuals. The geographic location of special interest in this study was a section of the Rio Grande Valley in New Mexico called the San Acacia constriction. This is a zone of secondary contact where two subspecies of pocket gophers meet and hybridize to a limited extent. Restricted hybridization between these hosts was predicted to influence genetic structure of the corresponding louse populations.

In the present study, genomic DNA was isolated from 118 chewing lice collected in 2011 from 3 localities surrounding the San Acacia constriction and from 39 samples of lice collected in 1992 from one of the same localities. A portion of the mitochondrial cytochrome-c oxidase subunit I (COI) gene was sequenced and used to construct a phylogenetic tree, which indicated two distinct haplotypes, with one of these occurring north of the host hybrid zone and the other occurring south of it. These two haplotypes likely diverged 78,000-200,000 years ago. Haplotype distribution coincides with the geographic break in suitable pocket gopher habitat imposed by the San Acacia

constriction, indicating that either geography or limits on host hybridization in this region cause an impediment to gene flow between northern and southern chewing louse populations. Eight novel microsatellite loci developed for this study revealed greater levels of genetic variation than were available in previous studies of chewing louse populations, which relied on allozymes. Like the mtDNA data, microsatellite data supported a distinct separation between northern and southern louse populations coincident with geography. Furthermore, distinct infrapopulations on different host individuals were detected in microsatellite genetic distance measures and AMOVA analyses, thus supporting previous predictions of louse population subdivision resulting from a life history whereby host pocket gophers serve as isolated islands of habitat for chewing lice with horizontal transmission of lice between unrelated hosts being relatively rare. Contrary to previous predictions, louse populations appeared to be in Hardy-Weinberg Equilibrium and showed little or no evidence of population bottlenecks or inbreeding. Despite a life history that has been thought to impose frequent bottlenecks on chewing louse populations, relatively stable genetic diversity was maintained over a 19.5-year, 175-generation time span between collection dates at the same sampling locality.

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has been approved as meeting the thesis requirement for the

Degree of Master of Science

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CHAPTER 1

INTRODUCTION

The Importance of Parasites

The field of parasitology is predominantly concerned with the study of symbiosis, which refers to a relationship in which two organisms live in intimate association, usually with one organism living in or on the body of another organism (Schmidt and Roberts 1989). According to classical definitions, a parasite is a symbiont that lives at the expense of its host causing harm in any number of ways (Schmidt and Roberts 1989). However, some parisitologists have pointed out problems with this definition since harm is relative and often difficult to quantify (Esch and Fernández 1993). There are many cases of symbiosis in nature that do not involve clear harm to the host but that are typically regarded as parasitism (Schmidt and Roberts 1989).

Parasitism is an extremely successful mode of life having evolved independently many times. By conservative estimate, nearly half of all living animals could be considered parasites (Price 1980). However, parasites have received comparatively little attention from biologists relative to free-living forms.

The constraint of being tied to another living organism for survival adds a layer of complexity to symbiont population structure that is not seen in free-living organisms. For this reason, the field of parasitology requires the definition of a unit of population structure not required by other disciplines in biology. The term “infrapopulation” describes all of the parasite individuals of a species on an individual host (Esch et al. 1975). Transmission of parasites among hosts (i.e., from one infrapopulation to another)

may be considered horizontal if it involves transmission between unrelated hosts or vertical if it involves transmission from parent to offspring (Stewart et al. 2005). Horizontal transmission would make inbreeding effective population size very large in parasites relative to vertical transmission. Additionally, mode of transmission is thought to impact the virulence of parasites, with vertical transmission potentially imposing a stronger advantage to parasites with lower virulence so these parasites don't kill their host before being transferred to a new host (Lipstich et al. 1996; Stewart et al. 2005).

Mode of transmission of parasites among hosts and degree of host specificity displayed by the parasite both can influence gene flow among parasite infrapopulations and populations, which has at least three important ramifications. First, restricted gene flow among parasite populations can lead to an especially intimate association between host and parasite, which could allow the parasite to share a macroevolutionary history with its host. This macroevolutionary pattern is reflected in Fahrenholz's rule which states "…the natural classification of some groups of parasites corresponds with that of their hosts" (Eichler 1948: 588), a pattern often referred to as cophylogeny or cospeciation. Second, gene flow among parasite populations and genetic drift within each individual infrapopulation also can influence the ability of a parasite to adapt to its local environment. Finally, small effective population (N_e) size and vertical transmission can initiate founder effect speciation, while large N_e could allow natural selection to cause adaptive or ecological speciation (Huyse et al. 2005). Therefore, an understanding of parasite population genetics is relevant to studies of speciation, cospeciation, and adaptation, yet parasite systems are critically understudied at the genetic level.

The Ectoparasitic Chewing Lice of Pocket Gophers

Ancient Association

One host-parasite system that has been studied extensively is pocket gophers and their ectoparasitic chewing lice. Pocket gopher and chewing louse phylogenies are more similar to one another than would be expected by chance, indicating a long history of association between these lineages (Demastes and Hafner 1993; Hafner et al. 1994; Hughes et al. 2007; Demastes et al. 2011). Although congruence between these phylogenies is not perfect, the pocket gopher-chewing louse system has been referred to as a textbook example of cospeciation (Esch and Fernández 1993; Page and Holmes 1998). The degree of phylogenic congruence displayed by pocket gophers and their lice is greater than that seen in other host-parasite systems such as birds and lice, and this congruence is likely the result of unique biological features of both pocket gophers and chewing lice (Clayton et al. 2004). Importantly, many of these same life history traits would be expected to impact the population genetics of these parasites.

Pocket Gophers

Pocket gophers (Rodentia: Geomyidae) are named for the fur lined pouches outside the mouth that are used to store food (Hall 1981). They are fossorial mammals that spend their entire lives in closed burrow systems. Features that have made them well suited to a subterranean lifestyle include reduced eyes and ears and powerful forelimbs and incisors used in digging (Hall 1981). Pocket gophers are restricted to friable soils with sufficient depth and food resources. The soil must be porous to allow for gas exchange, contain enough moisture to maintain the integrity of tunnel systems, and have sufficient depth to

aid in temperature regulation. Patch size and available resources typically determine population size. Because of these stringent requirements, pocket gophers occur in genetically distinct groups with patchy distributions (Hall 1981). Pocket gophers are asocial; multiple burrow occupancy happens only during the reproductive period allowing for brief contact during mating and while the mother nurses her young (Hall 1981).

Pocket gophers exhibit a strictly New World distribution from Canada to northern Columbia, and species distributions are largely parapatric or allopatric (Hall 1981). Six genera, 35 species, and more than 400 subspecies are included in the family (Hall 1981). All extant taxa are members of the subfamily Geomyinae, which contains two tribes Geomyini and Thomomyini. The Thomomyini tribe is made up of seven species of the genus *Thomomys*. One species of *Thomomys*, *T. bottae*, is the host of the chewing lice that are the focus of this study.

Chewing Lice

Chewing lice of pocket gophers are obligate parasites that live their entire life cycle in the fur of their host. They feed on skin detritus and apparently cause little, if any, harm to their host (Rust 1974). For this reason, these obligate symbionts could be considered relatively harmless parasites, or they could be considered commensals. Generation time is 40 ± 6 days with an average life span of approximately 30 days (Rust 1974). Chewing lice have several characteristics that seem well suited for a subterranean existence, including being eyeless, wingless, and having specialized antennal sensory organs. Chewing lice also have a well-developed head groove that is used in attachment to the

host and that may play a role in host specificity (Reed and Hafner 1997). Many of these traits would be expected to reduce dispersal ability. Therefore, transmission of lice is thought to be primarily vertical, from mother to offspring (Rust 1974), with horizontal transmission occurring at times, possibly with the direct host-to-host contact that occurs during mating (Demastes et al. 1998).

There are 122 species and subspecies of pocket gopher-dependent chewing lice, all belonging to the genera *Gemoydoecus* or *Thomomydoecus* (Mallophaga: Trichodectidae; Hellenthal and Price 1991; Page et al. 1995; Demastes et al. 2011). *Thomomydoecus* was elevated to generic status by Hellenthal and Price in 1984, an arrangement that was supported by Nadler and Hafner (1989), because these taxa exhibit genetic divergence typical of different genera of insects. The 17 recognized species of *Thomomydoecus* chewing lice are found only on pocket gophers in the genus *Thomomys* (Price and Hellenthal 1980). *Thomomydoecus* lice often co-occur on the same host individuals with *Geomydoecus* lice (Hellenthal and Price 1984). *Thomomydoecus* are more slender with a tapered body and are generally smaller than *Geomydoecus* (Hellenthal and Price 1991). One species of *Thomomydoecus* (*T. minor*) is the focus of this thesis.

Chewing Louse Population Genetics

Previous Research

Despite the attention given to cospeciation between pocket gophers and chewing lice, the fine scale population genetics of chewing louse populations that may allow cospeciation has been the subject of only two studies (Nadler and Hafner 1989; Nadler et al. 1990). Both of these studies used allozymes to examine genetics of lice. Data

representing four species of chewing lice from the genera *Geomydoecus* and *Thomomydoecus* indicated that these genera are, indeed, distinct genera with genetic divergence values similar to those seen between other insect genera (Nadler and Hafner 1989). Within species, allozymes showed limited polymorphism. Only two of eleven allozyme loci examined showed any genetic variation within *T. minor*, and two of the five infrapopulations were monomorphic for all loci (Nadler and Hafner 1989). Intraspecific variation was similarly limited for *Geomydoecus actuosi* populations examined (Nadler and Hafner 1989). However, sufficient genetic variation among individuals of *G. actuosi* exists to show that infrapopulations are subdivided from one another, even at the same locality, as evidenced by high and significant F_{ST} values between lice on different hosts ($F_{ST} = 0.039 – 0.162$; Nadler et al. 1990). Between-locality F_{ST} for *G. actuosi* was even higher ($F_{ST} = 0.24$), indicative of limited gene flow between lice at different localities. This result was similar to the population subdivision observed between pocket gopher host populations ($F_{ST} = 0.236$). Therefore, Nadler et al. (1990) proposed that louse gene flow is limited by gopher gene flow and cannot exceed gene flow of the host, but rather, must lag behind that of their host. Because *G. actuosi* louse infrapopulations exhibit low levels of population heterozygosity compared to other insect species, Nadler et al. (1990) suggested that founder events at initial host colonization and the seasonal population bottlenecks in louse populations observed by Rust (1974) both may serve to decrease genetic diversity in chewing lice.

Available Tools for Population Genetics

Molecular markers with different rates of substitution will capture population processes at different depths of evolutionary history (Wang 2010; Diniz-Filho et al. 2008). Since different types of data are only informative over specific temporal scales, it is important to apply the appropriate methods of analysis for the data being examined and the type of question(s) being addressed. Common types of data that have been used in population studies include allozymes, mitochondrial DNA (mtDNA) sequence variation, and microsatellite polymorphisms (Parker et al. 1998). There is a 10^4-fold difference in mutational rate between mtDNA and microsatellite DNA, with microsatellite DNA having the faster mutational rate; therefore, mtDNA is excellent for capturing molecular signatures of historical processes, and microsatellites give resolution to recent and ongoing processes on microevolutionary scales (Wang 2010).

Mitochondrial DNA haplotype sequences were examined in this research as a tool for identification of historical isolation in populations and to aid in estimation of time since divergence of those populations. Mitochondrial genes are located on a circular chromosome, which is inherited maternally. These genes contain no introns and have an accelerated rate of mutation compared to nuclear genes (Moritz and Brown 1987). The cytochrome-c oxidase subunit I (COI) gene of the mtDNA was chosen for amplification and sequence comparison for this study. The COI gene has often been used in research as a model gene (Lunt et al. 1996) because it is one of the most conservative protein-coding genes in the mitochondrial genome of animals (Folmer et al. 1994). The COI gene commonly is used to identify insect species, and it has been shown to be extremely

informative in population genetics and phylogenetic analysis (Folmer et al. 1994; Virgilio et al. 2010).

In the past twenty years, evidence of exceptions to maternal inheritance of mtDNA has accumulated (White et al. 2008). Heteroplasmy refers to the condition in which more than one mtDNA variant is found within a cell, tissue, or individual. Paternal leakage, which is the transmission of a paternal mtDNA genome along with the maternal mtDNA genome, is the cause of heteroplasmy, which is not entirely uncommon among insects (Harrison et al. 1985; Kvist et al. 2003; White et al. 2008). Heteroplasmy is increasingly being seen as an important contributing factor to genetic diversity in eukaryotes (Goto et al. 2011). Heteroplasmic mtDNA genome size variants have been observed in the hybrid offspring of two closely related species of crickets (Harrison et al. 1985). Heteroplasmy in this case was heritable; ten offspring of a heteroplasmic female all were found to be heteroplasmic as well. Once heteroplasmy is established in a lineage, it can take up to 500 generations to return to homoplasmy (White et al. 2008). Therefore, in the absence of multigenerational data, it is difficult to determine whether a heteroplasmic individual was the result of paternal leakage or if it has inherited heteroplasmy from previous generations (Kvist et al. 2003).

Microsatellite regions of the nuclear genome are also known as simple sequence repeats (SSRs) or short tandem repeats (STRs). These loci have proven useful in numerous studies of recent gene flow and population diversity owing to their high level of polymorphism (e.g., Roberts et al. 2004; Tapio et al. 2010; Caballero et al. 2011). These loci consist of nucleotide units that are from two to six nucleotides long, which are

repeated a variable number of times in different individuals (Goldstein and Polluck 1997; Ellengren 2004; Guichoux et al. 2011). The high polymorphism characteristic of many microsatellite regions within a population is caused by selective neutrality and a relatively high mutation rate that is two to three orders of magnitude higher than that of allozymes (Jarne and Lagoda 1996). Mutations in microsatellites may arise due to a replication slippage mechanism that results in insertion and/or deletion of repeat sequences relative to the template strand (Ellengren 2004). It is hypothesized that loci with perfect repeats result in higher polymorphism than do loci at imperfect repeats as a result of interference with slipped-strand mispairing by imperfect repeats, which increases their stability (Jeffreys et al. 1988).

Microsatellite loci are typically flanked by more stable sequences for which primers are designed to bind and assist in amplification by polymerase chain reaction (PCR). Once primers have been developed, it is important to test target loci for variation among individuals and for reliability. Undetected genotyping errors can lead to inaccurate allele frequencies that result in a deviation from Hardy-Weinberg Equilibrium (HWE), mis-assignment of population substructure, and over-estimation of inbreeding (Bonin et al. 2004). Microsatellite analyses are prone to problems resulting from allelic dropout, false alleles, possible contamination, or human error (Bonin et al. 2004). Given the potential for these problems, 5-10% of a microsatellite data set should be re-screened to check for accuracy of genotyping (Bonin et al. 2004; Pompanon et al. 2005; DeWoody et al. 2006); however only 26% of the studies surveyed by Guichoux et al. (2011) included an attempt to quantify genotyping errors. Other quality control measures include evaluation of blind

samples, use of standard laboratory protocols, use of negative controls for contamination screening, favoring of automatic scoring over scoring by hand, elimination of low quality DNA samples and suspicious markers, and analysis of error rates with a report of these findings in the final work (Bonin et al. 2004).

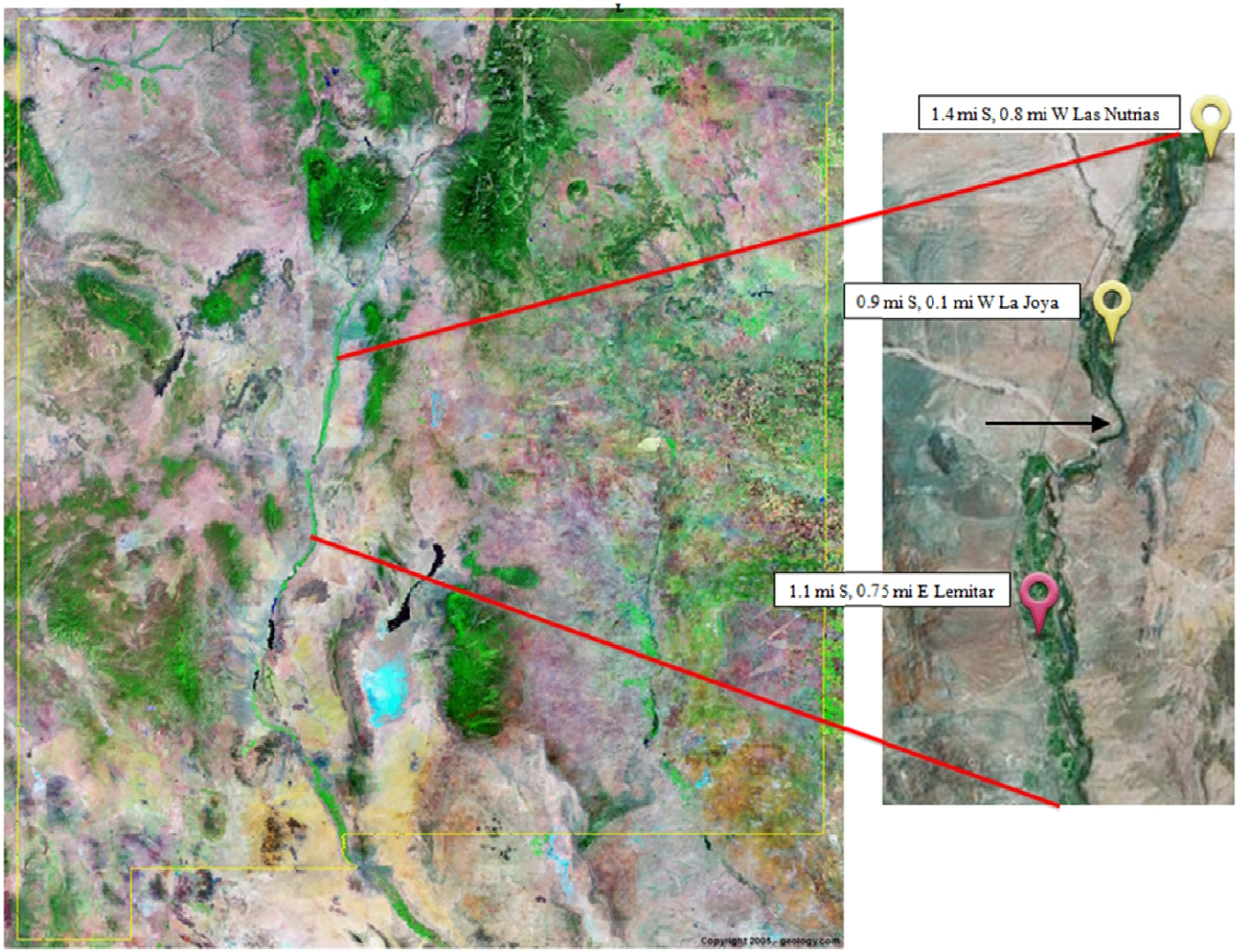
Specific Aims

The system of special interest in this research lies along the Rio Grande Valley of New Mexico with particular focus placed on a location 23 km north of Socorro in Central New Mexico (Figure 1). This area has been referred to as the San Acacia constriction for its proximity to the town of San Acacia, New Mexico (Hafner et al. 1998). This region of the Rio Grande Valley is only 300 meters wide compared to 15 km in width near Albuquerque. It is surrounded largely by open desert and provides limited suitable habitat for pocket gophers, resulting in an exceptionally patchy distribution of pocket gophers at the constriction (Smith et al. 1983). At this site, there is limited overlap and hybridization between two pocket gophers, *T. b. connectens* and *T. b. opulentus*, which come in from north and from south of this region, respectively (Smith et al. 1983). These two subspecies of gophers show only a 69% allozyme-based genetic similarity, and are easily distinguished by a sharp discontinuity in hind foot length, body size, pelage color, and number of bi-armed autosomes (Smith et al. 1983). Despite the genetic, morphological, and karyotypic differences displayed by these subspecies, Smith et al. (1983) regarded these gophers as members of the same species given what they thought was ongoing hybridization as evidenced by shared allozyme alleles.

The overarching goal of this study was to expand knowledge of chewing louse population genetics by examining populations of *T. minor* inhabiting the two subspecies of pocket gophers, *T. b. connectens* and *T. b. opulentus*, which come into contact and hybridize near San Acacia, New Mexico. Achievement of this goal required fine-scale genetic data, which previously have not been available for chewing lice. Therefore, an important first step in this study was the development of new microsatellite markers for *T. minor*.

The first objective of this study was to determine the degree of mitochondrial DNA and nuclear genetic variation in *T. minor* at sites spanning the San Acacia constriction to determine if lice from different subspecies of pocket gophers are genetically different from one another. Based on current taxonomy, there is no reason to suspect genetic differences between lice north and south of the San Acacia constriction (Hellenthal and Price 1991). However, given that pocket gopher hybridization is limited between *T. b. connectens* and *T. b. opulentus*, and because patterns of pocket gopher breeding should have an impact on louse population genetics, then some genetic isolation between lice north and south of this contact zone could be expected. Alternatively, *T. minor* lice may show genetic subdivision within the species, but that subdivision may not correspond with the host contact zone. This latter situation was observed for *Geomydoecus aurei* chewing lice, which are native to the northern subspecies of pocket gopher near San Acacia, but which are actively colonizing the southern subspecies of pocket gopher (Hafner et al. 1998).

Figure 1. Study Site. The map of New Mexico (left) outlined in yellow, with a corresponding view of the study sites along the Rio Grande Valley (right). Arrow indicates the "San Acacia constriction", an area where the Rio Grande Valley narrows, limiting suitable habitat for pocket gophers and their parasites (Smith et al. 1983; Hafner et al. 1998).



A second objective of this study was to determine if there is additional genetic subdivision between louse infrapopulations on neighboring pocket gophers or between lice collected at different localities. If pocket gopher hosts represent "islands" of habitat for lice, with vertical rather than horizontal transmission being the rule for lice moving from one host island to another, then population structure should reflect infrapopulation boundaries.

The final objective of this research was to determine if lice collected at different times from the same locality would show genetic differences. For this comparison, lice spanning a 19.5-year, 175-generation difference in time were compared. Few natural populations have been studied over similar numbers of generations, but severe bottlenecks are expected to reduce heterozygosity, to alter allele frequency distributions, and to cause the loss of alleles over time, leaving “ghost” alleles in more recent populations (e.g., Harper et al. 2003, Harper et al. 2006, Ugelvig et al. 2011). If seasonal population bottlenecks and population bottlenecks at host colonization have a large impact on chewing louse populations, as proposed by Nadler et al. (1990), then these processes should be evident at the genetic level.

CHAPTER 2

MATERIALS AND METHODS

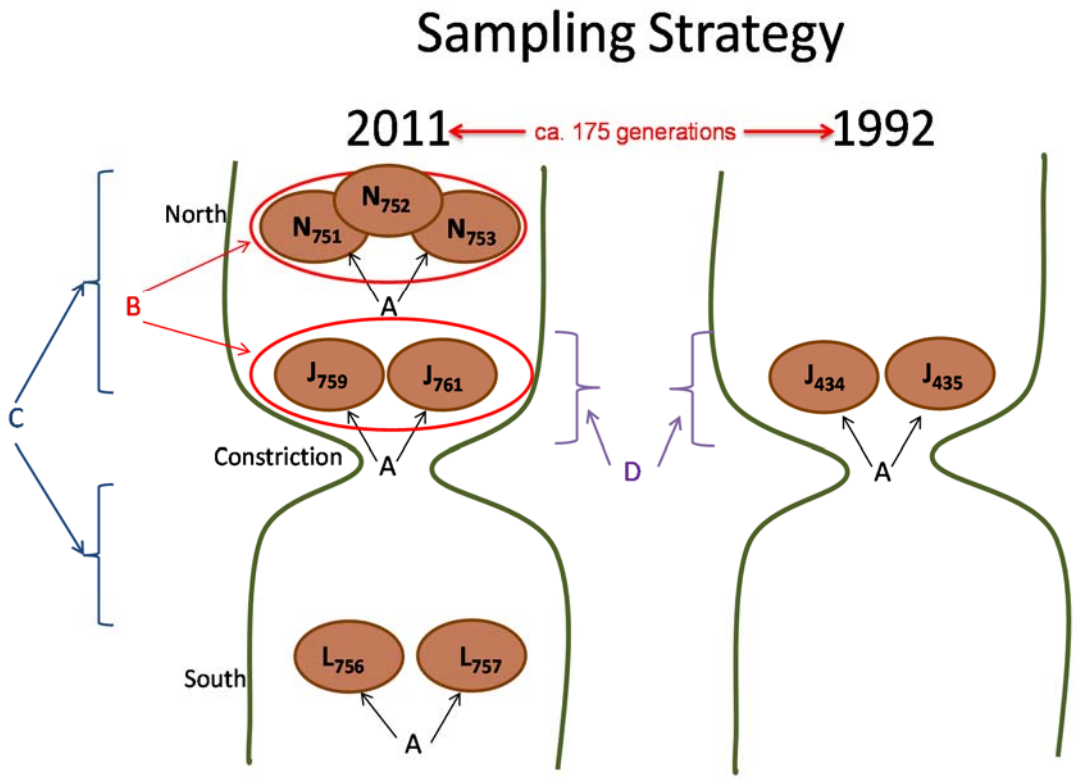
Fieldwork

Fieldwork was conducted at locations north and south of San Acacia constriction near San Acacia, New Mexico (Table 1; Figure 2). Locations were chosen to facilitate comparisons with chewing louse specimens collected in the course of previous studies. The New Mexico Department of Game and Fish (NMDGF) approved collection of specimens (permit # 3500). Procedures used in the field and laboratory followed all guidelines set by the University of Northern Iowa Institutional Animal Care and Use Committee and the American Society of Mammalogists (Sikes and Gannon 2011).

Table 1. Gopher and Louse Specimens. Specimen collection numbers are given by collection year along with collection locality and numbers of *T. minor* lice used (n) in this study. Individual lice were sequenced for mtDNA sequence variation and genotyped in microsatellite analyses; pooled lice were combined for microsatellite primer development.

1992 Gophers	2011 Gophers	Socorro Co. Locality	Individual Lice (n)	Pooled Lice (n)
	749	1.4 mi S, 0.8 mi W Las Nutrias	—	28
	750	1.4 mi S, 0.8 mi W Las Nutrias	—	84
	751	1.4 mi S, 0.8 mi W Las Nutrias	20	—
	752	1.4 mi S, 0.8 mi W Las Nutrias	10	—
	753	1.4 mi S, 0.8 mi W Las Nutrias	10	5
	754	1.4 mi S, 0.8 mi W Las Nutrias	—	7
	755	1.4 mi S, 0.8 mi W Las Nutrias	—	36
	756	1.1 mi S, 0.75 mi E Lemitar	19	—
	757	1.1 mi S, 0.75 mi E Lemitar	20	—
	759	0.9 mi S, 0.1 mi W La Joya	19	—
	761	0.9 mi S, 0.1 mi W La Joya	20	—
434		1 mi S La Joya	20	—
435		1 mi S La Joya	19	—

Figure 2. Visual Representation of Sampling Strategy. Ovals represent distinct louse populations on individual pocket gophers collected from each locality. Letters represent locality name and numbers represent pocket gopher specimen.



Gophers were collected using Macabee traps (Z.A. Macabee Gopher Trap Company, Los Gatos, CA) placed in tunnel systems after mounds were opened. The traps were secured by placing a wire engineer flag through a link of a chain attached to the trap to prevent the trap from being carried off by the gopher or a predator. Traps were checked approximately every twenty minutes. Once GPS location was recorded and sample numbering was complete (Appendix A), deceased gophers were individually placed in a sealed container with chloroform soaked cotton in order to quickly and efficiently

euthanize their parasitic chewing lice, causing lice to detach from the host's fur. Subsequent combing of gopher pelage allowed for collection of an entire louse infrapopulation. Combing was done in an area with little to no airflow to prevent loss of specimens. Collected lice and gopher tissues were placed in labeled 1.0 ml Nunc CryoTube vials (Nalge Nunc International, Denmark) and stored on dry ice until return to the laboratory at the University of Northern Iowa, where lice and gopher tissues were then stored in an ultra-cold freezer at (-80ºC).

Pocket gopher weight, measurements, and reproductive history were recorded. Tissues were collected and vouchers were prepared. Later, cleaned pocket gopher skulls were examined for defining characteristics of the cranium according to Hendrickson (1972) in order to estimate age of specimens (Appendix B). Dr. Mark Hafner aged pocket gopher skulls for host specimens 434 and 435 from Louisiana State University's 1989 New Mexico specimen collection.

Louse Preparation

Individual DNA Isolation

Lice from a single CryoTube vial were poured onto a clean ice pack in small portions for work under a dissecting microscope. *T. minor* lice were identified according to characteristics listed by Hellenthal and Price (1991) and separated from *Geomydoecus* lice, other parasitic inhabitants (such as mites), and dirt.

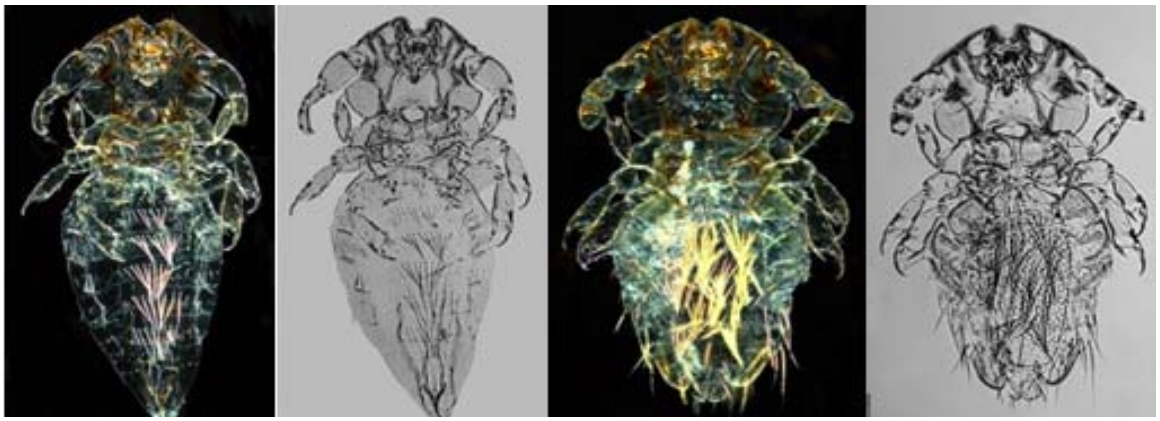
Lice (n = 157) from five gophers north of the constriction and two gophers south of the constriction (Table 1) were placed individually in 0.5 ml labeled centrifuge tubes indicating the louse number, host number, and collection locality. Genomic DNA was

extracted from individual lice using a QIAamp DNA Micro Kit (Qiagen, Valencia, California). Manufacturer's recommendations were followed with the following exceptions: Prior to DNA extraction, individual louse bodies were placed on a freezer block under the dissecting microscope and punctured a total of six times in the head, neck, and shoulders (punctured twice in each region) using a #2 insect pin. Carrier RNA was added to AL Buffer before addition of ethanol. All centrifuge times were increased by thirty seconds and incubation before elution was increased from one to five minutes. In the final step, each louse was eluted in 30 µl H_2O. Louse DNA yield was quantified for several test lice on a Qubit™ 2.0 Fluorometer (Invitrogen, Eugene, Oregon).

Louse Vouchers

Upon completion of DNA extraction, cleared louse bodies were stored in ethanol for preservation and dehydration. In preparation for slide mounting, ethanol was removed and the louse body was placed in xylene for 20 minutes. Under a dissecting microscope, the body was positioned ventral side up and permount was used to permanently attach a cover slip for storage (Appendix C). Slides were dried for three weeks before being stored in a microscope slide box. Photographs were taken of one male and one female for reference of *T. minor* morphology including sex characteristics (Figure 3).

Figure 3. Photographs of *Thomomydoecus minor* Voucher Specimens. Left two photos are of the same male specimen (753.3, Las Nutrias). Right two photos are of the same female specimen (753.5, Las Nutrias). Color photographs taken using dark field on Zeiss AxioScopeA1 compound microscope. Black and white photographs taken on a Zeiss Axiostar plus compound microscope.



Mitochondrial DNA

Mitochondrial DNA Sequencing

Following DNA extraction, a 710-bp fragment of the mitochondrial cytochrome oxidase subunit I gene (COI) was amplified using published universal insect primers LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al. 1994). Polymerase chain reactions (PCR) contained 1.0 µl of DNA (approximately 0.47-2.0 ng/µl), 0.5 µl of each primer (10µm), 10.0 µl Hotstart GoTaq Green Master Mix (Promega, Madison, WI), and 8.0 µl sterile water for a final volume of 20.0 µl per reaction. Thermocycler conditions were as follows for all samples: denaturation at 94ºC for 45 seconds, annealing at 45ºC for 45 seconds, and elongation at 72ºC for 45 seconds for a total of 40 cycles, followed by extension at 72ºC for 10 minutes.

PCR products were screened for amplification and contamination though gel electrophoresis using 1.2% agarose (Fischer Scientific, Fair Lawn, NJ) and 1X Sodium Boric Acid (SB) Buffer (Fischer Scientific, Fair Lawn, NJ). Successfully amplified PCR products were purified using ExoSAP-IT (USB, Cleveland, OH) to remove unincorporated primers and dNTPs. Cleaned PCR products were sent to Iowa State DNA Facility (ISU) for sequencing on an Applied Biosystems 3730xl DNA Analyzer.

Mitochondrial DNA Analysis

Sequences were screened for error and edited manually using Geneious Pro (version 5.4.6, Biomatters Ltd), yielding a 577 bp fragment of sequence common to all individuals. For outgroup comparison, Verity Mathis (Louisiana State University) provided a mitochondrial COI sequence from *T. genowaysi* collected in Chihuahua, Mexico in 2008 (*Thomomys umbrinus* pocket gopher host, #LSUMZ 36721). Average uncorrected sequence divergence between unique haplotypes was calculated using the software MEGA (version 5; Tamura et al. 2011).

The software jModelTest (version 2.1.1; Darriba et al. 2012) was used to determine the best-fit model for use in phylogenetic analyses of mtDNA. Likelihood scores were computed for 40 models, and the corrected Akaike Information Criterion (AICc) was implemented for model selection. The selected model was then used in phylogeny reconstruction using MEGA (version 5; Tamura et al. 2011) for maximum likelihood (ML) analysis with Nearest-Neighbor Interchange heuristic searches for 500 bootstrap replicates. Bayesian analysis was conducted using the MrBayes plugin (Ronquist et al. 2012) for Geneious Pro implementing the substitution model indicated by the same

jModelTest analysis described above. Chain length was set to 1,500,000 with 4 heated chains, a 0.1 heated-chain temperature, and a sub-sampling frequency of 300. Burn-in was set to 100,000 using unconstrained branch lengths for priors. Output was evaluated to assess the quality of runs using three criteria recommended in the program documentation: 1) ESS values of frequency histograms were a minimum of 100-200, 2) log likelihood of run_1 showed distribution of likelihood scores had reached stationarity, and 3) standard deviation (StdDev) for posterior output had a value ≤ 0.01.

Microsatellites

Microsatellite Development

To discover microsatellite loci in the genome of *T. minor*, a 454 sequencing approach was used. A pool of 160 *T. minor* lice was collected from 5 gophers from a single collection site for use in creation of a microsatellite library through genome sequencing (Table 1). A DNeasy Blood & Tissue Kit (Qiagen, Valencia, California) was used on the pool of lice. Manufacturer's recommendations were followed with the following exceptions: lice were used directly from the ultra-cold freezer and use of liquid nitrogen was eliminated. After four hours of incubation in ATL buffer, an additional 20 µl Proteinase K was added with additional crushing performed before continued incubation overnight at 56ºC. All centrifuge times were increased by thirty seconds at an increased speed from 6.0 to 6.6 rcf. Final elution in AE Buffer was decreased from 200 µl to 50 µl with incubation increased from one to five minutes. DNA concentration was measured using a Qubit™ 2.0 Fluorometer (Invitrogen, Eugene, Oregon).

Steven M. Bogdanowicz at Cornell University used the restriction enzyme Hinc III to digest genomic DNA, which was then ligated to a double stranded SNX linker. The ligation procedure was modified to generate *Pme I* sites if linkers ligated to themselves. Digested, ligated fragments were enriched for microsatellites by hybridization to 3' biotinylated di-, tri-, and tetra-nucleotide repeat probes. PCR amplified products were ligated to 1.0 µl of a Titanium Rapid Library MID adapter (10 µm adapter stock), and small fragments were removed with Ampure beads. Libraries were submitted to the Sequencing and Genotyping Facility at Cornell Life Sciences Core Laboratory Center for FAM-quantification and Titanium 454 sequencing.

Output fasta and excel files from Cornell University showed single sequence reads and contigs from analysis of 454 sequencing data. These files were analyzed at University of Northern Iowa. Loci were chosen for amplification if they had a tetrameric repeat structure with a minimum of five repeats. Loci chosen based on multiple sequence reads were given "names"; loci chosen based on single sequence reads were numbered based on sequencing read number. Primerselect software (Lasergene Core Suite package, DNAStar, Madison, WI) was used to identify suitable primers to amplify each locus. A long M13 tag (5’-CGAGTTTTCCCAGTCACGAC-3’) was added to the 5’ end of all locus-specific forward primers to allow concurrent amplification with a fluorescent primer (Schuelke 2000). A short M13 tag (5'-GTTTCTT-3') was added to all locus-specific reverse primers to promote adenylation and reduce stutter (Brownstein et al. 1996). Fluorescent tags (6-FAM, HEX, NED) were added to the 5’ end of universal M13 primers (5’-CGAGTTTTCCCAGTCACGAC-3’) to allow three-primer amplification of

PCR products and subsequent multiplex genotyping (Schuelke 2000). Fluorescent dyes were assigned to locus-specific primer pairs based on locus amplification lengths in order to maximize the number of loci that could be run together for genotyping at the ISU DNA Facility on an Applied Biosystems 3730 DNA analyzer.

Microsatellite Genotyping of Individual Lice

For all 157 louse individuals included in mtDNA analysis, nine microsatellite loci were amplified using microsatellite primers developed for *T. minor* (Table 2) in a dye-labeled, nested, 3-primer amplification technique (Schuelke 2000). All reactions contained 0.5µl DNA, 0.4 µl each primer, 0.4 µl designated fluorophore-labeled M13 primer (Table 2), 5.0 µl GoTaq Clear Master Mix (Promega, Madison, WI), and 3.3 µl sterile water for a 10.0 µl reaction. Thermocycler conditions were as follows for all samples: 10 cycles of denaturation at 94ºC for 40 seconds, annealing at 58ºC for 40 seconds, and elongation at 72ºC for 40 seconds followed by 30 cycles of denaturation at 94ºC for 40 seconds, annealing at 53ºC for 40 seconds, and elongation at 72ºC for 40 seconds. The final step was extension at 72ºC for 15 minutes.

Representative samples from each infrapopulation, including all negative PCR controls, were screened for amplification and contamination on 1.2% agarose gels following the same procedure used in mtDNA screening. Successfully amplified products were sent to the Iowa State University DNA Facility for analysis on an Applied Biosystems 3730 DNA Analyzer. For comparison, all microsatellite procedures were repeated on 12 sympatric *G. aurei* pocket gopher chewing lice from La Joya, New Mexico.

Output fsa files received from Iowa State University were scored using the software GeneMarker (version 1.90, SoftGenetics, State College, PA), coupled with visual inspection and editing. The software Convert (version 1.31; Glaubitz 2004) was used to reformat all data files for use in additional genetic analysis programs.

Locus Quality Evaluation

Genotyping errors occur when a genotype assigned following molecular analysis does not correspond to the actual genotype of the individual being assessed. Rates of error are determined by repeated genotyping of a subset of individuals. Any observed allelic difference in repeated samples is reported as a ratio of number of mistyped alleles over the total number of allelic comparisons performed.

In order to check the accuracy of genotyping assignments and estimate error rate, 15 randomly chosen DNA samples out of the 157 samples (10%) were chosen for accuracy screening. At least two lice per host individual were included in this re-assessment. New extractions could not be performed since louse bodies were used in entirety during original isolation of mitochondrial and genomic DNA, but new dye-labeled, 3-primer PCR was performed and amplified products were submitted to Iowa State University for analysis alongside a subset of previous PCR products.

The software Microchecker (version 2.2.3; Van Oosterhout et al. 2004) was used for identification of genotyping errors due to null alleles, large allele dropout, and stutter in preparation for further genetic analysis. For microchecker analysis, lice were assigned to populations in two ways: 1) by location and collection date, with La Joya lice collected in 2011 designated as a different population than lice collected from the same location

Table 2. Fluorescent Label Assignments for Multiplexing PCR. Primer pair sequences are included for each locus. Forward M13 tags are highlighted in blue and reverse M13 tags are highlighted in green for each sequence provided.

Locus Name	Length (bp)	Fluorescent Labels (Dye Set D)	Multiplex Group Assignment	Primer Pair Sequences (5' – 3') {M13 tag highlighted in blue for each forward primer and in green for each reverse primer}
4189	159	NED	1	CGAGTTTTCCCAGTCACGACAGAGATTTGAATTGAACGGAGGG GTTTCTTTCTTTGTTCCTTTGACATCGACC
3495	172	HEX	1	CGAGTTTTCCCAGTCACGACAATGAGTAAGTACGATCCAGCAC GTTTCTTAGTGAGTTAAATGCTAGGCTGATG
1569	179	6-FAM	1	CGAGTTTTCCCAGTCACGACTTTAACAAAGAGGAATCGGATGC GTTTCTTCATACTCCCTCACGATTCTGTCC
1451	332	6-FAM	1	CGAGTTTTCCCAGTCACGACCTCATGGTGATGGTCTTTGTCTC GTTTCTTGTGTTCGAGAAGCTGTATCATCC
Jan	174	NED	2	CGAGTTTTCCCAGTCACGACAATTGTGCTATGTCATCACTTGG GTTTCTTCTTCAAACTTGCATTTACCTCTTC
Allie	191	HEX	2	CGAGTTTTCCCAGTCACGACACCACATACACTATCAGCAATCC GTTTCTTTTAATTCAAGGACCAGTGGAGGG
Belle	201	6-FAM	2	CGAGTTTTCCCAGTCACGACATCCCTCTTTCTGTCGTTGGAAG GTTTCTTTGCGGTTCGAAATTCTCCAAG
4011	342	6-FAM	2	CGAGTTTTCCCAGTCACGACCATCCAACGGCAATTTCCTCC GTTTCTTGCAGAAGAAGGATATGAAAGACC
851	420	6-FAM	2	CGAGTTTTCCCAGTCACGACTTAATTCACACTCAATCCAGGCG GTTTCTTGTGTAGGCGTCCCACAAATTTG

previously, yielding a total of 4 populations, and 2) by gopher for a total of 9 infrapopulations.

Population Genetics Analysis

Arlequin (version 3.5.1.2; Excoffier and Lischer 2010) was used to assess linkage disequilibrium between all pairs of loci using 10^4 permutations and 2 initial conditions for the Expectation-Maximization algorithm (EM) used in point estimations of parameters given a set of variables (Dempster et al. 1977). Hardy Weinberg Equilibrium (HWE) also was assessed using Arlequin; the Markov chain Monte Carlo method (MCMC) was used to calculate *p*-values using 10^6 steps in the forecast chain and 10^5 dememorization steps to calculate departures from HWE. Global tests of Hardy-Weinberg heterozygote deficiency were assessed by infrapopulation in GENEPOP (version 1.2; Raymond and Rousset 1995; Rousset 2008). Markov chain parameters were set to 1000 dememorization steps and 100 batches with 1000 iterations per batch. Additionally, linearized pairwise F_{ST} values, R_{ST} (Slatkin 1995), and analysis of molecular variance (AMOVA) were assessed in Arlequin. The inbreeding coefficient F_{IS} was evaluated for significant heterozygosity deficit and excess using the program FSTAT (version 2.9.3.2; Goudet 2001); *p*-values for F_{IS} were calculated per locus and sample. In all statistical comparisons involving multiple tests of the same hypothesis, a Benjamini-Yekitieli (B-Y; Benjamini and Hochberg 1995) correction of the critical *p*-value was used (Narum 2006). These corrections were necessary for linkage disequilibrium tests, HWE, global tests of heterozygote deficiency, F_{ST}, and F_{IS}. This method was used in place of Bonferroni

corrections to provide a less stringent significance correction method that is more appropriate for evaluation of population genetic data (Narum 2006).

Structure (version 2.3.4; Pritchard et al. 2000; Falush et al. 2003; Hubisz et al. 2009) was used to implement a Bayesian algorithm to identify genetically homogenous clusters of individuals. Burn-in period was set at 3×10^5 followed by 3×10^6 MCMC repetitions. Population admixture and correlated allele frequencies were assumed. Five runs were evaluated for each cluster value. Infrapopulation identity was used as a prior for the LOCPRIOR model for some analyses in order to enhance clustering when genotypic signal is weak (Hubisz et al. 2009). For this model, assignment of louse individuals to populations was made by gopher for a total of 9 louse infrapopulations. Additionally, data were analyzed without the LOCPRIOR model by combining lice from all localities as a single population. Structure Harvester (web version 0.6.92; Earl and vonHoldt 2012) was used to implement the Evanno et al. (2005) method to evaluate the appropriate number of clusters (K) for each analysis by examining the mean log-likelihood scores and the ΔK for each cluster value.

Variation in population sample size has been shown to cause Structure analysis to group individuals into too few clusters, resulting in an inaccurate assessment of the value of K (Kalinowski 2010). Therefore, population structure was further assessed by clustering populations using the software POPTREE2 (Takezaki et al. 2010). For each of the nine louse infrapopulations studied, four genetic distance measures were calculated between louse infrapopulations, D_A (Nei et al. 1983), G_{ST} without sample bias correction (Nei et al. 1983), F_{ST} (Wright 1951), and R_{ST} (Slatkin 1995). To visualize population

similarity, Neighbor-joining (NJ; Saitou and Nei 1987) trees were built in PopTree2 for matrices of genetic distance using 1000 bootstrap replicates in each case.

The software Bottleneck (version 1.2.02; Piry et al. 1999) was used to assess the likelihood of recent reductions in effective population size using 10,000 replicates for a two-phase model. The Wilcoxon sign-rank test was used to assess heterozygote excess (Luikart et al. 1998). Multiple tests of the two-phase model were examined for heterozygosity excess in Bottleneck using the pre-set default parameters (TPM; Di Rienzo et al. 1994); 3,000 replicates were performed for each trial in which 70% of mutations were assumed to be single steps, and the variance among multiple steps was set at a value of 30.

CHAPTER 3

RESULTS

Mitochondrial DNA Analysis

Maximum likelihood analysis of mtDNA COI sequences using a Hasegawa-Kishino-Yano model (HKY; Hasegawa et al. 1985) resulted in a tree that indicated two clades with high bootstrap support. Bayesian analysis generated an identical tree with high posterior probabilities (Figure 4). These two groups of mtDNA sequences will hereafter be referred to as haplotype A and haplotype B. Haplotypes correspond tightly to geography: lice bearing A-group haplotypes were found at localities Las Nutrias and La Joya, north of the San Acacia constriction, and the B haplotype was found at Lemitar, south of the San Acacia constriction (Figure 5).

Average pairwise uncorrected nucleotide divergence between haplotypes in group A and haplotype B was 2.2%, with 0.2% divergence among haplotypes in group A (north) and no variation among lice south of the constriction (haplotype B). Sequence divergence between *T. minor* and *T. genowaysi* ranged from 5.1 to 5.7%.

Figure 4. Mitochondrial DNA Haplotype Tree. Representation of 149 *Thomomydoecus minor* lice plus one outgroup (*T. genowaysi*). Leading each line of data is a gopher collection number (corresponding to Table 1), followed by louse specimen number (or ranges of numbers) exhibiting a common sequence. Locality information is indicated by J (La Joya) N (Las Nutrias), or L (Lemitar). The tree with the highest log likehood is shown with bootstrap values above branches and posterior probabilities from Bayesian analysis below branches. Representative louse photos shown are *T. minor* (male on the left and female on the right).

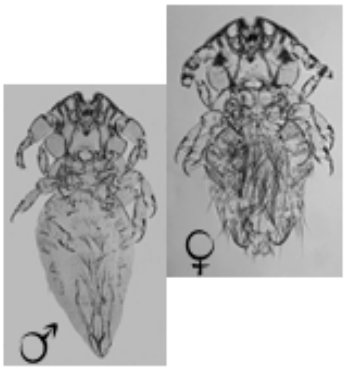
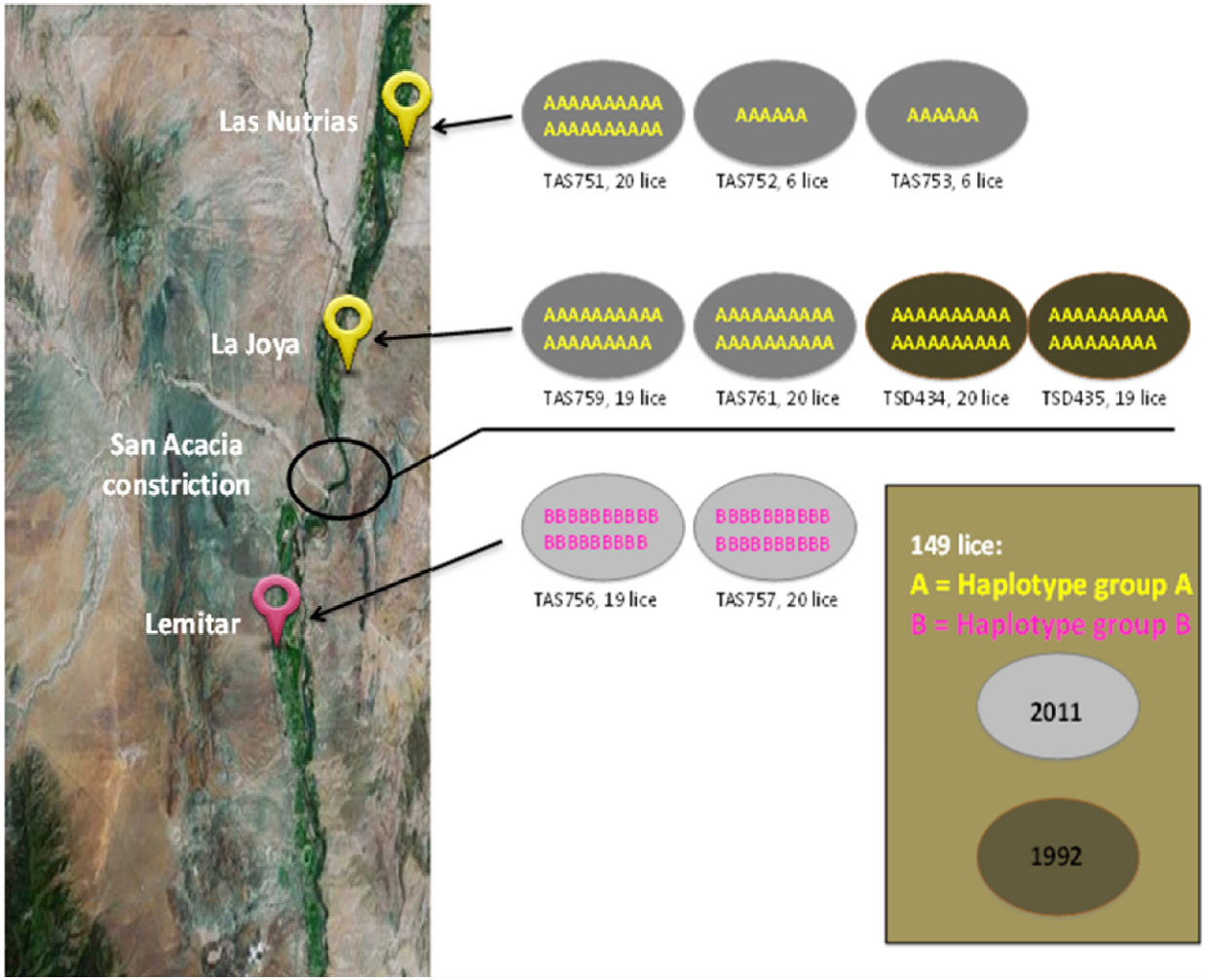


Figure 5. Visual Representation of Collection Localities and mtDNA Haplotypes. Haplotypes observed represent northern or southern sequences corresponding to Figure 4. Circles represent host individuals, and letters indicate individual lice with a particular haplotype (A or B).



Microsatellite Library Development

DNA extraction from pooled lice yielded a concentration of 58 ng/µl, which exceeded the 50 ng/µl requested by Cornell University for microsatellite development. From these lice, Cornell University provided a file of 19,542 sequence reads with 7,768 corresponding primer pair suggestions. Of these, twenty-five loci were originally chosen for testing based on presence of tetra-nucleotide repeats, which should exhibit less stutter from enzyme slippage during amplification than would shorter repeat stretches. Only loci with five (or more) but less than sixteen repeats were chosen to increase the chance of capturing polymorphism while reducing the drawbacks of increased allelic dropout and stutter that can accompany a higher number of repeat units (Guichoux et al. 2011). This approach yielded twenty-four loci that were subsequently tested for amplification and genetic variability using six lice (three from north of the San Acacia constriction and three from south of it). Loci were eliminated from further analysis if they had a high failure rate for initial amplification, displayed a high degree of stutter, lacked polymorphism, or were difficult to score. This resulted in nine microsatellite loci that were chosen for assessing genetic variation within and among louse populations.

Microsatellite Genotyping of Individual Lice

Locus Quality Evaluation

After microsatellite amplification was completed for 157 louse individuals, a subset representing 10% of these samples was re-amplified and re-scored for all nine loci. No genotyping errors were observed in which allele size was incorrectly identified. Five genotyping errors were identified in the individuals that were re-screened. In these 5

cases, an individual louse was originally scored as a homozygote and later re-scored as a heterozygote or vice versa, resulting in an estimated allelic error rate of 1.88% (Table 3).

Table 3. Calculation of Allelic and Locus Specific Genotyping Error Rates.

Loci Names	Miscalled Alleles	Error Rate/Allele	Miscalled Genotypes	Error Rate/Genotype
1451	0/30	0%	0/15	0%
4189	1/30	3.3%	1/15	6.6%
3495	0/28	0%	0/14	0%
1569	2/28	7.0%	2/14	14.3%
851	1/30	3.3%	1/15	6.6%
4011	0/30	0%	0/15	0%
Jan	1/30	3.3%	1/15	6.6%
Belle	0/30	0%	0/15	0%
Allie	0/30	0%	0/15	0%
Total Errors	5/266	1.88%	5/133	3.76%

Of the nine loci that were originally tested for microsatellite analysis in individual lice, only locus 4189 showed evidence of null alleles as assessed by Microchecker. Primers for this locus also did not amplify louse DNA as reliably as other did other primer pairs; 16 of 157 (10%) louse samples did not yield sufficient PCR product on initial amplification with primers for locus 4189. Therefore, this locus was deemed unreliable and was eliminated from further analysis. The eight remaining loci examined indicated no evidence of null alleles, allelic drop-out, or stutter. Furthermore, limited significant linkage disequilibrium was detected between any pairs of loci. The critical *p*-

value for these tests was adjusted to $p \leq 0.009$ using the B-Y correction suggested by Narum (2006). The two cases of significant linkage disequilibrium were loci 1451 and 4011 at Las Nutrias and loci Jan and Allie at La Joya in 1992. Given that there was no consistent pattern of linkage disequilibrium between loci at multiple localities, no other loci were excluded from downstream analysis.

Population Genetics

Loci 4011 and Belle had the highest polymorphism with five alleles each (Appendix D). For each locus, there were noticeable frequency differences between southern populations and northern populations. Loci 1451, 851, 4011, Belle, and Allie each showed one allele private to a single population.

Analysis of molecular variance (AMOVA) was used to assess population subdivision in four unique comparisons (Figure 6). Comparisons by host, locality, and host subspecies (Tests A-C) all explained substantial portions of the existing genetic variation (Table 4). Comparison D indicated that time was not a significant factor contributing to the genetic variation of the louse populations examined (Table 4).

Figure 6. Visual Representation of Sampling Strategy for AMOVA Analysis. Population subdivision in louse populations was analyzed in the following ways: (A) between louse infrapopulations on each gopher within sampling localities, (B) between louse infrapopulations at different sampling localities, (C) between louse infrapopulations on opposite sides of the San Acacia constriction, and (D) between louse infrapopulations sampled at the same locality 19.5 years apart.

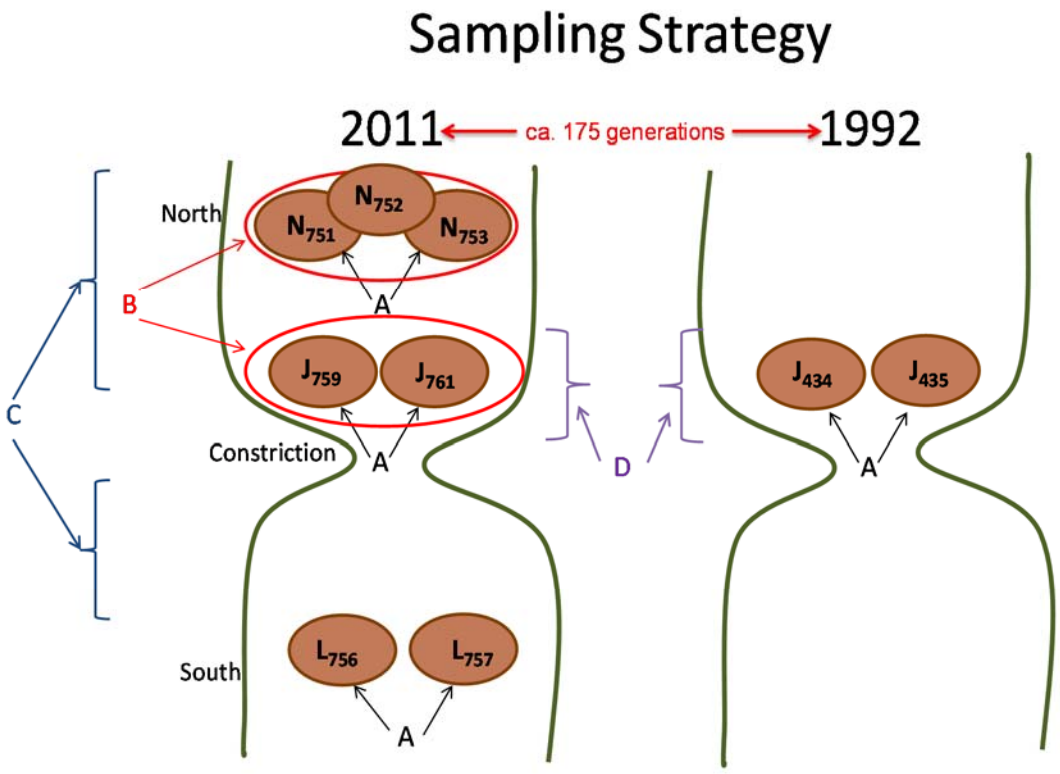
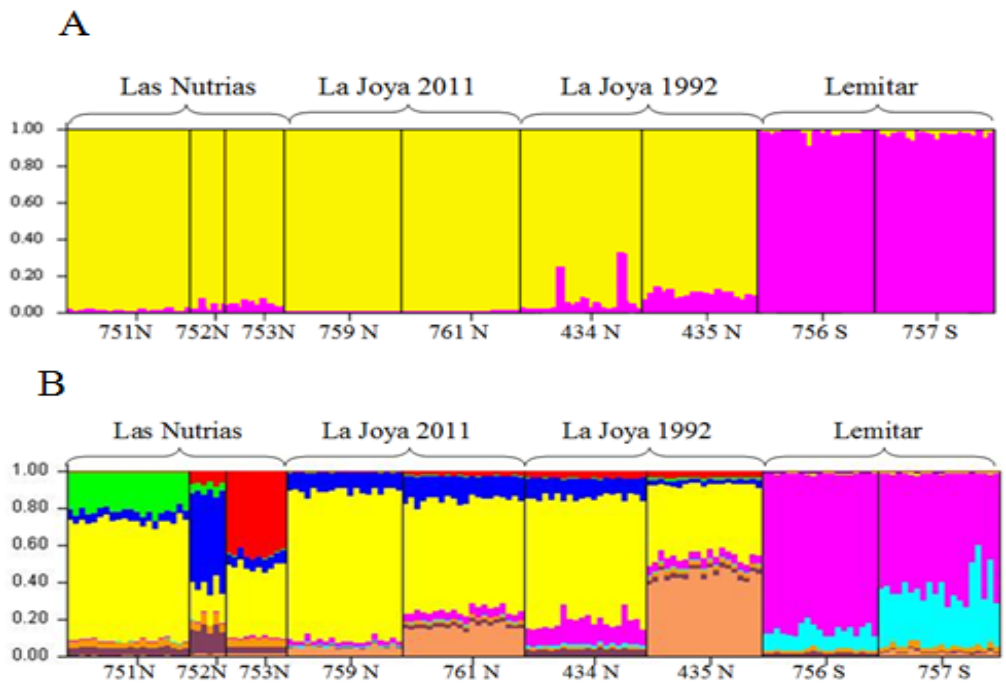


Table 4. Analysis of Molecular Variance (AMOVA) Results. Comparisons A, B, C, and D correspond with Figure 6. Component of genetic variation and *p*-values are given for comparisons of A-D.

	Percent variation	*p*-value	Population Comparisons
A	4.36	**<0.001**	751 vs. 752 vs. 753, 759 vs. 761, 434 vs. 435, and 756 vs. 757
B	8.77	**<0.001**	751, 752, and 753 vs. 759 and 761
C	42.98	**0.04**	751, 752, 753, 759, and 761 vs. 756 and 757
D	-1.08	0.66574	759 and 761 vs. 434 and 435

Structure Harvester (2012) indicated a peak in the log likelihood of the data set from Structure analysis output at $K = 2$, indicating two major clusters. One cluster consisted of louse individuals from north of the San Acacia constriction (n = 114), and the other cluster consisted of lice from south of the constriction (n = 39, Figure 7A). Further analysis in the absence of *a priori* louse population locality definitions detected these two major clusters in addition to detection of a much smaller log likelihood peak indicating more subtle clustering at $K = 9$, a number equivalent to the number of infrapopulations. With infrapopulation identities defined by host used as priors, structure analysis for $K = 9$ was suggestive of weak among-host population structure in chewing lice (Figure 7B).

Figure 7. Structure Analysis Results. Collection localities are listed above graphs and pocket gopher host numbers are listed at the bottom of graphs with N or S indicating direction north or south of the San Acacia constriction. Each individual louse is represented by a bar, and the proportion of each color in a bar represents a coefficient of cluster membership. (A) Two main clusters ($K = 2$) found for all nine infrapopulations defined a clear separation between northern and southern localities. (B) More subtle clustering was detected for nine groups indicating population structure by infrapopulations.



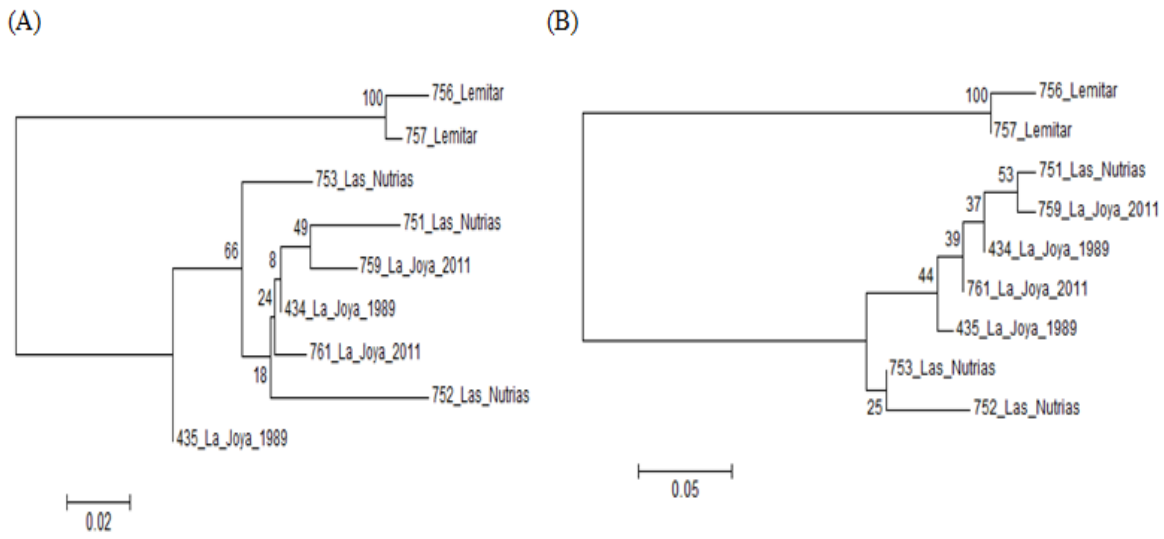
With louse populations defined by host individual, genetic distance (D_A) was low between southern populations (0.019) and showed a wide range among northern populations (0.012 - 0.097), but had consistently higher values between northern and southern populations (0.173 - 0.269; Table 5). Of 36 pairwise F_{ST} comparisons, 31 indicated a statistically significant impediment to gene flow (B-Y adjusted critical value $p < 0.01$; Table 5). All F_{ST} comparisons between northern and southern populations were

statistically significant, as were the majority of values between northern infrapopulations. When infrapopulations at the same locality were combined, significant F_{ST} values were observed between all localities (F_{ST} = 0.04 - 0.50, $p < 0.0001$), but not between populations collected at the same locality approximately 20 years later (F_{ST} = 0.00, p = 0.07). Pairwise comparisons of R_{ST} yielded similar patterns of significance. Neighbor-joining trees of D_A and F_{ST} values (Figure 8) showed distinct differences between louse infrapopulations north of the constriction versus south of the constriction. However, among the seven populations from north of the constriction, there was no grouping consistent with locality or time of collection. G_{ST} analysis yielded numbers very similar to F_{ST} and the same arrangement of populations in a neighbor-joining tree (data not shown).

Table 5. Distance Matrices for D_A and F_{ST}. Divergence of Alleles (D_A) statistics are given above diagonal and population subdivision statistics (F_{ST}) are given below. F_{ST} values in bold are statistically significant ($p < 0.01$) after B-Y correction for multiple tests. Shaded values represent differences between northern and southern populations.

	Las Nutrias; North			**Lemitar; South**		**La Joya '11; North**		**La Joya '92; North**	
	751N	752N	753N	756L	757L	759J	761J	434J	435J
751N	—	0.097	0.058	0.245	0.232	0.043	0.056	0.039	0.071
752N	**0.178**	—	0.073	0.255	0.254	0.077	0.061	0.048	0.095
753N	**0.097**	**0.096**	—	0.212	0.206	0.064	0.044	0.042	0.052
756L	**0.507**	**0.510**	**0.445**	—	0.019	0.269	0.231	0.195	0.174
757L	**0.436**	**0.429**	**0.370**	0.005	—	0.251	0.220	0.188	0.173
759J	**0.041**	**0.195**	**0.116**	**0.580**	**0.511**	—	0.025	0.019	0.046
761J	**0.078**	**0.153**	**0.086**	**0.489**	**0.427**	**0.035**	—	0.012	0.029
434J	0.030	**0.108**	**0.075**	**0.450**	**0.386**	0.013	0.007	—	0.030
435J	**0.118**	**0.193**	**0.077**	**0.424**	**0.368**	**0.089**	0.025	**0.049**	—

Figure 8. Neighbor-joining Trees for Distance Measures D_A and F_{ST}. Trees were generated for both (A) divergence distance; D_A and (B) F_{ST} pairwise distance. Bootstrap values are given above branches.



Global Hardy-Weinberg Equilibrium (HWE) tests for heterozygote deficits indicated that the observed number of heterozygotes was lower than expected in populations from southern localities (Table 6). A similar pattern of inbreeding was indicated by per population F_{IS} values which were significant in one southern infrapopulation and high (F_{IS} = 0.10 and 0.23), but not significantly different than zero for two of the seven northern infrapopulations and the other southern infrapopulation. F_{IS} values also were significant for lice from Lemitar when all lice from the same locality were considered as a single population (Table 7; $p \leq 0.024$ using B-Y correction method). Genetic diversity, as measured by expected heterozygosity, ranged from 0.29 to 0.38 (Table 7).

Table 6. Hardy-Weinberg Equilibrium Tests by Population. Values in bold are statistically significant ($p \leq 0.018$ based on B-Y correction method).

North	751N	752N	753N	759J	761J	434J	435J
***p*-value**	0.2311	0.0354	0.8426	0.9444	0.6233	0.0300	0.4465
South	756L	757L					
***p*-value**	**0.0089**	**0.0016**					

Table 7. F_{IS} and Heterozygosity Values. F_{IS} values are given for all loci and infrapopulations and for each locality. Expected heterozygosity is given by locality. Significant *p*-values ($p \leq 0.018$ for nine populations and $p \leq 0.024$ for four locations using B-Y correction method) are shown in bold.

	Las Nutrias; North			**La Joya; North**		**La Joya '92 ; North**		**Lemitar; South**	
	751N	**752N**	**753N**	**759J**	**761J**	**434J**	**435J**	**756L**	**757L**
1451	0.057	0.024	-0.200	-0.302	-0.149	-0.154	0.104	0.000	-0.027
3495	0.337	0.000	-0.125	-0.029	-0.056	-0.056	-0.029	-0.333	0.027
1569	-0.073	1.000	0.217	-0.200	0.240	0.022	0.297	-0.059	-0.027
851	-0.039	0.333	-0.047	0.064	0.088	0.542	0.036	0.654	-0.026
4011	-0.063	0.286	-0.071	NA	-0.188	0.360	-0.038	0.667	0.410
Jan	-0.027	0.000	-0.200	-0.029	-0.365	0.307	-0.053	-0.091	0.587
Belle	-0.013	0.000	0.169	-0.091	0.159	-0.152	-0.104	0.064	0.212
Allie	0.022	-0.111	-0.370	-0.059	0.224	0.022	-0.166	NA	1.000
AVERAGE	0.017	0.227	-0.073	-0.127	-0.033	0.107	0.007	0.151	**0.309**
F_{IS} By Locality			0.097		-0.051		0.082		**0.246**
Expected Heterozygosity			0.380		0.303		0.359		0.286

Allele frequency distribution tests performed in Bottleneck displayed shifted modes for three infrapopulations (Table 8). The Wilcoxon sign-rank test also was used to assess

recent reductions in effective population sizes, because this test retains high statistical power with as few as four loci and any number of individuals, although it should be noted that a minimum of fifteen individuals and ten polymorphic loci is recommended to achieve full statistical power (Luikart and Cornuet 1998). There was no significant heterozygote excess in any infrapopulation of lice after B-Y correction for multiple tests (Table 8), but two infrapopulations fell short of the recommended for minimum number of individuals (752, n=6; 753, n=10), and only eight polymorphic loci could be used in this analysis.

Table 8. Bottleneck results. Detection of possible recent reductions in effective population size by allele distribution modes indicated in bold. No significant bottlenecks in Wilcoxon test detected after B-Y correction for multiple tests.

	Shifted or L Shaped Distribution Mode	**Wilcoxon Het Excess *p*-value**	**Host Sex**	**Host Reproductive Status**	**Host Weight**	**Life Stage**
751N	Normal L shaped	0.67969	Male	Scrotal, T ≡ 15mm	≡ 210g	Adult
752N	Normal L shaped	0.90234	Female	Parous, swollen, no embs	≡ 174g	Subadult
753N	**Shifted**	0.09766	Female	Parous, swollen, no embs	≡ 160g	Subadult
759J	**Shifted**	0.59375	Female	Null, no embs	≡ 175g	Young
761J	**Shifted**	0.02734	Female	Parous, 4 embs, cr = 6mm	≡ 197g	Subadult
434J	Normal L shaped	0.27344	Female	Non-parous, closed	≡ 140g	Subadult
435J	Normal L shaped	0.37109	Female	Non-parous, no embs	≡ 174g	Adult
756L	Normal L shaped	0.76563	Female	Null, no embs	≡ 123g	Subadult
757L	Normal L shaped	0.27344	Female	Null, no embs	≡ 145g	Subadult

Aberrant Data

Eight of the 157 lice examined herein yielded mtDNA sequences that were incompatible with certain aspects of downstream analysis due to mixed mtDNA

sequences and/or conflicts between mtDNA sequences and morphological assessment (Appendix E). Comparative analysis of microsatellite alleles seen in *T. minor* and *G. aurei* indicated additional conflicting data from the nuclear genome for some of these individuals (Appendix F). Three distinct patterns of data conflict emerged among the eight aberrant individuals examined in this study.

The first type of data conflict came from one louse from Las Nutrias gopher 752, which showed a mixture of mtDNA sequences that repeatedly coamplified when COI primers were used. This mixture appeared to be a combination of a normal *T. minor* A haplotype combined with a noncoding COI-like sequence normally only otherwise seen in *G. aurei* (data not shown). Despite repeated amplification and sequencing, this specimen never yielded a completely readable mtDNA sequence. This specimen's morphological characteristics identify it as *G. aurei*, and it showed microsatellite alleles private to *G. aure*i for two loci (851 and 1451; Appendix F). Additionally, microsatellite PCR reactions that normally failed for *G. aurei* (locus 3495) also failed for this specimen. Therefore, given the resemblance of this specimen to *G. aurei*, it was not included in either mtDNA analyses or microsatellite analyses.

The second type of data conflict came from three lice, also from Las Nutrias gopher 752, which showed unmixed mtDNA sequences typical of *T. minor* haplotype B, but a *G. aurei* morphology and microsatellite characteristics similar to those of *G. aurei* (details in Appendix E). In light of their morphological and nuclear DNA resemblance to *G. aurei*, these three individuals also were eliminated from mtDNA analyses and from microsatellite analyses.

The last type of data conflict came from four lice from Las Nutrias gopher 753, which all showed a mixture of mtDNA sequences that repeatedly coamplified when COI primers were used. This mixture appeared to be a combination of normal *T. minor* A and B haplotypes (Appendix G). All four of these lice were identified as *T. minor* morphologically, and they showed normal *Thomomydoecus* microsatellite alleles. Thus, these individuals were included in microsatellite data analysis, but they were excluded from mtDNA analysis because individual haplotypes could not be identified from the mixture of sequences given the methodology used in this study.

CHAPTER 4

CONCLUSIONS AND DISCUSSION

Development of Genetic Markers for *Thomomydoecus minor*

This study represents the first examination of the population genetics of chewing lice using molecular techniques that are ideal for examining genetic variation among individuals within an infrapopulation as well as among lice from different host subspecies. Population-level assessments prior to this study were limited to allozyme studies of genetic variation, which showed little measurable variation within a species (Nadler and Hafner 1989). In the current study, genetic assessment was made possible using previously available universal insect mitochondrial DNA primers in conjunction with eight novel microsatellite loci developed for the purposes of this study. These genetic markers revealed substantial polymorphism within *T. minor* louse populations. Microsatellite data were more variable within *T. minor* (observed heterozygosity averaged over all loci = 0.31) than were allozyme data for the same species (observed heterozygosity averaged over all loci = 0.01; Nadler and Hafner 1989). This pattern of greater variability in microsatellite data than in allozymes is typical of many organisms (i.e., Atlantic salmon, Sánchez et al. 1996; fruit flies, Irvin et al. 1998; dusky grouper, De Innocentiis et al. 2001).

Importantly, the quality of the newly generated microsatellite markers was carefully checked as part of this study. In this study, 10% of samples were rescreened to evaluate repeatability, and all loci were subjected to analysis for null alleles and large-allele dropout. One of the nine loci used for genotyping was eliminated from population

genetic analysis because of probable null alleles. The eight remaining loci showed high repeatability with an allelic error rate of only 1.88%. When considering whether to discard loci with higher error rates in order to lower the overall genotyping error rate, it is important to keep in mind the level of precision required given the nature of the study; for example, studies of population structure are less affected by genotyping errors than are studies of parentage (Bonin et al 2004; Pompanon et al. 2005). The genotyping allelic error rate of 1.88% obtained for the microsatellite data described herein likely provides an acceptable assessment of population genetic parameters.

Population Genetics of *Thomomydoecus minor*

The Effects of the San Acacia Constriction

Within *T. minor*, substantial geographically structured genetic variation exists in both the nuclear and mitochondrial genomes. Mitochondrial A haplotypes occurred exclusively in locations north of the San Acacia constriction, and haplotype B occurred south of it (Figure 4). Separation of the haplotypes was complete with the exception of seven individual lice collected from two hosts from Las Nutrias, north of the San Acacia constriction (Appendix E). These seven lice showed evidence of southern mtDNA haplotype B, but these samples have been puzzling for their mixture of morphological and genetic characteristics (interpretation discussed below). Assessment of the frequency of southern haplotypes north of the San Acacia constriction will have to await further sampling as part of an extended study that is already underway.

Nuclear microsatellite data indicate genetic structure that is concordant with mtDNA and geography. Microsatellite allele frequencies for all loci tested differed noticeably

between individuals collected north of the San Acacia constriction vs. south of it (Appendix D). Basal clustering in Structure analysis showed clear support for two groups of lice with individuals falling in groups that mirror the grouping generated from mtDNA (Appendix H). Likewise, pairwise genetic distance measures D_A and F_{ST} supported division of louse populations into two distinct groups (Figure 8), with F_{ST} being high (0.37 - 0.58) and statistically significant in all comparisons between northern and southern populations. AMOVA analysis indicated that differences between northern and southern populations of lice accounted for 43% of the genetic variance ($p = 0.04$). Thus by all analyses, both mtDNA and nuclear microsatellite data indicated distinct genetic differences in louse populations from north of the San Acacia constriction and south of it.

Existence of two distinct genetic groups of *T. minor* that transition at or near the geographical location of the San Acacia constriction was not predicted based on morphology (Hellenthal and Price 1991), but it does make sense in light of host distribution and breeding behavior. Genetic differences in northern and southern populations of *T. minor* could be attributed to the barrier to gene flow caused by the constriction itself. Although the Rio Grande Valley is lush and provides suitable soils for pocket gophers along much of its length, the San Acacia constriction is prone to flooding and provides limited hospitable habitat along the river and adjacent bajadas, which results in patchy distribution of the host subspecies that meet there (Smith et al. 1983). Because host density is low at the San Acacia constriction, lice have few opportunities for switching from the northern subspecies of host to the southern one. Hybridization between the two host subspecies, which differ in their chromosome numbers and in

several allozyme loci, appears to be restricted (Smith et al. 1983). Therefore, restricted interaction between host subspecies also may play an important role in limiting opportunities for dispersal of lice from one subspecies of pocket gopher to another. It is difficult to tease the effect of geography and host distribution apart from the effect of host breeding behavior, but genetic data from *T. minor* clearly indicate one or both of these factors limits migration of *T. minor* across the San Acacia constriction.

Estimating the time of divergence between northern and southern *T. minor* is difficult. There is no fossil record available for these pocket gophers or their chewing lice that would help in estimating the timing of divergence within either group. However, sequence divergence values allow a rough approximation of divergence time. These approximations are complicated by significant heterogeneity in rate of molecular evolution among species of pocket gophers (Spradling et al. 2004) and among species of lice (Light and Hafner 2007). Therefore, any molecular-clock based estimates of time of divergence are especially susceptible to error. As a rough approximation, however, the observed sequence divergence between *T. bottae* and *T. umbrinus* for COI is 11.4% (based on data from Spradling et al. 2004), and the divergence time of these taxa is estimated to be 1.6 mya (Spradling et al. 2004). Therefore, the estimated rate of COI evolution in this group of gophers is 7.125% per million years. While this rate is faster than the 2% per million-year rate of substitution that is often used for animal mtDNA, mammals exhibit a 100-fold difference in mtDNA substitution rates, with rodents exhibiting a higher substitution rate than that of most other mammals (Nabholz et al 2008). Additionally, pocket gophers have a higher rate of mutation than most rodents

(Spradling et al. 2001). Given this 7.125% per million year rate of evolution, and the 5.5% COI sequence divergence observed between the host subspecies (Spradling 1997), these two host subspecies diverged approximately 770,000 years ago, a time frame that seems reasonable for subspecies and well within the Pleistocene as postulated by Smith et al. (1983). Northern and southern *T. minor* differ by only 2.2% of their COI sequence (less than the 5.5% observed in their corresponding hosts), likely indicating a more recent divergence in these lice than in their hosts. This discrepancy in host and parasite divergence times is rather dramatic given that all species of lice examined to date show a higher substitution rate than do their corresponding hosts (1.5-4 fold higher rates of evolution; Light and Hafner 2007). Therefore, *T. minor* may have an approximate rate of COI evolution of 11-28% per million years. While this rate is faster than the maximum 4.2% per million years rate of mtDNA evolution previously calculated in insects, substantial differences in mutation rate have been observed among insects (Papadopoulou et al. 2010), and lice exhibit a far higher rate of mtDNA substitution than is seen in other insect orders (Johnson et al. 2003). Thus, the calculated rate of evolution for chewing lice of 11-28% per million years places the divergence between northern and southern lice (which differ by 2.2% of their mtDNA COI sequence) at 78,000-200,000 years ago, well after divergence of their hosts.

The disparity in divergence times of the two host subspecies and the two haplotypes of *T. minor* indicates that they probably did not co-diverge, but that *T. minor* switched from one subspecies of *T. bottae* to another after host divergence. Whether this host switch happened in the vicinity of the San Acacia constriction is a matter of speculation until

broader sampling can be done, but genetic data from this study clearly indicate the importance of host distribution and/or interactions to louse gene flow.

Genetic Variation Within and Among Infrapopulations

Microsatellite data indicate that horizontal transmission of *T. minor* chewing lice from one infrapopulation to another (i.e., from one host individual to another) is somewhat limited (AMOVA test A; Table 4). This pattern is corroborated by several other measures of population differentiation. When *a priori* louse populations were left undefined, structure analysis showed weak sub-structure of 9 clusters consistent with the number of host pocket gophers (Figure 7B). Population pairwise comparisons of genetic distance measures D_A and F_{ST} (Table 5) also demonstrated that each gopher carries a somewhat isolated infrapopulation of lice, because differentiation was high and significant even between several infrapopulations of lice collected in the same field. Population subdivision by infrapopulation also was observed in another chewing louse, *G. actuosi*, as assessed using allozyme loci (Nadler et al. 1990). Pocket gophers are asocial animals except during brief mating encounters (Hall 1981), so transfer of lice likely is rare with mode of transmission being predominantly vertical from mother to offspring (Rust 1974).

Lice collected from different nearby localities (Las Nutrias and La Joya) exhibited evidence of reduced gene flow as measured by AMOVA and F_{ST}, but the proportion of genetic variation observed between these populations (8% of variance, Table 4) was not much greater than that between infrapopulations from the same field (4% of variance, Table 4). Neighbor-joining trees of distance measures (F_{ST} and D_A) illustrate that

neighboring host gophers do not necessarily have the most closely related louse infrapopulations (Figure 8). Therefore, these data provide additional support for the idea that horizontal transmission of these parasites, even among neighboring hosts, is limited.

Time was not a significant factor in the genetic variation observed among lice despite the 19.5 year time span and approximately 175 generations between collection dates (AMOVA Test D, Table 4; F_{ST} not significantly different from zero for 3 of 4 pairwise comparisons; Table 5). However, heterozygosity dropped slightly in louse populations at La Joya (H_e 1992 = 0.36, H_e 2011 = 0.30; Table 7). Two alleles recovered from the 1992 population were not recovered in the 2011 population (Appendix D), but one of these alleles was exceptionally rare in 1992 (Frequency = 0.025) in the initial sample. Therefore, it is not clear if these missing alleles represent sampling error or ghost alleles truly lost from the population. Few other studies of insect population genetics have been done using historical samples. In the studies that have been done, relatively stable genetic diversity was maintained in the populations observed over periods of time ranging from 20 to 100 years, even in the face of acute population declines (Harper et al. 2003; Harper et al. 2006; Mizuki et al. 2010; Ugelvig et al. 2011).

If chewing louse transmission occurs primarily from a female host to her offspring (vertical transmission), the potential for founder effects may be unusually large for this parasite (Nadler et al. 1990), with additional population bottlenecks also occurring seasonally (Rust 1974). Because *G. actuosi* chewing lice showed less allozyme variability than had been reported for other sexually reproducing insects, Nadler et al. (1990) suggested that seasonal bottlenecks and/or founder events may play an important

role in the genetics of chewing louse populations. However, in the current study based on highly variable microsatellite data, heterozygote deficits resulting in global departures from HWE were detected in only two of the nine infapopulations examined, and significant inbreeding was detected in only one infrapopulation and its locality when infrapopulations were pooled (Table 7). No evidence of bottlenecks was detected for infapopulations except as indicated by a shifted mode for allele frequency distribution in three infrapopulations, and these cases did not appear to be correlated with reproductive status of the host (Table 8). However, bottleneck analysis is somewhat hampered by population sample sizes in this study; only two infrapopulations were sampled south of the San Acacia constriction and two of the nine infrapopulations examined had very small sample sizes (n = 10), meaning that the recommended number of individuals for maximum statistical power in Bottleneck was not met by these two infrapopulations (Luikart and Cornuet 1998). Also, the data collected herein fell just short of the recommended number of loci for maximum statistical power in Bottleneck analyses (8 instead of 10; Luikart and Cornuet 1998). Therefore, it is possible that sampling more loci and increased population sample sizes could indicate population bottlenecks, but for now, genetic data in *T. minor* seem to reflect relatively stable population sizes and an absence of pronounced inbreeding despite the relative isolation of louse infrapopulations on individual hosts.

Interpretation of Mixed Sequences

Of the 157 individuals genotyped in this study, eight lice displayed "mixed" or otherwise aberrant genetic and morphological data. Laboratory error may account for

these mixed patterns, but laboratory error seems unlikely given the order in which sample were handled. Alternatively, several biologically interesting phenomena could account for these patterns, but determining the cause(s) of these patterns will require further sampling that is beyond the scope of this project.

All of the eight louse samples with aberrant data were from Las Nutrias, north of the San Acacia constriction. Seven of these eight samples showed mitochondrial haplotype B in some form (either alone, in a mixture with haplotype A, or in a mixture with *Geomydoecus* mtDNA; Appendix E). These cases were the only samples for which B haplotypes were found on the north side of the constriction, so it is puzzling why the B haplotype did not show up in otherwise normal samples. The apparent presence of haplotype B in the north may be the result of incomplete lineage sorting; in other words, both haplotypes A and B could have been present in ancestral louse populations from north of the constriction, and both haplotypes remain there today, with haplotype B in low frequency. Another explanation for the apparent presence of haplotype B in the north is host switching, whereby lice from south of the constriction have been transmitted across the constriction into northern louse populations, bringing haplotype B with them. Interestingly, *Geomydoecus* lice from the San Acacia constriction have switched hosts with the northern *Geomydoecus* species having invaded pocket gophers south of the San Acacia constriction; the northern species of *Geomydoecus* is now steadily expanding its range southward at a consistent rate (Hafner et al. 1998). The same host interactions that have allowed transmission of northern *Geomydoecus* onto southern pocket gophers could also be allowing southern *Thomomydoecus* haplotypes to expand northward.

Four of the eight aberrant samples displayed *Thomomydoecus* mtDNA in lice that were morphologically characterized as *Geomydoecus* and that also showed nuclear microsatellite evidence of being *Geomydoecus*. Chewing lice are known to feed on skin detritus of their pocket gopher hosts, but it is not known if chewing lice also feed on other lice, shed exoskeletons, eggs, or egg casings. So it is possible that these mixed patterns of morphology/nuclear DNA vs. mtDNA have arisen as a result of *Geomydoecus* individuals having fed on *Thomomydoecus* (or their eggs) that carried haplotype B. The fact that *Geomydoecus* mtDNA was not recovered in PCR amplification of three of these four individuals may be explained by the fact that the PCR primers used to amplify mtDNA in this study are more effective at amplifying *Thomomydoecus* DNA than *Geomydoecus* DNA.

If feeding behaviors don't explain the *Thomomydoecus* mtDNA sequences found in *Geomydoecus* individuals, then past intergeneric hybridization between *Geomydoecus* and *Thomomydoecus* may, although it seems rather unlikely given the genetic divergence between these genera. Mitochondrial DNA sequence divergence indicates that these sympatric species, *G. aurei* and *T. minor*, are quite differentiated (25% uncorrected sequence divergence from 545bp of COI; Spradling 1997). Given the estimated rate of COI evolution discussed above, divergence between these genera would have occurred 900,000 - 2.3 million years ago. Allozyme divergence values between *T. minor* and *G. aurei* are in line with divergence between other genera of insects (Nadler and Hafner 1989). Morphology of these lice also is quite different; while *Geomydoecus* males have genital sac spines, *Thomomydoecus* males do not (Price and Hellenthal 1980). The

function of the genital sac spines is not known, but because these structures are species specific and associated with the reproductive tracts of male *Geomydoecus*, it seems they may be functionally related to reproduction, and lack of such structures in *Thomomydoecus* males may indicate reproductive incompatability. Thus, intergeneric hybridization may not be impossible, but it likely is very rare that successful mating with live reproductive offspring would occur between these two genera of lice. Therefore, the mixed morphology and mtDNA sequences seen in these samples are more likely the result of *Geomydoecus* having fed on *Thomomydoecus* or their eggs.

The remaining four of eight aberrant samples occurred in lice that were identified as *Thomomydoecus* based on morphology, and their nuclear genotypes also indicated that they were *Thomomydoecus*. However, these four lice showed mixed (approximately at a 1:1 ratio) *Thomomydoecus* haplotypes A and B. Again, feeding habits could explain the presence of two *Thomomydoecus* haplotypes if one *Thomomydoecus* individual ate another with a different haplotype. A second possibility is past hybridization between two divergent lineages of *Thomomydoecus* with retained heteroplasmy. All four lice with mixed *Thomomydoecus* haplotypes came from the same gopher host and could be first order relatives given their microsatellite genotypes. Heteroplasmy is not a rare phenomenon in insects (Harrison et al. 1985; Kvist et al. 2003; White et al. 2008), particularly at hybrid zones (Kvist et al. 2003). Therefore, the mixed mtDNA sequences seen in these lice could be a result of hybridization with retained heteroplasmy.

Further sampling of louse individuals from north of the San Acacia constriction is already underway as part of an extension to this study. If the presence of haplotype B

north of the San Acacia constriction is confirmed, migration estimates will be examined. If mixed haplotypes and/or mixed morphology and genetics occur in further sampling, experiments will be designed to analyze the possible impact of feeding habits on genetic analysis in chewing lice, perhaps by isolating DNA from louse heads and louse bodies separately to determine whether mixed haplotypes result from hybridization (if so, mixed sequences should come from both the head and the body) or from feeding (in which case the mixed sequences should only come from the gut).

Conclusions

Microsatellite data and mitochondrial sequence data have proven useful tools for population genetic analysis in chewing lice. These tools have revealed significant genetic variation that corresponds with geography and host type and provides evidence for limited horizontal transmission of lice among host individuals. Comparisons across time indicated that the level of heterozygosity decreased only slightly in the populations examined, leaving overall genetic variation relatively stable despite 19.5 years and 175 generations between collection of samples.

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APPENDIX A
HOST AND PARASITE COLLECTION INFORMATION

Host Information for all *Thomomydoecus minor* Lice Analyzed. Host gophers from Las Nutrias and La Joya are *Thomomvs bottae connectens*. Host gophers from Lemitar are *Thomomvs bottae opulentus*.

1989 Host Pocket Gopher	2011 Host Pocket Gopher	Socorro Co. Locality New Mexico	GPS Coordinates Latitude/Longitude	Elevation (Feet)	Sex	Reproductive Status	Weight	Life Stage
	751	1.4 mi S US 85 and W Las Nutrias	N34°27'11.46" W106°47'13.11"	4669	Male	Scrotal T = 15mm	= 210g	Adult
	752	1.4 mi S US 85 and W Las Nutrias	N34°27'15.1" W106°47'00.10"	4704	Female	Parous, swollen, no embs	= 177g	Subadult
	753	1.4 mi S US 85 and W Las Nutrias	N34°27'16.9" W106°47'13.8"	4698	Female	Parous, swollen, no embs	= 150g	Subadult
	756	1.1 mi S US 85 and E Lemitar	N34°08'38.8" W106°53'33.5"	4618	Female	Null, no embs	= 123g	Young
	757	1.1 mi S US 85 and E Lemitar	N34°08'38.78" W106°53'33.1"	4593	Female	Null, no embs	= 145g	Subadult
	759	0.9 mi S US 85 and W La Joya	N34°09'54.9" W106°50'50.5"	4670	Female	Null, no embs	= 175g	Subadult
	761	0.9 mi S US 85 and W La Joya	N34°09'54.3" W106°50'53.1"	4664	Female	Parous, 4 embs, cr = 6mm	= 169g	Adult
434		1 mi S La Joya	NA	NA	Female	Non-parous, aged	= 140g	Subadult
435		1 mi S La Joya	NA	NA	Female	Non-parous, no embs	= 174g	Subadult

Host and parasite information for louse specimen used as an out group in genetic analysis.

APPENDIX B
LOUSE SPECIMEN VOUCHERS

2011 Louse specimens collected in New Mexico. Specimens are stored in the Evolutionary Biology Lab at the University of Northern Iowa. Sex of each individual louse is given and any significant problems with voucher preparation are noted.

Specimen ID	**Sex**	**Problems**	**Locality**
751.01_N	Female		Las Nutrias
751.02_N	Unknown	Body unusable, crystallized in precipitate	Las Nutrias
751.03_N	Unknown	Body unusable, crystallized in precipitate	Las Nutrias
751.04_N	Unknown	Body unusable, crystallized in precipitate	Las Nutrias
751.05_N	Female	Terminalia region missing	Las Nutrias
751.06_N	Male	Body dried twisted	Las Nutrias
751.07_N	Male	Mounted in two pieces	Las Nutrias
751.08_N	Female		Las Nutrias
751.09_N	Male		Las Nutrias
751.10_N	Female	Head only	Las Nutrias
751.11_N	Male		Las Nutrias
751.12_N	Unknown	Body lost	Las Nutrias
751.13_N	Female	Mounted in three pieces	Las Nutrias
751.14_N	Unknown	Body only	Las Nutrias
751.15_N	Female		Las Nutrias
751.16_N	Male		Las Nutrias
751.17_N	Male		Las Nutrias
751.18_N	Male		Las Nutrias
751.19_N	Male		Las Nutrias
751.20_N	Female		Las Nutrias
752.02_N	Male		Las Nutrias
752.03_N	Female		Las Nutrias
752.04_N	Unknown	Head only	Las Nutrias
752.07_N	Unknown	Body lost	Las Nutrias
752.08_N	Male		Las Nutrias
752.13_N	Unknown	Body lost	Las Nutrias
752.20_N	Male	**Geomydoecus*; Mounted in two pieces	Las Nutrias
752.22_N	Male	**Geomydoecus*	Las Nutrias
752.23_N	Unknown	**Geomydoecus;* Body lost	Las Nutrias
752.25_N	Male	**Geomydoecus*; Dried folded	Las Nutrias

Specimen ID	Sex	Problems	Locality
753.01_N	Male		Las Nutrias
753.02_N	Male		Las Nutrias
753.03_N	Male	Used for photo representation	Las Nutrias
753.04_N	Unknown	Head only	Las Nutrias
753.05_N	Female	Used for photo representation	Las Nutrias
753.06_N	Male	Dried twisted	Las Nutrias
753.07_N	Female	Dried folded	Las Nutrias
753.08_N	Female		Las Nutrias
753.09_N	Female		Las Nutrias
753.10_N	Male	Dried folded	Las Nutrias
756.01_L	Unknown	Body lost	Lemitar
756.02_L	Male	Head and pro-thorax region only	Lemitar
756.03_L	Male		Lemitar
756.04_L	Unknown	Body lost	Lemitar
756.05_L	Male		Lemitar
756.06_L	Female	Body dried twisted	Lemitar
756.07_L	Female		Lemitar
756.08_L	Male		Lemitar
756.09_L	Female		Lemitar
756.10_L	Unknown	Body lost	Lemitar
756.11_L	Female		Lemitar
756.12_L	Male	Head and pro-thorax region only	Lemitar
756.13_L	Male		Lemitar
756.14_L	Female		Lemitar
756.15_L	Male		Lemitar
756.17_L	Unknown	Body lost	Lemitar
756.18_L	Female		Lemitar
756.19_L	Male		Lemitar
756.20_L	Female		Lemitar
757.01_L	Male		Lemitar
757.02_L	Female		Lemitar
757.03_L	Male		Lemitar
757.04_L	Unknown	Body lost	Lemitar
757.05_L	Male		Lemitar
757.06_L	Female		Lemitar
757.07_L	Female		Lemitar
757.08_L	Female		Lemitar
757.09_L	Male		Lemitar
757.10_L	Female		Lemitar

Specimen ID	Sex	Problems	Locality
757.11_L	Female		Lemitar
757.12_L	Female		Lemitar
757.13_L	Male		Lemitar
757.14_L	Male		Lemitar
757.15_L	Male		Lemitar
757.16_L	Male	Mounted in two pieces	Lemitar
757.17_L	Male	Body dried twisted	Lemitar
757.18_L	Female		Lemitar
757.19_L	Female		Lemitar
757.20_L	Male	Body dried twisted	Lemitar
759.01_L	Male		La Joya
759.02_L	Female		La Joya
759.03_L	Unknown	Dried folded	La Joya
759.04_L	Unknown	Body lost, excess xylenes melted tubes	La Joya
759.05_L	Male		La Joya
759.06_L	Unknown	Body lost, excess xylenes melted tubes	La Joya
759.07_L	Unknown	Body lost, excess xylenes melted tubes	La Joya
759.08_L	Unknown	Body lost, excess xylenes melted tubes	La Joya
759.09_L	Male		La Joya
759.10_L	Female		La Joya
759.11_L	Male		La Joya
759.12_L	Male		La Joya
759.13_L	Male		La Joya
759.14_L	Female		La Joya
759.15_L	Female	Dried twisted	La Joya
759.17_L	Male		La Joya
759.18_L	Male		La Joya
759.19_L	Female		La Joya
759.20_L	Male		La Joya
761.01_J	Unknown	Mount failed	La Joya
761.02_J	Unknown	Mount failed	La Joya
761.03_J	Unknown	Mount failed	La Joya
761.04_J	Unknown	Mount failed	La Joya
761.05_J	Unknown	Louse destroyed from xylenes	La Joya
761.06_J	Male		La Joya
761.07_J	Female		La Joya

Specimen ID	Sex	Problems	Locality
761.08_J	Female		La Joya
761.09_J	Unknown	Body lost	La Joya
761.10_J	Female		La Joya
761.11_J	Female		La Joya
761.12_J	Female		La Joya
761.13_J	Male		La Joya
761.14_J	Male		La Joya
761.15_J	Unknown	Body lost	La Joya
761.16_J	Female		La Joya
761.17_J	Female		La Joya
761.18_J	Female	Body dried twisted	La Joya
761.19_J	Male	Head only	La Joya
761.20_J	Male		La Joya

1992 Louse specimens from host pocket gophers 434 and 435; La Joya, New Mexico. DNA extraction, mtDNA amplification and sequencing, and voucher mounting done by Courtney Calhoun, Biology undergraduate thesis.

Specimen ID	Sex	Problems	Locality
434.01_J	Male		La Joya
434.02_J	Male		La Joya
434.03_J	Male		La Joya
434.04_J	Male		La Joya
434.05_J	Male		La Joya
434.06_J	Male		La Joya
434.07_J	Male		La Joya
434.08_J	Male		La Joya
434.09_J	Unknown	Mount failed	La Joya
434.10_J	Unknown	Head only	La Joya
434.11_J	Female		La Joya
434.12_J	Male	Dried folded	La Joya
434.13_J	Male		La Joya
434.14_J	Unknown	Dried folded	La Joya
434.15_J	Female		La Joya

434.17_J	Female		La Joya
434.18_J	Male		La Joya
434.19_J	Male	Dried folded	La Joya
434.20_J	Unknown	Dried folded	La Joya
434.21_J	Female		La Joya
435.01_J	Male		La Joya
435.02_J	Male	Dried folded	La Joya
435.03_J	Male	Dried folded	La Joya
435.04_J	Female		La Joya
435.05_J	Female		La Joya
435.06_J	Male		La Joya
435.07_J	Male	Dried folded	La Joya
435.08_J	Male		La Joya
435.09_J	Male		La Joya
435.10_J	Male		La Joya
435.11_J	Male		La Joya
435.12_J	Female		La Joya
435.13_J	Female		La Joya
435.14_J	Male	Dried folded	La Joya
435.15_J	Female		La Joya
435.17_J	Male		La Joya
435.18_J	Male		La Joya
435.19_J	Unknown	Dried folded	La Joya
434.20_J	Female		La Joya

APPENDIX C
POCKET GOPHER HOST CRANIAL AGE CHARACTERS AS LISTED BY HENDRICKSON (1972)

Juvenile - Deciduous premolars (or if permanent premolars present, not in line with remainder of toothrow and showing no wear); temporal ridges not yet formed; bones of cranium porous and cranial sutures unfused; juvenile pelage.

Young - Permanent premolars functional; temoral ridge absent or faintly present; bones of cranium porous; exoccipital-supraoccipital and basisphenoid-basio sutures unfused; juvenile pelage or in process of molt from that pelage.

Subadult - Temporal ridges faintly to well developed; some bones of cranium still porous; exoccipital fused with supraoccipital, but sutures sometimes not completely obliterated; basisphenoid-basioccipital sutures unfused; usually in adult pelage.

Adult - Exoccipital-supraoccipital sutures obliterated; basisphenoid firmly ankylosed to basioccipital but suture sometimes not completely obliterated; remainder of cranial sutures well fused; pitting and sculpturing of basioccipital well developed; *Geomys bursarius lutescens*-(females) temporal ridges discernible to naked eye and detected by running thumbnail over cranial surface, width between ridges less than (or equal to) maximum width of nasals, or (males) temporal ridges well developed and width between them less than maximum width of nasals; *Geomys bursarius majusculus* (females) temporal ridges less than maximum width of nasals and sagittal crest sometimes formed, or (males) temporal ridges in contact and sagittal crest thus present.

Old adult - Skull extremely rugose; basisphenoid-basioccipital suture completely obliterated; sagittal crest well developed (forming a strong, blade-like structure in males of *G. b. majusculus*), characterized by strong vertical ridging in occipital region.

APPENDIX D
ALLELE FREQUENCIES BY POPULATION

Louse microsatellite allele frequencies by infrapopulation. Columns representing southern populations are highlighted in gray. Frequencies in bold are possible contributors to the disparity between northern and southern populations due to private possession of alleles or sharp contrast in frequency of allele in northern vs. southern populations.

		North							**South**		
Locus	**Allele Size (bp)**	**751N**	**752N**	**753N**	**759J**	**761J**	**434J**	**435J**	**756L**	**757L**	**Private allele**
1451	337	0.550	0.250	0.800	0.605	0.650	0.425	0.789	0.026	0.050	
	341	0.425	0.250	0.200	0.184	0.225	0.400	0.184	**0.974**	**0.950**	
	345	**0.025**	**0.500**	**-**	**0.211**	**0.125**	**0.150**	**0.026**	**-**	**-**	
	349	-	-	-	-	-	0.025	-	-	-	434J
3495	192	0.875	0.917	0.850	0.947	0.925	0.925	0.947	0.737	0.675	
	196	0.125	0.083	0.150	0.053	0.075	0.075	0.053	0.263	0.325	
1569	198	0.650	0.833	0.600	0.816	0.850	0.775	0.763	0.079	0.050	
	206	0.350	0.167	0.400	0.184	0.150	0.225	0.237	**0.921**	**0.950**	
851	436	0.300	-	-	-	-	-	-	-	-	751N
	440	0.125	0.667	0.650	0.222	0.200	0.300	0.395	**0.921**	**0.800**	
	444	0.575	0.333	0.350	0.778	0.800	0.700	0.605	0.079	0.200	
4011	360	**0.025**	**0.500**	**0.250**	**-**	**0.175**	**0.075**	**0.026**	-	-	
	364	-	0.083	-	-	-	-	-	-	-	752N
	368	0.075	0.083	0.050	-	-	0.050	0.053	0.158	0.056	
	372	0.900	0.333	0.700	1.000	0.825	0.875	0.921	0.710	0.694	
	376	-	-	-	-	-	-	-	**0.132**	**0.250**	
Jan	198	0.050	0.083	0.200	0.053	0.375	0.225	0.421	**0.895**	**0.775**	
	202	0.950	0.917	0.800	0.947	0.625	0.775	0.579	0.105	0.225	
Belle	223	0.025	-	0.100	-	-	-	-	-	-	
	227	0.950	0.917	0.800	0.895	0.825	0.850	0.632	0.263	0.275	
	231	-	-	-	-	-	-	-	-	0.025	757L
	235	0.025	0.083	0.100	0.105	0.175	0.150	0.368	**0.658**	**0.600**	
	239	-	-	-	-	-	-	-	**0.079**	**0.100**	
Allie	211	-	-	-	-	-	-	0.132	-	-	435J
	215	**0.225**	**0.167**	**0.450**	**0.263**	**0.250**	**0.225**	**0.079**	-	-	
	219	0.775	0.833	0.550	0.737	0.750	0.775	0.789	**1.000**	**0.850**	
	223	-	-	-	-	-	-	-	-	0.150	757L

APPENDIX E
ABERRANT DATA

Louse Speciman	mtDNA Sequence Type	Louse Voucher Identification (Morphology Explanation)	Microsatellite Data	Possible Expla
752.20	*Geomydoecus* non-coding nuclear + *Thomoydoecus* A haplotype	Male *Geomydoecus*	• Showed alleles private to *Geomydoecus* at loci 851 and 1451; • PCR reactions failed for 3495, which never worked in *Geomydoecus*	**Feeding Habits:** *Geomydoec* *Thomomydoecus* possessing PCR primers amplified both *Thomomydoecus* in gut **Hybridization:** Intergeneric *Thomomydoecus* mtDNA
752.22	*Thomomydoecus* B haplotype (clean sequence)	Male *Geomydoecus*	• Showed alleles private to *Geomydoecus* at loci 851, 1451, and Belle; • PCR reactions failed for 1569 and 3495, which never worked in *Geomydoecus*	**Feeding Habits :** *Geomydoe* *Thomomydoecus* possessing primers preferentially amplif in gut **Hybridization:** Intergeneric *Thomomydoecus* mtDNA
752.23	*Thomomydoecus* B haplotype (clean sequence)	Body lost (looked like *Geomydoecus* on initial inspection, but no voucher)	• Showed alleles private to *Geomydoecus* at loci 851 and 1451; • Showed alleles not seen in other lice tested for locus Jan • PCR reactions failed for 1569 and 3495, which never worked in *Geomydoecus*	See 755.22
752.25	*Thomomydoecus* B haplotype (clean sequence)	Male *Geomydoecus*	• Showed alleles private to *Geomydoecus* at loci 851 and 1451; • Showed allele not seen in other lice tested for loci 1569 and Belle; • PCR reactions failed for 3495, which never worked in *Geomydoecus*	See 752.22
753.2	Mixed *Thomomydoecus* sequence Both A & B haplotypes	Male *Thomomydoecus*	• *Thomomydoecus* alleles at all loci tested	**Hybridization:** between two of *Thomomydoecus* with reta **Feeding Habits :** *Thomomyd* haplotype fed on louse or egg with coamplification of gut I
753.7	Mixed *Thomomydoecus* sequence Both A & B haplotypes	Female *Thomomydoecus*	• *Thomomydoecus* alleles at all loci tested	See 753.2
753.8	Mixed *Thomomydoecus* sequence Both A & B haplotypes	Female *Thomomydoecus*	• *Thomomydoecus* alleles at all loci tested	See 753.2
753.10	Mixed *Thomomydoecus* sequence Both A & B haplotypes	Male *Thomomydoecus*	• *Thomomydoecus* alleles at all loci tested	See 753.2

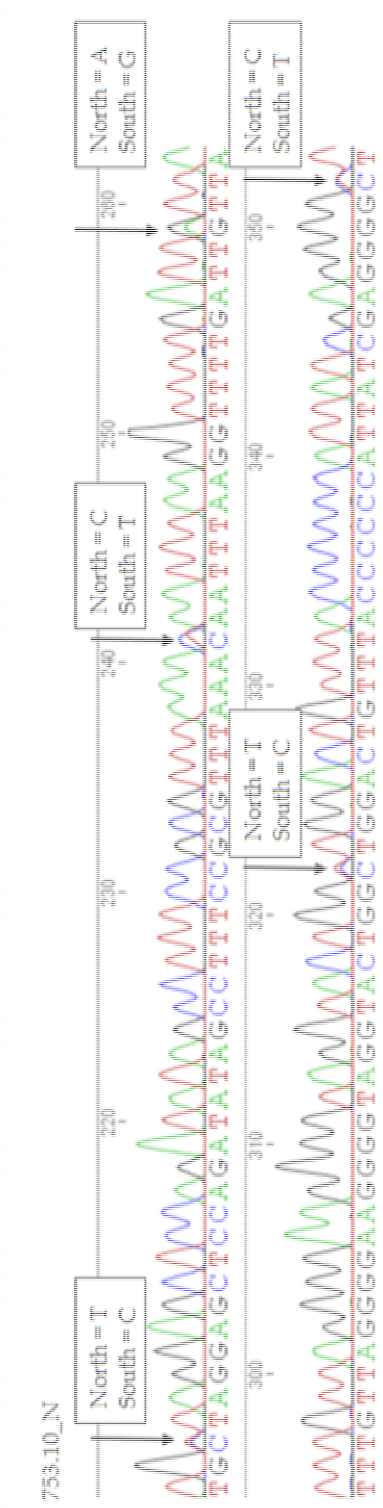
APPENDIX F
ALLELE COMPARISONS

Microsatellite alleles by locus for *T. minor*, *G. aurei*, and aberrant lice from host pocket gophers 752 and 753 (Appendix E)

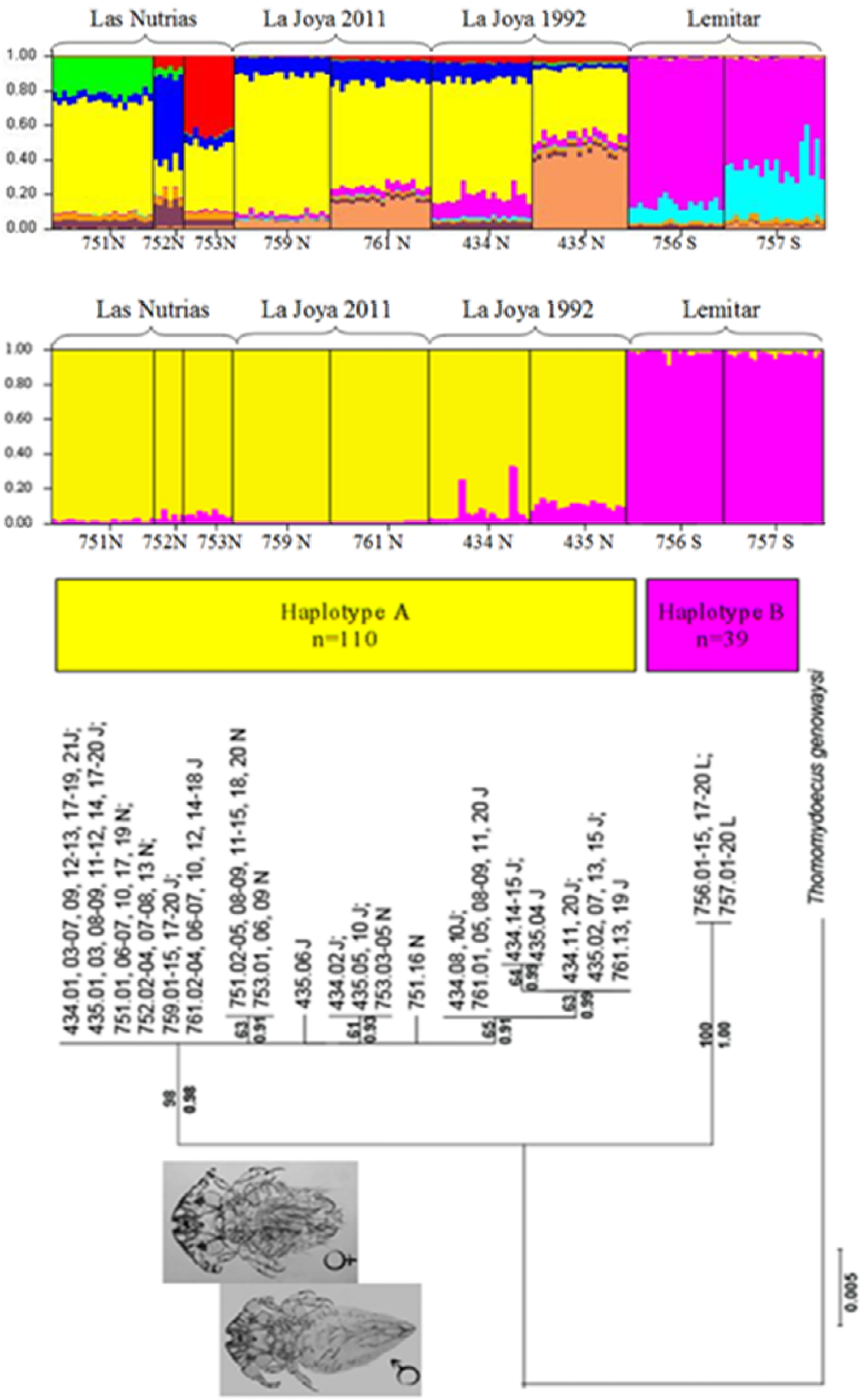
	1451	**3495**	**1569**	**851**	**4011**	**Jan**	**Belle**	**Allie**
T. minor	337	192	198	436	360	198	223	211
	341	196	206	440	368	202	227	215
	345			444	372		235	219
					376		239	223
G. aurei	353			512	368	198	227	223
	357				372	206	231	
	369						235	
							239	
							254	
752 Aberrant lice	357		196	512	360	194	235	223
	369		198		372	198	239	
			206			202	254	
							263	
753 Aberrant lice	337	192	198	440	360	198	227	215
	341	196	206	444	368	202		219
					372			

APPENDIX G
MIXED SEQUENCE DATA

Example of mixed sequence data (partial sequence) for individual 753.10. This sequence is consistent with a mixture of northern and southern haplotypes at all polymorphic sites.



APPENDIX H
MTDNA AND STRUCTURE RESULTS



Maximum-likelihood tree based on mtDNA COI gene and STRUCTURE population assignments based on 8 microsatellite loci both support two genetically distinct groups within *T. minor* that coincide with host subspecies and with the geographic barrier to gene flow at the San Acacia constriction of the Rio Grande Valley in New Mexico.