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Genetic analysis of a parasite contact zone in the southwestern United States

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GENETIC ANALYSIS OF A PARASITE CONTACT ZONE IN THE SOUTHWESTERN UNITED STATES

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Abstract

Studies of cophylogeny (the mirroring of the evolutionary histories of two independent species) lies at the interface of ecology and evolution and therefore is inherently interesting to biologists. The actual mechanisms that lead to patterns cophylogeny are poorly understood. Chewing lice (Trichodectidae) and pocket gophers (Geomyidae) are a model system for the study of cophylogeny. The question to be addressed in this study centers around the fine-scale interactions between pocket gophers and chewing lice in the Southwest United States and whether different louse species actually hybridize within a zone of potential contact. DNA microsatellite techniques, DNA sequencing, PCR, and gel electrophoresis were used to test this question. Based on the results of extensive testing, the evidence indicated hybridization between the two louse species examined. Furthermore, this hybridization was detected on a single host. These conclusions provide valuable insight for future endeavors and furthering the understanding of cophylogeny.

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Genetic analysis of a parasite contact zone in the Southwestern United States

Purpose

The purpose of this research was to analyze the genetic interactions among two species of parasites that come into contact and potentially hybridize in the Magdalena, New Mexico region. When this study began, there were no known hybridization between louse species in this area. The mechanisms of hybridization and cospeciation are not well understood, and the results of this study had potential to help understanding greatly. Two species of lice, *Geomydoecus aurei* (*G. aurei*) and *Geomydoecus limitaris* (*G. limitaris*) were studied using microsatellite techniques and mitochondrial DNA (mtDNA) sequencing. The microsatellite, also known as short tandem repeats (str), essentially identifies the nuclear DNA of the louse. Hybridization would result in the mtDNA and nuclear DNA signals differing or with hybrids possessing nuclear DNA elements from both of the parent species. This would indicate successful mating between individuals from both species. Furthering the understanding of issues such as hybridization and cospeciation would be beneficial for helping to figure out more ways to help climate change-related problems such as endangered species, among others.

Literature Review

Relevance

Investigating coevolution between chewing lice and pocket gophers is not as recent and unusual of a phenomenon as it may seem. The pattern of coevolution between the two species has been studied extensively, and it is well known that these patterns have developed through

coexistence over millions of years (Hafner, 1988). So much evidence has piled up to support this symbiotic relationship that it is the primary example used to explain cospeciation (the process of two unrelated species speciating in tandem) in textbooks (Page, 2003). This history of speciating in tandem produces phylogenies of the two groups that can be mirror images (cophylogeny). Researching the coevolution between chewing lice and pocket gophers pertains to important issues with the human race and planet earth. Investigating genetic variation and evolution, will increase understanding about the effects of earth's climate fluctuation, aiding and defending endangered populations, and observing how past genetic variations pertain to current situations (Demastes et al. 2016). This study concentrates on the genetic mechanisms that are responsible for generating the larger patterns of cospeciation.

Population Characteristics

Geographic isolation of the pocket gophers increases probability of coevolution with the other organisms below surface. They scarcely, if ever, see above ground, therefore they are isolated long term and have adapted specifically for darkness and underground environments. These closed-off subterranean circumstances which join the gophers and other organisms are prime conditions to form long term symbiotic relationships between the small bit of life that is below ground alongside the pocket gophers. The small population size also sets a perfect stage for cospeciation. As mentioned, the cospeciation between pocket gophers and chewing lice is a well-supported phenomenon. However, this conclusion merely provides a launchpad for new ideas and areas of research relating to these relationships (Page, 2003).

Chewing Lice: Behavior and Qualities

As explained in *Tangled Trees*, pocket gophers and chewing lice are an ideal combination for studying (Page, 2003). Chewing lice are insects that do not fly. They do not bite into the

flesh, rather they feed off dead skin and hair on the pocket gopher. They are ectoparasites, which means that they live on the outside of the pocket gophers. Chewing lice are much easier to study for a couple of reasons. First, ectoparasites are preserved in fossils more prominently than endoparasites, which enables investigators to delve into host-parasite relationships from millions of years ago. Ectoparasites are also much more abundant and easier to collect from their host. Furthermore, ectoparasites such as *Geomydoecus* tend not to leave their host for survival reasons. As such, they are an optimal specimen for study (Page, 2003).

History of Pocket Gopher-Chewing Lice Relationships

Different areas of research based on the pocket gopher-chewing lice focal point have ensued, ranging from taxonomical cophylogeny to DNA phylogenetics. Some researchers have chosen to work on the lice side of things, where there are ample areas of study pertaining to the genetic materials. It is noted that the phylogenetic trees of pocket gophers and their chewing lice are intriguingly similar. Speciation in each group seems to have occurred almost simultaneously. (Nadler et al., 1989). Similar evolutionary history such as this makes a good argument for cospeciation. A major boost to the research of chewing lice was provided in the 1980's when "qualitative and quantitative" aspects in a plethora of lice were observed and recorded. This comprehensive study conducted by Price and Hellenthal (1981) found that two main genera of lice that live symbiotically with pocket gophers, *Geomydoecus* and *Thomomydoecus*. Furthermore, a baseline of 16 *Geomydoecus* subspecies and 14 *Thomomydoecus* subspecies were established (Price and Hellenthal, 1981). These advances proved to be crucial, as future investigators now had an extremely detailed manuscript on each species and subspecies of chewing lice relating to pocket gophers. This investigation essentially served as a baseline taxonomy for future studies. It had also been noted that the lice of different hosts contained

significantly different allele loci when compared to lice of different hosts (Nadler et al., 1989). This seems to be another indication of cospeciation. These observations were eventually backed up with data. Using starch-gel electrophoresis, it was verified that there was genetic differentiation between *Thomomydoecus* and *Geomydoecus* (Nadler and Hafner, 1989).

Coevolution Hypothesis

The work done by Price and Hellenthal laid the foundation for more intricate studies of louse and gopher cospeciation. They followed up their experiment several years later and found similar results. It was observed that one louse species was typically found with one pocket gopher species; there was rarely more than one louse species found on a pocket gopher (Hellenthal and Price, 1984). With the morphological analysis and taxonomy in place, allozyme investigations were carried out. These studies further supported coevolution between chewing lice and pocket gophers. Allozyme techniques and starch-gel electrophoresis were used on both pocket gophers and chewing lice to build phylogenetic trees of both the host and parasite (Page, 2003). Overlap between the two phylogenies was prevalent, illustrating cospeciation (Hafner et al. 1988).

Coevolution: Supporting Evidence

While the allozyme and morphological data supported coevolution, another breakthrough study by Hafner led to the first DNA study of lice. He and his team were able to isolate the mitochondrial 379-bp sequence of the cytochrome *c* oxidase subunit 1 (CO1) from 15 pocket gopher taxa and 17 taxa of lice (Page, 2003). Using the tangible evidence from this DNA testing, Hafner et al., 1994, were able to conclude that pocket gophers and their chewing lice do indeed inhabit symbiotically with each other.

Localized Cospeciation

As the "big picture" of the host-parasite relationship solidified, more opportunities arose in niche research areas of the pocket gopher-lice cospeciation. Furthermore, it makes sense that coevolution on a broader scale must start from symbiotic relationships on a smaller, more local level. This approach was taken up by Demastes (1993), who looked at the phylogenetics between gophers and chewing lice in a specific field located in New Mexico. He used DNA fingerprint technology to investigate the popular opinion that parasite cophylogeny was passed from the mother to her offspring. In a surprising turn of events, the data showed that the mother and offspring inheritance were not necessarily correlated. Rather, the genome of the louse depended more on its locality than its parents (Page, 2003).

Research in Louse Gene Transfer

In a further investigation of the maternal inheritance patterns, Demastes et al (1998). "compared the distribution of louse populations with the distribution of mitochondrial DNA (mtDNA)" (Page, 2003). This is an intuitive experimental design, as it is a known fact that mitochondria are passed from the mother to her offspring. The results concluded no correlation between locality of louse population and the distribution of mtDNA (Page, 2003). This means that chewing lice are not explicitly handed down from mother to offspring, and the conclusion is supported by the previous DNA fingerprinting techniques. In essence, transfer of genetic material depends more on where the lice are distributed than on what the mothers pass down to their offspring. This was an important discovery because it shows that louse DNA can be freely transported within populations.

Present Research

Current and future directions of research are abundant, and many are investigating new aspects at this time. From the evolution of morphological features, such as rostral groove length, to endosymbionts, to coexistence of *Geomydoecus* and *Thomomydoecus*, there are plenty of areas to study. More recent experiments have shed light on details of specific *Geomydoecus aurei* lice and their location distribution, gene flow, and allele surfing (Demastes et al. 2016).

Microsatellite Techniques

More recent studies of microsatellite techniques have made them an attractive option to study the effects of evolution on small populations of lice. A microsatellite is essentially a genetically labeled polymorphic segment of the genome. They have found tremendous success in the scientific field to be used as genetic markers (Ellegren, 2004). Microsatellite sequences produce higher mutation rates because of the way its DNA Polymerase works. It produces substitutions and deletions among DNA bases (Eckert, 2009). This aids in showing variability in populations, especially in a localized area.

Gene Flow in Louse Populations

Another important area of louse study involves gene flow. The ability of alleles to move from generation to generation depends on a lot of factors, including population size, inbreeding, bottleneck effects, and mutation rates. Allelic richness measures how much diversity a population has in the long run. Genetic drift and gene flow play a huge role in the allelic richness in a population. If two populations get close to each other, they may begin to hybridize, while if one population splits into different groups for a long enough time, it may diverge into new species. This process may result in what is called the founder event, which plays a huge role of genetic drift- if a population is isolated for long enough, the alleles can become high frequency

in some areas but low frequency in other portions of the genome. The founder event and genetic drift can drastically affect allelic richness in populations (Greenbaum, 2014).

DNA Fingerprinting

One way to assess gene flow and genetic similarities in louse populations is via DNA fingerprinting. It has been widely used as a fast way to analyze genetic variation, with the abilities to detect genetic differences on the allelic level of homozygosity (Lynch, 1990). The general premise of DNA fingerprinting is simple, but yet effective. DNA is extracted from a source and then the desired DNA fragments are isolated using restriction enzymes. The isolated DNA is amplified using a polymerase chain reaction, and then run on a gel (van Embden et al., 1993). Advances in technology have allowed for much quicker analysis of results and comparison of genetic variability. DNA fingerprinting is a prime example of this, and it has been crucial in the investigation and comparison of louse species.

Allele Surfing

From lab-grown bacteria to eukaryotes in the wild, the process of "allele surfing" has been documented. The increased occurrence of founder effects allow for rare alleles to reach high levels in a population (Demastes et al., 2018). This gives the impression of a wave front, hence the "surfing" part of the name. Genetic differentiation can occur as a result of high levels of allele surfing, leading to large genetic changes in populations.

Hybridization

At times, when a species is in the middle of consistent speciation, zones of overlap may occur. Here, distinct species can mate and produce hybrids. This area is called a "hybrid zone" (Hewitt, 1988). Hybridization could reveal how genes and genomes are passed down to offspring. There are two official ways by which hybrid zones can occur, primary and secondary.

A primary hybrid zone means that evolved with consistent geographic distribution, and the two populations are always together. Secondary hybrid zones occur when two distinct, geographically isolated populations come together and hybridize. Secondary hybrid zones are thought to be how most hybrid zones originated (Hewitt, 1988). There is also reason to believe that hybrid offspring may be superior in certain environments. Further research is needed to support this hypothesis (Hewitt, 1988).

Hypothesis to be Tested

1. There is contact between the Northern *Geomydoecus aurei* and Western *G. limitaris* in the Magdalena region of New Mexico.
2. *G. aurei* and *G. limitaris* are near enough relatives that hybridization will occur in areas of contact.

Methodology

Louse DNA Isolation

The process of isolating and genotyping louse DNA starts with the louse itself (in Figure 1). There is an entire box stored in the lab freezer labeled, “Magdalena Lice.” The box contains tubes stuffed with dirt, hair, and lice taken off a pocket gopher in a specific region of Magdalena, New Mexico. Each vial is assigned a number (e.g. 119), and that number corresponds with a specific area on a map of Magdalena. The first goal was to isolate DNA from each number in order to identify their species. To do this, several lice were picked from each numbered tube. The tube was emptied out onto a plastic weigh boat underneath a dissecting microscope. The dirt, hair, lice, and even ticks were subsequently separated in order to isolate the desired lice.

Figure 1: Isolated louse underneath as observed under a dissecting microscope, shown grasping hair of pocket gopher.

Adult females were the optimal lice, as they are larger and have rounded abdomens, whereas males have sharper abdomens. Adolescents are smaller. Somewhere between one and twenty lice were usually isolated from each tube using a toothpick under the microscope. Each louse was individually placed in its own tube and labeled “119.1,” “119.2,” “119.3,” and so on with the first part of the number indicating the pocket gopher host and the number following the decimal the specific louse from that host. The remains of the original tube were then dumped and scraped from the weigh boat back into the vial to

conserve all materials. Next, each louse was taken from its respective test tube and placed on a microscope slide underneath the microscope. Using a stitching pin, each was perforated once in the head and once in the abdomen. This allowed the DNA of each louse to leak out from the body once it was mixed with the proper materials. The louse was then put back into its individual, labeled test tube. Once all lice had been perforated, they were mixed with 180 μL of ATL and 20 μL of Proteinase K (Qiagen Inc., Valencia, CA). The tubes were vortexed, centrifuged, and then placed into a 56°C water bath overnight. This process degraded some of the louse body and extracted the DNA from the louse, mixing it with the liquid.

DNA Extraction

The next day, each louse body was extracted from its respective test tube with a toothpick and put into another test tube. The isolated liquid in the tube was now what contained the DNA. 50 μL of 100% ethanol were added to the new test tube to preserve the louse body, which was then mounted on a slide. It could later serve as a reference if it was necessary to look back at the morphology. The next step of the process was to isolate the DNA from the liquid in the"old" test tube. To do this, *Qiagen DNEasy Blood & Tissue* kit (Qiagen Inc., Valencia, CA) was used. 200 μL of AL buffer from the kit were added to the tubes, followed by 1 μL of yeast tRNA. The tubes were vortexed and incubated in the 56°C water bath again until the precipitate dissolved. Once the precipitate was gone, the tubes were taken out of the water bath and 200 μL of 100% ethanol was added. The tubes were vortexed and then pipetted into a fresh, labeled Qiagen column. The columns were then spun in the ultracentrifuge at 8000 rpm for 1 minute. The collection tube was discarded, and basket kept (it contains the DNA). The basket was placed in a fresh collection tube, and 500 μL AW1 was added. The columns were spun at 8000 rpm for 1 minute, the collection tube was again thrown out, and the basket placed in a new collection tube.

500 μL AW2 was added and spun at 11000 rpm for 3 minutes. The collection tube was thrown out, but this time the basket was placed in a fresh, tough tag-labeled 1.5 mL tube with a lid. The basket was left to air dry for 1 minute, and then 30 μL AE buffer was added. All tubes (with baskets still on top) were placed in the water bath for 3 minutes. They were then ultracentrifuged at 8000 rpm for 1 minute. The newly isolated liquid in the tube was the DNA. The basket was discarded and the isolated DNA was stored in the freezer. The louse bodies were mounted on slides and labeled with their unique identifier (e.g. 119.2) that linked them to the host and to the DNA analyses.

Amplifying Desired DNA Fragments

After the DNA had been isolated, we prepared it for a Polymerase Chain Reaction (PCR) to amplify and make tons of copies of our desired DNA fragment: a 379 base pair region of the mitochondrial cytochrome oxidase subunit 1 (CO1) gene. To do this, we prepared 10 μL reactions, which meant each individual well of the PCR strip had a total of 10 μL in it. This breaks down into 1 μL DNA, 1 μL Primer mix, 5 μL MM, and 3 μL water. First off, we created a "cocktail," which had all of the ingredients (except DNA) to mix in for the PCR. This included water, G2 *MasterMix* (MM), and the primer pair, which in this case was designed to amplify CO1. The cocktail was vortexed, centrifuged, and then 9 μL of it were pipetted into each well of the PCR strip. Next, 1 μL of the DNA isolated from each louse was pipetted into its respective well. DNA was not added to one well at the end of the strip in order to serve as a control. The strip was then placed into a PCR machine and run on the "Sheree CO1" program, which consisted of the following progression: 95°C for 2 minutes; (94°C for 40 seconds → 53°C for 40 sec → 72°C for 40 sec) x30 cycles, then 72°C for 45 min, and finally hold at 15°C.

Gel Electrophoresis

The PCR strip was taken out of the freezer in order to run a gel electrophoresis. This separated the bands of DNA out by charge. First off, 1.2% agarose gel solution was mixed with 100 mL of 1x SB buffer and heated to a boil three times. Once fully liquid, 20 mL of agarose solution was mixed with 2 μL of ethidium bromide and then poured into a casting tray to make a gel. Either a wide or skinny comb was inserted to form the wells. The agar set for 15 minutes. Once the agar gel was solidified, 1 μL dots of loading dye were pipetted onto a strip of parafilm paper. For a wide comb, 5 μL of PCR solution was added to each dot of loading dye on the plastic, but for a skinny comb, 2.5 μL of PCR solution were added to the loading dye. 250 mL of 1x SB buffer was poured into the bottom of the RAGE gel electrophoresis chamber, and then the gel mold was snapped into the electrophoresis chamber. 250 mL of deionized water was poured over the top of the gel. Subsequently, 2.5 μL (skinny comb) or 5 μL (wide comb) of the 100-base pair ladder was loaded. Each dye/PCR solution dot on the parafilm was then pipetted into the wells of the gel. Once all DNA had been loaded, the gel electrophoresis could begin. The lid was placed on top and the electricity was turned on at a voltage of 210. The gel was let run for 6 minutes before the electricity was turned off and the gel was transferred to the photo table for pictures. There was a camera fixed on the lid of the contraption, and the UV light was turned on before a picture was taken. The picture should show clear fluorescence with bands of each DNA sample. The gel's picture is printed, labeled, dated, and put in the lab notebook. If it illustrated a successful DNA isolation, the original PCR strip of louse DNA could be sent to Iowa State for DNA genotyping.

Genotyping

After the desired louse mtDNA gene region was successfully isolated and amplified, it could be sent to Iowa State University to be genotyped. To do this, a multi-channel pipette was used to transfer the DNA into a specific PCR plate. The lids were deliberately tamped down to avoid leakage of DNA liquid. The plate was centrifuged and packaged with paper towel in order to keep it safe. An online order request was filled out, a tracking number assigned, and the DNA mailed to Iowa State. The lab then digitally posted the genetic results in the form of the raw data. In collaboration with geneticist Dr. Theresa Spradling, these results allowed for conclusions of what species the lice are.

Microsatellite Testing

The above process was used for sequencing the mtDNA in the Magdalena lice. The abstract also mentioned work on microsatellite tests to look for hybridization. This search for hybridization has been the main focus of the most recent semester. Fortunately, the process was identical with the exception of the primers. Rather than using the CO1, there were four mixtures ("groups") of primers used. Group 1 (primers 3702, 4103, and 6020), Group 2 (primers 4282, 4911), Group 3/5 (primers 4863, 4737, 43595, 739, and 51656), and Group 4 (primers 33816 and 29676) were all tested. Each group's primer mixture highlighted different parts of the louse genome. The other difference was that for the microsatellite testing, PCR plates with 33 DNA samples that we had previously isolated, rather than just a small PCR strip, were used. They were run on the "Sheree M13SAT2" program of the PCR as follows: 95°C for 2 minutes; (94°C for 40 seconds → 53°C for 40 sec → 72°C for 40 sec) x30 cycles, then 72°C for 45 min, and finally hold at 15°C. These same 33 samples were tested with each primer group to look for

commonalities and differences that might indicate genetic hybridization. In microsatellite experiments, the results provided information regarding specific genes and base pair repeats.

Results

In the spring semester of 2018, the CO1 primer was used to investigate louse mtDNA. This allowed for the distinction of one species from another. These lice cannot be distinguished from one another morphologically, so analyzing DNA provided the answers. It was discovered that the *G. aurei* occupied the Northern region of Magdalena, while *G. limitaris* inhabited the Western region. After analyzing many lice, a general region of overlap was found 6 miles South and four miles West of Magdalena, New Mexico, in the Southwestern United States. A clear line between the two species could be traced. There was also signs of a contact zone with hybridization (Figure 1).

Hybridization

These discoveries led to a new question: Now that the species of the lice and where they are located is known, do they hybridize with each other? The convergence zone provides the perfect area to look for hybridization. Microsatellite techniques (also known as STR's) are the best way to investigate genetic hybridization. Microsatellite techniques differ from the previous CO1 primers because it focuses on specific portions of the genome, such as repeats. This can reveal genetic characteristics that might differ or overlap between the two species. The first step in looking for hybridization is to establish baseline genetic characteristics of both the *G. aurei* and *G. limitaris* species. By running microsatellites of lice from the Northern region that are known to be "pure" *G. aurei* and running microsatellites of lice from the "pure" Western *G. limitaris* region, trends and common characteristics of each were observed.

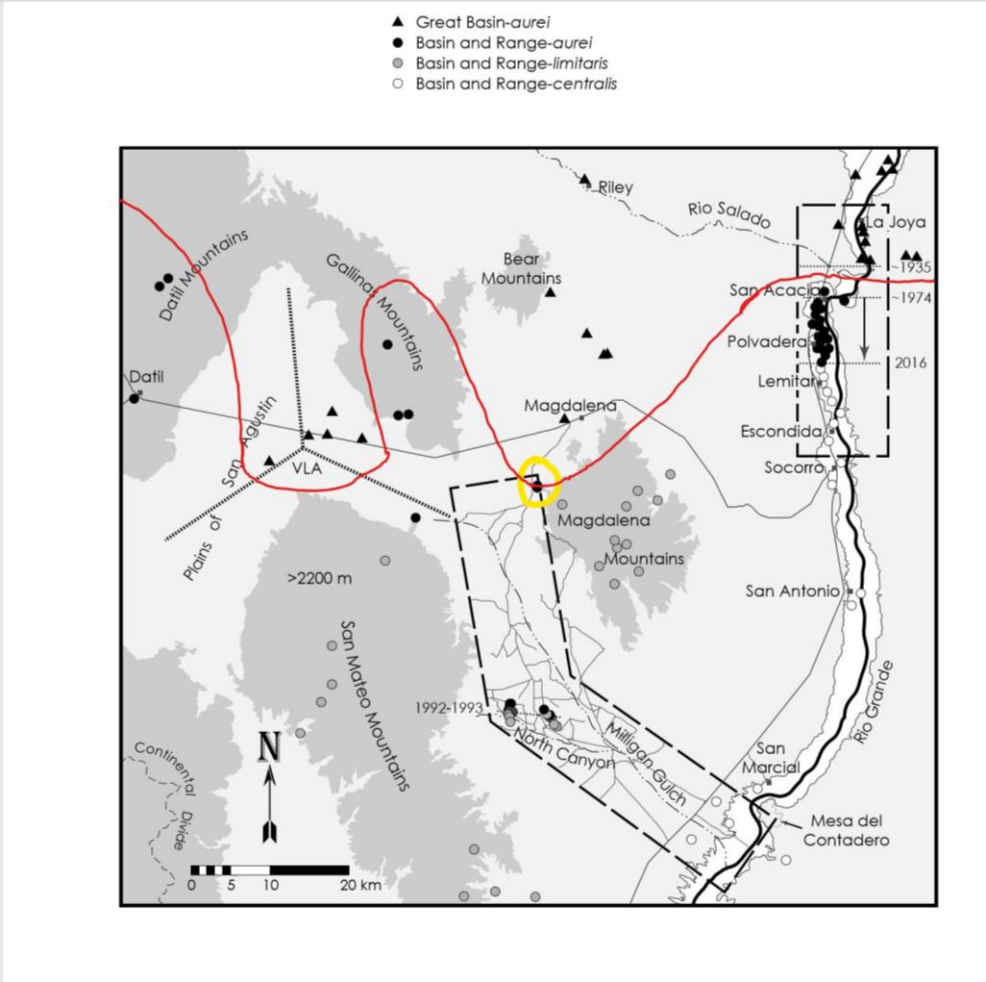

Figure 2: Map of Magdalena Area. The red line indicates separation between G.aurei and G. limitaris. The yellow circle indicates the contact zone.

Microsatellite Testing for Hybridization

Once these baselines were put in place, the hypothetical hybridization zone, or overlap area, could be tested. Microsatellite testing of these lice will allow for the comparison of species in the overlapping region to see if they have hybridized or not. If these lice share traits from both species, then hybridization was observed. If there is no genetic overlap and sharing of traits, then no hybridization will have occurred. Upon analysis, it was discovered that louse 115 contained both *G. limitaris* and *G. aurei* mtDNA types (Figure 4). There is a 115 louse in the blue and pink clades. But the STR analysis (Figure 3) shows all of the *G. aurei* to the left of that vertical line and all of the *G. limitaris* to the right. Therefore, even though those two lice come from different maternal lines, the nuclear genes of *G. limitaris* have swamped out the aurei genes. This validated the hypothesis and provides the opportunity for future research in a multitude of areas.

Conclusion

At the beginning of these experiments, there was a question as to whether or not a true contact zone existed in the Magdalena, New Mexico area. There was also no evidence of hybridization. We were successful in identifying a zone of contact, even finding both species occurring on a single host individual. The DNA fingerprinting methods employed allowed for identification of DNA in each louse species. After the nuclear DNA and mtDNA had been sequenced for all of the collected lice, any mismatches were identified. A difference in the mtDNA and nuclear DNA of a louse indicated hybridization. Such a phenomenon was found in one louse, supporting the original hypotheses. The hybrid louse had inherited its mtDNA from its mother and its nuclear DNA from its father. The findings of this research open the door for future studies on hybridization, especially in the Southwestern United States region. The collection of more louse hybrids would provide opportunities for much more comprehensive

studies into its mechanisms. Discovering the second known louse hybrid will hopefully serve as a launchpad for future endeavors in the field.

Additional Figures

PCA

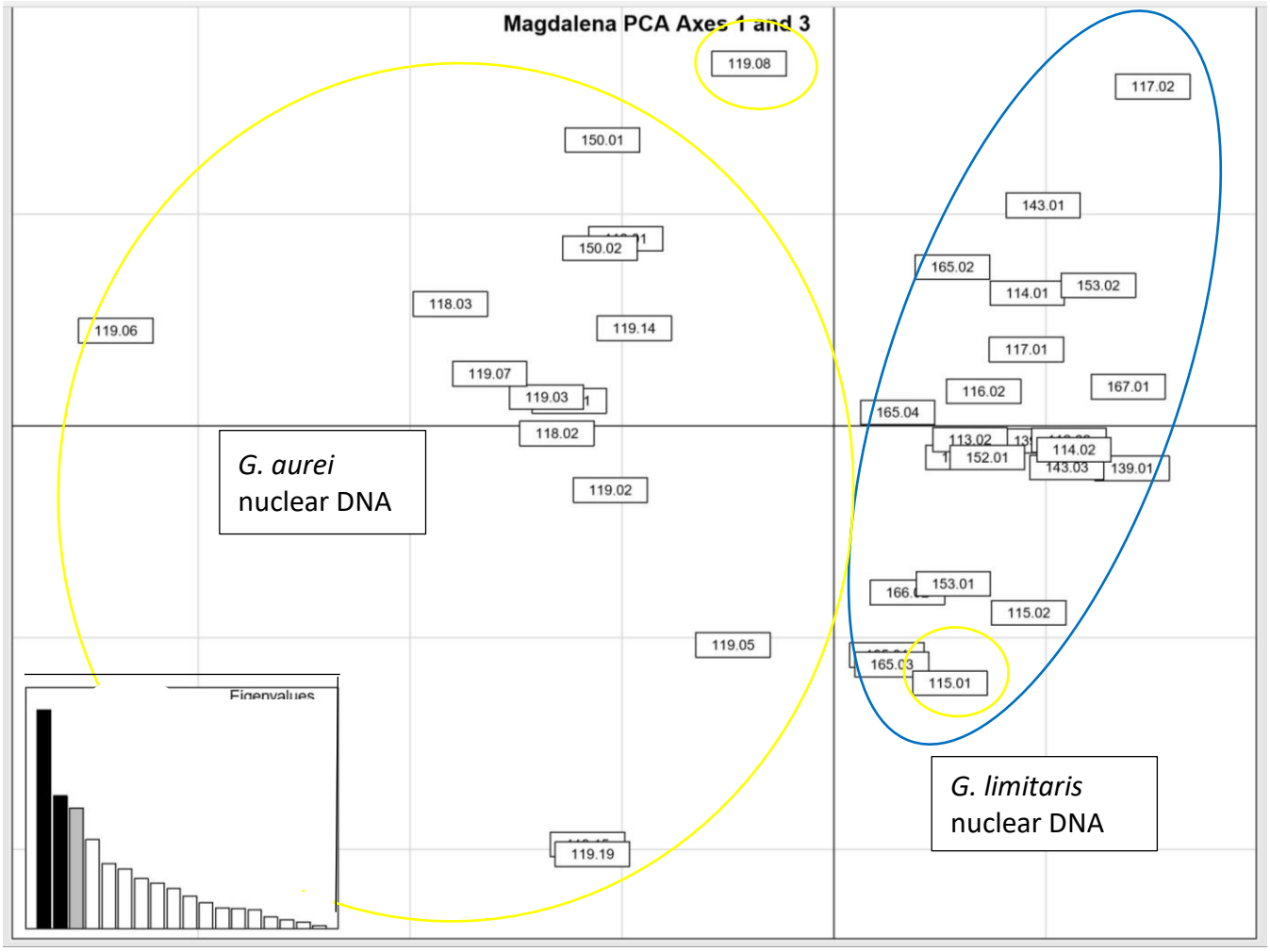

Figure 2: Principal component analysis (PCA) of the nuclear allele data for Geomydoecus examined (x axis=PC 1, y axis = PC 3). Chewing lice depicted to the left side of the vertical dividing line possess nuclear DNA indicative of G. aurei, those to the right, G. limitaris. Chewing lice with maternally inherited mtDNA of G. aurei are circled in yellow, and limitaris in blue. Note that individual 115.01 exhibits a *G. limitaris* nuclear genome with a mtDNA haplotye from *G. aurei.* This is the result of repeated back crossing involving the maternal contribution from at least one *G. aurei* female coupled with nuclear dilution (swamping out) from *G. limitaris*.

Phylogenetic Tree

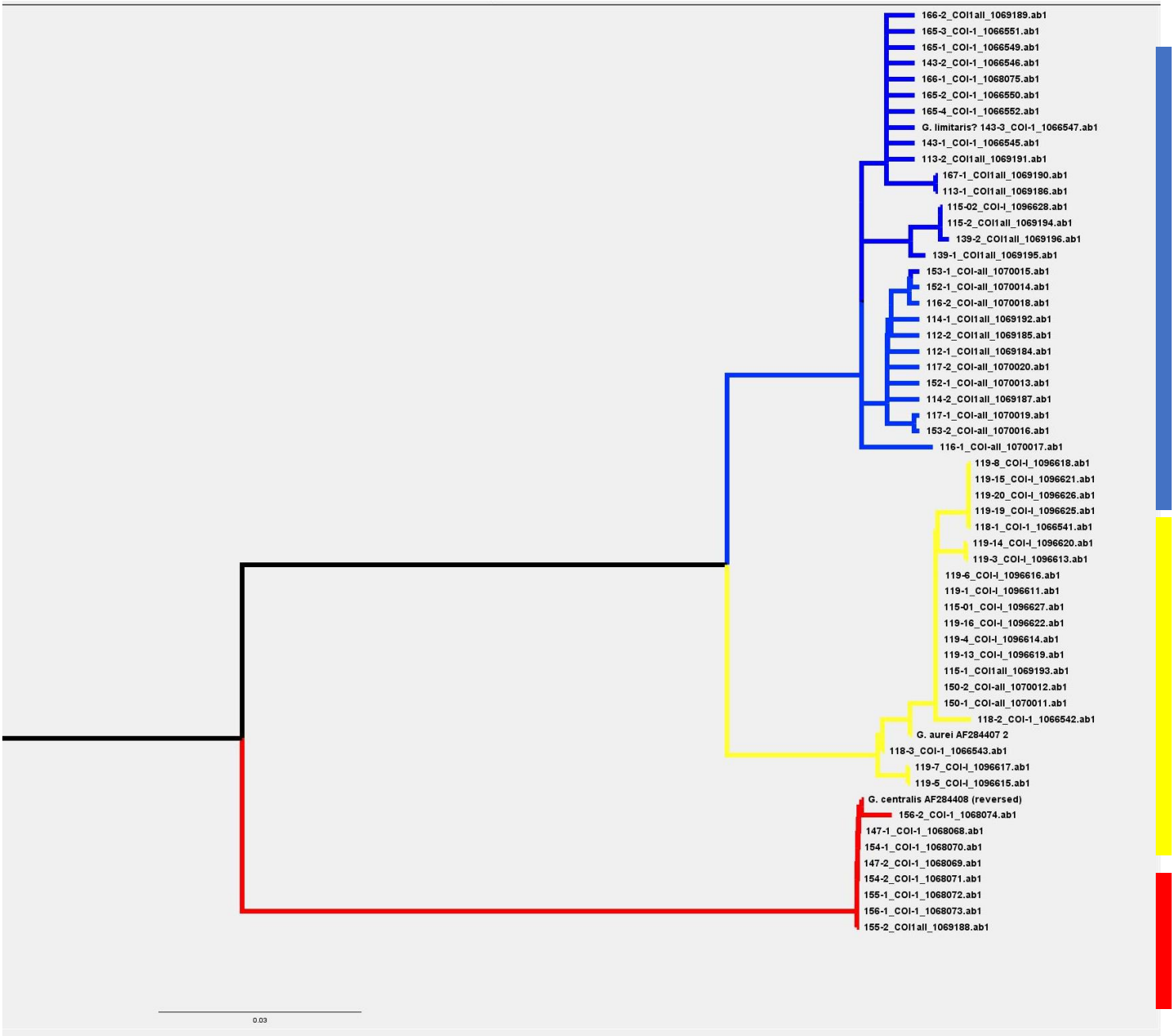

Figure 3: Magdalena louse phylogenetic tree based on mtDNA sequence data. Blue clade represents *G. limitaris*, yellow clade represents *G. aurei*, and red clade represents *G. centralis*. Note that lice from pocket gopher host 115 are present in *G. aurei* and *G. limitaris* simultaneously, which indicates contact of the species on a single host. The *T. minor* (not shown) roots the tree.

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Appendix A

Detailed Specimen Information: The following contains all lice worked with. Note that 115.1 is highlighted because the mtDNA is aurei, from the mother, while the microsatellite frequencies indicated limitaris DNA, illustrating hybridization.

Sample #	Latitude Code	Locality	mtDNA	Msat
112.1	10	9 mi S	limitaris	limitaris
112.2	10	9 mi S	limitaris	limitaris
113.1	11	12.5 mi S	limitaris	limitaris
113.2	11	12.5 mi S	limitaris	limitaris
114.1	9	8 mi S	limitaris	limitaris
114.2	9	8 mi S	limitaris	limitaris
115.1	7	6 mi S	aurei	limitaris
115.2	7	6 mi S	limitaris	limitaris
116.1	7	6 mi S	limitaris	limitaris
116.2	7	6 mi S	limitaris	limitaris
117.1	8	7 mi S	limitaris	limitaris
117.2	8	7 mi S	limitaris	limitaris
118.1	3	2 mi S	aurei	aurei
118.2	3	2 mi S	aurei	aurei
118.3	3	2 mi S	aurei	aurei
139.1	12	15.2 mi S	limitaris	limitaris
139.2	12	15.2 mi S	limitaris	limitaris
143.1	16	23 mi S	limitaris	limitaris
143.2	16	23 mi S	limitaris	limitaris
143.3	16	23 mi S	limitaris	limitaris
150.1	5	5 mi S	aurei	aurei
150.2	5	5 mi S	aurei	aurei
152.1	5	5 mi S	limitaris	limitaris
153.1	5	5 mi S	limitaris	limitaris
153.2	5	5 mi S	limitaris	limitaris
165.1	13	22.3 mi S	limitaris	limitaris
165.2	13	22.3 mi S	limitaris	limitaris
165.3	13	22.3 mi S	limitaris	limitaris
165.4	13	22.3 mi S	limitaris	limitaris
166.1	13	22.3 mi S	limitaris	limitaris
166.2	13	22.3 mi S	limitaris	limitaris
167.1	13	22.3 mi S	limitaris	limitaris
119.1	4	2 mi N, 2 mi W	aurei	aurei
119.2	4	2 mi N, 2 mi W	aurei	aurei
119.3	4	2 mi N, 2 mi W	aurei	aurei
119.4	4	2 mi N, 2 mi W	aurei	aurei
119.5	4	2 mi N, 2 mi W	aurei	aurei
119.6	4	2 mi N, 2 mi W	aurei	aurei
119.7	4	2 mi N, 2 mi W	aurei	aurei
119.8	4	2 mi N, 2 mi W	aurei	aurei
119.9	4	3 mi N, 2 mi W	aurei	aurei
119.1	4	4 mi N, 2 mi W	aurei	aurei
119.11	4	5 mi N, 2 mi W	aurei	aurei
119.12	4	6 mi N, 2 mi W	aurei	aurei
119.13	4	7 mi N, 2 mi W	aurei	aurei
119.14	4	8 mi N, 2 mi W	aurei	aurei
119.15	4	9 mi N, 2 mi W	aurei	aurei
119.16	4	10 mi N, 2 mi W	aurei	aurei
119.17	4	11 mi N, 2 mi W	aurei	aurei
119.18	4	12 mi N, 2 mi W	aurei	aurei
119.19	4	13 mi N, 2 mi W	aurei	aurei
119.2	4	14 mi N, 2 mi W	aurei	aurei