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MATING FREQUENCIES AND ECOLOGICAL MODELING OF HARVESTER ANT: POGONOMYRMEX COMANCHE

Rachel Romo
University of Texas at Tyler

MATING FREQUENCIES AND ECOLOGICAL MODELING OF HARVESTER ANT: *POGONOMYRMEX COMANCHE*

by

Rachel Romo

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RACHEL ROMO

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Approvals:

Thesis Chair: Katrin Kellner, Ph.D.

Member: Kate Hertweck, Ph.D.

Member: Jon Seal, Ph.D.

Chair, Department of Biology

Dean, College of Arts and Sciences

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Abstract

MATING FREQUENCIES AND ECOLOGICAL MODELING OF HARVESTER ANT: *POGONOMYRMEX COMANCHE*

Rachel Romo

Thesis Chair: Katrin Kellner, Ph.D.

The University of Texas at Tyler
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There is little known about the life history of the imperiled harvester ant *Pogonomyrmex comanche.* Due to the conservation status, there is a need to learn and understand how these ants are dispersing their genetics within the region. This study focused on resolving uncertainties about genetic diversity, mating frequencies, and habitat associations influencing distribution of this species. It's well known and studied that queens of different species within this genus take part in multiple mating; but to determine if *P. comanche* follows the other species, microsatellite markers were used to conduct paternal analysis on the colonies collected from Camp Swift in Bastrop, Texas. The genetic relationship between populations collected for this study was determined through the construction of a rooted maximum likelihood tree and a haplotype network, as well as through the analysis of molecular variance (AMOVA). Both the tree and the haplotype showed similar pairings between the collection sites with high bootstrap support of greater than 80. These results show that there is no gene flow between the populations. The AMOVA and neutrality results were indicative of high differentiation among the populations under neutral conditions. In determining the mating habits of this species, microsatellite markers were used in conjunction with the software matesoft. The results showed that at least 72% of the colonies collected from Camp Swift in Bastrop TX participate in multiple mating. The ecological model identified the best habitats for the species across the known range of Texas, Oklahoma,

Kansas and Louisiana. The most important climate variable for their occurrence is minimum temperature. These ants are important ecological engineers as they are a major promoter of seed dispersal. The more we know about these ants and their life histories, the better we can create and implement conservation actions.

Chapter 1
Harvester Ant Ecology and Conservation Concerns

Introduction

Global biodiversity has become an increasing concern for the scientific community. Most scientists would agree that the major driving force in the decrease of biodiversity is climate change (Wake & Vredenburg 2008). Climate change is propagated through anthropological effects such as urbanization, agricultural expansion, and grazing (Bellard et al. 2014). In order to combat the effects of over expansion and habitat degradation, conservationists are concerned with cataloguing threatened species. The IUCN Red List of Threatened Species is the best-known conservation status list and ranking system in the world. For the past 50 years the IUCN Global Species Program has been working with the IUCN Species Survival Commission to assess the conservation status of species on the global scale (IUCN). The ranking system used is broken down into three categories: vulnerable, endangered and critically endangered. As of now, there is a total of 16,938 known endangered species found on the Red List. Out of those species, 141 are ant species (AntWiki 2014).

Colonies of ants, bees, wasps, and termites are prevalent in many terrestrial ecosystems (Wilson 1971). The key to their success rests upon their regulation of internal conflicts, control of diseases, and their collective intelligence in foraging, nest building and defense (Boomsma & Franks 2006). Each ant species has a unique life history because all species have a eusocial system set in place (Korb 2016). For a species to be considered eusocial, three major characteristics must be present: reproductive division of labor, overlapping generations, and cooperative care of the young (Wilson & Holldobler 2005). Within each colony there is a mated female, known as the ‘queen’, that produces

both male and female reproductives. The female reproductives are known as ‘gynes’ and they will one day venture out of the nest to mate and create a new colony (Enzmann, Gibbs, & Nonacs 2014). In addition to these offspring, the queen will also produce sterile female workers that will take care of the brood, forage, and defend the colony (Ingram et al. 2013; Cahan & Gardner-Morse 2013).

The infertile workers that make up the colony have an age-based job distribution. As they age, they climb up through the nest performing various jobs at each level until they reach the top and become foragers for food (Gordon 2008). This is process known as task allocation or division of labor, and it occurs in all ant colonies (Gordon 2008; Manfrendini et al. 2014). There are several factors that influence the task allocation within these colonies, including morphology, genetic variation, and even pheromone usage (Manfrendini et al. 2014). Pheromones play a major role in reproduction, foraging, and general interactions with nest mates (Wyatt 2003). The pheromones can signify rank, attract fellow nest mates to join in on a job, or repel other individuals from the nest (Evison, Fenwick, & Hughes 2012). Through the use of these tools, ants have a highly sophisticated community that impacts the environment around them.

Ants hold a unique and important role in every ecosystem in which they are found. They have the ability to form relationships with the organisms that they interact with; specifically, parasitic and mutualistic relationships (Folgarait 1998). Morton Wheeler conducted a study to observe the parasitic interaction between two species, *Formica consocians* and *Formica incerta*. Over several months of observation, it was found that *F. consocians* were using the nests of *F. incerta* to rear their offspring (Wheeler 1904). Harvester ants form significant symbiotic relationships with plants in the

form of seed predation and dispersal (Macmahon, Mull & Crist 2000). In fungus gardening ants, a mutualistic relationship is seen as the ants take care of the fungus keeping it alive so that they may obtain their nutrients from it (Seal & Tschinkel 2007).

In forming these relationships, they also have a tremendous impact on the soil in which they nest, as well as the plant communities with which they interact. For example, leaf cutter ants propagate soil fertility through the disposal of their refuse material. By taking their nutrient-rich waste material to areas around the nest, they enhance the plant life around them (Farji-Brener & Werenkraut 2015). Harvester ants impact their environment by collecting seeds from various plants in their area. Depending on the size of the colony and the affinity the colony has for a particular seed, it's been estimated that the species can have up to a 10% removal rate of seeds in their area (Macmahon, Mull & Crist 2000). Ants have also been found to add nutrients to climate impacted soils. In an experiment geared towards analyzing how ants add value to soils experiencing hotter temperatures, it was found that the soils occupied by ants had higher soil respiration and a greater amount of displaced soil than those without ants (Toro, Ribbons & Ellison 2015). These traits of burrowing, excavating, disposing of waste, and seed predation have great impacts on the colony itself as well as the environment that they choose to nest.

Characteristics of the genus Pogonomyrmex

Pogonomyrmex is a genus of harvester ants found in mostly arid regions from northern to southern America (Macmahon, Mull & Crist 2000). The species found within this genus are monogynous, meaning they are founded by an individual queen with a lifespan of about 15 to 50 years depending on various conditions (Porter & Jorgensen

1988). This genus has a very specific nest structure, with population densities ranging from 20 to 150 colonies per hectare and nest diameters ranging from 0.5 to 2 m and sometimes reaching a diameter of 5.5 m (Mayo 2015). This genus has unique external nest forms that aid in their identification in the field. They will either be found by a flattened disk covered with small pebbles around, a flattened disk covered by small pebbles and a tall cone of pebbles, or a crater with a central entrance (Mayo 2015). The internal nest structure consists of downward branching tunnels and chambers that house the queen, the brood, the collected seeds, and the working ants of the colonies (Hendricks & Hendricks 1999).

Species within this genus conduct nuptial flights. Bert Holldobler did extensive research on the mating habits of four species in the genus *Pogonomyrmex*: *P. barbatus, P. rugosus, P. desertorum, and P. maricopa*. He found that within each species, the males will preemptively mark their perennial mating site with species specific pheromone to attract the gynes. Once the gynes arrive at the mating site they secrete their own pheromone that alerts the males in the area to begin the mating process. During the mating process there is a strong male competition that takes place between 2 – 5 males for one female (Holldobler 1976). There are many unknowns to the reproductive success of this species due to many unaccounted factors, such as age of the gynes when they take the nuptial flight, the dispersal rates, the success of that gyne to start a colony, and the amount of gynes fertilized at each nuptial flight (Ingram 2013).

Characteristics of Pogonomyrmex comanche

P. comanche is an endangered species predominantly located in the prairie like fine-grained sandy soils of Arkansas, Louisiana, Kansas, Oklahoma, and Texas (Cole 1968). According to the Texas Natural Diversity Database (TXNDD), *P. comanche* has a state rank of S2 (imperiled) and a global rank of G2G3 (imperiled/endangered). Within the Californicus complex, there are roughly 5 other *Pogonomyrmex* species that do not fall on the TXNDD list: *P. anzensis*, *P. badius*, *P. californicus*, *P. magnacanthus*, and *P. maricopa* (Mayo 2015). In the regions of Texas, Oklahoma, Louisiana and Arkansas this species is found in the Cross Timbers or Post Oak Savannah Ecoregion, which lends to their discontinuous distribution (Mayo 2015; Wheeler 1914). The nest shape of *P. comanche* follows the general characteristics of the genus, a disk-like crater with a single entry and exit opening. Aside from the general characteristics, there is not much information on the nature or general history of this species. In order to initiate a conservation effort, there needs to be a general understanding of how this species mates and what factors contribute to the overall distribution.

The use of microsatellites in conservation efforts

Microsatellites, or short tandem repeats, are non-coding repetitive motifs about 1 to 6 nucleotides found within DNA (Oliveira et al. 2006). In the field of conservation, microsatellites provide information that allow scientists to identify conservation units and to investigate the genetic processes that take place in a population such as gene flow and genetic drift (Heywood & Iriondo 2003). The initial development of microsatellites can lead to considerable investment of time and money (Freeland, Kirk, & Petersen 2011). To

begin, random fragments of DNA are cloned into a library and screened with a microsatellite probe; clones that contain microsatellites are then isolated and sequenced (Freeland, Kirk, & Petersen 2011). The sequences and primers created from this process can sometimes be used on closely related species to generate data. One important feature of microsatellites occurs during replication. A malfunction in polymerase can create high degrees of length polymorphisms within populations of a single species (Butler et al. 2014). The variations found within a population can be biallelic, and due to their heritable quality, they create trackable variation within members of the same population (Oliveira et al. 2006). High levels of polymorphisms make microsatellites suitable for inferring relatively recent population genetic events (Freeland, Kirk, & Petersen 2011).

Mitochondrial DNA

Within animals, mitochondrial DNA (mtDNA) contains 13 protein coding genes, 22 types of tRNA, and 2 types of rRNA (Freeland, Kirk, & Petersen, 2011). Inside this small genome, the sequences are subject to high rates of mutation, limited recombination, non-repetitive, and maternally inherited, making them great for determining genetic relatedness between individuals of a population (Davolos & Maclean 2005). One of the biggest obstacles in using mtDNA is selecting the appropriate marker for analysis (Łukomska-Kowalczyk et al. 2016). The mostly commonly used mtDNA markers for animals are Cytochrome Oxidase I and Cytochrome Oxidase II (Freeland, Kirk, & Petersen, 2011). With the limitations in morphology-based identification systems, this approach has become increasingly popular in molecular systematics. The use of micro-genomic identification system allows for the identification and differentiation of various

species (Cywinska et al. 2003). DNA barcoding uses objective DNA to identify species and has begun to take hold as a useful tool in biological systematics. Through the use of short sequences of specific genomic regions, each species has a unique barcode making it possible to detect genetic variation between species (Cui et al. 2017).

Ecological modeling

In recent years species distribution models (SDMs) has become an important tool for ecologists and biogeographers (Saupe et al. 2012; Mota-Vargas & Rojas-Soto, 2016). The model has been developed to assist with a vast range of fields such as: biogeography, conservation biology, climate change research, and habitat management. SDMs are commonly used to predict the geographic ranges of individuals based off of species occurrence probability and environmental estimates (Elith & Leathwick 2009) To understand the habitat limitation for this species, an SDM was created. Through the use of statistical functions and GIS, SDMs can link the spatial distribution of a species to the spatial variation in environmental parameters (Guisan & Zimmermann 2000; Miller 2012). These models commonly use correlations between the selected environmental elements and coordinates of known locations of the species. With all of these variables accommodated, species distributions and potential habitats can be predicted from the outputs of the SDMs (Linder et al. 2012; Elith & Leathwick 2009).

Aims

To accomplish the analysis of the life history of *P. comanche*, three aims were put in place. First, in order to assess the genetic diversity and population differentiation in regard to habitat fragmentation and dispersal potential, a population genetic analysis was conducted using mitochondrial gene markers. Second, an intra- and intercolonial genetic analysis was performed using microsatellite markers from two closely related species to investigate the mating biology and dispersal potential of females within Camp Swift in Bastrop, TX. Third, a species distribution model was created using location points, climate and soil variables and MaxEnt to identify habitat suitability in the ARK-LA-TX-OK region.

Chapter 2
Mitochondrial Analysis

Materials and Methods

Specimen collection

On October 14, 2016, 21 colony samples were collected from Camp Swift in Bastrop TX. From each colony 10-20 individuals were collected with metal tweezers and placed into 100% ethanol for preservation. Each vial was labeled with a number to signify a different colony. May 19 -20, 2017 10 individuals from six colony samples were collected from Miller County Sandhills Natural Area in Arkansas; and 10 individuals from five colonies were collected from Arkansas Oak Natural Area. The individual ants were collected in the same manner conducted at Bastrop, Texas. From a separate trip to Norman, Oklahoma on May 23, 2017, 5 individuals from 4 colonies were collected and preserved in ethanol. On July 20, 2017, 4 individuals from 12 colonies were collected and preserved in ethanol from Lindsey Park in Tyler, Texas. On September 28, 2017, 1 colony was located at Camp Maxey in Lamar county. Two individuals were collected and placed in ethanol for preservation. From all the locations sampled, all colonies would be used in mitochondrial analysis; but only the colonies from Bastrop TX would be used for microsatellite analysis.

DNA extraction

Eight individuals were randomly selected for DNA extraction from the Bastrop colonies; for all other colonies two 2 individuals were randomly selected. The DNA extraction was done using QIAmp DNA micro kit (QIAGEN).

Mitochondrial DNA Analysis

The extracted DNA was used for PCR sequencing with a cytochrome c oxidase subunit I, C1-J- 2195 (alias CO1-RLR) (5¢-TTGATTTTTTGGTCATCCAGA AGT-3¢) (Crozier & Crozier, 1993). The polymerase chain reaction amplifications were performed with 10 μl of reaction volume. The reaction mix contained 2 μl genomic DNA, 3.7 μl H2O, 1 μl 10X Buffer, 0.8 μl dNTP, 0.8 μl MgCl2, 0.4 μl BSA, 0.6 μl COI primer, 0.6 μl COII primer, 0.1 μl Taq. All loci were amplified using the following parameters: 2 mins at 94 °C, 38 cycles of 1 min at the denaturation temperature of 94 °C, 1 min at the annealing temperature 50 °C, and then 2 mins at the extension temperature of 68 °C. After this cycling, an extension was held for 5 min at 72 °C until it's holding temperature of 12 °C.

The PCR products were sent off to the DNA sequencing facility in Austin TX in 10 μl quantities (https://icmb.utexas.edu/dna-sequencing-facility). Along with the samples, 2 μl of a 2 μM concentration of the COI primer was included for each sample.

Raw mitochondrial data processing

Geneious R10 (http://www.geneious.com, Kearse et al., 2012) was used to trim and align all 100 *P. comanche* sequences and 1 *P. barbatus* sequence obtained from a previous collection trip was used as the outgroup. The sequences were trimmed from about 1,360 base pairs to 776 base pairs to eliminate the noise at the beginning and end of the sequences. To assess the nucleotide base calls, the chromatogram was visually assessed to make adjustments on uncalled nucleotides. Once all the sequences were cleaned and reviewed, they were aligned using MUSCLE (Edgar 2004) with a maximum number of 8 iterations and the resulting alignment was exported as a fasta file. Because

all of the collection sites are grouped into four general regions, they were assigned a region to perform the phylogenetic and haplotype analysis. This allowed for a broader look at the overall movement of genes within the areas collected.

Phylogenetic analysis of mitochondrial DNA

The fasta file with all the sequences was converted to a phylip file format to be used in constructing a phylogenetic tree. jmodeltest (Darriba et al. 2012) was run to determine which evolutionary model fit the data best. HKY+I+G was assigned and the program PhyML (Guindon et al. 2010) was used to generate a maximum-likelihood tree with 1000 bootstrap replicates. The output from this process was visualized in FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Haplotype network analysis

The aligned COI sequences used to construct the phylogenetic tree were also used to generate the haplotype network. This process was completed using R (RStudio Team 2016) utilizing the packages ape (Paradis et al. 2004), adegenet (Jombart & Ahmed 2011), and pegas (Paradis 2010). All the individuals were grouped together by their location so that the haplotype would show the relation between individuals in each population (Table 1.).

Table 1. A list of the number of colonies collected from each collection site and the GPS coordinates associated with them. Abbreviations used in the phylogeny provided as well as the region (central Texas, North East Texas, Arkansas, and Oklahoma) in which each collection site is located.

Region	Abbreviation	Location (Place, County, State)	GPS Coordinates	Number of Colonies
CTX	Cald	Ecolab Caldwell, Bastrop, TX	29°50.319'N -97°25.994'W	1
CTX	BTX	Camp Swift, Bastrop, TX	30.291937°N -97.270956°W	18
CTX	JSB	Jarvis, Wood, TX	29.9285° N -97.3636°W	1
NETX	JP	Ecolab Red Rock, Bastrop, TX	32.5907°N -95.1833°W	1
NETX	Hawk	N/A, Hawkins, TX	N/A	1
NETX	LP	Lindsey Park, Smith, TX	32.3105° N -95.3777° W	11
NETX	CM	Camp Maxey, Lamar, TX	33.781655, N -95.534978 W	1
AK	AORN	Arkansas Oak Nature Reserve, Nevada, AR	33.65808° N -93.17229° W	6
AK	Mill	Miller County Sandhills, Miller, AR	33.19354°N -94.02781°W	7
OK	Norm	N/A, Norman, OK	N/A	5

Analysis of Molecular Variance

Arlequin3.5 (Schneider et al. 2000) was used to conduct two AMOVAs on the aligned COI sequences obtained from the collection sites (Table 1). The first AMOVA was used to quantify the genetic diversity within each collection site (calculating the differences between the colonies at each collection site) and among the collection sites (calculating the genetic diversity between each location). The second AMOVA was conducted on the regions. With four major regions the genetic diversity was calculated among each region (CTX, NETX, AK, and OK), and within each region (calculating the genetic diversity found among each collection site located within the region). Arlequin

was set with the following parameters for both AMOVAs: 1000 permutations, determining the minimum spanning network among haplotypes, computing distance matrix and pair-wise difference with a gamma value of 0.

Population expansion analysis

To test for population expansion the Tajima's D neutrality, Harpending's raggedness, and Fu's F statistics were calculated use the software DNAsp 6.11.01 (Julio 2017). Tajima's D and Fu's F statistic test have a null hypothesis that a population is operating under neutrality. A negative value to these tests signifies an excess of low frequency polymorphisms which could indicate a recent bottleneck or recent expansion. A positive value signifies low levels of polymorphisms which indicates population contraction. Harpending's raggedness statistic tests the null hypothesis of a star-like expanding phylogeny. A significant p-value signifies that the population does not follow the null hypothesis, whereas a nonsignificant p-value signifies an outward expanding population.

Results

DNA sequences with 776 bp of the mitochondrial COI gene were obtained from a total of 100 specimens of *P. comanche*. Sequences have been deposited to NCBI Genbank under accession numbers MH193071 - MH193170.

Phylogenetic Analyses

Assessing the population structure of the four major regions of P. comanche ants, Arkansas (AR), central Texas (CTX), North East Texas (NETX) and Oklahoma (OK). They phylogeny (Figure1) generated on the four regions show that there are strong geographic patterns between both the AR and OK colonies with NETX colonies. CTX colonies can be seen throughout the tree isolated into singular clade as well as being closely related to the other regions.

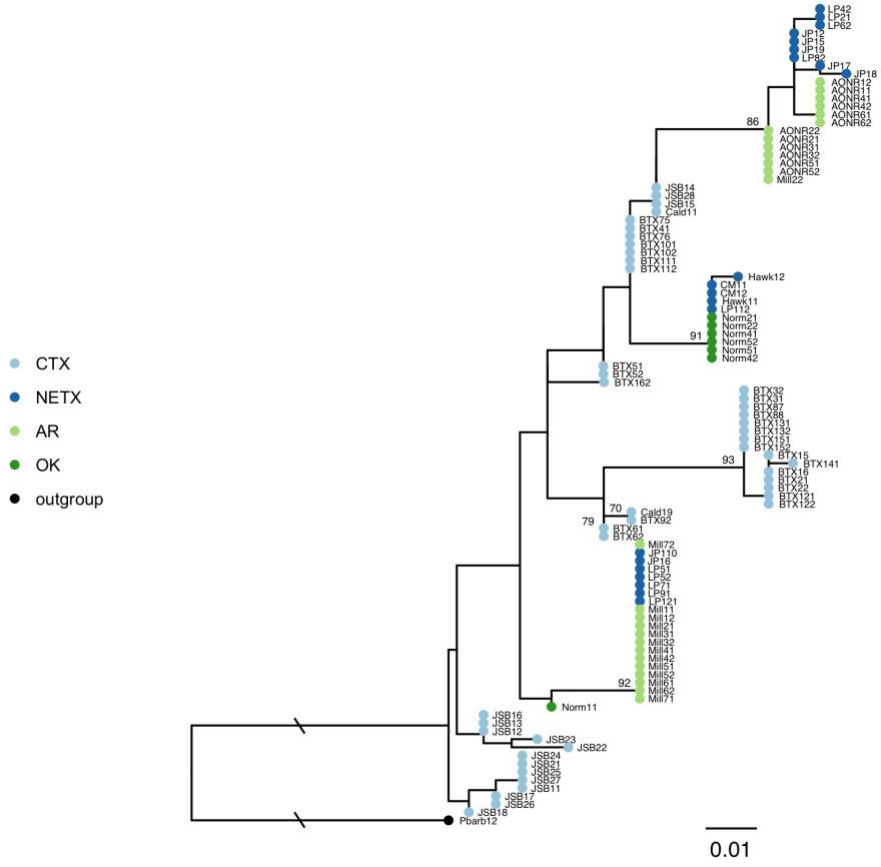

Figure 1. Maximum-likelihood phylogenetic analysis on 10 populations of *Pogonomyrmex comanche* and an outgroup of *Pogonomyrmex barbatus*. Numbers at each node indicate bootstrap values from a maximum likelihood (ML) analysis. The values are out of 1000 replicates. The following collection sites are grouped by regions: CTX (central Texas), NETX (North East Texas), AR (Arkansas), OK (Oklahoma); AONR (Arkansas Oak Nature Reserve), BTX (Bastrop, Texas: Camp Swift), Cald (Bastrop, Texas: Caldwell County), Hawk (Hawkins, Texas), JSB (Bastrop, Texas: Ecolab Red Rock), JP (Wood, Texas), LP (Tyler, Texas: Lindsey Park), Mill (Miller, Arkansas: Miller County Sandhills), and Norm (Norman, Oklahoma).

Haplotype Analysis

From the 100 *P. comanche* mitochondrial sequences separated into their 4 major regions, there are 25 unique haplotypes. There are 14 unique haplotypes within the CTX region showing the most diversity of all four regions. The other three regions share identical haplotypes, or they are separated by a few variations within the haplotype.

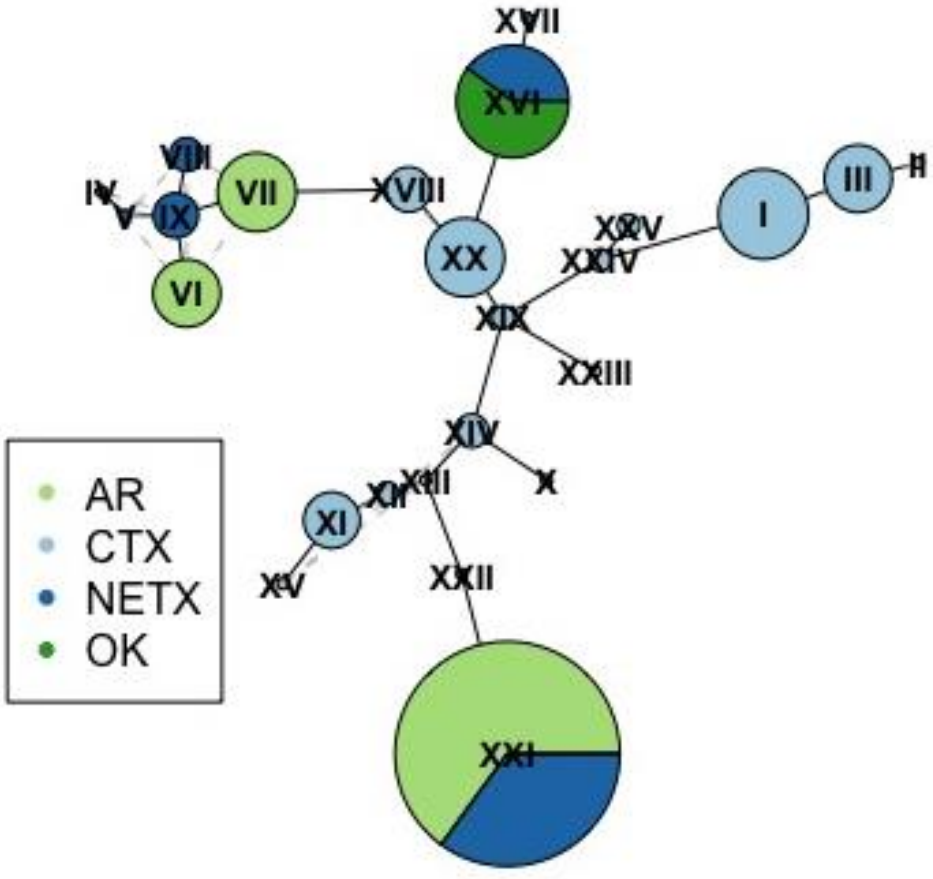

Figure 2. Haplotype network of mitochondrial DNA from collected locations of *Pogonomyrmex comanche* grouped together in four regions. A different color is associated with each location, and the size of the circle is indicative of the number of individuals with that specific sequence.

AMOVA Analysis

Two AMOVAs (Analysis of Molecular Variance) were calculated through the use F-statistics to assess the population structure. The AMOVA provides the percentage of molecular variation that was found among individuals of a population, as well as between the populations themselves. The AMOVA conducted for the collection sites had an F_{st} reported for the variation among populations is 0.547 and a significant P-value = 0.000. The AMOVA conducted for the regions had an F_{st} reported for the variation among the four regions is … and a p-value of ….

Table 2. AMOVA results from100 mitochondrial sequences for *P. comanche* species analyzing the genetic variation among and within each collection site.

Source of Variation	d.f.	Percentage of Variation
Among Populations	10	54.70
Within Populations	89	45.30
Total	99	
Fixation Index	F_{ST} : 0.54704	
Va and FST	P (rand. value > obs. Value) = 0.0000	
	P (rand. value = obs. Value) = 0.0000	
	P-value = 0.0000 ± -0.0000	

Table 3. AMOVA results from 100 mitochondrial sequences for P. comanche species analyzing the genetic variation among and within each region.

Source of Variation	d.f.	Percentage of Variation
Among Populations	3	21.26
Within Populations	96	78.74
Total	99	
Fixation Index	F_{ST} : 0.21265	
Va and FST	P (rand. value > obs. Value) = 0.0000	
	P (rand. value = obs. Value) = 0.0000	
	P-value = 0.0000 ± -0.0000	

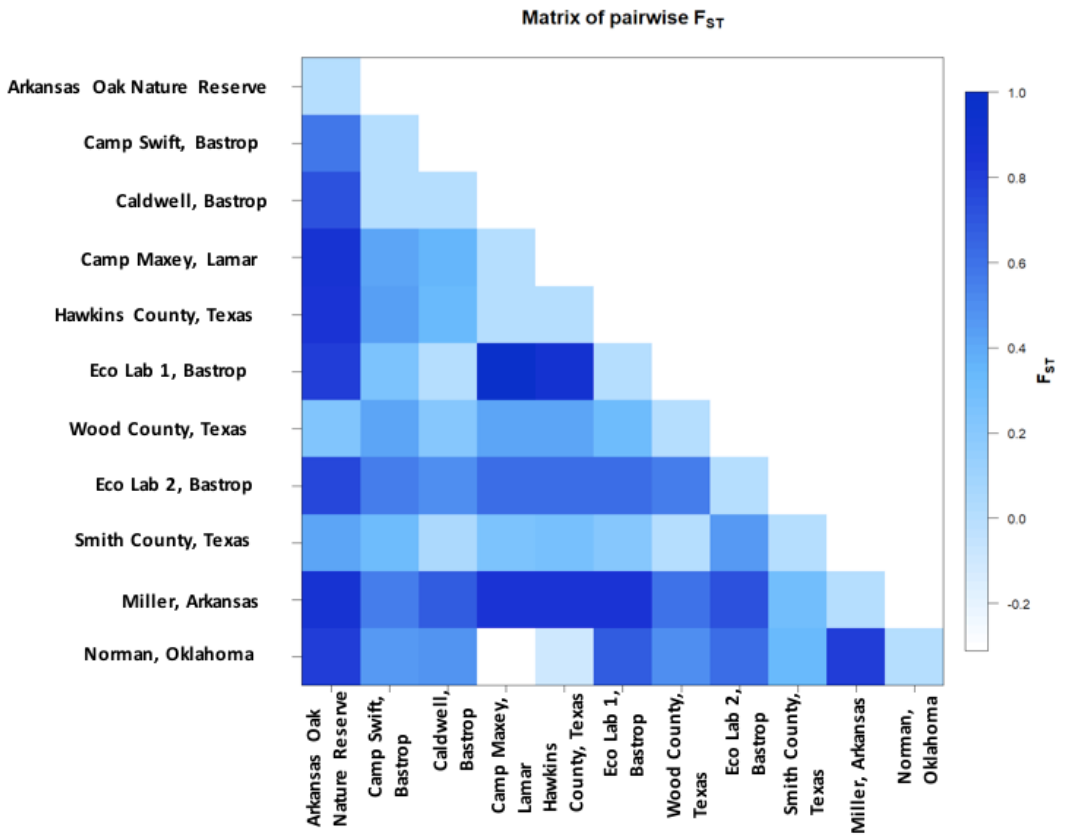

Figure 3. Matrix of pairwise F_{st} on the mitochondrial data from the AMOVA conducted on the collection locations of *P. comanche*. This figure shows the genetic similarity between each collection location where 1 (dark blue) indicates no genetic similarity and 0 (white) indicates identical sequences are present between the individuals of the collection sites.

Population Expansion Analysis

Statistical evidence shows that the populations of *P. comanche* ants are operating under neutrality because both the Tajima's D and Fu's F have negative values with insignificant p-values (Table 3). Harpending's raggedness statistics indicates that the populations are not expanding because the p-value is significant, therefore, this null hypothesis is rejected.

Table 4. Values from the three neutrality tests run on the mitochondrial DNA.

Tajima's D Test	Fu's F	Raggedness Statistic
P = 0.91	P = 0.602	P = 0.0000
D = -0.14	Fs = -0.62	R = 0.037

Chapter 3
Microsatellite Analysis

Materials and Methods

P. barbatus and P. rugosus microsatellite primers and PCR protocol

Of the 11 primers used, 9 of them were created for *P. barbatus* (Gadau et al. 2000) and the other two were created for *P. rugosus* (Volney & Gordon 2000) (Table 1). To test if the primers would work with this species a trial run of 11 markers was conducted. Through this trial it was determined that markers 1, 2, 3, 4, 8, and 9 from *P. barbatus* (PB1, PB2, PB3, PB4, PB8, PB); and marker 2 from *P. rugosus* were monomorphic when tested on *P. comanche*. Markers 5, 6, 7, and 10 from *P. barbatus* (PB5, PB6, PB7, and PB10); and marker 1 from *P. rugosus* (PR1) did work with previously obtained specimens of *P. comanche*. However, primers PB5, PB6, and PB10 were unable to be fully amplified when tested with the 144 specimens from Camp Swift, leaving only PB7 and PR1 to yield usable results. The markers used did not have a fluorescent label attached; because of this, the PCR was modified to use the universal fluorescent-labeled M-13 primer. This process involved using the same 11 markers discussed previously, but with the addition of a specific sequence placed at the beginning of the sample. This sequence was added to enable the fluorescent marker FAM M-13 to locate the sequence and bind, giving each sample the fluorescence needed to be visualized. Each PCR was performed in accordance with Shuelke (2000) with three primers: the sequence specific forward primer with M-13(-21) tail at the 5' end, the sequence specific reverse primer, and the universal fluorescent-labeled M13(-21) primer. To attach the fluorescent marker, the PCR settings were adjusted so that during the first cycle the forward primer with the M13(-21) sequence can attach to the accumulating

sequences. Once the M13(-21) forward primer is attached, a second annealing temperature is used to bind the fluorescent FAM M13(-21) marker to the M13 sequences (Shuelke 2000). The forward M13(-21) primer sequence is 5'-TGT AAA ACG ACG GCC AGT, and the FAM labeled fluorescent M13(-21) marker sequence is: FAM - TGT AAA ACG ACG GCC AGT (Schuelke, 2000).

The polymerase chain reaction amplifications were performed with 13.65 μl of reaction volume. The reaction mix contained 1.5 μl genomic DNA, 2.87 μl H2O, 1 μl 10X Buffer, 1 μl dNTP, 1.5 μl MgCl2, 0.5 μl BSA, 0.6 μl M13 Labeled primer, 0.10 μl Taq, 1 μl reverse primer, 0.03 μl forward primer with tail. All loci were amplified using the following parameters: 30 cycles of 5 mins at the denaturation temperature of 94 °C (plus an additional 30 secs), 45 seconds at the annealing temperature 58 °C, and then 30 seconds at the extension temperature of 74 °C. This amplification process required an additional step to bind the M13 tail which required 8 cycles of 30 secs at the denaturation temperature 94 °C, 45 secs at the annealing temperature 53 °C, and then 45 secs at the extension temperature 72 °C (plus an additional 10 min.).

The PCR products were sent off to The University of Texas at Austin Genomic Sequencing and Analysis Facility in 2 μl quantities (https://icmb.utexas.edu/dna-sequencing-facility). Fragment analysis was performed on the samples and the standard Liz600 was added to each sample at the facility to provide a ladder for comparison.

Raw microsatellite data clean-up

Geneious R10 (http://www.geneious.com, Kearse et al., 2012) was used to analyze the sequences received from the DNA sequencing facility. Before any alleles could be marked, the ladder was checked to make sure it was called correctly. For each

sample, the ladder was adjusted to ensure the most accurate results. With all the ladders present and adjusted, the locus info was set to identify the peak calls on each sample. This allowed for the peaks on each sample to be categorized into a specific sequence range. If the peaks fell within this range, they were collected into bins, and exported into a spreadsheet to be analyzed.

Microsatellite analysis

To ensure the primers selected for use would provide usable data, the program Genetic Data Analyzer (GDA) (Lewis & Zaykin 2001) was used to calculate expected and observed heterozygosity of each individual collected at Camp Swift. The software Arlequin3.5 (Schneider et al. 2000) was used to test for genetic differentiation within and among colonies in Camp Swift. The software MATESOFT v. 1.0 (Moilanen, Sundstrom & Pedersen 2006) was used to examine for evidence of polygyny and polyandry, and to calculate queen mating frequencies.

Results

Out of the 11 primers tested, 2 were used in this analysis: primer PB7 and PR1. GDA reported from the 144 samples collected in Camp Swift, primer PB7 has 9 alleles and primer PR1 has 7 alleles. The expected heterozygosity was 0.718 and the observed heterozygosity was 0.636.

MATESOFT determined that 5 out of the eighteen colonies were determined to be polygynous, these colonies were excluded for the rest of the analyses. From the remaining colonies, the double mating estimate (D. Est) and the double mating observed

(D. obs) showed that 72% of the colonies exhibit monogyny and have mated with at least two males. The mating frequency was 1.6 with a non-detection error (probability of two males having the same phenotype) of 0.

The AMOVA results indicated that there is no real genetic diversity between the colonies collected at Camp Swift, with an Fst of 0.16646 and a significant p-value of 0.0000.

Table 5. AMOVA results for P. comanche colonies collected from Camp Swift using 2 polymorphic loci.

Source of Variation	Percentage of Variation
Among Populations	16.64604 (Within Camp Swift)
Within Populations	83.35396 (Within colonies because they are half sisters)
Fixation Index F_{ST} : 0.16646	
Va and FST	P (rand. value > obs. Value) = 0.0000
	P (rand. value = obs. Value) = 0.0000
	P-value = 0.0000 ± -0.0000

Chapter 4
Ecological Modeling

Materials and Methods

Study design and modeling

The SDM was performed on the species *Pogonomyrmex comanche*. The study focused on the climate and soil data associated with the known occurrence points of this species. Out of 37 variables, 19 climate variables were obtained from (www.bioclim.org) and 18 soil variables were obtained from the State Soil Geographic (STATSGO) Data Base (United States Department of Agriculture, 1994). To establish which variables best suited the occurrence points present, all 37 layers were converted to raster format in GrassGIS (http://grass.osgeo.org.). All the rasters were sampled to have a common resolution of 30 seconds in the NAD 1983 UTM Zone 15 N projection using a geographic (XY) coordinate system with meters as the unit. The environmental variables were cut to the extent of ARK-LA-TX-OK. Using R, they were run through a correlation test and from the results any variable with a value higher than 0.65 or lower than -0.65 was eliminated. From this initial elimination, 18 layers were kept and uploaded to MaxEnt with the GPS coordinates of *Pogonomyrmex comanche*.

MaxEnt (Steven et al. 2018) was used to produce a geographic model of habitat suitability by assigning a logistic score to each grid cell. These scores are then interpreted as the degree of suitability of that particular location for the species, given the environmental variables provided. Upon review of the Jackknife test gain values, there were variables present that reduced the fit of the model. These variables were removed, leaving a total of 6 variables. Five uncorrelated continuous environmental layers: mean diurnal range (mean of monthly (max temp – min temp)), temperature seasonality

(standard deviation *100), min temperature of coldest month, mean temperature of driest quarter, and annual precipitation; and 1 soil variable: share of map unit with hydric soils. The analysis encompassed four states: Texas, Arkansas, Oklahoma, and Louisiana (ARK-LA-TX-OK), the occurrence data for the colonies were obtained from field collections (Table 1); the only state not represented was Louisiana.

Model validation

The MaxEnt model was validated using a "n-1" cross-validation or a jackknife approach (Pearson et al. 2007). This approach subdivides the dataset into two: the training data and the test data. Training data uses species occurrence locations to develop the model and test data uses species occurrence locations that were not used in model development. As described by Pearson et al. (2007), the jackknife method builds a model by removing one observed locality or (n-1); where n is the observed species localities. For a species with n observed localities, there are n separate models built for testing. From there, the predicative performance is assessed based on the ability of each model to predict the single locality excluded from the training data set.

The model was validated using the test area under the operator receiving curve, or AUC. The AUC measures the probability that a randomly chosen presence site will be ranked above a randomly chosen pseudoabsence site (Philips & Dudik 2008).

Results

Model validation

The test AUC value for the model run with 18 layers was 0.738. When the unfit variables were removed, the test AUC for the model was 0.804. To be considered a sufficiently useful model, the AUC value must be greater than 0.75 (Elith 2002). The second model run with fewer layers is a sufficiently useful model.

Areas of high suitability

The species distribution model is considered to be fairly good, with a produced Area Under the Curve (AUC) value of 0.804. The AUC value is used to determine how accurate the predictive model is given the variables and known occurrence points provided to MaxEnt. Based on the model (Figure 4), most of the area that *P. comanche* could potentially be found is in the Texas Post Oak Savannah comprising 31 counties; along the borders of Texas Hill Country and the Oak Prairie Wild Life District, and along the border of Texas and Louisiana in the Pineywoods wildlife district.

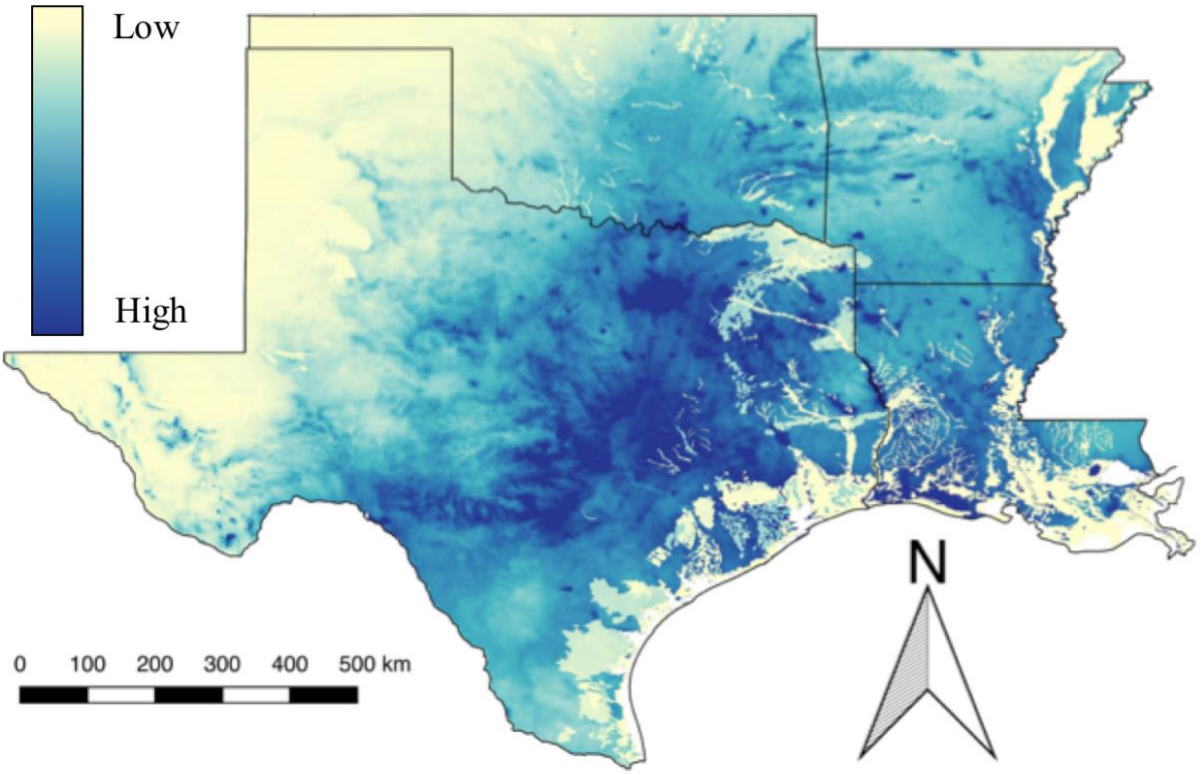

Figure 4. Species distribution model using 5 environmental variables and 1 soil variable with known occurrence points of *Pogonomyrmex comanche*.

The importance of individual environmental variables to the model

For the model, Bio6 (minimum temperature of coldest month) (Figure 5) was the most important contributing variable according to the Jackknife Test Gain.

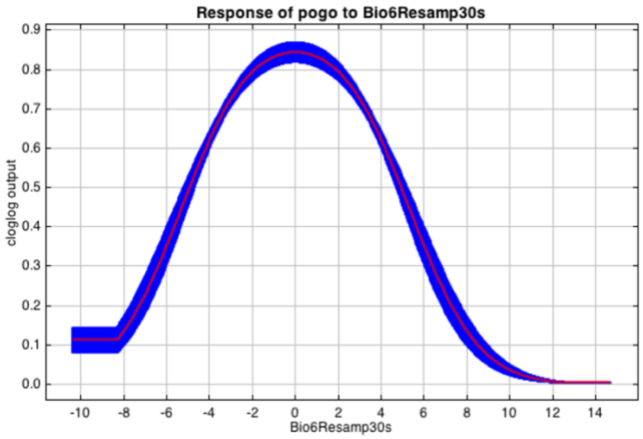

Figure 5. The response curve for the minimum temperature of coldest month from the MaxEnt model run for *P. comanche* occurrence points. Showing the minimum monthly temperature occurrence over the given year. The curves show how the predicted probability of presence changes as each environmental variable is varied, keeping all other environmental variables at their average sample value.

For the species *Pogonomyrmex comanche*, the most suitable habitats have the following characteristics:

(a) Bio2 - mean diurnal range between 3 and 11 degrees Celsius (See Figure S3 in Appendix);

(b) Bio4 - tempexrature seasonality of 500% (See Figure S4 in Appendix);

(c) Bio6 - minimum temperature of 0 degrees for the coldest month (Figure 4);

(d) Bio9 - mean temperature of 28 for the driest quarter (See Figure S5 in Appendix);

(e) Bio12 - annual precipitation of 200 (See Figure S6 in Appendix),

(f) And Ifhydric – the amount of water that saturates soils -0.1 and 0 units (See Figure S7 in Appendix).

Outside of these ranges and values the habitat suitability declines considerably.

Table 6. Summary information of each variable when only that variable is in use for the SDM of the species *P. comanche*

Species	Test AUC	Annual precipitation	Mean diurnal range	Temp seasonality	Min. temp	Mean temp	Shared hydric soil
P. comanche	0.8044	0.0205	0.1615	0.0384	0.2606	0.0167	0.113

Chapter 5

Discussion

Population structure, dispersal potential, genetic diversity, and habitat availability are the major concerns when looking at threatened species (Suni & Gordon 2010). These issues need to be fully studied in order to understand the cause of population decline. One of the major attributes of species decline is habitat loss and destruction; without a strong genetic diversity among the existing populations, the chances of reviving a species is abysmal. With *Pogonomyrmex comanche*, there is little known about the genetic diversity among and within the current populations. This research set out to discover if this species was genetically diverse enough to be revived as well as if there are any suitable habitats left for them to colonize.

Mitochondrial Analysis

The genetic diversity of the collected populations of *P. comanche* appears to be stable when looking at all the collection sites. The relationship between each colony was mapped out through a maximum-likelihood phylogeny and a haplotype network. The phylogeny (Figure 1) shows that the populations from central Texas are the most recently diverged from the outgroup *Pogonomyrmex barbatus*. Within the tree, the central Texas populations appear throughout, but they never occur in the highly supported monophyletic groupings that the other regions share. The haplotype (Figure 2) supports the idea that the central Texas populations are oldest because they are located in the center of the structure and have no shared haplotypes with the other regions. The Arkansas populations and the Oklahoma populations share identical haplotypes with the

North Eastern populations. It would appear that these populations share a close genetic relation that stems from the central Texas populations.

The AMOVA analysis on the mitochondrial COI sequences showed that at each collection site (Table 2), the genetic diversity within and among the site is high enough that these collection sites are fairly unique from one another. When looking at the diversity of the 4 designated regions (Table 3), the diversity among the four is low, but within each region there is high genetic diversity. This could indicate that overall, these populations are recently derived from one another, but each region is genetically unique driving the overall genetic diversity of this species. The pairwise F_{st} (Figure 3) breaks down the genetic similarity of each collection site. According to the figure, the populations from central Texas have little genetic similarity with the populations from Arkansas and Oklahoma, but the relation with the North Eastern populations are a little stronger. Which adds to the idea that the central Texas colonies are the oldest and potentially the starting point of this species expansion.

The status of population expansion was addressed through neutrality tests conducted on the mitochondrial COI sequences. It was determined through Tajima's D and Fu's F that the populations are operating under neutrality, meaning there are no major selection pressures causing the changes in the sequences. This indicates that each haplotype is equally represented among the populations, and homozygosity is not a current threat to their survival. Not only are these populations evolving neutrally, but they are not recent expansions. The Harpending's raggedness statistic shows that these populations are not exhibiting a star-like expansion, but that they are established and stable populations. From these analyses it's apparent that these populations are not recent

arrivals, and they are significantly diverged from one another, aiding in the thought that genetic diversity is not the major cause of decline within this species.

Microsatellite Analysis

P. comanche follows the trend of polyandry that is found within the genus of *Pogonomyrmex*. Most species found in this genus are known to be highly polyandrous; where the queens, on average, mate with 5 to 18 males (Holbrook et al. 2007). Although this research shows that 72% of colonies within Camp Swift are monogynous, mating with at least two males, the use of only two primers lowers the power of these results. From the data obtained, it's apparent that the workers are half-sisters due to multiple-mating, which is in accordance with other species found in *Pogonomyrmex*. Increased genetic diversity within a colony has many hypothesized advantages: reducing disease transmission (Holbrook et al. 2007), impacting task allocation and the division of labor within the nest (Curry et al. 2009), and it can provide a buffer against environmental variability (Page et al. 1995).

For the colonies at Camp Swift, the AMOVA indicates that there is not much variation within the population or between the colonies. This indicates that neighboring colonies within this area are most likely related, which could be a sign that the queens are not dispersing very far after the nuptial flight. This also shows that there is not much, if any, gene flow occurring for this population.

To fully understand the mating potential of the queens in *P. comanche*, more primers would need to be developed and a larger sample size obtained to accurately represent the individuals within Camp Swift. Although the primers used were highly

polymorphic, there is not enough evidence to fully support or understand the role of polyandry within this species. The small insight obtained from this study is a good starting points, but genetic monitoring should be continued over time to make sure that the genetic diversity within this species stays consistent. Understanding how dispersal potential and polyandry aid to the survival of this species is key to maintaining their populations.

Ecological Modeling

Habitat destruction and climate change are considered to be the leading causes of species decline (Wake & Vredenburg 2008; Bellard et al. 2014), it is no surprise that this threat also faces *P. comanche* populations. There is a growing use of species distribution models to understand species-environment interactions (Chapman et al. 2017). The species distribution model created here shows that the most suitable habitats for P. comanche coincide with the most populated and colonized areas of Texas. Leading to the idea that this species is in decline due to habitat loss and destruction. The soil and climate variables used to generate this model indicate that temperature and dry soil are the most important variables to this species.

With an AUC of 0.804 the model fitness is considered to be fairly good. The most important variable, mean temperature of the coldest month, has the most useful information by itself compared to the other variables used. From this model and the layers used, warmer temperatures with less variation over time are best suited for this species. This species can survive in drier conditions as they obtain water through the metabolism of the seeds they eat (Mayo 2015), which explains the rain and soil variables

used for this model. These variables might change as climate change continues to shape the environment, and as these variables get updated more models can be run to track the distribution of this species.

The next step in validating this model would be to go out and look in the areas where it is predicted to have high suitability. Through my collection experience this species thrives in open grasslands with little to no tree coverage. Locating untouched areas that fit this description is imperative when discussing conservation efforts. Without suitable habitats, this species is doomed to decline regardless of the strong genetic diversity it possesses.

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Appendix

Table 7. List of microsatellite primers used with the forward and reverse sequences.

Locus	Sequence (5'-3')	Annealing temp	Site range	alleles	Species	Reference
PB1	F: CTGGAGGCAAAGAGTCACATC	57	228-286	39	*P. barbatus*	Volny&Gordon 2002
	R: CTTCTTGAATCGGTCGCTACG					
PB2	F: GTCTCATCATCTCCCTCGTTG	57	288-361	26	*P. barbatus*	Volny&Gordon 2002
	R: CGGTAATCGGCAGCAAGTG					
PB3	F: GCGACGACCAAGATGCTTC	57	289-328	33	*P. barbatus*	Volny&Gordon 2002
	R: GAGGATGAGCCACGGTGAGAC					
PB4	F: GTGGGACAACCTGTCAGAACG	57	218-275	34	*P. barbatus*	Volny&Gordon 2002
	R: CCTCGGGAATGCCTATATGAG					
PB5	F: CGCCGATGTCGCTATACC	57	221-242	20	*P. barbatus*	Volny&Gordon 2002
	R: CTCAGAAGACGCAGGAACG					
PB6	F: GCTGACGACGACTCAAATC	57	127-177	28	*P. barbatus*	Volny&Gordon 2002
	R:GATCTTATCACCACTGCTCAT					
PB7	F: CACAAGAATCAGCGACGAC	57	141-185	19	*P. barbatus*	Volny&Gordon 2002
	R: GCCAATAACACAGCCGTG					
PB8	F: GAGATGGGCAAGGAACAGGAC	57	268-297	17	*P. barbatus*	Volny&Gordon 2002
	R: GGAAGAATCTGCGGAGTGC					
PB9	F: GTCGTGAAATAATAATCAGTACG	50	320-358	25	*P. barbatus*	Volny&Gordon 2002
	R: GAACACAATAGAAATCCAGC					
PR1	F:AATCTCAGCAGGCGAGAAAG	54	335-431	9	*P. rugosus*	Gadau et al. 2003
	R: GAGAGCGTAGACGGAAATGC					
PR2	F: ACATTCGGCATACAAATGGG	55	222-249	8	*P. rugosus*	Gadau et al. 2003
	R: AGTGCGACGTTTTTCATTCC					

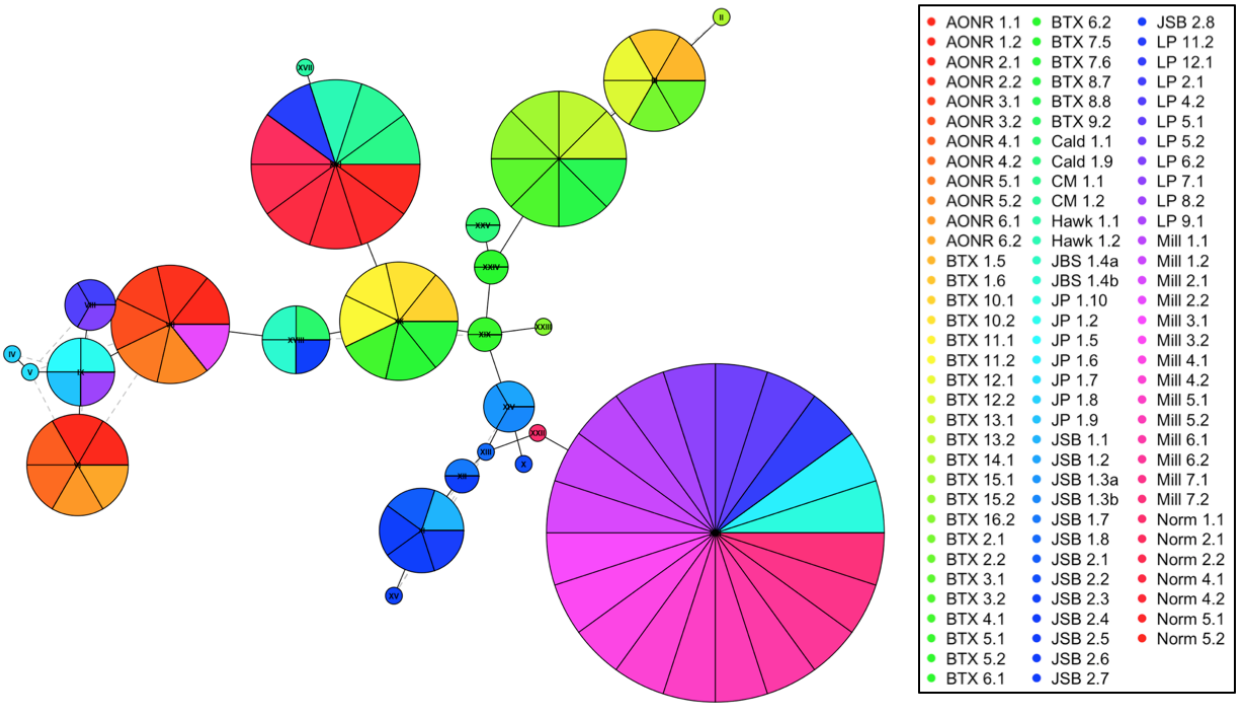

Figure S1. A haplotype network of *P. comanche* ants collected from Texas, Arkansas, and Oklahoma. Each segment of a circle represents an individual with a corresponding key for the color. Each individual is listed by an abbreviated identifier followed by the colony it was collected from and which individual it is from the colony. AONR (Arkansas Oak Nature Reserve), BTX (Bastrop, Texas: Camp Swift), Cald (Bastrop, Texas: Caldwell County), Hawk (Hawkins, Texas), JSB (Bastrop, Texas: Ecolab Red Rock), JP (Wood, Texas), LP (Tyler, Texas: Lindsey Park), Mill (Miller, Arkansas: Miller County Sandhills), and Norm (Norman, Oklahoma).

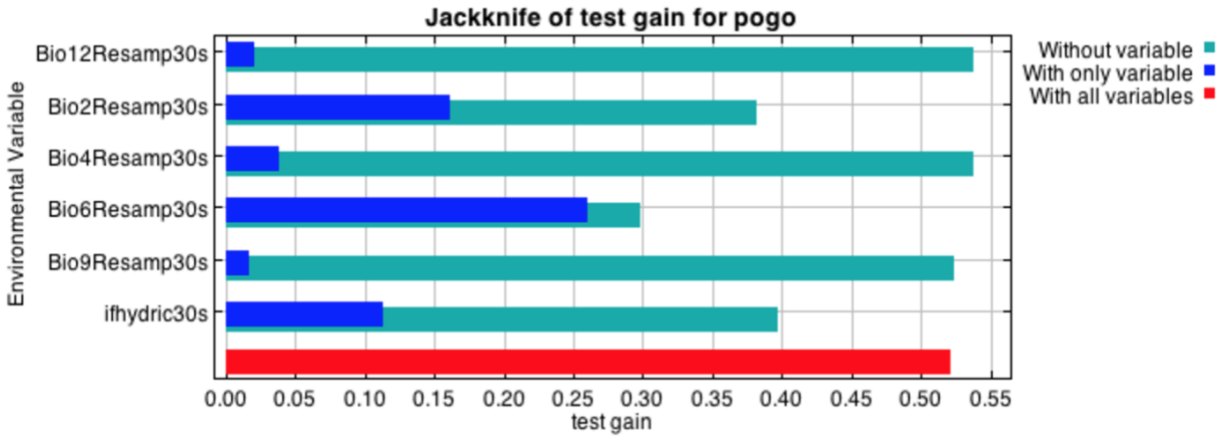

Figure S2. The Jackknife test using test gain data. The red bar shows the model AUC when all variables are used, the light blue bars show the AUC when the model is run without that particular variable, the dark blue bar shows the AUC when only that variable is used to run the model.

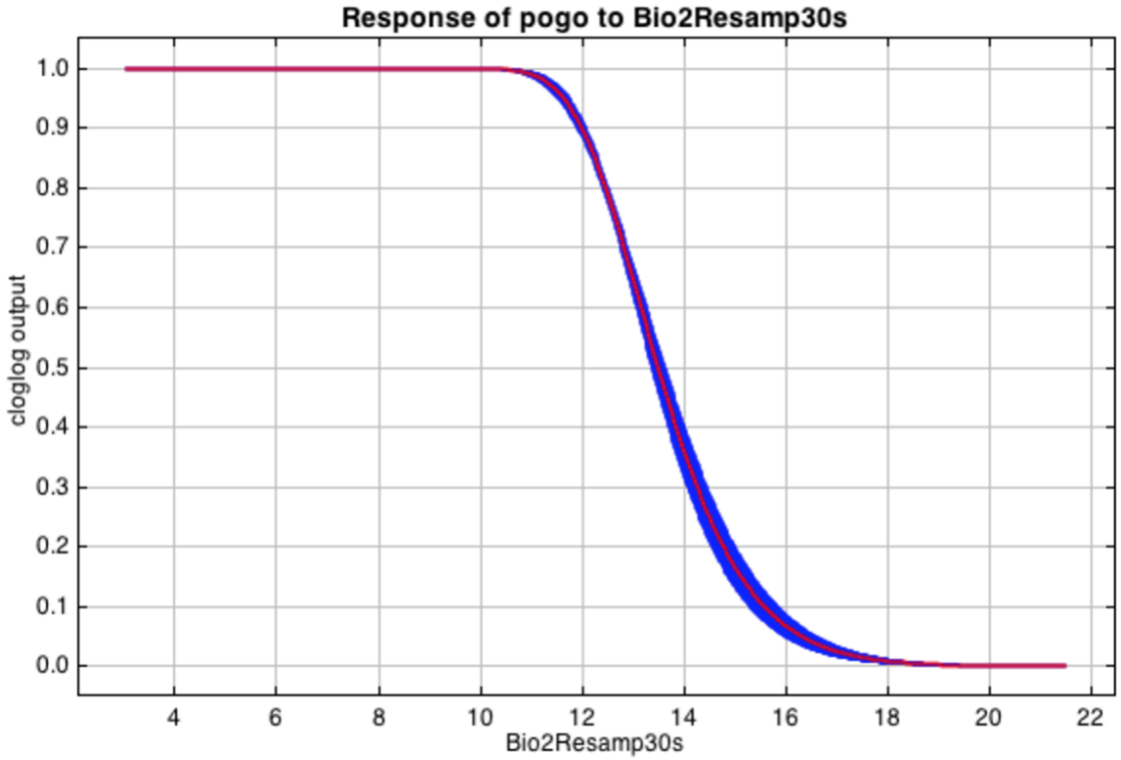

Figure S3. The response curve for the mean diurnal range of temperature for the MaxEnt model of *P. comanche* occurrence points. Showing the mean of the maximum and minimum monthly temperature ranges. The curves show how the predicted probability of presence changes as each environmental variable is varied, keeping all other environmental variables at their average sample value.

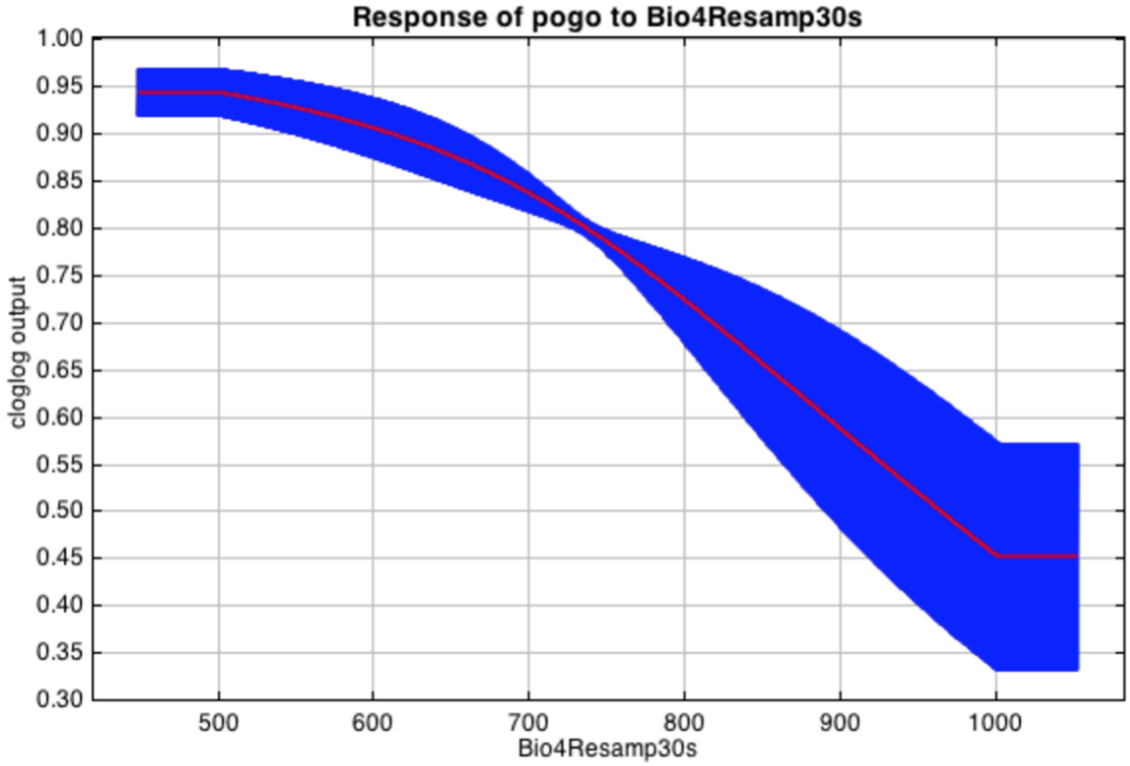

Figure S4. The response curve for the temperature seasonality (standard deviation *100) for the MaxEnt model of *P. comanche* occurrence points. Showing the amount of temperature variation over a given year based on the standard deviation of the average monthly temperatures. The curves show how the predicted probability of presence changes as each environmental variable is varied, keeping all other environmental variables at their average sample value.

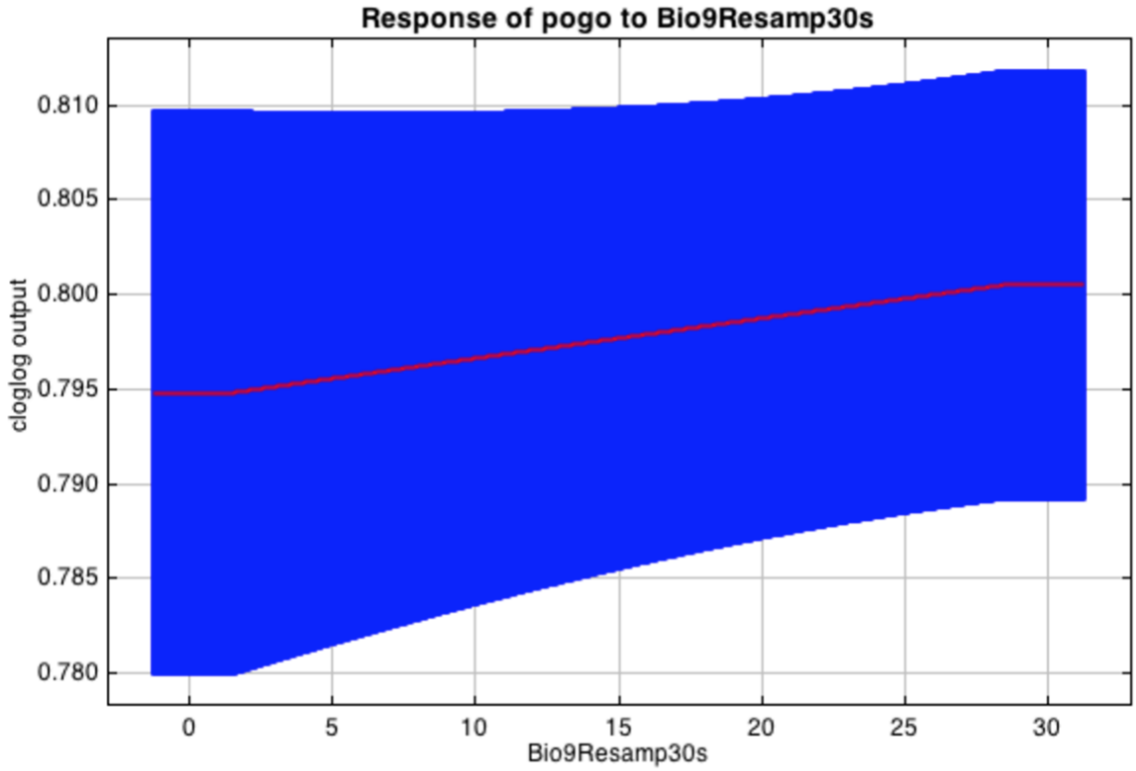

Figure S5. The response curve for mean temperature of driest quarter from the MaxEnt model of *P. comanche* occurrence points. This is a quarterly index that approximates the mean temperatures (degrees Celsius) that prevail during the driest quarter of the year. The curves show how the predicted probability of presence changes as each environmental variable is varied, keeping all other environmental variables at their average sample value.

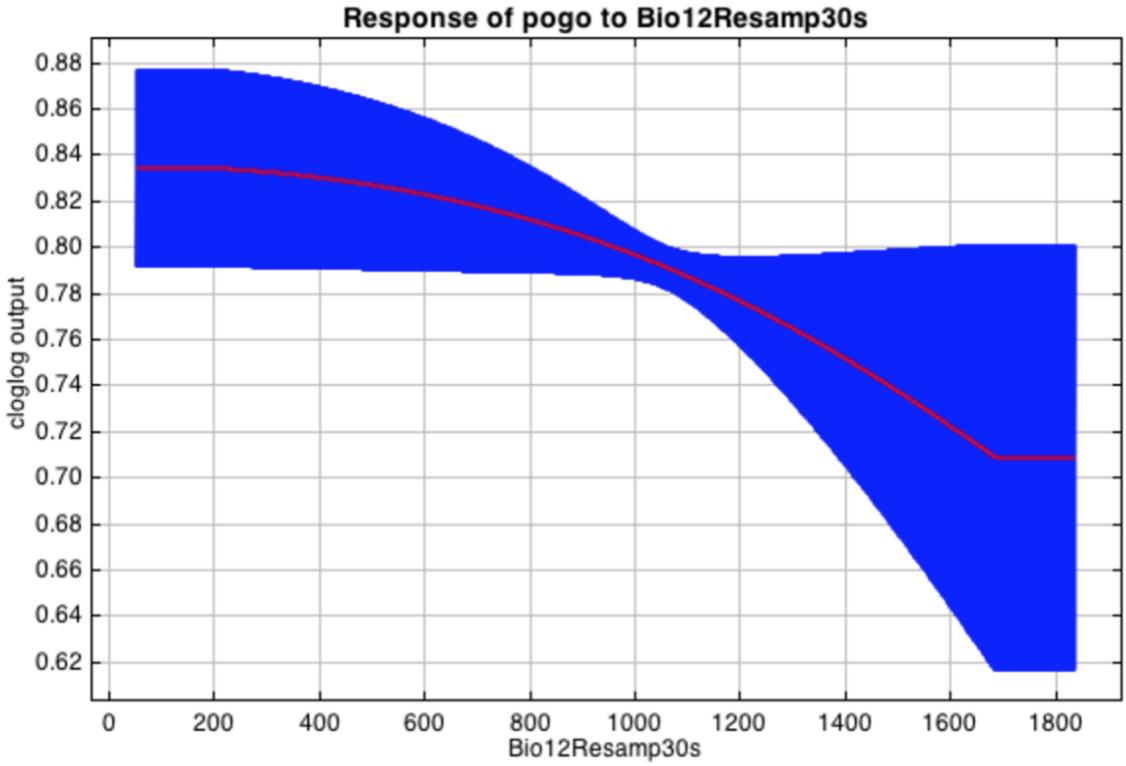

Figure S6. The response curve for annual precipitation from the MaxEnt model for *P. comanche* occurrence points. Showing the sum of all total monthly precipitation values. The curves show how the predicted probability of presence changes as each environmental variable is varied, keeping all other environmental variables at their average sample value.

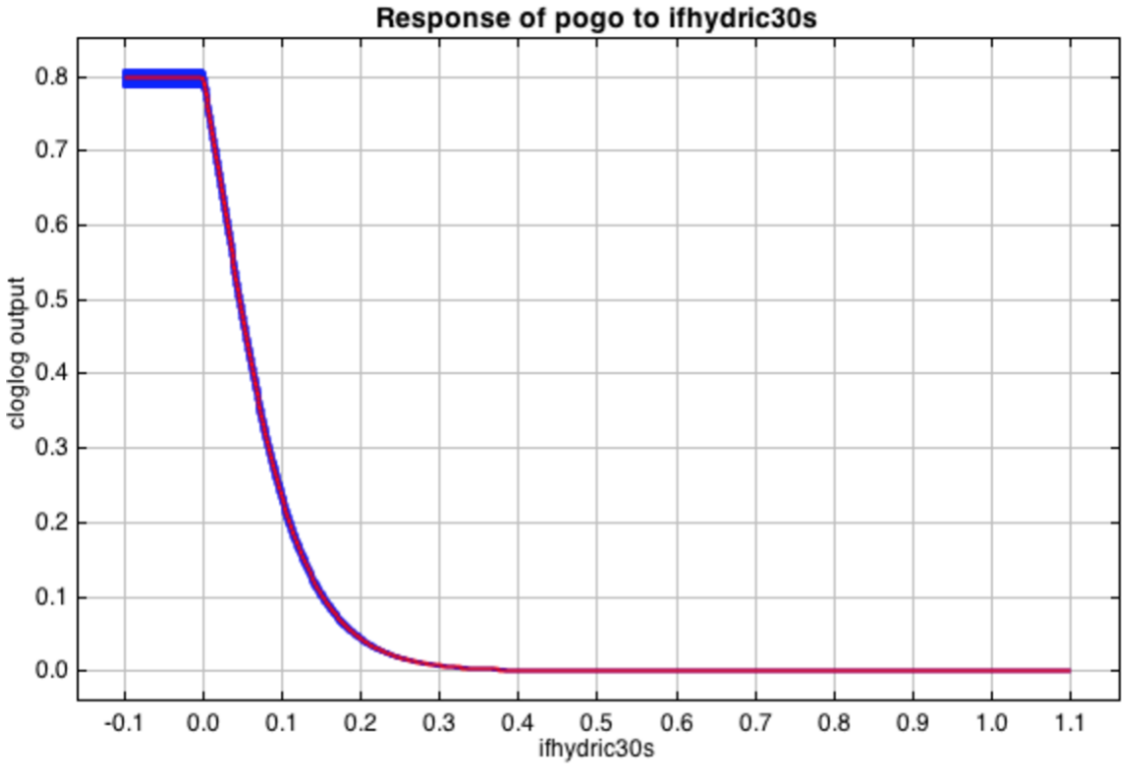

Figure S7. The response curve for the share of map unit with hydric soils from the MaxEnt model for *P. comanche* occurrence points. Showing the amount of soil saturation for the points provided. The curves show how the predicted probability of presence changes as each environmental variable is varied, keeping all other environmental variables at their average sample value.