A PRELIMINARY PHYLOGEOGRAPHIC STUDY OF THE DIPLURID GENUS *MICROHEXURA*

By

Mia M. Martens

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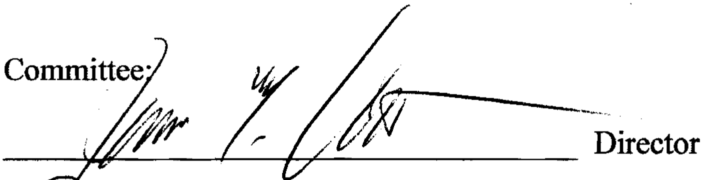

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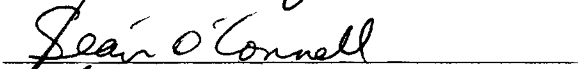

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A thesis presented to the faculty of the Graduate School of Western Carolina University in partial fulfillment of the requirements for the degree of Master of Science.

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Mia M. Martens

Director: Dr. James Costa
H.P. and Katherine P. Robinson Professor of Biology
Department of Biology

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The Sequence

A cross between a bug and a woolly worm
Was here to seep away the sap.
First, all the firs began to squirm,
Then fell to take a long nap.

A change came to the forest,
Darkness turned to light.
The firs couldn't persist
Due to this adelgid blight.

Before too long, there was no shade.
The light made the moss begin to dry,
And as their populations began to fade,
The spruce-fir moss spider started to sigh.

Habitat loss and lack of lumber
Caused questions for spiders to heed.
What is our minimum number?
How many of us do we need?

Come; let's closely compare these three things.
How's the weather, things like rain and snow?
What's the rate of death to new beings?
And finally, let's look at our gene flow.

Now with this, we compare all our info.
Put into program to get PVA.
This seems to be the only way to go,
So we can live to see another day.

---Mia M. Martens

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Abstract

A PRELIMINARY PHYLOGEOGRAPHIC STUDY OF THE DIPLURID GENUS *MICROHEXURA*

Mia M. Martens, M.S.

Western Carolina University December 2005

Director: Dr. James T. Costa

Microhexura spiders are the northernmost members of the family Diplurid, and are the smallest mygalomorph spiders, ranging from 2.5 to 5.6 mm in adult body length. There are two species, *Microhexura idahoana* and *Microhexura montivaga* (the Spruce-fir Moss Spider). *Microhexura montivaga* was placed on the endangered species list in 1995. It has been found in only seven spruce-fir forest dominated sites and occurs at or above 1646 meters. Part of the U.S. Fish and Wildlife's recovery plan for the Spruce-fir Moss Spider is to genetically compare these populations.

In this study, 13 *M. montivaga* specimens, including five from Mt. Buckley (Sevier Co., TN), five from Mt. LeConte (Sevier Co., TN), and three from Roan Mountain (Mitchell Co., NC). Six *M. idahoana* specimens from Wasco Co., Oregon (used as a comparison for *M. montivaga*) were also used for genetic analysis. One leg was removed from each field-caught spider. The spider was then observed to ensure that it was not killed during the procedure, and then released. Legs were transported to Western Carolina University where they were stored in a -80°C freezer. Subsequent

DNA extractions, PCR, and sequencing of the CO1 gene were then done on the legs. Once sequences were obtained and aligned, base frequencies, a genetic similarity matrix showing pairwise distances within and between populations, neighbor-joining trees, cladograms (showing branch lengths), and an estimate of divergence was made using the molecular clock rate of 2.3% divergence per million years.

Pairwise distances of *M. montivaga* populations showed an increase in genetic diversity between populations as geographic distance increased, with specimens from Roan Mountain (the population farthest away geographically from the other two locations) showing a greater genetic distance when compared to the pairwise distance between Mt. Buckley and Mt. LeConte. The neighbor-joining trees also showed Roan Mountain specimens to be separated in their own clade. The molecular clock data suggested that Roan Mountain specimens had been isolated from Mt. Buckley and Mt. LeConte specimens since the Pliocene era (3.48 million years ago), whereas Mt. Buckley had been isolated from Mt. LeConte during the Pleistocene (17,826 years ago). *Microhexura idahoana* diverged from *M. montivaga* during the Miocene (8.37 million years ago).

These data suggest managing the Spruce-fir Moss Spider as isolated populations with little gene flow. In order to save the Spruce-fir Moss Spider, a more extensive phylogeographic study and a plan of action on how to prevent future habitat loss and restore current habitat are needed.

Introduction

Conservation Genetics

The aim of conservation genetics is to preserve genetic diversity. In this way, biodiversity can be preserved as well. Biodiversity can be measured on a fine scale by looking at the amount of genetic diversity within populations. Avise (1994) claims, "Biodiversity (the ultimate subject of conservation interest) *is* genetic diversity." Thus, in order to preserve biodiversity, it is important to preserve genetic diversity. Biodiversity is valued for the commercial profits it may provide, moral reasons, such as the idea that species have their own intrinsic value, and what Norton (1988) refers to as "amenity value." This is when a variety of organisms enhance our life in an indirect, and admittedly subjective way, such as the beauty of a forest with many different trees as opposed to a forest of only pine trees. Biodiversity is also very important to ecosystem health. Without a wide range of species, the food web and intricate relatedness of the ecosystem may suffer.

Conservation genetics is the use of empirical genetic data for management applications (Avise and Hamrick 1996). The goals of conservation genetics are to minimize the loss of genetic variation and to preserve evolutionary processes (such as hybridization, introgression and speciation). Beardmore (1983) defines four types of genetic variation; variation resulting from mutations, inflow of genes from other

populations or species (migration), variation caused by stochastic processes, and variation caused by some form of selection.

In order to preserve biodiversity, scientists must become aware of the impact of isolation on genetic variability. When isolated, there is no movement of individuals from one population to another; therefore the variation resulting from the inflow of genes from other populations is lost.

Genetic surveys of populations provide an advantage over directly observing the movement of populations because it is not always feasible to study dispersal directly, either because the dispersal has occurred over a long time range or because species are too elusive to track (Templeton et al. 1990). If the dispersal occurred over a long time range and has stopped, it is not possible to observe the movement. Species that may be too elusive to track include those found in very small or endangered populations. Because of these populations being endangered, it would be more beneficial to be able to study only a few of the individuals, rather than the whole population.

In small populations, the amount of genetic drift and inbreeding depression is substantial. Inbreeding, when relatives mate with each other, can lead to inbreeding depression. As populations become smaller, the likelihood of inbreeding increases. Inbreeding can cause a sequence of events of which Caughley (1994) summarizes as follows:

1. The frequency of mating between close relatives rises.
2. Heterozygosity is reduced in offspring, reducing the ability of the population to respond to environmental change.
3. Semi lethal recessive alleles are expressed in a homozygous condition.

4. As a result of this expression, fecundity is reduced and mortality is increased (inbreeding depression).

5. The population becomes even smaller, amplifying the sequence initiated in step 1.

Thus, inbreeding can lead to loss of heterozygosity which can then lead to reduced numbers of offspring, lower immune responses, physical deformities and abnormalities, and reduced longevity (Dyke 2003). Inbreeding also increases the effect of genetic drift, which is likely to reduce a population's genetic variability (Templeton and Read 1994).

Genetic drift is defined as "changes in the genetic composition of a population due to random sampling in small populations" (Frankham et al. 2003). For many endangered or threatened species, genetic drift stems from a population bottleneck resulting from some event that eliminates a majority of the population. Thus, the survivors represent a small genetic sample of the original population. Often the event that causes the population bottleneck is habitat destruction. When habitat is destroyed, populations break up into small fragmented subpopulations. Without inflow of genes from other populations, genetic drift as well as inbreeding depression becomes more prominent.

The amount of genetic variability within an endangered or threatened population can have management implications. Frankel (1983) described the goal of management as "not so much to increase the population size directly as it is to reduce the rate of erosion of the genetic variance by the introduction of genotypes from other sources." It is not enough to just increase the numbers of a species present if they do not possess genetic

diversity. Without genetic diversity, many problems occur that eventually lead to lower fitness. In a study of felids, for example, Sarno et al. (2000) found that loss of genetic variability correlated with diminished physiological and reproductive abilities related to fitness. However, increased numbers of individuals can be a start on the path to recovery.

Molecular Approaches

Scientists have discovered techniques that allow for genetic surveys of populations, permitting accurate assessment of population-level inbreeding. A good way to measure genetic variability is through the use of molecular markers, such as DNA sequences. A beneficial use of molecular data is the ability to directly compare the relative levels of genetic differentiation. Loew (2000) states, "DNA sequencing provides the greatest resolution of genetic divergence by actually determining the identity and sequence of all bases within a target region." By knowing the sequence of all bases in a specific region, it is possible to compare the sequences of individuals from different populations and thus determine the degree of genetic variance among and within the populations.

Overall, in order to preserve genetic diversity, and thus biodiversity, it is helpful to first determine the amount of genetic variability present. Molecular markers using mitochondrial DNA (mtDNA) sequencing allow us to determine this. Mitochondrial DNA is an especially useful molecule for conservation genetics. It is maternally inherited and studies have found that the mitochondrial genome evolves 5-10 times faster

than the nuclear genome (Simon et al. 1994). There are three hypotheses as to why animal mitochondrial DNA evolves more rapidly; mtDNA is not complexed with histone proteins that might constrain rates of nuclear DNA evolution, there is a high mutation rate, and there is a relaxation of functional constraint within the mtDNA (Avise 2000).

Mitochondrial DNA has a relaxation of functional constraint because it doesn't code for proteins that are directly involved in its own replication or transcription. Its mutation rate is higher due to a lack of repair mechanisms and exposure to more free radicals in the oxidizing mitochondrial environment.

A popular mitochondrial gene used in gene flow analyses is the cytochrome oxidase I (COI) gene. This gene provides the oxidizing environment conducive to mutations as well as having silent base substitutions. This is beneficial because the silent regions are freer to vary (Gray 1989). Thus, the CO1 gene of the mtDNA can be very informative when comparing genetic variability.

Scientists can access information about the fundamental molecular basis of evolutionary change through DNA sequencing (Avise 1994). An aspect of evolutionary change is the date of divergence between populations. A molecular clock examines the number of base substitutions to determine how long ago populations have diverged. Evolutionary change can be positive or negative. For extremely small populations, the evolutionary change tends to result in the loss of genetic variability.

Biogeography of Microhexura

Loss of genetic variability and the resulting potential negative fitness effects are highly relevant for a species in western North Carolina and eastern Tennessee: the Spruce-fir Moss Spider (*Microhexura montivaga*, family Dipluridae), which was placed on the endangered species list in 1995 (Fridell 2001). This spider species is thought to exist in isolated populations that may have suffered bottlenecks due to habitat destruction and climate change (Costa 2002). *Microhexura montivaga* live in well-drained moss mats that grow on rocks or boulders in well-shaded Fraser fir and red spruce forests at high elevations in the southern Appalachian mountains (Fridell 1994).

The spruce-fir forest has undergone many changes in distribution. The vegetation of an area has been historically traceable using fossilized pollen grains as markers (Moore et al. 1991). The fauna that depends on this vegetation naturally follow any shifts in latitude and elevation. Currently, these forests dominate the south's highest elevations. They consist of red spruce (*Picea rubens*), Fraser fir (*Abies fraseri*), and balsam fir (*Abies balsamea*). The forests occur above 1350m in the southern Appalachians, but at lower elevations at higher latitudes. The mountains of the southern Appalachians span a large region, but the amount of area above 1350m is actually very limited. In the words of White et al. (1993), "... high elevation forests are found in a series of discontinuous and irregularly shaped 'islands'." The mountains where these forests exist do not have tree lines, but are contiguous with deciduous forests. The southern Appalachians tend to have fir-dominated forest at the highest elevations, followed by spruce dominated stands, then the deciduous forest at lower elevations.

However, during the last ice age (Pleistocene era), the spruce-fir forests covered much more of the landscape. The last great ice age was marked by both glacial and interglacial periods occurring at about 100,000 year intervals (Wilson et al. 2000). It began 2.6 million years ago and is characterized by the period when major glaciers advanced and retreated over the northern hemisphere. Average temperatures shifted between interglacial and glacial periods by as much as 18˚C (Pittillo et al. 1998). The last glacial period before the retreat was characterized by the Laurentide ice sheet that covered most of northern North America and retreated about 10,000 to 90,000 years ago (Nesje and Dahl 2000). Even though the glaciers did not reach the southern Appalachians, the effect they had on the climate still greatly influenced the flora and fauna of the mountains. Temperatures were much colder and the high peaks of the mountains became a tundra environment during glaciation (Davis 1976). This environment could not support even the species which preferred cold climates. These species were then forced downward both in elevation and latitude, and as a result, their distribution ranges were increased. Palynological evidence shows that spruces were present on the coast as far south as South Carolina before the glacial retreat (Pittillo et al. 1998).

As the last glacier retreated and temperatures increased, the cold-loving spruce-fir forest and the fauna associated with it were forced to find "refuges" at higher elevations or latitudes. This is the refugia hypothesis, commonly used to describe the movement distributions of flora and fauna in response to the climate changes brought about by the Pleistocene era (Davis 1976). The use of a molecular clock, a molecular technique used

to determine approximate dates of divergence between populations (Bromham and Penny 2003) would be useful in determining if this view holds true for *Microhexura*.

Climate change and the migration of the spruce-fir forest is not the only reason for the isolation and decline of *M. montivaga*, however. Their decline can also be attributed to habitat destruction. Fraser firs have suffered high mortality due to past land use history, air pollution, and infestation of the balsam woolly adelgid (*Adelges piceae*, Homoptera: Adelgidae) (U.S. Fish and Wildlife Service 1998).

Intense logging of the spruce-fir forest occurred between 1900 and 1925 (Pyle and Schafale 1988). Spruce has a high strength to weight ratio which makes it ideal for timber. It also makes good pulp and is preferred for such pulp products as newsprint and high quality writing papers (White et al. 1993). These qualities of spruce led to high exploitation and subsequent loss of the forests.

Non-human factors have also influenced the loss of spruce-fir forests. In 1956, the Fraser fir began to be destroyed by an exotic species introduction, the balsam woolly adelgid. The adelgid, which is native to central Europe, migrated south from New England to the United States in the early 1900s. Mature Fraser firs usually die within 5-7 years of infestation (White et al. 1993). There have been many attempts to control this destruction, but none have been successful so far. The mortality of the Fraser firs results in loss of forest canopy, increased light, decreased moisture, and consequent mortality of the moss mats. *Microhexura montivaga* apparently requires the high and constant humidity of these moss mats (U.S. Fish and Wildlife Service 1998), so with the decline of the moss mats, the spider population suffers as well.

Biology of Microhexura montivaga *and its relatives*

Microhexura spiders are the northernmost members of the family Dipluridae, and are the smallest mygalomorph spiders, ranging from 2.5 to 5.6 mm in adult body length (Coyle 1981). Mygalomorphs are more commonly referred to as tarantulas. With the exception of *Microhexura*, members of this family are usually large, averaging more than an inch in length. Typical mygalomorphs are morphologically different from other spiders based on several characteristics, some of which include the presence of a second pair of book lungs, paraxial (rather than diaxial) chelicerae, absence of anterior median spinnerets or their homolog, the reduction or absence of anterior lateral spinnerets, and the presence of a subsegmentation of the basal segment of the posterior lateral spinnerets (Raven 1985).

The mygalomorph group is one of two suborders of spiders. These orders are Opisthothelae and Mesothelae. Mygalomorphae (as well as Araneomorphae, the "true" spiders) both belong to the order Opisthothelae (Platnick and Gertsch 1976). The Mygalomorphae are then further divided into the typical and atypical tarantulas. *Microhexura* is one of the atypical tarantulas.

The atypical tarantulas live much farther north than do the typical tarantulas. One of the characteristics of atypical tarantulas is their small number of dorsal sclerites at the base of the abdomen. In contrast to the Mygalomorphae as a whole, the atypical tarantulas are moderate in length, rarely exceeding an inch. They have lost their anterior median pair of spinnerets, but retain the anterior lateral pair of spinnerets. This is

important because it shows up in two primitive genera in the family Dipluridae (Gertsch 1979). These characteristics have been important in establishing the argument for monophyly of the Dipluridae, though Goloboff (1993) argues there are too few characteristics, some of which are vaguely defined, to fully support the monophyly hypothesis.

The family Dipluridae shares three characteristics that suggest monophyly. First, the posterior lateral spinnerets are very elongate, composed of three similarly sized sections, but with a secondary reduction in *Microhexura.* These sections of the spinnerets are widely separated — the second distinguishing feature of the Dipluridae. The last distinguishing characteristic is the lowered caput plus elevated thoracic region (Raven 1985).

The genus *Microhexura* consists of two species, *M. montivaga* and its sister species, *Microhexura idahoana*, the latter of which is found in conifer forests in the Pacific Northwest (Coyle 1981). The two are distinguished by geographic distribution and by features of the male genitalia (Coyle 1981). The first *Microhexura montivaga* was described in 1925 based on a collection by Crosby and Bishop in 1923 from the top of Mt. Mitchell, the highest mountain in eastern North America.

The Spruce-fir Moss Spider makes thin, tube-shaped webs between the moss and the rocks. No prey have been found in the webs, but it is thought that the abundant springtails may provide food (Coyle 1981). Males mature in September and October and females lay eggs in June.

Microhexura montivaga has been found in only seven sites: Mount Mitchell, Grandfather Mountain, Mount Collins, Clingman's Dome, and Roan Mountain in North Carolina, and Mount Buckley and Mount LeConte in Tennessee (Fridell 2001). All of these sites have elevations at or above 1646 meters and are dominated by spruce-fir forests. It is thought that the populations found at these sites have been isolated since the Pleistocene glacier's retreat. This isolation would result in loss of genetic variability, which has serious consequences, especially when a population's habitat is being destroyed.

For any project or study aimed at conserving *M. montivaga*, genetic diversity, and biodiversity, it is essential to realize what Avise (1994) so expertly claims, "Not only must societies find ways to preserve existing genetic diversity, but they also must seek sustainable environments for life in which the evolutionary processes fostering biotic diversity are maintained." Thus, it is important to know what processes are in force, including what is affecting the habitat, the climate changes that have taken or are taking place, and the degree of isolation of the populations and the resulting gene flow patterns.

An analysis of *M. montivaga* and *M. idahoana* DNA sequences can provide knowledge of the genetic variation present in the two species. This can then be compared with DNA sequences within and among populations of *M. montivaga*. This genetic information can then be combined with other information, such as the evolutionary history, habitat needs, and current conditions of the land in which *M. montivaga* lives.

The Recovery Plan for the Spruce-fir Moss Spider (U.S. Fish and Wildlife Service 1998) advises that, "Long-term management of Spruce-fir Moss Spider populations will

require knowledge of the genetic composition of each population and the number of individuals necessary to maintain genetic viability as well as an understanding of the factors that affect viability." The data gained from a preliminary phylogeographic analysis of *M. montivaga* can be used as a basis for further research that can determine the number of individuals necessary to maintain healthy populations.

Materials and Methods

Sample Collection

I investigated natural populations of both Microhexura species (*Microhexura idahoana* and *Microhexura montivaga*). Dr. Fred Coyle collected eight *M. idahoana* spiders in August of 2001 at Wapinita Pass, Wasco Co., Oregon, and ten individuals at Blue Box Pass, Wasco Co., Oregon (Table 1). For this study only six of these individuals were used. These individuals were kept alive until transported back to Western Carolina University in Cullowhee, NC, where they were placed in labeled collection tubes and stored in a -80°C freezer. One leg from each *M. idahoana* specimen was removed in the lab using forceps and a scalpel and then used for subsequent DNA extraction, amplification and sequencing.

In the spring of 2004, one leg was removed from each of ten *M. montivaga* spiders, five from each of two localities: Mt. Buckley and Mt. LeConte in Tennessee (Table 1). In the summer of 2004, three spiders were collected from Roan Mountain (Mitchell Co., NC). The spiders were captured alive using Petri dishes and collection tubes, and then chilled on ice to slow movement. One leg was then removed from each individual using a hand lens, forceps, and a scalpel. The legs were directly placed in a liquid nitrogen tank for transport to the laboratory, where they were stored in a -80°C freezer until used for DNA extraction, amplification, and sequencing. The amputated

spiders were allowed to recover in the Petri dishes and observed for approximately thirty minutes before release.

Table 1 ***Microhexura idahoana*** **and** ***Microhexura montivaga*** **specimens, collection locality, and primers used**

Specimens	Location Collected	Primers Used*
WP04, WP05	Wapinita Pass, Wasco Co., Oregon, beside Pacific Crest Trail about 100m E of Highway 26, 3920' elevation, in a hemlock-fir-cedar forest	C1-J-1751SPID & C1-N-2776
BBP03, BBP06, BBP08, BBP09	Blue Box Pass, Wasco Co., Oregon, about 30m W of Highway 26, 4080' elevation, in a hemlock-fir-cedar forest	C1-J-1751SPID & C1-N-2776
BKL01 – BKL05	Mt. Buckley, Sevier Co., Tennessee, 6535' elevation, in fir forest	C1-J-1751SPID & C1-N-2776
CNT02 – CNT06	Mt. LeConte, Sevier Co., Tennessee, 6593' elevation, in fir forest	C1-J-1751SPID & C1-N-2776
RN1, RN2, RN3	Roan Mtn., Mitchell Co., North Carolina, 6287' elevation, in fir forest	PMT1 & C1-N-2776

* See Table 2

DNA Extraction

DNA was isolated from one leg of each individual spider using Qiagen's DNeasy® tissue extraction kit (Qiagen Inc., Valencia CA) with a slight modification of

step 1 (Hedin 2004). For step 1, instead of cutting tissue and adding 180µl ATL, the tissue was ground using a modified mortar and pestle in 90µl ATL. The pestle was made using a 1mL pipette tip with the end melted to fit a 1.5ml centrifuge tube (used as the mortar). Remaining residue was washed off the pestle and into the 1.5mL centrifuge with an additional 90µl ATL. Steps 2 through 7 were identical to the kit's protocol. For steps 8 and 9, a 2mL centrifuge tube was used and elution with 200µl of AE was performed twice. A speedvac was then used to concentrate the remaining volume down to 30 to 60µl.

DNA was quantified by gel electrophoresis on a 1% agarose gel run for 90 min at approximately 47 volts. Bands were visualized using 20µl of ethidium bromide added to the agarose gel and a UV light box attached to a camera interfaced with a computer (EDAS 290, New Haven CT) with the viewing program Kodak 1D (New Haven CT).

Mitochondrial DNA Amplification and Sequencing

A region of the mitochondrial DNA (mtDNA) cytochrome oxidase subunit 1 (CO1) gene was amplified by PCR from the DNA extracted from one leg of each specimen. Three primers obtained from Operon (Huntsville AL) were used (Table 2). PCR assays for all samples other than RN1, RN2, and RN3 were conducted with 1µl of each template DNA in a total reaction volume of 50µl (Hedin 2002). The PCR reaction mix contained 0.2mM of each dNTP, 5µl per reaction of 10x TAQ salts (125µl of 4M KCL, 100µl of 1M TrisHCL, 25µl of 1M $MgCl_2$, 750µl deionized water, 0.001g gelatin), 0.2 pmol/µl of each primer (Operon), and 0.02 U/µl Taq DNA polymerase. PCR cycling

conditions consisted of an initial denaturation of 45s at 94°C; followed by 60s annealing at 42°C, and 90s elongation at 72°C for 30 cycles; with a final hold at 4°C.

Table 2 COI primers used in mtDNA amplification of *Microhexura* specimens

Primer	Sequence
C1-J-1751SPID	5'-GAGCTCCTGATATAGCTTTTCC-3'
PMT1	5'-GGTCAACAAATCATAAAGATATTGG-3'
C1-N-2776	3'-GGATAATCAGAATATCGTCGAGG-5'

PCR amplification products were quantified by gel electrophoresis as previously described, then cleaned and stabilized using the Millipore Montage kit (Millipore Corp., Billerica MA). PCR products were then quantified by comparing band intensity and width (run on a 1% agarose gel) of 1µl of PCR template and 1µl of k/Hind III ladder (Amersham Life Science Inc., Arlington Heights IL) used as standard.

Sequencing PCR assays were then performed using 2pmol/µl primer, 8µl of Big Dye® Terminator v3.1 Ready Reaction Mix (Applied Biosystems, Foster City CA), 15-40ng of PCR template, and the necessary amount of deionized water to bring the total reaction volume to 20µl. Separate sequencing PCR assays were performed using each forward and reverse primer in order to increase the length of sequencing read.

Sequencing products were cleaned using the AutoSeq G-50 kit (Amersham Biosciences Corp., Piscataway NJ), then dried using a speedvac. The sample was then resuspended in 5µl of formamide blue dextran solution, heated at 96°C for 5 min, and

resuspended in 5µl of formamide blue dextran solution, heated at 96°C for 5 min, and placed on ice. An ABI Prism 377 GeneScan automated sequencer was used to sequence the CO1 fragment (Applied Biosystems, Foster City CA). The sequencer requires the use of a polyacrymalide gel, which was made using Long Ranger® Singel® XL Packs (Cambrex, Pittsburgh PA). When loading the gel, 1.5µl of PCR products were used in each lane.

It was very difficult to amplify DNA from the Roan Mountain samples (RN1, RN2, and RN3). After trying unsuccessfully several times with the same PCR methods and primers used with the other samples, the Roan Mountain DNA was sent to M. Hedin's lab (San Diego State University) where different conditions and primer (PMT1) were used. The extracted DNA was first diluted one to ten. Next, three 25µl reactions were performed and combined. The same reaction mix was used as with the other samples, except for the different volume and primer. PCR cycling conditions consisted of 30 cycles of 92°C denaturation for 30s, 44°C annealing for 45s (with annealing increasing 0.2° per cycle) and elongation at 72°C for 90s. Combined products were purified via PEG (polyethylene glycol) precipitation.

Sequence Editing, Comparison, and Data Analysis

The computer program SeqEd was used to edit all sequences, except for RN1, RN2, and RN3. These were edited using Sequencher. The edited sequences were then compared using an unweighted pair group method analysis (UPGMA) with the computer program PAUP* (Phylogenetic Analysis Using Parsimony). Using complete edited

compared using an unweighted pair group method analysis (UPGMA) with the computer program PAUP* (Phylogenetic Analysis Using Parsimony). Using complete edited sequences, PAUP* was used to obtain base frequencies and a genetic similarity matrix showing distances within and between populations. In addition to this, PAUP* was used to produce a 50% majority rule consensus maximum likelihood tree (both heuristic and branch-and-bound search options). A strict consensus tree was also obtained combining maximum likelihood and maximum parsimony procedures. The phylogenetic data were obtained from truncation of the Roan sequences (eliminating all bases before character 250).

Approximate dates of divergences between populations were estimated using a molecular clock rate of 2.3% divergence per million years, as calibrated for mitochondria of arthropods (Brower 1994).

Results

Genetic diversity indices

Cytochrome oxidase DNA sequences obtained ranged from 840 to 1214 base pairs, with an average of 971.6 base pairs (Table 3). Before truncation of the Roan Mountain data, the percentages of nucleotides were calculated with an average of 22.6% A's, 12.6% C's, 21.0% G's, and 43.9% T's (Table 3). Complete edited and aligned sequences are given in the Appendix. Within population genetic distances between individuals were found (Table 4), showing an average distance ranging between 0.0000 and 0.00347. Between population distances were also found (Table 5), ranging between 0.0041 and 0.0800 for *M. montivaga* populations (with the distance between the Roan Mountain population and that of Mt. LeConte and Mt. Buckley being an order of magnitude greater than that of Mt. Buckley compared to Mt. LeConte) and 0.1881 and 0.1957 for *M. montivaga* populations compared to M. *idahoana*.

Table 3 Percentage of nucleotides found in each sequence and total length in base pairs (bp) of sequence obtained

Individual	%A	%C	%G	%T	# bp
RN1	22.8	13.3	21.8	42.0	1214
RN2	23.5	14.0	22.6	39.9	960
RN3	23.0	13.4	22.0	41.6	1206
BKL01	23.3	12.2	20.0	44.4	950
BKL02	23.3	12.6	20.0	44.1	951
BKL03	23.2	12.8	19.7	44.3	963
BKL04	23.2	12.5	20.1	44.2	947
BKL05	22.7	13.0	20.7	43.7	840
CNT02	23.1	12.6	20.1	44.2	954
CNT03	23.0	12.5	20.0	44.6	962
CNT04	22.6	13.0	20.4	44.0	925
CNT05	22.8	13.0	20.4	43.8	925
CNT06	23.0	12.5	20.4	44.1	947
BBP03	21.5	11.9	21.7	45.0	951
BBP06	21.6	12.0	21.6	44.8	953
BBP08	21.5	11.9	21.5	45.2	960
BBP09	21.5	11.9	21.8	44.8	946
WP04	21.5	11.9	21.5	45.2	960
WP05	21.5	11.9	21.8	44.8	946
MEAN	22.6	12.6	21.0	43.9	971.6

Table 4 Within Population uncorrected DNA distances, showing minimum, mean (bold), and maximum distance estimates

Population	Distance Among Individuals
M. idahoana	0.00000/**0.00000**/0.00000
M. montivaga (Mt. Buckley)	0.00000/**0.00045**/0.00124
M. montivaga (Mt. LeConte)	0.00110/**0.00347**/0.00665
M. montivaga (Roan Mtn.)	0.00000/**0.00000**/0.00000

Table 5 Between population DNA distances (bold) and standard deviations derived from the maximum likelihood model

	M.idahoana	*M.montivaga* (Mt.Buckley)	*M.montivaga* (Mt.LeConte)
M.montivaga (Mt.Buckley)	**0.1935**/0.0038	-	
M.montivaga (Mt.LeConte)	**0.1957**/0.0020	**0.0041**/0.0008	-
M.montivaga (Roan Mtn.)	**0.1881**/0.0061	**0.0776**/0.0019	**0.0800**/0.0011

Phylogenetic analysis

The 50% majority rule consensus maximum likelihood tree (Figure 1) showed all Roan specimens (RN1, RN2, RN3) to be basal in comparison to all other specimens, followed by the *M. idahoana* specimens which were grouped together into a monophyletic clade. The Buckley specimens (BKL01-BKL05) also grouped together, though not resolved into one clade, and all the LeConte specimens (CNT02-CNT06) were monophyletic. The strict consensus tree combining both maximum likelihood and maximum parsimony procedures (Figure 2) clearly shows the Roan specimens to be separate from all other specimens (*M. idahoana* as well as *M. montivaga*). The *M. idahoana* clade remains distinct from the Buckley and LeConte (*M. montivaga*) specimens. The consensus tree (Figure 2) also shows Buckley and LeConte populations as sister groups.

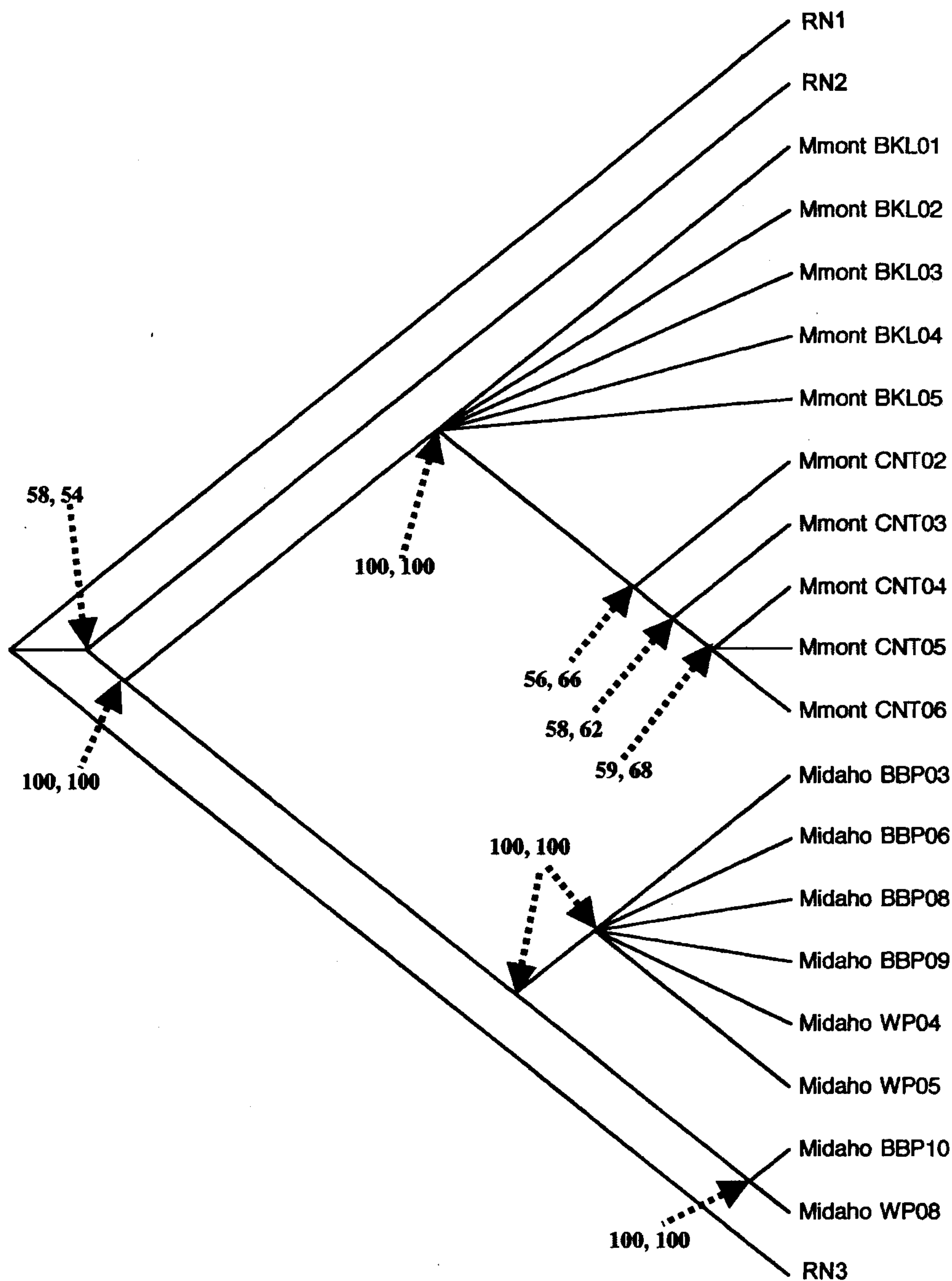

Figure 1 50% majority rule consensus maximum likelihood tree for *Microhexura* mt-CO1 data with bootstrap values (heuristic values listed first, followed by branch-and-bound values)

M. montivaga
(Roan)

Buckley

LeConte

M. montivaga

M. idahoana

Figure 2 Strict consensus tree of maximum parsimony and maximum likelihood approaches.

Estimates of divergence times

A molecular clock rate of 2.3% divergence per million years, as calibrated for mitochondria of arthropods (Brower 1994) was applied to between-population distances. These suggest that *M. idahoana* diverged from *M. montivaga* around 8.37 million years ago (during the Miocene era). When comparing *M. montivaga* populations, it was found that the Mt. Buckley and Mt. LeConte populations diverged around 18,000 years ago (corresponding to the Pleistocene era) and Roan Mountain specimens diverged from Mt. Buckley and from Mt. LeConte approximately 3.4 million years ago (both of which correspond to the Pliocene era).

Discussion

In order to properly describe population structure, a minimum of thirty individuals should be sampled and compared. However, since *M. montivaga* is an endangered species, the possibility of hurting or killing individuals was of concern, thus our sampling permit was limited to five individuals non-destructively sampled from each site. Of those sampled, it was important to observe any negative effects resulting from the sampling technique. When the legs were removed, it was noted that they consistently broke off between the coxa and trochanter joint and that no bleeding occurred. Another important observation indicating that the removal of one leg was not fatal was the discovery of a spider with only seven legs that appeared to be functioning normally.

As expected, within population distance estimates were very low for all of the populations. This low divergence within populations can be used as a comparison for the between population distances. The genetic distance between populations yielded interesting data. There is not much geographic distance between Mt. LeConte and Mt. Buckley (around 10 miles), whereas Roan Mountain is much farther away (approximately 60 miles). The distance estimates between the Roan Mountain population compared to both Mt. Buckley and Mt. LeConte populations showed that the Roan population was an order of magnitude more divergent. This genetic distance between the Roan population and Mt. Buckley and Mt. LeConte populations thus supports the hypothesis that with increasing geographic distance there is increasing genetic distance between populations,

and therefore less breeding between the populations of Roan Mountain and those of Mt. Buckley and Mt. LeConte. This corresponds to evidence suggesting that even though many members of the spider infraorder Araneomorphae are able to disperse great distances by a process of aerial ballooning (releasing silken threads which are then picked up by the wind and carry the spiders along), primitive mygalomorph taxa rarely disperse this way (Coyle 1983). Since the Mt. Buckley and Mt. LeConte populations showed considerably less genetic divergence, this also lends support to the distance hypothesis, since these two populations are very close geographically.

Unlike the Mt. Buckley and Mt. LeConte specimens, the Roan specimens were not found on a steep northern slope, but rather in a flatter area on top of the mountain. Also, instead of finding them in mats consisting only of moss, the mats were mixed with liverwort. Also, the Roan specimens presented considerable difficulty in amplification and would not yield significant product with the same primers as with the other *M. montivaga* specimens and, even using a different primer, only three of five individuals could be sequenced. This alone indicates that there could be significant genetic divergence, probably occurring along the sequence where the primers anneal.

The maximum likelihood and consensus trees (Figure 1 and Figure 2) showed appropriate grouping based on collection locality. However, the Roan population grouping was very interesting. All three Roan specimens consistently came out as an unresolved group basal to the rest of the specimens. These data, the difficulty of DNA amplification, and the difference in microhabitat of the Roan population, suggest that the Roan individuals may be a cryptic species.

The molecular clock data indicate isolation of the Roan population from that of the Mt. Buckley and Mt. LeConte populations around 3.4 million years ago, corresponding to the Pliocene era. Divergence times between Mt. Buckley and Mt. LeConte was much more recent (approximately 18,000 years ago), during the Pleistocene era. The divergence dates of the Mt. Buckley population from the Mt. LeConte population support the Pleistocene refugium hypothesis. However, the earlier divergence date of the Roan population fails to support the hypothesis. This could indicate that there was an expansion of range during the last ice age, but that there were other barriers to breeding besides distance.

The divergence dates of the Mt. Buckley and Mt. LeConte populations are consistent with several other studies examining the refugium hypothesis. Catley (1994) did a study of Hypochilidae (Araneae) spiders in New Mexico and California and provided data corresponding to divergence times during the Pliocene era. A study done on another mygalomorph, the trapdoor spider *Aptostichus simus*, revealed estimated separation times ranging from 2 to 6 million years (Bond et al. 2001).

There have been studies showing earlier divergence times similar to that of the Roan population from the Mt. Buckley and Mt. LeConte populations. A study done on a high-elevation salamander (*Desmognathus wrighti*) found in the same range and requiring similar habitat as that of *M. montivaga*, also failed to support the Pleistocene refugium theory (Crespi et al. 2003). Hedin (2001) found that for the ancient spider genus *Hypochilus*, the divergence estimates ranged from 10 to 12 million years ago, which corresponds to the divergence of *M. idahoana* from *M. montivaga* during the Miocene

era. Our study found that the populations remained fragmented throughout the Pleistocene era.

These studies undermining the Pleistocene refugium view have all analyzed species found south of the regions actually covered by the expanding and retracting ice sheets of the last ice age. When looking at a species found in the boreal forest of North America that was directly fragmented by the advancing glaciers, the data found are different. Weir and Schluter (2004) found that speciation in boreal birds did occur during the Pleistocene era as a result of population fragmentation stemming from glaciation events.

There are many possible reasons why the Pleistocene refugia view is not supported when looking at divergence times between Roan Mountain specimens compared to Mt. Buckley and Mt. LeConte. As mentioned previously, the fact that the dates of divergence were older than the Pleistocene era can be explained by the rate of a single nucleotide substitution occurring only every 54,000 years given 800 base pairs sequenced, which is much longer than the 10,000 year vicariance event of the Pleistocene (Masta 2000). Also, it seems that many of the species distributed in the more southern areas that were not as directly affected by the Pleistocene ice sheets remained fragmented.

The effectiveness of the molecular clock rate must also be examined. Since there is no molecular clock rate established for arachnids, the broader molecular clock rate calibrated for arthropods had to be used. When establishing this rate Brower (1994) calibrated it using only insects and crustaceans. There are data that suggest that arachnid

mitochondrial DNA may differ significantly from that of other arthropods (Masta 2000). When comparing *M. montivaga* to *M. idahoana*, it must be taken into account that the populations of *M. montivaga* are endangered and thus significantly smaller in number. This can be significant as Bromham and Penny (2003) stated: "If a population undergoes a marked reduction in the population size due to an environmental catastrophe, this event might be accompanied by a burst of fixation of nearly-neutral alleles. In this way, population fluctuations might add to the sloppiness of the molecular clock." In other words, the *M. montivaga* populations could have a much higher substitution rate than *M. idahoana*.

From the information gained in this study it is clear that a more intensive genetic analysis using more individuals and sequences from other genes (besides CO1) would be useful. The hypothesis that the Roan Mountain population could be a cryptic species should be further examined. As far as managing the endangered *M. montivaga* populations, it would be wise to manage them as isolated populations which have low gene flow and a small genetically effective size, due not to climate change from the Pleistocene era, but other factors. Rather than glaciation effects, more recent events such as the habitat loss resulting from past logging and the recent adelgid plague seem to be more likely causes of the decline of *M. montivaga*. Thus, habitat restoration may be the most effective means of preventing the remaining isolated *M. montivaga* populations from slipping below the viability threshold.

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Appendix

Appendix

Matrix showing raw sequences.
Missing data characterized by "-" and matching data characterized by "." Differences in bases shown by the letter. N represents unresolved bases, Y represents either a T or C, and M represents either an A or C.

```
RN1    TTTGGTGTTTGGTCGGGGATGGTGGGTACTGCGATAAGAGTAATTATCCGGGTAGAAC
RN2    ------------..............................................
RN3    ---.......................................................
BKL01  ----------------------------------------------------------
BKL02  ----------------------------------------------------------
BKL03  ----------------------------------------------------------
BKL04  ----------------------------------------------------------
BKL05  ----------------------------------------------------------
CNT02  ----------------------------------------------------------
CNT03  ----------------------------------------------------------
CNT04  ----------------------------------------------------------
CNT05  ----------------------------------------------------------
CNT06  ----------------------------------------------------------
BBP03  ----------------------------------------------------------
BBP06  ----------------------------------------------------------
BBP08  ----------------------------------------------------------
BBP09  ----------------------------------------------------------
WP04   ----------------------------------------------------------
WP05   ----------------------------------------------------------
```

```
RN1    TTGGTCAGGGGGGTAGATTACTAGGAGATGATCAATTATATAATGTTATTGTAACTTC
RN2    ..........................................................
RN3    ..........................................................
BKL01  ----------------------------------------------------------
BKL02  ----------------------------------------------------------
BKL03  ----------------------------------------------------------
BKL04  ----------------------------------------------------------
BKL05  ----------------------------------------------------------
CNT02  ----------------------------------------------------------
CNT03  ----------------------------------------------------------
CNT04  ----------------------------------------------------------
CNT05  ----------------------------------------------------------
CNT06  ----------------------------------------------------------
BBP03  ----------------------------------------------------------
BBP06  ----------------------------------------------------------
BBP08  ----------------------------------------------------------
BBP09  ----------------------------------------------------------
WP04   ----------------------------------------------------------
WP05   ----------------------------------------------------------
```

```
RN1    TCATGGTTTAGTAATAATCTTTTTTATAGTAATGCCAATTATAATTGGCGGATTCGGA
RN2    ..........................................................
RN3    ..........................................................
BKL01  ----------------------------------------------------------
BKL02  ----------------------------------------------------------
BKL03  ----------------------------------------------------------
BKL04  ----------------------------------------------------------
BKL05  ----------------------------------------------------------
CNT02  ----------------------------------------------------------
CNT03  ----------------------------------------------------------
CNT04  ----------------------------------------------------------
CNT05  ----------------------------------------------------------
CNT06  ----------------------------------------------------------
BBP03  ----------------------------------------------------------
BBP06  ----------------------------------------------------------
BBP08  ----------------------------------------------------------
BBP09  ----------------------------------------------------------
WP04   ----------------------------------------------------------
WP05   ----------------------------------------------------------
```

```
RN1    AATTGGCTAGTGCCTTTAATGTTAGGGTCTCCAGACATGGCATTTCCTCGTATAAATA
RN2    ..........................................................
RN3    ..........................................................
BKL01  ----------------------------------------------------------
BKL02  ----------------------------------------------------------
BKL03  ----------------------------------------------------------
BKL04  ----------------------------------------------------------
BKL05  ----------------------------------------------------------
CNT02  ----------------------------------------------------------
CNT03  ----------------------------------------------------------
CNT04  ----------------------------------------------------------
CNT05  ----------------------------------------------------------
CNT06  ----------------------------------------------------------
BBP03  ----------------------------------------------------------
BBP06  ----------------------------------------------------------
BBP08  ----------------------------------------------------------
BBP09  ----------------------------------------------------------
WP04   ----------------------------------------------------------
WP05   ----------------------------------------------------------
```

```
RN1    ATTTAAGTTTTTGGTTGTTACCCCCTTCTATTATAATGTTGTTAATATCATCTTTTAG
RN2    ..........................................................
RN3    ..........................................................
BKL01  --------------------------...........A.....M.....G........
BKL02  -------------------...T..............A...........G........
BKL03  -----------------CC...T..............A...........G........
BKL04  -----------------------..............A...........G........
BKL05  ---------------------.T..............A...........G........
CNT02  --------------------..T..............A...........G........
CNT03  --------------------..T..............A...........G........
CNT04  --------------------..T.....C........A...........G........
CNT05  --------------------..T..............A...........G........
CNT06  -----------------------...Y..........A...........G........
BBP03  ----------------------T..G...T...CT...A.A..TT.T.........TT
BBP06  -----------------------..G...T...CT...A.A..TT.T.........TT
BBP08  ----------------------T..G...T...CT...A.A..TT.T.........TT
BBP09  -----------------------..G...T...CT...A.A..TT.T.........TT
WP04   ----------------------T..G...T...CT...A.A..TT.T.........TT
WP05   -----------------------..G...T...CT...A.A..TT.T.........TT
```

```
RN1     AGGTTCTGGTGTTGGGGCTGGCTGAACTATTTATCCCCCTTTATCTTCTATTCTAGGG
RN2     ..........................................................
RN3     ..........................................................
BKL01   ...............T..A..T..............T...C......T....T....T
BKL02   ...............T..A..T..............T...C...........T....T
BKL03   ...............T..A..T..............T...C...........T....T
BKL04   ...............T..A..T..............T...C...........T....T
BKL05   ...............T..A..T..............T...C...........T....T
CNT02   ...............T..A..T..............T...C...........T....T
CNT03   ...............T..A..T..............T...C...........T....T
CNT04   ...............T..A..T..............T...C...........T....T
CNT05   ...............T..A..T..............T...C...........T....T
CNT06   ...............T..A..T..............T...C...........T....T
BBP03   ......A.........A.G..T..............T........A......T.G..T
BBP06   ......A.........A.G..T..............T........A......T.G..T
BBP08   ......A.........A.G..T..............T........A......T.G..T
BBP09   ......A.........A.G..T..............T........A......T.G..T
WP04    ......A.........A.G..T..............T........A......T.G..T
WP05    ......A.........A.G..T..............T........A......T.G..T
```

```
RN1     CATAATGATTGAGGAATAGACTTTGCTATTTTTTCTTTACACTTGGCCGGAGCTTCCT
RN2     ..........................................................
RN3     ..........................................................
BKL01   ....................T....................T.....T........T.
BKL02   ....................T....................T.....T........T.
BKL03   ....................T....................T.....T........T.
BKL04   ....................T....................T.....T........T.
BKL05   ....................T....................T.....T........T.
CNT02   ....G...............T....................T.....T........T.
CNT03   ....................T....................T.....T........T.
CNT04   ....................T....................T.....T........T.
CNT05   ....................T....................T.....T........T.
CNT06   ....................T....................T.....T........T.
BBP03   ....TA...G.G..G.....T...A................T.....G..G.....T.
BBP06   ....TA...G.G..G.....T...A................T.....G..G.....T.
BBP08   ....TA...G.G..G.....T...A................T.....G..G.....T.
BBP09   ....TA...G.G..G.....T...A................T.....G..G.....T.
WP04    ....TA...G.G..G.....T...A................T.....G..G.....T.
WP05    ....TA...G.G..G.....T...A................T.....G..G.....T.
```

```
RN1    CTATTATAGGGGCTATTAATTTTATTAGTACTATTATTAACATGCGTTCTTCAGGGAT
RN2    ..........................................................
RN3    ..........................................................
BKL01  ............................C..........................A..
BKL02  ............................C..........................A..
BKL03  ............................C..........................A..
BKL04  ............................C..........................A..
BKL05  ............................C..........................A..
CNT02  ............................C..........................A..
CNT03  ............................C..........................A..
CNT04  ............................C..........................A..
CNT05  ............................C..........................A..
CNT06  ............................C..........................A..
BBP03  ...........................C.......T.A..T..A.......T...A..
BBP06  ...........................C.......T.A..T..A.......T...A..
BBP08  ...........................C.......T.A..T..A.......T...A..
BBP09  ...........................C.......T.A..T..A.......T...A..
WP04   ...........................C.......T.A..T..A.......T...A..
WP05   ...........................C.......T.A..T..A.......T...A..
```

```
RN1    AAGAATGGAGCGTATATCTTTGTTTGTATGGTCTGTGTTGCTGACTGCTATTTTACTA
RN2    ..........................................................
RN3    ..........................................................
BKL01  ...T.........G.T....................T..................T..
BKL02  ...T.........G.T....................T..................T..
BKL03  ...T.........G.T....................T..................T..
BKL04  ...T.........G.T....................T..................T..
BKL05  ...T.........G.T....................T..................T..
CNT02  ...T.........G.T....................T..................T..
CNT03  ...T.........G.T....................T..................T..
CNT04  ...T.........G.T....................T..................T..
CNT05  ...T.........G.T....................T..................T..
CNT06  ...T.........G.T....................T..................T..
BBP03  GGA............TC....A.....T........T..AT.A......G........
BBP06  GGA............TC....A.....T........T..AT.A......G........
BBP08  GGA............TC....A.....T........T..AT.A......G........
BBP09  GGA............TC....A.....T........T..AT.A......G........
WP04   GGA............TC....A.....T........T..AT.A......G........
WP05   GGA............TC....A.....T........T..AT.A......G........
```

```
RN1    TTATTATCACTTCCTGTTTTAGCCGGGGCTATCACTATGTTGTTGACAGATCGAAATT
RN2    ..........................................................
RN3    ..........................................................
BKL01  ..............C........T..A.....T.....A.......G...........
BKL02  ..............C........T..A.....T.....A.......G...........
BKL03  ..............C........T..A.....T.....A.......G...........
BKL04  ..............C........T..A.....T.....A.......G...........
BKL05  ..............C........T..A.....T.....A.......G...........
CNT02  ..............C........T..A...G.T.....A.......G...........
CNT03  ..............C........T..A.....T.....A.......G...........
CNT04  ..............C........T..A.....T.....A.......G...........
CNT05  ..............C........T..A.....T.....A.......G...........
CNT06  ..............C........T..A.....T.....A.......G...........
BBP03  ........T.....A........T...........A..A..A..A..T.....G....
BBP06  ........T.....A........T...........A..A..A..A..T.....G....
BBP08  ........T.....A........T...........A..A..A..A..T.....G....
BBP09  ........T.....A........T...........A..A..A..A..T.....G....
WP04   ........T.....A........T...........A..A..A..A..T.....G....
WP05   ........T.....A........T...........A..A..A..A..T.....G....
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RN1    TTAACACTTCTTTTTTTGACCCTGCTGGAGGAGGGGATCCTGTGTTATTTCAACATTT
RN2    ..........................................................
RN3    ..........................................................
BKL01  ....T..C...........T......................................
BKL02  ....T..C...........T......................................
BKL03  ....T..C...........T......................................
BKL04  ....T..C...........T......................................
BKL05  ....T..C...........T......................................
CNT02  ....T..C...........T......................................
CNT03  ....T..C...........T......................................
CNT04  ....T..C...........T...........G..........................
CNT05  ....T..C...........T...........G..........................
CNT06  ....T..C...........T...........G..........................
BBP03  .......C.................A..G.................G.....G.....
BBP06  .......C.................A..G.................G.....G.....
BBP08  .......C.................A..G.................G.....G.....
BBP09  .......C.................A..G.................G.....G.....
WP04   .......C.................A..G.................G.....G.....
WP05   .......C.................A..G.................G.....G.....
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RN1    GTTTTGGTTTTTTGGTCACCCAGAGGTTTATATTTTGATTTTACCGGGGTTTGGTATA
RN2    ..........................................................
RN3    ..........................................................
BKL01  ................................................A.........
BKL02  ................................................A.........
BKL03  ................................................A.........
BKL04  ................................................A.........
BKL05  ................................................A.........
CNT02  ................................................A.....G...
CNT03  ................................................A.....G...
CNT04  ................................................A.....G...
CNT05  ................................................A.....G...
CNT06  ................................................A.....G...
BBP03  A.....A...........T.....A...........A.....G..T........A...
BBP06  A.....A...........T.....A...........A.....G..T........A...
BBP08  A.....A...........T.....A...........A.....G..T........A...
BBP09  A.....A...........T.....A...........A.....G..T........A...
WP04   A.....A...........T.....A...........A.....G..T........A...
WP05   A.....A...........T.....A...........A.....G..T........A...
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RN1    ATTTCTCATGTAATTAGAAGAGGAGTAGGTAAGCGAGAACCTTTTGGCTCTTTAGGGA
RN2    ..........................................................
RN3    ..........................................................
BKL01  ..C.......................T..A............................
BKL02  ..C.......................T..A............................
BKL03  ..C.......................T..A............................
BKL04  ..C.......................T..A............................
BKL05  ..C.......................T..A............................
CNT02  ..C.......................T..A............................
CNT03  ..C.......................T..A............................
CNT04  ..C.......................T..A............................
CNT05  ..C.......................T..A............................
CNT06  ..C.......................T..A............................
BBP03  G......................T.CT.....A..T...........TGTG.....T.
BBP06  G......................T.CT.....A..T...........TGTG.....T.
BBP08  G......................T.CT.....A..T...........TGTG.....T.
BBP09  G......................T.CT.....A..T...........TGTG.....T.
WP04   G......................T.CT.....A..T...........TGTG.....T.
WP05   G......................T.CT.....A..T...........TGTG.....T.
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RN1    TAGTTTTTGCTATAGTAGGAATTGGGGGAATAGGTTTTGTAGTATGAGCTCATCATAT
RN2    ..........................................................
RN3    ..........................................................
BKL01  ..A..........G...........A..G...........C.................
BKL02  ..A..........G...........A..G...........C.................
BKL03  ..A..........G...........A..G...........C.................
BKL04  ..A..........G...........A..G...........C.................
BKL05  ..A..........G...........A..G...........C.................
CNT02  ..A..........G...........A..G...........C.................
CNT03  ..A..........G...........A..G...........C.................
CNT04  ..A..........G...........A..G...........C.................
CNT05  ..A..........G...........A..G...........C.................
CNT06  ..A..........G...........A..G...........C.................
BBP03  ..A...A...G...A.T..T.....A..............T..T...........C..
BBP06  ..A...A...G...A.T..T.....A..............T..T...........C..
BBP08  ..A...A...G...A.T..T.....A..............T..T...........C..
BBP09  ..A...A...G...A.T..T.....A..............T..T...........C..
WP04   ..A...A...G...A.T..T.....A..............T..T...........C..
WP05   ..A...A...G...A.T..T.....A..............T..T...........C..
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RN1    GTTTTCTGTTGGGATGGATGTGGACACTCGAGCTTACTTTACTGCAGCGACTATAATT
RN2    ..........................................................
RN3    ..........................................................
BKL01  A...........A..A.....T..T.....G...........................
BKL02  A...........A..A.....T..T.....G...........................
BKL03  A...........A..A.....T..T.....G...........................
BKL04  A...........A..A.....T..T.....G...........................
BKL05  A...........A..A.....T..T.....G...........................
CNT02  A...........A..A.....T..T.....G...........................
CNT03  A...........A..A.....T..T.....G.......................G...
CNT04  A...........A..A.....T..T.....G.......................G...
CNT05  A...........A..A.....T..T.....G.......................G...
CNT06  A...........A..A.....T..T.....G.......................G...
BBP03  .........A..A........A..T.....G.....T........T............
BBP06  .........A..A........A..T.....G.....T........T............
BBP08  .........A..A........A..T.....G.....T........T............
BBP09  .........A..A........A..T.....G.....T........T............
WP04   .........A..A........A..T.....G.....T........T............
WP05   .........A..A........A..T.....G.....T........T............
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RN1   ATTGCTGTTCCTACTGGAATTAAAGTATTTAGATGAATAGCTACTTTACACGGTTCTT
RN2   ..........................................................
RN3   ..........................................................
BKL01 .................G.................G..............T.......
BKL02 .................G.................G..............T.......
BKL03 .................G.................G..............T.......
BKL04 .................G.................G..............T.......
BKL05 .................G.................G..............T.......
CNT02 ...................................G..............T.......
CNT03 .................G.................G..............T.......
CNT04 .................G.................G..............T.......
CNT05 .................G.................G..............T.......
CNT06 .................G.................G..............T.......
BBP03 ........A.....A..G.....G........T...G.G...........T..A....
BBP06 ........A.....A..G.....G........T...G.G...........T..A....
BBP08 ........A.....A..G.....G........T...G.G...........T..A....
BBP09 ........A.....A..G.....G........T...G.G...........T..A....
WP04  ........A.....A..G.....G........T...G.G...........T..A....
WP05  ........A.....A..G.....G........T...G.G...........T..A....
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RN1   TTTATAAGATAGATTCATCTATTATGTGGTGTATTGGGTTTGTCTTTTTATTTACTTT
RN2   ...........................................---------------
RN3   ..........................................................
BKL01 .......A...........A.................A.....A..............
BKL02 .......A...........A.................A.....A..............
BKL03 .......A...........A.................A.....A..............
BKL04 .......A...........A.................A.....A..............
BKL05 .......A...........A.................A.....A..............
CNT02 .......A...........A.................A.....A..............
CNT03 .......A...........A.................A.....A..............
CNT04 .......A...........A.................A.....A..............
CNT05 .......A...........A.N...N...........A.....A..............
CNT06 .......A...........A.................A.....A..............
BBP03 A...C..A..G.....T...T.G..A..A...G....T.....T...C..........
BBP06 A...C..A..G.....T...T.G..A..A...G....T.....T...C..........
BBP08 A...C..A..G.....T...T.G..A..A...G....T.....T...C..........
BBP09 A...C..A..G.....T...T.G..A..A...G....T.....T...C..........
WP04  A...C..A..G.....T...T.G..A..A...G....T.....T...C..........
WP05  A...C..A..G.....T...T.G..A..A...G....T.....T...C..........
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RN1    AGGCGGTTTAACTGGCGTAGTTTTGGCTAATTCCTCTTTAGATATTGTTCTTCATGAT
RN2    ----------------------------------------------------------
RN3    ..........................................................
BKL01  ...T...........T........A..............G..............C...
BKL02  ...T...........T........A..............G..............C...
BKL03  ...T...........T........A..............G..............C...
BKL04  ...T...........T........A..............G..............C...
BKL05  ...T...........T........A..............G..............C...
CNT02  ...T...........T........A..............G..............C...
CNT03  ...T...........T........A..............G..............C...
CNT04  ...T...........T........A..............G..............C...
CNT05  ...T.........NNN.G..NNN.A..............G..............C...
CNT06  ...T...........T........A..............G..............C...
BBP03  ...G.....G.....G..G..............T............A...........
BBP06  ...G.....G.....G..G..............T............A...........
BBP08  ...G.....G.....G..G..............T............A...........
BBP09  ...G.....G.....G..G..............T............A...........
WP04   ...G.....G.....G..G..............T............A...........
WP05   ...G.....G.....G..G..............T............A...........
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RN1    ACTTATTATGTAGTAGCTCATTTTCATTACGTTCTTAGAATGGGAGCTGTTTTTGCTA
RN2    ----------------------------------------------------------
RN3    ..........................................................
BKL01  ...........G..G..............T...........A................
BKL02  ...........G..G..............T...........A................
BKL03  ...........G..G..............T...........A................
BKL04  ...........G..G..............T...........A................
BKL05  ...........G..G..............T...........A.......---------
CNT02  ...........G..G..............T...........A................
CNT03  ...........G..G..............T...........A................
CNT04  ...........G..G..............T...........A................
CNT05  ...........G..G..............T...........A................
CNT06  ...........G..G..............T...........A................
BBP03  .............................T..G........A................
BBP06  .............................T..G........A................
BBP08  .............................T..G........A................
BBP09  .............................T..G........A................
WP04   .............................T..G........A................
WP05   .............................T..G........A................
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RN1     TTTTAGCTGGTTTGACTTATTGATTTCCTTTATTTTTTGGTGTAATTTTAAGATTTAA
RN2     ----------------------------------------------------------
RN3     ..........................................................
BKL01   .............A..............A............A...........A....
BKL02   .............A..............A............A...........A....
BKL03   .............A..............A............A...........A....
BKL04   .............A..............A............A...........A....
BKL05   ----------------------------------------------------------
CNT02   .............A..............A............A...........A....
CNT03   .............A..............A............A...........A....
CNT04   .............A..............A............A...........A....
CNT05   .............A..............A............A...........A....
CNT06   .............A..............A............A...........A....
BBP03   ....G..G..GG.TG.......G....................GG........A....
BBP06   ....G..G..GG.TG.......G....................GG........A....
BBP08   ....G..G..GG.TG.......G....................GG........A....
BBP09   ....G..G..GG.TG.......G....................GG........A....
WP04    ....G..G..GG.TG.......G....................GG........A....
WP05    ....G..G..GG.TG.......G....................GG........A....
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RN1     ACATTCTAGGGTTCAGTTCATACTAATGTTTATTGGGGTGAATTTAACTTTTTT
RN2     ------------------------------------------------------
RN3     ................................----------------------
BKL01   ............C.....T...T..........C..A..A........------
BKL02   ............C.....T...T.......N..C..A..A..------------
BKL03   ............C.....T...T..........C..A..A............--
BKL04   ............C.....T...T..........C..A..A..------------
BKL05   ------------------------------------------------------
CNT02   ............C.....T...T..........C..A..A......--------
CNT03   ............C.....T...T.............A..A..............
CNT04   ............C...--------------------------------------
CNT05   ............C...--------------------------------------
CNT06   ............C.....T...T..........C..A..A..------------
BBP03   GT......A......A..TG..A.T..A..............Y..---------
BBP06   GT......A......A..TG..A.T..A....................------
BBP08   GT......A......A..TG..A.T..A..........................
BBP09   GT......A......A..TG..A.T..A.............-------------
WP04    GT......A......A..TG..A.T..A..........................
WP05    GT......A......A..TG..A.T..A.............-------------
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