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# Fine-scale genetic structure and parentage in Urocitellus beldingi

Marissa R. Lafler
*University of San Francisco*, mrlafler@usfca.edu

**Fine-scale genetic structure and parentage in *Urocitellus beldingi***

**by**

**Marissa R. Lafler**

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**Dedication**

To Anna, born just days ago:

May you find beauty and joy in the natural world.

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Thanks to my parents for their unconditional love and support, even though they don't understand what I do.

And finally, thanks to my husband, Alex Povilus, for his love and patience, without whom I wouldn't have had the strength to accomplish this.

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**Abstract**

*Urocitellus beldingi* (Belding's ground squirrel), previously known as *Spermophilus beldingi,* is a social, montane rodent that occupies alpine and subalpine meadows in the Sierra Nevadas (Helgen et al. 2009). The Tioga Pass meadow (Mono Co. CA) population has been studied behaviorally and demographically for several decades. Microsatellites were developed for this species and conditions for polymerase chain reaction (PCR) were defined. Nine microsatellite loci amplified adequately and were in Hardy-Weinberg equilibrium, displaying an average heterozygosity of 0.67±0.19. These nine microsatellite loci were analyzed via PCR to elucidate the fine scale genetic structure, offering insight into population health, stochastic events, and dispersal. Two populations were identified, corresponding to the elevation differences between the sites ($F_{ST}$ = 0.054605 $p<0.00001$), with a migration rate ($N_m$) of 8.66. These populations appear to maintain a stable size and are not adversely affected by inbreeding. *Urocitellus beldingi* maternity can be deduced by observing female burrows usage during gestation and which offspring first emerge from the burrow associated with that particular female. Paternity, however, can only be posited by observation of mating because the species exhibits multiple mating in both sexes, though not all matings necessarily result in offspring. Multiple paternity has been previously verified through allozyme analysis. Microsatellites were used to verify maternity and determine paternity. Analysis of ten litters demonstrated an average of 1 father per 1.35 pups in a litter. Sibship deduced from maternity and paternity analysis was then compared with previously collected behavioral

data. While previous observations indicate preference of fullsib play partners in juveniles, our findings do not corroborate this, though the results were not significant.

## Introduction

*Urocitellus beldingi*, or Belding's ground squirrel, is a diurnal, social, montane rodent whose native range extends from northern Oregon to central California, and east into southwestern Idaho and the northwest corner of Utah. The squirrels inhabit alpine and subalpine meadows approximately 2000m to 3500m above sea level (Morton and Sherman 1978). The squirrels in the Tioga Pass meadow, just to the east of Yosemite National Park in Mono Co, CA, have been studied behaviorally and demographically since the late 1960s (Sherman 1977, Morton and Sherman 1978, Hanken and Sherman 1981, Holekamp 1984, Bushberg and Holmes 1985, Nunes, 1997, 2004a, 2004b, etc.).

*Urocitellus beldingi* are burrowing animals who either dig their own burrows or use old burrows from other animals, such as pocket gophers or moles (Morton and Sherman 1978). The squirrels generally do not reuse burrows from season to season, but rather find or dig a new burrow and fill in the entrance to their old burrow (Sherman 1976). Burrows may have one or multiple entrances, and are used for sleeping, shelter from predators, housing young while nursing, and hibernation, which lasts approximately eight months a year (Sherman and Morton 1984). Their active period, generally from May to August, is predicated on temperature, which determines emergence and immergence dates (Morton and Sherman 1978). The squirrels, especially mating males, may emerge before the snowpack has completely subsided; the emergence date appears to be linked to surface air temperature and snowpack depth, thus the exact time span of hibernation varies from year to year based on weather (Sherman 1976, Morton and

Sherman 1978, Jenkins and Eshelman 1984). Because the squirrels are active during the warmest period of the year, they are active during the growing season in their habitat and are thus not limited by the availability of food (Morton and Sherman 1978, Nunes et al. 1998). In the spring, sexually mature adult males emerge first, followed by sexually receptive females, then non-mating females, and finally by yearling males, who typically do not mate (Morton and Sherman 1978). Mating males typically emerge about ten days to two weeks before sexually receptive females (Morton and Sherman 1978).

Male squirrels are reproductively viable for approximately one month, whereas females are only receptive for a few hours of one day a year shortly after emergence from their hibernacula (Morton and Sherman 1978). In the event of lack of female receptivity, such as during the spring 1977 snowstorm observed by Morton and Sherman (1978), the testes of some males decreased in size or reascended completely and only descended again once the storm had subsided and sexually receptive females had emerged. Copulation occurs above ground, making it easily observable, thus it is possible to make predictions about paternity of litters via behavioral observation (Hanken and Sherman, 1981). There is, however, some evidence that copulation may occur below ground, as males have been observed entering a female's burrow after her, emerging some time later, and doing a characteristic post-mating display of posting and chirps, which is also observed in the California ground squirrel (*Spermophilus beecheyi*) (Sherman 1976, Holekamp and Nunes 1989, Nunes personal comm.). Females and males may mate with multiple mates, and litters with multiple paternity occur (Morton and Sherman 1978,

Hanken and Sherman, 1981). Females produce one litter per year, with an average litter size of four pups in the Tioga Pass meadow population (Sherman 1976, Jenkins and Eshelman 1984). Litter size appears to be dependent on altitude; populations inhabiting lower altitudes appear to have larger litters than their high-altitude counterparts (Jenkins and Eshelman 1984). Maternal age also affects litter size, with yearling females and 5+ year old females having smaller litters than 2-4 year old females (Sherman 1976, Jenkins and Eshelman 1984). Female squirrels live on average 3.9 years, and males live on average 2.3 years, though a female squirrel of eleven years has been observed, and it is not uncommon to see squirrels much older than three or four years (Morton and Sherman 1978, Jenkins and Eshelman 1984). Females reach sexual maturity earlier than males; while it is not uncommon for females to breed as yearlings after their first hibernation, males typically do not breed until another season has passed (Morton and Sherman 1978). Female mate selection occurs, with females preferring larger, older males who win more fights (Sherman 1976). When females mate with multiple males, subsequent matings are typically with more successful males than prior matings (Sherman 1976). Similarly, larger males and those more experienced at intraspecific combat have more mating success (Sherman and Morton, 1984).

Belding's ground squirrel exhibits sexual dimorphism, with males being the larger and heavier of the sexes (Jenkins and Eshelman 1984, Nunes et al. 2004a). Belding's ground squirrels also exhibit sexually dimorphic dispersal patterns; males disperse earlier and farther than females and do not inhabit the same areas from season to season, as

females do (Sherman 1976, Morton and Sherman 1978, Holekamp 1984, Nunes et al. 1998). By the end of the first summer, most juvenile males will have dispersed from their natal burrow, but not all juvenile females will have dispersed. The average dispersal distances are greater for males than females, with males being found over 1km from their natal burrows, while females are generally found within 100m from their natal burrows (Sherman 1976, Holekamp 1984). The shorter distance of female dispersal tends to result in matrilines, burrow patterns in which closely-related female kin occupying burrows whose territories border those of their mothers or other female littermates (Sherman 1976, Sherman 1977, Holekamp 1984).

Designated by the IUCN as a species of least concern, *U. beldingi* is a generalized omnivore that has been observed feeding on grasses, seeds, forbes, insects, and occasionally, dead mammals or birds (Morton and Sherman 1978, Jenkins and Eshelman 1984, Linzey and Hammerson 2008). While both males and females have been observed killing conspecific juveniles, only males have been observed cannibalizing conspecific juveniles (Morton and Sherman 1978). It is estimated that as much as 29% of juvenile mortality is the result of intraspecific aggression (Jenkins and Eshelman 1984).

The squirrel populations east of Yosemite do not appear to be limited by the availability of food; estimates of the usage of available biomass by the squirrels range from 2-65%, thus, with the exception of stochastic events, starvation is not a common cause of mortality (Morton and Sherman 1978). Predation by coyotes, badgers, weasels, and raptors, overwintering mortality, in which the animals simply are not observed the

following summer, and intraspecific aggression are the primary sources of mortality (Morton and Sherman 1978, Jenkins and Eshelman 1984, Sherman and Morton 1984). Juvenile squirrels are especially vulnerable to predation (Sherman 1976). Mating males (i.e. those older than one year) are adversely affected by male-male aggression during the mating period and may suffer life-threatening or debilitating injuries, whereas yearling males who do not compete for mates are largely ignored by conspecifics (Sherman 1976, Morton and Sherman 1978). Since State Highway 120 borders the western side of the meadow, automobile impact is also a notable source of mortality for the Tioga pass population (Nunes, personal comm.).

The squirrels engage in intraspecific signaling; they are often observed posting and emitting warning chirps to other squirrels, indicating the presence of threatening stimuli (Sherman 1977, Sherman 1984). Such signaling increases the likelihood that closely-related individuals will survive an attack from a terrestrial predator, or in the case of an aerial predator, directly increases the likelihood of the signaling individual of escaping the predator.

Despite the aforementioned demographic and behavioral research done on *U. beldingi*, to date, only a small study of the population structure using the cytochrome B gene in mtDNA has been done (Curtain unpubl.). Allozyme analysis of pups has been performed to determine whether multiple paternity occurs in this species (Hanken and Sherman 1981). Both of these techniques have their limitations; mtDNA is passed down only through females, and allozymes have slow mutation rates in comparison with other

markers, such as microsatellites (Queller et al. 1993, Jarne and Lagoda 1996).

Molecular markers have become very important tools for investigating questions regarding population structure and parentage (Primmer 2009). Microsatellites, non-coding regions of the genome consisting of repeats of a base pair motif (e.g. TAGA), have been used in many species to investigate such questions (e.g. humans, Litt and Luty 1989; cod, Bentzen et al 1996; crocodiles, Dever and Densmore 2001; squirrels, Garner et al. 2005; honeybees, Oxley et al; soybeans, Li et al. 2011, etc.). The repeated unit is between two and nine base pairs long, and may be repeated a few times or a hundred times. Microsatellites are numerous and distributed throughout the genome almost evenly (Weissenbach et al. 1993). Because they are not highly conserved, they are useful markers for fine-scale population structure and parentage questions (Jarne and Lagoda 1996, Storfer et al. 2009). They are abundant, heterozygous, biparentally inherited, neutral mendelian markers (Litt and Luty 1989, Weissenbach et al. 1993, Queller et al 1993, Kohn et al. 2006). Because of their high mutation rate, they are more variable than more highly conserved markers, such as allozyme or mitochondrial markers, which are sometimes not sensitive enough to accurately estimate relatedness between individuals (Queller et al. 1993, Jarne and Lagoda 1996). When enough polymorphic loci are analyzed, a unique genetic fingerprint of an individual can be created based on the specific combination of alleles for the loci (Wright et al. 1994). Because of these factors, they have been used as analytical tools for investigating fine-scale genetic structure of a population, metapopulation structure, and parentage (Wright et al. 1994, Jarne and

Lagoda, 1996, Pestsova et al. 2000).

Polymerase chain reaction (PCR) is a technique that allows for fast and accurate amplification of a region of interest within the genome from a small sample of DNA, allowing for analysis of that region (Mullis et al, 1986, Saiki et al 1988). Specifically designed primers anneal to flanking regions that surround the target region (Mullis et al, 1986, Saiki et al 1988). This method can be used to amplify specific microsatellite loci within a genome. Characterizing microsatellites and their PCR conditions provides a basis on which future research can be built for both the species for which the microsatellites were identified and other closely-related species. Because PCR is able to amplify small quantities of DNA, enough DNA to analyze microsatellites can obtained in minimally invasive ways without sacrificing or impairing an individual.

When performing PCR on numerous microsatellite loci, the number of loci multiplied by the number of samples can quickly create a large number of reactions required to analyze the samples completely. To reduce the number of total reactions necessary, multiplex PCR may be used. Multiplex PCR is a technique in which multiple PCRs are performed in one tube. By amplifying multiple loci in one tube, the amount of reagents, DNA, and time required to process samples is reduced (Morral and Estivill 1992, Luikart et al. 1999, Eggelston et al. 2002, Markoulatos et al. 2002). In order to identify products of different primers in the final mix, one primer for each locus is typically labeled with a fluorescent tag that is recognized by the analyzer (Ziegle et al. 1992, Luikart et al. 1999).

Polymorphic loci, such as microsatellites, can be used to calculate gene flow by analyzing the allelic frequency. Gene flow can be measured by calculating $F_{ST}$ or $R_{ST}$, both of which are inbreeding coefficients (Hudson et al. 1992). It can also be measured more directly by investigating the number of migration events ($N_m$) between populations, measuring genetic divergence as a function of number of migrants in a generation (Hudson et al. 1992, Palsbøll et al. 2006). Immigration and emigration can be important sources for genetic diversity, especially in small populations (Tamura and Hayashi 2007).

Previous gene flow work on *U. beldingi* involved the use of mtDNA markers, which are more susceptible to genetic drift and other stochastic effects than microsatellites and also only effectively measure gene flow in females, thus are likely to demonstrate lower levels of gene flow (Swei et al. 2003). The use of microsatellites to investigate population questions and gene flow could give a more accurate picture of the population. Furthermore, dispersal patterns and population structure in rodents tends to vary greatly, and assumptions cannot necessarily be drawn across species lines, necessitating investigation in as many species as possible (Booth et al. 2009). This information could determine whether populations in the Mono Co. area comprise a metapopulation, a population comprised of several subpopulations connected by low levels of dispersal.

Because this is a social species in which the dispersal pattern of females keeps kin in close contact, thus enabling altruistic behavior, understanding how the social bonds between related individuals form is important. One way this may occur is through play.

Juveniles of mammalian species engage in play for a variety of hypothesized reasons, including development of social and motor skills, expenditure of excess energy, and strengthening of social bonding in play partners (Bekoff 1972). This holds true for *U. beldingi* (Nunes 1997, 1998, 1999, 2004a, 2004b). Insight to the nature of play in juveniles and how that affects the development of social structures in *U. beldingi* may be useful in explaining similar behavior in congeneric species (Holmes 1997).

While *U. beldingi* is listed as a species of least concern, it is worthwhile to study a such a species because information gained from a healthy population of communal rodents can be applied to other species that may not be as easy to study as Belding's ground squirrel (Linzey and Hammerson 2008). Investigating how this species behaves will enable researchers to better understand how endangered spermophilids and social animals interact, without the risk of adversely affecting small populations of endangered species. This species can also be used as a model for other species with similar natural histories (e.g. Michener and Michener 1977). Some *Urocitellus* species are endangered, and microsatellites characterized in *U. beldingi* may be useful in assessing population structure in those species (Martin et al. 2003, Garner et al. 2005).

The objectives of this research are fourfold: first, to characterize the microsatellite loci and determine the proper PCR conditions; second, to determine the nature of the population structure in the study area; third, to investigate the mating system and paternity of the species using a highly-variable, neutral molecular marker; and fourth, to investigate what role play plays in the ontogeny of *U. beldingi* juveniles.

**Characterization of microsatellites for *Urocitellus beldingi* (Belding's ground squirrel), a social, montane rodent living in the High Sierra**


## Abstract

*Urocitellus beldingi* (Belding's ground squirrel) is a social, montane rodent that lives in the High Sierra in central and northern California. Though the species has been studied behaviorally and demographically via field observation for over four decades, genetic analysis has been limited to a small population study using mtDNA and allozyme analysis of paternity. We developed primers and polyemerase chain reaction (PCR) conditions for eleven microsatellite loci specific to *U. beldingi*. One locus failed to amplify adequately, and another was out of Hardy-Weinberg equilibrium (HWE). The nine microsatellites that amplified adequately and were in HWE exhibited a range of 4 to 15 alleles and an average heterozygosity of 0.67±0.19. These microsatellites will continue to be useful in monitoring the health of these squirrels and can potentially be applied to other, closely-related species.

## Introduction

*Urocitellus beldingi* is a social, montane rodent that lives in the High Sierra of central and northern California (Sherman and Morton 1984). The populations in and near Yosemite, specifically in Dana Meadows and Tioga Pass meadow, have been studied behaviorally and demographically for over four decades (Sherman 1976, Sherman and Morton 1984, Bushberg and Holmes 1985, Nunes et al. 2004, etc.). The species exhibits multiple mating in both sexes, and previous allozyme analysis has indicated that litters can be multiply sired (Hanken and Sherman 1981). Mitochondrial DNA analysis of the cytochrome B gene of *U. beldingi* population structure has been conducted, indicating that the Tioga Pass meadow population is not adversely affected by inbreeding (Curtain unpubl.).

Microsatellites are useful markers for fine-scale population structure and parentage questions because they are not highly conserved; they evolve on an ecological time scale of several generation as opposed to an evolutionary timescale of thousands of generations (Jarne and Lagoda 1996, Storfer et al. 2009). Microsatellites are non-coding regions of the genome consisting of repeats of a base pair motif (e.g. TAGA), which is between two and nine base pairs long, and may be repeated a few times or a hundred times. They are also numerous and distributed throughout the genome almost evenly (Weissenbach et al. 1993). They are abundant, heterozygous, biparentally inherited, neutral mendelian markers (Litt and Luty 1989, Weissenbach et al. 1993, Queller et al 1993, Kohn et al. 2006). Since they have a higher mutation rate, they are more variable

than more highly conserved markers, such as allozyme or mitochondrial markers, which are sometimes not sensitive enough to estimate relatedness between individuals accurately (Queller et al. 1993, Jarne and Lagoda 1996). When enough polymorphic loci are selected, a unique fingerprint of an individual can be identified based on the specific combination of alleles for the loci (Wright et al. 1994). These factors make them useful analytical tools for investigating fine-scale genetic structure of a population, metapopulation structure, and parentage (Wright et al. 1994, Jarne and Lagoda, 1996, Pestsova et al. 2000). Microsatellite data can obtained in minimally invasive ways without sacrificing or impairing an individual and has been used to determine genetic diversity and parentage in a variety of species across many taxa (e.g. humans, Litt and Luty 1989; cod, Bentzen et al 1996; crocodiles, Dever and Densmore 2001; squirrels, Garner et al. 2005; honeybees, Oxley et al; soybeans, Li et al. 2011, etc.).

Polymerase chain reaction (PCR) is a technique that can be used to amplify specific microsatellite loci within a genome (Mullis et al. 1986, Saiki et al. 1988). Characterizing microsatellites and their PCR conditions provides a basis on which future research can be built for both the species for which the microsatellites were identified and other closely-related species. When performing PCR on numerous microsatellite loci, the number of loci multiplied by the number of samples can quickly create a large number of reactions required to thoroughly analyze the samples. To reduce the number of total reactions necessary, multiplex PCR may be used. Multiplex PCR is a technique in which multiple loci are amplified in one tube, reducing the amount of reagents, DNA, and time

required to process samples (Morral and Estivill 1992, Luikart et al., 1999, Eggelston et al. 2002, Markoulatos et al. 2002). In order to identify products of different primers in the final mix, one primer for each locus is typically labeled with a fluorescent tag that is recognized by the analyzer (Ziegle et al 1992, Luikart et al., 1999).

Our objectives were to test the amplification of microsatellite primers developed for this species and to characterize the loci. These tools and techniques can be employed to investigate questions about the fine-scale genetic structure of the squirrels found in the Tioga Pass Meadow and surrounding areas and also to confirm maternity and assign paternity in order to determine full and half sibship, as has been demonstrated in many other species (e.g. dispersal in gerbils; Meyer et al. 2009, parentage in dogs; Koskinen and Bredbacka 1999). These markers may also be useful in investigating such questions in other *Urocitellus* species.

## Methods

Squirrels were sampled in 2002-2005 and 2010 from four sites in Mono Co. CA. A tissue sample measuring approximately 1mm x 3mm was collected from the medial edge of the ear and stored in a cryo tube packed on ice until it could be transferred to a -20ºC freezer. DNA was later extracted using the Qiagen DNeasy™ Blood & Tissue kit (Germantown, MD) according to the specified protocol for for purification of total DNA from animal tissues.

Genetic Identification Services (GIS) was employed to develop microsatellites for this species (GIS personal comm.). They designed the primers for the 67 loci which they

identified using DesignerPCR v. 1.03 (GIS personal comm.). To create the library, DNA fragments from *U. beldingi* were ligated into the *HindIII* site of the pUC19 plasmid, which was then transformed into *E. coli*. The microsatellites range from two to four base pairs in size, and are 129-316 base pairs in length (Table 1). Of those loci, 17 were clearly polymorphic, and 11 exhibited polymorphism without extraneous peaks in the samples provided. We focused on these eleven loci for polymerase chain reaction (PCR) condition development.

In order to multiplex the reaction, the primers were grouped into five batches according to their annealing temperatures (Table 1). The 5' end of the forward primers were tagged the fluorescent markers 5Hex, 56Fam, and Ned in order to differentiate the fragments when later analyzed. The TaKaRa™ PCR kit (Otsu, Japan) was used to test whether the primers would amplify and in cases when multiplexing was unnecessary.

Preliminary analysis showed that A116 and D107 exhibited alleles one base pair off of other prominent alleles that could have been the result of band stuttering. These two primers were then modified on the 5' end of the reverse primer with a PIGtail. The PIGtail is a GTGTCTT sequence that ensures the addition of a terminal adenine to the PCR product in order to reduce band stuttering, which is characterized by the inconsistent addition of a terminal adenine, causing alleles that differ in size by one base pair (Brownstein et al. 1996).

The Qiagen Type-it™ kit (Germantown, MD) was used to multiplex reactions. All concentrations were prepared according to the manufacturer's instructions, with 1µl of

each sample added to the final mix, forward and reverse primers both used in 0.2µM concentrations, 12.5µl of the supplied master mix, and the balance of the 25µl sample consisting of water. Each 1µl of a sample resulted in 0.1-10ng of DNA, which necessitated more PCR cycles. The protocol utilized for each of the five multiplex batches was as follows: 95°C hot start for 5 minutes, 35 cycles of 95°C for 30 seconds melting, X°C for 60 seconds annealing, where X is the annealing temperature of the particular batch (Table 1), 72°C for 30 seconds extension, followed by 60°C for 30 minute final extension period. The PCR products were analyzed via gel capillary electrophoresis on the ABI 3730xl DNA Analyzer, with the 400HD ROX size standard at the University of California, San Francisco (UCSF). The TaKaRa™ kit was also used when singleplexing the reactions was appropriate. This PCR protocol also involved a 25µl reaction, however, 0.5µM final concentration of the primers was used. Per the instructions, 2.5µl of the buffer provided, 2µl of dNTPs, and 1 unit of TaKaRa Ex Taq™ polymerase were used, and the balanced was filled with water. The PCR protocol for this kit involved a 94°C hot start, 40 seconds at 94°C, 40 seconds at the appropriate annealing temperature, and 30 seconds at 72°C extension, with a 3 minute 72°C final extension.

DNA from 285 individuals was analyzed from four sites in Mono Co., CA. The population genetic parameters for the loci were estimated using Arlequin 3.11 software (Excoffier 2005). Tests for linkage disequilibrium and deviation from Hardy-Weinberg equilibrium (HWE) were conducted on each locus and across all loci. HWE was calculated by Arlequin according to the method developed by Guo and Thompson (1992),

**Table 1**. Characterization of the primers, including fluorescent labels and multiplex information. Sizes ranged from 129-316 base pairs, and allele numbers per locus ranged from 4-15.

| Locus Name | Primer Sequence | Repeat Motif | No. Alleles | Fragment Size | Allelic Range | Multiplex group | Annealing T °C |
|---|---|---|---|---|---|---|---|
| A116 | **F:** FAM-TCTGTCTCACCTCCTGTGTC<br>**R:** GCAAACTCACCTCTAAGATGG | CA | 12 | 300-316 | 16 | 2 | 57.0 |
| A120 | **F:** FAM-TTCCTCTGAGCCTCCCTAAC<br>**R:** AACCCAAGTGAGAAGTAACGG | CA | 13 | 143-182 | 39 | 3 | 57.0 |
| B6 | **F:** FAM-CACCCTCCACCTTTTAGAAG<br>**R:** TCCAATGAACTTTTCCATCTC | AAC | 4 | 129-147 | 18 | 1 | 55.0 |
| B12 | **F:** HEX-CCAGCCTACTTTGTTGTTCC<br>**R:** CACCAGGACAGCACACATAC | AAC | 4 | 213-225 | 12 | 3 | 57.0 |
| B108 | **F:** HEX-GGAGCGTCAATGGAGAGG<br>**R:** GGCAGAAGGCAGAACTGG | AAC | 7 | 279-298 | 19 | 5 | 58.5 |
| C4 | **F:** FAM-ACTTGCATCCTTCTAGCTCTG<br>**R:** TCCCATTCTTTGTAAACTACCC | AAAG | 15 | 225-281 | 56 | 4 | 56.0 |
| C11 | **F:** HEX-CCTGTTCTGGTGACTTCTG<br>**R:** AGGTCCACTGTAATGCTTG | AAAG | 12 | 206-229 | 23 | 1 | 55.0 |
| D4 | **F:** HEX-AGCAAGACCCTAAGCAAC<br>**R:** AGCACCCTGTTACAAAGG | TAGA | 14 | 271-343 | 72 | 4 | 56.0 |
| D106 | **F:** FAM-GGACCAGAGTGGTACTTCTGTG<br>**R:** AGCACCCAGAGACTGTGACTTA | TAGA | 4 | 164-176 | 12 | 5 | 58.5 |
| D107 | **F:** NED-CAACTCTGAATCCCTCACAG<br>**R:** TCCAAGCTGAATCCTCTACTAG | TAGA | 20 | 169-207 | 38 | 4 | 56.0 |
| D108 | **F:** HEX-CACCAACTGTAAACCTGTTCTG<br>**R:** CAACGTCAGTGAGACTGTGTC | TAGA | 7 | 199-223 | 24 | 2 | 57.0 |

with a burnin of 1000 and 100,000 steps in the Markov chain. Linkage disequilibrium was calculated according to the method described by Slatkin and Excoffier (1996).

## Results

Of the eleven polymorphic loci, nine were ultimately used for population structure and parentage analysis (Table 2). C11 demonstrated poor amplification; 16% of the samples failed to amplify over multiple tries with varying primer and DNA concentrations, and thus the locus was discarded. Significant deviations from HWE were detected at two loci. Locus A120 deviated from HWE in half of the sites (Table 2), which was enough to cause the samples to deviate from HWE when all sites were analyzed together. Locus D106 deviated from HWE only when all sites were combined ($p=0.04687$). Because there is so little gene flow between the lower and upper elevations, we decided to keep D106 as a marker, as both populations independently were in HWE. It was also in HWE in the upper elevation populations, which was analyzed on 234 samples. That the locus was out of HWE when the samples were combined may be a result of isolation and genetic drift. Locus A120 was discarded because it deviated significantly ($p<0.001$) in the upper elevation samples, which comprised the largest population. The nine microsatellites that amplified adequately and were in HWE exhibited a range of 4-15 alleles. They exhibited a combined heterozygosity of $0.67\pm0.19$ (Table 2).

Significant linkage disequilibrium ($p<0.05$) was found between various loci (Table 3), though not consistently. Linkage disequilibrium differed in the upper

elevations, lower elevations, and when all samples were combined. Because it was the largest population and therefore had the most statistical power, the upper elevations analyzed together are the reported significant linkage disequilibria.

Locus B12 exhibited a peak of 210 base pairs, which was of uniform size and area in every sample and larger in size and area than every other allele identified, regardless of the homozygosity or heterozygosity of the other alleles. The size excludes primer dimerism, as the primers are both 20 base pairs long. Because it was one short tandem repeat off of the other alleles identified for this locus, it could not be discounted as an artifact, because it could be a genuine 210 allele, or it could mask a 210 allele. The data suggest, however, that it is not an allele at the same locus. When the peak was discounted and the samples were subsequently scored as homozygous or heterozygous, which is consistent with the peak areas given by the ABI 3730xl DNA Analyzer, the data are in HWE and consistent with homozygous and heterozygous data. If the 210 peak is scored as a true allele at that locus, then all samples are heterozygous, and some samples display three peaks. It was concluded that the primers are annealing correctly to both a microsatellite locus that is variable, and, with greater affinity, to one which is highly conserved.

Loci A116 and D107 exhibited many alleles that did not fall cleanly into the sizes predicted by the microsatellite repeat, which was originally thought to be the result of band stuttering. The GTGTCTT PIGtail did not alleviate this problem, but it also did not change the size of the alleles upon reanalysis. It is likely that these alleles deviate from

**Table 2**. Observed and expected heterozygosities of the microsatellites. "High" and "low" refer to the elevation sites; "pooled" refers to all locations combined. Locus C11 was excluded because it failed to amplify adequately. The average heterozygosity calculation was performed without locus A120 because it deviated significantly ($p<0.05$) from HWE in the larger upper elevation. B106 deviated significantly when elevations were pooled, but because it was in HWE in the both the upper and lower sites independently the locus was kept.

| Locus Name | High Ho | High He | P-value | Low Ho | Low He | P-value | Pooled Ho | Pooled He | P-value |
|---|---|---|---|---|---|---|---|---|---|
| A116 | 0.75862 | 0.77093 | 0.21707 | 0.62745 | 0.59619 | 0.83767 | 0.73498 | 0.74789 | 0.06871 |
| A120 | 0.78696 | 0.82958 | 0.00071 | 0.56863 | 0.59678 | 0.84712 | 0.74733 | 0.81514 | 0.00000 |
| B6 | 0.21030 | 0.20871 | 0.23890 | 0.44000 | 0.34667 | 0.09204 | 0.25088 | 0.23883 | 0.46568 |
| B12 | 0.67382 | 0.66502 | 0.41181 | 0.54902 | 0.60396 | 0.58261 | 0.65141 | 0.65737 | 0.18884 |
| B108 | 0.53680 | 0.51860 | 0.07414 | 0.62000 | 0.63374 | 0.47361 | 0.55160 | 0.54887 | 0.14868 |
| C4 | 0.90086 | 0.88792 | 0.60589 | 0.80392 | 0.80217 | 0.17836 | 0.88339 | 0.89039 | 0.06018 |
| D4 | 0.84783 | 0.83175 | 0.60813 | 0.68627 | 0.82566 | 0.19417 | 0.81851 | 0.84043 | 0.12877 |
| D106 | 0.58696 | 0.57899 | 0.17756 | 0.66667 | 0.67696 | 0.10267 | 0.60142 | 0.60919 | 0.04687 |
| D107 | 0.84000 | 0.85823 | 0.12886 | 0.81633 | 0.83000 | 0.12509 | 0.83577 | 0.85882 | 0.06402 |
| D108 | 0.69397 | 0.70588 | 0.38518 | 0.82353 | 0.74335 | 0.06932 | 0.71731 | 0.72686 | 0.17845 |
| Avg het | 0.67213 | 0.66956 | | 0.67035 | 0.67319 | | **0.67170** | 0.67985 | |
| Stdev | 0.21160 | 0.21348 | | 0.12992 | 0.15342 | | **0.19243** | 0.20191 | |

the predicted simple stepwise mutation model of microsatellite allele formation. However, only a handful of samples were analyzed with the PIGtail, so it is not possible to say that no band stuttering is occurring.

**Table 3**. Linkage disequilibrium between the loci. Those combinations indicated with a + exhibited significant linkage disequilibrium ($p<0.05$).

| Locus | A116 | B6 | B12 | B108 | C4 | D4 | D106 | D107 | D108 |
|---|---|---|---|---|---|---|---|---|---|
| A116 | | - | - | - | - | - | - | + | - |
| B6 | | | - | - | - | - | - | + | - |
| B12 | | | | - | + | - | - | + | + |
| B108 | | | | | + | - | - | - | - |
| C4 | | | | | | - | - | - | + |
| D4 | | | | | | | + | + | - |
| D106 | | | | | | | | - | - |
| D107 | | | | | | | | | + |
| D108 | | | | | | | | | |

## Discussion

These nine loci used with the multiplex method will allow for a detailed population genetic analysis of *U. beldingi.* Utilization of these markers may also be possible in cross-species amplification for *Urocitellus* species.

If the 210 peak in locus B12 is present in other species, this could both indicate an important region of the genome, and help elucidate the phylogeny of this genus. It is possible that the extra observed peak is the result of a transposition event (Pestsova et al. 2000). The extraneous peak in B12 may be the result of a duplication event and subsequent loss of function of a highly conserved region of the genome (Angers et al. 2002). Because it is so highly conserved, it could be a useful molecular marker in elucidating the phylogenetic tree of Spermophilidae, whose phylogeny is not completely

resolved (Helgen et al. 2009). This fragment should be sequenced and analyzed in other Spermophilidae species to determine its presence and usefulness as a genetic marker.

Elimination of the the extraneous peak in the B12 amplifications could be achieved by a couple of methods. More information regarding the sequence of the extraneous peak and the genuine alleles is necessary before one could proceed. Once this information is known, using either a nested PCR or trying a different primer set may result in amplification of only the polymoprhic microsatellite region (Mullis et al 1986).

Linkage disequilibrium was calculated for each Arlequin run conducted, but results were inconsistent. We chose to use the results from the upper elevation samples in the higher sample run, as the upper elevation comprised the largest population, and we observed limited migration between the two populations. Because there was such inconsistency between runs, further analysis of linkage disequilibrium should be conducted to determine to what extent this occurs for these microsatellites.

We have characterized nine polymorphic microsatellite loci in *U. beldingi* and developed PCR parameters to optimize amplification and analysis of the fragments. They can be multiplexed to reduce the amount of time and reagents required to fingerprint an individual or conduct population or parentage studies. In the future, these loci should be tested for cross-species amplification in other *Urocitellus* species.

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**Fine-scale genetic analysis of *Urocitellus beldingi* (Belding's ground squirrel), a social, montane rodent living in the High Sierra**


## Abstract

*Urocitellus beldingi* (Belding's ground squirrel) is a social, montane rodent that inhabits meadows in the mid to high elevations in the Sierra Nevadas. The Tioga Pass Meadow (Mono Co. CA) population has been studied behaviorally and demographically for several decades. Over the course of eight years, squirrels samples were collected from four sample sites at two different elevations, separated by approximately 6.5 km. We used polymerase chain reaction (PCR) on nine microsatellite loci to elucidate the fine scale genetic structure, offering insight to population health, stochastic events, and dispersal. Two populations were identified ($F_{ST}$ = 0.05460, $p<0.00001$), corresponding to the elevation differences. The smaller site of the higher elevation experienced a population crash between 2004 and 2010 and is hypothesized to be a sink population, with the source likely being the larger Tioga Pass Meadow. $N_e$ was determined to be 170 for the upper elevations and 40 for the lower elevations. $N_m$ between the two was 8.66. Our data confirm previous studies that indicate a healthy, stable population in the Tioga Pass Meadow region that is not adversely affected by inbreeding

## Introduction

*Urocitellus beldingi*, or Belding's ground squirrel, is a social ground squirrel whose native range extends from northern Oregon to central California, and east into southwestern Idaho and the northwest corner of Utah, in alpine and subalpine meadows, from approximately 2000m to 3500m above sea level (Jenkins and Eshelman 1984). Over the course of four decades, the Tioga Pass meadow (which measures approximately 1km by 500m) population of *U. beldingi* has been studied behaviorally and demographically (Sherman 1977, Morton and Sherman 1978, Hanken and Sherman 1981, Holekamp 1984, Bushberg and Holmes 1985, Nunes, 1997, 2004a, 2004b, etc.). Hanken and Sherman (1981) used allozymes to demonstrate that multiple paternity occurs, and found that 55-75% of litters were multiply sired. They were unable to determine the exact number of sires for most of their litters. Nunes (2004a) monitored glucocorticoid levels in relation to play. These studies have been used primarily to augment behavioral observations made in the field. While these molecular methods have been employed, to date, only a small, cursory study involving the cytochrome B gene in mtDNA has been conducted (Curtain unpubl.)

Previous research has indicated that the squirrels disperse either during the end of their natal summer or as yearlings during the subsequent summer (Sherman 1976, Holekamp 1984, Holekamp 1986). The exact causes for dispersal are not known, but food availability, density, and ectoparasite load do not appear to be the causes (Holekamp 1986). On average, males disperse earlier and farther than females, with males averaging

272 ± 19m from their natal burrows after two years, whereas females averaged 33 ± 7m from their natal burrows (Sherman 1976, Holekamp 1984). In the upper limits of observed dispersal, males have been observed ranging over 1800m from their natal burrows, but females generally do not disperse farther than 100m (Sherman 1976). Furthermore, males do not occupy the same territory from year to year, but rather move each season (Sherman 1976). Such behavior, where females remain close to their natal burrows, leads to matrilines, and also increases the likelihood of a female to engage in alarm calling in response to a terrestrial predator, as such alarm calling has been found to be altruistic (Sherman 1977).

Microsatellites are useful markers for fine-scale population structure and parentage questions because they are not highly conserved; they evolve on an ecological time scale of several generation as opposed to an evolutionary timescale of thousands of generations (Jarne and Lagoda 1996, Storfer et al. 2009). They are non-coding regions of the genome consisting of repeats of a base pair motif (e.g. TAGA), which is between two and nine base pairs long, and may be repeated a few times or a hundred times. When enough polymorphic loci are analyzed, a unique DNA fingerprint of an individual can be identified based on the specific combination of alleles for the loci (Wright et al. 1994). Microsatellites are numerous and distributed throughout the genome almost evenly (Weissenbach et al. 1993). They are abundant, biparentally inherited, neutral mendelian markers (Litt and Luty 1989, Weissenbach et al. 1993, Queller et al 1993). Because of their high mutation rate, they are more variable than more highly conserved markers,

such as allozyme or mitochondrial markers (Jarne and Lagoda 1996, Storfer et al. 2009). These factors make them useful analytical tools for investigating fine-scale genetic structure of a population, metapopulation structure, and parentage (Wright et al. 1994, Jarne and Lagoda, 1996, Pestsova et al. 2000). Microsatellite data can obtained in minimally invasive ways without sacrificing or impairing an individual and has been used to determine genetic diversity and parentage in a variety of species across many taxa (e.g. humans, Litt and Luty 1989; cod, Bentzen et al 1996; crocodiles, Dever and Densmore 2001; lions, Spong et al. 2002; squirrels, Garner et al. 2005; honeybees, Oxley et al; soybeans, Li et al. 2011, etc.).

Polymerase chain reaction (PCR) is a technique that allows for fast and accurate amplification of a region of interest within the genome from a small sample of DNA (Mullis et al, 1986, Saiki et al 1988). Specifically designed primers anneal to flanking regions which surround the target region (Mullis et al, 1986, Saiki et al 1988). This method can be used to amplify specific microsatellite loci within a genome. Characterizing microsatellites and their PCR conditions provides a basis on which future research can be built for both the species for which the microsatellites were identified and other closely-related species.

Gene flow can be measured by calculating $F_{ST}$ or $R_{ST}$, both of which are inbreeding coefficients calculated by analyzing allele frequencies of polymorphic loci (Hudson et al. 1992). It can also be measured more directly by investigating the number of migration events ($N_m$) between populations, measuring genetic divergence as a

function of number of migrants in a generation (Hudson et al. 1992, Palsbøll et al. 2006). Immigration and emigration can be important sources for genetic diversity, especially in small populations (Tamura and Hayashi 2007).

Previous gene flow work on *U. beldingi* involved the use of mtDNA markers, which are more susceptible to genetic drift and other stochastic effects than microsatellites and also only effectively measure gene flow in females, thus are likely to demonstrate lower levels of gene flow (Swei et al. 2003). The use of microsatellites to investigate population questions and gene flow could give a more accurate picture of the population. Furthermore, dispersal patterns and population structure in rodents tends to vary greatly, and assumptions cannot necessarily be drawn across species lines, necessitating investigation in as many species as possible (Booth et al. 2009). This information could determine whether populations in the Mono Co. area comprise a metapopulation, a population comprised of several subpopulations connected by low levels of dispersal.

Nunes (1997, 1998, 1999, 2004a, 2004b) has observed and sampled the squirrels from various points in the Tioga meadow, the north side of Tioga Lake, across Hwy 120, near Yosemite, and at Lee Vining and Aspen campgrounds. His work has investigated reasons for dispersal (1997, 1998) and how play affects young squirrels (1999, 2004a, and 2004b). The two sites campsites are 2200m in elevation, and 6.5 km from the Tioga Pass meadow. Each of the campgrounds measures 100m by 50m, and they are separated by <1km. The objectives of this research were to elucidate the population structure of

our sample sites determine whether the squirrels in these areas comprise a metapopulation, what the population boundaries are, whether the population is affected by inbreeding, and determine whether there was structuring within the Tioga Pass meadow.

## Methods

Squirrels were sampled from four distinct sites in Mono Co. CA on State Highway 120, east of the Tioga Pass entrance to Yosemite National Park. Four different meadows were sampled (Figure 1). Two sites are at an upper elevation of 3000m (Tioga Pass meadow and North Tioga lake), and the other two sites (Aspen Campground and Lower Lee Vining Campground) are at approximately 2200m. The upper and lower elevation sites are separated by approximately 6.5 km. The terrain between the sites consists of forest and meadow and does not include impassible elevation changes. The lower elevations and North Tioga lake were sampled in 2004 and 2010, and Tioga Pass meadow was sampled between 2002-2005 inclusive and in 2010. In the interim, while not sampled genetically, the squirrels captured in the Tioga Pass meadow population were tagged, so it was possible to identify squirrels marked 2006-2009 upon recapture in the 2010 season.

Tomahawk chipmunk live traps (Tomahawk, WI) baited with peanut butter were used to trap the squirrels. Trapped individuals were uniquely marked with hair dye and ear tags, both of which are common, minimally invasive methods of allowing for immediate visual identification of individuals both within that field season and over the

course of the individual's life (Sherman 1976, Sherman and Morton 1984). Each squirrel also had a sliver of its ear tissue from the medial edge, measuring approximately 1mm by 3mm, clipped for later DNA extraction. Tissue samples were kept on ice in the field until they could be transferred to -20° C, where they were stored until DNA extraction.

The Qiagen DNeasy™ Blood & Tissue kit (Germantown, MD) was used to isolate DNA from ear tissue. Extractions were performed according to the protocol for purification of total DNA from animal tissues, with an overnight digestion incubation and two elutions in the final step to maximize yield. DNA was isolated from 160 of the animals sampled between 2002 and 2005, and all 125 of the individuals sampled in the 2010 season, which included 13 squirrels trapped in the lower elevations and 112 trapped in the upper elevations (Table 4). To determine paternity and sibship, DNA was isolated from as many mother and pup groups and males as possible.

**Table 4**. Geographic and temporal distribution of the squirrels sampled. Upper elevations include the Tioga Pass meadow and North Tioga lake; lower elevations include Aspen and Lower Lee Vining campgrounds. The sites are separated by approximately 6.5km.

| Location | 2002-2005 | 2010 | Total |
|---|---|---|---|
| Lower Elevations | 38 | 13 | 51 |
| Upper Elevations | 122 | 112 | 234 |
| Total | 160 | 125 | 285 |

Microsatellites specific to *U. beldingi* were previously isolated, and primers were designed by Genetic Identification Services (GIS) (Chatsworth, CA). Subsequent characterization of the primers resulted in nine polymorphic loci which were used in this study. The microsatellites ranged from two to four base pairs in size, and were

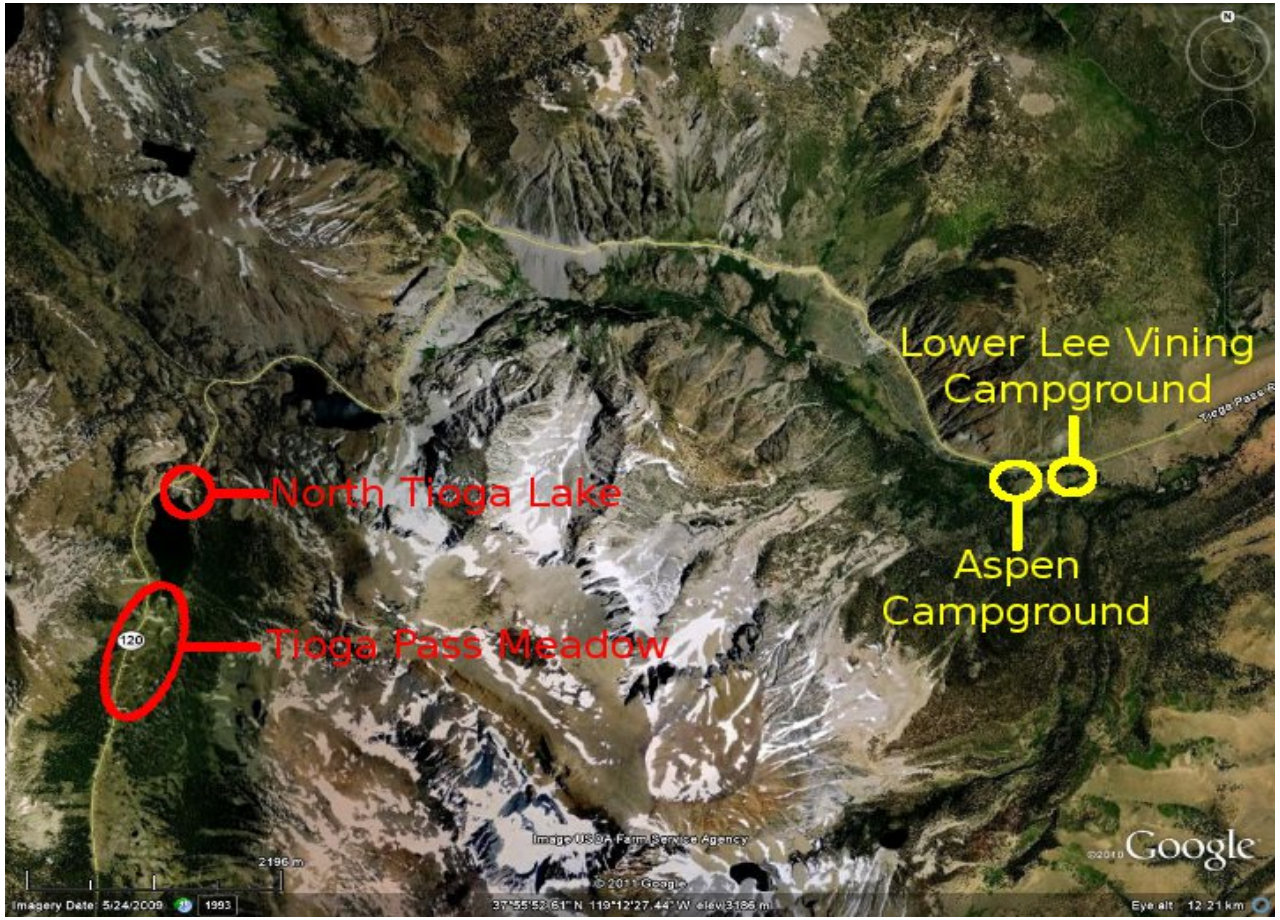


**Figure 1**. Satellite map of the sample sites (Mono Co, CA). Red sites are at approx. 3000m elevation, yellow sites are approx. 2200m elevation. The two elevations are separated by approximately 6.5 km.

between 129 and 316 base pairs in length (Table 1). The forward primers were fluorescently tagged so that the PCRs could be multiplexed, and the 5' end of the reverse primer reverse primers for A116 and D107 were PIGtailed with GTGTCTT to avoid band stuttering (Brownstein et al. 1996).

Because of the number of DNA samples and microsatellite loci used in this study, the number of PCR reactions required was large. Multiplex PCR was utilized to expedite the processing of these samples and minimize the amount of reagents needed. By multiplexing the reactions, as many as ten or eleven markers can be run in a single well, greatly expediting the process of amplifying the markers of interest (Morral and Estivill 1992, Luikart et al., 1999, Eggelston et al. 2002, Markoulatos et al. 2002). The Qiagen Type-it™ kit (Germantown, MD) was used to multiplex reactions. All concentrations were prepared according to the instructions for the Qiagen Type-it™ PCR kit (Germantown, MD), with 1µl of each sample added to the final mix, forward and reverse primers both used in 0.2µM concentrations, 12.5µl of the supplied master mix, and the balance of the 25µl sample consisting of water. Each 1µl of a sample resulted in 0.1-10ng of DNA, which necessitated more PCR cycles. The protocol utilized for each of the five multiplex batches was as follows: 95°C hot start for 5 minutes, 35 cycles of 95°C for 30 seconds melting, X°C for 60 seconds annealing, where X is the annealing temperature of the particular batch, 72°C for 30 seconds extension, followed by 60°C for 30 minute final extension period. When only singleplex PCR was required, the TaKaRa PCR kit was used. The protocol also involved a 25µl reaction, but in this case, 0.5µM final

concentration of the primers was used. Per the instructions, 2.5µl of the buffer provided, 2µl of dNTPs, and 1 unit of TaKaRa Ex Taq™ polymerase were used, and the balanced was filled with water. The PCR protocol for this kit involved a 94°C hot start, 40 seconds at 94°C, 40 seconds at the appropriate annealing temperature, and 30 seconds at 72°C extension, with a final 3 minute 72°C extension. All multiplexed samples were analyzed via gel capillary electophoresis on the ABI 3730xl DNA Analyzer, with the 400HD ROX size standard at the University of California, San Francisco (UCSF). This technique allows for finer resolution of fragment size with higher accuracy and precision than agarose gel electrophoresis, especially when analyzing multiplex PCR samples (Vemireddy et al. 2007).

Allele frequencies at each locus were calculated using Arlequin 3.11 (Excoffier 2005). Arlequin was also used to calculate $F_{ST}$ and $R_{ST}$ (Slatkin 1995). The test for deviations from Hardy-Weinberg equilibrium (HWE) was performed by Arlequin, with a burnin of 1000 and 100,000 steps in the Markov chain (Guo and Thompson 1992). The migration rate ($N_m$) was estimated with Arlequin using the method explained by Slatkin (1991). Structure was used with a burnin period of 10,000 and 100,000 MCMC reps after the burnin to determine the number of populations (Pritchard et al 2000). Effective population size ($N_e$) was determined using the program NeEstimator using linkage disequilibrium, as described by Hill (1981) (Ovenden et al. 2007).

## Results

$F_{ST}$ values for the upper elevations in comparison to the lower elevations show

significant separation between the two sites ($F_{ST}$= 0.05775, p<0.00001), and an estimation of migration indicated that $N_m$= 8.66. $R_{ST}$ analysis yielded a lower value, though still significant ($R_{ST}$= 0.02230, p<0.00001), with $N_m$= 21.9. No difference was detected between the two campgrounds in the lower elevation sites, or between the Tioga Pass Meadow and North Tioga Lake upper elevation sites. No significant difference was found between male and females (p<0.05). Structure confirmed two populations defined by elevation indicated by Arlequin (Figure 2). Those squirrels sampled in the lower elevations fell cleanly into one population, indicated by the predominance of the green bar, while the squirrels sampled in the upper elevations fell cleanly into another population, indicated by the predominance of the red bar. NeEstimator indicated an effective population size ($N_e$) of 170 for the upper elevation sites and 40 for the lower elevation sites. The loci exhibited an average heterozygosity of 0.67±0.19 and ranged from 4-15 alleles per locus. With one exception the loci were in HWE. Locus A120 deviated from HWE significantly in the high elevations (Table 2), but the low elevation loci were in HWE. Because the high elevation samples greatly outnumbered the low elevation samples, the locus was discarded.

Locus B6 exhibited four alleles, one of which was found in only one individual. In each sample that amplified, locus B12 displayed an extraneous peak that was higher and larger in area than all other alleles. Furthermore, if considered a true peak, it made each sample heterozygous, and implied some samples had three alleles, which violates the axiom that each diploid individual possesses two copies of the microsatellite. When

the extraneous peak was discarded and samples were scored as homozygotes or heterozygotes, the locus was in HWE. Loci A116 and D107 exhibited alleles that were one base pair off of each other, which could be indicative of band stuttering. Despite the PIGtail, the alleles remained, indicating that they are not the result of band stuttering, but rather deviation from the standard stepwise mutation model (SMM) of microsatellite evolution (Slatkin 1995).

## Discussion

Our findings provide a fuller picture of the natural history of Belding's ground squirrel. The populations appear to be of a stable size and are not exhibiting any inbreeding depression. Heterozygosity estimates calculated from microsatellite loci are congruent with observed haplotype diversity estimates calculated from cytochrome B sequence data (Curtain, unpublish). The heterozygosity exhibited in these *U. beldingi* populations appears to be consistent with those exhibited by other rodents (e.g. Degus, Quan et al. 2008; European wood mouse, Booth et al. 2009; Eastern Chipmunk, Chambers and Grant 2010; Red Viscacha rat, Ojeda 2010).

$R_{ST}$ rather than $F_{ST}$ might have been a more appropriate measure of the populations, because $F_{ST}$ has been demonstrated to overestimate genetic similarity in comparison to $R_{ST}$ (Slatkin 1995). This, however, assumes that the microsatellite alleles are formed by the stepwise mutation model (SMM) (Slatkin 1995). The SMM assumes that each allele is created by the mutation of an existing allele by either the addition or removal of one repeat motif (Kimura and Ohta, 1978). Because of the deviation of these

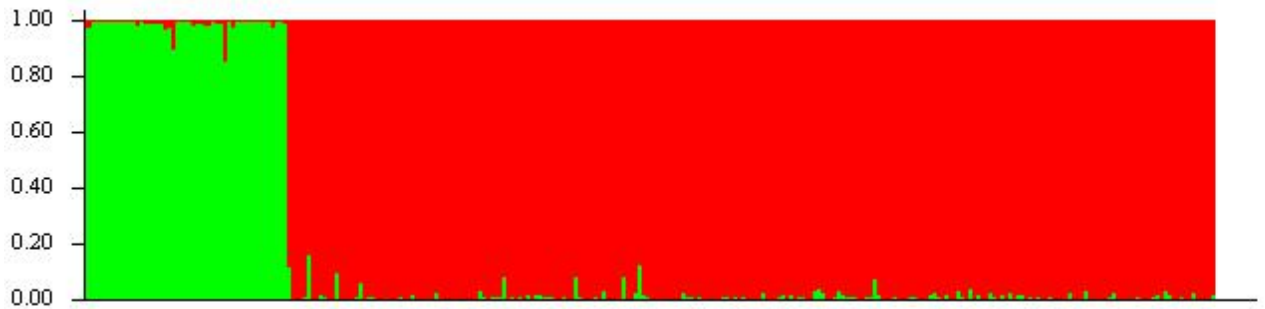


**Figure 2**: Structure plot indicating the presence of two populations. The green bar represents the probability of assigning the individual to the lower elevation population; likewise, the red bar represents the probability of assigning the individual to the upper elevation population. The split between the predominance of green and red corresponds exactly to the location in which the individual was captured.

microsatellites from the SMM, FST, rather than RST, was ultimately used, as it is more appropriate in such situations (Rousset 1996). We know that our microsatellites deviate from the SMM because their alleles are not separated by the size of the repeated motif. A116 and D107 exhibited numerous alleles one base pair in size smaller or larger than another allele. Several loci exhibited alleles that were separated by "n x motif unit" where n is a positive integer. Several loci also exhibited alleles that were separated by "n x motif unit +1". All of these conditions deviate from the assumption of the SMM. Because of this, RST is not the most appropriate metric, but rather FST (Rousset 1996).

Because only small, unmarked males were found in the North Tioga Lake site in the 2010 season, these could be yearling males that had dispersed to that location from another site, possibly the main Tioga Pass Meadow, as this site hosts a robust population and is within average male dispersal distance. Because of the size and proximity of the Tioga Pass Meadow, it is possible that it serves as a source population for the North Tioga Lake site, which is discontinuously inhabited by *U. beldingi*. Sherman (1976) found evidence of low levels of migration between Tioga Pass meadow and Dana meadow, and as the distance between those meadows is similar to the distance between the Tioga Pass meadow and the North Tioga lake site, it is plausible that migration occurs between the latter two sites. Such migration would create gene flow between the two sites.

While the distance between the two populations is greater than the largest recorded distance a squirrel has been observed moving in a season, males, who disperse up to ten times farther than females, are known not to inhabit the same area from season

to season (Sherman 1976). It is therefore possible that a male, over the course of multiple seasons, could move from one population to the other. Overall, gene flow appears to remain limited between the upper and lower elevations.

It is known that on average females disperse shorter distances than males, which leads to the formation of matrilines (Sherman 1976). Because of this burrow pattern, some genetic structuring within the Tioga Pass Meadow may exist. To determine whether such structuring exists, future research should focus on identifying the primary burrow used by the females, especially overnight. These burrows would be marked with a GPS device and distance between the burrows could be correlated to genetic data collected concurrently.

Fewer squirrels were observed in the 2010 collection season than in previous seasons. It is possible that the inclement weather that extended into late May of 2010 affected the overwintering mortality rate of the animals, as occurred in the 1977 snowstorm documented by Morton and Sherman (1978). Because emergence date appears to be at least partially dependent on surface air temperature, the fair weather followed by a late season snowstorm may have led to the death of some animals by starvation or exposure.

As the lower elevations are campgrounds cleared out of the forest, and since squirrels do not prefer to make their burrows in forested areas, the fragmented habitat and small population size may be susceptible to loss of genetic diversity. This may be exacerbated by low gene flow from the larger Tioga Pass meadow population. In the

future, this population should be monitored for genetic diversity, and efforts should be made to determine if other nearby subpopulations may also be part of this population. Overall, this information adds to the corpus of dispersal and population structure work that has been done on *U. beldingi*.

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**Parentage and Play analysis in Belding's Ground Squirrel**


## Abstract

*Urocitellus beldingi* (Belding's ground squirrel) is a social, montane rodent that inhabits meadows in the mid to high elevations in the Sierra Nevadas. The Tioga Pass Meadow (Mono Co. CA) population has been studied behaviorally and demographically for several decades. While *U. beldingi* maternity can be deduced by observing female burrows usage during gestation and first emergence of offspring from the burrow associated with that female, paternity can only be posited by observation because the species exhibits multiple paternity, and not all matings result in offspring. Microsatellites were used to verify maternity and determine paternity, which was correlated with previously collected behavioral data. We observed multiple paternity at a rate of 1 father per 1.35 offspring in a litter. While previous observations indicate preference of fullsib play partners in juveniles, our findings were not able to resolve this preference.

## Introduction

*Urocitellus beldingi*, or Belding's ground squirrel, is a social, montane rodent that lives in meadows in the High Sierra of central and northern California (Sherman and Morton 1978). The populations in and near Yosemite National Park, specifically in Dana Meadows and Tioga Pass Meadow, have been studied behaviorally and demographically for over four decades (Sherman 1976, Sherman and Morton 1984, Bushberg and Holmes 1985, Nunes et al. 2004a, etc.). The species exhibits multiple mating in both sexes with evidence arising from both field observations of mating and allozyme analysis (Sherman 1976, Morton and Sherman 1978, Hanken and Sherman 1981). Allozyme analysis has indicated that litters can be multiply sired, often by two or three fathers (Hanken and Sherman 1984).

Extensive research on play behavior in juvenile *U. beldingi* has been conducted (Nunes 1999, 2004a, 2004b). Previous studies have involved cross-fostering pups (Nunes et al. 2004b), removing pups from the natal burrow of their biological mother and placing them in a different natal burrow. The pups were marked by toe clips, and their play behavior was then observed in order to determine the level and type of interaction between differing levels of relatedness and association as juveniles.

Multiple mating in females may be driven by selection of subsequent mates as being superior to earlier mates, in order to increase genetic diversity and thus improve reproductive success, or as a method of "bet hedging", where a female may not be able to discern which males are best (Stockley et al. 1993). There is also some evidence that

females that mate multiply have increased offspring survival than do monogamous females (Stockley 2003). This may be driven by genetic similarity between the mates, with those individuals who are more closely-related producing less viable offspring (Tregenza and Wedell 2000).

Observational field methods are a reliable way of determining maternity. Timing one's work with the first emergence of a litter from its natal burrow and correlating that with the pattern of usage established by the female associated with the burrow provides an accurate and non-invasive way of assigning maternity (Sherman 1976). The same does not hold true for paternity. Since multiple paternity exists in *U. beldingi*, and fathers do not engage in any parental care, paternity can only be narrowed down to those males observed mating with the female in question during that season (Sherman 1976, Morton and Sherman 1978). The number of matings does not necessarily correspond to the number of fathers represented in the litter and gives no information about the number of pups associated with any given father.

Microsatellites can be used to elucidate parentage (Koskinen and Bredbacka 1999, Jones et al. 2010), and when one parent is known, the other parent can be deduced with high exclusion. It is also possible to utilize microsatellite data of siblings to further refine parentage assignment for members of a litter (Usha et al 1995, Heyen et al. 1997, Luikart et al. 1999, Jones et al. 2010). This technique has been employed successfully in both domestic animals, where some mating and parentage information may be known *a priori* (e.g. Usha et al. 1995), and in free-living animals, where little to no mating information

can be assumed (e.g. Nielsen and Nielsen 2007 di Pierro et al. 2010). While allozyme analysis of multiple mating was performed in this species, microsatellites have a higher mutation rate than allozymes, and therefore may be more sensitive (Voelker et al. 1979, Hanken and Sherman 1981, Jarne and Lagoda 1996, Haig 1998).

Belding's ground squirrels are able to differentiate kin from non-kin and have been observed being less agonistic toward full-siblings than half-siblings (Holmes 1986, Holmes 1994, Mateo et al. 2000). This recognition persists from year to year in both females and males, despite males dispersing and rarely coming into contact with related individuals (Mateo et al. 2000).

There are several hypothesized reasons that juvenile mammals engage in play, including development of social and motor skills, expenditure of excess energy, and strengthening of social bonding in play partners (Bekoff 1972). This holds true for *U. beldingi* (Nunes 1997, 1998, 1999, 2004a, 2004b). Holmes (1997) found a preference for litter-mate playmates over non-litter-mate playmates, suggesting that they recognize close kin and use play to strengthen such relationships. Our objectives are to refine estimates of multiple paternity and determine whether the squirrels' ability to differentiate between both kin versus non-kin and fullsibs versus halfsibs would yield a preference for fullsib playmates.

## Methods

Tissue samples were collected from squirrels trapped in 2002-2005 and in May-July of 2010. Animals were trapped in Aspen Campground, Lower Lee Vining

Campground, Tioga Pass Meadow, and a small meadow on the north side of Tioga Lake, henceforth North Tioga Meadow. The squirrels were trapped using Tomahawk chipmunk size live traps (Tomahawk, WI) baited with peanut butter. Traps were placed according to observation of areas squirrels frequented, with a large emphasis on making the trap and peanut butter an attractive option to the squirrel upon emergence from or return to its burrow. Because the squirrels are diurnal, traps were placed around 7:30 AM and retrieved around 6 PM, which coincides with their active period. Traps were closed during the night to prevent squirrels from entering them in the evening and being above ground through the night, which can lead to hypothermia. Eighty traps were set and monitored approximately once every 20 minutes to allow the squirrels to return to normal activity after our presence ceased, but not enough time to allow them to overheat in the full daytime sun exposure. Once captured, they were sexed and marked with ear tags for permanent identification and hair dye for observational studies, and a tissue sample was taken. The tissue sample consisted of a sliver of tissue approximately 1mm by 3mm taken from the medial edge of one ear. Samples were stored in cryo tubes and were stored on ice until they could be transferred to -20°C, where they were kept until extraction. Squirrels were not weighed, and as our study period occurred after mating and gestation, reproductive status was not possible to determine. If animals were recaptured, they were released at their point of capture and not handled a second time.

All 125 of the squirrels squirrels sampled in 2010 were analyzed in order to increase statistical power. This included 13 squirrels trapped in the lower elevations and

112 trapped in the upper elevations (Table 4). Of the animals sampled between 2002 and 2005, 160 were chosen for analysis. All of the squirrels from the lower elevations were analyzed, and among the squirrels of the upper elevations, as many mother and pup groups were preserved as possible. As many males as possible were included to increase the probability of later assigning paternity to a known individual. The rest of the available samples were chosen randomly due to time and financial constraints**.**

To define paternity, we used nine microsatellite loci to produce fingerprints for the individuals to be used as exclusion criteria. The microsatellites were identified and developed by Genetic Identification Services (GIS) specifically for *U. beldingi* (GIS personal correspondence). Both the TaKaRa™ PCR kit (Otsu, Japan) and the Qiagen Type-it™ PCR kit (Germantown, MD) were used to process the samples, the former being used when singleplex PCR was appropriate, the latter being used when multiplex PCR was appropriate. Tissue extraction was performed using the Qiagen DNeasy™ Blood & Tissue kit (Germantown, MD) according to their protocol for purification of total DNA from animal tissues.

Squirrel maternity was inferred by observing burrow usage of the females after mating, and both during and after gestation, and observing the first emergence of pups from the burrow. No positive assumptions regarding paternity were made *a priori*, though some males were excluded based on certain criteria. Exclusion factors involved temporal and spatial parameters. Given that the average dispersal distance of *U. beldingi* is 100m for females and 1000m for males, parental and filial relationships were not

considered between squirrels from higher elevations (Tioga Pass meadow and North Tioga lake) and lower elevations (the campgrounds). Similarly, squirrels identified as juveniles in the season in which the offspring in question was born were not considered for parental and filial relationships, because females do not mate until at least their yearling year, and males do not typically mate until their second year.

The computer program Colony (Jones and Wang 2010) was used to determine full and half sibship, confirm maternity, and assign paternity. Colony uses a full-pedigree likelihood method to determine relation, using information from all known relations. Colony analysis was performed on litters with a known mother. Squirrels without litter and maternal information were not analyzed for parentage. We analyzed 38 pups in 10 litters of known mothers. One litter had several pups represented with existing DNA samples, but the mother's sample did not amplify and so was inferred by the program. Because the relationship between the pups and the mother was known, this litter was included in the analysis.

## Results

Of the litters analyzed, Colony analysis indicated multiple paternity at a rate of 1 father per 1.35 pups. Males were also identified as fathering pups in litters of more than one female. We did not see significant difference in the number of mates that resulted in offspring for males or females (t-test $p = 0.14$) (Fig 3). As expected in such a mating system that involves multiple paternity, many more halfsib relationships exist in the pups we analyzed than fullsib relationships (Figure 4).

The play data consisted of 10 interactions involving 7 individuals. They do not support the hypothesis that juvenile squirrels preferentially seek out full siblings for play interaction over half siblings ($p>0.80$) (Fig. 5).

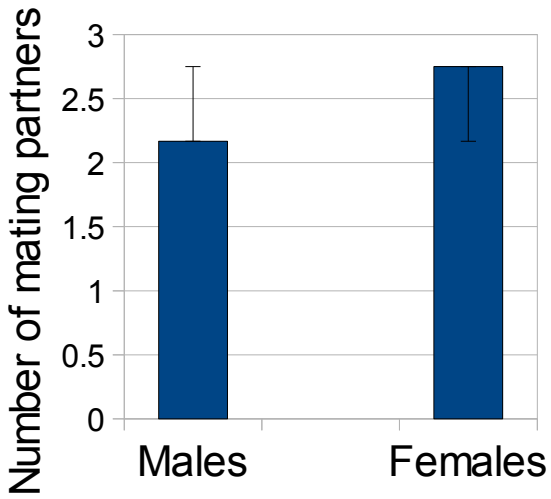


**Figure 3**. Average number of mating partners found in the analyzed litters. The data, analyzed by t-test and $p<0.05$ significance level, were not significant, though the tendency of females mating with more males than vice versa corroborates previous research.

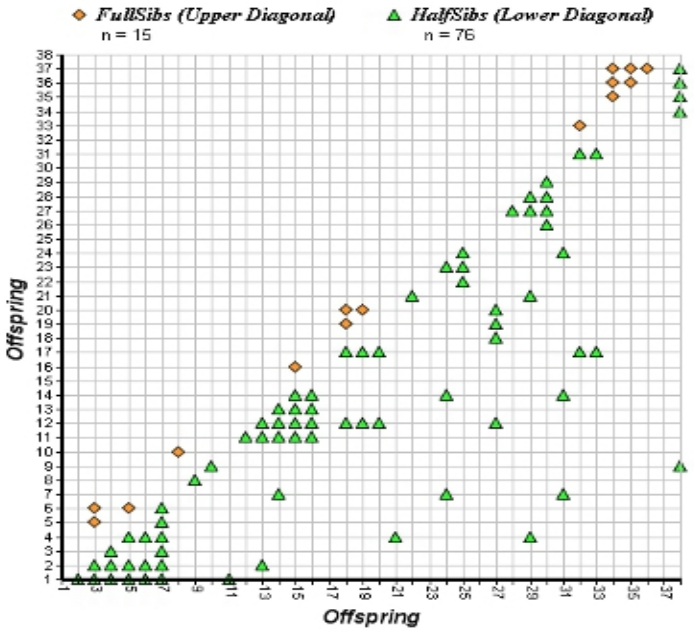


**Figure 4.** Colony output of halfsib and fullsib relationships based on 38 pups from 10 litters. The number of relationships is greater than the number of individuals because each point represents the relationship, not the individual.

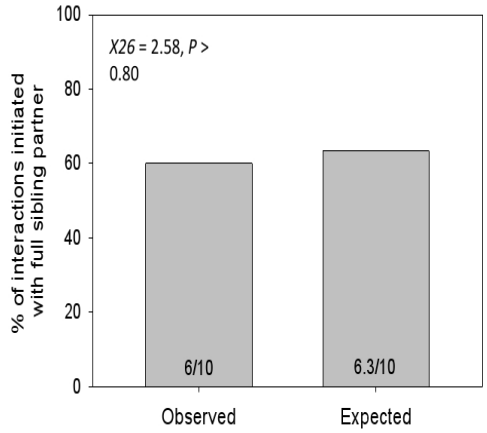


**Figure 5**. Chi-squiared analysis of expected and observed play interactions with fullsib pairs. No significant difference was found in play partner choice.

## Discussion

*Urocitellus. beldingi* exhibits promiscuous mating in both sexes. These data confirm previous allozyme and observational work that such a mating pattern in *U. beldingi* leads to multiply sired litters, with males also having the capacity to sire offspring in multiple litters in a single season. Because of the limitations in processing and analyzing samples, some males which may have been represented in the squirrels sampled between 2002 and 2005 were not analyzed, and therefore the parentage data is incomplete. Further investigation in these populations and years should begin with the analysis of the remaining samples.

If some of the microsatellite loci have higher rates of evolution than others, such mutations may result in the false exclusion of actual fathers, thus increasing the apparent rate of multiple paternity (Queller et al. 1993). This would also decrease the apparent number of offspring of those males that were reproductively successful. Sherman (1976) found that in 1975-76, 33% of females were monogamous. We cannot comment about whether genetic data validate this observation because of the incompleteness of mother/pup sampling.

Hanken and Sherman (1981) found that evidence that females mated with more partners than did males. Though our data were not statistically significant, we found this tendency to hold true. It should be noted that the mating data we observed only included those pairings that yielded offspring. More matings that did not yield offspring may have occurred in the field, but we were unable to make these observations.

Play data were inconclusive because of the small number of field observations that could be aligned with genetic data. Some samples were lost in the interim, and some litters lacked observational data. Further investigation into this would aid in supporting or refuting the hypothesis that play, especially between full female siblings, serves to reinforce social bonds that ultimately aid in sociality, such as territory defense or predator alerting. It may be that there will be no difference found between fullsibs and halfsibs regarding play preference. Sherman (1977) found that female squirrels were more likely to engage in alarm calling for terrestrial predators when in the presence of related female kin. Because the promiscuous mating system produces many more halfsibs than fullsibs, alarm calling may be more advantageous if halfsibs are strongly identified as kin. Furthermore, female squirrels do not disperse as far as males, rather choosing to stay close to their natal burrows, creating matrilines (Sherman 1976, Holekamp 1984, Nunes *et al*. 1997). This increases the number of related individuals within an area that would benefit from alarm calling of a female calling near her burrow.

Conspecific infanticide has been observed in *U. beldingi* in both males and females, though only males engage in cannibalism (Sherman 1976, Morton and Sherman 1978). Polyandry may decrease the likelihood of cannibalism of their litter by males, and while females have been observed killing the pups of other females, they have not been observed killing the pups of related females (Morton and Sherman 1978). Thus, having many half siblings and ambiguous paternity may increase the likelihood of the survival of one's litter.

Phenotype recognition has been observed in this species (Holmes 1986). Squirrels have been observed touching noses and engaging in olfactory recognition (Holmes 1997, Nunes et al. 1999). It is posited that the major histocompatibility complex (MHC) affects mate choice, with individuals preferring dissimilar mates (Tregenza and Wedell 2000). MHC also affects the olfactory sense, perhaps explaining why the squirrels touch noses (Tregenza and Wedell 2000). Future research may involve attempting to determine the manner in which kin recognize each other from season to season, and whether this supports the MHC hypothesis.

Sherman (1976) observed 33% of females engaging in monogamous mating, and that when females engaged in multiple mating, subsequent mates were larger, older, or had won more fights than previous mates. That multiple mating in females is not ubiquitous in this species, and because of the trend noted, the "best male" hypothesis, wherein a female is able to identify fitter males and preferentially mate with them, seems most plausible (Stockley et al. 1993). These findings provide the basis on which future research into multiple mating and paternity and the purpose of play in *U. beldingi* ontogeny can be built.

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## Conclusion

While *U. beldingi* is a species of least concern, other ground squirrel species face the possibility of extinction. This species, and the Tioga Pass meadow population in particular, may serve as a model species or population for social rodents. This knowledge adds to the corpus of ground squirrel work and may aid in developing conservation strategies for similar *Urocitellus* species.

Having microsatellites characterized for a species may eliminate the need to develop novel markers for other closely related species, which could save time and money for other researchers. Marker B12 could be used to resolve the phylogeny of Spermophilidae. While such a peak occurs rarely, microsatellites have been amplified successfully in closely-related species for which they were not developed (e.g. Luikart et al. 1999, Chambers and Grant 2010).

Our results corroborate previous research regarding population structure, immigration and emigration patterns, and the mating system present in this species, lending support through the use of neutral molecular markers. This offers further insight to the nature of the populations in the eastern Yosemite area and also the characteristics of the mating patterns exhibited by Belding's ground squirrel.

Future research should focus on investigating additional microsatellite loci identified by GIS and determining whether they are appropriate markers for population or parentage studies in this or closely related species. Furthermore, there exist several hundred unanalyzed samples dating from 2002-2005, and analysis of those could add

more insight to the population structure or parentage patterns in the populations, especially the Tioga Pass meadow population. More extensive trapping of the population coupled with field observations of the individual's location and burrow would assist in demonstrating whether or not genetic structuring based on matrilines is noticeable in the Tioga Pass meadow. Intensive trapping of males coupled with field observations of matings and sampling of litters could help determine whether females do mate with more partners than males, and also whether copulations take place underground, out of sight of field observers. The lower elevation population should continue to be monitored for genetic diversity, and further investigation should seek to determine whether there are additional areas that contribute to the population.

This work, combined with the substantial corpus of *U. beldingi* research that has proceeded it, has led to a fuller picture of the natural history of the species. It has the potential to help predict the fate of this and similar species and determine the function of play and how kin recognition occurs.

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