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Pathologic findings in Western gray squirrels (*Sciurus griseus*) from a notoedric mange epidemic in the San Bernardino Mountains, California ☆

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ABSTRACT

Notoedric mange, caused by the contagious, burrowing mite *Notoedres centrifera*, has been associated with several large-scale population declines of western gray squirrels (*Sciurus griseus*) and has been a significant obstacle to population recovery in Washington State where the species is listed as threatened. In 2009, residents and wildlife rehabilitators in the isolated San Bernardino Mountains of southern California reported a dramatic die-off of western gray squirrels, in what had been a previously dense and robust population. Individuals were observed suffering from abnormal neurologic behaviors (ataxia and obtundation) and severe skin disease. Full necropsy of five squirrels from the epidemic showed that all had moderate to severe infestation with mange mites and severe dermatitis characterized by hyperkeratosis, acanthosis, intralesional mites, intracorneal pustules and superficial bacteria. Mites from affected squirrels were evaluated by light and electron microscopy and identified as *N. centrifera* based on morphologic criteria. Additionally, the internal transcribed spacer-2 region of the mite was cloned, sequenced and accessioned in GenBank. The cause for the abnormal neurologic behavior was not confirmed on postmortem examination. However, we hypothesize that mange can cause incoordination and obtundation as a result of malnutrition and dehydration, and intense pruritis may induce abnormal or erratic behavior that could be mistaken for neurologic signs. While we have characterized the severe impact this disease can have on individual animals, more work is needed to understand the impact on squirrel populations, particularly in view of the anecdotal reports of dramatic population declines that may take decades to recover.

1. Introduction

The western gray squirrel (*Sciurus griseus*) is a native arboreal squirrel that inhabits oak and conifer forest ranging from southern California to northern Washington (Verts and Carraway, 1998). There are three subspecies; *S. griseus anthonyi* in the mountains of southern California, *S. griseus nigripes* in the central California coast, and *S. griseus* in northern California ranging from the Sierra Nevada Mountains north up throughout Oregon and Washington (Ingles, 1947). Major threats to western gray squirrel populations include habitat degradation/fragmentation, predation, disease and competition with native and non-native species of squirrels that are expanding in range including the eastern gray squirrel (*S. carolinensis*), the fox squirrel (*S. niger*) and the California ground squirrel (*Otospermophilus beecheyi*; Linders and Stinson, 2006).

Notoedric mange is a parasitic skin disease caused by the sarcoptiform mite *Notoedres centrifera,* formerly *N. douglasi* (Lavoipierre, 1964; Klompen, 1992). Infestation with these mites can cause alopecia and crusting of the skin and lead to secondary bacterial infection, emaciation and death, though spontaneous recovery can occur (Carlson et al., 1982; Nebraska Game and Parks Commission, 1991; Cornish et al., 2001). *Notoedres centrifera* has been reported in the western gray squirrel, the eastern gray squirrel, the fox squirrel, the southern flying squirrel (*Glaucomys volans*), the eastern chipmunk (*Tamias striatus*) and the black giant squirrel (*Ratufa bicolor*; Carlson et al., 1982; Klompen, 1992; Cornish et al., 2001). Notoedric mange is the most important known disease of the western gray squirrel, contributing to at least four documented large scale die-offs in California, Oregon and Washington since its

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earliest reports in the 1920s (Bryant, 1921, 1926; Payne, 1940; Cornish et al., 2001; Linders and Stinson, 2006; Vander Haegen et al., 2007). Notoedric mange caused the near extinction of a population in the Yosemite Valley, taking over 20 years to recover (Bryant, 1926; Michael, 1940; Payne, 1940). Notoedric mange has also contributed to population declines of Western gray squirrels in Washington State, where the squirrel is listed as threatened (Cornish et al., 2001). In contrast, notoedric mange may occur at a low prevalence in some squirrel populations without causing large die-offs (Asserson, 1974) and is probably endemic in some squirrel populations.

The San Bernardino Mountains, located in southern California, are considered a "sky island" whose plant and animal community differs dramatically from the surrounding semi-arid habitat (Grinnell, 1908). In 2009, residents and wildlife rehabilitators in the San Bernardino Mountains reported a dramatic die-off of Western gray squirrels. Residents reported dead squirrels at the base of trees, live squirrels exhibiting unusual and erratic behavior, and squirrels with hair loss and wounds. Although prospective surveillance was not performed, cases were initially identified at the western end of the San Bernardino Mountains, followed by reports progressing eastward to Lake Arrowhead and later Big Bear Lake, urban areas at 1550 m and 2050 m elevations, respectively, that had high densities of Western gray squirrels (Villepique, unpublished data).

In this report, we confirm the cause of the outbreak as the mange mite *N. centrifera* and report the full necropsy findings from five squirrels with moderate to severe mange. Lastly, we publish the first genetic sequence for the internal transcribed spacer-2 (ITS-2) region for mite identification by PCR, which will aid future research and diagnostics.

2. Materials and methods

2.1. Study area

The San Bernardino Mountains (34.1256° N, 116.8764° W) are located in southern California approximately 150 km east of Los Angeles, CA, USA. Elevation ranges from 800 to 3500 m. The climate is Mediterranean with most precipitation falling as snow above 2000 m and as rain below this elevation (Minnich, 1988). The foothill regions are primarily chaparral, with a transition to forest composed of Jeffery pine (*Pinus jeffreyi*), Ponderosa pine (*Pinus ponderosa*) and incense cedar (*Calocedrus decurrens*) above 1800 m (Grinnell, 1908). There are several communities within the mountain range, including Lake Arrowhead and the Big Bear Lake area, which are year-round tourist destinations, and have permanent residences, vacation homes and campgrounds. Though there have been no recent studies on local Western gray squirrels numbers, squirrels were reportedly dense in those mountain communities, as evidenced by the presence of sheet metal "squirrel guards" on trees and bird feeders at many residences (Stephenson and Villepique, unpublished data).

2.2. Animal collection and necropsy

Between April and June 2011, we examined five squirrels from the Big Bear Lake area that were observed by local residents, wildlife rehabilitators and biologists and subsequently reported to the California Department of Fish and Wildlife. All animals were observed pre-mortem and exhibited abnormal neurologic signs ranging from erratic behavior to obtundation. Animals were either euthanized ($n = 3$) or died shortly after capture ($n = 2$), and all appeared to be suffering from severe skin disease. Veterinary pathologists performed full post-mortem examinations including necropsy and histopathologic examination on all animals at the California Animal Health and Food Safety Laboratory, San Bernardino, CA, USA (CAHFS). Tissue sample collection and tests performed varied at the discretion of the pathologists. Tissues collected included skin, skeletal muscle, brain, spinal cord, trachea, lung, heart, liver, kidney, spleen, esophagus, stomach, small and large intestines, testicle or ovary, thyroid, thymus (when present), and adrenal gland. Grading of the skin lesions as moderate to severe on gross and microscopic examination was loosely adapted from the classification of Pence et al. (1983). Criteria used on gross examination were extent of lesion distribution and degree of encrustation and thickening of the skin. Criteria on microscopic examination were presence or absence of mites/mite density noted at 100× magnification in up to four randomly selected fields, and extent and severity of associated inflammatory lesions. Additional testing performed at CAHFS included West Nile Virus qRT PCR on three squirrels, *Salmonella* PCR on liver and/or intestinal tissue on four squirrels, aerobic bacterial culture on liver, lung and occasionally other tissues on all squirrels, and screening for heavy metal and selenium levels by inductively coupled plasma-atomic emission spectrometry on liver tissue from four squirrels. Virus isolation was performed on tissue pools from two squirrels at the Veterinary Medical Diagnostic Laboratory, College of Veterinary Medicine, University of Missouri-Columbia, Columbia, MO, USA. Fluorescent antibody testing for rabies virus on fresh brain tissue from four of the squirrels was performed at San Bernardino County Department of Public Health Laboratory, San Bernardino, CA, USA.

2.3. Mite collection and identification

Mites were collected from the skin of all five squirrels postmortem by scraping the skin on the head or forelimb with a sterile surgical blade. Scraped material was examined microscopically under 400× magnification. In order to visualize the mites, specimens were fixed with Karnovsky's fixative in 0.1 M sodium phosphate buffer (Sorenson's), and then washed using 0.l M sodium phosphate. Dehydration was accomplished in increasing concentrations of ethanol through 100% ethanol followed by critical point drying in a Tousimis 931.GL Autosamdri critical point dryer (Tousimis Research Corp, Rockville, MD, USA). The mites were mounted on aluminum stubs and sputter-coated with gold using a PELCO SC-7 coater (Ted Pella, Redding, CA, USA). The samples were viewed on an FEI XL30 TMP scanning electron microscope (Eindhoven, The Netherlands). Mites were identified as *N. centrifera* based on body size, absence of dorsal scale-like formations (as seen on *N. cati* and *N. musculi*), the pattern and location of dorsal striae, and morphology of dorsal setae (Lavoipierre, 1964; Klompen, 1992).

2.4. PCR, cloning and sequencing

A small section of affected skin tissue from three of the squirrels was used for DNA extraction using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the animal tissues (spin-column) protocol with overnight incubation with proteinase K at 56 °C. Amplification of the ITS-2 gene for sarcoptid mites was performed using external primers RIB-18 (5′- GGG CTG CAG TAT CCG ATG GCT TCG T-3′) and RIB-3 (5′- CGG GAT CCT TC(A,G) CTC GCC G(C,T)T ACT-3′) yielding an approximately 450 bp product (Zahler et al., 1999). PCR reactions were performed using GoTaq Green Master Mix (Promega, Madison, WI, USA) per manufacturer instructions. The final 25 µl reaction volume contained 1.0 M of each primer, 4.5 µl water and 3 µl of DNA. Thermal cycling conditions were 92 °C for 3 min; then 45 cycles at 92 °C for 60 s, 64 °C for 60 s, and 72 °C for 90 s; followed by 7 min at 72 °C. Water-containing negative control reactions were included in each run. Results of PCR were assessed by electrophoresis and UV-transillumination of

Table 1
Summary of data from five Western gray squirrels (*Sciurus griseus*) from Big Bear, California with notoedric mange including squirrel identification number (ID #), date of death, age at death, sex, body condition at death, if the animal was euthanized (Euth), and results of diagnostic tests performed as part of necropsy examination.

ID #	Date	Age	Sex	Body condition	Euth	West nile virus	*Salmonella* PCR	Virus isolation	Rabies	Intestinal parasites
1	4/18/11	6 wk	M	Poor	No	Neg (kidney)	NA	Neg (tissue pool)	Neg (brain)	Neg
2	4/29/11	A	M	Poor	Yes	Neg (brain, kidney)	Neg (liver)	Neg (tissue pool)	Neg (brain)	Coccidia
3	4/29/11	A	F	Fair	Yes	Neg (kidney)	Neg (liver, sm int)	NA	Neg (brain)	Coccidia Nematodes *Aspiculuria* sp.
4	5/6/11	A	F	Good	Yes	NA	Neg (liver, colon)	NA	NA	Coccidia
5	6/6/11	A	F	Good	No	NA	Neg (liver, sm int)	NA	Neg (brain)	Coccidia Nematodes

Tissue that assay was performed on is indicated in parentheses.
Neg, negative result; NR, not recorded; NA, test not performed on this animal; M, male; F, female; A, adult; sm int, small intestine; wk, week.

GelStar (Lonza, Rockland, ME, USA) stained 1% agarose gels. Bands of the expected size were excised and cleaned with a Qiagen gel extraction kit per manufacturer instructions. Three gel-extracted amplicons were then cloned using the pGEM-T easy vector system (Promega). Cloned products were sequenced in both forward and reverse directions in an ABI 3730 sequencer (Davis Sequencing, Davis, CA, USA). *In silico* analysis of the sequences was performed using BLAST search of GenBank (NCBI, National Institutes of Health, Bethesda, MD, USA). The sequences were then submitted to GenBank for accession.

3. Results

3.1. Necropsy

Of the five squirrels examined, four were adult (three female and one male), and the fifth was a juvenile male approximately six weeks old (Table 1). Three of the squirrels were euthanized due to concern for suffering and zoonotic disease, one died of trauma, and in the last case of a six-week-old squirrel that died, the only significant finding was severe skin lesions. Squirrel #5 had multiple skull fractures and associated hemorrhage and cause of death was determined to be severe trauma (e.g. fall from a tree). Body condition ranged from poor to good, with one animal suffering from emaciation (squirrel #2). All animals had moderate to severe mange, based on both gross and microscopic examination, which affected the skin of the head (including pinnae), neck, front legs and thorax, caudoventral abdomen and dorsomedial thighs. Lesions were often confluent involving greater than 50% of the body and grossly, the skin appeared thickened and crusted with areas of alopecia. Histopathologic examination demonstrated similar skin lesions in all five individuals. Lesions consisted of moderate to severe, irregular acanthosis with rete ridge formation, extensive orthokeratotic and parakeratotic hyperkeratosis with serocellular crusting, intracorneal pustules, numerous variably-sized, intracorneal and intraepidermal tunnels, and superficial bacteria (Fig. 1). Tunnels contained numerous mites, abundant brown globular material (waste products) and small numbers of round to oval eggs (Fig. 2). The superficial dermis had infiltrates of small numbers of leukocytes. Increased mite burden correlated with more severe gross and microscopic lesions. Additional findings included minimal focal non-suppurative interstitial pneumonia (squirrel #3), reactive lymphoid tissue (lymph nodes and spleen) and mild pulmonary neutrophilic leukocytosis (squirrel #4). Sections of brain and spinal cord were examined microscopically in all cases and no significant abnormalities were found. All four squirrels that were tested for rabies and three that were tested for West Nile Virus were negative, and virus isolation on tissue pools from the two tested squirrels was negative (Table 1). Of the four squirrels tested for *Salmonella* by PCR, all were negative. Most bacterial isolates were rare mixed flora with no predominance of any colony type on culture, and no histologic evidence of bacterial infection in tissues from which they were cultured, and so considered contaminants, although, *Staphylococcus aureus* was isolated from the lung and liver of squirrel #4 which may have been significant. Various intestinal parasites were detected in four of the squirrels. The livers of all squirrels had selenium levels slightly above the reference range, and all had one or more heavy metals above the reference range (Table 2).

3.2. Mite identification

Mites from all five squirrels were identified as *N. centrifera* based on the morphologic criteria described above. Additionally, four mites from one squirrel were visualized by electron microscopy for increased accuracy of body size measurement and better visualization of dorsal striae and dorsal setae. All were consistent with *N. centrifera*. Mites measured between 210–230 μm in length and 160–190 μm in width.

3.3. ITS-2 sequence

Sequencing of the three cloned products produced two 514 bp and one 483 bp regions of the *N. centrifera* genome. *In silico* analysis of the sequences showed 69% homology to *N. cati* (AF251801.1), which was the most closely related organism. The sequences contained a partial sequence for the 5.8S ribosomal RNA gene, on the 5′ end, the complete ITS-2, and a partial sequence for 28S ribosomal RNA gene at the 3′ end. The GenBank accession numbers are-KF278482, KF278483, and KF278484.

4. Discussion

Notoedric mange is a devastating disease that can cause significant morbidity and mortality in an animal population (Pence et al., 1995; Cornish et al., 2001; Riley et al., 2007). In this paper we have characterized the pathologic changes in five Western gray squirrels with moderate to severe notoedric mange. Anecdotal evidence suggests that prior to the outbreak the squirrel population was dense in urban areas with residents reporting seeing several Western gray squirrels in their yards every day. After the outbreak, most residents reported seeing a western gray squirrel only once every several weeks (Stephenson, Foley, and Clifford, unpublished data). While mange was the proximate cause of death in only one of these cases, all of the animals were suffering such debilitation that they likely would have died of mange, had euthanasia not been performed first. Severe mange may cause death in squirrels by inhibiting the animal's ability to forage leading to severe malnutrition and dehydration, by secondary bacterial infection and septicemia, or by hindering the animal's ability to avoid predation. Cornish et al. (2001) reported the cause of death in two squirrels from the Washington State epidemic as emaciation. Bryant (1921, 1926) hypothesized that lesions around the eyes interfered with normal foraging behavior.

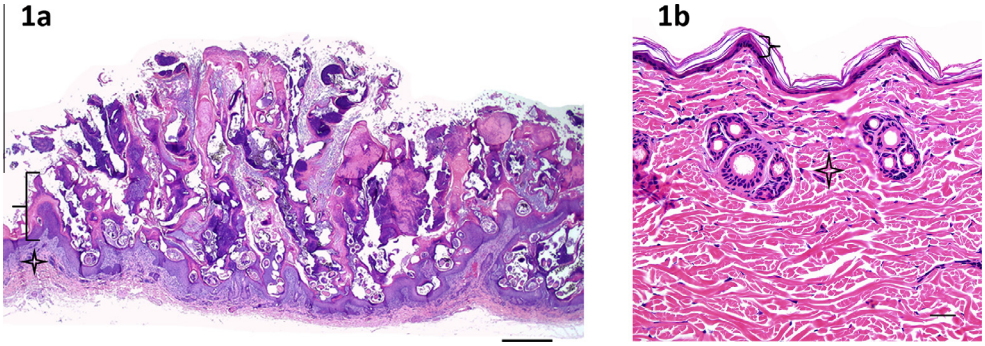

Fig. 1. (a). Histologic sections of skin of free-ranging Western gray squirrels (*Sciurus griseus*). (a) Severe notoedric mange characterized by, irregular acanthosis with rete ridge formation, extensive orthokeratotic and parakeratotic hyperkeratosis with serocellular crusting, intracorneal pustules, and numerous variably-sized, intracorneal and intraepidermal tunnels. H&E stain. Bar = 1000 μm. (b) Unaffected skin for comparison. H & E stain. Bar = 500 μm. [Brace = epidermis; star = dermis.]

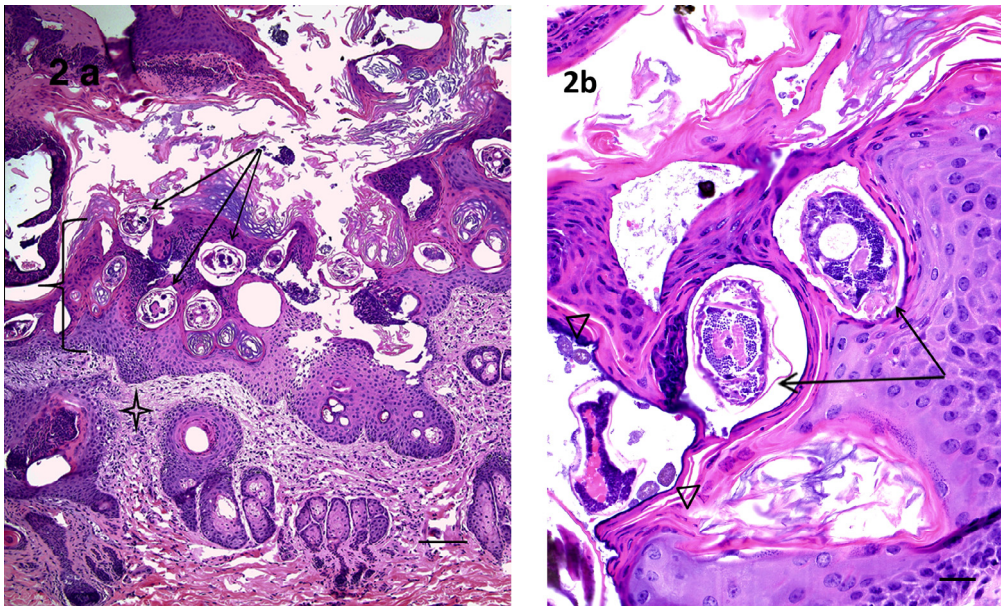

Fig. 2. Histologic section of skin of a free-ranging western gray squirrel (*Sciurus griseus*) with notoedric mange. (a) Intraepidermal tunnels containing numerous mites [arrows]. H&E stain. Bar = 500 μm. [Brace = epidermis; star = dermis.] (b) High magnification demonstrating intralesional mites [arrows] and small numbers of round to oval eggs [arrowheads]. H&E stain. Bar = 100 μm.

Table 2
Results of heavy metal and selenium screening on liver tissue from four Western gray squirrels (*Sciurus griseus*) with notoedric mange from Big Bear, California.

ID #	Lead (<2)	Manganese (160–230)	Iron (63–120)	Mercury (<0.2)	Arsenic (<0.5)	Zinc (26–40)	Copper (3.0–6.0)	Cadmium (<0.1–1.2)	Selenium (0.2–0.52)
1	ND	2.8	90	ND	ND	**60**	**29**	ND	**1.20**
2	ND	3.3	**1200**	ND	ND	**84**	**6.4**	3.7	**0.79**
3	ND	3.5	**260**	ND	ND	36	**6.7**	ND	**0.66**
4	ND	2.2	**220**	ND	ND	36	3.6	ND	**0.64**

All concentrations measured in parts per million. Reference ranges given in parentheses below analyte (Puls, 1988).
Bold font indicates that the concentration is above the reference range.
ID #, squirrel identification number; ND, not detected.

In the present study, all of the squirrels were observed pre-mortem and all were reported to be displaying abnormal neurologic behaviors, ranging from ataxia and erratic behavior to obtundation. The cause of these neurologic signs was not determined by post-mortem examination. Some of the animals were tested for West Nile Virus and rabies as possible causes of neurologic signs in squirrels, but all were negative (Table 1). Virus isolation performed on tissue pools from two animals failed to identify any viral elements. Other causes of neurologic behavior could include head trauma, other viral encephalitides, bacterial, fungal or parasitic central nervous system infections, toxins or hypoglycemia (Schueler, 1973; Kiupel et al., 2003; Heinz-Taheny et al., 2004; Carrasco et al., 2006). In this group of animals no lesions were noted on microscopic examination of the brain and spinal cord. Severe mange can cause intense pruritis, malnutrition and weakness, the clinical signs of which may be interpreted as neurologic disease, and are considered to be the most likely explanation for the abnormal behavior of the observed squirrels.

The cause of death for squirrel #5 was a fractured skull resulting from blunt trauma, possibly resulting from a fall from a tree. This is a common finding during notoedric mange outbreaks (Bryant, 1921; Cornish et al., 2001) and may be the result of incoordination or weakness secondary to mange. While mange may lead to secondary bacterial infection and septicemia, this was not the likely cause for neurologic signs in most cases, as there was evidence of possible septicemia in only one squirrel. In this animal *S. aureus* cultured from the liver and lung may represent terminal septicemia secondary to severe dermatitis. All four squirrels that were tested were above the normal reference range for selenium and at least one heavy metal, although most of the elevations were slight, and likely of very little if any clinical significance (Table 2).

Notoedric mange can have significant impacts at the individual level, but also at the population level as well. As with other types of parasitism, pregnant animals are likely more susceptible to infestation with mange, especially in late gestation and early lactation when nutritional requirements are prioritized for reproduction, rather than immune function (Coop and Kyriazakis, 1999; Fthenakis et al., 2001). Pregnant and lactating animals have increased mite burdens, which are often passed onto the highly vulnerable young. Mite infestation in neonatal and juvenile production animals causes lower growth rates, higher nutritional requirements and higher mortality (Arends et al., 1990; Soulsbury et al., 2007). A study of sarcoptic mange in coyotes showed that females with severe mange had lower ovulation and pregnancy rates than uninfested coyotes (Pence and Windberg, 1994). By decreasing reproduction rates and increasing juvenile mortality, notoedric mange could have a large population impact. Western gray squirrels historically were very common throughout their range but since the 1920s have undergone significant declines (Bryant, 1921; Payne, 1940; Cornish et al., 2001; Vander Haegen et al., 2007). While notoedric mange is likely a contributor to these declines, there are other factors including degradation and fragmentation of habitat due to increased urbanization, competition with the non-native eastern gray and fox squirrel, predation and possibly other diseases (Ingles, 1947; Linders and Stinson, 2006). Anecdotal reports suggest that large die-offs might follow mast crop failure leading to nutritional stress, which could cause both increased transmission and increased mortality (Cornish et al., 2001). Moreover, squirrel density may increase transiently around sparse food resources, facilitating disease transmission. Notoedric mange may represent a form of top-down population regulation in squirrel species (Cornish et al., 2001; Linders and Stinson, 2006; Vander Haegen et al., 2007). Large die-offs caused by notoedric mange are of particular concern in the native western gray squirrel especially in Washington State where the populations have suffered dramatic declines and range reductions (Linders and Stinson, 2006).

In this paper we identify *N. centrifera* as the etiologic agent of a disease in five squirrels from an outbreak in Big Bear, California and we show the marked effects that notoedric mange can have on individual squirrels. We also report sequences of the ITS-2 region of *N. centrifera* for future identification by PCR and genetic studies. While we have shown the significant effects that notoedric mange can cause in individuals, further research is needed to fully understand the population impact since anecdotal reports suggest that this disease can cause dramatic die-offs which may take the population decades to recover (Bryant, 1921; Payne, 1940; Cornish et al., 2001).

Note

Nucleotide sequence data reported in this paper are available in the GenBank databases under the accession numbers KF278482, KF278483, and KF278484.

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