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Unraveling Parasite Identification in Invasive and Endangered Squirrel Species: Potential for Parasite Spillover

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Unraveling parasite identification in invasive and endangered squirrel species: potential for parasite spillover

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Abstract Invasive species can impact ecosystem health by introducing parasites during their establishment in new areas. However, the dynamics of parasite loads between invasive and native species remain poorly understood. In the 1940s, Abert's squirrels (*Sciurus aberti*) were introduced to the Pinaleno Mountains in southeastern Arizona where they now co-occur with endemic endangered Mt. Graham red squirrels (*Tamiasciurus fremonti grahamensis*). The study aimed to identify and quantify parasites in both Abert's and Mt. Graham red squirrels, with a focus

on investigating possible parasite spillover between these two species and examining how host-related factors affect parasite presence. PCR amplification and morphological analyses revealed two endoparasite species: *Citellinema* sp. and *Trypanoxyuris* (*Rodentoxyuris*) sp. In Abert's squirrels, ectoparasites such as *Opisodasys robustus*, *Anomiopsyllus* sp., and *Neohaematoinnum sciurinus* were encountered, while Mt. Graham red squirrels carried *Orchopeas caeden* and a mite from the Glycyphagidae family. Our findings suggest new host and geographic record for the Glycyphagidae mite in Mt. Graham red squirrels of Arizona and indicate possible spillover of *Trypanoxyuris* (*Rodentoxyuris*) sp. from Abert's squirrels to Mt. Graham red squirrels. Additionally, sex influenced presence of ectoparasites in Abert's squirrels. We can

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offer a better understanding of factors that place species at risk of extinction with enhanced insights into probable parasite transmission routes and the role that parasites play in biological invasion.

Keywords Ectoparasites · *Tamiasciurus fremonti grahamensis* · Spillover · Helminths · *Sciurus aberti* · Presence

Introduction

Invasive species can influence ecosystem health via introduction of vectored parasites as these introduced species establish residence in a new geographic area (Prenter et al. 2004; Dunn et al. 2012). Parasites may play a key role in either enhancing or limiting the impacts of biological invasions, affecting native species positively or negatively (Dunn et al. 2012; Lymbery et al. 2014). Parasite-mediated competition can be explained by three principal processes. Spillback occurs when invasive species acquire native parasites in the area of introduction and amplify local parasite circulation, resulting in higher infection levels in native species (Goedknegt et al. 2017; Romeo et al. 2019). Spillover occurs via transmission of a parasite endemic to one host species to a new host (Daszak et al. 2000; Dunn et al. 2012; Borremans et al. 2019). In this scenario, the impact of parasites carried by invasive species can be severe if native species are naïve to the introduced parasites (Prenter et al. 2004; Romeo et al. 2015). Finally, the enemy-release hypothesis is a concept in which species introduced to a new area lose their natural parasites and are less impacted by parasitic infections, improving their fitness as a consequence (Lymbery et al. 2014). These processes can create a competitive advantage for the invader making its invasion successful (Dunn et al. 2012; Mazzamuto et al. 2016; Romeo et al. 2021).

The squirrel poxvirus (SQPV) is one of the best-known models of spillover and disease-mediated competition resulting from the introduction of the eastern gray squirrel (*Sciurus carolinensis*) from the USA into the UK and Ireland (Rushton et al. 2006a; McInnes et al. 2012; Romeo et al. 2015). Introduced eastern gray squirrels are unaffected carriers of SQPV and spread the disease to the native Eurasian red squirrels (*Sciurus vulgaris*) that are lethally affected by the infection (Tompkins et al. 2002; Prenter et al.

2004). As a result, native red squirrel populations have declined or have been extirpated in all areas where the introduced eastern gray squirrel is present (Tompkins et al. 2002; Santicchia et al. 2020). Despite the attention spillover has received, the mechanisms used by these parasitic invaders remain poorly understood, and studies demonstrating the impact of foreign parasites on native hosts are surprisingly few (Romeo et al. 2021).

In this study we investigated the relationship between the introduced Abert's squirrel (*Sciurus aberti*) and the native, federally endangered Mt. Graham red squirrel (*Tamiasciurus fremonti grahamensis*) on Mt. Graham in the Pinaleno Mountains in southern Arizona, with a focus on their respective parasite populations. The Pinaleno Mountains are the northern extent of the Madrean Sky Island Complex and have the highest peak in the complex at 3,269 m (Hoffmeister 1956; Warshall 1996). The federally endangered Mt. Graham red squirrel is the southernmost and rarest subspecies of the southwestern red squirrel (*Tamiasciurus fremonti*) (Hope et al. 2016). Mt. Graham red squirrels were initially thought to have been extinct in the 1960s, but a few squirrels were rediscovered in the 1970s (Minckley 1968; Sanderson and Koprowski 2009). This subspecies is restricted to mixed-conifer and spruce-fir forests at elevations above 2,400 m while being isolated from other southwestern red squirrel populations for at least 10,000 years (Allen 1894; Merrick et al. 2021). Mt. Graham red squirrels are sensitive to changes in the environment and are short-lived, typically about three years in the wild, and reproduce once or sometimes twice per year (Munroe et al. 2009; Goldstein et al. 2017; Merrick et al. 2021). Over the last three decades, the population has experienced large fluctuations due to low habitat quality, multiple insect outbreaks, three major wildfires in short succession, human activities, and introduced species (Sanderson and Koprowski 2009; USFS 2011; Merrick et al. 2021).

In the 1940s, 69 Abert's squirrels were trapped at Fort Valley north of Flagstaff, Arizona and introduced in the Pinaleno Mountains by the Arizona Game and Fish Department for hunting purposes (Hoffmeister 1956; Davis and Brown 1988; Edelman and Koprowski 2009). Abert's squirrels are native to New Mexico, Colorado, Utah, northern Arizona, parts of Wyoming, and north-central Mexico

(Davis and Brown 1988; Davis and Bissell 1989; Allred 2011). Abert's squirrels are active year-round and often are sympatric with other squirrels in their natural range (Keith 1965; Cudworth and Koprowski 2010). Abert's squirrels were expected to be constrained to lower-elevation pine forests. However, in the last 75 years, Abert's squirrels have extended mountain-wide and now overlap with Mt. Graham red squirrels near the summit of the Pinaleno Mountains dominated by spruce-fir forests (*Picea engelmannii*, *Abies lasiocarpa* var. *arizonica*) (Rushton et al. 2006b; Allred 2011; Merrick et al. 2021). Where co-occurring, Abert's squirrels may further constrain Mt. Graham red squirrels' recovery via higher population density, competition for food resources, niche overlap, direct competition between squirrels species, and novel parasites (Steele and Koprowski 2001; Rushton et al. 2006b; Goldstein et al. 2018; Bergman et al. 2021).

No parasitological studies have been conducted on neither Abert's nor Mt. Graham red squirrels in this area. We first assessed the parasitological diversity of these species and investigated the potential role that parasites may play in providing Abert's squirrels an additional competitive advantage. We hypothesized that if spillover has occurred in the last > 75 years, parasites identified in Abert's squirrels would also occur in native Mt. Graham red squirrels hence we would see a similar community composition of endo/ectoparasites between the two host species. If spillover did not occur, then the community composition would be different. We also investigated if the presence or absence of parasites was influenced by host-linked factors (sex and body mass).

Methods

Study area—Spanning more than 2,100 m, the Pinaleno Mountains span a diverse range of forest types, from spruce-fir woodlands at their highest points to arid desert grasslands at their base (Hoffmeister 1956; O'Connor et al. 2014; Merrick et al. 2021). Dominated by Engelmann spruce and corkbark fir above 2,985 m, the forest transitions to a mix of Douglas-fir (*Pseudotsuga menziesii*), southwestern white pine (*Pinus strobiformis*), and aspen (*Populus tremuloides*) at lower elevations (O'Connor et al. 2014). The surrounding forest mainly comprises dry mixed

conifer stands, including white fir (*Abies concolor*) and ponderosa pine (*Pinus ponderosa*) (O'Connor et al. 2014). Rising from the arid desert landscape, the Pinaleno Mountains ascend to higher elevations, creating a forested sky island within the surrounding desert scrub (Wood et al. 2007). Precipitation primarily occurs during two main periods: snowfall from December to April and monsoon rains spanning July to September (Wood et al. 2007). Small mammals that co-exist in the same areas of Mt. Graham red squirrels include rock squirrels (*Otospermophilus variegatus*), spotted ground squirrels (*Xerospermophilus spilosoma*), Mexican wood rats (*Neotoma mexicana*), deer mice (*Peromyscus maniculatus*), Mt. Graham pocket gopher (*Thomomys bottae grahamensis*), western pocket gopher (*T. bottae mearnsi*), western harvest mouse (*Reithrodontomys megalotis*), brush mouse (*P. boylii*), long-tailed vole (*Microtus longicaudus*), eastern cottontail (*Sylvilagus floridanus*), and desert cottontail (*S. audubonii*) (Hoffmeister 1986).

Study system—Abert's squirrels are larger (500–750 g), non-territorial, occasional scatterhoarders, have larger home ranges and practice social nesting (Edelman and Koprowski 2007; Debridge and Koprowski 2019). Abert's squirrels prefer open forests, which often result from fire and insect infestations, as on the Pinaleno Mountains (Minor and Koprowski 2015; Gwinn and Koprowski 2016). In contrast, Mt. Graham red squirrels are solitary, are territorial, and have smaller home ranges (Koprowski 2005; Debridge and Koprowski 2019). Mt. Graham red squirrels are much smaller in size (200–250 g), and aggressively defend larderhoarders at the center of their territory aggression and have unique territorial vocalizations (Debridge and Koprowski 2019).

Removal and collection—Examinations were conducted on Abert's squirrels from the years 2002 to 2019. A total of 15 individuals were collected from road-killed and through an experimental removal conducted by Debridge and Koprowski (2019). While the main approach involved euthanizing a substantial portion of these squirrels using firearms, under the 2019 Arizona Game and Fish Department-funded removal program (Bergman et al. 2021). The program targeted areas of proposed action that overlapped with the habitat of the Mt. Graham red squirrel on the Pinaleno Mountains (Bergman et al. 2021). The project personnel employed suppressed firearms to mitigate potential noise disturbance. Shooting activities

were strictly limited to daylight hours and confined to locations where discharging firearms is both legal and safe (Bergman et al. 2021). The personnel received specialized training to distinguish between Abert's squirrels and Mt. Graham red squirrels and were current with all the necessary firearms handling training requirements (Bergman et al. 2021). During collection of population data for Mt. Graham red squirrel, researchers opportunistically (on a monthly basis there are consistently at least two people on the mountains for a 20-d period) collected Mt. Graham red squirrels in situations where they were road-killed, partially consumed by a predator, or found deceased due to natural causes in close proximity to their middens between 2002 to 2020. Each carcass was placed in an individual plastic bag, sealed, and stored at -20°C for later examination. We performed post-mortem examinations for all carcasses.

A total of 113 Abert's squirrels (male=56, female=57) were examined for ectoparasites, and only adult specimens were considered for both endoparasites and ectoparasites. Due to observed damages to the intestinal tracts in 13 individuals, the endoparasite assessment included 100 individuals (male=50, female=50). Male and female Abert's squirrels were sampled equally and randomly. We examined Abert's squirrels during their breeding season, between late February to early June (Allred 2011). For Mt. Graham red squirrels, a sample of 24 individuals (male=11, female=13) was examined for ectoparasites, but only 22 individuals (male=11, female=11) could be included in the endoparasite assessment due to damage to their intestinal tracts. Due to limited sample size, all Mt. Graham red squirrels were sampled regardless of the date collected or sex. However, to ensure the reliability and quality of the data, we excluded any bodies in decomposition from our analysis. It is worth noting that ectoparasites tend to leave shortly after host death, while endoparasites may be affected by the decomposition process.

Endoparasites

Collection -The gastrointestinal tract from the esophagus to the rectum was removed during necropsy. The stomach, small intestine, and large intestine were examined for endoparasites following standard parasitological procedures as described in Romeo et al. (2013). The content collected from each section was

examined using a dissecting microscope (AmScope, California, USA) at 45X magnification. For later identification, the helminths were counted and stored in Tris-EDTA (TE) pH 8.0. Helminths were stored in separate tubes for each squirrel's intestinal tract section (e.g., small intestine, large intestine, stomach).

Morphological methods -A total of 12 nematodes were photographed from six Abert's squirrels (7,2,1,1,1 from each individual), and a total of eight nematodes from three red squirrels (4, 2, 2 from each individual) for morphological identification. Nematodes were cleared and studied in temporary mounts of lactophenol. Most features were studied at 100X and 200X magnification. Measurements were taken with an optical micrometer. The morphological traits used for identification included the tightly coiled body, the pattern of cuticular ridges (synlophe), the asymmetrical bursa, a notably reduced dorsal ray, and the shape and size of the spicules in males, as detailed in identification keys by Hall (1916) and Dikmans (1938). Morphological analysis was also used to validate our molecular identifications.

Molecular methods—DNA was extracted from individual nematodes collected from both squirrels species. Individual nematodes were isolated and ground using a pestle motor mixer for ≤ 2 min. DNA extraction of nematodes followed the manufacturer's recommendation (Thermo Fisher User guide 2016) of the GeneJet Genomic DNA purification kit (#K0722, Thermo Scientific). With the exception overnight incubation for the digestion step: incubation ranges from 45 min to overnight, depending on the amount of DNA in the reaction. To improve DNA binding, the elution buffer was incubated at 56°C for about 30 min. Molecular identification involved the amplification of three markers using the polymerase chain reaction (PCR): 18S small subunit (SSU) ribosomal DNA, 28S large subunit (LSU) ribosomal DNA, and the internal transcribed spacer 2 (ITS2). The ITS2 marker was selected due to it being helpful in strongylid identification, although Pafčo et al. (2018) used the primers in metabarcoding. Optimal PCR conditions were determined for each primer by adjusting annealing temperatures, time of cycles, primer concentrations, and DNA concentrations (Table S1). This process was followed to refine our end product for optimal results. The GeneJet PCR Purification Kit (#K0702, Thermo Scientific) was used for the PCR purification following the manufacturer's

recommendation (Thermo Fisher User guide 2015) for PCR purification. We incubated the elution buffer at 56 °C for about 15 min as the manufacture recommended for maximal recovery of end product.

Sequencing and phylogenetic analyses—PCR products were Sanger sequenced at the University of Arizona Genetics Core. Nucleotide sequences were edited, aligned, and analyzed using Geneious software version 9.0 (Kearse et al. 2012) and compared with sequences available in GenBank dataset using the Basic Local Alignment Search Tool (BLAST) tool (Camacho et al. 2009). For the phylogenetic analysis, the ITS2 sequences obtained in this study were aligned with sequences of Trichostrongyloidea (n=8) and Heligmosomidae (n=22) available in the GenBank database identified as closely related by BLAST. In addition, the 28S rDNA sequences obtained in this study were aligned with sequences of Heligmosomidae (n=8), Herpetostrongylidae (n=4), Herpetostrongylinae (n=2), Trichostrongyloidea (n=1), Mackerrastrongylidae (n=2), *Parastrostrongylus bettongia* available in GenBank database. Lastly, the 18S rDNA sequences obtained in this study were aligned with sequences of Oxyuridae sequences (n=19) available in GenBank database. Phylogenetic analyses were performed with the maximum likelihood method using IQ-TREE multicore version 1.5.5 (Nguyen et al. 2014) with ultrafast bootstrap (1,000 replicated) to test the topology robustness (Minh et al. 2013). Jmodeltest (Posada 2008) was used to select the best model of evolution for each gene based on the Akaike information criterion. The produced phylogenetic trees were edited using FigTree version 1.4.4 and Inkscape version 1.0.2.

Ectoparasites

Collection—We combed the entire body of each squirrel with a flea comb above a white surface and examined areas on the body where clusters most commonly occur (e.g., on the face, near ears, near eyes, neck area, underside regions). Each squirrel was combed for a fixed amount of time (2 min), ensuring uniformity across all individuals to avoid bias. The flea comb was properly sanitized between squirrels to prevent any cross-contamination. In addition to the post-mortem examinations, the sealed bags containing the squirrel carcasses were thoroughly inspected to recover any ectoparasites present. Ectoparasites

were counted and stored in 70% ethanol for later identification.

Morphological methods—Ectoparasites were examined using a dissecting microscope (WILD Heerburgg, Gais Schweiz, Switzerland) for whole specimens and DM compound scope (Lecia Microsystems, Wetzlar, Germany) for slide-mounted and dissected material. Specimens were placed in cold 10% potassium hydroxide (KOH) to remove soft internal tissues. When cleared, specimens were passed through water and ethanol alcohol (EtOH) progressions to halt the clearing process until dehydrated in 100% EtOH. Once cleared, specimens were sorted using the dissecting scope, counted, and males/females selected for slide-mounting and identification. Slide-mounted specimens were placed in Hoyer's medium or glycerol under a coverslip on a slide, then viewed under high magnification to examine critical external morphological characters, including the female spermatheca and male aedeagus (Ewing and Fox 1943; Hubbard 1947; Fleas of the World 2021).

Statistical analyses for endo/ecto parasites—To describe the parasitic infections and infestations, we calculated prevalence and mean intensity for both endo and ecto parasites (Margolis et al. 1982). Prevalence was obtained by dividing the total number of infected/infested hosts by the total number of sampled hosts (Margolis et al. 1982; Mergo and Crites 1984). Both molecular (Sanger sequences) and morphological confirmed identifications were included in calculating prevalence and mean intensity. Mean intensity was calculated by dividing the total number of individuals of a particular parasite species in an individual of a host species by the number of infected/infested individuals of the host species in the sample (Margolis et al. 1982; Mergo and Crites 1984). The incorporation of Sanger sequences offers an additional dimension by facilitating the identification and enumeration of specific parasite taxa. Results are presented as mean \pm SE.

We examined factors influencing the probability of squirrels being infected and infested by endoparasites and ectoparasites using binomial generalized linear models (GLMs) with a logit-link function (McCullagh and Nelder 1989). Separate models were fitted for Abert's and Mt. Graham red squirrels. We initially considered a random effect (i.e. random intercepts) of year in all our models for Abert's squirrels, but the

random variance was consistently estimated as zero, so we reported results from our GLMs. When modeling endo/ectoparasite presence in Abert's squirrels and Mt. Graham red squirrels, we included body mass and sex as covariates. We conducted all statistical analyses using R software version 4.1.1 (R Core Team 2021).

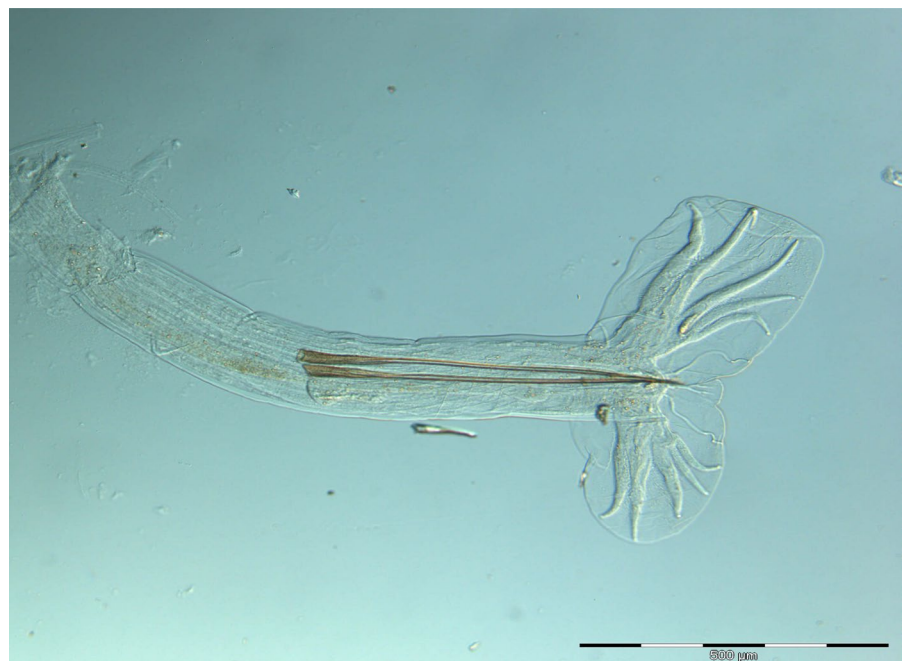
Results

Endoparasites—65% (26 males, 39 females) of the Abert's squirrels ($n=100$) necropsied harbored at least one endoparasite; 36% (five males, three females) of Mt. Graham red squirrels ($n=22$) harbored at least one endoparasite. Helminths were effectively found in small intestine in Abert's squirrels and the large intestine in Mt. Graham red squirrels. Based on the presence of spicules, needle-like mating structures exclusively found in male nematodes (Fig. 1) within samples from both squirrel species, we identified the initial nematode as *Citellinema quadrivittati*. These spicules represent a distinctive morphological feature characteristic of *Citellinema* spp. As documented by Hall in 1916, *Citellinema quadrivittati* is characterized by spicules with a length of 695 μm . In our analysis, we determined a

range of spicule lengths from 498 μm to 693 μm , with a mean length of 592 μm . PCR amplification of the ITS2 rDNA marker (Fig. 2) was performed to further characterize the recovered nematodes. A 95% to 99% sequence similarity was observed between our ITS2 rDNA sequences in both squirrels and that available on the NCBI database of *Citellinema* spp. The phylogenetic relationship of our ITS2 rDNA marker revealed the recovered nematodes grouped with other *Citellinema* spp. Nematodes recovered from both host squirrels clustered in a single group but showed strong divergences between samples (Fig. 2). To verify if the observed divergence in the analysis of ITS2 rDNA sequences were due to intraspecific variation, we compared it with the divergence of the 28S rDNA sequences of nematodes isolated from both squirrels (Fig. 3). The analysis of the 28S rDNA marker displayed 97% to 100% similarity among the nematode samples, suggesting that only one species of *Citellinema* was isolated.

The second nematode species detected in both squirrels species was identified as *Trypanoxyuris* (*Rodentoxyuris*) sp. In Abert's squirrels, molecular identification through PCR amplification of the 18S rDNA marker was employed (Fig. 4) and observed a 99% similarity with sequences of this genus available on the NCBI database. Our analysis revealed

Fig. 1 Image of the spicules, needle-like mating structures found in males *Citellinema quadrivittati* in Mt. Graham red squirrels (*T. f. grahamensis*) and Abert's squirrels (*S. aberti*): spicule length was 626.1 μm . Image taken under 400 \times magnification of *C. quadrivittati* from a Mt. Graham red squirrel from the Pinaleño Mountains in southeastern Arizona, USA



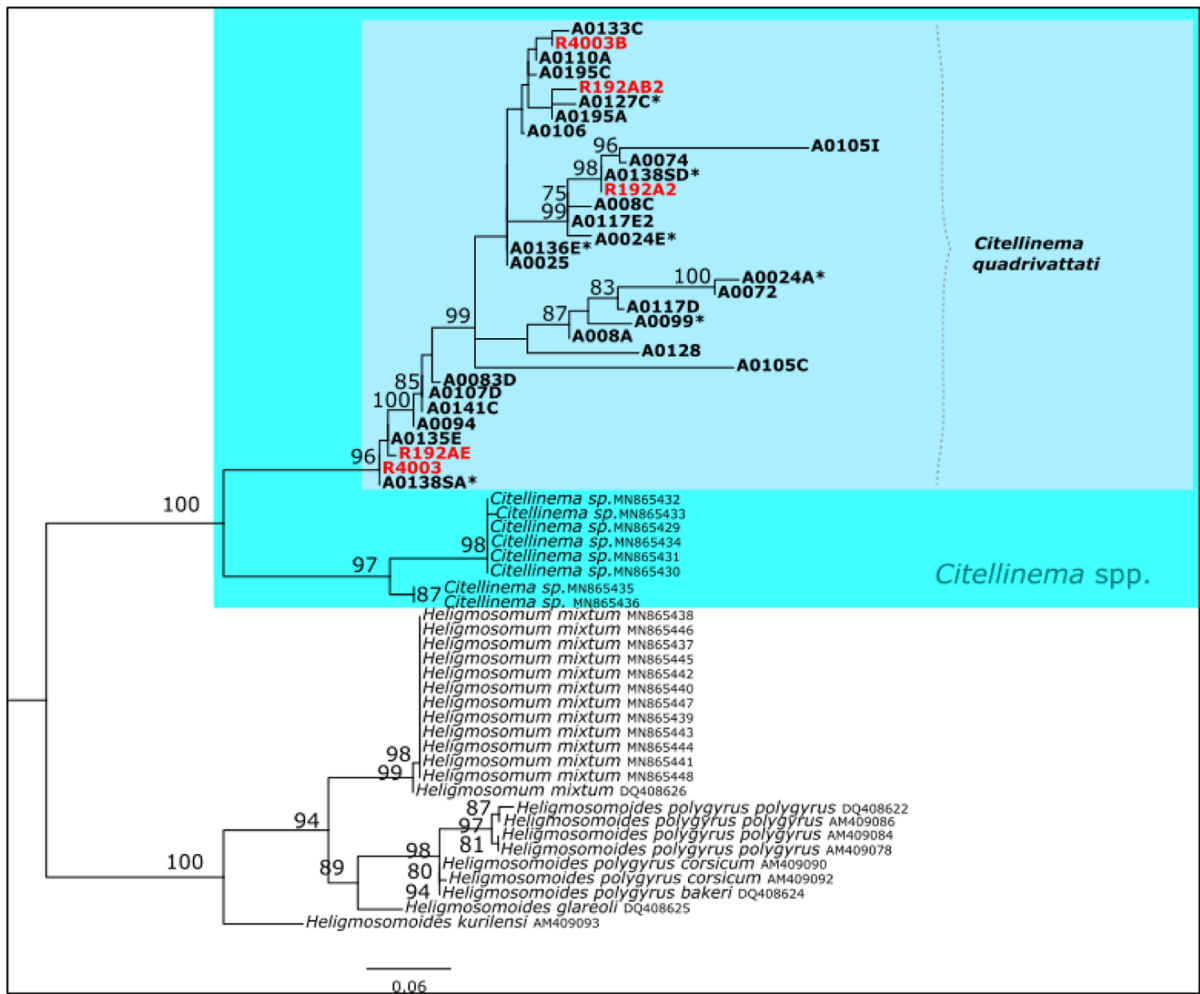


Fig. 2 Phylogenetic tree of ITS2 rDNA based on maximum likelihood method of Abert’s squirrel (*S. aberti*) and Mt. Graham red squirrels (*T. f. grahamensis*) from the Pinaléño Mountains in southeastern Arizona, USA. According to AIC the best-fit model for ITS2 sequencing was HKY + G4. Aqua colored rectangle shows *Citellinema* spp. Smaller blue rectangle shows *Citellinema quadrivattati* samples. Asterisks repre-

sent other samples from that host which have been morphologically identified as *Citellinema quadrivattati*. Taxa colored red are Mt. Graham red squirrels and bold taxa are Abert’s squirrels. Nodes (numbers) are associated with Bootstrap values based on 1,000 replicates. Bootstrap values less than 70 are not shown. The scale bar at the bottom left indicates the number of nucleotide substitutions (0.06)

Trypanoxyuris spp. as a monophyletic sister-group to *Enterobius* spp. (Fig. 4) as the recovered nematodes were closely related to *Trypanoxyuris* (*Rodentoxyuris*) *sciuri*. For nematodes from Mt. Graham red squirrels, experts assessments (Hugot 1984) confirmed the presence of a pinworm, most likely *Trypanoxyuris* (*Rodentoxyuris*) sp., based on an initial image (Fig. 5). However, due to the limited number of specimens collected during the endoparasite assessment, challenges arose in obtaining further photographic evidence and amplifying the 18S rDNA sequence

for this specific nematode, resulting in unsuccessful results in Mt. Graham red squirrels.

The most abundant intestinal trichostrongylid in Abert’s squirrels and Mt. Graham red squirrels was *Citellinema* sp. (Abert’s squirrel n=48: Sanger sequences and morphological confirmations; Mt. Graham red squirrel n=5: Sanger sequences). Abert’s squirrels exhibited an overall prevalence of 48% and a mean intensity of 2.6 ± 0.05 (Table 1), while Mt. Graham red squirrels had a prevalence of 23% and a mean intensity of 3.4 ± 0.11 (Table 1). Abert’s

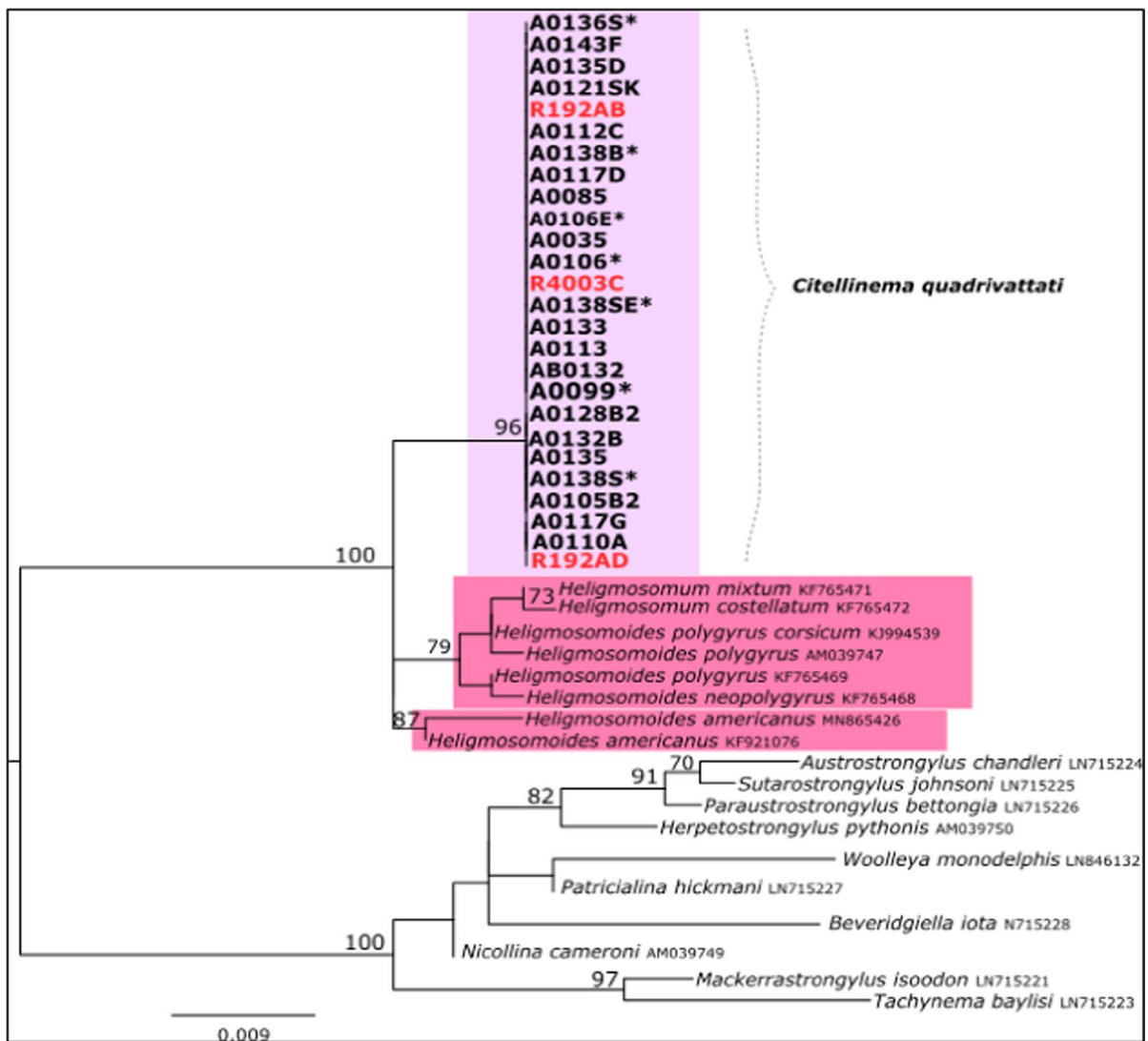


Fig. 3 Phylogenetic tree of 28S rDNA based on maximum likelihood method of Abert's squirrel (*S. aberti*) and Mt. Graham red squirrels (*T. f. grahamensis*) from the Pinaleno Mountains in southeastern Arizona, USA. Analysis showing that samples from this study are grouping together. According to AIC the best-fit model for 28 s sequencing was TPM3u+I. Dark pink rectangles represent sister group *Heligmosomum* spp. The lighter pink rectangle symbolizes a representative

subset of samples of *Citellinema quadrivattati*. Asterisks represent other samples from that host which have been morphological identified as *Citellinema quadrivattati*. Taxa colored red are Mt. Graham red squirrels and bold taxa are Abert's squirrel. Nodes (numbers) are associated with Bootstrap values based on 1,000 replicates. Bootstraps values less than 70 are not shown. The scale bar at the bottom left indicates the number of nucleotide substitutions (0.009)

squirrels harbored 127 confirmed nematodes of *Citellinema* sp., while Mt. Graham red squirrels harbored 17. Regarding *Trypanoxyuris* (*Rodentoxyuris*) sp. in Abert's squirrels (n=2: Sanger sequences), the overall prevalence appeared lower at 2%, with a mean intensity of 1.5 (Table 1). A smaller number of PCR products underwent Sanger sequencing for

this nematode. Abert's squirrels had three confirmed *Trypanoxyuris* (*Rodentoxyuris*) sp. None of the host related factors tested affected the presence of endoparasites in Abert's squirrels nor in Mt. Graham red squirrels ($p > 0.05$) (Table 2).

Ectoparasites—Abert's squirrels and Mt. Graham red squirrels did not share any ectoparasite

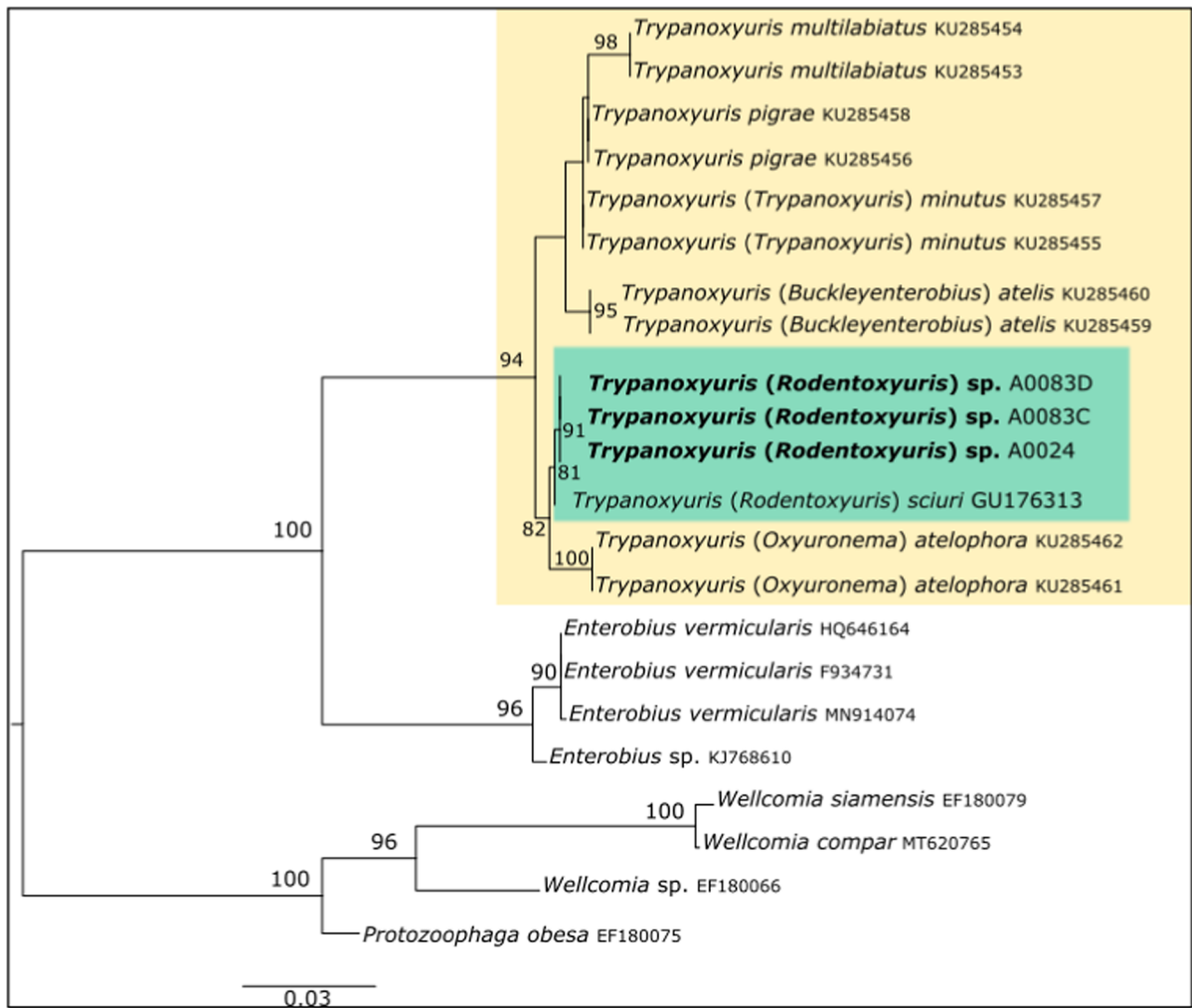


Fig. 4 Phylogenetic tree of 18S rDNA based on maximum likelihood method of Abert's squirrel (*S. aberti*) from the Pinaleno Mountains in southeastern Arizona, USA. According to the AIC the best-fit model for 18 s sequencing was TVM+I+G4. The light-yellow rectangle represents the *Trypanoxyuris* spp., while the green rectangle includes three samples from this study that are grouped together within the *Trypanoxyuris* spp. category. Furthermore, one sequence labeled

Trypanoxyuris (Rodentoxyuris) sp. from the GenBank database closely aligns with our sequences within the green rectangle. Bold taxa are the Abert's squirrel. None of the PCR amplification attempts for Mt. Graham red squirrels (*T. f. grahamensis*) yielded successful sequences. Nodes (numbers) are associated with Bootstrap values based on 1,000 replicates. Bootstraps values less than 70 are not shown. The scale bar at the bottom left indicates the number of nucleotide substitutions (0.03)

species. Among the 113 Abert's squirrels, a total of 53 males and 36 females (79%) harbored an ectoparasite. Abert's squirrels hosted two species of fleas: *Opisodasys robustus* and *Anomiopsyllus* sp. The *Anomiopsyllus* fleas were not common, only occurring in two individual Abert's squirrels. One host was a reproductive male collected in March 2014, and the other host was a reproductive female collected in May 2019. Additionally, one species

of louse was found on 37% of Abert's squirrels: *Neohaematoinuss sciurinus*. The most abundant ectoparasite was the flea *Opisodasys robustus* with an overall prevalence of 51% and a mean intensity of 5.2 ± 0.37 (Table 1). The final model revealed that male Abert's squirrels, rather than females, were more likely to be parasitized by ectoparasites ($p < 0.001$). However, body mass did not affect the presence of ectoparasites in Abert's squirrels

Fig. 5 Image taken under 400× magnification of a male oxyurid: most likely a *Trypanoxyuris* (*Rodentoxyuris*) sp. from a Mt. Graham red squirrels (*T. f. grahamensis*), from the Pinaleño Mountains in southeastern Arizona, USA



Table 1 Macroparasite species harbored by Abert's squirrels and Mt. Graham red squirrels in the Pinaleño Mountains in southeastern Arizona, USA. Both molecular (Sanger sequences) and morphological confirmed identifications were included in calculating prevalence and mean intensity for both host species. Endo=number of squirrels sampled for endo-

parasites; ecto=number of squirrels sampled for ectoparasites. Ectoparasite counts are set in italic when the number of infected hosts < 10. SE=Standard Error. We were not able to obtain sequence data for pinworms (*Trypanoxyuris* (*Rodentoxyuris*) sp.) in Mt. Graham red squirrels although they were present

Macroparasite species	Abert's squirrel (n = 113) endo = 100 ecto = 113		Mt. Graham red squirrel (n = 24) endo = 22 ecto = 24	
	Prevalence (%)	Intensity ± SE	Prevalence (%)	Intensity ± SE
Helminths				
<i>Citellinema</i> sp.	48	2.6 ± 0.05	23	3.4 ± 0.11
<i>Trypanoxyuris</i> (<i>Rodentoxyuris</i>) sp.	2	<i>1.5</i>	Present	-
Arthropods				
<i>Opisodasys robustus</i>	51	5.2 ± 0.37	-	-
<i>Anomiopsyllus</i> sp.	> 1	<i>1</i>	-	-
<i>Neohaematoinnum sciurinus</i>	37	5.9 ± 0.04	-	-
<i>Orchopeas caedens</i>	-	-	37	6.0 ± 0.18
Glycyphagidae	-	-	> 1	<i>1</i>

($p > 0.05$) (Table 2). Among the 24 Mt. Graham red squirrels examined, a total of four males and four females (33%) harbored an ectoparasite. The flea *Orchopeas caedens* had an overall prevalence of 37% and a mean intensity of 6.0 ± 0.18 (Table 1). A solitary mite specimen, belonging to the family Glycyphagidae, was recovered from a female squirrel captured during the spring of 2005. In Mt. Graham

red squirrels none of the tested factors influenced ectoparasite presence ($p > 0.05$) (Table 2).

Discussion

This study finds shared endoparasites in both squirrel species, primarily *Citellinema* sp., along with

Table 2 Generalized linear model results of ectoparasites and endoparasites presence/absence of Abert's squirrel and Mt. Graham red squirrels. Both host species are from the Pinaleno Mountains in southeastern Arizona, USA. SE = Standard Error

	Coefficients	Estimates	SE	Z value	P
Abert's squirrels					
Ectoparasite	Sex (male)	2.595	0.845	3.070	<0.001
	Body mass	-0.002	0.004	-0.460	0.646
Endoparasite	Sex (male)	-0.866	0.523	-1.657	0.097
	Body mass	0.005	0.004	1.097	0.273
Mt. Graham red squirrels					
Ectoparasite	Sex (male)	-0.048	0.900	-0.053	0.958
	Body mass	-0.031	0.022	-1.431	0.152
Endoparasite	Sex (male)	0.673	0.976	0.689	0.491
	Body mass	-0.041	0.026	-1.572	0.116

distinct ectoparasite communities. The presence of *Citellinema quadrivittati*, a nematode belonging to the Trichostrongylidae superfamily, was observed in both Abert's squirrels and Mt. Graham red squirrels. *Citellinema quadrivittati* is widely distributed and typically inhabits the digestive systems, specifically the stomach and/or small intestine, of plant-feeding vertebrates (Mehlhorn 2016). Transmission of these nematodes occurs through oral uptake of larvae by a suitable host (Anderson 2000; Melhorn 2016). Conversely, the pinworm *Trypanoxyuris (Rodentoxyuris)* sp. exhibited a lower prevalence in both squirrel species. *Trypanoxyuris (Rodentoxyuris)* sp. are members of the order Oxyurida and are common in lizards, tortoises, marsupials, rodents, and primates (Anderson 2000). Transmission of the pinworm usually require fecal-oral transmission, but hosts can also digest eggs from the environment or ingest during grooming (Anderson 2000; Romeo et al. 2015; Santicchia et al. 2020). Importantly, both these nematodes have been identified in other Sciuridae species, indicating a lengthy co-evolutionary history as generalist parasites of squirrels (Anderson 2000; Mehlhorn 2016; Santicchia et al. 2020) (Table S2). To gain a better understanding of spillover dynamics, it's crucial to consider the intricate life cycles of macroparasites involving multiple host species (Hatcher and Dunn 2011). This becomes particularly relevant in warmer climates, where elevated temperatures can expand the habitats of reservoir hosts, increase vector populations, intensify biting frequencies, enhance survival rates,

and accelerate parasite transmission through vectors (Froeschke et al. 2010).

Before the introduction of Abert's squirrels to the Pinaleno Mountains, limited knowledge existed about parasites in Mt. Graham red squirrels. It is plausible that either or both squirrels may have already carried the endoparasites prior to the introduction. In New Mexico, *C. quadrivittati* occurs in sympatric populations of Abert's squirrels and southwestern red squirrels, but only Abert's squirrels harbor the pinworm, *Enterobius sciuri* (Patrick and Wilson 1995). The pinworm *E. sciuri* occurs in other squirrels in North America (*Sciurur niger*, *Glaucomys volans*, *S. carolinensis*) (Rausch and Tiner 1948; Parker 1968; Parker and Holliman 1971; Davidson 1976). The taxonomy of *E. sciuri* remains unclear and it is difficult to discern differences between *E. sciuri* and *Trypanoxyuris (Rodentoxyuris)* sp. (Hugot 1984, 1999; Cameron 1932; Popiolek et al. 2009). In North America, neither *E. sciuri* nor *Trypanoxyuris (Rodentoxyuris)* sp. has been reported in the red squirrel (*T. hudsonicus*) or southwestern red squirrel (*T. fremonti*) in any areas where overlap occurs with other squirrel species (Patrick and Wilson 1995; Hope et al. 2016). Other rodent species present on Mt. Graham alongside Abert's and Mt. Graham red squirrels (e.g., rock squirrels, spotted ground squirrels, Mexican wood rats, and deer mice) could potentially host incidental parasites and act as reservoirs for certain parasites. Given that these rodent species are native to this area and coexistence within this ecosystem, these occurrences may not strictly qualify as spillover. Unfortunately, this particular region lacks prior research on the parasite fauna of these rodents, in contrast to other regions in the USA with more thoroughly documented parasite profiles (Table S3).

Detecting *Trypanoxyuris (Rodentoxyuris)* sp. in both host species depicts the potential for parasite transmissions between Abert's squirrels and Mt. Graham red squirrels through parasite spillover. In Italy, Romeo et al. (2021) demonstrated that the spillover of a foreign nematode from an introduced species can lead to reduced survival in a native mammal, adding to the growing body of evidence indicating potential detrimental effects on native species' fitness caused by less-studied forms of competition from invasive or introduced species (Romeo et al. 2021). Similarly, in North America, the nematode *S. robustus* is suspected to contribute to competition between two

species of North American flying squirrels (*Glaucomys*) (Krichbaum et al. 2010). Studies suggest that *S. robustus* can diminish the survival and productivity of northern flying squirrels (*G. sabrinus*) while having no effect on southern flying squirrels (*G. volans*) (Weigl 1969; Krichbaum et al. 2010). Although instances of rare pathogenicity from foreign parasites through spillover are documented in only a few cases in the literature, the majority of interactions between parasites and their hosts result in a balanced coexistence, with parasites rarely causing significant harmful impact (Seilacher et al. 2007; Barnard and Behnke 1990). While our results do suggest the possibility of parasite spillover from Abert's squirrels to Mt. Graham red squirrels, caution is warranted as present data do not conclusively demonstrate a negative impact on Mt. Graham red squirrel individuals. The parasites we have encountered are relatively ubiquitous and may not necessarily have damaging consequences for the fitness of Mt. Graham red squirrels.

The presence of distinct ectoparasite species in Abert's squirrels and Mt. Graham red squirrels suggests that spillover has not taken place. Transmission of fleas, lice, and mites between hosts takes place through direct contact, the fecal–oral route, and mutual grooming (Bitam et al. 2010; Duboscq et al. 2016; Sundar et al. 2017). The fleas *O. robustus* and *Anomopsyllus* sp. detected on Abert's squirrels were previously reported in Abert's squirrels in New Mexico (Baker 1898; Jordan 1925; Holland 1965; Barnes et al. 1977; Patrick and Wilson 1995), and the flea identified in Mt. Graham red squirrels, *O. caedens*, has been previously reported in southwestern red squirrels and Abert's squirrels in New Mexico (Jordan 1925; Patrick and Wilson 1995; Hope et al. 2016). The louse, *Neohaematopinus sciurinus* (Superfamily: Anoplura) found on our Abert's squirrels has been previously reported in Abert's squirrels native to Colorado (Mjöberg 1910) and on *Sciurus niger*, *S. carolinensis*, and other squirrels outside the United States (Mjöberg 1910; Ferris 1923; Durden and Musser 1994) (Table S2). The mite identified on Mt. Graham red squirrel belonged to the Glycyphagidae family and was previously found on Douglas' squirrels (*Tamiasciurus douglasii*) in Oregon (Pence and Webb 1977). Our results suggest a new host and geographic record for the Glycyphagidae mite from Mt. Graham red squirrels in Arizona.

The variation in parasite loads between Abert's squirrels and Mt. Graham red squirrels could stem from distinct behavioral and social traits demonstrated by these species. Notably, the Abert's squirrel population on Mt. Graham engages in more communal nesting, a behavior that enhances the likelihood of parasite transmission and acquisition (Edelman and Koprowski 2007). The presence of co-occurring small mammals could lead to increased parasite transmission among species, potentially affecting the overall parasite loads and diversity in Abert's and Mt. Graham red squirrels. Previous studies have revealed a lower incidence of endo/ecto parasites in Abert's squirrels (Patrick and Wilson 1995). Our analysis revealed a trend: male Abert's squirrels exhibited a higher susceptibility to ectoparasite infestations in comparison to their female counterparts. This finding may arise from interactions, home ranges, seasonality, habitat preferences, and breeding patterns, resulting in sex-specific discrepancies in parasite loads among Abert's squirrels (Hillegrass et al. 2008; Merrick and Koprowski 2016; Mazzamuto et al. 2020). This pattern can also be attributed to breeding season behaviors: male Abert's squirrels, engaging in increased social interactions, contrast with females that choose solitary nesting to prioritize lactation resources (Edelman and Koprowski 2007). In the context of vertebrates, males tend to carry heavier parasite burdens (Isomursu et al. 2006; Perez-Orella and Schulte-Hostedde 2005; Hillegrass et al. 2008). This well-documented pattern reinforces the importance of considering gender-based susceptibility in the context of host-parasite interactions.

Conclusions

Our study raises questions about squirrel host-parasite co-evolution and emphasizes the necessity for specific nematode DNA primers. Long-term research is essential for a thorough exploration of the role of parasites and their transmission in biological invasions (Prenter et al. 2004). Understanding parasite transmission routes and their consequences helps identify factors that jeopardize species survival. Management strategies should prioritize controlling introduced species that carry foreign parasites, as exemplified by the Abert's squirrel removal program (Bergman et al.

2021), and monitoring parasite loads in native ranges remains vital (Kelly et al. 2009). Habitat conservation efforts are pivotal in reducing parasite transmission by addressing crowding and disease susceptibility between introduced and native species (Tompkins and Poulin 2006). Proactive monitoring and comprehensive research on parasite infection and transmission enable timely responses to potential spillover effects (Romeo et al. 2021). In addition, it is important to adopt an all-encompassing perspective when investigating the impact of climate on parasite distribution and survival, as this approach could reveal insightful mechanisms underlying observed variations in parasite patterns. With the increasing global movement of non-native species, vigilance becomes imperative in managing the introduction of exotic pathogens or vectors to new regions or hosts (Daszak et al. 2000). As ecosystems continually evolve with novel interactions, the collaboration between invasion ecologists and parasitologists is vital in addressing emerging challenges (Dunn and Hatcher 2015).

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Declarations

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