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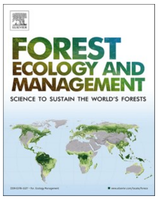

Using high-throughput sequencing to investigate summer truffle consumption by chipmunks in relation to retention forestry

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ABSTRACT

Intensive management of forests for wood modifies forest biodiversity, affecting the composition of plant and animal communities as well as microorganisms such as bacteria and fungi. Ectomycorrhizal fungi (EMF) form mutualistic relationships with trees, but there is evidence that the abundance and diversity of EMF declines under intensive forest management. The dispersal of some EMF, including that of truffles, depends on small mammals consuming their fruiting bodies and dispersing spores through feces. To better understand provisioning of this ecosystem service within intensively managed forests, we applied high-throughput, DNA amplicon sequencing to scat collected from Townsend's chipmunks (*Neotamias townsendii*) inhabiting retention patches in recently clearcut-conifer stands. Across two summers, we collected 165 chipmunk scat samples from 43 clearcut-conifer stands in the Pacific Northwest (USA). We identified 81 truffle species representing 16 families, which included many rare and uncommon taxa. Retention patch sizes varied from 9 to 222 trees (0.002–0.83 ha) but we did not detect an effect of retention patch size on the richness of truffles in chipmunk scat samples. However, truffle richness was slightly higher in scat samples collected from chipmunks in retention patches compared to individuals sampled in adjacent clearcuts. Furthermore, the abundance of certain truffle species in chipmunk scat varied in relation to retention patch size and location (riparian or upland), suggesting retention forestry practices may influence truffle community composition. Throughout the sampling season, we detected a 44% decline in truffle species richness and a significant reduction in abundance (i.e., sequence reads) for > 50% of common truffle taxa. Our application of high-throughput sequencing of scat effectively captured variation in truffle species consumption by chipmunks. Our results confirm that common small mammals play an important role in the dispersal of EMF across recently logged forests (<8 years since harvest) and indicate that retaining relatively small (~10 tree) green-tree patches may promote truffle dispersal by providing small mammal habitat.

1. Introduction

Consumption of ectomycorrhizal fungi (EMF) by small mammals aids in dispersal of EMF spores to roots of tree seedlings (Maser et al. 1978; Stephens and Rowe 2020; Elliott et al. 2022), where the fungi facilitate uptake of soil nutrients in return for carbohydrates (Smith and Read 1997). This symbiotic relationship between trees and EMF is critical to forest regeneration (Parladé and Alvarez 1993; Grove et al. 2019). Truffle forming fungi, most of which are ectomycorrhizal, fruit underground and evolved to depend on small mammals for dispersal via consumption and spore defecation (Fogel and Trappe 1978; Colgan and Claridge 2002). Thus, small mammals constitute a crucial link between trees and EMF (Maser et al. 2008). Although this mechanistic relationship between small mammals, trees, and truffles has been extensively documented (Maser et al. 1978; Schickmann et al. 2012, Stephens et al. 2021), understanding of factors related to the species composition of truffles in small mammal diets remains limited.

Despite their importance to tree growth, EMF communities are

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sensitive to forest harvesting practices. Although many truffles are considered pioneer species that aid in forest regeneration (e.g., *Rhizopogon*; Baar et al. 1999), after clearcutting, young regenerating forests have a lower diversity and altered species composition of EMF compared to older stands (Twieg et al. 2007). In recently harvested stands, changes in EMF community composition occurs in relation to distance from intact forest, which is potentially linked to decreases in tree regeneration within clearcut interiors (Grove et al. 2019). However, negative effects of clearcutting on EMF communities can be ameliorated by retaining a portion of trees within the clearcut area (Luoma et al. 2004; Lazaruk et al. 2005; Kranabetter et al. 2013; Sterkenburg et al. 2019). As small mammals are adapted to locate truffles (Stephens et al. 2020) and may consume truffles in proportion to availability (Stephens et al 2017), investigating patterns of small-mammal consumption of truffles in harvested forests may provide insight into effects of forest management practices on EMF communities (McIntire 1984; Jacobs and Luoma 2008). Small mammal diets can further reveal the potential for EMF recolonization of clearcuts, as they can disperse spores of truffles consumed elsewhere in their home ranges (e.g., older stands).

Conifer forests of western Oregon and Washington (i.e., Pacific Northwest), USA, are fragmented by industrial logging activities, and most of the old-growth forests in this region have been replaced by conifer plantations since the early 1900′s. Management regimes in these plantations typically are intensive, relying on even-aged forest rotations (~50 year), clearcut silviculture, site preparation, and post-harvest planting of native conifer seedlings, principally the ectomycorrhizal-associate Douglas-fir (*Pseudotsuga menzeisii)*. Regional policies require a small percentage of trees, typically > 5%, be retained at the time of logging to promote structural complexity within relatively homogenous, regenerating forests (Gustafsson et al. 2012, Fedrowitz et al. 2014, Mori and Kitagawa 2014). Tree retention practices vary widely in the Pacific Northwest, ranging from aggregated groups connected to riparian corridors to dispersed trees isolated from surrounding forests (Aubry et al. 2009; Sultaire et al. 2021a).

Populations of small mammal species respond to the spatial pattern of retention trees (Sullivan and Sullivan, 2001; Gitzen et al. 2007). For instance, Townsend's chipmunk (*Neotamias townsendii*) densities are highest in small, dispersed patches, and are comparatively low elsewhere in clearcut forests (Sultaire et al. 2021a). Although not a specialist consumer of fungi, Townsend's chipmunk is considered an avid mycophagist (Maser et al. 1978; Jacobs and Luoma 2008) and chipmunks are one of the primary consumers of truffles in recently harvested forests (Stephens et al. 2021). However, our understanding of how consumption of truffles by chipmunks changes with variation in retention patches is nascent. As truffle-sporocarp (i.e., fruiting body) production declines at low levels of green-tree retention in logged forests (Luoma et al. 2004), there is reason to expect that chipmunk consumption of truffles may also decline in small compared to larger retention patches.

In this study, we investigated the richness and composition of truffle species in the diet of chipmunks inhabiting recent clearcuts with retention by applying high-throughput DNA amplicon sequencing to chipmunk scat. In addition to documenting the taxonomic breadth of truffles consumed by chipmunks in managed forest landscapes, we further apply our technique to explore relationships between the truffle community observed in chipmunk diet and variation in retention forestry practices. Because live trees promote EMF persistence in logged forests (Luoma et al. 2004, Sterkenberg et al. 2019) and truffle taxa have variable sensitivities to disturbance, we expected the diversity and composition of truffles in chipmunk diets would vary in relation to the retention-patch characteristics where they were sampled. Specifically, we predicted that chipmunks sampled in larger retention patches and those connected to larger riparian corridors would consume a higher taxonomic richness of truffles, and abundance of common truffle species. In addition to providing a novel technique to identify truffle species consumed by small mammals with a high taxonomic resolution, our findings reveal the diversity of truffles present, and highlight an important ecosystem service provisioned by small mammals, in managed forests.

2. Materials and methods

2.1. Study area and experimental design

The experimental design consisted of 50 recently clearcut (<8 years) conifer stands (20–55 ha) spread across the Coast and Cascade Ranges in western Oregon and Washington, USA (Fig. 1). Stands were in either their second or third harvest rotation since the old growth was harvested (approximately 50–100 years). The 50 stands were grouped spatially into 10 blocks with five stands in each block (Fig. 1). Forest harvesting rules in both states require protection of riparian buffers for fish-bearing streams and retaining approximately five trees/ ha outside of riparian protection buffers. Across the 50 stands, these required retention trees were grouped in patches of various sizes and distances to riparian buffers, representing five distinct treatments. Two treatments contained only one patch, either in the upland portion of a stand or connected to a riparian zone. Two other treatments contained two smaller patches, one in the upland area, and one connected to a riparian zone. These treatments differed by the presence of mechanically topped trees within patches (i.e., created snags; Kroll et al. 2012). The fifth treatment contained four distinct patches of < 15 trees each and also contained created snags in each patch. For this study, we did not consider stand-scale treatment assignments and instead focused on the size and location (riparian or upland) of individual patches within treatments. We also did not consider the effects of created snags present within the two treatments. Patch size varied from 9 to 222 trees, with a mean patch size of 42 trees (sd = 36). The area of patches varied from 0.002 ha to 0.832 ha ($\bar{x}$ = 0.15, sd = 0.17), with most patches were smaller than 0.09 ha (median patch size). Retention patches primarily consisted of Douglas-fir, with western hemlock (*Tsuga heterophylla*) more common at wetter sites in addition to minor components of western red cedar (*Thuja plicata*) and noble fir (*Abies procera*; Table S1). Red alder (*Alnus rubrum*) was a common deciduous species along streams and was also present in some upland patches (Table S1). The exact ages of stands before harvest were not available but even-aged rotations in the Pacific Northwest are generally 40–60 years.

Within these stands we live-trapped chipmunks and collected scat samples from the differently sized and located retention patches. In addition to clearcut stands with retention, we also sampled an unharvested conifer stands within nine out of the 10 blocks (nine stands total) that were approximately 50 years of age. This age is characteristic of harvested conifer stands in the region, thus we term them rotation-aged stands as they are near the end of even-aged harvest schedules. Further details on live-trapping protocols can be found in Sultaire et al. (2021a). Briefly, we used two types of livetraps: larger Tomahawk (Model 202, 48.3 × 15.2 × 15.2 cm, Tomahawk Live Trap Co., Tomahawk, Wisconsin) and smaller Sherman traps (Model LFA, 7.6 × 8.9 × 22.9 cm; H. B. Sherman Traps Inc., Tallahassee, Florida), both of which captured chipmunks. Trapping effort varied with patch size, such that small patches (<15 trees) had 12 Sherman and 6 Tomahawk traps per patch, patches in the split treatments contained 24 Sherman traps and 12 Tomahawk traps per patch, and patches in the two aggregated treatments contained 48 Sherman and 24 Tomahawk traps per patch. Trapping grids of consistent size as grids in patches were also deployed in adjacent clearcuts, at a random distance ($\bar{x}$ = 49.5 m, sd = 27.5 m), and compass bearing from the patch center. We live-trapped in treatment stands for four consecutive nights across two consecutive summers (2018–19) and the timing of sampling within each stand was rotated between early summer and late summer between the two years. Townsend's chipmunks are the most common mycophagous rodent in recently-clearcut stands of the Pacific Northwest (Gashwiler 1970; Cole et al. 1998), with densities approaching 20/ha in retention patches

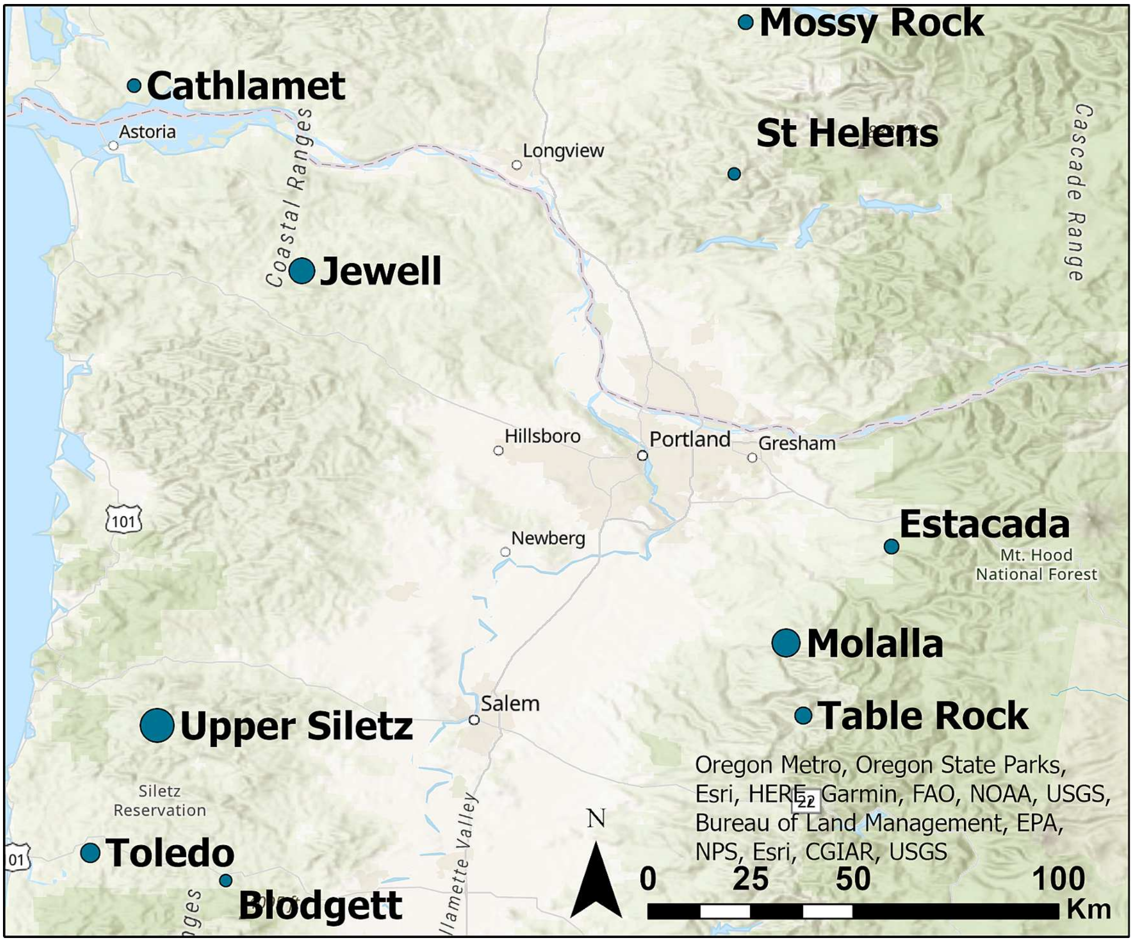

Fig. 1. Map of study area in the northwest Oregon and southwest Washington, U.S.A., showing locations of ten experimental blocks that contained tree retention treatments in clearcut harvested stands. The size of the points is proportional to the number of chipmunk scat samples collected from the block (range 6 – 24).

(Sultaire et al. 2021a). Mycophagous species associated with interior forest, such as red-backed voles (*Myodes* spp.) and flying squirrels (*Glaucomys oregonensis*), were rarely captured in our plots (Sultaire et al. 2022).

We applied uniquely-identifying ear tags to all Townsend's chipmunks captured, and collected scat samples from individuals on the first day of capture (Carey et al. 1999). We limited sample collection to only the first capture because we expected that the digestive tract of animals repeatedly captured would be contaminated with bait and less likely to reflect fungi present in chipmunk diets (Carey et al. 1999). Samples were only collected from chipmunks that defecated while handling, to prevent contamination from traps that previously caught animals. This opportunistic nature of sample collection meant that samples were not collected from some of the stands live-trapped for chipmunks. As a result, imbalances in the number samples from each retention patch type existed, which we accounted for in analyses.

2.2. *MiSeq library preparation*

Genomic DNA was extracted from chipmunk feces with a cetyltrimethylammonium bromide (CTAB) chloroform extraction method (Gardes and Bruns 1993). This method was chosen based on a comparison of three methods for DNA extraction (Fig. S1) from chipmunk scat with a representative subset of samples: a fast extraction method as in (Liber et al. 2021), a conventional CTAB method, and commercial DNeasy PowerSoil DNA extraction kit (Qiagen, USA). In these preliminary analyses we found that the CTAB method generated the highest fungal biodiversity (i.e., higher richness) and a deeper sequencing depth (i.e., more sequence reads; Fig. S1). Illumina MiSeq libraries were prepared targeting the internal transcribed spacer region 1 (ITS1) of the nuclear ribosomal (nr) DNA with the ITS1F (Gardes and Bruns 1993) and ITS2 (White et al. 1990) PCR primer pairs. We prepared amplicon libraries as described previously (Lundberg et al. 2013; Benucci et al. 2019; Benucci et al. 2020). Briefly, an enrichment PCR step (10 cycles) was carried out with generic ITS1F-ITS2 primers to enrich target fungal rDNA; second (10 cycles), and 1–6 random nucleotide frameshifts were incorporated into amplicons to increase the diversity of clusters during sequencing. During step three (15 cycles), barcode primers with a 10-nucleotide index and Illumina adapters were PCR-ligated to amplicons for sequencing. Negative (no DNA added) and positive, synthetic fungal mock community control samples were also included in the library (Palmer et al. 2018). Final amplicon libraries were visualized using a QIAxcel Advanced machine with a DNA Fast Analysis Kit (Qiagen, USA) to assess PCR success. DNA libraries were then normalized with the SequalPrep Normalization Plate Kit (ThermoFisher Scientific, USA), pooled, concentrated (approx. 20:1) with Amicon Ultra 0.5 mL 50 K filters (EMDmillipore, Germany) and cleaned with Agencourt AMPure XP magnetic beads (Beckman Coulter, USA). The ITS amplicon library was then sequenced on an Illumina MiSeq sequencer using the v3 600 cycles kit (Illumina, USA) at the Michigan State University Research Technology Support Facility.

2.3. *Bioinformatic analysis*

Raw ITS sequence reads were evaluated for quality with FastQC (Andrews, 2010) and then demultiplexed with QIIME (Caporaso et al.

2010). Demultiplexed sequences were removed from Illumina adapters, and sequencing primers were removed with Cutadapt (Martin 2011), quality filtered (max expected errors $\leq$ 1), trimmed to equal length (Edgar and Flyvbjerg 2015; Edgar 2016) and de-replicated. Singleton sequences were removed and sequences were then clustered into operational taxonomic units (OTUs) at 97% sequence similarity with the UPARSE (Edgar, 2013) algorithm in USEARCH (Edgar and Flyvbjerg 2015). Taxonomic assignments were performed with CONSTAX2 (Liber et al. 2021) against the eukaryotic UNITE database (PlutoF Biodiversity Platform) version 4–2–20. Raw, demultiplexed, sequence data reads are available in the Sequence Read Archive (Leinonen et al. 2011) and accessible with the BioProject number PRJNA828603.

Bioinformatic analysis, and further filtering to retain only truffle taxa generated a total of 181 OTUs, of which 83 were classified as *Barssia oregonensis* despite having > 10% sequence variation between the sequences. To confirm these classifications, we used the Basic Local Alignment Search Tool (BLAST) to compare all OTU representative sequences against NCBI GenBank database. We obtained low coverage and percent similarity for most of the OTU representative sequences that were classified as *Barssia oregonensis* compared to the only 2 *Barssia oregonensis* sequences accessioned in GenBank. Only eight species of Barssia have been described worldwide, of which *B. oregonensis* is the only *Barssia* species known from North America. In order to provide a conservative estimate of *Barssia* species diversity detected in our study we clustered *Barssia* OTUs at 94% sequence similarity, which likely underestimated species diversity. This resulted in 19 sequence clusters. We aligned representatives of the 19-sequence cluster with *Barssia* sequences available in GenBank and those of the sister genus *Balsamia* in Mesquite v3.70 (Maddison and Maddison 2009). Aligned sequences were used to infer a Maximum likelihood phylogeny in RAXML (Fig. S3; Stamatakis 2014).

2.4. Statistical analysis

Contaminant OTUs based on sequences present in negative control samples were removed from the datasets with the R package *decontam (*Fig. S2; Davis et al. 2018). Given that small mammals show preference for truffle-forming species (Lehmkuhl et al. 2004, Stephens et al 2020), we further filtered for only truffle taxa based on the FunGuild database (Nguyen et al. 2016). We quantified truffle species richness at the scale of each individual scat sample and considered the number of species with greater than one sequence per sample to calculate species richness (i.e, removed singletons). To relate truffle-species richness in each sample with retention-forestry variables, we used Poisson mixed-effects models. We included stand- and block-level random intercepts to account for non-independence of samples collected from the same stand and within the same experimental block. The block level random effect also helped account for variation in truffle richness across the study area when assessing the effect of retention variables. We fit two models: 1) a model that included all samples collected and related truffle richness to the different plot types sampled (upland patches, riparian patches, clearcuts, and rotation-age) and 2) a model that related truffle richness from only samples collected in retention patches to retention patch size. Patch size was quantified as the number of green trees retained in a patch and was log-transformed to conform to species-area relationship convention (linear-log relationship). In addition to patch size and location, we also included ordinal date of sample collection as an explanatory variable to account for changes in species richness across the 4-month sampling season in both models. We fit the mixed-effects model in R package lme4 version 1.1.21 (Bates et al. 2015).

To explore differences in truffle species composition among the plot types and differently sized retention patches we used latent variable models (Niku et al. 2019). Latent variable models estimate species-specific relationships with each environmental variable included in the model, and model residual correlation between species as latent variables (Niku et al. 2019). Ordination plots can be generated by plotting species or site loadings on each latent variable. We fit models using the negative-binomial distribution that associated sequence counts of each species to the retention variables. The negative binomial distribution better accounted for over-dispersion of OTU read numbers compared to the Poisson distribution. We also fit two models, 1) a null model that included all samples, and 2) a model with only samples collected from patches to examine species-specific relationships to retention patch size and location. The null model explored differences in community composition among plot types using an ordination plot while the covariate model allowed us to explore species-specific relationships to patch size and location. To aid in model fitting, we included only the 21 truffle species that occurred in $\geq$ 10 samples. Consistent with the richness mixed-effects model, we included ordinal date of sample collection as a fixed-effect and patch size was log-transformed. A stand-level random effect was also included in the LVMs to account for repeated samples from the same stand. LVMs were fit in the R package gllvm version 1.1.3 (Niku et al. 2019).

3. Results

Between 2018 and 2019, we collected 165 fecal samples from different chipmunks. These samples were collected from 43 different stands, including 38 recent clearcuts with retention and five unharvested rotation-aged stands, and from all 10 experimental blocks (Table 1). On average, we collected 3.8 samples/stand (ranging from 1 to 12). We collected 83 samples from upland patches, 35 samples from riparian patches, 34 samples from clearcuts, and 13 samples from five rotation-aged stands. Across the 165 samples, we identified 264 fruiting EMF species belonging to 25 families and 40 genera, of which 81 species from 16 families and 22 genera were truffles.

Truffle forming EMF were particularly common in sampled chipmunk scat, showing high species diversity in several genera, some of which were higher than previously reported from the literature (e.g., *Barssia*; Fig. 2). *Rhizopogon,* the most common genus, was detected in at least 85% of the samples from each block and was detected in 100% of the samples from several blocks (Fig. 3). In total 14 different *Rhizopogon* species were identified (Fig. 2; Table S3). Other genera were infrequently identified at both the scales of individual scat sample and experimental block scales (i.e., *Balsamia*, *Tuber*; Fig. 3). The most common *Rhizopogon* species was *Rhizopogon vinicolor,* which was present in 151 samples (~92%) and 36 stands (~84%) (Table S2). One *Barssia* OTU was also frequently detected and was present in 61 samples from all 10 blocks (Fig. 3). More broadly, our results indicated greater species diversity in *Barssia* than previously identified, with 19 OTUs identified at the 94% similarity threshold (Table S2) and confirmed through phylogenetics (Fig. S3). Other common species sampled included *Alpova diplophloeus* (36 samples), *Genea harknessii* (24 samples), *Russula ellipsospora* (24 samples), and *Hymenogaster crassirhachis* (22 samples).

The average number of truffle species per fecal sample (i.e., species richness) was 7 (range 0 to 17). When considering different plot types, truffle species richness was not different from scat samples taken from

Table 1
Summaries for chipmunk scat samples collected from the 4 plot types, number of stands samples were collected from, and the timing of sample collection in northwest Oregon and southwest Washington, USA 2018–2019. Aside from rotation-aged stands, all stands contained retention patches (either riparian, upland, or both), and clearcut plots. We accounted for the nonindependence of samples collected from the same stands in analysis with a stand-level random effect.

Plot Type	Samples	Stands	Date range	Mean date
Upland Patch	83	25	May-29: Aug-28	Jul-16
Riparian Patch	35	17	May-29: Sep-06	Jul-03
Clearcut	34	20	Jun-11: Aug-19	Jul-12
Rotation-aged	13	5	May-31: Aug-30	Jul-16
Total	165	43	May-29: Sep-06	Jul-12

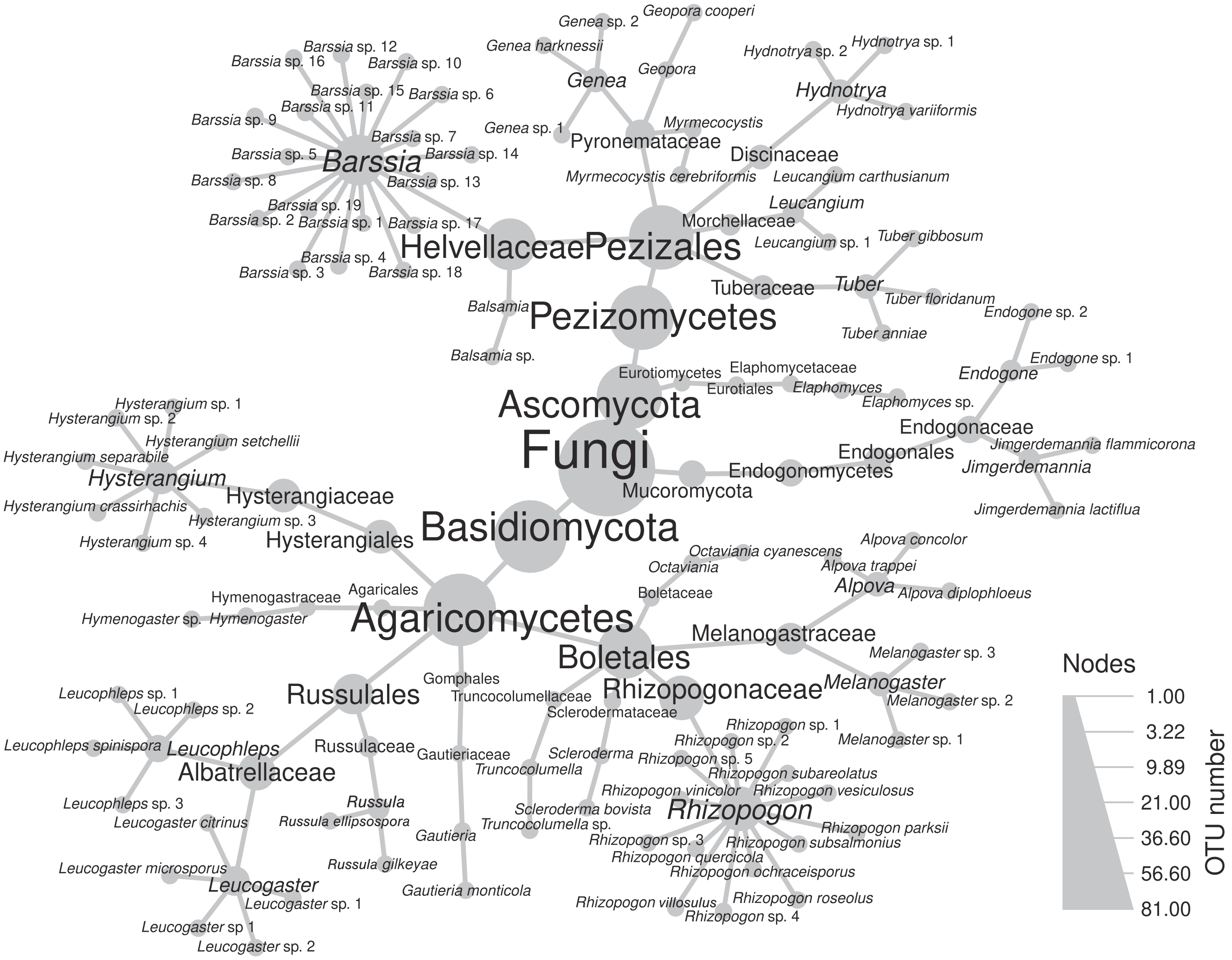

Fig. 2. Taxonomic tree depicting the overall diversity of truffle-forming fungi found in chipmunk scat. Node size is proportional to the number of taxonomic groups present at each node. Terminal nodes represent fully classified species (full specific name reported) or OTUs unclassified at species level (genus name plus sp. reported, e.g., Barssia sp.1, Barssia sp.2, etc.).

upland and riparian patches ($\beta_{\text{riparian}} = 0.13$; 95% CI = $-0.05, 0.31$; Fig. 4a) or upland patches and rotation-aged forest ($\beta_{\text{RotAge}} = 0.13$; 95% CI = $-0.20, 0.43$; Fig. 4a). However, we did detect a weak trend towards lower truffle richness in samples from clearcut plots compared to other plot types ($\beta = -0.24$; 95% CI = $-0.43, -0.06$; Fig. 4a). For the model that only considered samples from retention patches, the relationship between sample scale truffle richness and retention patch size was positive but not significant ($\beta = 0.07$; 95% CI = $-0.04, 0.19$), with predicted richness increasing by < 2 taxa across the entire patch size gradient (Fig. 4c). Ordinal date of sampling had the strongest relationship with truffle richness, with the number of truffle species in each sample declining by > 5 taxa (Fig. 4b), approximately 54% on average, throughout the sampling season ($\beta = -0.15$; 95% CI = $-0.29, -0.10$).

The latent variable model fit with data from all samples did not indicate differences in species composition among plot types when considering the number of sequence reads of common species in each sample (Fig. S4). However, when considering only samples collected from retention patches, some species-specific responses to retention were identified (Fig. 5). The number of sequence reads within samples had a positive relationship with retention patch size for *Hymenogaster* sp. (OTU 26 in reposited data), *Rhizopogon villosulus*, and *Rhizopogon vesiculous* (Fig. 5b). However, the number of sequence reads also decreased for three species in response to retention patch size: *Genea harnknessii*, an unidentified *Rhizopogon* (OTU 19) and a *Truncocolumella* sp. (OTU 20, Fig. 5b). *Genea harknessii* was also more common in samples collected in upland patches compared to riparian patches, as were the species *Rhizopogon vesiculus* and *Hysterangium crassirhachis* (Fig. 5c). *Rhizopogon parksii* and an unidentified *Melanogaster* sp. (OTU 16) were the only species with significantly higher sequence reads in samples taken from riparian patches (Fig. 5c). Consistent with declines in species richness throughout the sampling season, the number of sequence reads for 14 species decreased later in the season while reads of *Alpova diplophloeus* and a *Rhizopogon* sp. increased (OTU 18, Fig. 5a).

4. Discussion

Intensively managing forests for wood production strongly alters biodiversity in forested landscapes (Jones et al. 2022; Stokely et al. 2022) and may have cascading effects on ecosystem function and productivity. Ectomycorrhizal fungi are a critical component of forest biodiversity that positively affect tree growth, and small mammals contribute to this ecosystem service by dispersing EMF spores (Maser et al. 1978). To understand potential influences of forest management on this ecosystem service, we investigated how the richness and composition of truffles in chipmunk scat varied among different retention-forestry practices via a novel genetic technique. We confirmed that Townsend's chipmunks consume a diversity of EMF taxa, particularly truffle-forming fungi. For instance, we detected truffle genera

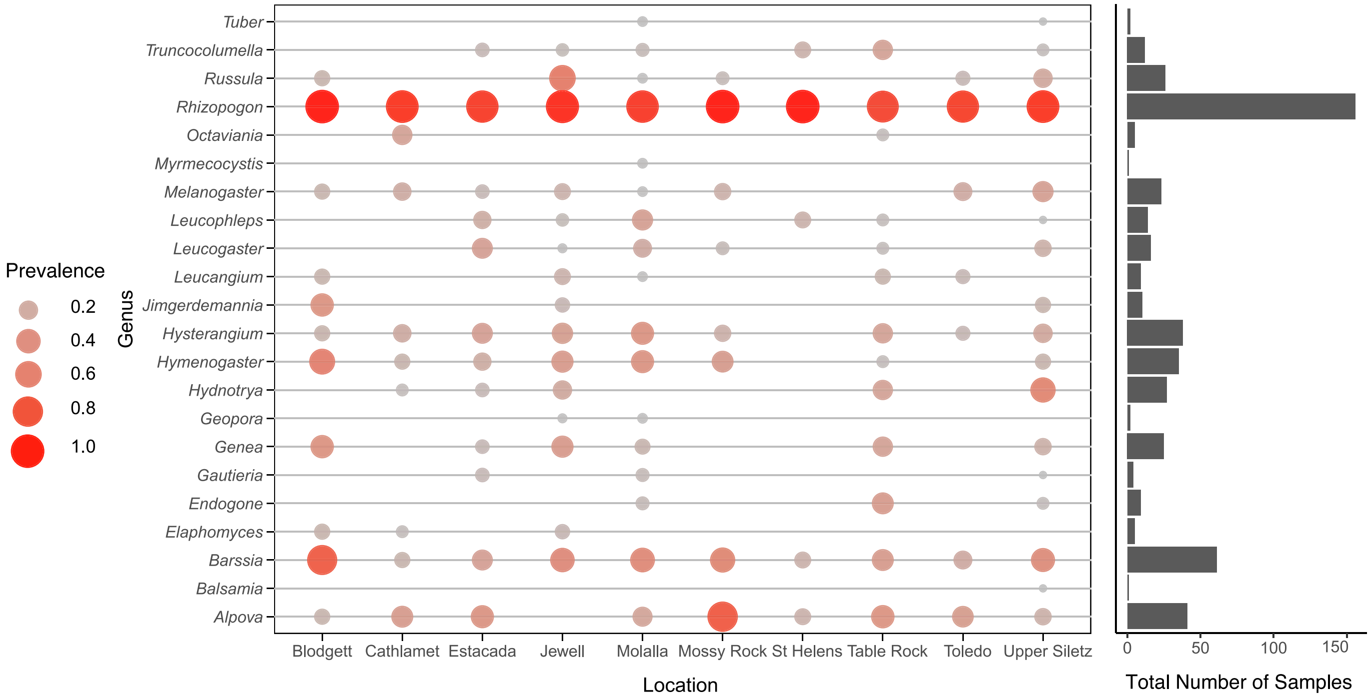

Fig. 3. Genus prevalence (i.e., the proportion of samples a genus was found) of truffle genera detected in each study block (left panel) and total number of samples that genus was detected across all study blocks (right panel). Increasing bubble size and color gradient represent higher prevalence, with small gray color circles indicating the absence of a genus and larger red circles indicating 100% prevalence of a genus (i.e., genus detected in all samples from that block).

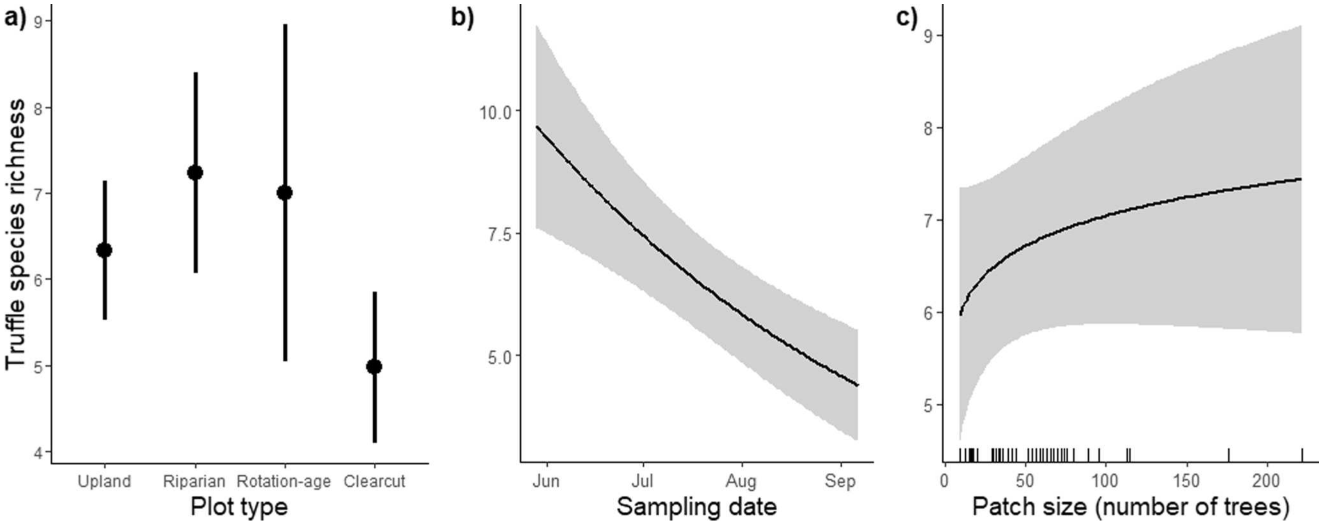

Fig. 4. Predicted relationships between sample-scale truffle species richness in chipmunk scat samples and a) plot type, b) date of scat sample collection, and (c) retention patch size. The ticks on the x-axis in panel c) represent sizes of patches where samples were collected. The patch size effect was not statistically significant. Shaded regions are the 95% confidence intervals for the predictions.

belonging to Ascomycota (e.g., *Barssia*, *Balsamia*, *Elaphomyces*, *Geopora, Hydnotrya, Genabea, Genea, Leucangium, Tuber*), Basidiomycota (e.g., *Alpova*, *Hymenogaster*, *Hysterangium*, *Gautieria*, *Russula*, *Leucogaster*, *Leucophleps*, *Melanogaster*, *Octaviana*, *Rhizopogon*, *Scleroderma*) and Mucoromycota (*Endogone*, *Jimgerdemania*). Although not considered in our analyses, mushroom forming EMF were also frequently detected in chipmunk scat (e.g., *Cortinarius*, *Hebeloma*, *Hygrophorus*, *Inocybe*, *Lactarius*, *Octaviana*, *Paxillus*, *Suillus*). We further detected many genera of ectomycorrhizal coral and resupinate fungi (e.g., *Amphinema*, *Athelia*, *Clavulina*, *Piloderma*, *Tomentella*, *Trechispora*), which do not produce large fruiting bodies targeted by small mammals and were likely incidentally ingested by chipmunks (Borgman-Winters 2022). Furthermore, our methods were able to identify multiple species within several truffle genera (e.g., 14 *Rhizopogon* sp., 19 *Barssia* sp.), providing higher taxonomic resolution than often possible using spore morphology, and in many cases novel diversity previously unaccounted for. Thus, our findings build on previous studies by demonstrating that the diversity of truffles in small mammal diets can be successfully assayed from high-throughput amplicon sequencing of chipmunk scat, as opposed to morphological identification of spores which provides less taxonomic resolution (Castellano et al. 1989).

Despite the large number of truffle taxa detected and the large variation in retention patch sizes we sampled, we did not find an effect of retention patch size on taxonomic richness of truffles in chipmunk

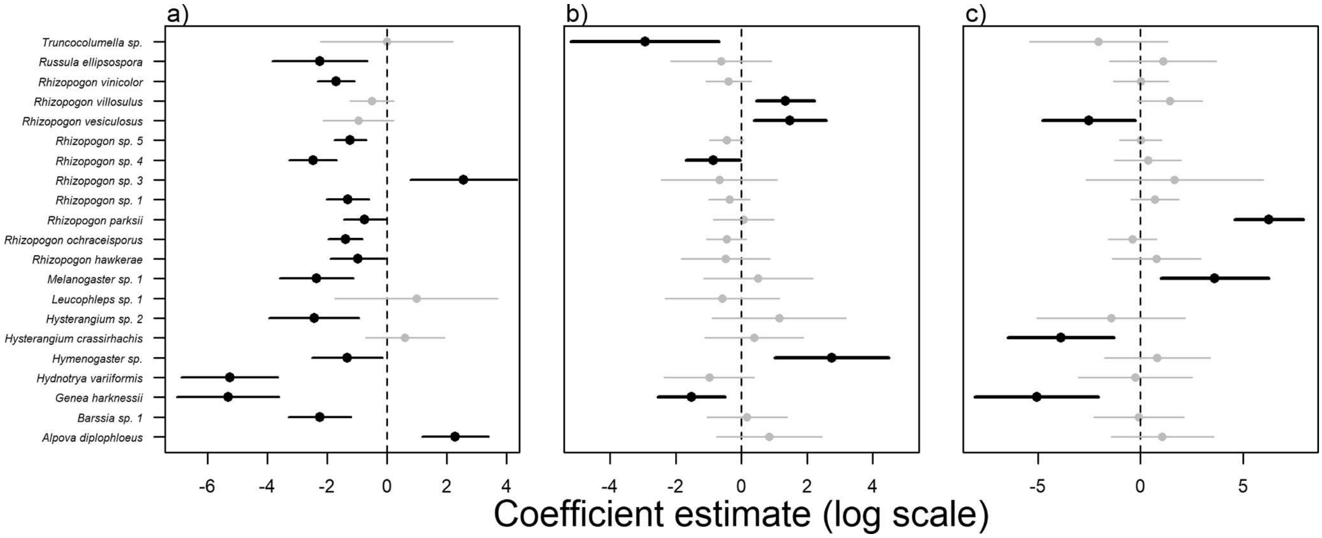

Fig. 5. Negative binomial coefficients relating counts of the 21 truffle species most commonly found in 117 chipmunk scat samples to a) date of sample collection, b) retention patch size, and c) riparian patches compared to upland patches. Points indicate point estimates and horizontal lines are 95% confidence intervals. Points plotted in black indicate effects for which 95% confidence intervals do not overlap zero. Not shown is the riparian effect on *Leucophleps* sp, which was large and negative but with small uncertainty.

diet. Across the full gradient of patch sizes, from nine to 222 trees, predicted richness of truffles in chipmunk diets increased by fewer than two species when mean sample species richness was 7. The abundance (i.e., number of reads) of only a few common truffle species in chipmunk diet varied in relation to retention patch size, and likely contributed to the weak effect of retention patch size on truffle species richness. However, truffle-species richness in chipmunk diet was slightly elevated for chipmunks sampled in retention patches compared to samples taken from clearcuts, indicating that retention forestry promotes truffle diversity in intensively managed forests. Increasing species richness with increasing habitat area, known as the species-area relationship, is a well-documented ecological rule (Preston 1962; Lomolino 2000). Our results did not adhere to this relationship and contrast with previous soil sampling studies that found EMF diversity had a positive relationship with retention amount (Luoma et al. 2004, Sterkenberg et al. 2019).

There are several potential explanations for the lack of relationship we found between retention patch size and truffle richness in chipmunk diets. First, we did not directly sample truffles present in retention patches but instead from mobile animals whose movement transcends the size of individual patches (Sultaire et al. 2021a). Hence, chipmunk movement impacted our truffle richness estimates (Komur et al. 2021), and chipmunks were likely ingesting truffle spores outside of sampled patches. This is a key aspect of our results, as small patches either contain nearly as many truffle species as large patches or chipmunks are dispersing spores from elsewhere into small patches. Stronger effects of sampling date on truffle richness in chipmunk scats supports this contention as phenological variation in truffle fruiting would reduce their availability at the landscape scale, whereas patch size would only affect local availability. Slightly lower truffle richness in the diets of chipmunks sampled in clearcuts adjacent to patches, however, indicates fine-scale effects of retention (<100 m) on chipmunk truffle consumption, and potential benefits of retention patches to truffle diversity. In support of this conclusion, recaptured chipmunks moved 26 m on average between captures and 22% moved between patches and clearcuts within stands over 4-day trapping periods; indicating chipmunks were most often recaptured within the same plot but movements between plots were not uncommon.

Furthermore, variation in the truffle taxa consumed among chipmunk individuals is plausible, yet we quantified truffle richness at the scale of the individual scat sample. Aggregating samples collected from the same patches confirmed the presence of greater truffle diversity than consumed by individual chipmunks, but variation in the number of samples collected among patches precluded straightforward inference of patch-scale richness from aggregated samples. These factors make comparisons to results of EMF community studies that analyze small mammal scats and those employing soil sampling difficult. Our results are more consistent with other studies that employed dietary analysis of small mammal scats and found minimal differences in truffle occurrence in small mammal diets across different retention amounts and configurations in the Pacific Northwest (Jacobs and Luoma 2008).

We also failed to detect an effect of retention patch location on the richness of truffles in chipmunk scats, but low sample size from riparian patches (n = 35) hindered our ability to detect such an effect. However, taxa-specific responses indicated lower consumption of three truffle species in riparian retention compared to upland retention, with consumption of only two truffle species (e.g., *Rhizopogon parksii*) higher in riparian patches (Fig. 5c). With the caveat that chipmunk diet reflects the truffle community of an area larger than patches, our results indicate that retention placement does not strongly influence the abundance of most truffle species consumed by chipmunks. However, a few truffle taxa may be more common in upland retention (e.g., *Rhizopogon vesiculous*, *Genea harknessii*). This finding is consistent with limited responses to retention location of other taxonomic groups in the Pacific Northwest (Sultaire et al. 2021b, Sultaire et al. 2022) but contrasts with distinct communities in riparian and upland forests found for other taxonomic groups and regions (Baker et al. 2006; Palmer and Bennet 2006). Significant differences in responses of congeneric taxa to retention placement (i.e., *Rhizopogon parksii* vs. *Rhizopogon vesiculous*), demonstrates the presence of interspecific habitat associations that would have been difficult to detect without using molecular methods to identify species.

Despite limited evidence for effects of tree retention on truffle consumption, variation in the richness of truffle taxa found in chipmunk diets across the summer sampling period indicates our technique captured variation in the presence of fruiting truffle species. Predicted richness of truffles detected in each chipmunk's diet decreased by more than five species from May to September. Taxon-specific responses to sampling date mirrored community richness responses, with several taxa declining in abundance (14 species) in chipmunk diet throughout the summer and only two species increasing. These findings are consistent with previous studies showing declines in truffle biomass in the Pacific Northwest throughout the summer months (Fogel 1976) and declines in taxonomic richness of truffles consumed by flying squirrels (*Glaucomys oregonensis*) during summer in the Pacific Northwest (Lemkuhl et al. 2004). Previous studies have found a strong correlation between truffle spore counts in chipmunk scat and fruiting phenology (Stephens et al. 2017). Thus, we interpreted the estimated decline in truffle richness across the summer sampling season as evidence that our molecular technique effectively captured fruiting patterns of truffle taxa during our sampling. However, this result could also be related to increases in other food resources later in the summer (i.e., mast; Tevis 1953), or preference by chipmunks for alternative truffle species that become available at different times of the year. Many truffle taxa fruit primarily in the fall (e.g., *Truncocolumella*, Maser et al. 1986; *Gautieria*, Cazares et al. 1999) and although represented in our samples, peak fruiting windows were likely missed for these taxa. A temporal sampling extent that encompasses the full range of truffle fruiting phenology will better demonstrate the ability of our molecular techniques to detect truffle fruiting patterns.

Townsend's chipmunks are most abundant in structurally-complex stands with abundant downed wood (Carey 1995; Carey et al. 1999, Waldien et al. 2006), which in clearcuts, is provided by small patches subject to high levels of blowdown (Sultaire et al. 2021a). In addition to supporting high chipmunk densities, our results show that chipmunks occurring in these patches consume as many truffle species as chipmunks occurring in larger patches and more than individuals sampled in surrounding clearcuts. From the perspective of forest harvest management, this indicates that high densities of chipmunks in small retention patches (Sultaire et al. 2021a) provide for EMF dispersal in conifer plantations. EMF spore dispersal by chipmunks could be particularly beneficial under the silvicultural system studied, which relies on artificial regeneration, as small mammals like chipmunks may inhibit natural regeneration by consuming conifer seeds (Tevis 1952).

Although we did not detect a strong effect of retention patch size on the abundance of most common taxa in chipmunk diet, our results show the presence of many truffle taxa that appeared rare and were detected in < 10 samples. Rare taxa often make unique contributions to ecosystem function (Burner et al. 2022), but these same taxa are the most difficult to sample (Jeliazkov et al. 2022). Given the ability of small mammals to seek out soil fruiting fungi (Carey et al. 2002; Stephens et al. 2020), they are likely effective at sampling rare truffle taxa. Modern molecular techniques increase our ability to detect these species in small mammal diets. In future studies, sample sizes will likely need to be increased to better infer occurrence patterns for rare taxa. For example, truffles of the genera *Tuber* and *Gautieria,* which are commonly found in diets of small mammals sampled in less intensively managed forests, rarely occurred in our samples, with only 4 total occurrences of *Tuber* and 5 occurrences of *Gautieria* (Fig. 3). Common pioneer truffle genera, including *Rhizopogon*, were commonly sampled. EMF communities can take several decades past typical harvest rotations to recover from logging disturbance (Spake et al. 2015), and the rarity of many truffle taxa in our samples could reflect their general absence in intensively managed forest landscapes. However, it is important to note that we only sampled scat from one small mammal species in this study, and diets of small mammals are known to be variable among species (Carey et al. 1999; Jacobs and Luoma 2008; Komur et al. 2021). Future application of high throughput sequencing techniques will benefit from sampling scat of additional mycophagous species, many of which are rare in intensively managed forests (Sultaire et al. 2022). Such approaches may help to guide forestry practices to optimize biodiversity and economic goals.

5. Conclusions

Through amplicon sequencing of mycophagous, small mammal scat, we detected a diversity of EMF, particularly truffles, present in intensively-managed conifer plantations. The application of high-throughput amplicon sequencing to chipmunk scat demonstrated here improves our ability to detect the diversity of truffles consumed by small mammals at a high taxonomic resolution. This method further demonstrates that some fungi considered to be rare, or rarely collected (e.g., *Barssia*), are instead quite common components in the diets of small mammals.

Although we found no relationship between truffle richness in chipmunk scat and the size of the retention patch where a chipmunk was captured, truffle richness was slightly elevated in scat collected from individuals captured in retention patches compared to those captured in adjacent clearcuts. These results support the retention of small tree groups that promote high chipmunk density (Sultaire et al. 2021a). Tracking truffle consumption by small mammals in these small patches over time will reveal whether small patches support this ecosystem service further into harvest rotations. In the short term (<10 years), our results indicate that adopting the strategy of retaining several small (~10 tree) retention patches has the potential to increase EMF dispersal by chipmunks in recently harvested conifer plantations.

6. Open research statement

Raw, demultiplexed, sequence data reads are available in the Sequence Read Archive (Leinonen et al., 2011) and accessible with the BioProject number PRJNA828603.

CRediT authorship contribution statement

Sean M. Sultaire: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Gian Maria Niccolò Benucci:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Data curation, Investigation. **Reid Longley:** Methodology, Visualization, Formal analysis, Writing – review & editing, Investigation. **Andrew J. Kroll:** Conceptualization, Methodology, Writing – review & editing, Resources, Funding acquisition, Supervision. **Jake Verschuyl:** Methodology, Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Gregory Bonito:** Methodology, Writing – review & editing, Supervision, Resources, Project administration. **Gary J. Roloff:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw, demultiplexed, sequence data reads are available in the Sequence Read Archive (Leinonen et al., 2011) and accessible with the BioProject number PRJNA828603.

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Appendix A. Supplementary data

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