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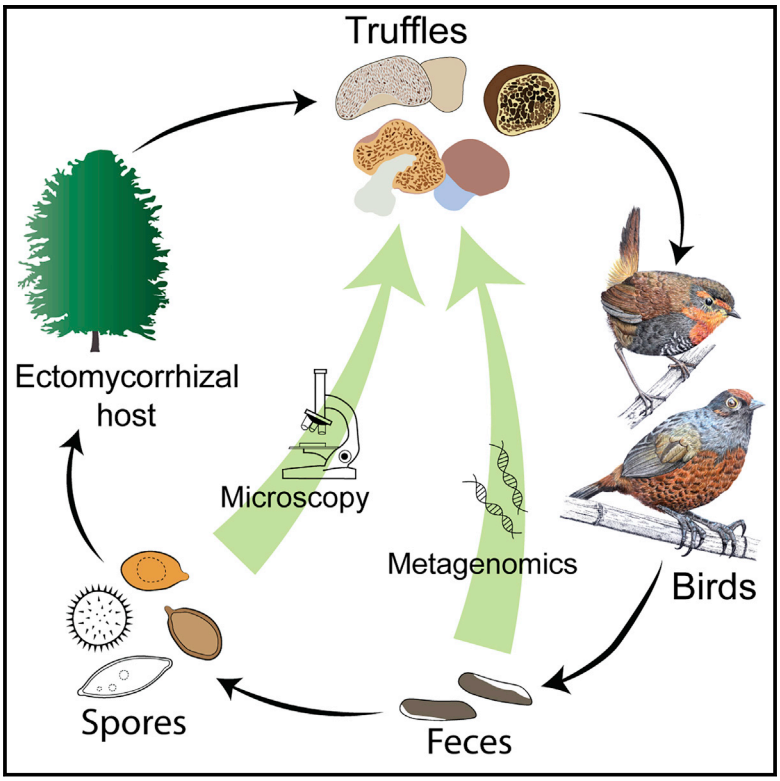
Discovering the role of Patagonian birds in the dispersal of truffles and other mycorrhizal fungi

Graphical abstract



Authors

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In brief

Caiafa et al. explore the role of Patagonian birds in the dispersal of mycorrhizal fungi. Metagenomic analyses of fecal samples show that birds consume a wide diversity of truffles and other mycorrhizal fungi. Epifluorescence microscopy reveals that a high proportion of fungal spores remain viable after passage through the birds' digestive systems.

Highlights

- Bird fecal samples are analyzed using metagenomics and epifluorescence microscopy
- Metagenomic results show that birds consume diverse truffles and other fungi
- Bird-dispersed ectomycorrhizal fungi are associated with Nothofagaceae trees
- Patagonian birds disperse viable spores of mycorrhizal fungi

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Discovering the role of Patagonian birds in the dispersal of truffles and other mycorrhizal fungi

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SUMMARY

Dispersal is a key process that impacts population dynamics and structures biotic communities. Dispersal limitation influences the assembly of plant and microbial communities, including mycorrhizal fungi and their plant hosts. Mycorrhizal fungi play key ecological roles in forests by feeding nutrients to plants in exchange for sugars, so the dispersal of mycorrhizal fungi spores actively shapes plant communities. Although many fungi rely on wind for spore dispersal, some fungi have lost the ability to shoot their spores into the air and instead produce enclosed belowground fruiting bodies (truffles) that rely on animals for dispersal. The role of mammals in fungal spore dispersal is well documented, but the relevance of birds as dispersal agents of fungi has been understudied, despite the prominence of birds as seed dispersal vectors. Here, we use metagenomics and epifluorescence microscopy to demonstrate that two common, widespread, and endemic Patagonian birds, chucao tapaculos (*Scelorchilus rubecula*) and black-throated huet-huets (*Pteroptochos tarnii*), regularly consume mycorrhizal fungi and disperse viable spores via mycophagy. Our metagenomic analysis indicates that these birds routinely consume diverse mycorrhizal fungi, including many truffles, that are symbiotically associated with Nothofagaceae trees that dominate Patagonian forests. Epifluorescence microscopy of fecal samples confirmed that the birds dispersed copious viable spores from truffles and other mycorrhizal fungi. We show that fungi are a common food for both bird species and that this animal-fungi symbiosis is widespread and ecologically important in Patagonia. Our findings indicate that birds may also act as cryptic but critical fungal dispersal agents in other ecosystems.

INTRODUCTION

Dispersal of propagules to new habitats is a fundamental process that affects metapopulation dynamics and structures communities.[1] For many theoretical frameworks in ecology, such as the theory of island biogeography,[2] the neutral theory,[3] the meta-community theory,[4] and the historical contingency theory,[5] dispersal is recognized as a critical process that generates variability in community assembly. Dispersal ability is highly variable between species[6,7] and therefore generates predictable patterns of community assembly.[8,9] The "everything is everywhere" hypothesis[10] postulates that dispersal is unimportant in structuring microbial communities and that these communities are instead determined by environmental factors. However, there is ample evidence that dispersal limitation impacts microbial diversity at different scales and influences community assembly.[11–14]

Mycorrhizal fungi represent a key microbial guild. They promote plant growth by facilitating the uptake of nutrients such as nitrogen and phosphorus,[15,16] and they consequently shape plant populations and communities.[17] Dispersal limitation has been shown to have strong impacts on diversity patterns of mycorrhizal fungi and consequently on their plant hosts.[12,13,18,19]

Many mycorrhizal fungi produce fruiting bodies to disperse their spores. Most fruiting bodies that grow above ground (mushrooms) shoot their spores into the air and rely on wind to disperse them. In contrast, some fungi have lost the ability to forcibly discharge their spores and instead produce enclosed belowground fruiting bodies (truffles) (Figure 1).[20,21] Because truffles cannot spread their spores alone, they rely on animals that eat

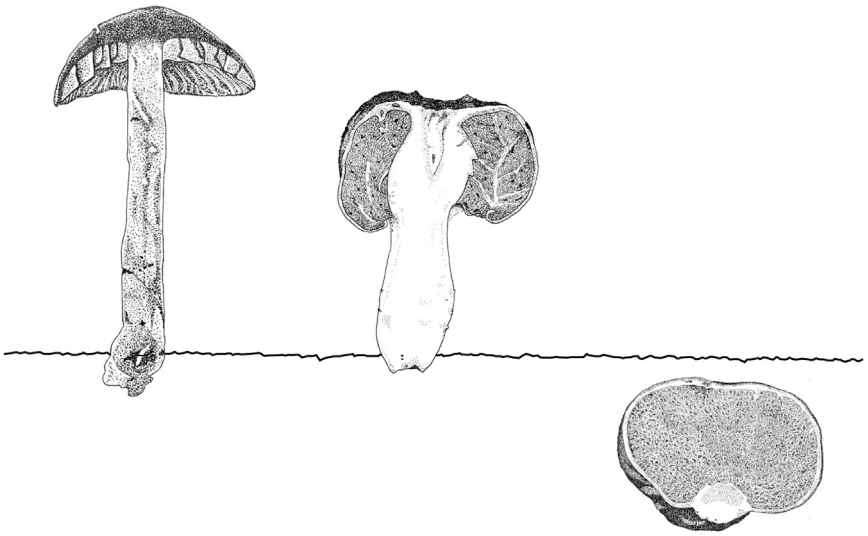

Figure 1. Common morphologies of aboveground and belowground fungi

Mushrooms (left) are aboveground fruiting bodies that can forcibly discharge their spores and rely primarily on wind for dispersal. Secotioid fungi (center) may be either aboveground or belowground but have lost the ability to shoot their spores into the air. Truffles (right) are belowground fruiting bodies that have lost the ability to shoot their spores into the air. Secotioid fungi and truffles rely almost exclusively on animals for spore dispersal, but mushrooms may also be dispersed by animals. Illustrations by Pamela Ciudad Martin.

them for spore dispersal.[22] Although aboveground mushrooms shoot their spores into the air, most spores travel only a few meters from their source, so long-distance dispersal is relatively rare.[19,23] Animal-facilitated spore dispersal through consumption, called mycophagy, is an important process that increases the chances that mycorrhizal fungi, both mushrooms and truffles, will colonize the roots of young plants.[23–25]

Birds are widely recognized as important dispersal vectors of seeds and are crucial for plant establishment in new locations,[26] but we know almost nothing about the importance of birds for fungal dispersal.[27] Some birds can disperse fungal spores over short or long distances via their feathers[28–31] and disperse root-like vegetative fungal tissue (i.e., rhizomorphs) when building nests,[32,33] but there are only a few documented examples of mycophagy in birds.[34–36] Some ground-foraging birds may occasionally consume fungi,[37] and there is evidence that some birds consume fungi during periods of low fruit abundance.[38,39] Most documented examples of bird mycophagy seem to be incidental, but new evidence indicates that these interactions may be far more common than previously thought.[27] Many mammals are known to consume mushrooms and truffles, pass the spores through their digestive tracts, and deposit them in new locations. However, this phenomenon has been documented primarily in mammals from North America, Europe, and Australia.[18,22,24,40] Insects and other invertebrates have also been occasionally shown to disperse fungal spores, although relatively few studies have examined dispersal of non-pathogenic fungi.[25,41,42] Mycophagy by animals from other world regions remains mostly unexplored.[22,43]

We examined the ecological importance of fungal spore dispersal via mycophagy by birds in Patagonian temperate forests, an understudied and endangered biome characterized by high rates of endemism and accelerating human impacts.[44,45] Patagonia's forests are dominated by trees in the family Nothofagaceae (Southern Beech) that form ectomycorrhizal (ECM) associations with diverse fungi, including many truffle species.[46–49] These forests are widespread in southern South America, from central Chile to Tierra del Fuego (Figure 2A), and in some cases Nothofagaceae species constitute 100% of the trees.[50] Chucao tapaculos (*Scelorchilus rubecula*) and black-throated huet-huets (*Pteroptochos tarnii*) are endemic passerine birds that are locally common throughout Patagonia.[51] Both bird species are territorial ground-foragers that are sensitive to forest fragmentation.[52,53] *S. rubecula* have territories of circa (ca.) 1 hectare, whereas *P. tarnii* have territories of ca. 4 hectares,[54] although juveniles often move much further when seeking new territories.[55,56] Both bird species are widely distributed in Patagonia from sea level at the Pacific Coast up to 1,500 m above sea level in the Andes, ranging from 34°S to 48°S (ca. 1,500 km) in *S. rubecula*[51,57] and from 37°S to 50°S (ca. 1,500 km) in *P. tarnii* (Figure 2A) [51,58]. We have observed both *S. rubecula* and *P. tarnii* foraging on the ground at forest sites with high densities of truffles and other mycorrhizal fungi. Although relatively little is known about these birds' diets, available data indicate that they primarily consume invertebrates, berries, and seeds.[59–61] Here we describe the symbiotic mycophagy of *S. rubecula* and *P. tarnii* and a diverse but cryptic community of mycorrhizal fungi in Patagonian forests using a combination of metagenomics and epifluorescence microscopy. We also discuss the importance of bird mycophagy for the dispersal of mycorrhizal fungi, including both arbuscular mycorrhizal (AM) and ECM fungi as well as truffles.

RESULTS

Sampling and sequencing of fungi from bird fecal samples and soil

We documented the fungi consumed by *S. rubecula* and *P. tarnii* by analyzing their feces using microscopy and molecular methods. We collected 169 fecal samples in the field over three expeditions (May 2018, April–May 2019, and October 2019). We successfully amplified fungal ITS1 sequences from 141 samples. The bird species identity was visually confirmed for 33 *S. rubecula* and 13 *P. tarnii* feces. In cases where visual confirmation was impossible, we used bird-specific PCR and DNA sequencing to identify an additional 31 *S. rubecula* and 31 *P. tarnii* samples (Data S1A). Samples yielding sequences of non-target birds (n = 14) or samples where the bird species could not be confirmed (n = 19) were excluded from the analyses. Samples with low quality sequences or possible lab contaminants were also excluded (n = 6).

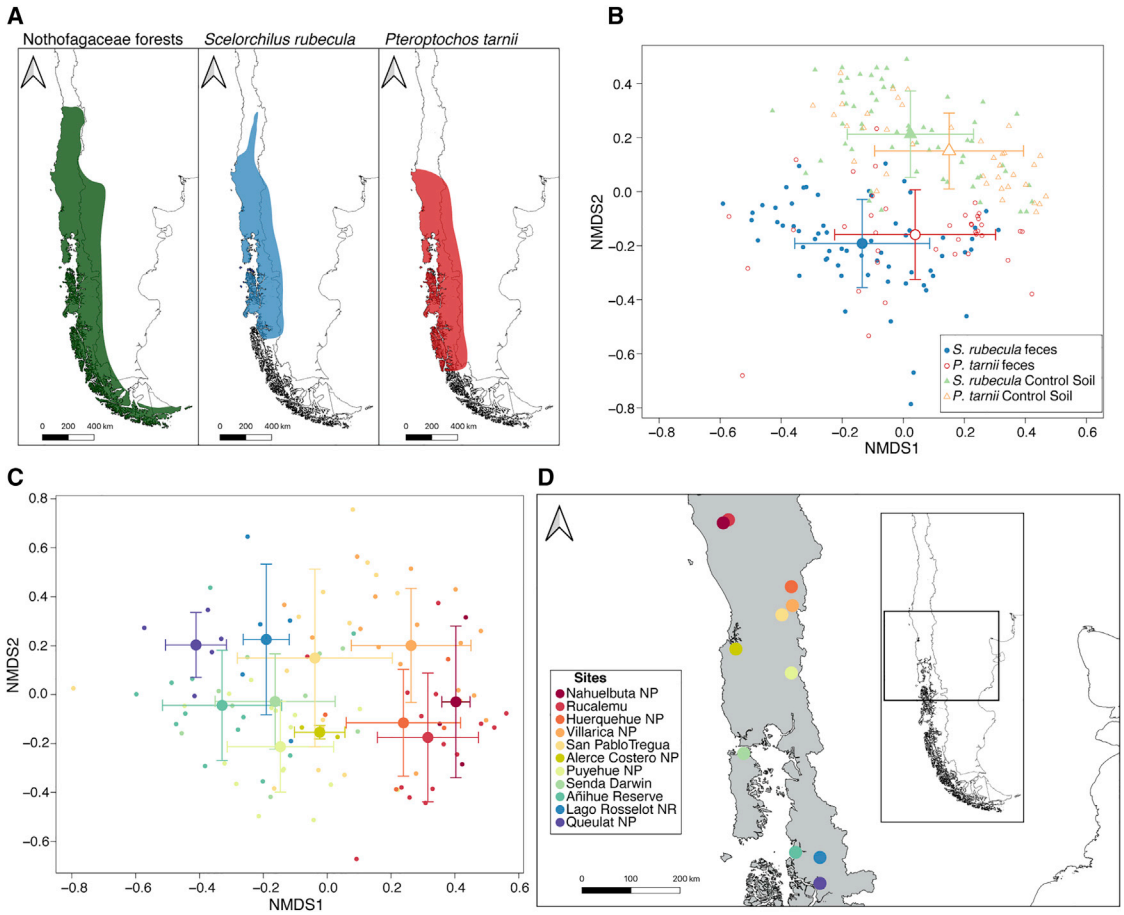

Figure 2. Geographic distribution of Nothofagaceae forests, bird species, and sampling sites and non-metric multi-dimensional scaling (NMDS) ordinations of the fungal communities from different sample types and locations

(A) Distribution of Nothofagaceae forests and of the two bird species, *Scelorchilus rubecula* and *Pteroptochos tarnii*. Distribution of Nothofagaceae forests estimated according to Moreira-Muñoz.[45] Distribution of *S. rubecula* and *P. tarnii* according to Jaramillo[46] and IUCN.[52,53]

(B) NMDS of fungal communities from feces of *Scelorchilus rubecula* (blue) and *Pteroptochos tarnii* (red) and their corresponding control soil samples using a modified Raup-Crick dissimilarity metric (Stress = 0.1512932). For NMDS using Bray-Curtis dissimilarity metric, refer to Figure S1A.

(C) NMDS of fungal communities from feces of both bird species across the 11 sampling sites, excluding OTUs present in soils from the same sampling points using a modified Raup-Crick dissimilarity metric (Stress = 0.1722587). For NMDS using Bray-Curtis dissimilarity metric, refer to Figure S1B. Colors in the NMDS ordination correspond to the locations shown in (D).

(D) Color-coded map of the study area in southern Chile.

We proceeded with fungal-specific PCR, metagenomic sequencing, and analysis from 102 samples (63 from *S. rubecula* and 39 *P. tarnii*). We also used similar fungal-specific molecular methods to sequence and analyze 97 soil samples that were collected directly adjacent to fecal samples. These served as controls to ensure that the fungal communities in fecal samples were not contaminated by nearby soil (60 soil samples from nearby *S. rubecula* feces and 37 soil samples from nearby *P. tarnii* feces). All fungal sequences that were present in both a fecal sample and the corresponding soil sample were considered possible contaminants and were removed from the analysis (see below). Overall, we obtained 10,580,643 ITS1 reads from fecal and soil samples after all bioinformatic processing, including 10,189–121,641 reads per fecal sample and 17,791–170,075 reads per soil sample. 10,539,209 (99.6%) reads were identified as fungal; all non-fungal sequences (0.4%) were excluded. All successfully sequenced samples contained fungal sequences. Fungal sequences were clustered into 10,081 operational taxonomic units (OTUs), from which 7,163 OTUs were present only in a fecal sample but not the corresponding soil. The number of OTUs varied from 7 to 806 OTUs per fecal sample. From the fungal OTUs present only in the fecal samples, we assigned 331 OTUs to symbiotic ECM fungi (4.6%), 69 OTUs (0.7%) to AM fungi, and 1,756 OTUs (24.5%) to nonmycorrhizal fungi guilds (e.g., animal pathogens, plant pathogens, endophytes, or saprobes). Trophic mode remained unknown for 5,007 OTUs (69.9%). After excluding sequences from soil and possible contaminants, we obtained 4,015,432 reads from which 733,198 (18.3%) were assigned to ECM fungi, 18,337 (0.5%) were assigned to AM fungi, 1,587,579 (39.5%) were assigned to nonmycorrhizal guilds, and 1,676,318 (41.7%) were assigned to fungi of unknown trophic modes.

Fungal community structure in bird fecal samples and soil

Our results show that feces from both *S. rubecula* (adonis; r^2 = 0.40, *pseudo-F* = 77.25, p < 0.0001) and *P. tarnii* (adonis; r^2 = 0.29, *pseudo-F* = 29.61, p < 0.0001) had significantly different fungal communities than the surrounding soils (Figures 2B and S1A). This difference indicates that birds likely impact soil fungal communities by bringing different fungi that are not already present in nearby soils. The multivariate dispersion between fecal and soil samples was only different in *S. rubecula* (betadisper; F = 23.87, p < 0.0001) but not in *P. tarnii* (betadisper; F = 0.6, p = 0.24). The fungal communities in *S. rubecula* feces were also distinct from the fungal communities in *P. tarnii* feces (adonis; r^2 = 0.12, *pseudo-F* = 13.66, p < 0.0001) with small differences in multivariate dispersion (betadisper; F = 8.31, p = 0.048). When OTUs from soils were excluded and fecal samples were analyzed alone, it became clear that fungal communities are strongly influenced by geography (adonis; r^2 = 0.60, *pseudo-F* = 13.86, p < 0.0001; betadisper; F = 12.30, p < 0.0001; Figures 2C and S1B). Study sites are distributed along a latitudinal gradient (Figure 2D), so the consumption of fungal species was likely determined by their availability (because fungi also follow a clear latitudinal trend[62]).

Detection of mycorrhizal fungi and truffles from bird fecal samples

We detected 331 ECM fungi OTUs from 31 different ECM lineages (Figures 3A and 3B; Data S1B) that are categorized as independent evolutionary origins of the ECM symbiosis.[63] ECM lineages are denoted with a slash followed by the lineage name in lower case, non-italicized font (e.g., /cortinarius). ECM fungi were present in 95.2% of feces from *S. rubecula* and 82.1% of feces from *P. tarnii*. The most frequently detected ECM lineages were /cortinarius (74.6% of *S. rubecula* and 43.6% of *P. tarnii* samples), /inocybe (54.0% of *S. rubecula* and 36.0% of *P. tarnii* samples), /tomentella-thelephora (39.7% of *S. rubecula* and 17.9% of *P. tarnii* samples), and /russula-lactarius (27.0% of *S. rubecula* and 17.9% of *P. tarnii* samples) (Figure 3A). The high frequency of /cortinarius and /inocybe OTUs in the feces of both birds is likely due to the high availability of these fungi in Patagonia.[48] Both ECM lineages have some species that form aboveground mushrooms and others that form belowground truffles.

Similarly, the most diverse ECM lineages (i.e., the highest number of OTUs) were /cortinarius, /inocybe, /tomentella-thelephora, and /russula-lactarius (Figure 3B). The high richness of /cortinarius, /inocybe, and /tomentella-thelephora is consistent with previous surveys of fruiting bodies, soil, and mycorrhizal roots of Nothofagaceae in Patagonia,[47–49] but our results show higher richness of /russula-lactarius and lower richness of /clavulina than previous studies. This may be due to the preferential consumption of certain fungi by the birds. Specimens of /russula-lactarius from Patagonia make large obvious fruiting bodies, including truffles, whereas /clavulina from Patagonia are rare and form minute coral-shaped mushrooms.[48,65] We hypothesize that the presence of spores from fungi with inconspicuous resupinate or coralloid fruiting bodies (such as species in the /tomentella-thelephora, /sebacina, or /clavulina lineages) is likely due to secondary consumption. Soil invertebrates are known to consume and disperse mycorrhizal fungi,[25,42] and both *S. rubecula* and *P. tarni* regularly consume soil invertebrates.[59–61] It seems likely that *S. rubecula* and *P. tarni* act as secondary dispersers by consuming invertebrates that previously consumed mycorrhizal fungi, but more work is needed to confirm this hypothesis. We noted wide variation in the proportion of ECM fungi sequences across samples, varying from 0% to nearly 100% in some samples (Figure 3C). Interestingly, >75% of all ECM fungi sequences belonged to /cortinarius (Figure 3D), although there were also abundant sequences from /inocybe, /russula-lactarius, and /descolea.

Among the 331 ECM OTUs, we identified 45 species of truffle fungi from the feces of both birds (Figure 4A). Of those, 20 were only found in *S. rubecula* feces, 8 were only found in *P. tarnii* feces, and 14 were found in feces of both birds (Figure 4A). Since truffles fruit underground and do not actively disperse their spores, the presence of truffle spores and DNA in the feces indicates that dispersal of these spores could not be accidental. We also visually confirmed the birds actively foraging for truffles and consuming them (Figure S2). Finding taxa such as *Cortinarius dombeyi*, *Descolea brunnea*, *Cystangium nothofagi*, and *Hysterangium* species in the feces was not surprising considering that these taxa are among the most common truffles in Patagonia.[48] It is worth noting that some of the truffles detected in bird feces (e.g., *Russula* sp. 1) represent undescribed species that match herbarium voucher specimens,[48] whereas others (e.g., *Inocybe anfractuosa*, *I. illariae*, and *Cortinarius quadrisporus*) were recently described.[66,67] Our findings indicate that the analysis of bird feces could be used to document hidden diversity of mycorrhizal fungi, an approach that has proven useful with mammal scats.[68]

In addition to the ECM fungi detailed above, we also detected 69 AM fungi OTUs from five different orders (Figure 5; Data S1C). AM fungi were present in 39.7% of *S. rubecula* and 48.7% of *P. tarnii* feces. Most AM fungi do not produce visible fruiting bodies,[69,70] but interestingly, two of the detected OTUs matched with truffle-forming AM species, suggesting birds may also consume truffles of AM fungi (Figures 5C and 5D). It is important to note that we likely underestimated the number of AM fungi species because our methods were not optimized to detect them.[71,72]

Due to our opportunistic sampling, we also detected DNA from mycorrhizal fungi and truffles in feces from non-target birds (Data S1D), including the chestnut-throated huet-huet (*Pteroptochos castaneus*), the Magellanic tapaculo (*Scytalopus magellanicus*), and the austral thrush (*Turdus falcklandii*). These samples were excluded from the analyses due to the small sample size from these bird species, but these chance observations indicate that the phenomenon of bird mycophagy in Patagonia is not restricted to *S. rubecula* and *P. tarnii*. The results also indicate that bird mycophagy may be far more common than previously thought and that many other birds may also act as important vectors for spore dispersal.

Microscopic examination of spores and spore viability assays from bird fecal samples

We used light and epifluorescence microscopy to directly examine representative fecal samples to estimate both the spore load and spore viability. We selected a subset of samples with

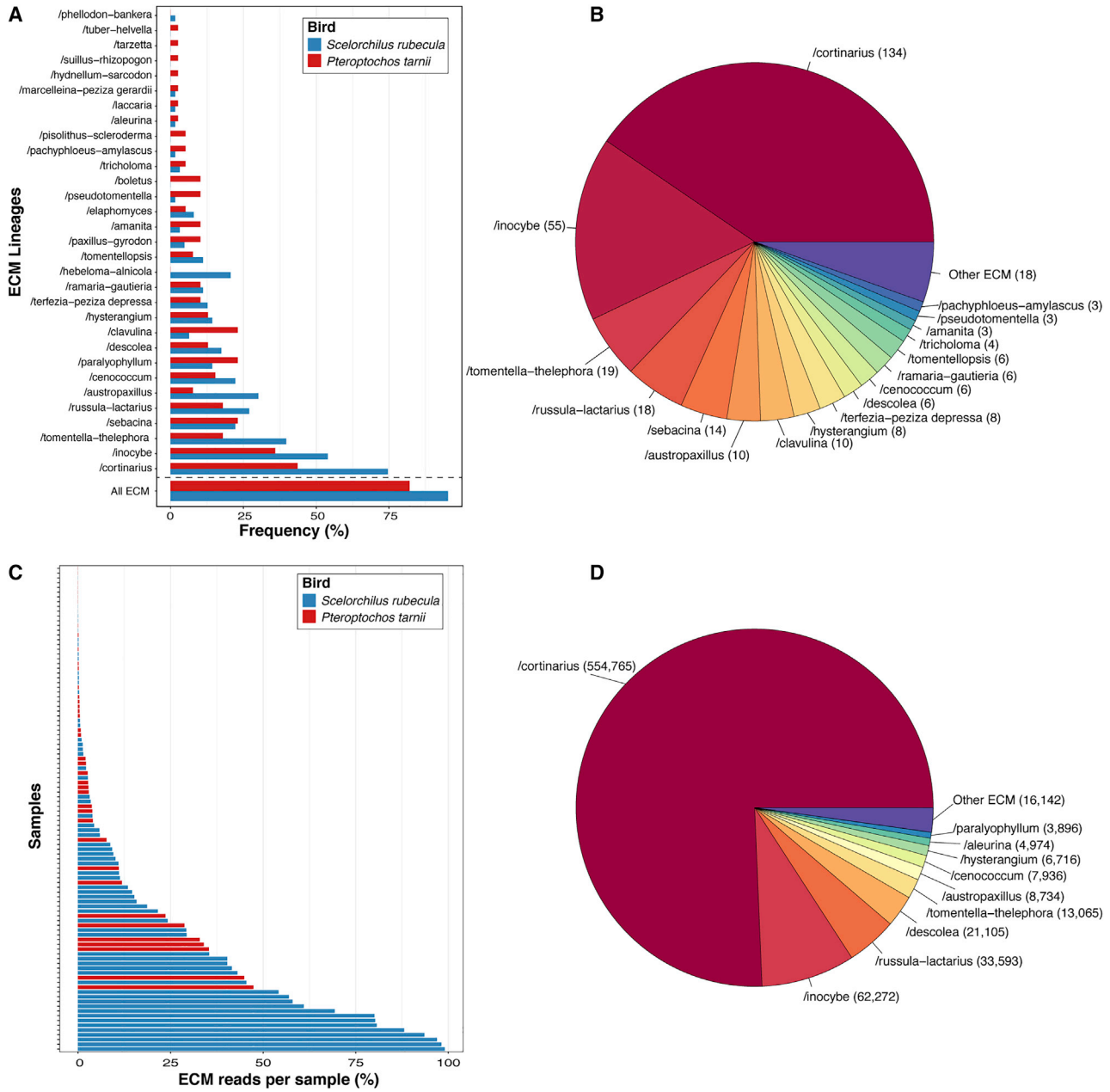

Figure 3. Mycorrhizal fungi detected in the fecal samples from *Scelorchilus rubecula* and *Pteroptochos tarnii*

Mycorrhizal fungi were detected by Illumina sequencing of ITS1 rDNA. OTUs classified as ECM fungi were grouped into ECM lineages according to previous studies.[58,64]

(A) Frequency at which different ECM lineages were detected in feces of both birds. Results indicate that >75% of the feces from both birds contained spores of at least one ECM fungus. On average, the feces of *Scelorchilus rubecula* contained more ECM fungi spores than those of *Pteroptochos tarnii*.

(B) Relative number of OTUs per ECM lineage. Consistent with previous surveys of soil, roots, and fruiting bodies of ECM fungi,[42–44] the /cortinarius, /inocybe, /russula-lactarius, and /tomentella-thelephora lineages were the most diverse.

(C) Proportion of sequence reads identified as ECM fungi from different fecal samples from *S. rubecula* (blue) and *P. tarnii* (red). Although the proportion of sequences assigned to ECM fungi was highly variable across samples, nearly 100% of the sequences were identified as ECM fungi from some fecal samples.

(D) Relative abundance of sequences assigned to different ECM lineages. Similar to previous studies of roots and soils in the region,[42,44] /cortinarius accounts for more than 75% of the ECM reads, followed by /inocybe, /russula-lactarius, and /descolea.

the highest proportion of ECM fungi based on metagenomics results to estimate spore load (n = 7; all of these samples were feces of *S. rubecula*). Spore load of selected samples varied from 1.5×10^3 to 2.5×10^4 spores/milligram of feces (Table S1). Light microscopy of fecal samples documented a wide array of different spore morphologies, including spores that were

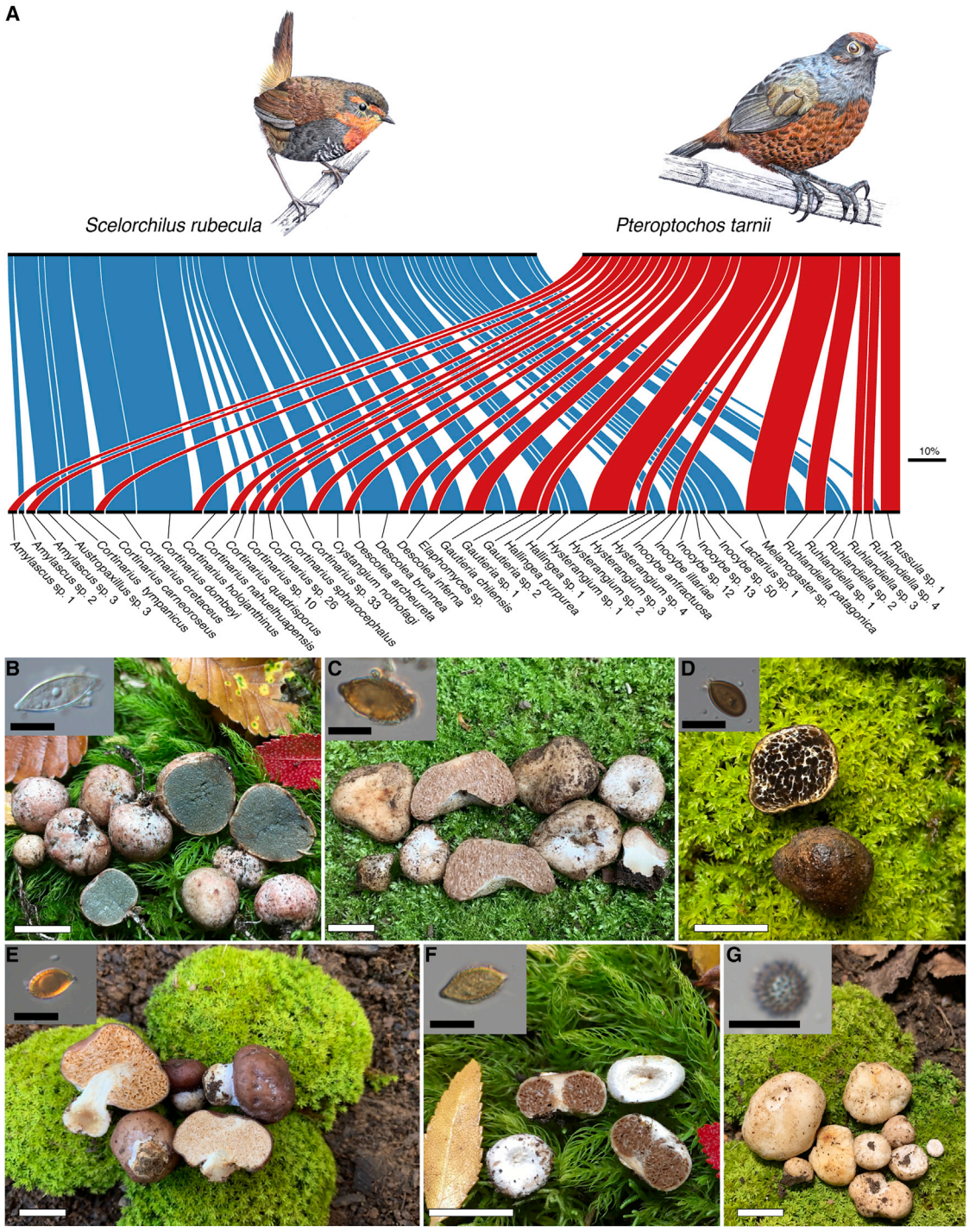

Figure 4. Truffles consumed by *Scelorchilus rubecula* and *Pteroptochos tarnii*

(A) Bipartite network of the two bird species (upper level) and the truffle OTUs identified from fecal samples of each bird species (lower level). Truffle OTUs labeled with the best available taxonomic name (Data S1B). Width of the lines are proportional to the relative frequency (e.g., number of fecal samples) in which each truffle OTU was detected in the feces. Sample size was n = 63 for *Scelorchilus rubecula* and n = 39 for *Pteroptochos tarnii.*

(B) Fruiting bodies and a spore of *Hysterangium* sp. found in fecal samples (white bar = 1 cm; black bar = 10 μm).

(C) Fruiting bodies and a spore of *Descolea brunnea* found in fecal samples (white bar = 1 cm; black bar = 10 μm).

(D) Fruiting body and a spore of *Melanogaster* sp. found in fecal samples (white bar = 1 cm; black bar = 10 μm).

(E) Fruiting bodies and a spore of *Cortinarius dombeyi* found in fecal samples (white bar = 1 cm; black bar = 10 μm).

(F) Fruiting bodies and a spore of *Descolea inferna* found in fecal samples (white bar = 1 cm; black bar = 10 μm).

(G) Fruiting bodies and a spore of *Cystangium nothofagi* found in fecal samples (white bar = 1 cm; black bar = 10 μm).

Illustrations of *Scelorchilus rubecula* and *Pteroptochos tarnii* by Pamela Ciudad Martin.

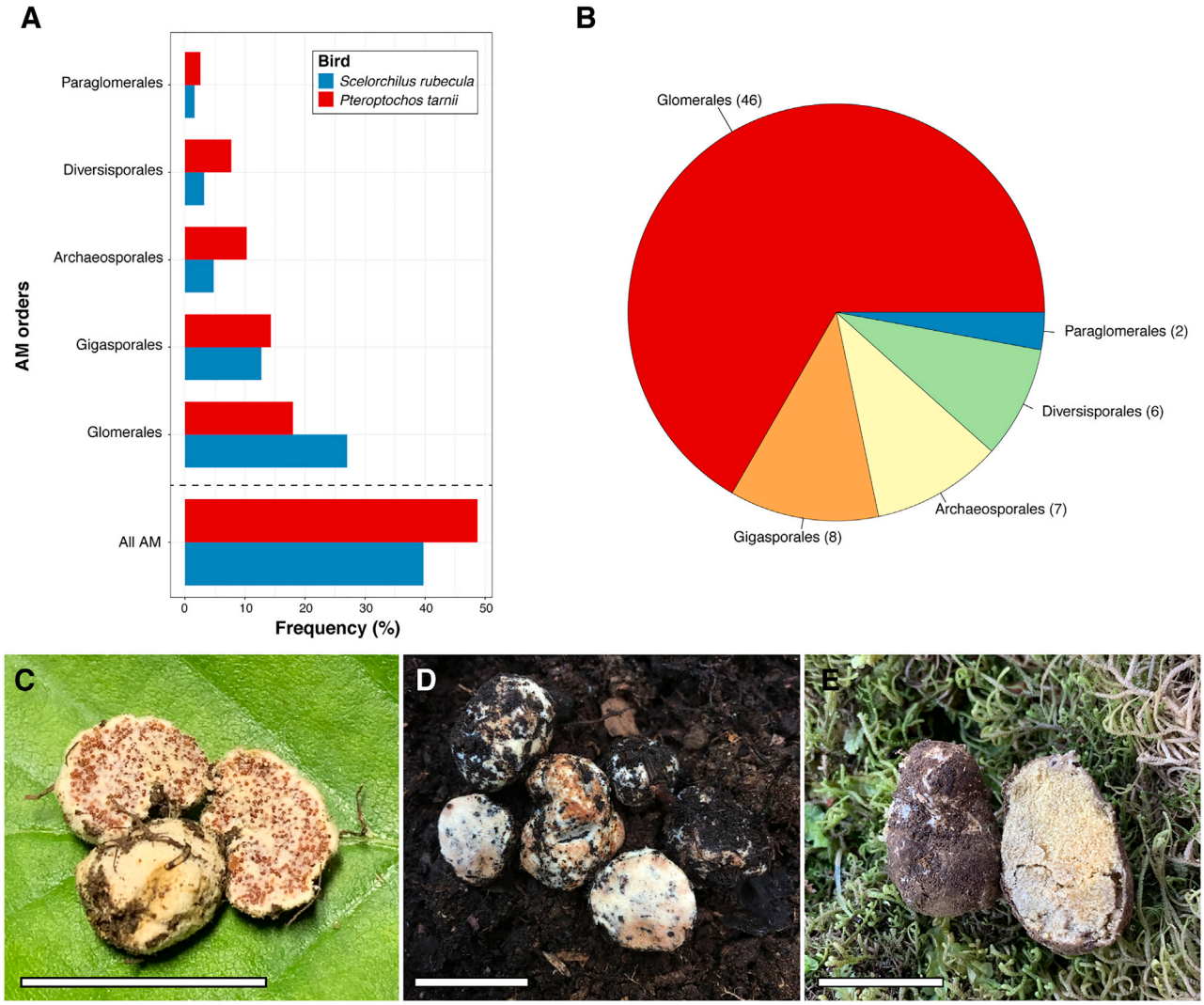

Figure 5. AM fungi detected in fecal samples from *Scelorchilus rubecula* and *Pteroptochos tarnii*

(A) Frequency of different AM fungi in the two bird species. Nearly 40% of feces from *Scelorchilus rubecula* contained AM fungi, whereas approximately 48% of feces from *Pteroptochos tarnii* contained AM fungi.

(B) Pie chart displaying the relative number of OTUs per order of AM fungi. The most diverse order was Glomerales.

(C–E) Truffles of AM fungi commonly found in Patagonian forests (bars = 1 cm). Specimen shown in (C) matched with OTUs detected in fecal samples.

morphologically identifiable as truffles and other ECM fungi.[73,74] These include truffle species of *Cortinarius*, *Descolea*, *Gautieria*, *Hysterangium*, *Melanogaster*, and others (Figures 4B–4G). In samples that were examined microscopically (n = 17 for *S. rubecula*; n = 10 for *P. tarnii*), we were able to find spores of morphologically identifiable taxa that were expected based on the metagenomics results. To examine the viability of the spores, we used propidium iodide as a stain. This is a DNA-intercalating fluorochrome that, due to its size, can only stain spores with damaged walls, but not spores with intact walls.[75] Epifluorescence microscopy determined that between 20% and 70% of the spores did not fluoresce when stained with propidium iodide, indicating that these spores are intact and likely remain viable (Figures 6 and S3). Our results show that many spores remain intact and likely viable after passage through bird guts, and they could likely germinate to form new individuals or act as sexual propagules. Furthermore, since samples were transported and stored in the lab for up to 1.5 years prior to microscopy, these results likely underestimate the true spore viability.

DISCUSSION

We document for the first time that two widespread and common Patagonian birds, *S. rubecula* and *P. tarnii,* regularly consume fungi and act as vectors for fungal spores, including a high diversity of mycorrhizal fungi (Figures 3 and 5) and truffle species (Figures 4 and S2). These interactions were common in mixed forests and Nothofagaceae-dominated forests over a wide geographic area spanning more than 700 km across the ranges of both bird species (Figure 2A). Based on previous observations

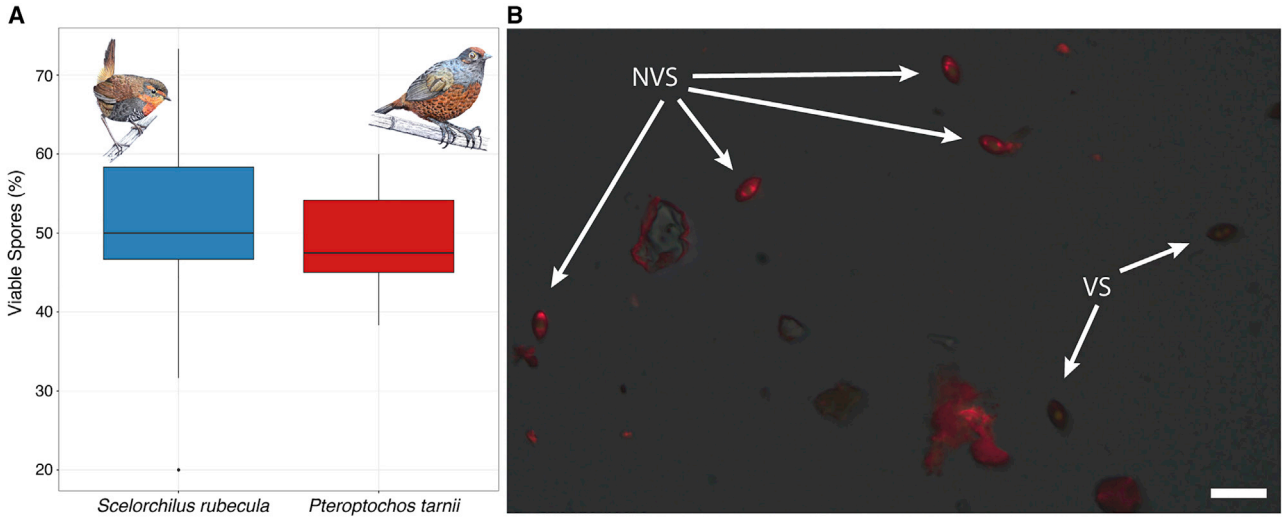

Figure 6. Viability tests of truffle spores found in fecal samples

Fecal samples were stained with propidium iodine and observed with brightfield and fluorescence microscopy. Propidium iodine is a DNA-intercalating fluorochrome that stains damaged cells.[70] Propidium iodine can therefore be used as a viability test where non-viable spores (NVS) will stain orangish red, whereas viable spores (VS) remain unstained. Spore load of fecal samples is shown in Table S1.

(A) Proportion of viable spores detected in fecal samples of *Scelorchilus rubecula* and *Pteroptochos tarnii*.

(B) Micrograph of *Cortinarius* spores found in fecal samples and stained with propidium iodine. NVS fluoresce orangish red, whereas VS do not fluoresce (scale bar: 20 μm).

at other sites further south of our sampling areas, we suspect that this interaction occurs across the entire 1,500 km range where these birds occur in Nothofagaceae forests. Our molecular results are supported by the spore viability assays we conducted; we determined that a high proportion of the fungal spores we visualized were likely viable and capable of germination (Figures 6 and S3). We also determined that at least some samples contained a spore load comparable to mycophagous mammals in previous studies.[68,74] Our results indicate that bird mycophagy may be much more common and important in some species and ecosystems than previously realized. Some birds may regularly consume fungi and also serve as critical vectors for fungal spores.

Some mammals regularly consume fungi and are well known as spore dispersal agents,[22,24,40,68,76] but only a few studies have previously detected bird mycophagy and suggested that birds are important fungal spore dispersers.[27,34,38] However, most studies of animal mycophagy (including almost all previous studies of birds) suffered from significant limitations; they often relied on a small number of samples, lacked molecular data that allow detailed identification of the fungi, and ignored the issue of spore viability. Thus far, there are only a few studies of animal mycophagy from Patagonia, and all of these studies have focused on mammals.[77–81]

ECM Nothofagaceae trees are dominant throughout Patagonia, and both *S. rubecula* and *P. tarnii* are common and disperse spores across a vast geographic area (Figures 2A and 2D). The widespread nature of our observations suggests that bird mycophagy is likely an important mechanism to disperse spores and facilitate the colonization of roots by mycorrhizal fungi. By dispersing viable ECM fungi spores, *S. rubecula* and *P. tarnii* could contribute to the establishment of ECM associations in new locations and facilitate movement of species between forest patches. Some of the ECM fungi consumed by *S. rubecula* and *P. tarnii*, such as *Cystangium nothofagi* and species of *Cortinarius*, *Descolea*, and *Inocybe*, are important ECM fungi that colonize the roots of seedlings and saplings of several species of Nothofagaceae.[82–84] The results of our viability tests show that nearly half of the fungal spores in fecal samples are able to survive intact after passage through the digestive systems of the birds. It is important to note that not all structurally intact spores are capable of germinating and that our estimates cannot assess the germination rate itself, only the structural integrity of the spores. Our results show that *S. rubecula* and *P. tarnii* also disperse AM fungi, which are associated with diverse plant species in Patagonian forests.[85] Although we did not look for seeds in this study, previous research has shown that birds can co-disperse seeds and AM propagules and thereby facilitate dispersal of both mycorrhizal partners over long distances.[86]

Previous diet studies considered both *S. rubecula* and *P. tarnii* as insectivorous, frugivorous, and granivorous.[59–61] During hundreds of hours of nest monitoring, De Santo et al.[87] observed *S. rubecula* and *P. tarnii* feeding their chicks European earthworms (*Lumbricus terrestis*) and native horsehair worms (Gordioidea) collected from forest litter during hours of ground foraging each day. While no publications reported fungi consumption, probably due to methodological constraints such as low-magnification microscopy that does not allow for the detection of fungal spores,[27,60] *S. rubecula* individuals consumed mushrooms and truffles growing in their territories when available (data not shown). Both bird species also vigorously scrape through litter to reach the soil when foraging, and we have directly observed them uncovering truffles (data not shown; Figure S2). Considering the diverse diet of both bird species,

Figure 7. Visual cues in Patagonian truffles and indirect evidence of bird mycophagy

(A) White truffles of *Cystangium nothofagi* mixed with berries of *Gaultheria mucronata* (above) and purple truffles of *Hallingea purpurea* mixed with berries of *Empetrum rubrum* (below) (bar = 1 cm). Some truffles strongly resemble native Patagonian berries in their shape, size, and color, suggesting that birds may use visual cues to find them. Photo by Camille Truong.

(B) Truffles of *Cystangium nothofagi* under white light (bar = 1 cm).

(C) Truffles of *Cystangium nothofagi* under UV light (395 nm) (bar = 1 cm). Some truffles reflect UV light, which might facilitate their detection by birds such as *Scelorchilus rubecula* and *Pteroptochos tarnii*.

(D and E) Specimens of *Cortinarius* species with visible peck marks and missing pieces of spore mass (bars = 1 cm); evidence of direct observation of bird mycophagy is available in Figure S2.

S. rubecula and *P. tarnii* are likely preferential or opportunistic mycophagists rather than obligate mycophagists.[22] Although previous studies suggested that obligate mycophagous animals were the main dispersers of fungal spores,[88,89] recent research suggests that generalist foragers that consume fungi may be important fungal dispersers, particularly when these animals are abundant or widespread.[74] Considering that both *S. rubecula* and *P. tarnii* are common, widely distributed in Patagonian forests, and habitual ground-foragers, they likely have a strong influence on fungal communities in these forests. Furthermore, spore dispersal by generalist animals can have important ecological impacts, such as facilitating succession or promoting exotic invasions. For example, Ashkannejhad and Horton[18] showed that the fecal pellets of herbivorous deer (*Odocoileus hemionus*) contained sufficient spore loads from mushrooms (*Suillus*) and truffles (*Rhizopogon*) to inoculate pine seedlings with ECM fungi. Similarly, exotic animals such as boars (*Sus scrofa*), red deer (*Cervus elaphus*), and fallow deer (*Dama dama*) consume and disperse exotic ECM fungi, which subsequently promote pine invasions in Patagonia.[80] Although these animals have a larger fecal spore load than *S. rubecula* and *P. tarnii*, the high abundance and wide range of both birds ensures that they impact fungal communities of Patagonia, particularly since only one or two spores are needed to start a new fungal individual.[90]

There is general agreement that truffles (and also some other fungi) make themselves conspicuous by emitting strong odors and that this increases detection by mycophagous mammals with highly developed olfaction.[22,91,92] However, olfactory detection is much less important in avian foraging than eyesight,[93,94] especially in omnivorous birds and those in the order Passeriformes (perching birds), which includes *S. rubecula* and *P. tarnii*.[95,96] This suggests that *S. rubecula* and *P. tarnii* probably rely at least partially on visual cues to find the truffles and other fungi that they eat. While some truffles in the Nothofagaceae forest emit odors, numerous species only produce weak or indetectable odors but, at the same time, exhibit a wide variety of shapes, colors, and sizes. We noted that some truffles consumed by *S. rubecula* and *P. tarnii* strongly resemble native Patagonian berries and seeds in their shape, size, color, and

location on the forest floor (Figure 7A). We also found that some truffles, such as the common species *Cystangium nothofagi*, reflect UV light and might therefore be more easily detected by birds like *S. rubecula* and *P. tarnii*[97,98] (Figures 7B and 7C). Indeed, visual cues that attract birds are hypothesized to have facilitated the diversification and spread of truffles in New Zealand, where there are no native ground-dwelling mammals.[99] During fieldwork, we also regularly saw evidence of bird mycophagy, including direct visual observations of bird mycophagy of *C. nothofagi* by *S. rubecula* (Figure S2) as well as truffles and mushrooms with peck marks and missing pieces of spore mass (Figures 7D and 7E).

Our results show that bird mycophagy is common and widespread in Patagonian forests and that birds act as important dispersal agents of viable fungal spores. Since mycorrhizal fungi can shape plant populations and are dispersal-limited, our findings reveal the direct impact that birds have on community assembly in Patagonian fungi. Further, as bird populations in Patagonia are increasingly threatened by forest fragmentation,[45] acknowledging the important role that birds play in the dispersal of mycorrhizal fungi is crucial to better understand the potential impact of fragmentation on birds, fungi, and plant communities. Finally, the fact that this widespread and common interaction in Patagonia had been previously unnoticed suggests that birds could also be important mycophagists and dispersers of fungal spores in other systems.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2021.10.024.

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AUTHOR CONTRIBUTIONS

M.V.C., M.A.J., and M.E.S. conceived and designed the study and obtained funding for the project. M.V.C. and M.E.S. performed sampling in the field. I.A.D. provided help during fieldwork and obtained necessary permits. I.A.D. and K.E.S. provided their expertise on the ecology of South American birds. M.A.J. and M.V.C. designed molecular protocols. M.V.C. conducted molecular work and microscopy. A.C.W. designed the epifluorescence microscopy protocol and contributed microscopy resources. M.V.C. wrote the first draft of the article. M.E.S. contributed resources and supervised the project. All authors read and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. The author list of this paper includes contributors from the location where the research was conducted who participated in the data collection, design, analysis, and/or interpretation of the work.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Feces of *Scelorchilus rubecula*	This paper	N/A
Feces of *Pteroptochos tarnii*	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Propidium iodide 1.0 mg/mL	Invitrogen	Cat# P3566
Deposited data		
Illumina reads from fecal and soil samples	This paper	NCBI SRA: PRJNA718579
CO1 and NADH II sequences from birds	This paper	See Data S1A
Software and algorithms		
AMPtk v1.4.1	Palmer et al.[100]	https://amptk.readthedocs.io/en/latest/
FUNGuild	Nguyen et al.[101]	http://www.funguild.org
R version 3.6.3	R Core Team[102]	https://www.r-project.org

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Marcos V. Caiafa (mvcaiafa@gmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All metagenomic data have been deposited at NCBI SRA (Sequence Read Archive). Accession numbers are listed in the key resources table.
- This paper does not report original code
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Focal species

We studied fecal samples from individuals of *Scelorchilus rubecula* and *Pteroptochos tarnii.* All birds were trapped under the research permit #1817/2019 from the Chilean Agricultural and Livestock Service (SAG). The following protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida (protocol #201810239).

METHOD DETAILS

Fieldwork

Fecal samples were collected in 11 sites across Chilean Patagonia (Figure 2D) over three expeditions (May 2018, April-May 2019, and October 2019). Sites spanned 700 km along the distribution of *S. rubecula* and *P. tarnii*, which are widely distributed in Patagonia (Figure 2A). Fresh fecal samples were passively collected from the ground, using sterile techniques, often after direct observation of fecal deposition. In cases where feces were taken from the ground, soil from beneath the fecal sample was sampled as a control (e.g., to ensure that the detected fungi are from the fecal sample and not from surrounding soil). In cases where we could not directly observe fecal deposition by the target bird, the species-level identity of the bird was confirmed using DNA barcoding of the mitochondrial Cytochrome *c* oxidase subunit I (CO1) and NADH dehydrogenase subunit 3 (NADH III) regions using bird-specific primers.[103,104] When possible, we obtained samples by capturing birds using clap-net traps.[105] Birds were placed in clean cloth bags after trapping and held until they produced a fecal sample, which was then aseptically collected. In these cases, no soil control sample was

collected. Each fecal sample was homogenized using a sterile spatula and sub-sampled with half of the sample retained for microscopy and the other half for molecular work. Sub-samples used for microscopy were dried with silica gel and kept at 4 C whereas samples used for molecular work were stored in molecular grade 95% ethanol until DNA extraction.

DNA extraction and amplification

We dried the ethanol from fecal samples and extracted total genomic DNA using the QIAGEN DNeasy Power Soil kit according to the manufacturer's instructions (QIAGEN, Germantown, MD, USA). DNA from soil samples was extracted using the same kit. To identity the fungal components of the diets of *S. rubecula* and *P. tarnii*, we used high-throughput amplicon sequencing (HTAS) to perform our metagenomic analysis, using previously optimized protocols.[100] We amplified the nuclear rDNA internal transcribed spacer region 1 (ITS1) using the primers ITS1F[106] and ITS2[107] modified with Illumina Nextera v2 adapters (Illumina, San Diego, CA, USA). PCR conditions were 94°C (1 min) followed by 30 cycles at 94°C (30 s), 52°C (30 s), 68°C (30 s) and a final extension at 68°C (7 min), using 5–10 ng of DNA, DNA-free water, 5X Green GoTaq buffer (Promega, Madison, WI, USA), 10 mM of each primer, 20 mg/mL BSA (New England BioLabs, Ipswich, MA, USA), 10 mM dNTPs (Promega, Madison, WI, USA) and 5 units/μl GoTaq® Polymerase (Promega, Madison, WI, USA). Positive and negative controls were used during extraction, PCR, and sequencing. A single-copy mock community consisting of single-copy non-biological (synthetic) ITS sequences combined in equimolar amounts (SynMock[100]) was also used as a positive sequencing and bioinformatics control. PCR products were stained with SYBR Green I (Lonza Bioscience, Rockland, ME, USA) and run in a 1.5% agarose gel. Amplicons were indexed using a dual-indexing approach with an eight-cycle PCR reaction using indices from the Illumina Nextera v2 Kit (Illumina, San Diego, CA, USA). Indexed samples were then cleaned and size-selected at $\geq$ 300 bp with Zymo Select-A-Size Clean & concentrator kits (Zymo Research, Irvine, CA, USA). Resultant products were then quantified using an Invitrogen Qubit 4.0 Fluorometer and Qubit 1X dsDNA HS Assay kit. Samples were then equilibrated at 5000 pM and combined. Libraries were sequenced on three separate Illumina MiSeq runs with v3 2 × 300 bp sequencing kits. Sequencing was performed at the University of California Riverside Institute for Integrative Genome Biology and the Interdisciplinary Center for Biotechnology Research at the University of Florida.

Bioinformatics

Illumina MiSeq sequencing data were processed using AMPtk v1.4.1.[100] Sequences were de-multiplexed using the unique index combinations and the forward and reverse primers were stripped using VSEARCH v2.14.1.[108] Sequences < 125 bp were discarded and reads > 300 bp were trimmed to 300 bp. Samples with fewer than 5000 reads were excluded and reads from each of the three Illumina runs were pre-processed separately and then concatenated before clustering. Sequences were quality-filtered with expected errors less than one[64] and clustered at 97% similarity into operational taxonomic units (OTUs) using UPARSE.[109] The 97% cutoff is commonly used to approximate fungal species.[110] After clustering, we used SynMock[100] to account for observed rates of index bleed using the filtering module of AMPtk. We assigned OTU-level taxonomy using the taxonomic algorithm in AMPtk and performing BLAST searches via the National Center of Biotechnology Information (NCBI). Due to the scarcity of Patagonian sequences, we also performed BLAST searches using a local database of Patagonian fungi at the University of Florida. Finally, we identified the ECM and AM fungal OTUs using FUNGuild,[101] a python-based tool that allows the user to taxonomically parse fungal OTUs by their ecological guilds. If FUNGuild failed to assign a guild due to taxonomic uncertainty, we considered these OTUs as ECM when the closest BLAST hit matched an ECM species hypothesis with > 90% similarity and > 90% coverage in the curated fungal sequence database UNITE.[111] We then grouped ECM OTUs into ECM lineages based on Tedersoo et al.[63,112] This annotation is consistent with common usage and is more conservative and straightforward than using fungal genus names.[63,111,112] We also used FUNGuild to assign a morphology (e.g., mushroom, secotioid, truffle, etc.)[113,114] to each OTU (Figure 1). When FUNGuild failed to assign a definitive morphology, we assigned the morphology based on known morphology of the closest BLAST hit with > 98% similarity. We used a conservative approach in order to ensure that sequences obtained from fecal samples were not environmental contaminants. We paired fecal and soil samples and then excluded all OTUs that were present in both feces and soil from the same sampling point.

Data analyses

All analyses were performed using R version 3.6.3.[102] To visualize the fungal communities from different sample types and locations in ordination space, we performed nonparametric multidimensional scaling (NMDS) using the Vegan package[115] and the metaMDS function with the modified Raup-Crick dissimilarity metric.[116] This metric has proven to be appropriate for zero-weighted datasets. To test whether the sample type (fecal sample or soil), bird identity (*S. rubecula* or *P. tarnii*) or geographical location had an effect on the fungal community, we performed a nonparametric permutational multivariate ANOVA (PERMANOVA)[117] using the adonis function from the vegan package.[115] We tested the differences in multivariate dispersion between different sample types[118] using the betadisper function in the vegan package.[115] Multivariate dispersion indicates the homogeneity of the dispersion in each of the tested groups. Therefore, significant values in the PERMANOVA, but not significant values in the multivariate dispersion, suggests that observed variation is due to differences in the community composition but not the dispersion of the groups.

Microscopy

To visualize fungal spores, ~0.1 g of the dried fecal sub-sample was re-hydrated in 1 mL of DI water and shaken for two h on a vortex. The suspension was then washed through a 48 μm sieve with DI water. Spores were observed and photographed with a Zeiss Axio

Imager.A2 microscope (Carl Zeiss, Oberkochen, Germany) equipped with differential interference contrast (DIC) using a Axiocam 305 camera and ZEN 3.1 software. We identified fungal spores from the fecal samples using available literature.[73,119] Note that many AM fungi have spores larger than our 48 μm sieve and where therefore not likely to be visualized using our methods.[120]

To determine spore viability, we used propidium iodide as a vital stain. Propidium iodide is a standard reagent used for assessing cell viability and is effective to differentiate viable from nonviable spores in the truffle *Rhizopogon roseolus*.[75] Epifluorescence microscopy was conducted using an Olympus BX41 microscope (Olympus, Tokyo, Japan) equipped with a filter system Semrock CY3-4040B (Semrock, Rochester, NY, USA) comprising an excitation filter of 507–549 nm, a dichroic mirror of 568 nm, and an emission filter of 569–620 nm. Photographs were taken using an Olympus DP74 camera and the Olympus cellSens Standard 3.1 imaging software. A stock solution of propidium iodide 1 mg/mL (Invitrogen, Eugene, OR, USA) was used. We prepared a 1:1 mix of sample and stain (10 μL) and incubated in the darkness for at least one minute. Propidium iodide can only penetrate spores with damaged cell walls but not spores with intact walls. We therefore considered all spores that did not fluoresce when stained with propidium iodide as viable (See Figure S3). Although we generally did not quantify spore load, we selected and quantified the spore load of seven fecal samples from *S. rubecula* with a high proportion of ECM fungi based on metagenomics results. Microscope slides of representative fecal samples are available in the fungal collection at the Florida Museum of Natural History at the University of Florida (FLAS).

QUANTIFICATION AND STATISTICAL ANALYSIS

To test the effect of sample type (fecal sample or soil), bird identity (*S. rubecula* or *P. tarnii*) or geographical location on the fungal communities, we performed a nonparametric permutational multivariate ANOVA (PERMANOVA)[117] using the adonis function from the vegan package.[115] We tested the differences in multivariate dispersion between different sample types[118] using the betadisper function in the vegan package.[115]