Universidad Pública de Navarra
Nafarroako Unibertsitate Publikoa

IS-FOOD
Research Institute on
Innovation & Sustainable
Development in Food Chain

Tesis doctoral

La expansión de *Brachypodium rupestre* en el Pirineo occidental: Hongos endófitos y pérdida de servicios ecosistémicos

The expansion of Brachypodium rupestre in western Pyrenees: Fungal endophytes and loss of ecosystem services

María Durán Lázaro

Memoria presentada para optar al grado de
Doctora en Agrobiología Ambiental con Mención Internacional

Pamplona, 2021

https://doi.org/10.48035/Tesis/2454/42921

Dña. Rosa Maria Canals Tresserras, Titular de Universidad en el Departamento de Agronomía, Biotecnología y Alimentación, y miembro del Grupo de Investigación en Ecología y Medio Ambiente de la Universidad Pública de Navarra,

Dña. Leticia San Emeterio Garciandía, Investigadora Doctora en el Instituto de Innovación y Sostenibilidad en la Cadena Agroalimentaria (IS-FOOD)

HACEN CONSTAR:

Que el trabajo descrito en la presente memoria, titulado: "La expansión de *Brachypodium rupestre* en el Pirineo occidental: Hongos endófitos y pérdida de servicios ecosistémicos" que presenta Dña. **María Durán Lázaro** para optar al título de Doctora con mención de "Doctora Internacional", ha sido desarrollado bajo su dirección en el Área de Producción Vegetal del Departamento de Agronomía, Biotecnología y Alimentación de la Universidad Pública de Navarra y reúne todos los requisitos necesarios para su defensa.

Pamplona - Iruña, 2021

CANALS TRESSERRAS ROSA MARIA - 37290821R
Firmado digitalmente por CANALS TRESSERRAS ROSA MARIA - 37290821R
Fecha: 2021.11.22 14:10:16 +01'00'

SAN EMETERIO GARCIANDIA LETICIA - 33446156Q
Firmado digitalmente por SAN EMETERIO GARCIANDIA LETICIA - 33446156Q
Fecha: 2021.11.22 10:46:59 +01'00'

Fdo. Rosa Maria Canals Tresserras

Fdo. Leticia San Emeterio Garciandía

Los resultados obtenidos en esta tesis han sido presentados en las siguientes publicaciones y congresos:

Durán, M., San Emeterio, L., Canals, R.M., 2021. Comparison of culturing and metabarcoding methods to describe the fungal endophytic assemblage of *Brachypodium rupestre* growing in a range of anthropized disturbance regimes. Biology 10, 1246. doi: 10.3390/biology10121246

Durán, M., San Emeterio, L., Múgica, L., Zabalgogeazcoa, I., Vázquez de Aldana, B.R., Canals, R.M., 2021. Disruption of traditional grazing and fire regimes shape the fungal endophyte assemblages of the tall-grass *Brachypodium rupestre.* Frontiers in Microbiology 12:679729. doi:10.3389/fmicb.2021.679729

Durán, M., Canals, R.M., Sáez, J.L, Ferrer, V., Lera-López, F., 2020. Disruption of traditional land use regimes causes an economic loss of provisioning services in high-mountain grasslands. Ecosystem services 46 101200. doi:10.1016/j.ecoser.2020.101200

Durán, M., Canals, R.M., San Emeterio, L., Múgica, L., Ibarrola, A., 2021. Especies en expansión favorecidas por el Cambio Global y sus efectos en los servicios Ecosistémicos de aprovisionamiento. 8º Congreso Forestal Español, Lleida (España) 20-24 Septiembre 2021

Ibarrola, A., Múgica, L., San Emeterio, L., **Durán, M**., Canals, R.M., Saez, JL., Echeverría, L., Lizarza, J., Goñi, M., Gárriz, I., Sarriés, V., Cittadini, A., Insausti, K., 2021. Open2Preserve: Modelo de gestión sostenible para la preservación de espacios abiertos de montaña. 8º Congreso Forestal Español, Lleida (España) 20-24 Septiembre 2021

Durán, M., Canals, R.M., Múgica, L., Jiménez, JJ., San Emeterio, L., 2021. Invasive behaviour of a native tall-grass in mountain grasslands: Linking plant and soil spatial patterns. Eurosoil Congress 2021, Geneve (Switzerland) 23-27 August 2021

San Emeterio, L., Múgica, L., **Durán, M**., Ibarrola, A., Canals, R.M., 2021. Preserving soil function while using pyric herbivory to maintain high-valuable open landscapes: The Open2Preserve Project. Eurosoil Congress 2021, Geneve (Switzerland) 23-27 August 2021

Durán, M., Canals, R.M., 2019. Proyecto Open2preserve: Modelo de gestión sostenible para la preservación de los paisajes abiertos de montaña. 3rd International colloquium on climate change in mountain areas. Pyradapt, Jaca (Spain) 22-23 October 2019

Durán, M., San Emeterio, L., Múgica, L., Canals, R.M., 2019. Mycobiome diversity of *Brachypodium rupestre* from high and low diverse grasslands. 4th International *Brachypodium* Conference, Huesca (Spain) 25-28 June 2019.

Canals, R. M., **Durán, M.**, Múgica, L., San Emeterio, L., 2019. Formando futuras investigadoras: introduciendo la ciencia ecológica y ambiental al mundo escolar. Ecosistemas, 28(2), 116-119. doi:10.7818/ECOS.1794

Durán, M., Canals, R.M., 2018. Preliminary study on the patterns of *Epichloë* infection in some grasses of high mountain diverse and degraded grasslands. 10th International Symposium on Fungal Endophytes of grasses, Salamanca (Spain) 18-21 June 2018

Terrádez, J., Arauzo, I., Canals, R.M., **Durán, M**., Puig, J., 2018. Agropastoralismo de montaña. OPCC-CTP, El cambio climático en los Pirineos: impactos, vulnerabilidades y adaptación. ISBN: 978-84-09-06268-3

Durán, M., Canals, R.M., San Emeterio, L., Ferer, V., Múgica, L., 2017. Grassland ecosystems vulnerability to invasions under Global Change. 2nd International colloquium on climate change in mountain areas. Pyradapt, Biarritz (France) 7-8 November 2017

85

El compendio de artículos en los que María Durán Lázaro ha participado como integrante del grupo de ecología y medio ambiente de la UPNA han sido:

INVESTIGACIONES PARALELAS A LA REALIZACIÓN DE LA TESIS

Plant-herbivory feedbacks and selective allocation of a toxic metal are behind the stability of degraded covers dominated by *Brachypodium pinnatum* in acidic soils

Canals, R.M., San Emeterio, L., **Durán, M.** and Múgica, L.

Soil bacterial functional diversity mirrors the loss of plant diversity by the expansion of a native tall-grass in high mountain grasslands

Canals, R.M., Múgica, L., **Durán, M**. and San Emeterio, L.

Formando futuras investigadoras: Introduciendo la ciencia ecológica y ambiental al mundo escolar

Canals, R.M., **Durán, M**., Múgica, L. y San Emeterio, L.

Relating the spatial distribution of a tall-grass to fertility islands in a temperate mountain grassland.

San Emeterio, L., **Durán, M.**, Múgica, L. Jiménez, J.J. and Canals, R.M.

INVESTIGACIÓN DOCTORAL

Capítulo 1: Disruption of traditional land use regimes causes an economic loss of provisioning services in high-mountain grasslands

Durán, M., Canals, R.M., Ferrer, V., Sáez, J.L. and Lera-López, F.

Capítulo 2: Disruption of traditional grazing and fire regimes shape the fungal Endophyte Assemblages of the tall-grass *Brachypodium rupestre*

Durán, M., San Emeterio, L., Múgica, L., Zabalgogeazcoa, I., Vázquez de Aldana, B.R. and Canals, R.M.

Cápítulo 3: Comparison of culturing and metabarcoding methods to describe the fungal endophytic assemblage of *Brachypodium rupestre* growing in a range of anthropized disturbance regimes

Durán, M., San Emeterio, L. and Canals, R.M.

INVESTIGACIONES EN CURSO

Efecto de las quemas recurrentes en la **biología reproductiva** de *Brachypodium rupestre* en pastos pirenaicos

Diversidad genética de las poblaciones de *Brachypodium rupestre* en el valle de Aezkoa

Colaboración BIOFLORA:
Estudio genómico y taxonómico del grupo:
B. pinnatum* - *B. rupestre* - *B. genuense

María Durán Lázaro ha sido beneficiaria de una de las “Ayudas de nueva solicitud para la Formación de Personal Investigador en la Universidad Pública de Navarra” en la convocatoria del año 2017. También ha recibido soporte económico de las “Ayudas de movilidad de doctorado 2021” para la realización de una estancia internacional en la Universidad de Camerino (Italia).

Los proyectos que han dado soporte financiero a los estudios relacionados con esta tesis han sido:

- Interreg Sudoe Programme, European Regional Development Fund, Open2preserve Project (SOE2/P5/E0804).

- Libera 2017, SEO y Ecoembes - Apadrinamiento de espacios naturales. La degradación de los ecosistemas pascícolas de la Selva de Irati: Comportamiento invasivo de especies endémicas bajo condiciones de cambio global.

- Libera 2018, SEO y Ecoembes - Apadrinamiento de espacios naturales. La degradación de los ecosistemas pascícolas: ZEC Roncesvalles-Selva de Irati.

- Proyecto de investigación en centros escolares (Gobierno de Navarra, 2018). Formando futuras investigadoras: Introduciendo la ciencia ecológica y ambiental al mundo escolar.

AGRADECIMIENTOS

No existen suficientes palabras para agradecer a todas las personas que han dedicado un pequeño o grandísimo esfuerzo para que esta tesis saliera adelante. Con especial cariño profesional, pero también personal, a mi querido grupo de pastos de la UPNA. A mis directoras de tesis Rosa y Leticia, por su dedicación, apoyo y paciencia. Gracias por depositar vuestra confianza en mí y guiarme en la realización de este trabajo. A mi compañera Leire que tanta ayuda me ha prestado, con la que tantas horas he pasado y la que tanta paz siempre me ha transmitido. Al resto de compañeras del grupo, Amaia y Sadys; y a las compañeras con las que he compartido despacho y cafés en estos últimos años: Jana, Itziar, Amaia y Rasmi. Y con especial cariño hacia la incombustible italiana de Recanati, Aurora. Gracias a todas y cada una de ellas, por las que siento un gran aprecio y admiración.

Gracias a todos los componentes del grupo de ecología y medio ambiente de la UPNA (Juan, Bosco, Javier, Antonio) y al resto de personas de la universidad que en algún momento me han prestado su ayuda (Natxo, Julio, Isabel, Vicky, Beatriz, Olaia, Esther, Pedro, etc.). También a Vicente Ferrer, a los estudiantes con los que he compartido campo o laboratorio en algún momento y a la gente del valle de Aezkoa por su amabilidad e interés por nuestros trabajos.

Gracias a todo el equipo del IRNASA de Salamanca (César, Victoria, Ángel y Eric), en especial al trato profesional y personal recibido por Beatriz e Íñigo que tanto me han enseñado y ayudado en el apasionante y oculto mundo de los hongos endófitos. Un enorme agradecimiento también al equipo de BIOFLORA del campus de Huesca, a todos sus componentes (Mª Ángeles, Mafer, Luis, Antonio y Ernesto) y a la admirable Pilar Catalán que tanta ayuda e interés ha mostrado por nuestros estudios. Y con mención internacional, agradecer enormemente a todo el equipo italiano de Andrea Catorci el recibimiento y la acogida que me brindaron en Camerino, por enseñarme los preciosos pastos de los Apeninos y por hacerme sentir como en casa. Gracias al esfuerzo que hicieron Alessandro, Tiziana, Arianna, Fabio, Ricardo y Federico, mostrándome su trabajo.

Mi más sincera gratitud hacia todas las personas con las que compartí mis primeras vivencias científicas en el Idab (son tantas que no me atrevo a mencionarlas por si me olvido de alguien). Muy especialmente a todo el equipo de Javier Pozueta, grandes profesionales y personas con las que compartí muchos años de mi vida (Paco, Edurne, Abdel, las Maites, Goi, Samuel, Pablo, Curro, Adriana, etc.). De este centro de investigación, no sólo me llevo experiencia profesional, también me llevo grandes amigos para toda la vida. A mis chicas de la resistencia (Ángela y Kinia), porque no hay mejor palabra que describa lo valientes que fuimos.

No puedo olvidarme, en esta etapa de mi vida, de todas las personas que he ido conociendo desde que llegué a Navarra, desde las que compartieron conmigo escasos pero intensos momentos hasta la nueva familia que fuimos creando en Pamplona. A los co-fundadores de esa familia, Toni y Andrea, por ser como son, por todo lo compartido y por haberse aprendido el nombre científico del *Brachypodium pinnatum*.

Gracias a mis amigos valencianos por estar siempre ahí pese a la distancia que un día decidí poner de por medio, y pese a que ninguno sepa bien qué hago en Navarra. A mis malilleras Marta y Cris, a sus paterneros Juan y Engui, y a toda esa prole de chicas que viene detrás (Lara, Kendra y mi ahijadita Manuela).

A toda mi familia consanguínea y política, a la que tanto quiero pero tan pocas veces se lo digo. A la primera, por esa unión tan bonita que tenemos con las montañas del Pirineo, y a la segunda por no odiarme por llevarme tan lejos a su joyita, a ese compañero de viaje que tengo en la vida. Gracias a él (a mi Juan) por estar ahí siempre, en cualquier circunstancia y en cualquier lugar. Gracias a mi hermano y a mis padres por inculcarme inconscientemente este amor tan desmedido que siento por la naturaleza. Con especial recuerdo de mi padre al que tanto añoro.

"Que tus pies te lleven donde tu corazón te llame…

… y mis pies me llevaron a las montañas"

RESUMEN

Los ecosistemas pascícolas son uno de los biomas terrestres más extensos y diversos del planeta. El hombre los ha gestionado y moldeado con el principal objetivo de abastecerse de alimento, pero el resto de bienes que nos proporcionan (materiales, medicamentos, agua, etc.) y los procesos en los que están implicados (regulación del aire y el agua, biodiversidad animal y vegetal, valor paisajístico, etc.) se engloban en lo que actualmente se conoce como servicios ecosistémicos.

La distribución primigenia de los ecosistemas pascícolas ha dependido esencialmente del clima, pero también del régimen de perturbaciones dominante en cada momento de la historia del planeta. Desde tiempos prehistóricos, las grandes manadas de herbívoros salvajes junto a los regímenes naturales de incendios contribuyeron a la creación y el mantenimiento de estas comunidades vegetales. Gradualmente, el hombre fue adquiriendo el control de la gestión del territorio. Los herbívoros domesticados adquirieron el rol de las grandes manadas y los incendios naturales pasaron a ser quemas controladas por la mano del hombre enfocadas a frenar los procesos de matorralización y forestación.

Los ecosistemas pascícolas que quedaron enclavados en las zonas más altas de las montañas han dado lugar a comunidades florísticas de alta diversidad condicionadas por el clima y el estrecho y ancestral vínculo que han mantenido los humanos y la gestión de la ganadería. La población del valle de Aezkoa, situado en el Pirineo navarro, mantiene un tradicional sistema productivo basado en la ganadería extensiva. Los animales son trasladados durante el periodo estival a las zonas altas de pastos dónde obtienen un alimento natural, de excelente calidad y bajo coste. La gestión sostenible de estos pastos mantiene un paisaje en mosaico (conjunto de zonas arboladas, de matorral y herbáceas) de alto valor ambiental y paisajístico. Con el objetivo de protegerlo y conservarlo, en el año 2011, se declaró la zona ZEC Roncesvalles-Selva de Irati. La calidad de sus pastos y su posición fronteriza con el vecino valle francés del Cize ha favorecido un uso conjunto de los mismos a través de acuerdos ancestrales que perduran hasta nuestros días, en lo que se conocen como facerías.

Las transformaciones a gran escala promovidas por el cambio global también afectan a la socioeconomía de las áreas remotas de montaña. El despoblamiento de las zonas rurales, el envejecimiento de su población y la reducción o los cambios de gestión del sector primario se han visto reflejados en un drástico descenso del número de animales pastantes. La desaparición de esta perturbación consumidora de biomasa favorece la acumulación de biomasa leñosa y herbácea. A su vez, en algunas áreas, los ganaderos que quedan en activo tienden a incrementar el uso del fuego como herramienta suplementaria para eliminar esta acumulación de biomasa. Esta alteración de los regímenes tradicionales de fuego y pastoreo tiene un efecto directo en la composición florística de las cubiertas vegetales. Concretamente en el valle de Aezkoa se ha desencadenado una importante pérdida de biodiversidad florística ligada a procesos de matorralización dominados por la especie *Ulex gallii* y a la expansión de los lastonares, cubiertas herbáceas dominadas por la gramínea perenne *Brachypodium rupestre.*

B. rupestre, junto a *B. pinnatum* y *B. genuense*, son especies autóctonas de la región euroasiática que forman parte integrante de los pastos calcáreos. La alteración de las condiciones abióticas del medio en el que se desarrollan y, principalmente, el cese de las actividades ganaderas que limitan su desarrollo, ha favorecido su expansión a lo largo de todo el continente europeo. La expansión de este grupo de especies genera densas cubiertas herbáceas de escasa calidad pascícola, baja diversidad florística y alta persistencia temporal, en lo que se conoce como un estado estable alternativo (*alternative stable state*).

En los pastos de puerto del valle de Aezkoa coexisten cubiertas de baja diversidad florística

dominadas por *B. rupestre* y sometidas a regímenes de quemas recurrentes con presencia residual de ganado, junto con cubiertas de alta diversidad florística óptimamente pastadas y con baja periodicidad de quemas. Estos distintos estados son un escenario perfecto para evaluar la alteración de los sistemas biológicos debido a los cambios que acontecen en el actual contexto de cambio global.

En esta tesis se plantean dos líneas de investigación que han quedado recogidas en los tres trabajos científicos que la conforman:

Capítulo 1) La primera línea de investigación desarrolla un modo de cuantificar económicamente la pérdida del servicio ecosistémico de aprovisionamiento ligado a la expansión de *B. rupestre,* con el objetivo de sensibilizar a los sectores implicados y fomentar acciones medioambientales dirigidas a promover la conservación de los pastos de montaña, ecosistemas ambiental y económicamente valiosos. Para ello, se realizó una adaptación del método de valoración económica por sustitución y se llevaron a cabo inventarios florísticos en cubiertas de alta y baja diversidad florística. El registro de estos datos nos permitió generar unos valores pastorales transformables en cantidad de forraje disponible para los animales, tras un detallado cálculo de la superficie viable de ser pastada (200,05 ha tras excluir las zonas con grandes pendientes). Con censos de animales y con información detallada y exhaustiva del consumo de energía que tiene cada tipo de animal que pasta las estivas del valle (ovejas, vacas y caballos) se determinaron las raciones de alimento necesarias en una temporada de pastoreo estival (50007 raciones) y su coste monetario (21146€; 107€/ha). De este modo, se estimó el coste que supone la pérdida del valor de aprovisionamiento debido a la expansión de *B. rupestre* y que va ligado en este caso a la pérdida de riqueza y diversidad florística de las comunidades vegetales que lo constituyen.

Capítulo 2 y 3) La segunda línea de investigación engloba dos trabajos en los que se caracteriza la desconocida comunidad de hongos endófitos de individuos de *B. rupestre* desarrollándose en comunidades de alta y baja diversidad florística. El estudio de los microorganismos en su ambiente natural es una disciplina relativamente nueva, dado que su interés se ha centrado tradicionalmente en las enfermedades que afectan a los humanos, los animales o las plantas. Sin embargo, los grandes avances tecnológicos y científicos de las últimas décadas nos están permitiendo conocer su altísima diversidad y las complejas redes ecológicas en las que están involucrados. El conocimiento sobre los hongos endófitos, que crecen en el interior de casi todas las plantas sin desarrollar enfermedades, está demostrando numerosas ventajas adaptativas que estos pueden llegar a conferir a su huésped ante numerosas situaciones de estrés (salinidad, herbivorismo, patógenos, etc.).

En el primer trabajo se aplicó el método tradicional de cultivo para la identificación de hongos endófitos. Esta metodología consiste en depositar pequeños fragmentos de planta, previo proceso de esterilización de su superficie, sobre un medio de cultivo apropiado donde va creciendo el micelio de los hongos presentes en su interior. Este micelio es cuidadosamente aislado y clasificado en base a sus características morfológicas (color, textura, tasa de crecimiento, etc.). Una vez aislado el micelio de cada especie se procede a realizar la extracción de su ADN y la replicación de su región ITS completa (ITS1-5.8s-ITS2). Este material genético es enviado a una empresa especializada en la secuenciación de ADN. Tras la recepción del código genético, la secuencia se contrasta en bases de datos para su clasificación taxonómica (NCBI y UNITE). Los resultados más relevantes pusieron de manifiesto la enorme importancia de caracterizar las comunidades de los diferentes tejidos de la planta por separado, en el caso de *B. rupestre* se seleccionaron los tejidos de la vaina, el rizoma y la raíz. Cada uno de ellos arrojó comunidades endofíticas muy diferentes, principalmente entre el tejido aéreo (vaina) y los dos tejidos subterráneos (rizoma y raíz). Respecto a la alteración de la composición florística por el efecto del desacoplamiento de los regímenes de fuego y pastoreo, este primer trabajo no indicó diferencias significativas entre la riqueza y diversidad fúngica de las plantas recolectadas en ambas cubiertas. Sin embargo, sí se observó la alteración de la incidencia de algunas especies endófitas. Concretamente, *Omnidemptus graminis* en el tejido de la vaina y *Lachnum* sp. en el tejido de la raíz fueron significativamente más aisladas en las plantas recolectadas en las zonas de baja diversidad florística.

La caracterización endofítica de *B. rupestre* fue complementada con un segundo trabajo en el que se aplicó el método de *metabarcoding*. Esta novedosa técnica de secuenciación masiva analiza todo el ADN fúngico de una muestra, permitiendo la identificación de especies no cultivables. A pesar de su interés, es una técnica de alto coste económico, lo que influye en el tamaño muestral de los estudios que se realizan en la actualidad. Como en el primer trabajo, se recolectaron plantas de cubiertas de alta y baja diversidad florística. Además, de los mismos individuos que se enviaron al estudio de *metabarcoding*, se realizó el aislamiento de endófitos mediante el cultivo tradicional, con el objetivo de comparar los resultados de la caracterización con ambos métodos.

Ambas técnicas fueron capaces de detectar las tendencias de la riqueza y diversidad endofítica, en los tejidos (raíz > rizoma > vaina) y cubiertas analizadas (baja diversidad > alta diversidad florística) pero tan sólo el poder cuantitativo del *metabarcoding* permitió realizar una caracterización más completa. Este último análisis sí arrojó disimilitudes entre la comunidad endofítica del tejido de las raíces de ambos tipos de cubiertas. A pesar de las aparentes ventajas que parecen tener las técnicas de secuenciación masiva frente al cultivo tradicional, encontramos el gran escollo de una enorme y desconocida diversidad fúngica todavía sin clasificación, o clasificada a niveles taxonómicos altamente inclusivos. En este punto, la especie endófita más aislada del tejido aéreo, *Omnidemptus graminis*, no fue detectada por el *metabarcoding*, evidenciando las carencias aún existentes de este tipo de técnicas.

El conjunto de datos obtenidos por ambas metodologías nos permitió saber que la comunidad endofítica de *B. rupestre* en los pastos de Aezkoa está formado por unas pocas especies comunes y abundantes, y un numeroso grupo de especies raras o poco frecuentes. Los principales taxones que componen el micobioma de los tejidos subterráneos de *B. rupestre* pertenecen a las familias Hyaloscyphaceae, Mollisiaceae y Helotiaceae y los géneros más representativos fueron *Lachnum* sp., *Albotricha* sp., *Mollisia* sp., *Phialocephala* sp. y *Glarea* sp. La acertada caracterización de los diferentes tejidos por separado, nos permitió conocer las grandes diferencias existentes entre la escasa diversidad del tejido vegetal aéreo, la intermedia diversidad del tejido conector del rizoma y la alta diversidad que albergan las raíces. Algunas especies mostraron tendencia a ser más abundantes en plantas de una cubierta concreta. Este tipo de alteración puede estar evidenciando la variación de la diversidad florística ligada al desajuste de los regímenes de fuego y pastoreo, pudiendo existir perturbaciones que favorecen la incidencia de unas especies sobre otras. Los efectos de este incremento y el rol que pueden estar jugando en el proceso expansivo de *B. rupestre* necesitarán ser más profundamente estudiados en futuras investigaciones.

ABSTRACT

Grassland ecosystems are one of the most expanded and diverse terrestrial biomes on Earth. Humans have managed and shaped them with the main aim of providing food for domestic herbivores, but grasslands also provide other goods and are involved in many processes (air and water regulation, animal and plant diversity, etc.). Currently, these set of goods are called ecosystem services.

The primitive distribution of grassland ecosystems depended on the climate and on the dominant disturbances prevailing at each moment of the planet history. Since prehistory, large herds of wild herbivores and natural fire regimes contributed to the creation and maintenance of grasslands. Gradually, humans took control of landscape management and domesticated herbivores replaced wild herds and natural fires became human-controlled burns.

In high-mountain areas, natural grasslands are highly conditioned by the cold climate and by the ancestral management based on the use of pastures by domestic herbivores during the summer season. In the Aezkoa valley, located in the south-western Pyrenees, a traditional production system based on an extensive livestock farming is still occurring. Animals are moved to uplands during the summer period where they obtain natural, high-quality and low-cost food. Due to the location of the grassland surfaces, close to the French border, a shared grazing regime occurs with the neighbouring Cize valley based on an ancestral agreement called *facería*. This historical sustainable management maintains a mosaic landscape composed by forest, shrublands and natural grasslands with high environmental value. In order to preserve it, the zone was declared Special Area of Conservation (SAC Roncesvalles-Selva de Irati) in 2011.

The transformations related to the global change also affect the socioeconomy of the remote mountain areas. The depopulation and ageing of the rural zones and the changes occurring in the primary sector have led to a drastic decrease of the number of grazing animals. The decline of grazing favours the accumulation of woody and herbaceous biomass. In some situations, fuels build-ups encourage farmers and stakeholders to use controlled burnings at a high recurrence (every 1-2 years) to reduce fuel loads. The decoupling of traditional fires (bush-to-bush, high time lapses) and grazing regimes has a direct effect on the floristic composition of grassland communities. Specifically, in the Aezkoa valley, a significant loss of floristic diversity has been detected in relation to the shrub encroachment by *Ulex gallii* and the expansion of herbaceous covers dominated by the perennial tall-grass *Brachypodium rupestre*.

B. rupestre, *B. pinnatum* and *B. genuense* are native grasses in chalk grasslands of the Eurasian region. The decline of livestock grazing, among other factors cited in the literature, has favoured their spreading. This expansion generates grassland covers with low floristic diversity and low grazing quality, that are very stable in time, which is known as an alternative stable state. This type of degradative processes are found in some areas of the Aezkoa uplands, together with areas that maintain high floristic diversity under an optimal grazing management and a low frequency of burns. As a consequence, the area represents a perfect scenario for the study of the disruption of biological systems related to global change.

This thesis encompasses two research lines comprised in three scientific papers:

Chapter 1) First chapter quantifies economically the loss of the provisioning service related to the *B. rupestre* expansion, with the aim of raising awareness among stakeholders and promote environmental actions for grassland restauration. For this purpose, we adapted the substitution economic approach, that we based on field floristic surveys done in high-diversity and low-diversity grasslands. The data collected allowed the estimation of the pastoral values, that were related to energy values and eventually to animal feed rations (50007 rations). We estimated the available

surface for grazing (200.05 ha excluding steep slopes) and we compiled summer livestock censuses for determining the energy needs of each type of animal (sheep, cows and horses). Eventually, we estimated the economic cost of the loss of the provisioning service related to the *B. rupestre* expansion (21146€; 107€/ha).

Chapter 2 and 3) The second line of research encompasses two papers that characterize the fungal endophytic community of *B. rupestre* from plants developing in communities of high and low-floristic diversity. The study of microorganisms in their natural environment is a new discipline, considering that the research has usually focused on human, animal or plant diseases. The technological and scientific advances in the last decades have broadened our knowledge on the high diversity and the complex ecological networks in which they are involved. In particular, the study of fungal endophytes, which grow inside plants without developing diseases, is demonstrating the numerous adaptive advantages they confer to their host in different stressing situations (salinity, herbivory, pathogens, etc.).

In the first chapter, the traditional culturing method was applied for the identification of fungal endophytes. This methodology consists of plating small plant fragments, after surface sterilisation, on an appropriate culture medium where the mycelium grows from the inside. The mycelium is carefully isolated and classified according to its morphological characteristics (colour, texture, growth rate, etc.). From the isolated mycelium, its DNA is extracted and the complete ITS (IT1-5.8S-ITS2) region is replicated. The genetic material is sent to specialised enterprise for the DNA sequencing process. Then, sequences are contrasted in databases for their taxonomic classification (NCBI and UNITE). Data showed the importance of characterising the communities from the separately plant tissues (shoots, rhizomes and roots). Each of them harboured different endophytic communities, particularly between the aerial (shoot) and the underground tissues (rhizome and root). Regarding the disruption of the floristic composition due to the effect of the decoupling of fire and grazing regimes, this first work did not indicate significant differences between the richness and fungal diversity of the plants collected in both type of covers. However, alterations in the incidence of some endophyte species were observed. In particular, *Omnidemptus graminis* from the shoots and *Lachnum* sp. from the roots were significantly more isolated in plants collected in low-diversity grasslands, and was related to the disruption of the regime of disturbances and particularly, to the high burn recurrence occurring in these grasslands.

The endophytic characterisation of *B. rupestre* was complemented with a second study that aimed to compare the traditional culturing method with the novel metabarcoding techniques. The massive sequencing technique analyses all fungal DNA of a sample, including the identification of non-culturable species, however, the technique has a high economic cost, which influences the sample size. As in the first study, plants were collected from high and low-diversity grasslands. Both methods were able to detect trends in endophytic richness and diversity in the tissues (root > rhizome > shoot) and type of cover (low-diversity > high-diversity grasslands) but the metabarcoding technique allowed a higher identification of fungal species and detected significant differences in the endophytic community of the roots in both type of covers. Despite the advantages that massive sequencing techniques have on culturing methods, we find a great obstacle in the level of unknown and unclassified sequences, or classified at highly inclusive taxonomic levels. In addition, the most isolated endophyte from aerial tissues using the culturing method, *Omnidemptus graminis*, was not detected by metabarcoding, demonstrating the current shortcomings of the novel technique.

Data obtained by both methods allowed us to know that the endophytic community of *B. rupestre* in Aezkoa grasslands is composed by a few common and abundant species, and a large group of rare species. The main taxa harbouring the mycobiome of the underground tissues of *B. rupestre* belonged to the families Hyaloscyphaceae, Mollisiaceae and Helotiaceae and the most representative genera were *Lachnum* sp., *Albotricha* sp., *Mollisia* sp., *Phialocephala* sp. and *Glarea* sp. The accurate characterisation of the different tissues separately indicated the low diversity of the mycobiome of the aerial tissue, the intermediate diversity of the rhizome and the high diversity of the roots mycobiome.

CONTENIDOS

INTRODUCCIÓN GENERAL

1. Los ecosistemas pascícolas de montaña

El conjunto de ecosistemas pascícolas distribuidos por todo el mundo representa uno de los mayores biomas de la Tierra. Se estima que ocupan entre 41 y 56 millones de km^2, representan casi el 40% de la superficie terrestre (White y col., 2000; Sala y col., 2013) y son uno de los ecosistemas más diversos y productivos del mundo (Wilson y col., 2012). En Europa, concretamente los pastos permanentes (naturales o agrícolas, no sembrados durante diez o más años) ocupan alrededor 60 millones de hectáreas (van den Pol-van Dasselaar y col., 2019; Eurostat, 2020), y una gran parte de ellos se encuentran en zonas de montaña. Entre los pastos de montaña, los pastos de puerto son cubiertas herbáceas naturales que pueden ubicarse entre los pisos alpino y montano y que se caracterizan por una producción estacional, que se concentra en verano y es aprovechada de forma extensiva por ganado trashumante o trasterminante (Ferrer, 2016).

La distribución de los pastos en Europa en los últimos 2,5 millones de años ha venido marcada por la alternancia de periodos glaciales e interglaciales, por la influencia de la latitud y altitud en las diferentes regiones y por la actividad de los herbívoros. Durante las glaciaciones, la tundra esteparia dominaba Europa central y septentrional, mientras que los pastos xerotérmicos dominaban la cuenca mediterránea (Van Andel y Tzedakis, 1996; Pärtel y col., 2005). Tras la última gran glaciación, hace aproximadamente 12000-10000 años, las zonas boscosas fueron ampliándose a medida que las condiciones climáticas se volvían más favorables para el crecimiento vegetal (Galop y Catto, 2014). Sin embargo, numerosos enclaves permanecieron abiertos moldeados principalmente por el herbivorismo y por los regímenes naturales de incendios (Svenning, 2002; Canals, 2019). El conjunto de comunidades vegetales, muy diferentes entre sí, formó paisajes en mosaico (Remmert, 1991), compuestos por pastos, matorrales y masas arboladas que evolucionaban en función de la recurrencia e intensidad de las perturbaciones dominantes (Vera, 2000).

A medida que avanzaba el Holoceno, el hombre pasó a tener un papel más relevante en la creación del paisaje, y especialmente a partir del Neolítico, por la presión que comenzó a ejercer en el territorio. Las primeras sociedades de cazadores-recolectores evolucionaron hacia sociedades agrícolas-ganaderas ligadas a la domesticación de los herbívoros y al desarrollo de la agricultura (Hofmann, 1989; Canals, 2019). La distribución de los asentamientos humanos estuvo fuertemente influenciada por el clima de cada región y los recursos que cada zona era capaz de proporcionar (Kelly y col., 2013; Fernández-López de Pablo y col., 2019). Pese al frío clima, la población que quedó asentada en las zonas altas de montaña supo aprovechar los recursos de los que disponía. Los herbívoros domésticos fueron adquirieron las funciones de las grandes manadas de herbívoros salvajes y los incendios naturales pasaron a ser quemas controladas por la mano del hombre (Jones y Cushman, 2004; Savage, 2004). El fuego junto a la ganadería continúan siendo las principales perturbaciones que promueven el mantenimiento de los espacios abiertos en todo el mundo (Anderson y Hoffman, 2007; Leys y col., 2018).

La cordillera de los Pirineos, situada al norte de la península Ibérica, hace de frontera natural entre España y Francia. A pesar de la compleja orografía de los valles que la conforman y de su clima extremo, existen evidencias de actividad humana que se remontan a las primeras sociedades de cazadores-recolectores al inicio del Holoceno (8800-6500 a.C.), y a las sociedades agrícolas-ganaderas del Holoceno medio (5500-4500 a.C.) (Rius y col., 2012; Galop y Catto, 2014; Gassiot y col., 2014). En la actualidad, el modo de vida de sus habitantes es una representación del ancestral vínculo que han tenido los humanos, los herbívoros domesticados y las zonas de pasto como fuente natural de alimento para estos (García-Ruiz y Lasanta, 2018).

Además del importante suministro de alimento que proporcionan, los ecosistemas pascícolas contribuyen a la regulación de la calidad del aire y el agua, ofrecen espacios con una alta diversidad genética de plantas y animales, proporcionan beneficios inmateriales por su alto valor paisajístico y son seña de identidad cultural de la población humana que allí se asienta, además de otros innumerables recursos y bienes (productos farmacéuticos, regulación de suelos y nutrientes, almacenamiento de carbono, etc.) (Bengtsson y col., 2019). Este conjunto de bienes y procesos que los ecosistemas proporcionan a los seres humanos quedó acuñado con el término de servicios ecosistémicos, los cuáles se clasificaron en cuatro grandes tipos: servicios de abastecimiento, regulación, apoyo y culturales (www.millenniumassessment.org). El aislamiento de algunos de estos espacios junto al legado que el uso humano ha ejercido en ellos ha contribuido a convertirlos en uno de los principales puntos calientes (*hotspots*) de biodiversidad del planeta (Wilson y col., 2012). Sin embargo, pese a la importancia económica y ambiental que supone su conservación, numerosos problemas los amenazan.

2. Problemáticas de los ecosistemas pascícolas en un mundo en cambio

El concepto de cambio global hace referencia al conjunto de transformaciones a gran escala producto de las actividades de origen antropogénico que afectan a nuestro planeta (https://cambioglobal.uc.cl). Además de los impactos sobre los sistemas biofísicos (atmósfera, recursos hídricos, suelos, biodiversidad, etc.) también se alteran los sistemas socioeconómicos, y la socioeconomía de las zonas montañosas no es ajena a estos cambios globales (García-Ruiz y col., 2015). La estrecha relación entre la población rural y los recursos naturales de los que disponen se está viendo alterada por el despoblamiento, el envejecimiento de la población, la reducción de trabajadores dedicados al sector primario y la modernización de la ganadería, encaminada a una mayor intensificación de las explotaciones que deciden continuar su actividad empresarial. Estos cambios socioeconómicos se han visto fuertemente reflejados en un descenso del número de animales pastantes en áreas de montaña. La desaparición de la acción que implicaba el consumo de biomasa por parte de estos herbívoros ha alterado la composición florística de los pastos favoreciendo la expansión de algunas especies y la acumulación de su biomasa (Lasanta y Vicente-Serrano, 2006; Bracchetti y col., 2012; Komac y col., 2013).

En algunas zonas del Pirineo, este descenso de animales ha motivado un aumento de la solicitud de quemas controladas con el objetivo de frenar la acumulación de biomasa no pastada. Su aplicación resulta un método rápido y eficaz para reducir esta acumulación de combustible (Montane y col., 2009; Fernández-Giménez y Fillat, 2012). Sin embargo, quemas tradicionalmente de pequeñas dimensiones, enfocadas a quemar arbustos pie a pie han dado lugar a numerosas quemas recurrentes de amplias extensiones de pastos con elevada acumulación de biomasa (San Emeterio y col., 2016; Múgica y col., 2021). Este desacople de los regímenes tradicionales de fuego y pastoreo promueve diferentes situaciones en la dinámica de los pastizales.

En primer lugar, el cese de las perturbaciones consumidoras de biomasa debería conducir la comunidad vegetal hacia sistemas matorralizados a corto plazo y arbolados a largo plazo (Clements, 1936), sin embargo, esta dinámica evolutiva no parece ser la única opción del ecosistema (Chang y Turner, 2019). En algunas áreas se produce el establecimiento de densas cubiertas de matorral con baja capacidad de evolución hacia sistemas más desarrollados, como por ejemplo los dominados por la especie leñosa *Ulex gallii* (Múgica y col., 2018) o *Echinospartum horridum* (Komac y col., 2013). Por otro lado, encontramos cubiertas herbáceas en las que se han sustituido especies tolerantes al pastoreo por otras especies herbáceas con fuertes estrategias competitivas (Pardo y col., 2015), como *Agrostis curtisii* (Amezaga y col., 2004) o *Brachypodium rupestre* (Niedrist y col., 2009; Canals y col., 2017). En las peores situaciones, estas dos últimas opciones pueden llegar a establecer un estado estable alternativo no reversible o de compleja recuperación (Briske y col., 2005). La teoría de los estados estables alternativos (*Alternative stable states*) trata de explicar como en un ecosistema sometido a las mismas condiciones bióticas y abióticas pueden establecerse comunidades vegetales que albergan conjuntos de especies diferentes, capaces de persistir en el tiempo. En ellas, perturbaciones, cambios graduales y la existencia de procesos de retroalimentación, pueden impulsar la estabilidad de uno de estos estados, limitando el desarrollo de otros más evolucionados (Scheffer y col., 2001; Pausas y Bond, 2020).

2.1. El problema mundial de la expansión de gramíneas perennes altas

En la zona centro de Norteamérica, se acuñó el término de "*tallgrass prairies*" a aquellas superficies de pasto dominadas por gramíneas de elevada altura como *Sorghastrum nutants*, *Andropogon gerardii*, *Schizachyrium scoparium* o *Panicum virgatum*, entre otras (Samson y Knopf, 1994; Jones y Cushman, 2004). En la actualidad, el término "*tallgrass*" aglutina al grupo de gramíneas que tienden a dominar las cubiertas vegetales por la altura que son capaces de alcanzar, su expansión clonal, la acumulación de su propia biomasa y la capacidad de monopolizar los recursos del suelo (agua y nutrientes) y la luz (Wedin y Tilman, 1990; Burke y col., 1998; Grime, 2001). Sin embargo, podemos encontrarnos que, bajo determinadas circunstancias, especies autóctonas que forman parte natural del ecosistema sufran explosiones demográficas que les permite expandirse hacia nuevas áreas o dominar aquellas en las que ya estaban establecidas, ocasionando el desplazamiento de otras especies y generando una importante pérdida de diversidad florística, de manera similar al que siguen las especies invasoras exóticas (Valéry y col., 2009a). En la actualidad, los cambios del ambiente están principalmente relacionados con las actividades humanas, por ejemplo, la eutrofización, los cambios de usos del suelo, el calentamiento global, etc.

Este tipo de proceso degradativo se está observando en numerosas especies que ante situaciones de cambio en las condiciones del medio en el que se desarrollan, consiguen dominar la comunidad vegetal y desplazar al resto de especies (Veer y Kooijman, 1997; Valéry y col., 2009b; Pottier y Evette, 2010). En Europa por ejemplo, *Elymus athericus* y *Ammophila arenaria* en zonas costeras (Ketner-Oostra y Sýkora, 2004; Valéry y col., 2017) o *Arrhenatherum elatius* y *Calamagrostis epigejos* en pastos de la República Checa (Holub y col., 2012) y Hungría (Házi y col., 2011). Las fuertes estrategias competitivas que siguen estas especies para adaptarse al medio y sus rasgos funcionales determinan su potencial para establecerse y persistir bajo determinadas condiciones (Diaz y col., 1998; McIntyre y col., 1999; Grime, 2001).

2.2. La expansión de especies del género *Brachypodium* sp. en Europa

El género *Brachypodium* sp. está ampliamente distribuido por todo el mundo, y en Europa, es uno de los géneros con más representación de gramíneas altas. Contiene especies capaces de adaptarse a ambientes mediterráneos secos (*B. retusum*) y a zonas húmedas de sotobosque (*B. sylvaticum*). En los pastos calcáreos de Europa podemos encontrar las especies perennes *B. pinnatum, B. rupestre* y *B. genuense*. Gracias a los novedosos estudios de genética poblacional conocemos con más detalle la distribución de cada una de ellas, ya que sus diferencias morfológicas son mínimas y hasta hace poco su clasificación taxonómica variaba según el autor al que se consultase (Catalán y col., 2015). Mientras que *B. genuense* se clasificaba dentro de *B. pinnatum* (Valdés y Scholz, 2009), *B. rupestre* era considerada una subespecie de esta última (Aizpuru y col., 1999). Pese a la variabilidad genética que las separa, en todas ellas se observan patrones de expansión similares ligados al cese de las actividades ganaderas que limitaban su desarrollo (Poschlod y WallisDeVries, 2002). Tanto las circunstancias abióticas que favorecen la expansión, así como sus características fisiológicas y morfológicas han sido ampliamente estudiadas, y el compendio de resultados parece englobar múltiples factores desencadenantes del proceso de su expansión (figura 1).

Las cubiertas que forman *B pinnatum, B. rupestre* y *B. genuense* se caracterizan por una elevada altura y densidad de sus macollas que llega a alcanzar un recubrimiento total del suelo. Tienen un precoz y rápido rebrote primaveral, pudiendo registrar un nuevo rebrote otoñal condicionado por el clima. Este rápido desarrollo hace que pierdan muy pronto su digestibilidad y el ganado evite consumirlas (Peralta, 2010; Catorci y col., 2014a). Su fisiología les aporta una gran plasticidad que les permite adaptarse a numerosas situaciones ambientales, como por ejemplo a variadas condiciones lumínicas tanto de sombreo como de zonas altamente expuestas (Mojzes y col., 2003; Bąba y col., 2016). La abundante biomasa de sus macollas les permite captar un alto porcentaje de luz, privando de este recurso a las plantas que les rodean y favoreciendo que la acumulación de su propia biomasa seca dificulte la colonización de otras especies (During y Willems, 1984). Otra importante característica morfológica es la que le aporta la existencia de un potente rizoma que les permite almacenar nutrientes (Bobbink y col., 1989; Pottier y Evette, 2010) y reproducirse vegetativamente, permitiendo una estrategia reproductiva tanto sexual como asexual (de Kroon y Bobbink, 1997; Bąba y col., 2012). En lo que respecta a la reproducción sexual, tienen una producción regular de semillas ligada a una estrategia de colonización de territorios más distantes, mientras que la reproducción clonal va ligada a una estrategia de expansión más limitada, a cambio de una disminución de la variabilidad genética (Schläpfer, 1997).

Respecto a los cambios abióticos que podrían estar favoreciendo la expansión de estas especies, Bobbink y Willems (1987) fueron pioneros en su estudio tras observar una fuerte expansión de *B. pinnatum* en pastos de los Países Bajos, e intentaron comprender el mecanismo responsable de su exitoso avance. Estos autores argumentaron que la expansión era debida al aumento de las deposiciones atmosféricas de nitrógeno, ya que la planta es capaz de utilizar más eficazmente este recurso que otras especies (Bobbink y col., 1988; Hurst y John, 1999). También se empezó a relacionar su expansión con el abandono de los usos tradicionales del suelo (Bobbink y Willems, 1991, 1993; Buckland y col., 2001) o la limitación de fósforo en estos (Davis y col., 2000). Schläpfer y Fischer (1998) estudiaron como el frío clima del Reino Unido limitaba la expansión sexual de la especie, que podría verse incrementada con el cambio climático al aumentar sus latitudes de expansión.

Los estudios más recientes que encontramos relacionados con la expansión de este grupo de especies se han centrado en el Pirineo occidental (*B. rupestre*) y en los Apeninos centrales (figura 2). La cordillera italiana tampoco es ajena a los actuales cambios socioeconómicos, y el abandono de la gestión tradicional de esta zona rural promueve el avance de *B. rupestre* (Tardella y col., 2018, 2020; Bricca y col., 2020a, 2020b, 2021), y de *B. genuense,* distribuida en zonas de mayor altitud (Catorci y col., 2011, 2012, 2014b; Tardella y col., 2017).

Respecto a los trabajos realizados en el Pirineo navarro, estos han desvelado la capacidad de la gramínea para aprovechar mejor determinadas fuentes de nitrógeno (NH_4^+) que se liberan tras las quemas (Canals y col., 2014) o la eficiente redistribución de nutrientes entre sus tejidos vegetales a lo largo de su desarrollo, junto a la eficaz barrera que la acumulación de su propia biomasa aérea y subterránea puede suponer para el establecimiento de otras especies (Canals y col., 2017). También se ha observado la alteración de ciertos parámetros del suelo en comparación con cubiertas de mayor diversidad florística (riqueza y diversidad bacteriana, actividad ureasa, pH y nitratos) (Canals y col., 2019), la ventaja competitiva que es capaz de alcanzar en detrimento del arbusto *Ulex gallii* cuando su cobertura supera el 60% (Múgica y col., 2021) o como el continuado aporte de su propia biomasa al suelo puede generar 'islas de fertilidad' en las que favorecer su propia estabilidad y expansión (San Emeterio y col., 2021).

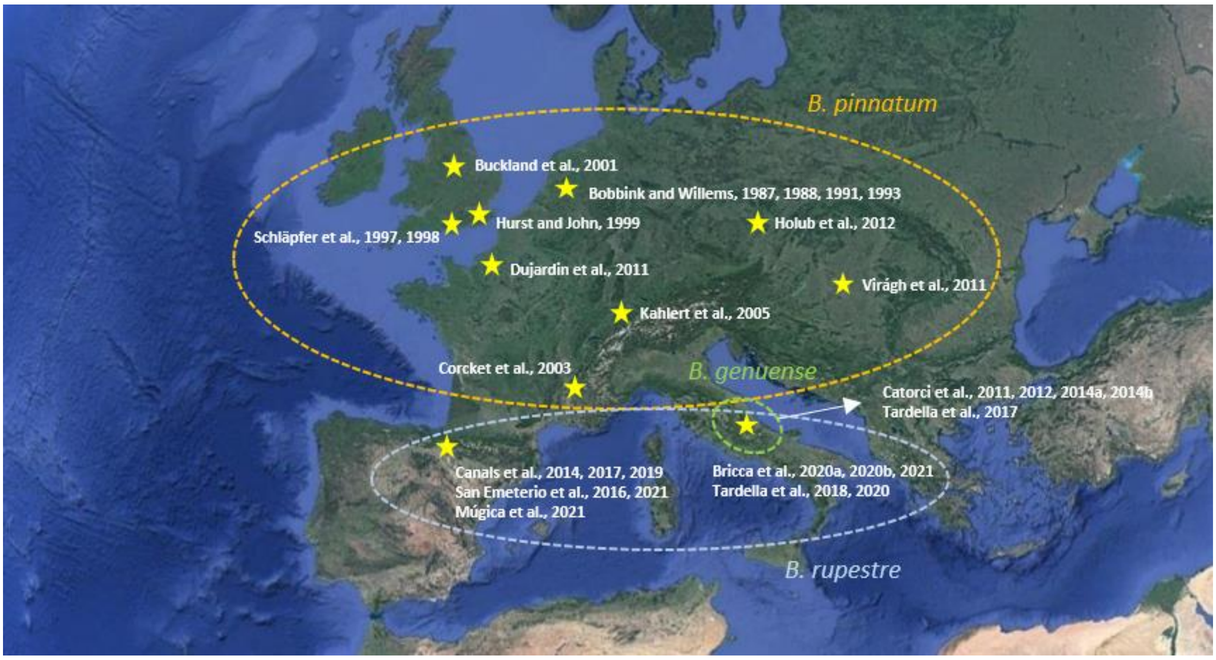


Figura 1. Estudios europeos ligados con el proceso de expansión de especies perennes del género *Brachypodium sp.*

A pesar de los concluyentes resultados que arrojan los estudios relacionados con la expansión de estas especies de *Brachypodium* sp. y el relevante papel de la ganadería para frenarla (Vitasović y col., 2011, 2014), resulta complejo transmitir estos conocimientos a los gestores del territorio que en la mayoría de los casos no son conscientes de la degradación ambiental que supone la expansión de esta gramínea.

Figura 2. Aspecto de las zonas invadidas por *B. rupestre* en abril en el Pirineo occidental (izquierda y centro) y de *B. genuense* en julio en los Apeninos centrales (derecha).

3. La biodiversidad que no vemos

La pérdida de biodiversidad, relativamente fácil de apreciar en su composición florística, resulta imperceptible a simple vista a otros niveles de organización biológica. En comparación con disciplinas como la zoología o la botánica, el estudio de los microorganismos es una disciplina más reciente que ha tenido un enfoque casi exclusivo hacia los microorganismos causantes de enfermedades. Sin embargo, este enfoque ha sido considerablemente ampliado a otras muchas materias como la agronomía o la biotecnología (Bud, 1991; Head y Prosser, 2007).

Respecto al estudio de los microorganismos en su ambiente natural, la ecología microbiana parece ir desentramando las complejas redes e interacciones en las que los microorganismos están implicados. Actualmente sabemos que la diversidad microbiana es la base de la dinámica de todos los ecosistemas de la Tierra (Tikhonovich y Provorov, 2011). La alteración de los ciclos vitales en los que están implicados (suelos, agua, atmósfera, etc.) puede tener imprevisibles consecuencias en los ecosistemas. Para ello, es necesario ampliar los conocimientos científicos acerca de las especies presentes en cada tipo de ecosistema, la interacción que tienen con el resto de organismos, el rol que pueden estar desempeñando y su respuesta al cambio global.

El novedoso concepto de holobionte hace referencia al conjunto de microorganismos que hay en el interior de un organismo huésped (Guerrero y col., 2013; Vandenkoornhuyse y col., 2015; Carthey y col., 2019; Simon y col., 2019). Para una planta, este microbioma puede incluir hongos, bacterias, virus y protistas, y desde hace décadas numerosos estudios reconocen la aportación que los microorganismos generan en numerosas funciones ecológicas, ampliando la capacidad de la planta a adaptarse a diferentes tipos de condiciones y situaciones de cambio ambiental. Concretamente, los microorganismos fúngicos que albergan las plantas en su interior están cobrando una gran relevancia en los últimos años (Rodriguez y col., 2009).

3.1. Los hongos endófitos

Casi todas las plantas albergan hongos en su interior. Se estima que la diversidad fúngica puede alcanzar los 3,8 millones de especies, sin embargo, tan sólo unas 120.000 han sido descritas (Hawksworth, 2001; Hawksworth y Lücking, 2017; Baldrian y col., 2021). Aunque el conocimiento de los hongos ha estado muy enfocado en los hongos fitopatógenos por sus efectos dañinos en los cultivos, muchos de ellos se desarrollan en el interior de las plantas sin producir síntomas de enfermedad. Estos hongos endófitos llegan a forjar en algunos casos interacciones biológicas positivas para ambos organismos (mutualismo) en la que principalmente la planta aporta alimento, hospedaje y protección, mientras que las ventajas que el hongo aporta a la planta están siendo objeto de interesantes estudios (Saikkonen y col., 1998, 2004; Arnold y col., 2003; Schardl y col., 2004; Schulz y Boyle, 2005).

Esta interacción planta-hongo, aparentemente mutualista en el momento de su identificación, puede pasar por diferentes etapas y no ser estática en el tiempo según el estado fisiológico, la estrategia de supervivencia o la etapa de desarrollo en el que se encuentren ambos organismos. Caracterizar la especie fúngica y averiguar su patrón de colonización, el nicho que tiende a ocupar en la planta, el tipo de crecimiento inter o intracelular, o la especificidad de la infección en órganos concretos (aéreos o subterráneos), nos puede permitir profundizar en el tipo de interacción ecológica y la etapa en la que se encuentra: inicio de la infección, estado latente, colonización local, virulencia, saprofitismo, colonización sistémica, etc. Conocer los detalles puede desvelar el momento evolutivo en el que se encuentran, saber qué limita la colonización del hongo para no desarrollarse como una enfermedad o la plasticidad del holobionte frente a la influencia de los factores externos (clima, herbivorismo, fuego, etc.) (*endophytic continuum concept*, Schulz y Boyle, 2005).

La mayoría de los trabajos se centran en los hongos endófitos de la familia Clavicipitaceae que crecen de forma sistémica en numerosas gramíneas y se dispersan por transmisión vertical colonizando las semillas (Bacon y col., 1977). El increíble desarrollo que ha tenido el estudio de esta familia (Schardl y col., 2014; Soto-Barajas y col., 2018), principalmente el del género *Epichloë* (o *Neotyphodium* en su estadio anamorfo) han permitido dar a conocer las ventajosas adaptaciones que pueden llegar a conferir a su huésped frente a numerosos estreses bióticos y abióticos como pueden ser la tolerancia a la sequía (Malinowski y col., 1997; Malinowski y Belesky, 2000; Zabalgogeazcoa y col., 2003), a la salinidad (Pereira y col., 2019), al herbivorismo (Brem y Leuchtmann, 2001; Clay y Schardl, 2002), etc. El desarrollo de estos y otros numerosos estudios está teniendo una importante repercusión en las ciencias agronómicas relacionadas con la mejora de los cultivos (Williams y col., 2007; Hume y Sewell, 2014).

El estudio de los hongos endófitos no sistémicos no ha desarrollado un recorrido tan largo, y sus funciones y roles son desconocidos en la mayoría de los casos (Saikkonen y col., 1998). Sin embargo, su estudio se está ampliando y en la actualidad también son objeto de interesantes trabajos (Rodríguez y Redman, 2008; Zabalgogeazcoa, 2008). Desde un punto de vista evolutivo, su alta diversidad en el interior de un mismo hospedador, unido al hecho de tener una transmisión horizontal, puede estar más relacionado con interacciones antagónicas, en lugar de simbióticas, teniendo la planta que defenderse para mantener la infección fúngica por debajo de un determinado umbral (Schulz y Boyle, 2005). En contraste con esta posible teoría, otros autores creen que las interacciones evolutivas y la ubicuidad del endofitismo podrían compensar los costes de la heterotrofía desempeñando funciones positivas en la planta (Yuan y col., 2010). A pesar de la escasez de estudios de hongos no sistémicos, poco a poco van saliendo a la luz interacciones positivas y ventajas adaptativas similares a las conferidas por los endófitos sistémicos en relación a la tolerancia a estreses bióticos y abióticos (Redman y col., 2002; Arnold y Lewis, 2005; Saikkonen y col., 2010; Sánchez-Márquez y col., 2012).

3.2. Técnicas de identificación de hongos endófitos

Hoy en día, diversas técnicas permiten el estudio de los hongos endófitos. Desde que se empezaron a estudiar estos microorganismos hasta la actualidad, extraordinarios avances biotecnológicos permiten mejorar su clasificación taxonómica, y poco a poco conocer su funcionalidad en los ecosistemas. Con anterioridad al siglo XXI, la diversidad fúngica se evaluó en función de la morfología de algunas de sus macro o micro estructuras (color, tasa de crecimiento, aspecto del micelio, etc.), lo que suponía numerosas limitaciones (complejidad del medio en el que cultivar el hongo, desconocimiento de las etapas completas de desarrollo, rasgos morfológicos limitados o muy variables, cepas estériles, etc.). Sin embargo, en las últimas décadas, novedosas técnicas moleculares, basadas en la secuenciación de ADN, se han ido desarrollando y empleando para profundizar en la diversidad de los hongos (Mueller y col., 2004).

El estudio de las secuencias de ADN ribosómico está generando una gran cantidad de información sobre numerosas especies fúngicas y sus relaciones evolutivas. Concretamente, la región ITS (*Internal Transcribed Spacer*) es el marcador universal de hongos y la más utilizada para analizar relaciones filogenéticas (Jorgensen y Cluster, 1988; Badotti y col., 2017). La región ITS consta de las secciones ITS1 e ITS2 que flanquean la región conservada 5.8S (Figura 3). Además de la región ITS, existen otras regiones que pueden ser secuenciadas (IGS: *intergenic spacer*), sin embargo, la abundancia y accesibilidad de las secuencias de la región ITS depositadas en grandes bases de datos científicos de acceso abierto (Benson y col., 2013; Nilsson y col., 2019), hacen que sea la región más indicada para la identificación de especies en estudios de diversidad fúngica (Lutzoni y col., 2004; Schoch y col., 2012).

Secuenciar la región ITS a partir de la extracción de ADN directamente de micelio fúngico es la metodología más común y tradicionalmente utilizada (White y col., 1990). Sin embargo, este método es dependiente del cultivo del hongo y las principales limitaciones que presenta se relacionan con la dificultad de obtener micelio de taxones que, por diversas razones, no tienen capacidad de desarrollarse en un medio de cultivo artificial. Por otra parte, hongos con diferentes tasas de crecimiento también pueden llegar a encriptarse o interferirse en el proceso de desarrollo miceliar (Vandenkoornhuyse y col., 2002; Vázquez-de-Aldana y col., 2019). Finalmente, también nos encontramos ante la complejidad de controlar la proliferación de contaminaciones bacterianas o de hongos del exterior de los tejidos, lo que indica que debe aplicarse una exhaustiva metodología de esterilización de las muestras (Schulz y col., 1993; Creer y col., 2016).

Algunos de los inconvenientes anteriormente citados parecen haberse resuelto con la aparición de las técnicas de caracterización genéticas (*metabarcoding*). Este conjunto de metodologías altamente tecnificadas permiten la identificación simultánea de todo el ADN existente dentro de una muestra (Baird y Hajibabaei, 2012; Creer y col., 2016). Además, el poder cuantitativo de los datos de *metabarcoding* permite profundizar en la abundancia de cada una de las especies, aportando un valor añadido a este tipo de datos (Thomas y col., 2016).

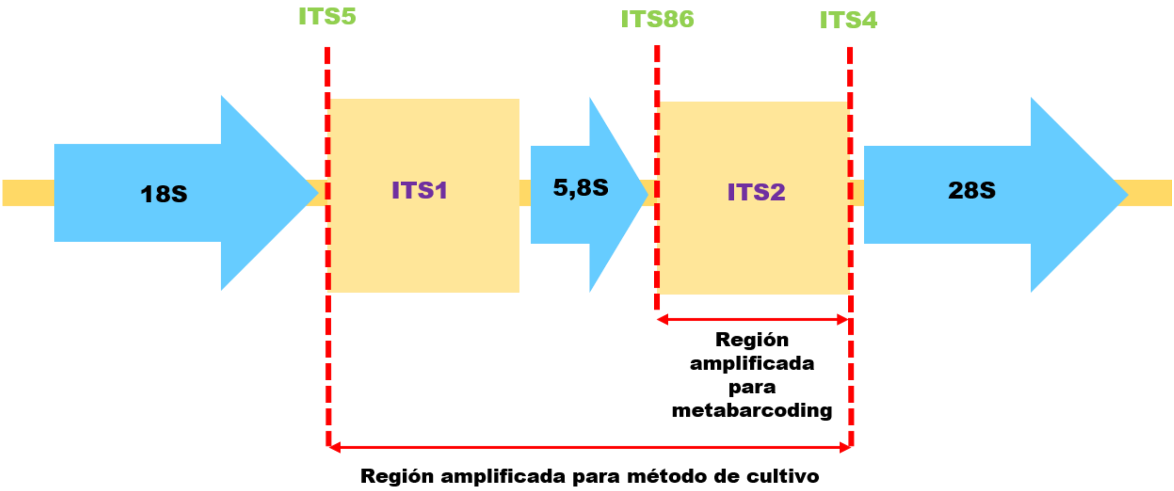


Figura 3. Ilustración de la disposición de la región ITS en el ADN fúngico, las regiones amplificadas para cada metodología y los primers utilizados (en verde).

A pesar de las ventajas que el *metabarcoding* parece tener sobre el método de cultivo tradicional, otros inconvenientes surgen con su aplicación. Más allá del actual elevado coste que implica su uso y que normalmente va ligado a una reducción del tamaño muestral del estudio (Stein y col., 2014), la clasificación taxonómica de todas las secuencias genéticas que van surgiendo, a veces trae consigo incongruencias que dificultan la clasificación de las especies. Por ejemplo, secuencias desconocidas que aún no tienen un taxón asignado, clasificaciones a niveles taxonómicos altamente inclusivos o modificaciones constantes de las asignaciones taxonómicas (Ebach y col., 2011). Conociendo las ventajas e inconvenientes de cada método, numerosos investigadores promueven la aplicación de ambas técnicas con el objetivo de comparar los resultados y combinarlos para mejorar los estudios (Hyde y Soytong, 2008; Chi y col., 2019).

4. El área de estudio: Los pastos de puerto del valle de Aezkoa

Al norte de la provincia de Navarra se localizan los valles más occidentales de la cordillera de los Pirineos. Su localización en la región eurosiberiana, la altitud de sus montañas y la influencia del mar Cantábrico (55 Km en línea recta) contribuyen a su clima frío y continental que junto a la gestión de su territorio ha propiciado un paisaje en mosaico compuesto por densas zonas boscosas, arbustivas y herbáceas que albergan una alta biodiversidad.

En las últimas décadas, la creciente concienciación ambiental ha propiciado el desarrollo de políticas medioambientales enfocadas a la conservación y protección de la naturaleza. A nivel Europeo, la Red Natura 2000, ha impulsado la creación de figuras de protección con el objetivo de conservar hábitats naturales y fauna y flora silvestres. La red está formada por las Zonas de Especial Conservación (ZEC) y por las Zonas de Especial Protección para las Aves (ZEPA). Por las características ambientales que conserva esta parte del Pirineo navarro, desde el año 2011 y a través del Decreto Foral 9/2011, del 7 de febrero, el Lugar de Importancia Comunitario (LIC) denominado “Roncesvalles-Selva de Irati” pasó a designarse como Zona de Especial Conservación (Código: ES0000126), quedando también aprobado su plan de gestión (figura 4). Esta zona ZEC cubre una superficie total de 18078,12 ha y el objetivo final planteado en el texto, en relación a los pastizales, es la de asegurar el estado de conservación de los mismos garantizando, al menos, su superficie actual, a través de un modelo de gestión y manejo que permita su conservación y mantenimiento, aplicando las medidas necesarias para garantizar la permanencia de una actividad ganadera sostenible que asegure la conservación de estas áreas de interés.

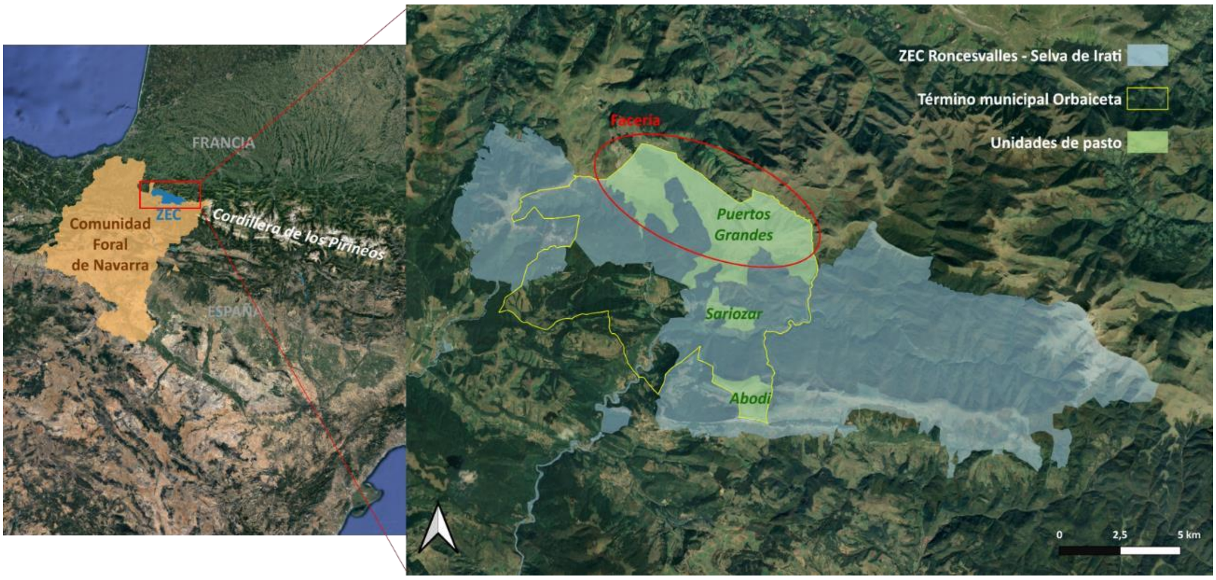


Figura 4. Localización geográfica de la zona ZEC Roncesvalles-Selva de Irati en la cordillera pirenaica y dentro de la Comunidad Foral de Navarra (izquierda). Localización de las unidades de pasto dentro del término municipal de Orbaizeta, que pertenece al Valle de Aezkoa (derecha).

Integrado casi en su totalidad dentro de los límites de la zona ZEC Roncesvalles-Selva de Irati encontramos el valle de Aezkoa. Este valle pirenaico está formado por 9 términos municipales y su actual población de 850 habitantes muestra un continuo descenso (Figura 5). Desde hace siglos, la gestión de la ganadería extensiva ha sido el pilar de su economía. Un aprovechamiento sostenible de sus tres principales unidades de pasto (Puertos grandes, Sariozar y Abodi, 2147 ha) han propiciado el equilibrio y mantenimiento de comunidades vegetales de alta diversidad florística.

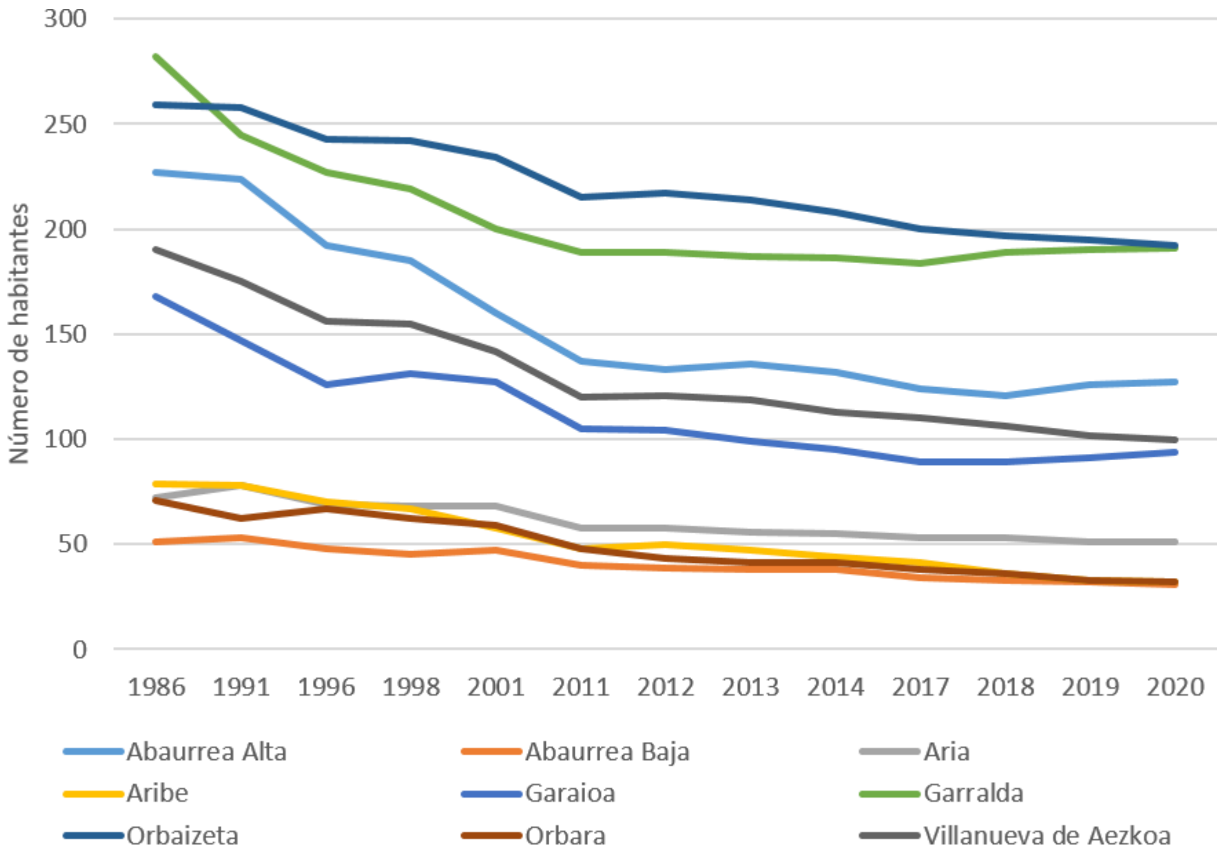


Figura 5. Evolución demográfica de los 9 términos municipales que integran el valle de Aezkoa.

El tipo de ganado que pasta este valle del Pirineo lo conforman ovejas, vacas y caballos. El ganado más representativo de la zona es el ovino. Tradicionalmente, la cría de ovejas de raza *Latxa* ha supuesto el medio de vida de numerosas familias. Su carácter montaraz, su agilidad y elevada rusticidad le permiten adaptarse a terrenos abruptos y climas fríos. Su principal producción está destinada a leche para la elaboración de quesos (Granado-Tajada y col., 2020). El ganado vacuno pertenece principalmente a las razas Pirenaica y Blonda de Aquitania, ambas especies muy rústicas, bien adaptadas a zonas con compleja orografía y clima y destinadas a la producción cárnica. Por último, encontramos al ganado caballar donde las especies Jaca Navarra y Burguete son las más representativas, esta última considerada en peligro de extinción. Su sistema de cría se basa en un estado de semilibertad, resguardándose únicamente en época de nevadas. Es un animal muy resistente a condiciones climáticas adversas y sus potros se destinan a la producción de carne (Ferrer y Canals, 2008; Ministerio de medio ambiente y medio rural y marino, 2019; Insausti y col., 2021). En las últimas décadas, numerosos factores socioeconómicos están alterando estos tradicionales sistemas productivos lo que está generando un continuo descenso de los censos de animales (Figura 6).

La alta calidad y cantidad de pasto disponible en el valle de Aezkoa también ha sabido ser aprovechada por ganaderos del limítrofe valle francés del Cize. El uso conjunto de superficies de pasto forjó las facerías. Esta figura, basada en antiguos acuerdos muy comunes a lo largo de todo el Pirineo, y anteriores al establecimiento de las modernas fronteras, establecían las bases para un uso compartido de los pastos por parte de ganaderos de ambas partes de la vertiente pirenaica a cambio del pago de un canon económico. En el caso que nos ocupa, se tienen registros de acuerdos entre el valle de Aezkoa y de Cize desde 1556 hasta la actualidad, siendo una de las pocas facerías que aún permanece vigente y actualizada (Razquin y col., 2012). Esta zona facera se localiza al norte del término municipal de Orbaizeta (Figura 4) y engloba territorios de la unidad de pasto de Puertos Grandes. Aunque es una facería recíproca, son mayoritariamente rebaños franceses los que aprovechan la superficie del lado español. En la campaña de pastos del año 2021 el canon pagado por el valle de Cize al valle de Aezkoa por el aprovechamiento de estas superficies alcanzó los 76.011,09€.

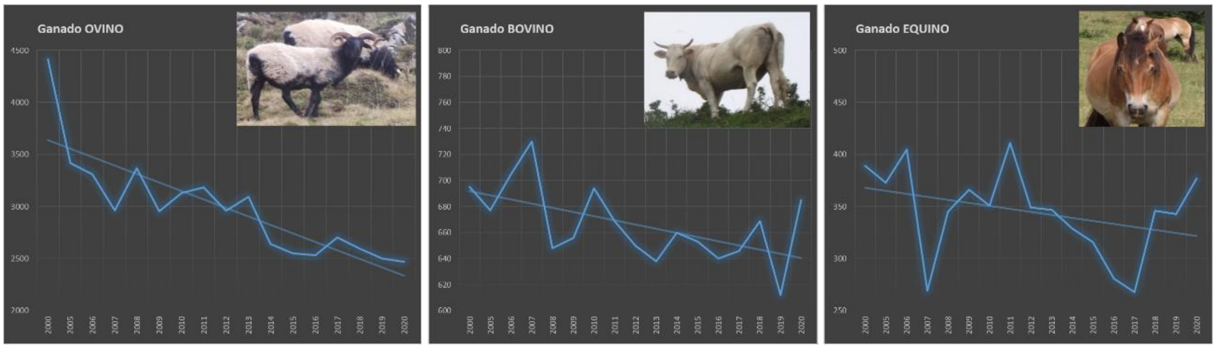


Figura 6. Evolución de los censos de ganado que pastan en el Monte Aezkoa desde el año 2000 hasta el 2020 (Fuente: Junta Central del Valle de Aezkoa).

En esta zona del Pirineo, la gestión de los pastos ha ido acompañada del uso del fuego como herramienta de control de la biomasa. Su aplicación tiene por objetivo frenar la matorralización y mejorar la productividad del siguiente rebrote vegetal. En la Comunidad Foral de Navarra, la realización de quemas prescritas está regulado por la Orden Foral 237/2017, del 4 de julio (posterior a la Orden Foral 222/2016, de 16 de junio). Sin embargo, resulta importante diferenciar las quemas que pueden llevar a cabo directamente los ganaderos en zonas con bajo riesgo de escape (quemas pastorales) y aquellas en las que la densidad de la vegetación entraña un riesgo potencial de expansión del fuego, y que por tanto requieren ser aplicadas por bomberos especializados (quemas prescritas) (Figura 7) (Canals y col., 2014; San Emeterio y col., 2016).

Figura 7. Quema pastoral (izquierda) y quema prescrita (derecha) realizadas en diferentes áreas de la zona ZEC Roncesvalles-Selva de Irati.

En el valle de Aezkoa, el descenso del número de animales en las últimas décadas ha ocasionado un incremento del uso del fuego para reducir la biomasa leñosa y herbácea no pastada. Pero, en algunos casos, se ha pasado de quemas tradicionales de pequeñas superficies (< 2 ha), dirigidas a quemar arbustos pie a pie y con baja recurrencia (cada 5-10 años), a quemas frecuentes (cada 1-2 años) de grandes extensiones (> 5 ha) en áreas con densas acumulaciones de combustible (San Emeterio y col., 2016; Múgica y col., 2021). Este desacople de los regímenes tradicionales de fuego y pastoreo ha conducido al establecimiento de diferentes tipos de pasto.

En el año 2008, la realización del proyecto de ordenación de los recursos pascícolas y forestales del monte Aezkoa (Ferrer y Canals, 2008), permitió identificar el problema de la expansión de los lastonares, cubiertas herbáceas de baja diversidad florística, dominadas por la gramínea *Brachypodium rupestre*. Estas cubiertas degradadas se asocian a lugares con un intenso desajuste de los regímenes tradicionales de fuego y pastoreo, caracterizadas por una escasa o inexistente presencia de herbívoros y una alta recurrencia de quemas pastorales (figura 8). En estas zonas los porcentajes de cobertura de *B. rupestre* superan el 75%, en comparación con las cubiertas de pasto más diversas, en las que la cobertura es inferior al 25%. En el gradual proceso de expansión, entre una cubierta diversa y degradada, encontramos zonas en fases intermedias caracterizadas por la paulatina aparición de rodales o parches de *B. rupestre* donde su cobertura oscila entre el 25 y el 75% (figura 9).

Los pastos de puerto del valle de Aezkoa son el escenario ideal para evaluar el efecto que el cambio global puede tener en este tipo de ecosistemas. Explorar la alteración de los sistemas biológicos ligada a cambios en las condiciones ambientales y de gestión humana nos puede ayudar a conocer su resiliencia. La presencia de determinados tipos de cubiertas florísticas, altamente dependientes del tipo de gestión a las que están sometidas, nos permite la posibilidad de estudiar un amplio espectro de alteraciones del medio (suelos, vegetación, microorganismos, etc.). En esta tesis se incluye un doble enfoque a la problemática de la expansión de *B. rupestre* en esta parte del Pirineo, concretamente a la alteración del servicio ecosistémico de abastecimiento (capítulo 1) y de la comunidad endofítica de la gramínea (capítulos 2 y 3).

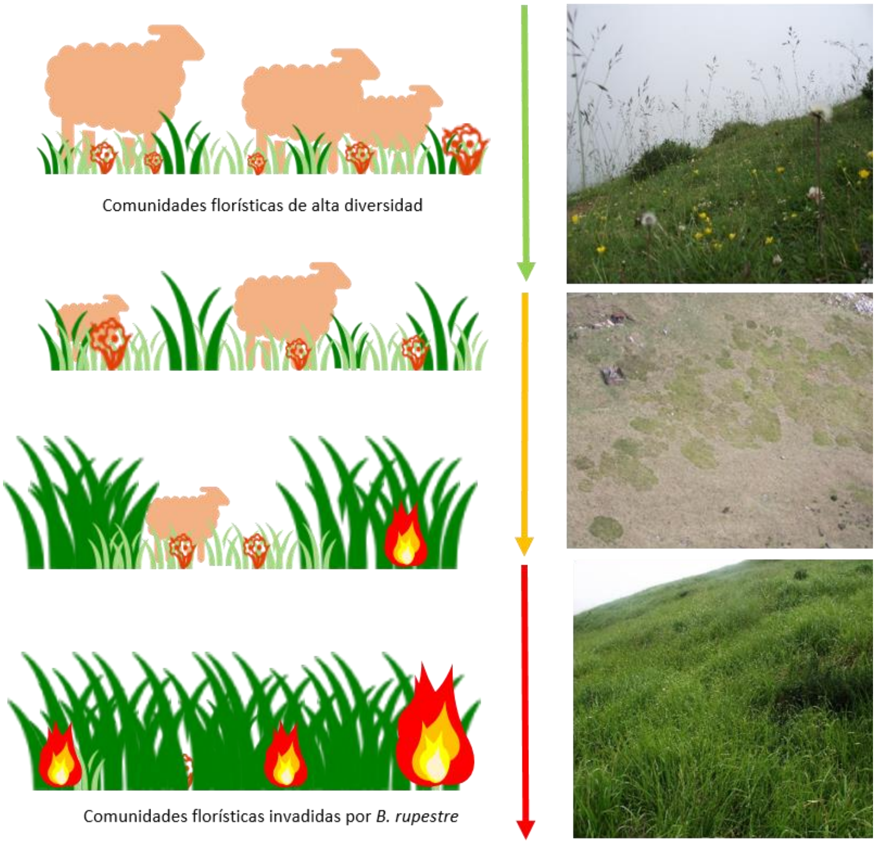


Figura 8. Relación entre la gestión de la ganadería y el fuego, y la diversidad de las comunidades florísticas.

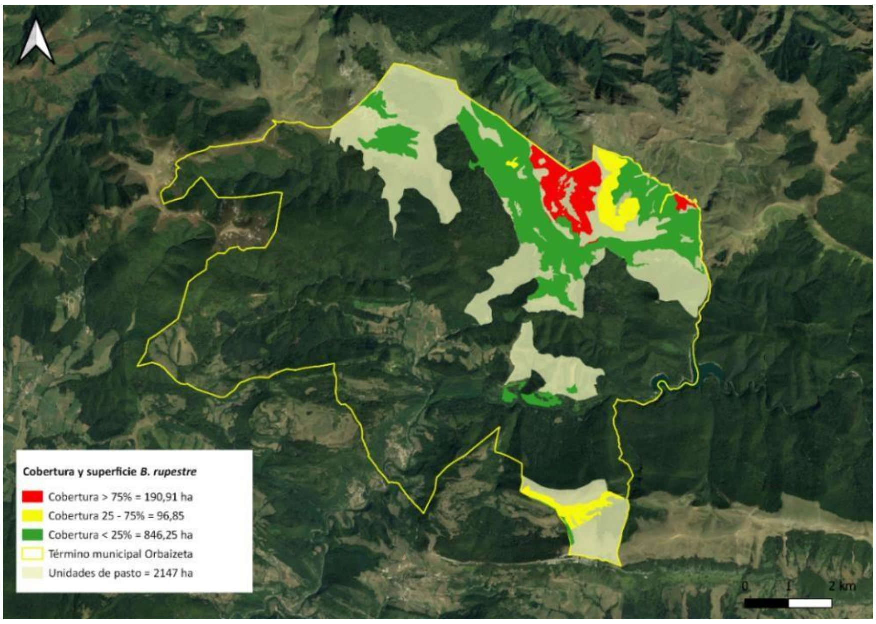


Figura 9. Zonas de pasto del valle de Aezkoa con presencia de *B. rupestre* clasificados por sus diferentes porcentajes de cobertura y la extensión que ocupan.

BIBLIOGRAFÍA

Aizpuru, I., Aseginolaza, C., Uribe-Echebarría, P.M., Urrutia, P., Zorrakin, I., 1999. Claves ilustradas de la flora del País Vasco y territorios limítrofes. Vitoria-Gasteiz: Servicio central de publicaciones del Gobierno Vasco. doi:10.1016/0022-4375(87)90074-0

Amezaga, I., Mendarte, S., Albizu, I., Besga, G., Garbisu, C., Onaindia, M., 2004. Grazing intensity, aspect, and slope effects on limestone grassland structure. Journal of Range Management 57, 606-612. doi:10.2307/4004016

Anderson, P.M.L., Hoffman, M.T., 2007. The impacts of sustained heavy grazing on plant diversity and composition in lowland and upland habitats across the Kamiesberg mountain range in the Succulent Karoo, South Africa. Journal of Arid Environments 70, 686-700. doi:10.1016/j.jaridenv.2006.05.017

Arnold, A.E., Lewis, L.C., 2005. Ecology and evolution of fungal endophytes and their roles against insects. In: Gical and evolutionary advances in insect-fungus associations. pp. 74-96.

Arnold AE, Lewis LC, 2005. Ecology and evolution of fungal endophytes and their roles against insects. In: Ecological and evolutionary advances in insect-fungus associations (Vega, F., Blackwell, M., Eds.) Oxford Univ. Press, pp. 74-96

Arnold, A.E., Mejía, L.C., Kyllo, D., Rojas, E.I., Maynard, Z., Robbins, N., Herre, E.A., 2003. Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences, 100 (26) pp. 15649-15654. doi: 10.1073/pnas.2533483100

Bąba, W., Kalaji, H.M., Kompała-Bąba, A., Goltsev, V., 2016. Acclimatization of photosynthetic apparatus of tor grass (*Brachypodium pinnatum*) during expansion. Plos One 11, 1-27. doi:10.1371/journal.pone.0156201

Bąba, W., Kurowska, M., Kompała-Bąba, A., Wilczek, A., Długosz, J., Szarejko, I., 2012. Genetic diversity of the expansive grass *Brachypodium pinnatum* in a changing landscape: Effect of habitat age. Flora 207, 346-353. doi:10.1016/j.flora.2012.01.011

Bacon, C.W., Porter, J.K., Robbins, J.D., Luttrell, E.S., 1977. *Epichloe typhina* from toxic tall fescue grasses. Applied and Environmental Microbiology 34, 576-581. doi:10.1128/aem.34.5.576-581.1977. doi:10.1128/aem.34.5.576-581.1977

Badotti, F., De Oliveira, F.S., Garcia, C.F., Vaz, A.B.M., Fonseca, P.L.C., Nahum, L.A., Oliveira, G., Góes-Neto, A., 2017. Effectiveness of ITS and sub-regions as DNA barcode markers for the identification of Basidiomycota (Fungi). BMC Microbiology 17, 1-12. doi:10.1186/s12866-017-0958-x

Baird, D.J., Hajibabaei, M., 2012. Biomonitoring 2.0: A new paradigm in ecosystem assessment made possible by next-generation DNA sequencing. Molecular Ecology 21, 2039-2044. doi:10.1111/j.1365-294X.2012.05519.x

Baldrian, P., Vetrovský, T., Lepinary, C., Kohout, P., 2021. High-throughput sequencing view on the magnitude of global fungal diversity. Fungal diversity. doi: 10.1007/s13225-021-00472-y

Bengtsson, J., Bullock, J.M., Egoh, B., Everson, C., Everson, T., O’Connor, T., O’Farrell, P.J., Smith, H.G., Lindborg, R., 2019. Grasslands-more important for ecosystem services than you might think. Ecosphere 10. doi:10.1002/ecs2.2582

Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Sayers, E.W., 2013. GenBank. Nucleic Acids Research 41, 36-42. doi:10.1093/nar/gks1195

Bobbink, R., Bik, L., Willems, J.H., 1988. Effects of nitrogen fertilization on vegetation structure and dominance of *Brachypodium pinnatum* (L.) Beauv. in chalk grassland. Acta Botanica Neerlandica 37, 231-242. doi:10.1111/j.1438-8677.1988.tb02132.x

Bobbink, R., den Dubbelden, K., Willems, J.H., 1989. Seasonal dynamics of phytomass and nutrients in chalk grassland. Oikos 55, 216-224. doi:10.2307/3565425

Bobbink, R., Willems, J.H., 1993. Restoration management of abandoned chalk grassland in the Netherlands. Biodiversity and Conservation 2, 616-626. doi:10.1007/BF00051962

Bobbink, R., Willems, J.H., 1991. Impact of different cutting regimes on the performance of *Brachypodium pinnatum* in dutch chalk grassland. Biological Conservation 56, 1-21. doi:10.1016/0006-3207(91)90085-N

Bobbink, R., Willems, J.H., 1987. Increasing dominance of *Brachypodium pinnatum* (L.) Beauv. in chalk grasslands: A threat to a species-rich ecosystem. Biological Conservation 40, 301-314. doi:10.1016/0006-3207(87)90122-4

Bracchetti, L., Carotenuto, L., Catorci, A., 2012. Land-cover changes in a remote area of central Apennines (Italy) and management directions. Landscape and Urban Planning 104, 157-170. doi:10.1016/j.landurbplan.2011.09.005

Brem, D., Leuchtmann, A., 2001. *Epichloë* grass endophytes increase herbivore resistance in the woodland grass *Brachypodium sylvaticum*. Oecologia 126, 522-530. doi:10.1007/s004420000551

Bricca, A., Catorci, A., Tardella, F.M., 2020a. Intra-specific multi-trait approach reveals scarce ability in the variation of resource exploitation strategies for a dominant tall-grass under intense disturbance. Flora 270, 151665. doi:10.1016/j.flora.2020.151665

Bricca, A., Tardella, F.M., Ferrara, A., Panichella, T., Catorci, A., 2021. Exploring assembly trajectories of abandoned grasslands in response to 10 years of mowing in sub-mediterranean context. Land 10,1158. doi:10.3390/ land10111158

Bricca, A., Tardella, F.M., Tolu, F., Goia, I., Ferrara, A., Catorci, A., 2020b. Disentangling the effects of disturbances from those of dominant tall grass features in driving the functional variation of restored grassland in a sub-mediterranean context. Diversity 12, 1-12. doi:10.3390/d12010011

Briske, D.D., Fuhlendorf, S.D., Smeins, F.E., 2005. State-and-transition models, thresholds, and rangeland health: A synthesis of ecological concepts and perspectives. Rangeland Ecology and Management 58, 1-10. doi:10.2111/1551-5028(2005)58<1:SMTARH>2.0.CO;2

Buckland, S.M., Thompson, K., Hodgson, J.G., Grime, J.P., 2001. Grassland invasions: effects of manipulations of climate and management. Journal of Applied Ecology 38, 301-309. doi:10.1046/j.1365-2664.2001.00603.x

Bud, R., 1991. Biotechnology in the Twentieth Century. Social Studies of Science 21, 415-457. doi:10.1177/030631291021003002

Burke, I.C., Lauenroth, W.K., Vinton, M.A., Hook, P.B., Kelly, R.H., Epstein, H.E., Aguiar, M.R., Robles, M.D., Aguilera, M.O., Murphy, K.L., Gill, R.A., 1998. Plant-soil interactions in temperate grasslands. Biogeochemistry 42, 121-143. doi:10.1023/A:1005987807596

Canals, R.M., 2019. Landscape in motion: Revisiting the role of key disturbances in the preservation of mountain ecosystems. Geographical Research Letters 45, 515-531. doi:10.18172/cig.3634

Canals, R.M., Múgica, L., Durán, M., San Emeterio, L., 2019. Soil bacterial functional diversity mirrors the loss of plant diversity by the expansion of a native tall-grass in high mountain grasslands.

Plant and Soil 243-257. doi:10.1007/s11104-019-04281-w

Canals, R.M., Pedro, J., Rupérez, E., San-Emeterio, L., 2014. Nutrient pulses after prescribed winter fires and preferential patterns of N uptake may contribute to the expansion of *Brachypodium pinnatum* (L.) P. Beauv. in highland grasslands. Applied Vegetation Science 17, 419-428. doi:10.1111/avsc.12088

Canals, R.M., San Emeterio, L., Durán, M., Múgica, L., 2017. Plant-herbivory feedbacks and selective allocation of a toxic metal are behind the stability of degraded covers dominated by *Brachypodium pinnatum* in acidic soils. Plant and Soil 415, 373-386. doi:10.1007/s11104-016-3153-1

Carthey, A.J.R., Blumstein, D.T., Tetu, S.G., Gillings, M.R., Gallagher, R.V, 2019. Conserving the holobiont. Functional Ecology 34, 764-776. doi:10.1111/1365-2435.13504

Catalán, P., López-Álvarez, D., Díaz-Pérez, A., Sancho, R., López-Herránz, M.L., 2015. Phylogeny and evolution of the genus *Brachypodium*. Plant Genetics and Genomics: Crops Models. doi:10.1007/7397

Catorci, A., Antolini, E., Tardella, F.M., Scocco, P., 2014a. Assessment of interaction between sheep and poorly palatable grass: A key tool for grassland management and restoration. Journal of Plant Interactions 9, 112-121. doi:10.1080/17429145.2013.776706

Catorci, A., Cesaretti, S., Gatti, R., Ottaviani, G., 2011. Abiotic and biotic changes due to spread of *Brachypodium genuense* (DC.) Roem. & Schult. in sub-Mediterranean meadows. Community Ecology 12, 117-125. doi:10.1556/ComEc.12.2011.1.14

Catorci, A., Cesaretti, S., Tardella, F.M., 2014b. Effect of tall-grass invasion on the flowering-related functional pattern of submediterranean hay-meadows. Plant Biosystems 148, 1127-1137. doi:10.1080/11263504.2014.980353

Catorci, A., Scapin, W., Tardella, F.M., Vitanzi, A., 2012. Seedling survival and dynamics of upper timberline in central Apennines. Polish Journal of Ecology 60, 79-94.

Chang, C.C., Turner, B.L., 2019. Ecological succession in a changing world. Journal of Ecology 107, 503-509. doi:10.1111/1365-2745.13132

Chi, W.C., Chen, W., He, C.C., Guo, S.Y., Cha, H.J., Tsang, L.M., Ho, T.W., Pang, K.L., 2019. A highly diverse fungal community associated with leaves of the mangrove plant *Acanthus ilicifolius* var. *xiamenensis* revealed by isolation and metabarcoding analyses. PeerJ. doi:10.7717/peerj.7293

Clay, K., Schardl, C., 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. The American Naturalist 160, 99-127. doi:10.1086/342161

Clements, F.E., 1936. Nature and structure of the climax. Journal of Ecology 24, 252-284. doi:/10.2307/2256278

Creer, S., Deiner, K., Frey, S., Porazinska, D., Taberlet, P., Thomas, W.K., Potter, C., Bik, H.M., 2016. The ecologist's field guide to sequence-based identification of biodiversity. Methods in Ecology and Evolution 7, 1008-1018. doi:10.1111/2041-210X.12574

Davis, M.A., Grime, J.P., Thompson, K., 2000. Fluctuating resources in plant communities: a general theory of invasibility. Journal of Ecology 88, 528-534. doi:10.1046/j.1365-2745.2000.00473.x

de Kroon, H., Bobbink, R., 1997. Clonal plant dominance under elevated nitrogen deposition, with special reference to *Brachypodium pinnatum* in chalk grassland. Ecology and Evolution of Clonal Plants 359-379.

Diaz, S., Cabido, M., Casanoves, F., 1998. Plant functional traits and environmental filters at a regional scale. Journal of Vegetation Science 9, 113-122. doi:10.2307/3237229

During, H.J., Willems, J.H., 1984. Diversity models applied to a chalk grassland. Vegetatio 57, 103-114. doi:10.1007/BF00047305

Ebach, M.C., Valdecasas, A.G., Wheeler, Q.D., 2011. Impediments to taxonomy and users of taxonomy: accessibility and impact evaluation. Cladistics 27, 550-557. doi:10.1111/j.1096-0031.2011.00348.x

Eurostat, 2020. Agriculture, forestry and fishery statistics; 2020 edition, Publications Office of the European Union; Luxembourg.

Fernández-Giménez, M.E., Estaque, F.F., 2012. Pyrenean pastoralists' ecological knowledge: Documentation and application to natural resource management and adaptation. Human Ecology 40, 287-300. doi:10.1007/s10745-012-9463-x

Fernández-López de Pablo, J., Gutiérrez-Roig, M., Gómez-Puche, M., McLaughlin, R., Silva, F., Lozano, S., 2019. Palaeodemographic modelling supports a population bottleneck during the Pleistocene-Holocene transition in Iberia. Nature Communications 10. doi:10.1038/s41467-019-09833-3

Ferrer, C., 2016. Diccionario de pascología. Ed. Sociedad española para el estudio de los pastos (SEEP).

Ferrer, V., Canals, R.M., 2008. Proyecto de ordenación de los recursos pascícolas forestales del monte Aezkoa nº 1 del CUP. Pamplona: Consultoría Belardi & Universidad Pública de Navarra.

Galop, D., Catto, N., 2014. Environmental history of European high mountains. Quaternary International 353, 1-2. doi:10.1016/j.quaint.2014.10.040

García-Ruiz, J.M., Lasanta, T., 2018. The Aragón Pyrenees as a cultural landscape. Pirineos. Revista de ecología de montaña 173. doi:10.3989/pirineos.2018.173005

García-Ruiz, J.M., López-Moreno, J.I., Lasanta, T., Vicente-Serrano, S.M., González-Sampériz, P., Valero-Garcés, B.L., Sanjuán, Y., Beguería, S., Nadal-Romero, E., Lana-Renault, N., Gómez-Villar, A., 2015. Geo-ecological effects of global change in the central spanish Pyrenees: A review at different spatial and temporal scales. Pirineos. Revista de ecología de montaña 170. doi:10.3989/Pirineos.2015.170005

Gassiot, E., Antón, D.R., Mañosa, A.P., Obiol, R.P., Brugués, R.J., Bal-Serin, M.C., Mazzucco, N., 2014. La alta montaña durante la Prehistoria: 10 años de investigación en el Pirineo catalán occidental. Trabajos de Prehistoria 71, 261-281. doi:10.3989/tp.2014.12134

Granado-Tajada, I., Rodríguez-Ramilo, S.T., Legarra, A., Ugarte, E., 2020. Inbreeding, effective population size, and coancestry in the *Latxa* dairy sheep breed. Journal of Dairy Science 103, 5215-5226. doi:10.3168/jds.2019-17743

Grime, J.P., 2001. Plant strategies, vegetation processes, and ecosystem properties, John Wiley & Sons, Chichester, UK.

Guerrero, R., Margulis, L., Berlanga, M., 2013. Symbiogenesis: The holobiont as a unit of evolution. International Microbiology 16, 133-143. doi:10.2436/20.1501.01.188

Hawksworth, D.L., 2001. The magnitude of fungal diversity: The 1.5 million species estimate revisited. Mycological Research 105, 1422-1432. doi:10.1017/S0953756201004725

Hawksworth, D.L., Lücking, R., 2017. Fungal diversity revisited: 2.2 to 3.8 million species. Microbiol Spectrum 5(4):Funk-0052-2016. doi: 10.1128/microbiolspec.FUNK-0052-2016

Házi, J., Bartha, S., Szentes, S., Wichmann, B., Penksza, K., 2011. Seminatural grassland management by mowing of *Calamagrostis epigejos* in Hungary. Plant Biosystems 145, 699-707. doi:10.1080/11263504.2011.601339

Head, I.M., Prosser, J.I., 2007. Microorganisms, macroorganisms and ecology. In: Symposium of the society for general microbiology. Environmental microbiology group and the British ecological society. doi:10.1111/j.1574-6941.2007.00395.x

Hofmann, R.R., 1989. Evolutionary steps of ecophysiological adaptation and diversification of ruminants: a comparative view of their digestive system. Oecologia 78, 443-457. doi:10.1007/BF00378733

Holub, P., Tuma, I., Zahora, J., Fiala, K., 2012. Different nutrient use strategies of expansive grasses *Calamagrostis epigejos* and *Arrhenatherum elatius*. Biologia 67, 673-680. doi:10.2478/s11756-012-0050-9

Hume, D.E., Sewell, J.C., 2014. Agronomic advantages conferred by endophyte infection of perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinacea* Schreb.) in Australia. Crop and Pasture Science 65, 747-757. doi:10.1071/CP13383

Hurst, A., John, E., 1999. The biotic and abiotic changes associated with *Brachypodium pinnatum* dominance in chalk grassland in south-east England. Biological Conservation 88, 75-84.

Hyde, K.D., Soytong, K., 2008. The fungal endophyte dilemma. Fungal Diversity 33, 133-173. doi:10.1016/S0006-3207(98)00089-5

Insausti, K., Beldarrain, L.R., Lavín, M.P., Aldai, N., Mantecón, Á.R., Sáez, J.L., Canals, R.M., 2021. Horse meat production in northern Spain: Ecosystem services and sustainability in high nature value farmland. Animal Frontiers 11, 47-54. doi:10.1093/af/vfab003

Jones, S.R., Cushman, R.C., 2004. The North American Prairie, Peterson Field Guides.

Jorgensen, R.A., Cluster, P.D., 1988. Modes and tempos in the evolution of nuclear ribosomal DNA: New characters for evolutionary studies and new markers for genetic and population studies. Annals of the Missouri Botanical Garden 75, 1238-1247. doi:10.2307/2399282

Kelly, R.L., Surovell, T.A., Shuman, B.N., Smith, G.M., 2013. A continuous climatic impact on Holocene human population in the Rocky Mountains. Proceedings of the National Academy of Sciences of the United States of America 110, 443-447. doi:10.1073/pnas.1201341110

Ketner-Oostra, R., Sýkora, K. V., 2004. Decline of lichen-diversity in calcium-poor coastal dune vegetation since the 1970s, related to grass and moss encroachment. Phytocoenologia 34, 521-549. doi:10.1127/0340-269X/2004/0034-0521

Komac, B., Kefi, S., Nuche, P., Escós, J., Alados, C.L., 2013. Modeling shrub encroachment in subalpine grasslands under different environmental and management scenarios. Journal of Environmental Management 121, 160-169. doi:10.1016/j.jenvman.2013.01.038

Lasanta, T., Vicente-Serrano, S.M., 2006. Factores en la variabilidad espacial de los cambios de cubierta vegetal en el Pirineo. Cuadernos de Investigación Geográfica 32, 57-80.

Leys, B.A., Marlon, J.R., Umbanhowar, C., Vannière, B., 2018. Global fire history of grassland biomes. Ecology and Evolution 8, 8831-8852. doi:10.1002/ece3.4394

Lutzoni, F., Kauff, F., Cox, C.J., McLaughlin, D., Celio, G., Dentinger, C., Padamsee, M., Hibbett, D., James, T.Y., Baloch, E., et al., 2004. Assembling the fungal tree of life: Progress, classification, and evolution of subcellular traits. American Journal of Botany 91, 1446-1480. doi:10.3732/ajb.91.10.1446

Malinowski, D., Belesky, P., 2000. Adaptations of endophyte-infected cool-season grasses to environmental stresses: Mechanisms of drought and mineral stress tolerance. Crop Science 40, 923-940. doi:10.2135/cropsci2004.0523

Malinowski, D., Leuchtmann, A., Schmidt, D., Nösberger, J., 1997. Symbiosis with *Neotyphodium uncinatum* endophyte may increase the competitive ability of meadow fescue. Agronomy Journal 89, 833-839. doi:10.2134/agronj1997.00021962008900050019x

McIntyre, S., Díaz, S., Lavorel, S., Cramer, W., 1999. Plant functional types and disturbance dynamics. Journal of Vegetation Science 10, 603-608. doi:10.1111/j.1654-1103.1999.tb00103.x

Millennium Ecosystem Assessment (MEA), 2005. Ecosystems and human well-being: Synthesis. Island Press, Washington, DC., 2005. doi:10.1196/annals.1439.003

Ministerio de medio ambiente y medio rural y marino, 2019. Razas de ganado del catálogo oficial de España.

Mojzes, A., Kalapos, T., Virágh, K., 2003. Plasticity of leaf and shoot morphology and leaf photochemistry for *Brachypodium pinnatum* (L.) Beauv. growing in contrasting microenvironments in a semiarid loess forest-steppe vegetation mosaic. Flora 198, 304-320. doi:10.1078/0367-2530-00102

Montané, F., Casals, P., Taull, M., Lambert, B., Dale, M.R.T., 2009. Spatial patterns of shrub cover after different fire disturbances in the Pyrenees. Annals of Forest Science 66, 612-612. doi:10.1051/forest/2009050

Mueller, G.M., Bills, G.F., Foster, M.S., 2004. Biodiversity of Fungi: Inventory and monitoring methods, Elsevier A. ed.

Múgica, L., Canals, R.M., San Emeterio, L., 2018. Changes in soil nitrogen dynamics caused by prescribed fires in dense gorse lands in SW Pyrenees. Science of the Total Environment 639, 175-185. doi:10.1016/j.scitotenv.2018.05.139

Múgica, L., Canals, R.M., San Emeterio, L., Peralta, J., 2021. Decoupling of traditional burnings and grazing regimes alters plant diversity and dominant species competition in high-mountain grasslands. Science of the Total Environment 790, 147917. doi:10.1016/j.scitotenv.2021.147917

Niedrist, G., Tasser, E., Lüth, C., Dalla Via, J., Tappeiner, U., 2009. Plant diversity declines with recent land use changes in European Alps. Plant Ecology 202, 195-210. doi:10.1007/s11258-008-9487-x

Nilsson, R.H., Larsson, K.H., Taylor, A.F.S., Bengtsson-Palme, J., Jeppesen, T.S., Schigel, D., Kennedy, P., Picard, K., Glöckner, F.O., Tedersoo, L., et al., 2019. The UNITE database for molecular identification of fungi: Handling dark taxa and parallel taxonomic classifications. Nucleic Acids Research 47, D259-D264. doi:10.1093/nar/gky1022

Pardo, I., Doak, D.F., García-González, R., Gómez, D., García, M.B., 2015. Long-term response of plant communities to herbivore exclusion at high elevation grasslands. Biodiversity and Conservation 24, 3033-3047. doi:10.1007/s10531-015-0996-3

Pärtel, M., Bruun, H.H., Sammul, M., 2005. Biodiversity in temperate European grasslands: origin and conservation. Grassland Science in Europe 10, 1-14. doi:10.1046/j.1472-765X.2002.01111.x

Pausas, J.G., Bond, W.J., 2020. Alternative biome states in terrestrial ecosystems. Trends in Plant Science 25, 250-263. doi:10.1016/j.tplants.2019.11.003

Peralta, J., 2010. Vegetación Potencial de Navarra. Comarca Agraria II: Pirineos. Memoria.

Pereira, E., Vázquez-de-Aldana, B.R., San Emeterio, L., Zabalgogeazcoa, I., 2019. A survey of culturable fungal endophytes from *Festuca rubra* subsp. *pruinosa*, a grass from marine cliffs, reveals a core microbiomes. Frontiers in Microbiology 9, 1-14. doi:10.3389/fmicb.2018.03321

Poschlod, P., WallisDeVries, M.F., 2002. The historical and socioeconomic perspective of calcareous grasslands - Lessons from the distant and recent past. Biological Conservation 104, 361-376. doi:10.1016/S0006-3207(01)00201-4

Pottier, J., Evette, A., 2010. On the relationship between clonal traits and small-scale spatial patterns of three dominant grasses and its consequences on community diversity. Folia Geobotanica 45, 59-75.

Razquin, M.M., Aranguren, E., Taüll, M., Fernández-Otal, J.A., Ferrer, V., Gascouat, P., Canals, R.M., 2012. International grassland communals (*facerías*) in the Pyrenees: history, current situation and future prospects. In: Canals, R.M., San Emeterio, L. (Eds.), Nuevos retos de la ganadería extensiva: un agente de conservación en peligro de extinción. SEEP, Pamplona.

Redman, R.S., Sheehan, K.B., Stout, R.G., Rodriguez, R.J., Henson, J.M., 2002. Thermotolerance generated by plant/fungal symbiosis. Science 298, 1581. doi:10.1126/science.1078055

Remmert, H., 1991. The mosaic-cycle concept of ecosystems, Remmert, H. (ed.) The Mosaic-cycle concept of ecosystems. Springer, Berlin, pp. 11-21.

Rius, D., Vannière, B., Galop, D., 2012. Holocene history of fire, vegetation and land use from the central Pyrenees (France). Quaternary Research 77, 54-64. doi:10.1016/j.yqres.2011.09.009

Rodríguez, R.J., Redman, R.S., 2008. More than 400 Million years of ecolution and some plants still can't make it on their own: Plant stress tolerance via fungal symbiosis. Journal of Experimental Botany 59, 1109-1114. doi:10.1093/jxb/erm342

Rodriguez, R.J., White, J.F., Arnold, A.E., Redman, R.S., 2009. Fungal endophytes: Diversity and functional roles. New Phytologist 182, 314-330. doi:10.1111/j.1469-8137.2009.02773.x

Saikkonen, K., Faeth, S.H., Helander, M., Sullivan, T.J., 1998. Fungal endophytes: A continuum of interactions with host plants. Annual Review of Ecology and Systematics 29, 319-43. doi:10.1146/annurev.ecolsys.29.1.319

Saikkonen, K., Saari, S., Helander, M., 2010. Defensive mutualism between plants and endophytic fungi? Fungal Diversity 41, 101-113. doi:10.1007/s13225-010-0023-7

Saikkonen, K., Wäli, P., Helander, M., Faeth, S.H., 2004. Evolution of endophyte-plant symbioses. Trends in Plant Science 9, 275-280. doi:10.1016/j.tplants.2004.04.005

Sala, O.E., Vivanco, L., Flombaum, P., 2013. Grassland ecosystems, encyclopedia of biodiversity: Second edition. Elsevier Ltd. doi:10.1016/B978-0-12-384719-5.00259-8

Samson, F., Knopf, F., 1994. Prairie conservation in North America. BioScience 44, 418-421. doi:10.2307/1312365

San Emeterio, L., Durán, M., Múgica, L., Jiménez, J.J., Canals, R.M., 2021. Relating the spatial distribution of a tall-grass to fertility islands in a temperate mountain grassland. Soil Biology and Biochemistry 163:108455. doi:10.1016/j.soilbio.2021.108455

San Emeterio, L., Múgica, L., Ugarte, M.D., Goicoa, T., Canals, R.M., 2016. Sustainability of traditional pastoral fires in highlands under global change: Effects on soil function and nutrient cycling. Agriculture, Ecosystems and Environment 235, 155-163. doi:10.1016/j.agee.2016.10.009

Sánchez-Márquez, S., Bills, G.F., Herrero, N., Zabalgogeazcoa, I., 2012. Non-systemic fungal endophytes of grasses. Fungal Ecology 5, 289-297. doi:10.1016/j.funeco.2010.12.001

Savage, C., 2004. Prairie: A natural history. David Suzuki Foundation.

Schardl, C.L., Leuchtmann, A., Spiering, M.J., 2004. Symbioses of grasses with seedborne fungal endophytes. Annual Review of Plant Biology 55, 315-340. doi:10.1146/annurev.arplant.55.031903.141735

Schardl, C.L., Young, C.A., Moore, N., Krom, N., Dupont, P.Y., Pan, J., Florea, S., Webb, J.S., Jaromczyk, J., Jaromczyk, J.W., Cox, M.P., Farman, M.L., 2014. Genomes of plant-associated Clavicipitaceae, 1st ed, Advances in Botanical Research. Elsevier Ltd. doi:10.1016/B978-0-12-397940-7.00010-0

Scheffer, M., Carpenter, S., Foley, J.A., Folke, C., Walker, B., 2001. Catastrophic shifts in ecosystems. Nature 413, 591-596. doi:10.1038/35098000

Schläpfer, F., 1997. Influence of management on cover and seed production of *Brachypodium pinnatum* (L.) Beauv. in a calcareous grassland. Bulletin of the Geobotanical Institute ETH 63, 3-10. doi: 10.5169/seals-377802

Schläpfer, F., Fischer, M., 1998. An isozyme study of clone diversity and relative importance of sexual and vegetative recruitment in the grass *Brachypodium pinnatum*. Ecography 21, 351-360. doi:10.1111/j.1600-0587.1998.tb00400.x

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America 109, 6241-6246. doi:10.1073/pnas.1117018109

Schulz, B., Boyle, C., 2005. The endophytic continuum. Mycological Research 109, 661-686. doi:10.1017/S095375620500273X

Schulz, B., Wanke, U., Draeger, S., Aust, H.-J., 1993. Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. Mycological Research 97, 1447-1450. doi:10.1016/S0953-7562(09)80215-3

Simon, J.C., Marchesi, J.R., Mougel, C., Selosse, M.A., 2019. Host-microbiota interactions: from holobiont theory to analysis. Microbiome 7:5. doi: 10.1186/s40168-019-0619-4

Soto-Barajas, M.C., Zabalgogeazcoa, I., González-Martin, I., Vázquez-de-Aldana, B.R., 2018. Near-infrared spectroscopy allows detection and species identification of *Epichloë* endophytes in *Lolium perenne*. Journal of the Science of Food and Agriculture 98, 5037-5044. doi:10.1002/jsfa.9038

Stein, E.D., Martinez, M.C., Stiles, S., Miller, P.E., Zakharov, E. V., 2014. Is DNA barcoding actually cheaper and faster than traditional morphological methods: Results from a survey of freshwater bioassessment efforts in the United States? Plos One 9. doi:10.1371/journal.pone.0095525

Svenning, J.C., 2002. A review of natural vegetation openness in north-western Europe. Biological Conservation 104, 133-148. doi:10.1016/S0006-3207(01)00162-8

Tardella, F.M., Bricca, A., Goia, I.G., Catorci, A., 2020. How mowing restores montane Mediterranean grasslands following cessation of traditional livestock grazing. Agriculture, Ecosystems and Environment 295, 106880. doi:10.1016/j.agee.2020.106880

Tardella, F.M., Bricca, A., Piermarteri, K., Postiglione, N., Catorci, A., 2017. Context-dependent variation of SLA and plant height of a dominant, invasive tall grass (*Brachypodium genuense*) in sub-Mediterranean grasslands. Flora 229, 116-123. doi:10.1016/j.flora.2017.02.022

Tardella, F.M., Malatesta, L., Goia, I.G., Catorci, A., 2018. Effects of long-term mowing on coenological composition and recovery routes of a *Brachypodium rupestre*-invaded community: insight into the restoration of sub-Mediterranean productive grasslands. Rendiconti Lincei. Scienze Fisiche e Naturali 29, 329-341. doi:10.1007/s12210-018-0711-x

Thomas, A.C., Deagle, B.E., Eveson, J.P., Harsch, C.H., Trites, A.W., 2016. Quantitative DNA metabarcoding: improved estimates of species proportional biomass using correction factors derived from control material. Molecular Ecology Resources 16, 714-26. doi:10.1111/1755-0998.12490

Tikhonovich, I.A., Provorov, N.A., 2011. Microbiology is the basis of sustainable agriculture: An opinion. Annals of Applied Biology 159, 155-168. doi:10.1111/j.1744-7348.2011.00489.x

Valdés, B., Scholz, H., 2009. *Brachypodium genuense* (DC.) Roem. & Schult. Euro+Med PlantNase-the information resource for Euro-Mediterranean plant diversity. http://ww2.bgbm.org/euroPlusMed/PTaxonDetal.asp?UUID=142AB795-174E-40A3-9787-9B1D435403FA.

Valéry, L., Fritz, H., Lefeuvre, J.C., Simberloff, D., 2009a. Invasive species can also be native. Trends in Ecology and Evolution 24, 585. doi:10.1016/j.tree.2009.07.003.

Valéry, L., Fritz, H., Lefeuvre, J.C., Simberloff, D., Valéry, L., Fritz, H., Lefeuvre, J.C., Simberloff, D., 2009b. Ecosystem-level consequences of invasions by native species as a way to investigate relationships between evenness and ecosystem function. Biological Invasions 11, 609-617. doi:10.1007/s10530-008-9275-5

Valéry, L., Radureau, A., Lefeuvre, J.C., 2017. Spread of the native grass *Elymus athericus* in salt marshes of Mont-Saint-Michel bay as an unusual case of coastal eutrophication. Journal of Coastal Conservation 21, 421-433. doi:10.1007/s11852-016-0450-z

Van Andel, T.H., Tzedakis, P.C., 1996. Palaeolithic landscapes of Europe and environs, 150,000-25,000 years ago: An overview. Quaternary Science Reviews 15, 481-500. doi:10.1016/0277-3791(96)00028-5

van den Pol-van Dasselaar, A., Bastiaansen-Aantjes, L., Bogue, F., O'Donovan, M., Huygue, C., 2019. Grassland use in Europe. A syllabus for young farmers.

Vandenkoornhuyse, P., Baldauf, S.L., Leyval, C., Straczek, J., Young, J.P.W., 2002. Extensive fungal diversity in plant roots. Science 295, 2051. doi:10.1126/science.295.5562.2051

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., Dufresne, A., 2015. The importance of the microbiome of the plant holobiont. New Phytologist 206, 1196-1206. doi:10.1111/nph.13312

Vázquez-de-Aldana, B.R., Cuesta, M.J., Zabalgogeazcoa, I., 2019. Cultivation and growth dynamics of endophytic fungi in a solid culture medium based on sugar beet pulp. Journal of the Science of Food and Agriculture. doi:10.1002/jsfa.10030

Veer, M.A.C., Kooijman, A.M., 1997. Effects of grass-encroachment on vegetation and soil in Dutch dry dune grassland. Plant and Soil 192, 119-128. doi:10.1023/A:1004280300820

Vera, F.W.M., 2000. Grazing Ecology and Forest History. CABI.

Vitasović, I., Tardella, F.M., Grbeša, D., Škvorc, Z., Catorci, A., 2014. Effects of abandonment on the functional composition and forage nutritive value of a North Adriatic dry grassland community (Ćićarija, Croatia). Applied Ecology and Environmental Research 12, 285-299. doi:10.15666/aeer/1201_285299

Vitasović, I., Tardella, F.M., Ruščić, M., Catorci, A., 2011. Assessment of floristic diversity, functional composition and management strategy of North Adriatic pastoral landscape (Croatia). Polish Journal of Ecology 59, 765-776.

Wedin, D.A., Tilman, D., 1990. Species effects on nitrogen cycling: a test with perennial grasses. Oecologia 84, 433-441. doi:10.1007/BF00328157

White, R., Murray, S., Rohweder, M., 2000. Pilot analysis of global ecosystems: Grassland ecosystems, world resources institute. Washington, DC. doi:10.1016/B978-0-12-384719-5.00259-8

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A guide to methods and applications, Ed M.A. Innis (San Diego, CA: Academic Press). pp. 315-322.

Williams, W.M., Easton, H.S., Jones, C.S., 2007. Future options and targets for pasture plant breeding in New Zealand. New Zealand Journal of Agricultural Research 50, 223-248. doi:10.1080/00288230709510292

Wilson, J.B., Peet, R.K., Dengler, J., Pärtel, M., 2012. Plant species richness: The world records. Journal of Vegetation Science 23, 796-802. doi:10.1111/j.1654-1103.2012.01400.x

Yuan, Z. lin, Zhang, C. long, Lin, F. cheng, 2010. Role of diverse non-systemic fungal endophytes in plant performance and response to stress: progress and approaches. Journal of plant growth regulation 29, 116-126. doi:10.1007/s00344-009-9112-9

Zabalgogeazcoa, I., 2008. Review. Fungal endophytes and their interaction with plant pathogens. Spanish Journal of Agricultural Research 6, 138-146. doi:10.5424/sjar/200806s1-382

Zabalgogeazcoa, I., Vázquez-de-Aldana, B.R., García-Ciudad, A., García-Criado, B., 2003. Fungal endophytes in grasses from semi-arid permanent grasslands of western Spain. Grass and Forage Science 58, 94-97. doi:10.1046/j.1365-2494.2003.00352.x

OBJETIVOS

OBJETIVOS

A pesar de los resultados que arrojan los estudios relacionados con la expansión de gramíneas altas perennes del género *Brachypodium* sp., y el relevante papel que la ganadería extensiva tiene para frenar el proceso degradativo que ocasionan, la concienciación y transmisión del conocimiento científico a los gestores del territorio resulta difícil y compleja. Dado que en la sociedad actual el valor ambiental que generan los ecosistemas carece aún de precio de mercado, encontrar una metodología que permita cuantificar la degradación ambiental, aunque sea relacionada con un único servicio ecosistémico, permite despertar conciencia y justificar acciones de mejora y de restauración. El proceso de degradación de los pastos de montaña debido a la expansión de *B. rupestre* y la pérdida del servicio ecosistémico de aprovisionamiento, ligado a la pérdida de diversidad florística, se describe con detalle en el capítulo 1 de esta memoria.

OBJETIVO 1: Cuantificar económicamente la pérdida del servicio ecosistémico de abastecimiento de alimento de calidad para el ganado en los pastos de puerto del valle de Aezkoa.

El estudio del micobioma de *B. rupestre* resulta ser una novedosa e inexplorada línea de investigación. Caracterizar la comunidad endofítica de esta gramínea en su ambiente natural, sometida a condiciones climáticas extremas y bajo fuertes y variables regímenes de perturbación de fuego y pastoreo, permitirá conocer las especies fúngicas presentes e iniciar una investigación para discernir si alguna de ellas pudiera estar aportando alguna adaptación ventajosa en su proceso expansivo, además de contribuir a entender y predecir la resiliencia de los ecosistemas de alta montaña al cambio global.

Para una adecuada caracterización es necesario tener en cuenta los diferentes tejidos de las plantas. La divergencia de microambientes en los que se desarrolla la parte vegetal aérea en comparación con sus tejidos subterráneos, puede conducir al establecimiento de comunidades endofíticas distintas. Nos encontramos por tanto con una gramínea desarrollándose en un clima extremo de montaña y formada por tejidos que crecen en microhábitats afectados de forma diferente por los regímenes de pastoreo y fuego. Para caracterizar el micobioma de *B. rupestre* se utilizaron los métodos de cultivo tradicional y de *metabarcoding*. Ambos procedimientos se describen detalladamente en el capítulo 2 y 3 de esta memoria. Adicionalmente, en el tercer capítulo, se realizó una comparativa de metodologías para determinar la capacidad de caracterización de ambas técnicas.

OBJETIVO 2: Caracterizar la comunidad endofítica de *Brachypodium rupestre* bajo las condiciones naturales en las que se desarrolla en los pastos de puerto del valle de Aezkoa mediante el método de cultivo tradicional y de *metabarcoding*.

OBJETIVO 3: Analizar la respuesta de la comunidad endofítica de *Brachypodium rupestre* a los cambios en la composición florística promovidos por la alteración de los regímenes tradicionales de pastoreo y fuego en los pastos de puerto del valle de Aezkoa.

OBJETIVO 4: Comparar la capacidad de caracterización de la comunidad endofítica de *Brachypodium rupestre* mediante el método de cultivo tradicional y de *metabarcoding*.

CHAPTER 1

1515

Disruption of traditional land use regimes causes an economic loss of provisioning services in high-mountain grasslands

ABSTRACT

Mountain ecosystems face many challenges related to global change. Most high-altitude grasslands in the Pyrenees, despite representing valuable assets recognised in the European conservation heritage, are at risk due to the decline of traditional extensive ranging. This research intends to quantify economically the loss of the provisioning service of high-quality food for livestock of an upland area on the western side of the range. The area is experiencing degradation due to the expansion of the native tall-grass *Brachypodium rupestre*, favoured by disruption of traditional grazing and anthropogenic fire regimes. We implement the substitution economic approach and use floristic and husbandry data to determine that the loss of food rations for livestock results in an unitary cost of 107€/ha, per year, amounting to 21146€ for the whole degraded area, according to the most conservative estimate. The study also finds evidence that the decline in grassland value is closely associated with the digestibility to herbivores of *B. rupestre* during the growing season. This approach may be an effective tool to raise awareness of the problem among local and regional stakeholders and encourage further environmental actions to prevent the degradation.

1. INTRODUCTION

Natural grasslands in high-mountain areas are one of the main biodiversity hotspots on Earth and provide an invaluable array of ecosystem services (ES), which include provisioning (food, water, raw materials and medicinal resources), regulating (air and water quality, carbon sequestration and storage, erosion prevention and soil fertility, pollination, biological control and water regulation), supporting (habitats for species and maintenance of genetic diversity) and cultural (recreation, tourism, walking) services (MEA, 2005; Pan et al., 2014; Sollenberger et al., 2019). With respect to the cultural heritage, high mountain grasslands represent the preservation of a legacy of the ancestral interaction between humans and herbivores (Wilson et al., 2012). For millennia, shepherds emulated the annual movements of nomadic wild herbivores, and high-altitude grasslands turned into a crucial component of the transhumance system as providers of food for domestic herbivores during the mild season (Galop and Catto, 2014; González-Sampériz et al., 2017).

Despite the fact that traditional pastoralism has contributed to the multifunctionality and quality of the ES provided by mountain grasslands (Sebastià et al., 2008; Gómez-García et al., 2009; Bernués et al., 2014), in recent decades the system in most high-altitude European ranges has been collapsing, affecting in different ways the provision of ES (Gehrig-Fasel et al., 2007; Schirpke et al., 2017). The Pyrenean mountain range is experiencing this situation (Vicente-Serrano et al., 2004; Lasanta et al., 2015; Canals, 2019). Rural depopulation, low people dedicated to the primary sector and, in the last fifty years, farm modernisation via intensification, replicating lowland breeding schemes, has led to the abandonment of many mountain grasslands. Extensive ranging has been partially replaced by intensified productive systems, which entail larger periods of livestock housing and feeding with fodder and concentrates (Petz et al., 2014; García-Ruiz et al., 2015; Lasanta et al., 2015; Guadilla-Sáez et al., 2019). The decline of grazing in grasslands has resulted in rapid vegetation succession, revealed by pine afforestation (*Pinus uncinata*, *Pinus sylvestris*) and shrub encroachment (*Buxus sempervirens*, *Genista scorpius*, *Ulex europaeus* and *Juniperus communis*, among others) (Komac et al., 2013; Gartzia et al., 2014).

In the particular case of the western Pyrenees, the amount of non-grazed summer biomass of shrubs and grasses has encouraged a more intense use of controlled burning to reduce necromass accumulation. As a consequence, the long-established practice of pastoral fires has been replaced by larger and more frequent burns (Múgica et al., 2018). The unbalanced regime of anthropic disturbances (traditional high grazing - low burning practices *vs*. current low grazing - high burning practices) has resulted in a dramatic change in species composition and in the spread of the native tall-grass *Brachypodium rupestre* (Host) Roem. & Schult. The species represents a guild of tussocky perennial grasses of tall stature, with high leaf dry-matter content and extensive lateral spread via rhizomes (Kahlert et al., 2005), well-adapted to intense regimes of disturbance and with the capacity to displace other native species, leading to a loss of grassland diversity (Niedrist et al., 2009; Tardella et al., 2017). In the case of *B. rupestre*, its poor palatability and digestibility to livestock results in a limited grazing and a build-up of much necromass by the end of the season, which perpetuates the vicious circle of fire use (Canals et al., 2014).

The consequences of the spread of *B. rupestre* are comparable to that of exotic invaders-successful development and species replacement, diversity loss and functional degradation (Valéry et al., 2009a), however, since the species is a native component of grasslands, there is a confusing perception of the problem at the local level (Canals, 2019). How stakeholders perceive the changes and their effects on ES is a crucial question, since they are the genuine drivers of change in the rural areas (Lamarque et al., 2011, 2014; Meyfroidt et al., 2013). Through the quantification of the costs associated to the loss of the ES we may be able to raise local stakeholders' and policy makers' awareness of the issue (Burkhard et al., 2012) and, to that end, we have planned this research. We assess how the expansion and dominance of *B. rupestre* caused by the disruption of the historical disturbance regime affects the provisioning value (food for herbivores) of high-altitude grassland areas. The opportunity cost of grasslands has been determined by applying value equivalency analysis, following the resource equivalency approach. Data for the study were obtained from field floristic surveys, detailed determinations of animal energy requirements and current costs of the feed rations through a practical farm-approach. To our knowledge, this is the first study addressing the loss of economic value of high-mountain grasslands due to the decline of traditional grazing practices.

2. MATERIAL AND METHODS

2.1. The study area

The Aezkoa valley (Navarra county, Spain) is the most western valley on the southern side of the Pyrenean range (42.53′ - 43.3′ N, 1.8′ - 1.17′ W), and encompasses an area of 198 km^2 populated by 873 inhabitants in nine municipalities (Figure 1). The climate is snowy and cold in winter, and mild and foggy in summer. The annual temperature averages 9.3°C and the accumulated precipitation reaches 1856 mm per year (Irabia climatic station; http://meteo.navarra.es). Natural grasslands develop at the highest altitudes of the valley (800 - 1400 m.a.s.l.) and comprise diverse communities of perennial grasses (*Festuca* gr. *rubra*, *Agrostis capillaris*, *Brachypodium rupestre*, *Danthonia decumbens*), forbs (*Achillea millefolium*, *Potentilla erecta*, *Gallium saxatile*) and legumes (*Trifolium repens*, *Lotus corniculatus*), and, also, in some territories, large, degraded communities dominated by the tall-grass *B. rupestre*. All these grasslands, diverse and degraded, are included in the Site of Common Interest (SCI) Roncesvalles-Selva de Irati (code ES0000126), and represent 11.9% of the total area protected (2147 ha over a total of 18078 ha), which encompasses valuable forests (e.g. *Fagus sylvatica*, *Abies alba*) and shrubland communities (e.g. *Erica* spp., *Ulex gallii*). The regional law 9/2011, 7th February, declared the area a Special Area of Conservation (SAC) and approved the management plan that regulates the uses of the territory. Regarding high-altitude grasslands, the general objective of the plan is to guarantee grazing activity that maintains the natural value of grassland communities. Neither ploughing nor sowing are permitted in the most valuable communities but mechanical clearing and prescribed burns are authorised to preserve the mosaic of shrublands and grasslands.

2.2. High-altitude grassland and its management

High altitude grasslands included in the SAC are distributed in three major grazing units, known as *Puertos Grandes* (1692 ha), *Abodi* (237 ha) and *Sariozar* (218 ha) (Figure 1). The areas belong to the commons of the valley and are managed by a representative organisation, the *Junta General del Valle de Aezkoa*, which oversees the use of the common resources (i.e. water, timber, fodder) through specific regulations (for grasslands, regional law 121/2001, 5th October). Since ancient times, extensive ranging of livestock has been a pillar of the economy of the valley. In the highlands, the abundance of megalithic remains attests to the ancient importance of summer pastoralism and the transhumance movements. During winter, sheep flocks were kept on lowland grasslands close to the Atlantic coast and moved to the high mountains in summer. Over the last century, livestock breeding in the Aezkoa valley has progressively declined, the sharpest fall occurring in the recent decades, from 130 breeders established in 2003 with 11500 sheep, 3928 cows and 540 horses (Ferrer and Canals,

2008) to 98 breeders with 5570 sheep, 2110 cows and 760 horses fifteen years later (2017). In addition, the long transhumance movements have almost disappeared and herds of the native breeds of sheep (*Latxa*), cattle (*Pirenaica*) and horses (*Burguete*), remain in the valley bottom from October to April, grazing in lowland areas and/or feeding on stored fodder and concentrate, and move to high-altitude grasslands from May/June to September/October for extensive free grazing.

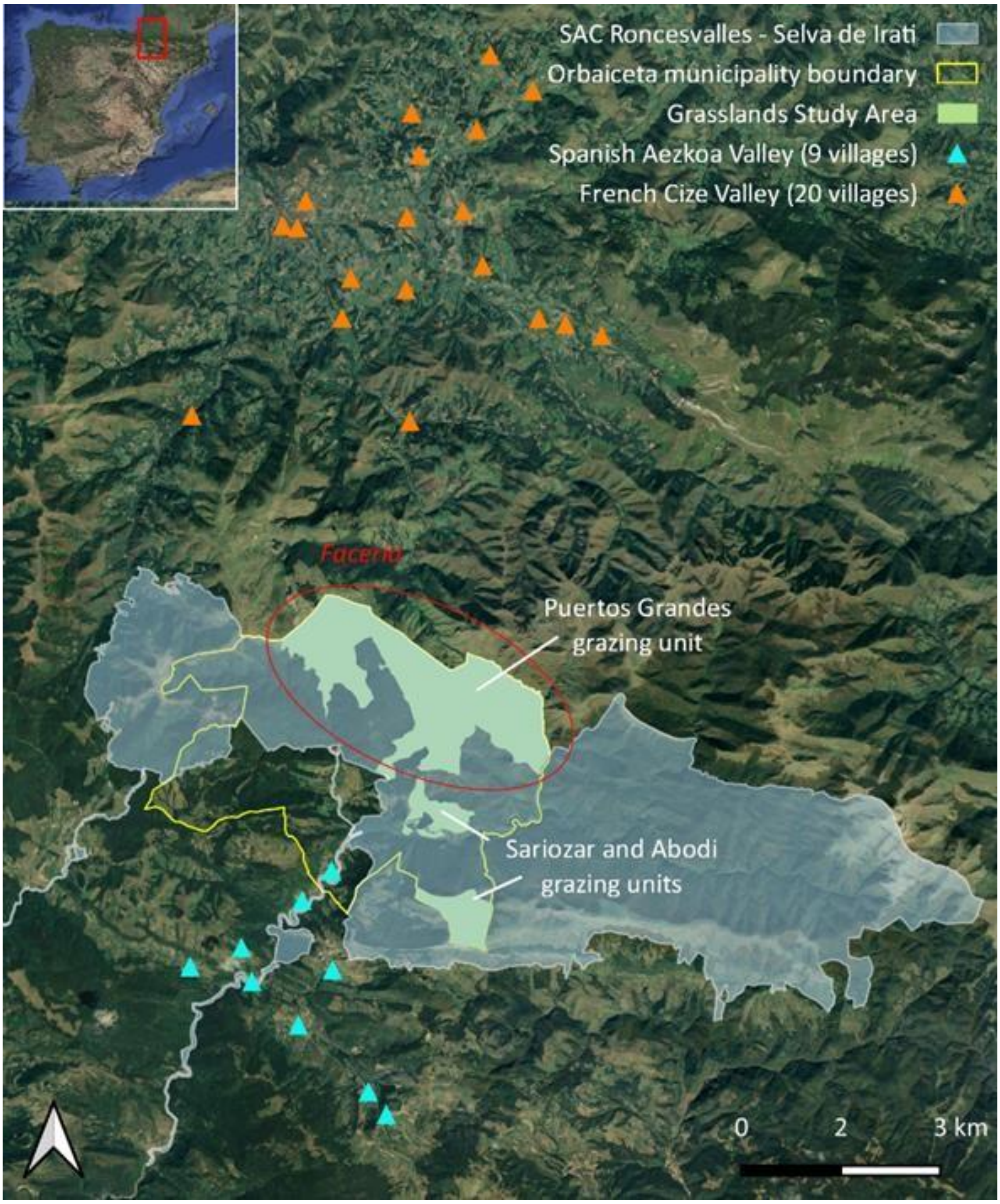


Figure 1. Grassland study area and grazing units of *Puertos Grandes*, *Sariozar* and *Abodi*. Geographic locations of the villages included in the *facería* agreement and delimitation of the Site of Common Interest Roncesvalles-Selva de Irati (ES0000126).

The largest grassland area, *Puertos Grandes*, located in the northernmost part of the valley, close to the French border, has inherited a historical use regime that deserves particular mention. The grazing of most of the area is shared with livestock from the French valley of Cize. The joint use of highland areas by different valleys, known as *facería* (Figure 1), relies on old agreements which were very common through the Pyrenees before the boundaries between the modern countries were established in the mid XIX century (Bayonne International Boundary Treaty signed in 1856). The first records of the existence of treaties date back to 1280 CE (1556 CE for the Aezkoa-Cize agreement) and, although many of them are outdated or no longer in force, a few remain and have been modernised, as is the case for the Aezkoa-Cize treaty (Razquin et al., 2012). Cross-border grazing in *Puertos Grandes* is regulated through annual agreements between the two local representative organisations, *Junta General del Valle de Aezkoa* and *Comision Syndicale du Pays de Cize*. Modern regulations include livestock records, health controls and the payment of a tax per animal for the use of foreign pastures (Table 1). Compared to Aezkoa, the valley of Cize is larger (341 km2) and has a bigger population, with 6880 inhabitants in 20 villages. It has developed a successful farming economy based on the production of high-quality sheep cheeses supported by nearly 400 breeders (owners of 60000 sheep, 3500 cows and 750 horses). As a consequence, for more than fifty French farmers, *Puertos Grandes* is a valuable summer pasture and they are willing to pay for its use. For the Aezkoa stakeholders, it delivers useful yearly revenue and a valorisation of their own grasslands.

Table 1. French livestock numbers and taxes paid by the syndicate of Cize for the use of high altitude grasslands included in the old agreement (*facería*) with the Aezkoa valley over the years 2008 to 2018. Sheep numbers include ewes and lambs (except in 2013 only ewes) since taxes are due on both, unlike cattle and horses. €: taxes per animal, n: number of animals.

Year	€.animal^{-1}	Sheep	Cattle	Horses	Total income
2008	€	5.48	44.54	57.9	61328.6
	n	8050	328	45	
2009	€	5.53	44.92	58.4	61878.3
	n	8050	328	45	
2010	€	5.58	45.3	58.89	62714.1
	n	7870	350	50	
2011	€	5.76	46.07	59.89	64450.2
	n	7870	350	50	
2012	€	5.9	47.18	61.34	66484.8
	n	7870	360	50	
2013	€	6.02	48.17	62.63	57478.1
	n	5855	390	55	
2014	€	6.05	48.49	62.91	69984.7
	n	7870	390	55	
2015	€	6.02	48.13	62.63	69591.6
	n	7870	390	55	
2016	€	6.02	48.27	62.75	70387.7
	n	7870	400	59	
2017	€	6.09	48.8	63.44	71191.3
	n	7870	400	59	
2018	€	6.16	49.37	64.18	73203.6
	n	7870	415	66	

The use of controlled burns to rejuvenate pasturelands and decrease shrub encroachment is a deeply-rooted practice in the western Pyrenees. Traditionally, pastoral fires consisted of very localised shrub-to-shrub burnings that reduced any shoots that remained ungrazed at the end of the season (San Emeterio et al., 2016). However, the decline in grazing activity in the last century, associated not only with a drop in livestock numbers, but also significant changes in herbivore species (fewer sheep compared to cows and horses), and pastoral practices (less continuous presence of shepherds and less shepherding of animals to different altitudes), has encouraged the use of large-scale burns applied every 2-3 winters in the most extreme cases (Múgica et al., 2018). Burns are recurrent on the French side, where the use of fire by shepherds is less restricted, and these fires frequently affect grasslands that span the border. Since they are conducted in winter time in remote, high-altitude areas, and affect *facería* lands, which are grazed predominantly by French livestock, there is little intervention by the local Spanish authorities. In fact, some Spanish shepherds support the practice too since fires reduce shrub encroachment and appear to boost the establishment of a dense herbaceous cover instead. The immediate purpose of the burning is accomplished, and there is little concern about the degradation caused by the loss of plant species and the expansion of *B. rupestre* in the repeatedly-burned areas.

2.3. Methodological approach the value equivalence analysis

Diverse and degraded grasslands can be distinguished on the basis of their botanical composition. The aggressive expansion of *B. rupestre* is associated with a gradual loss of grassland species diversity as well as a decrease in forage quality and energy supply capacity. We first quantified the decline of forage quality in degraded grasslands compared to diverse ones, and then determined the number of livestock food rations affected by this decrease. Thereafter, we converted this loss to monetary units by considering the expenses associated with hypothetical indoor feeding during the same period. This approach is based on the substitution methodology, described in detail by Champ et al. (2017). The method provides an economic value based on the replacement or opportunity cost associated with the environmental resource lost, considering the least costly alternative, and requires a deep knowledge of the environmental resource being evaluated.

The substitution methodology includes two different types of analysis, the replacement cost and the resource equivalency. For this study, we started by considering the replacement cost analysis, which relies on quantifying the cost of providing an identical alternative resource or service, and assuming this replacement cost to be an appropriate measure of the environmental value of the loss of the resource. This analysis, which has been previously applied in high-mountain areas (i.e. forest providing protection from natural hazards, Notaro and Paletto, 2012), has two relevant limitations: a) the difficulty in finding a perfect substitute and b) the risk of underestimating the monetary value when artificial technologies do not produce all the services provided by the ecosystem. Because of these constraints, we abandoned this methodology, since feeding livestock indoors is based on different fodder than outdoors (cereal straw, meadow hay and silage, concentrates), and more importantly, because plant diversity and forage quality in the highlands decrease in the absence of grazing, that is, indoor livestock feeding generates a negative externality in ES.

Eventually, we adopted the resource equivalency approach, which considers economic estimates to compensate the victim (i.e. farmers) for the loss of the environmental resource (Chapman et al., 2018; Lipton et al., 2018). Losses related to the damaged environmental resource include the quantity of the resource itself (i.e. biomass of fodder) and an evaluation of the services provided by the resource (provisioning value, biodiversity value, cultural value, etc.), although in our case we focused our attention on the provisioning value for livestock. This methodology has been applied in the US and in Europe (according to the EU Directives requiring compensation) to evaluate the damage to specific ES caused, for example, by oil spills (Roach and Wade, 2006) and severe wildfires (Hanson et al., 2013). To our knowledge, this is the first time that the analysis has been applied to the valuation of grassland degradation in high-altitude areas, although it has been utilised in other valuable ecosystems such as marine environments (Bas et al., 2016; Bradford, 2017) and semiarid and tropical forests (Majdalawi et al., 2016; Pavanelli and Voulvoulis, 2019).

Estimates of the environmental loss can be expressed in a range of different units such as the discounted service acre-year (habitat equivalency analysis, HEA), the loss of species-year (resource equivalency analysis, REA) and the loss of economic value (value equivalency analysis, VEA). We used VEA analysis because it is appropriate in situations when the damaged and the restored resource are not perfect substitutes, and when compensation may have distributional consequences (Cole, 2013). The VEA analysis is less data- and time-consuming than other techniques (Roach and Wade, 2006), and requires injury parameters that quantify the loss of the environmental resource (the amount of adversely affected habitat and the degree of damage). In a second step, the analysis estimates the economic value per hectare of the service adversely affected. Some relevant VEA limitations are that the analysis considers the value of the ES to be constant over time and space (Flores and Thacher, 2002; Chapman et al., 2018), and that it is quite sensitive to a variety of factors, such as price-change scenarios (Shaw and Wlodarz, 2013). To cope with these constraints, we developed a sensitivity analysis focused on the seasonal variation in the energy supplied by grasslands and the variability of the economic cost of the replacement ration.

2.4. Pastoral forage value and estimates of the energy supplied by grasslands

Around 53% of the grasslands in the area of study have the perennial tall-grass *B. rupestre* as a native component of the plant community (1134 ha over 2147 ha), but its cover is very variable between sites. Based on the thematic vegetation mapping of the Aezkoa commons (Ferrer and Canals, 2008), eight grassland areas with different amounts of *B. rupestre* cover were chosen for the study (Figure 2). In summer 2014, the sites were visited, described in terms of the main environmental variables (altitude, slope, aspect, grazing level), and inventoried by means of detailed floristic surveys using the point quadrat method. To that end, 50 equidistant points were located across two 20 m-transect lines per site, and the species that came into contact with a metallic rod placed vertically at each point were identified. In total, 16 transect lines and 800 points were inventoried. Floristic data were subjected to Principal Component Analysis (PCA), and the most representative diversity indexes (species richness and Shannon-Wiener index) were determined using the Species and Diversity Richness Program (Seaby and Henderson, 2006). One-way ANOVA was used to determine significant differences in cover. All statistical analyses were implemented in the R software (R Core Team, 2015).

The Pastoral Forage Value (PFV) of the grasslands was also estimated from the floristic data. The PFV index was originally developed to estimate the forage value of multi-species mountain grasslands in the Massif Central (Daget and Poissonet, 1971). Since then, it has been successfully applied in other mountain areas to classify and evaluate regional pastoral resources -in the Pyrenees (Barrantes et al., 2013) and in the Alps (Argenti and Lombardi, 2012; Pittarello et al., 2016)- with different purposes, such as ecological evaluation and sustainable management planning. The PFV ranges from 0 to 100 and is based on the assignment of an Index of Specific Quality (ISQ) to each species present in an inventory. The ISQ values vary between 0 (zero quality) and 5 (high quality) and have been established empirically considering the palatability, digestibility, morphology and productivity of each species (Jouglet, 1999). PFV is calculated for each transect according to the following equation, where SCi is the specific contribution of species i in a transect line:

$$\text{PFV} = 0.2\sum_{i=1}^{n}(\text{SC}i\ *\ \text{ISQ}i)$$

The PFV is linked to the Forage Units (FU) by applying a conversion coefficient (k) adjusted on the basis of regional specificities of altitude and climate for each area (k ranging from 30 to 66). FU represents the net energy supplied by 1 kg of barley, and is a reference parameter commonly used for comparing feedstuffs:

$$\text{Forage Units } (\text{FU.ha}^{-1}\ 1) = \text{PFV} * \text{k}$$

Comparing the energy supplied by diverse and degrading grasslands permits us to estimate the energy loss, in FU.ha^{-1}, caused by the expansion of *B. rupestre* (see Figure b in the annex for a methodological outline).

2.5. Estimation of animal rations

A ration is the amount of balanced feed that an animal receives each day, and it depends on the animal's intake capacity and energy and nutrient requirements. We specified indoor feeding rations and calculated the number of rations necessary to feed animals and compensate for the loss of good-quality upland grassland. Based on animal species, breed and the reproductive stage (maintenance, pregnancy, lactation), we estimated the level of food intake (in kg dry matter/day) and the daily energy supply (Forage Units/day) required to fulfil each animal's production and maintenance demands. We used the software INRAtion-PrévAlim (INRA, 2017-2019) to design and optimise the rations, considering the most common feedstuffs used by local farmers and their market prices. Two different situations were considered: rations based on farm-produced products and rations with a higher proportion of purchased products. Both situations occur in the valley, depending on the farmer's access to agricultural land. The public county farmer consultancy INTIASA provided the feedstuff prices. According to the premise of the resource equivalency analysis, we selected the cheapest ration within each category (type of animal and level of energy required).

2.6. Sensitivity analysis

We completed the economic evaluation by performing a sensitivity analysis, which focused on two different assumptions: a) the variation in the predicted energy supplied by high-altitude grasslands over the growing season, and b) the variation in the financial cost of the replacement ration for housed animals. The first assumption takes into account the variation in digestibility of *B. rupestre* during the spring and summer, which results in a loss of the energy supplied to herbivores. The references consulted consider a peak ISQ of 1 for *B. rupestre*, but this value drops gradually during the grazing season as the species losses digestibility and starts to be rejected by grazers (ISQ = 0). The second assumption considers the rising cost of the replacement ration when increasing the proportion of external foodstuff, a situation that occurs in years when the weather is unfavourable for fodder production or on farms with limited access to agricultural lands.

Figure 2. Top: Areas chosen for the floristic survey. Red points represent grasslands suffering degradation and green points represent diverse grasslands. Bottom left: *B. rupestre* dominated grassland. Bottom right: Diverse grassland.

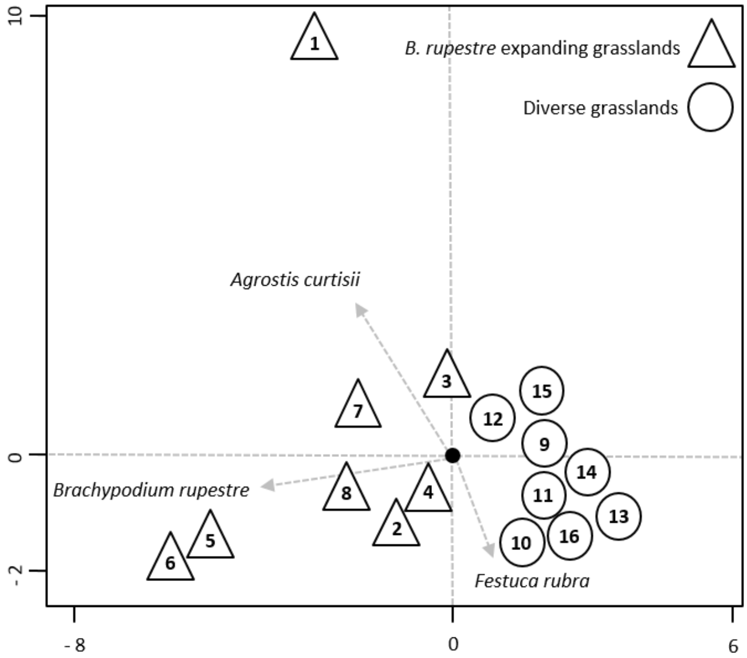
3. RESULTS

3.1. Grassland classification. Diversity and forage evaluation

The principal component analysis (PCA) allowed sites to be separated according to their floristic composition (see Table a in the annex for complete floristic data). The proportion of variance explained by the first axis of the PCA was 67.8% (9.2% for the second axis, Figure 3) and reflected the progressive decrease of cover of *B. rupestre* in the inventories. Grasslands on the left side of the ordination, with estimated covers of *B. rupestre* from 25% to >75%, represented areas where the species was expanding in large clumps, causing a sharp decline in plant species richness and diversity in the most extreme cases. Grasslands on the right side had covers of *B. rupestre* <25% and were significantly much more diverse. Figure 4 details the significant variation in species richness (F = 25.8, p < 0.001) and Shannon-Wiener diversity index (F = 18.46, p < 0.001) between diverse and degrading grasslands. On average, inventories made in degrading areas listed half the number of species present in diverse areas (22 *vs.* 11 species) and the diversity values dropped from 2.51 to 1.55. As the frequency of *B. rupestre* increased, codominant grasses such as *Festuca* gr. *rubra* and *Agrostis capillaris* disappeared from grasslands and legumes (*Lotus corniculatus*, *Trifolium repens*) declined, as did a significant cohort of small-size and rosette forbs (e.g. *Cruciata glabra*, *Hieracium pilosella*, *Hypochoeris radicata*, *Plantago lanceolata*).

Of the total area where *B. rupestre* was present, 75% were diverse grasslands and 25% grasslands experiencing *B. rupestre* expansion (Table 2). Both types of grasslands developed at a similar range of altitudes (800-1300 m.a.s.l.) and on similar parent substrates, but on a variety of slopes. To obtain an estimate of the effective grazing areas, topographic and grassland maps were overlaid to generate a new map that grouped diverse and degrading grasslands within the following categories: areas with slopes greater than 50% (15°C) unsuitable for grazers, areas with slopes between 30 and 50% (7°C-15°C) only suitable for sheep, and flatter areas (<30%), appropriate for all herbivores (see Figure a in the annex). Diverse grasslands were more frequent on gentle slopes (<30% slope), where grazing was more intense. Around 36% of the area of diverse grasslands, and 14% of degrading grasslands, were on gentle slopes, while steep slopes (>50%), inappropriate for the grazing of domestic herbivores, occupied 22% of diverse grasslands and 31% of degrading grasslands (Table 2).

To determine the energy supplied by grasslands, an averaged Index of Specific Quality (ISQ) of 0.5 was applied to *B. rupestre*. In the highlands, *B. rupestre* develops early in the season and tussocks of the species exhibit a more rapid loss of digestibility than other common grasses. In addition, the areas where *B. rupestre* expands are less visited by grazers, and there is little chance that new, more digestible shoots will resprout, thus increasing plant quality. Table 3 summarises the pastoral values and the available energy of grasslands determined from the floristic inventories. The diverse grasslands had a mean forage energy of 1243.53 FU/ha, so the estimated energy losses in degrading areas were 587.96 FU/ha and 355.45 FU/ha depending on the level of grassland degradation. As a percentage, 47% and 29% of the energy available in diverse grasslands was lost due to the *B. rupestre* expansion in highly invaded (>75% cover) and less invaded (25-75%) areas, respectively. Taking into consideration the area suitable for sheep flocks (30-50% slopes) and for mixed flocks (<30% slopes), Table 3 presents the total amount of FU lost due to the degradation occurring in grasslands.



Location	N. inventory	Estimated *B. rupestre* cover (%)
Arpea	6	83
Arpea	5	79
Errozate	1	45
Abodi	8	43
Errozate	2	41
Abodi	7	41
Armorieta	4	37
Armorieta	3	29
Zabaltea	10	21
Ezkanda	12	21
Ezkanda	11	13
Zabaltea	9	13
Azalegi	15	11
Urkulu	14	7
Urkulu	13	4

Figure 3. PCA ordination based on the floristic inventories. The proportion of variance explained by the first axis of the PCA is 67.8%. On the right, estimated cover of *B. rupestre* based on the floristic inventories.

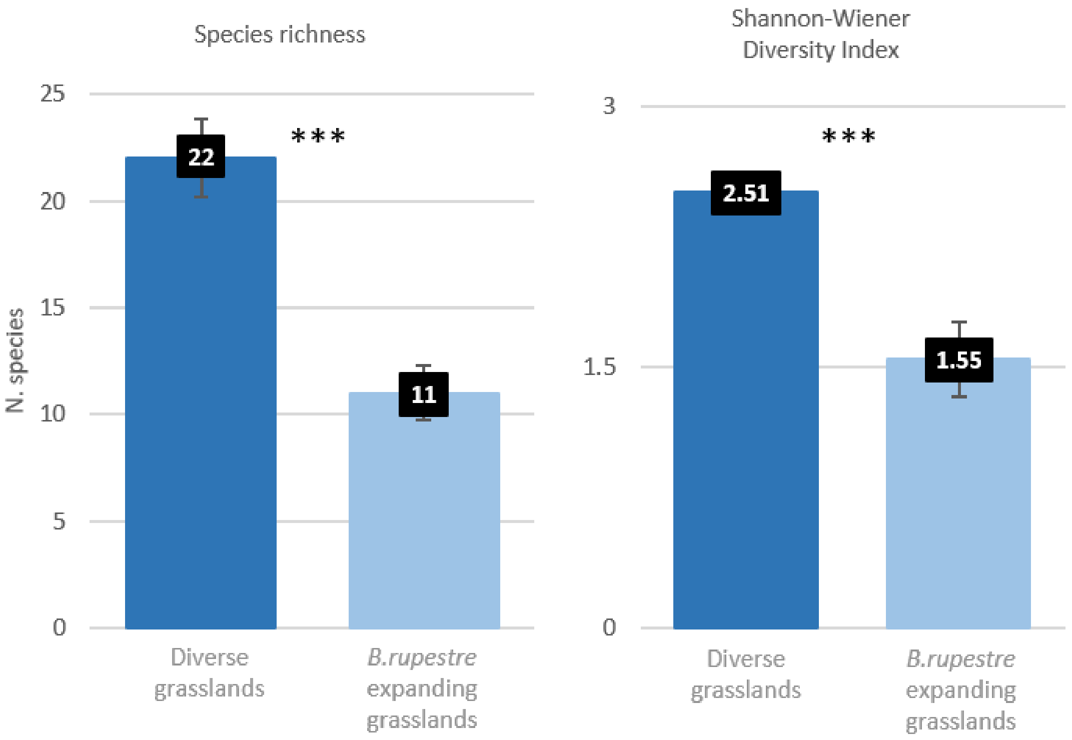


Figure 4. Species richness ($p < 0.001$) and Shannon-Wiener index ($p < 0.001$) in diverse and increasingly *B. rupestre*-dominated grasslands based on floristic inventories.

Table 2. *B. rupestre* estimated cover, areas and slopes in the Aezkoa high altitude grasslands.

Type of surface	***B.rupestre* cover**	**Surface (ha)**	**Surface (%)**	**Slope**	**Surface (ha)**	**Surface (%)**	**Type of grazing**
Degraded areas	>75%	190.91	16.83	<30	33.76	2.97	Mixed
				30-50	97.76	8.62	Sheep
				>50	59.39	5.23	No grazing
Expansive processes present	25-75%	96.85	8.54	<30	6.66	0.58	Mixed
				30-50	61.87	5.45	Sheep
				>50	28.32	2.49	No grazing
Diverse grasslands	<25%	846.25	74.60	<30	308.01	27.16	Mixed
				30-50	352.54	31.08	Sheep
				>50	185.70	16.37	No grazing

Table 3. Pastoral values, grassland available energy estimates (in forage units, FU/ha), energetic losses of grasslands being degraded (compared to the energy available in diverse grasslands) and energetic losses for sheep and mixed flocks (depending on the affected hectares and the frequency of the livestock species on the different slopes).

***B.rupestre* cover**	**Inventory**	**Location**	**PFV**	**Altitude**	**k**	**FU/ha**	**FU/ha average**	**FU/ha lost**	**FU for sheep**	**FU for mixed flocks**
>75%	6	Arpea	14.33	935	60	860.00	655.56	587.96	57479.95	19849.87
	5	Arpea	7.52	943	60	451.13				
25-75%	1	Errozate	25.31	1062	50	1265.31	888.08	355.45	21991.69	2367.30
	8	Abodi	13.28	1307	50	663.79				
	2	Errozate	19.50	1109	50	975.21				
	7	Abodi	8.47	1331	50	423.73				
	4	Armorieta	19.23	869	60	1153.85				
	3	Armorieta	14.11	796	60	846.58				
< 25%	10	Zalbetea	21.88	1069	50	1094.21	1243.53			
	12	Ezkanda	18.23	1017	50	911.35				
	11	Ezkanda	23.08	1022	50	1154.11				
	9	Zalbetea	20.79	1038	50	1039.50				
	15	Azalegi	20.83	1086	50	1041.40				
	16	Azalegi	36.06	1064	50	1802.82				
	14	Urkulu	31.56	1264	50	1577.78				
	13	Urkulu	26.54	1338	50	1327.07				

3.2. Animal energy requirement and cost feedstuff replacements

Table 4 summarises the energy requirements and the cost of rations calculated for horses, cattle and sheep at different reproductive stages. The feedstuff costs, expressed in fresh matter and based on February 2019 prices, are based on the most common fodders used by local farmers and include the costs of transportation for the products sourced externally (i.e. cereal straw, where production areas are 70 km away from the valley). For cattle and sheep, we evaluated different rations that delivered the energy requirements but differed in price depending on the proportion of farm-produced (meadow hay and silage) and external forage (cereal straw, ground corn, concentrate feed and lucerne hay). For horses, a unique ration was determined since it is customary to feed them only on farm products. The total daily cost per ration (€) was calculated as the product of the number of kilograms and the cost per kilogram of each foodstuff category.

3.3. Economic loss of provisioning value per unit area

The monetary cost per hectare lost to grazing (Table 5) was determined through a careful overview of the energy needs of the different flocks/herds during their stay in the high altitude grasslands. Based on the information collected during previous local farmer surveys (Ferrer and Canals, 2008), we compiled information regarding the proportion of livestock species and their energetic requirements according to their reproductive stage during the summer period. Most sheep farmers concentrated births in the autumn (November) to supply market lamb demands during Christmas and maintained a lactation period for cheese production until late spring. The annual ewe replacement rate averaged 20% and the sexual maturity required for mating occurred during the 16th month of life. Therefore, a standard sheep flock comprised 80% pregnant ewes (five-months pregnancy period) and 20% replacement ewes. For cattle, the most common practice concentrated births in the autumn and fattened growing calves in feedlots, which meant that most cows were pregnant (nine-months pregnancy) and without calf during the summer. Cow replacement rates on farms averaged 13% and heifers were first mated at 2.5 years. According to this, a standard herd comprised 74% adult pregnant cows and 26% heifers (one- and two-year-old). With respect to mares, most remained in the highlands after giving birth with their feeding foals, and became pregnant again (eleven-months pregnancy period). Since the annual horse replacement rate was 10% and the first servicing occurred at 3 years, a standard herd comprised 70% pregnant mares with feeding foals, 30% one- to three- year-old mares and a small proportion (1-2%) of stallions. For the energetic requirements of mares, we took into account a sole energy value because the higher needs of pregnant and lactating mares balanced out the lower needs of young mares.

Table 5 presents the standard flock/herd composition for each slope category, the number of rations lost according to the animals' energy requirements and the unitary costs per ha, based on the cheapest rations calculated in Table 4 for each type of animal and level of energy required. We approached the animal species distribution in the territory by assuming that 30% of the area suffering degradation was unsuitable for grazing (>50% slope) and that, in the remaining area, 80% was suitable for sheep flocks and 20% for mixed grazers (flock composition in Table 5, second and third columns). Since the total degraded area with a potential for grazing was 200.05 ha (Table 2), the resulting loss of provisioning value was 21146€ (16731€ and 4415€ for the intermediate and gentle slopes, respectively) and the averaged unitary cost was 107€/ha per year.

Table 4. Daily dry matter intake, energy per ration and total cost of different rations designed to feed horses, cattle and sheep with different energy requirements. Rations are based on the most common feedstuffs used by the Aezkoa farmers and are mostly based on farm-produced fodders. In grey, the cheapest rations for each physiological state. Source: INTIA based on February 2019 local feedstuff prices.

Animal	Horse	Cattle				Sheep				
Energy requirements	Low	Low		Medium to high		Low	Low to medium	Low to medium	Medium	Medium
Main forage source	Farm produced	Farm produced	Farm produced	Farm produced	Farm produced	Farm produced	Farm produced	High proportion purchased forage	Farm produced	High proportion purchased forage
Fodder 1	Meadow hay 85% dm	Meadow silage 30% dm	Meadow hay 85% dm	Meadow silage 50% dm	Meadow hay 85% dm	Meadow hay 85% dm	Meadow silage 60% dm	Meadow hay 85% dm	Meadow silage 60% dm	Meadow hay 85% dm
Fodder 2		Cereal straw 88% dm		Cereal straw 88% dm	Concentrate feed medium quality 88% dm		Cereal straw 88% dm	Luzerne hay 85% dm	Meadow hay 85% dm	Luzerne hay 85% dm
Fodder 3				Concentrate feed medium quality 88% dm			Concentrate feed medium quality 91% dm	Concentrate feed medium quality 91% dm	Ground corn 95% dm	Concentrate feed medium quality 91% dm
Fodder 4										Cereal straw 88% dm
kg fodder 1	15.0	30.0	14.0	14.0	12.0	1.2	2.4	1.4	2.6	0.7
kg fodder 2		2.0		3.5	2.0		0.2	1.0	0.6	1.3
kg fodder 3				1.0			0.7	0.4	0.8	1.0
kg fodder 4										0.35
Daily dry mater intake (kg)	12.8	10.8	11.9	11.0	12.0	1.0	2.3	2.4	2.8	2.9
FU fodder 1	0.65	0.75	0.65	0.82	0.65	0.80	0.90	0.80	0.90	0.80
FU fodder 2		0.50		0.50	0.95		0.50	0.70	0.80	0.70
FU fodder 3				0.95			1.00	1.00	1.27	1.00
FU fodder 4										0.50
Energy (FU) per ration	8.29	7.63	7.74	8.12	8.30	0.82	1.93	1.91	2.78	2.32
Cost (€/kg) fodder 1	0.12	0.04	0.12	0.08	0.12	0.12	0.09	0.12	0.09	0.12
Cost (€/kg) fodder 2		0.06		0.06	0.23		0.06	0.15	0.12	0.15
Cost (€/kg) fodder 3				0.23			0.27	0.27	0.25	0.27
Cost (€/kg) fodder 4										0.06
Total cost (€) per ration	1.80	1.42	1.68	1.56	1.90	0.14	0.42	0.43	0.51	0.57

Table 5. Standard flock/herd composition based on slope and flock energy needs, ration costs, number of lost rations and monetary costs. Bold numbers indicate average values per species.

Slope	Herbivore	Flock composition	Energy (FU) per ration	Cost (€) per ration	Flock FU/ration	Flock €/ration	N. of lost rations	Monetary cost (€)	Cost (€) per ha
30-50%	100% sheep	80% pregnant ewes	1.93	0.42	1.71	0.36	46475	16731	104.81
		20% young ewes	0.82	0.14					
		Sheep flock	1.71	0.36					
<30%	28% sheep	Sheep flock	1.71	0.36	6.29	1.25	3532	4415	109.23
	53% cow	74% pregnant cows	8.12	1.56					
		26% heifers	7.63	1.42					
		Cattle herd	7.99	1.52					
	19% horse	70% pregnant-lactating mares	8.29	1.80					
		30% young mares							
		Horse herd	8.29	1.80					

3.4. Sensitivity analysis

The sensitivity analysis showed that the unitary costs per hectare degraded varied between 55€ - before the loss of digestibility of the species and considering the cheapest replacement ration - and 173 € - after the loss of digestibility of the species and the more expensive replacement ration (Figure 6). The major effect was caused by the drop in digestibility of *B. rupestre* over the season, which largely decreased the pastoral value and the forage units of degrading grasslands because of the high cover attained by the species in these areas ($p\text{-value} < 0.01$; Figure 5). In contrast, the energy losses were not significant in diverse grasslands ($p\text{-value} > 0.05$), where *B. rupestre* was present at lower percentages. The drop in digestibility resulted in high economic value losses in the degrading areas, 106€/ha and 114€/ha respectively, considering the cheapest and the more expensive replacement rations. Comparatively, the economic losses due to changes in the cost of the ration varied within much smaller ranges, namely 4-12€/ha (Figure 6).

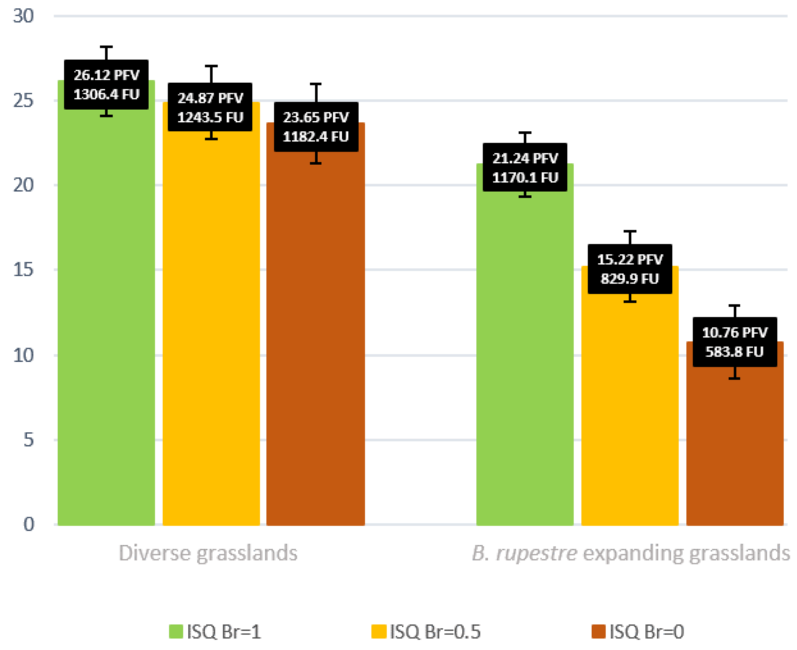


Figure 5. Loss of Pastoral Forage Value (PFV) and Energy Forage Value (FU) of the different grasslands due to the change in the digestibility of *B. rupestre* over the growing season and the resulting change in ISQ index from 1 to 0.

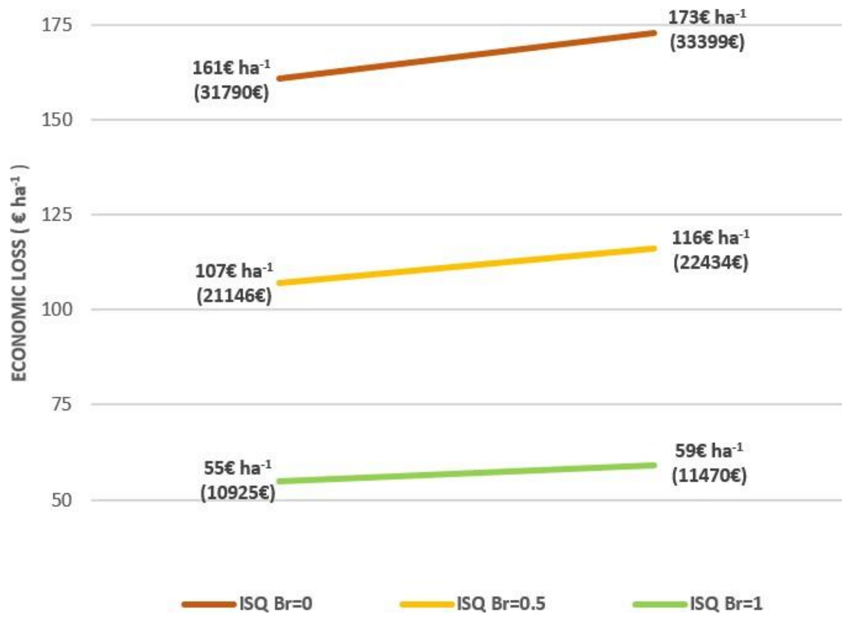


Figure 6. Results of the sensitivity analysis, which considers the increase in the cost of the animal feed rations and the loss of digestibility of *B. rupestre* over the growing season. Numbers in parentheses indicate the total predicted annual economic losses.

4. DISCUSSION

4.1. Loss of provisioning value of high-altitude grasslands

In the study area, each hectare of grassland facing the expansion of *B. rupestre* due to the decline in grazing and the recurrent burning regime, undergoes a loss of provisioning value of 107 €/ha per year on average, based on the cheapest replacement feed for the animals, i.e. using forage produced on-farm. The loss of provisioning value of high-altitude grasslands has severe consequences for neighbouring lowland regions and may also have an influence on processes occurring at larger spatial scales (Schirpke et al., 2019). As highlands are abandoned, there can be expected to be greater demand and competition for agricultural areas in the valley, which may result in scarcity of supply for farmers with small farms or low productive land, and a consequent increase in fodder prices. In extensive livestock production systems, where economic revenues are generally limited, the level of dependence of external food supplies is critical to farm profitability. Market prices for raw materials providing energy (based on cereal crops) and protein (based on legume crops) in animal rations are very volatile and experience significant year-to-year fluctuations, since they depend on global and futures markets more than on real production costs (Dronne, 2018). This situation of external dependence means an increase in the vulnerability of extensive livestock production systems (de Roest et al., 2018).

Moreover, the decline in the grazing of high-altitude grasslands is associated with changes in the traditional production systems that may have a significant influence on animal welfare and on the type and quality of the livestock products. The access of animals to an array of summer grazing areas allows them to move freely and practice optimal dietary selection, which improves their overall condition (Villalba et al., 2011; Gregorini et al., 2017). In addition, the current conditions of climate change and the frequent occurrence of heat weaves in valleys make summer grazing in the highlands particularly valuable (Lees et al., 2019; Joy et al., 2020). With regard to livestock products, breeding systems based on grazing have proved to be comparatively healthier and ensure better quality products than indoor feeding systems (Elgersma, 2015; Cabiddu et al., 2019). In the area of study, most farms with autochthonous sheep breeds produce milk which is transformed into cheese marketed under different prestigious names such as *Idiazabal* and *Ossau-Iraty* (from the Spanish and French sites respectively). In particular, there is a specific label to distinguish the high-quality cheese produced at altitude during the summer grazing of lactating sheep (*Fromage des estives*, in the French central and western Pyrenees). The decline of high mountain grazing areas would severely compromise this traditional, healthy and highly valuable production system.

4.2. Restoration efforts in mountain areas

The decline of traditional grazing in high-altitude grasslands makes plant communities more vulnerable to processes related to global change such as shrub encroachment, afforestation and the spread of perennial competitive tall-grasses (Dullinger et al., 2003; Niedrist et al., 2009). Most of these processes involve damage to the biodiversity, both at the landscape level - due to the loss of mosaics - and the community level - because of the loss of specific species of grasslands and ecotones (Canals and Sebastià, 2000; Sharma et al., 2014). The replacement of each particular plant species in a community is associated with a loss of land-use legacy, that is, the adaptive history of the species which evolved under an extreme environment (cold temperatures, long snow cover, intense solar radiation) and an ancestral regime of summer herbivory (by wild and domestic herbivores). In the case of the expansion of *B. rupestre*, the degradation of the original grassland community has been documented in this study in terms of the loss of species richness in the floristic inventories, from 22 to 11 species, on average.

The recovery of degraded areas in highlands is associated with specific problems, mostly related to the slope, the altitude and the accessibility. In this study, 25% of the grassland area is experiencing the expansion of *B. rupestre*, but the chances of undertaking effective restoration measures are limited. Nearly 8% of this area is located on steep slopes, where grazing is not feasible, and 14% on intermediate slopes (30-50%), suitable only for small ruminants, where shepherding is difficult and requires a great deal of effort. Repeated prescribed burning of the steepest areas (unable to support grazing), intended to restore the grassland, is useless and has negative environmental effects. These regimes should be avoided, unless other environmental benefits (i.e. firebreaks, access areas) can be demonstrated. Conversely, efforts should focus on the areas of grassland more likely to recover, by supporting regular and managed grazing on them. Our results highlight the need to develop restoration efforts at a very local scale, based on the suitability and appropriateness of each particular location.

The sensitivity analysis reveals how crucial it is to focus herbivory during the period in which *B. rupestre* remains digestible, in order to exert some control on the species and to provide a relatively good energy supply to herbivores. In grasslands where *B. rupestre* is expanding, its decline in digestibility over the growing season results in a high economic loss, varying between 106€/ha and 114€/ha per year. *B. rupestre* develops a large biomass early in the season and its digestibility falls very rapidly. As the species becomes indigestible, the chance of grazers defoliating the species is lower in degraded grasslands than in high-diversity communities. In the latter, the species has a significant chance of defoliation because of the vicinity of palatable species, and consequently, high plant tissue renewal is expected (Underwood et al., 2014). In contrast, degraded areas are visited less by grazers and a thick layer of necromass accumulates, which reduces the chance of grazing (Canals et al., 2017). Despite the fact that these results highlight the value of early-season grazing, its implementation is complex in the practice. The occurrence of late snowfall events in mid-spring, very frequent in the highlands, and the length of the milking period in the valleys have a key influence on shepherds' decisions about moving animals to higher pastures.

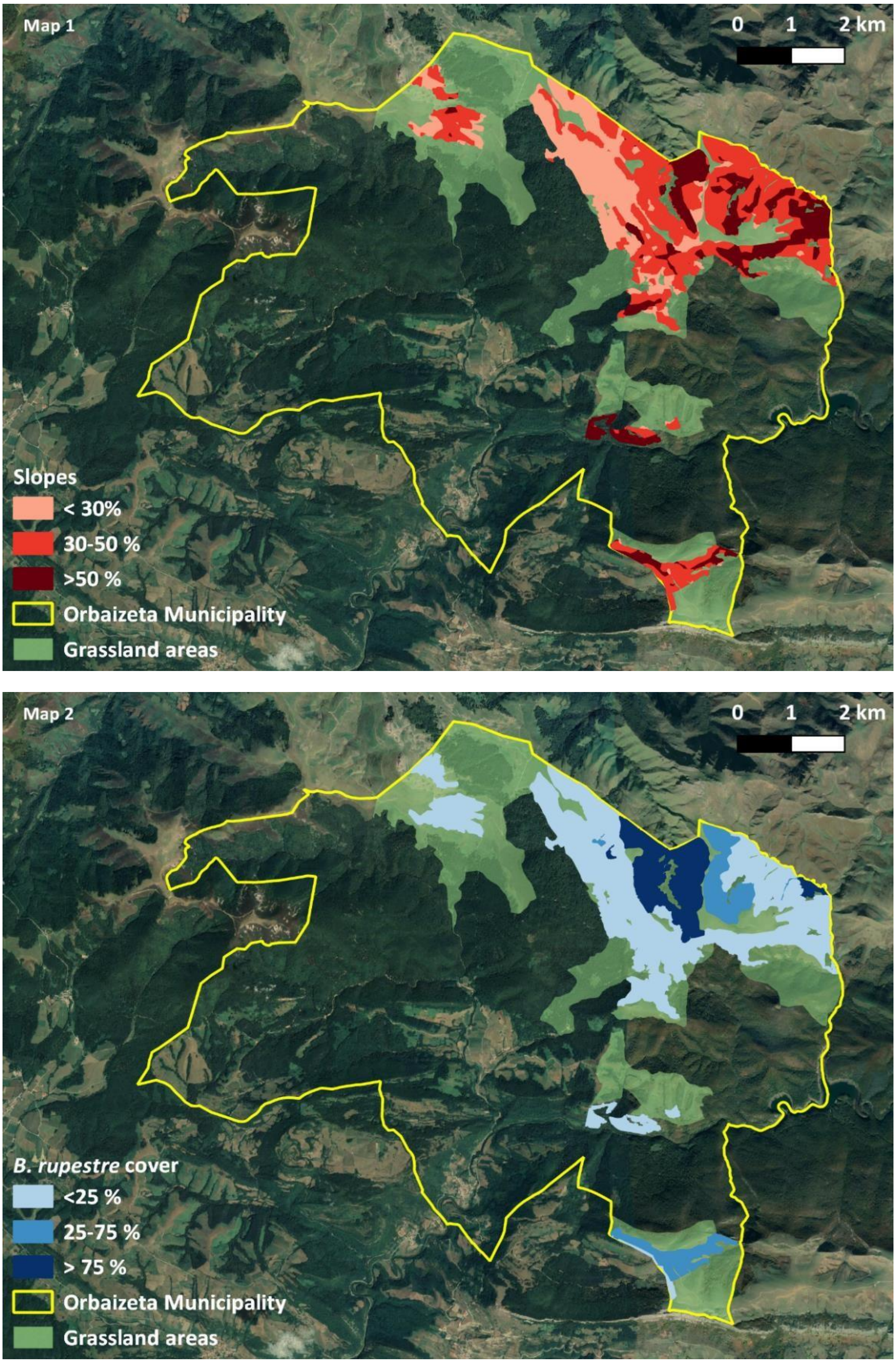
5. CONCLUSIONS

Monetary valuation of nature and of ES is a useful tool to provide a basis for discussion among stakeholders, land managers and policymakers, but needs to be implemented with caution (de Groot et al., 2012; Divinsky et al., 2017; Victor, 2020). In this research, the loss of the economic value of the provisioning service of high-altitude grasslands by means of the VEA method was conducted on the basis of careful field work to estimate the energy supply of grasslands and on the consideration of real market-based substitutes. Among ES, the evaluation of the provisioning value of grasslands is probably the most effective approach to raise awareness among rural communities, and particularly among those dedicated to the primary sector, of the consequences of the decline in the use of highland grazing resources. In mountain valleys, the chances of developing other economic alternatives to agriculture and livestock farming are limited, and any decrease in these activities may boost rural depopulation, which is currently a significant problem in Europe (Gretter et al., 2018; Canals, 2019). In this particular study, the loss of provisioning value may also affect the economic conditions of the modern international agreement between the Aezkoa and the Cize valleys, and local organisations representing farmers' interests on both sides of the border should definitely tackle this environmental challenge.

Mountain grasslands have many assets of interest for human societies and one limitation of the resource equivalence methodology is that it fails to estimate the costs imposed on society of the environmental damage caused (Cole, 2013). Our study reveals the parallels between the loss of plant diversity and the loss of provisioning value in high-altitude grasslands. Biodiversity has been positively related to many ecosystem services, including atmospheric regulation, carbon sequestration, pest regulation and pollination (Harrison et al., 2014; Chen et al., 2018), although there are other ES not directly associated with biodiversity that are also crucial for human societies (i.e., climate regulation, hydrological cycle, erosion control) and deserve consideration. To obtain a complete picture of the positive externalities provided by high-altitude grasslands, the resource equivalence methodology used in this research should be complemented by other techniques (revealed and stated preference approaches) that can help to highlight and explain the consequences of the decline in traditional disturbance regimes in high-altitude grasslands.

ANNEX

Figure a. Range of slopes (map 1), range of *B. rupestre* covers (map 2), and crossed cartography of slopes and *B.rupestre* covers (map 3) of the grasslands in the study area.



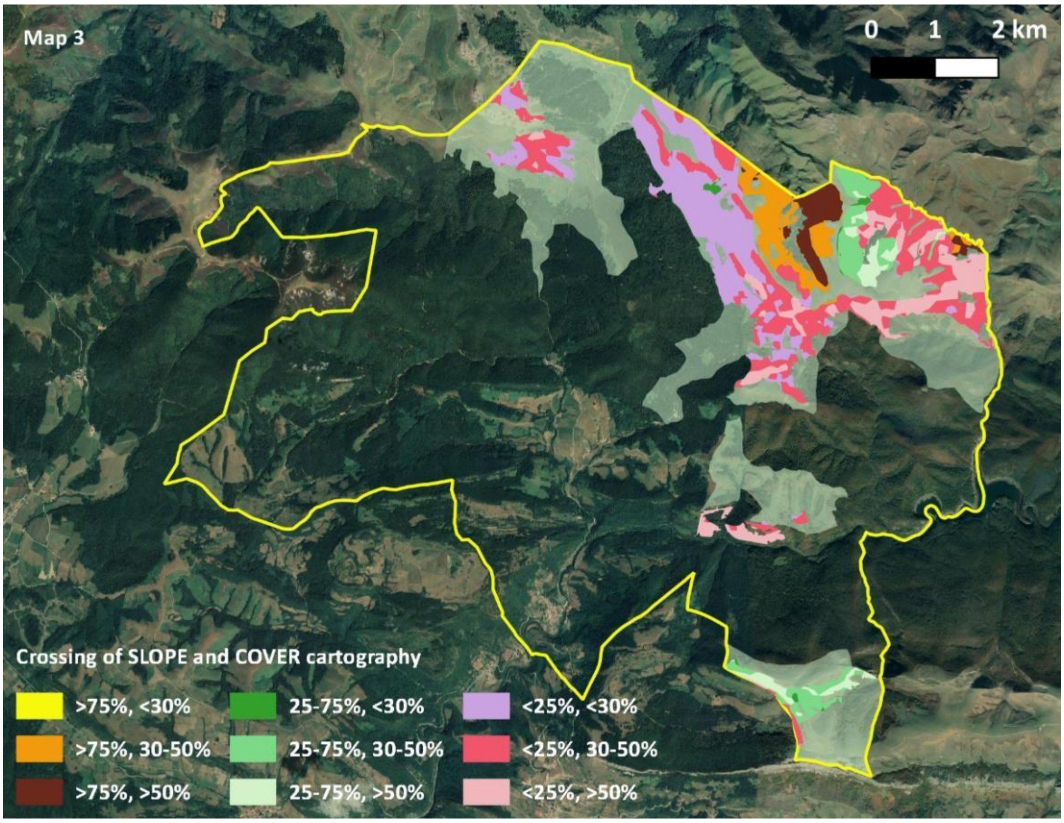

Map 3
0 1 2 km
Crossing of SLOPE and COVER cartography
>75%, <30%
>75%, 30-50%
>75%, >50%
25-75%, <30%
25-75%, 30-50%
25-75%, >50%
<25%, <30%
<25%, 30-50%
<25%, >50%

Figure b. Outline of the methodological approach followed to estimate the loss of animal food rations and the monetary costs per unit of degraded surface.

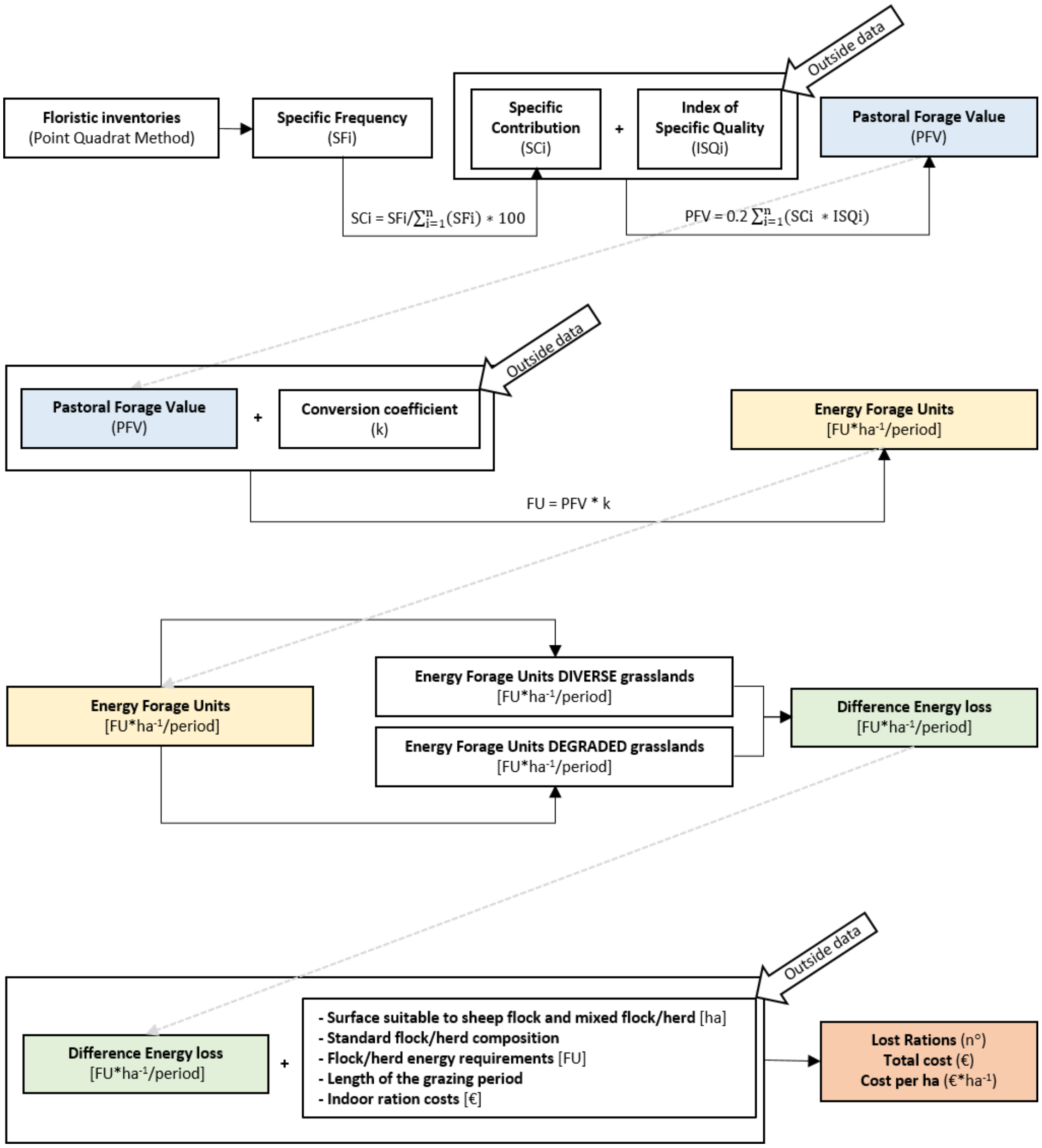


Table a. Specific Frequencies of the species (SFi) at each floristic inventory (n.16) in the different sites (n.8). Species specific contributions (SCi) are calculated from SFi as explained in the annexed Figure b.

	Errozate		Armorieta		Arpea		Abodi		Zalbetea		Ezkanda		Urkulu		Azalegi	
N.inventory	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Achillea millefolium	0	0	0	0	1	0	0	0	0	0	0	0	2	0	0	12
Agrostis capillaris	0	9	10	1	3	0	1	3	2	7	11	6	18	36	3	27
Agrostis curtisii	29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Avenula sulcata	10	1	3	0	0	0	0	0	0	0	0	0	1	0	0	0
Bellis perennis	0	0	0	0	0	0	0	0	2	0	0	1	0	0	4	1
Brachypodium rupestre	44	50	42	48	50	50	48	50	17	32	19	29	5	10	17	12
Briza media	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0
Campanula sp.	0	0	0	1	0	0	1	0	0	0	0	1	1	2	4	0
Carex caryophyllea	6	4	1	9	1	0	0	0	9	3	7	4	5	3	13	2
Carex flacca	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0
Cerastium fontanum	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Cirsium eriophorum	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0
Cirsium filipendulum	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Cirsium palustre	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	3
Cruciata glabra	0	0	1	3	0	0	1	1	0	0	0	0	2	1	1	0
Cynosurus cristatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	3
Danthonia decumbens	8	0	1	2	0	0	0	0	3	6	10	10	0	0	3	1
Deschampsia flexuosa	0	17	3	0	0	0	7	5	0	0	1	3	9	2	0	0
Eryngium bourgatii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Erica vagans	0	0	0	0	0	0	13	7	1	0	0	4	0	0	1	0
Euphrasia minima	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Festuca rubra	0	17	18	30	7	9	8	17	35	41	38	22	37	25	34	34
Galium saxatile	0	17	0	1	0	0	0	0	1	16	17	7	10	11	0	4
Globularia nudicaulis	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Hieracium pilosella	0	0	2	1	0	0	0	0	5	3	5	3	2	0	7	5

Hypericum pulchrum	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Hypochaeris radicata	0	0	0	0	0	0	0	0	4	1	5	2	6	12	0	0
Lathyrus linifolius	0	0	0	0	0	0	2	3	1	2	0	0	0	0	2	1
Leontodon hispidus	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
Linum catharticum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Lotus corniculatus	0	0	0	5	0	0	0	0	1	1	0	4	3	5	4	0
Luzula campestris	0	0	4	0	0	0	0	0	0	4	0	0	3	2	0	0
Nardus stricta	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Plantago lanceolata	0	0	1	2	0	0	0	0	7	0	0	1	1	3	6	2
Plantago media	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0
Polygala serpyllifolia	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Polygala vulgaris	0	0	0	0	0	0	0	1	0	1	0	1	0	3	0	1
Potentilla erecta	0	4	11	5	1	0	11	9	6	15	11	15	10	5	1	1
Potentilla montana	0	0	7	7	0	0	4	6	3	2	0	6	1	0	8	7
Pteridium aquilinum	0	0	36	12	0	0	0	0	0	0	0	0	0	0	0	1
Ranunculus bulbosus	0	0	1	0	0	0	0	0	3	0	1	0	1	0	2	1
Rhododendron ferrugineum	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Rubus sp.	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Stachys officinalis	0	0	0	0	0	0	0	0	2	0	1	0	0	0	2	0
Taraxacum sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
Thymus polytrichus	0	0	1	0	0	1	13	8	8	0	1	4	1	0	15	1
Trifolium repens	0	2	0	2	0	0	0	1	2	9	8	5	4	6	5	16
Ulex gallii	1	0	4	1	0	0	7	4	1	4	7	10	9	4	5	0
Veronica officinalis	0	0	0	0	0	0	0	0	2	3	3	1	0	0	0	7
Bare soil	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0

REFERENCES

Argenti, G., Lombardi, G., 2012. The pasture-type approach for mountain pasture description and management. Italian Journal of Agronomy. 7, 293-299. doi:10.4081/ija.2012.e39.

Barrantes, O., Reiné, R., Ferrer, C., 2013. Changes in land use of Pyrenean mountain pastures - Ski runs and livestock management - between 1972 and 2005 and the effects on subalpine grasslands. Arctic Antarctic and Alpine Research 45 (3), 318-329. doi:10.1657/1938-4246-45.3.318.

Bas, A., Jacob, C., Hay, J., Pioch, S., Thorin, S., 2016. Improving marine biodiversity offsetting: a proposed methodology for better assessing losses and gains. J. Environmental Management. 175, 46-59. doi:10.1016/j.jenvman.2016.03.027.

Bernués, A., Rodriguez-Ortega, T., Ripoll-Bosch, R., Alfnes, F., 2014. Socio-cultural and economic valuation of ecosystem services provided by mediterranean mountain agroecosystems. Plos One 9. doi:10.1371/journal.pone.0102479.

Bradford, M.J., 2017. Accounting for uncertainty and time lags in equivalency calculations for offsetting in aquatic resources management programs. Environmental Management. 60 (4), 588-597. doi:10.1007/s00267-017-0892-6.

Burkhard, B., Crossman, N., Nedkov, S., Petz, K., Alkemade, R., 2013. Mapping and modelling ecosystem services for science, policy and practice. Ecosystysem Services. 4, 1-3. doi:10.1016/j.ecoser.2013.04.005.

Cabiddu, A., Delgadillo-Puga, C., Decandia, M., Molle, G., 2019. Extensive ruminant production systems and milk quality with emphasis on unsaturated fatty acids, volatile compounds, antioxidant protection degree and phenol content. Animals 9, 17. doi:10.3390/ani9100771.

Canals, R.M., 2019. Landscape in motion: Revisiting the role of key disturbances in the preservation of mountain ecosystems. Geographical Research Letters 45, 515-531. doi:10.18172/cig.3634

Canals, R.M., Múgica, L., Durán, M., San Emeterio, L., 2019. Soil bacterial functional diversity mirrors the loss of plant diversity by the expansion of a native tall-grass in high mountain grasslands. Plant Soil 445 (1-2), 243-257. doi:10.1007/s11104-019-04281-w.

Canals, R.M., Pedro, J., Rupérez, E., San-Emeterio, L., 2014. Nutrient pulses after prescribed winter fires and preferential patterns of N uptake may contribute to the expansion of *Brachypodium pinnatum* (L.) P. Beauv. in highland grasslands. Applied Vegetation Science 17, 419-428. doi:10.1111/avsc.12088

Canals, R.M., San Emeterio, L., Durán, M., Múgica, L., 2017. Plant-herbivory feedbacks and selective allocation of a toxic metal are behind the stability of degraded covers dominated by *Brachypodium pinnatum* in acidic soils. Plant and Soil 415, 373-386. doi:10.1007/s11104-016-3153-1

Canals, R.M., Sebastià, M., 2000. Analyzing mechanisms regulating diversity in rangelands through comparative studies: a case in the southwestern Pyrennees. Biodiversity and Conservation 9, 965-984. doi:10.1023/A:1008967903169

Champ, P.A., Boyle, K.J., Brown, T.C., 2017. A primer on nonmarket valuation. The economics of non-market goods and resources. Springer, Dordrecht.

Chapman, D., Cole, S., Özdemiroglu E., 2018. Economic valuation for equivalency analysis. Equivalency methods for environmental liability: Assessing damage and compensation under the European environmental liability directive.

Chen, S.I., Shahi, C., Chen, H.Y.H., Kumar, P., Ma, Z., McLaren, B., 2018. Trade-offs and synergies between economic gains and plant diversity across a range of management alternatives in boreal forests. Ecological Economics. 151, 162-172. doi:10.1016/j.ecolecon.2018.05.014.

Cole, S., 2013. Equity over efficiency: a problem of credibility in scaling resource-base compensation? Journal of Environmental Economics and Policy 2, 93-117. doi:10.1080/21606544.2013.764616.

Daget, P., Poissonet, J., 1971. Une mèthode d'analyse phytologique des prairies. Annals of Agronomiques 22 (1), 5-41.

de Groot, R., Brander, L., van der Ploeg, S., Costanza, R., Bernard, F., Braat, L.,Christie, M., Crossman, N., Ghermandi, A., Hein, L., Hussain, S., Kumar, P.,McVittie, A., Portela, R., Rodriguez, L.C., ten Brink, P., van Beukering, P., 2012. Global estimates of the value of ecosystems and their services in monetary units. Ecosystem Services. 1 (1), 50-61. doi:10.1016/j.ecoser.2012.07.005.

de Roest, K., Ferrari, P., Knickel, K., 2018. Specialisation and economies of scale or diversification and economies of scope? Assessing different agricultural development pathways. Journal of Rural Studies. 59, 222-231. doi:10.1016/j.jrurstud.2017.04.013.

Divinsky, I., Becker, N., Bar (Kutiel), P., 2017. Ecosystem service tradeoff between grazing intensity and other services - a case study in Karei-Deshe experimental cattle range in northern Israel. Ecosystem Services. 24, 16-27. doi:10.1016/j.ecoser.2017.01.002.

Dronne, Y., 2018. Agricultural raw materials for food and feed: the world. Inra Production Animal. 31, 165-180. doi:10.20870/productionsanimales.2018.31.3.2345.

Dullinger, S., Dirnbock, T., Greimler, J., Grabherr, G., 2003. A resampling approach for evaluating effects of pasture abandonment on subalpine plant species diversity. Journal Vegetation of Science. 14, 243-252. doi:10.1658/1100-9233(2003)014[0243:arafee]2.0.co;2.

Elgersma, A., 2015. Grazing increases the unsaturated fatty acid concentration of milk from grass-fed cows: a review of the contributing factors, challenges and future perspectives: effect of grazing on milk fatty acid composition. European Journal of Lipid Science and Technology. 117 (9), 1345-1369. doi:org/10.1002/ejlt.201400469.

Ferrer, V., Canals, R.M., 2008. Proyecto de ordenación de los recursos pascícolas forestales del monte Aezkoa nº 1 del CUP. Pamplona: Consultoría Belardi & Universidad Pública de Navarra.

Flores, N., Thacher, J., 2002. Money, who needs it? Natural resource damage assessment. Contemporany Economy. Policy 20, 171-178. doi:org/10.1093/cep/20.2.171.

Galop, D., Catto, N., 2014. Environmental history of European high mountains. Quaternary International 353, 1-2. doi:10.1016/j.quaint.2014.10.040

García-Ruiz, J., López-Moreno, J., Lasanta, T., Vicente-Serrano, S., González-Sampériz, P., Valero-Garcés, B., Sanjuán, Y., Beguería, S., Nadal-Romero, E., Lana-Renault, N., Gómez-Villar, A., 2015. Geo-ecological effects of gobal change in the central spanish Pyrenees. A review of different spatial and temporal scales. Pirineos 170, 1-44.

Gartzia, M., Alados, C.L., Pérez-Cabello, F., 2014. Assessment of the effects of biophysical and anthropogenic factors on woody plant encroachment in dense and sparse mountain grasslands based on remote sensing data. Progress in Physical Geography: Earth and Environment 38 (2), 201-217. doi:10.1177/0309133314524429.

Gehrig-Fasel, J., Guisan, A., Zimmermann, N.E., 2007. Tree line shifts in the Swiss Alps: climate change or land abandonment? Journal of Vegation Science 18 (4), 571-582. doi:10.1111/j.1654-1103.2007.tb02571.x.

González-Sampériz, P., Aranbarri, J., Pérez-Sanz, A., Gil-Romera, G., Moreno, A.,Leunda, M., Sevilla-Callejo, M., Corella, J.P., Morell´on, M., Oliva, B., Valero-Garcés, B., 2017. Environmental and climate change in the southern central Pyrenees since the last glacial maximum: a view from the lake records. Catena 149, 668-688.

Gregorini, P., Villalba, J.J., Chilibroste, P., Provenza, F.D., 2017. Grazing management: setting the table, designing the menu and influencing the diner. Animal Production Science. 57 (7), 1248. doi:10.1071/AN16637.

Gretter, A., Ciolli, M., Scolozzi, R., 2018. Governing mountain landscapes collectively: local responses to emerging challenges within a systems thinking perspective. Landscape Restoration. 43 (8), 1117-1130. doi:10.1080/01426397.2018.1503239.

Guadilla-Sáez, S., Pardo-de-Santayana, M., Reyes-García, V., 2019. The role of traditional management practices in shaping a diverse habitat mosaic in a mountain region of northern Spain. Land Use Policy 89. doi:10.1016/j.landusepol.2019.104235

Gómez-García, D., García-González, R., Fillat, F., 2009. Multifunctionality of mountain grasslands: Towards a multidisciplinary interpretation of grassland systems in the Pyrenees of Aragon. In: Barrantes, O., Broca, A., Ferrer, C. (Eds.), Reiné R. SEEP, Huesca-Spain.

Hanson, D.A., Britney, E.M., Earle, C.J., Stewart, T.G., 2013. Adapting habitat equivalency analysis (HEA) to assess environmental loss and compensatory restoration following severe forest fires. Forest Ecology and Management. 294, 166-177. doi:10.1016/j.foreco.2012.12.032.

Harrison, P.A., Berry, P.M., Simpson, G., Haslett, J.R., Blicharska, M., Bucur, M., Dunford, R., Egoh, B., Garcia-Llorente, M., Geamana, N., Geertsema, W., Lommelen, E., Meiresonne, L., Turkelboom, F., 2014. Linkages between biodiversity attributes and ecosystem services: a systematic review. Ecosystem Services. 9, 191-203. doi:10.1016/j.ecoser.2014.05.006.

INRA (2017-2019) INRAtion PrévAlim. Logiciel de rationnement pour bovins, ovins et caprins. In: INRA (ed). <https://www.inration.educagri.fr/>, France.

Jouglet, J., 1999. Les végétations des alpages des Alpes françaises du sud. Guide technique pour la reconnaissance et la gestion des milieux pásturés d'altitude. Cemagref et Aten, France.

Joy, A., Dunshea, F., Leury, B., Clarke, I., DiGiacomo, K., Chauhan, S., 2020. Resilience of small ruminants to climate change and increased environmental temperature: a review. Animals 10 (5), 867. doi:10.3390/ani10050867.

Kahlert, B., Ryser, P., Edwards, P., 2005. Leaf phenology of three dominant limestone grassland plants matching the disturbance regime. Journal of Vegetation Science. 16, 433-442. doi:10.1111/j.1654-1103.2005.tb02383.x.

Komac, B., Kefi, S., Nuche, P., Escós, J., Alados, C.L., 2013. Modeling shrub encroachment in subalpine grasslands under different environmental and management scenarios. Journal of Environmental Management 121, 160-169. doi:10.1016/j.jenvman.2013.01.038

Lamarque, P., Meyfroidt, P., Nettier, B., Lavorel, S., 2014. How ecosystem services knowledge and values influence farmers' decision-making. Plos One 9. doi:10.1371/journal.pone.0107572.

Lamarque, P., Tappeiner, U., Turner, C., Steinbacher, M., Bardgett, R.D., Szukics, U., Schermer, M., Lavorel, S., 2011. Stakeholder perceptions of grassland ecosystem services in relation to knowledge on soil fertility and biodiversity. Regional Environmental Change 11 (4), 791-804. doi:10.1007/s10113-011-0214-0.

Lasanta, T., Nadal-Romero, E., Arnáez, J., 2015. Managing abandoned farmland to control the impact of re-vegetation on the environment. The state of the art in Europe. Environment Science Policy 52, 99-109. doi:10.1016/j.envsci.2015.05.012.

Lees, A., Sejian, V., Wallage, A., Steel, C., Mader, T., Lees, J., Gaughan, J., 2019. The impact of heat load on cattle. Animals 9. doi:10.3390/ani9060322.

Lipton, J., Özdemiroglu, E., LeJeune, K., Peers, J., 2018. Resource equivalency methods in the European Union: A ’toolkit’ for calculating environmental liability. Equivalency methods for environmental liability: Assessing damage and compensation under the European environmental liability directive. Springer.

Majdalawi, M.I., Raedig, C., Al-Karablieh, E.K., Schlueter, S., Salman, A., Tabieh, M., 2016. Integration of different environmental valuation methods to estimate forest degradation in arid and semi-arid regions. International Journal of Sustainable Development and World Ecology. 23 (5), 392-398. doi:10.1080/13504509.2015.1124934

MEA, 2005. Ecosystems and Human well-being: Sinthesis. Island Press, Washington DC, USA.

Meyfroidt, P., Lambin, E.F., Erb, K.H., Hertel, T.W., 2013. Globalization of land use: distant drivers of land change and geographic displacement of land use. Current Opinion in Environmental Sustainability 5 (5), 438-444. doi:10.1016/j.cosust.2013.04.003.

Múgica, L., Canals, R.M., San Emeterio, L., 2018. Changes in soil nitrogen dynamics caused by prescribed fires in dense gorse lands in SW Pyrenees. Science of the Total Environment 639, 175-185. doi:10.1016/j.scitotenv.2018.05.139

Niedrist, G., Tasser, E., Lüth, C., Dalla Via, J., Tappeiner, U., 2009. Plant diversity declines with recent land use changes in European Alps. Plant Ecology 202 (2), 195-210. doi:10.1007/s11258-008-9487-x.

Notaro, S., Paletto, A., 2012. The economic valuation of natural hazards in mountain forests: an approach based on the replacement cost method. Journal of Forest Economy 18 (4), 318-328. doi:10.1016/j.jfe.2012.06.002.

Pan, Y., Wu, J., Xu, Z., 2014. Analysis of the tradeoffs between provisioning and regulating services from the perspective of varied share of net primary production in an alpine grassland ecosystem. Ecological Complexity 17, 79-86. doi:10.1016/j.ecocom.2013.11.001.

Pavanelli, D.D., Voulvoulis, N., 2019. Habitat Equivalency Analysis, a framework for forensic cost evaluation of environmental damage. Ecosystem Services 38, 100953. doi:10.1016/j.ecoser.2019.100953.

Petz, K., Alkemade, R., Bakkenes, M., Schulp, C.J.E., van der Velde, M., Leemans, R., 2014. Mapping and modelling trade-offs and synergies between grazing intensity and ecosystem services in rangelands using global-scale datasets and models. Global Environment Change 29, 223-234. doi:10.1016/j.gloenvcha.2014.08.007.

Pittarello, M., Probo, M., Lonati, M., Lombardi, G., Vandvik, V., 2016. Restoration of subalpine shrub-encroached grasslands through pastoral practices: effects on vegetation structure and botanical composition. Applied Vegetation Science 19 (3), 381-390. doi:10.1111/avsc.12222.

R Core Team, 2011. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Razquin, M.M., Aranguren, E., Taüll, M., Fernández-Otal, J.A., Ferrer, V., Gascouat, P., Canals, R.M., 2012. International grassland communals (facerías) in the Pyrenees: history, current situation and future prospects. In: Canals, R.M., San Emeterio, L. (Eds.), Nuevos retos de la ganadería extensiva: un agente de conservación en peligro de extinción. SEEP, Pamplona.

Roach, B., Wade, W.W., 2006. Policy evaluation of natural resource injuries using habitat equivalency analysis. Ecological Economics 58 (2), 421-433. doi:10.1016/j.ecolecon.2005.07.019.

San Emeterio, L., Múgica, L., Ugarte, M.D., Goicoa, T., Canals, R.M., 2016. Sustainability of traditional pastoral fires in highlands under global change: Effects on soil function and nutrient cycling. Agriculture, Ecosystems and Environment 235, 155-163. doi:10.1016/j.agee.2016.10.009

Schirpke, U., Kohler, M., Leitinger, G., Fontana, V., Tasser, E., Tappeiner, U., 2017. Future impacts of changing land-use and climate on ecosystem services of mountain grassland and their resilience. Ecosystem Services. 26, 79-94. doi:10.1016/j.ecoser.2017.06.008.

Schirpke, U., Tappeiner, U., Tasser, E., 2019. A transnational perspective of global and regional ecosystem service flows from and to mountain regions. Scientifics Reports 9 (1) doi:10.1038/s41598-019-43229-z.

Seaby, R., Henderson, P.A., 2006. Species Diversity and Richness. Version 4. Pisces Conservation Ltd., Lymington, England.

Sebastià, M.T., Canals, R.M., Marks, E., Llurba, R., Hopkins, A., Gustafsson, T.,Bertilsson, J., Dalin, G., Nilsdotter-Linde, N., Sporndly, E., 2008. Low-intensity livestock systems in Europe: an opportunity for quality products, recreation revenues and environmental conservation. In: Biodiversity and animal feed: Future challenges for grassland production proceedings of the 22nd General meeting of the European grassland federation, pp. 892-901.

Sharma, L.N., Vetaas, O.R., Chaudhary, R.P., Måren, I.E., 2014. Ecological consequences of land use change: forest structure and regeneration across the forest-grassland ecotone in mountain pastures in Nepal. Journal of Mountain Science 11 (4), 838-849. doi:10.1007/s11629-013-2849-4.

Shaw, D., Wlodarz, M., 2013. Ecosystems, ecological restoration, and economics: does habitat or resource equivalence analysis mean other economic valuation methods are not needed? Ambio 42, 628-643. doi:10.1007/s13280-012-0351-9.

Sollenberger, L.E., Kohmann, M.M., Dubeux Jr., J.C.B., Silveira, M.L., 2019. Grassland management affects delivery of regulating and supporting ecosystem services. Crop Science 59 (2), 441-459. doi:10.2135/cropsci2018.09.0594.

Tardella, F.M., Bricca, A., Piermarteri, K., Postiglione, N., Catorci, A., 2017. Context-dependent variation of SLA and plant height of a dominant, invasive tall grass (*Brachypodium genuense*) in sub-Mediterranean grasslands. Flora 229, 116-123. doi:10.1016/j.flora.2017.02.022

Underwood, N., Inouye, B.D., Hambäck, P.A., 2014. A conceptual framework for associational effects: when do neighbors matter and how would we know? The quarterly review of biology 89 (1), 1-19. doi:10.1086/674991.

Valéry, L., Fritz, H., Lefeuvre, J.C., Simberloff, D., 2009a. Invasive species can also be native. Trends in Ecology and Evolution 24, 585. doi:10.1016/j.tree.2009.07.003.

Vicente-Serrano, S.M., Lasanta, T., Romo, A., 2004. Analysis of spatial and temporal evolution of vegetation cover in the spanish central Pyrenees: role of human management. Environmental Management 34 (6), 802-818. doi:10.1007/s00267-003-0022-5.

Victor, P.A., 2020. Cents and nonsense: a critical appraisal of the monetary valuation of nature. Ecosystem Services 42, 101076. doi:10.1016/j.ecoser.2020.101076.

Villalba, J.J., Provenza, F.D., Clemensen, K.A., Larsen, R., Juhnke, J., 2011. Preference for diverse-pastures by sheep in response to intraruminal administrations of tannins, saponins and alkaloids. Grass Forage Science 66, 224-236. doi:10.1111/j.1365-2494.2010.00779.x.

Wilson, J.B., Peet, R.K., Dengler, J., Pärtel, M., 2012. Plant species richness: The world records. Journal of Vegetation Science 23, 796-802. doi:10.1111/j.1654-1103.2012.01400.x

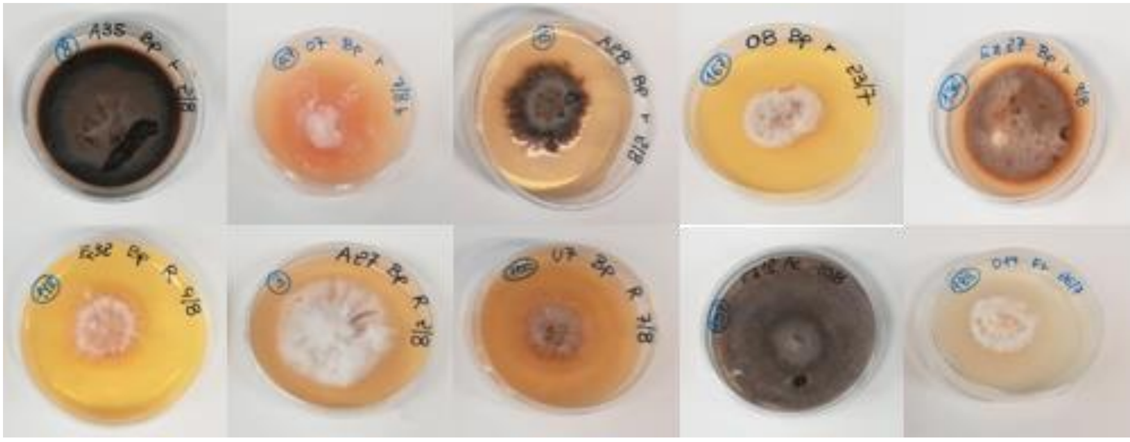
CHAPTER 2

2325

Disruption of traditional grazing and fire regimes shape the fungal endophyte assemblages of the tall-grass *Brachypodium rupestre*

ABSTRACT

The plant microbiome is likely to play a key role in the resilience of communities to the global climate change. This research analyses the culturable fungal mycobiota of *Brachypodium rupestre* across a sharp gradient of disturbance caused by an intense, anthropogenic fire regime. This factor has dramatic consequences for the community composition and diversity of high-altitude grasslands in the Pyrenees. Plants were sampled at six sites, and the fungal assemblages of shoots, rhizomes and roots were characterized by culture-dependent techniques. Compared to other co-occurring grasses, *B. rupestre* hosted a poorer mycobiome which consisted of many rare species and a few core species that differed between aerial and belowground tissues. Recurrent burnings did not affect the diversity of the endophyte assemblages, but the percentages of infection of two core species - *Omnidemptus graminis* and *Lachnum* sp. - increased significantly. The patterns observed might be explained by (1) the capacity to survive in belowground tissues during winter and rapidly spread to the shoots when the grass starts its spring growth (*O. graminis*), and (2) the location in belowground tissues and its resistance to stress (*Lachnum* sp.). Future work should address whether the enhanced taxa have a role in the expansive success of *B. rupestre* in these anthropized environments.



1. INTRODUCTION

The study of the plant microbiome is a powerful tool that contributes to the understanding and prediction of the resilience of plant communities to global change (Vandenkoornhuyse et al., 2015). In extreme environments, where ecosystems are fragile and very sensitive to changes, the plant microbiota is crucial to understand the adaptive capacity of plant communities. Low temperatures, soil acidity, low rates of mineralization, and low nutrient availability are some of the common traits of artic and alpine ecosystems, where the study of the composition and function of the plant-microbial consortium has been addressed in the last decades (Li et al., 2012; Poosakkannu et al., 2014; Rudgers et al., 2014; Bråthen et al., 2015; Kotilínek et al., 2017).

Current research reports that for a particular ecosystem, the composition and diversity of the fungal microbiome of a plant host depends on its potential for carbon provision (plant abundance and biomass), on the fungal propagule availability, and on environmental factors, mainly climate, rather than on the taxonomy of the plant host (Higgins et al., 2014; Ranelli et al., 2015; Glynou et al., 2016; Kivlin et al., 2019). The first factor, potential for carbon provision, gives support to the resource-diversity hypothesis, the highest plant carbon determines the highest guest diversity (Wang et al., 2020), and this effect is modulated by the richness and diversity of the neighboring host plants and their own fungal populations (Hiiesalu et al., 2017; Vannier et al., 2020). Regarding the climate, the historical and current precipitation regime is known to play a key role in fungal endophyte composition (Lau et al., 2013; Liu et al., 2017), and its consequences in a scenario of climate change are a growing subject of study in recent years (Giauque and Hawkes, 2016; Koide et al., 2017; Slaughter et al., 2018).

In addition to the climate change, plant communities in some cold environments are being affected by other significant mechanisms of change (Pauchard et al., 2009). Many high altitude grasslands undergo profound changes due to the disruption of the historical regime of disturbances that created and preserved them (Lasanta-Martínez et al., 2005; Komac et al., 2013). Fire and herbivory are two crucial disturbances shaping the landscape of natural grasslands worldwide (Archibald et al., 2005; Anderson and Hoffman, 2007). Extensive grazing by livestock in highlands has sharply declined in Europe in the last decades. Grazing is considered the major driver of plant guild composition and diversity in grassland communities (Milchunas et al., 1988; Canals and Sebastià, 2000; Frank, 2005; Eldridge and Delgado-Baquerizo, 2018). Grazing has profound effects on soils, promoting root exudation and carbon flow exchange to the rhizosphere (Dawson et al., 2000; Hamilton and Frank, 2001), enhancing soil microbial biomass and activating the soil biogeocycles (Bardgett and Wardle, 2003; Shaw et al., 2016), increasing and redistributing nutrients availability (Augustine et al., 2003; Liu et al., 2017) and affecting soil aeration by compaction (Jing et al., 2014). Through these effects on plants and soils, grazing may affect the structure of the fungal microbiome of plants (Schulz and Boyle, 2005; Wang et al., 2020).

Regarding fire, some high-altitude areas are currently experiencing an intensified regime of anthropic fires to reduce shrub encroachment and biomass build-ups caused by the relaxation of grazing activities (Köhler et al., 2005; Múgica et al., 2018). The effects of prescribed fires on soil properties have been documented in a range of contrasted habitats (Alcañiz et al., 2018), including high-altitude grasslands (in the Pyrenees, Armas-Herrera et al., 2016; San Emeterio et al., 2016). Although prescribed burnings do not reach the high temperatures of wildfires, their short and mid-term effects (thermal shocking, aboveground plant combustion, ash deposition, nutrient mineralization...) may drive profound changes in the plant community composition if the fire regime increases in recurrence (Uys et al., 2004). Regarding microbial soil communities, even low-intensity fires may depress soil microbial biomass (Múgica et al., 2018), with complex consequences on the C and N cycles (Soong and Cotrufo, 2015; Shaw et al., 2016; Pellegrini et al., 2020). The composition of soil fungal communities has been documented to be disrupted by fires as well (Artz et al., 2009; Egidi et al., 2016; Semenova-Nelsen et al., 2019). Few studies have addressed the effects of fire on plant fungal assemblages (Bellgard et al., 1994; Eom et al., 1999; Mataix-Solera et al., 2002), although changes in species richness have been detected in foliar endophytes of burned trees (Huang et al., 2016), and specific plant-fungal mutualisms and pyrophilous fungal species have been described (Baynes et al., 2012; Raudabaugh et al., 2020).

The western Pyrenees encompass large areas affected by the decoupling of traditional fire and grazing practices. As a result of decreasing herbivore pressure and increasing burnings, a native perennial grass is expanding. *Brachypodium rupestre* (Host) Roem. & Schult (Schippmann, 1991; Schippmann and Jarvis, 1988) (= *B. pinnatum* subsp. *rupestre* (Host) Schübl. & Martens according to some authors; Aizpuru et al., 1999), dominates grasslands and causes a severe loss of sympatric species (Canals et al., 2014; Canals, 2019), a phenomenon also observed in other European mountain ranges (Buckland et al., 2001; Holub et al., 2012; Tardella et al., 2018). In this natural setting, many questions arise concerning the response of the plant holobiont to the changed disturbance regime. To what extent the composition of the *B. rupestre* mycobiome responds to the altered disturbance regime? Does the species harbor a specific fire-adapted mycobiome in frequently burnt areas? Or on the contrary, is there sufficient inertia to maintain a similar mycobiome in a common climatic environment (cold temperatures, high rainfall and humidity), independently of the current disturbance regime? Since taxon-specific endophytes (such as *Epichloë* sp.) may confer a higher adaptive advantage in stressful situations than broadly distributed non-systemic endophytes (Hill et al., 1989; Malinowski and Belesky, 2000; Pereira et al., 2019; Harrison and Griffin, 2020), is the mycobiome of the plant responding to these expectations?

The main objective of this research was to characterize the culturable fungal endophyte community of *B. rupestre* across a well-defined gradient of disturbance. The gradient encompassed sites with different burning recurrences and levels of herbivory, which have led to different patterns of grassland structure and diversity. We also studied the fungal endophyte community of the aerial tissues of the most frequent companion species of *B. rupestre* in grasslands, *Festuca rubra* and *Agrostis capillaris*. The purpose was to estimate whether the major diversity of coexisting plant species (and potential fungal propagules) in diverse grasslands had a positive influence in the fungal assemblage of *B. rupestre*.

2. MATERIALS AND METHODS

2.1. Study site and sampling design

The study area was located in the Aezkoa Valley in the western Pyrenees (43◦3r N1◦13r W; 800-1900 m.a.s.l.), and occupies an area of 198 Km^2. Because of the influence of the Atlantic ocean (55 km away in a straight line) the weather is cold and snowy during winter, and mild and foggy during summer. The mean annual temperature is 9.3°C and mean annual precipitation is 1856 mm, according to data collected during 1989-2019 at the nearest climatic station, Irabia at 822 m.a.s.l. Soils are mainly derived from sandstones and calcareous clays, acidic with high organic matter and loamy or clay-loamy textures. The landscape is a mosaic of beech forests, gorse shrublands dominated by *Ulex gallii* Planch and *Erica vagans* L., and grasslands dominated by perennial grasses such as *Festuca rubra gr.*, *Agrostis capillaris* L., *Brachypodium rupestre* (Host) Roem. & Schult, *Danthonia decumbens* (L.) and *Avenula sulcata* (J. Gay ex Boiss) Dumort. Other species are *Galium saxatile* L., *Potentilla erecta* (L.) Räeusch, *Potentilla montana* Brot., *Hypochaeris radicata* L., and the legume *Trifolium repens L.* The area is part of the European Protected Areas Network (Natura 2000) and was declared Special Area of Conservation (Roncesvalles-Selva de Irati, code ES0000126) in 2011 (Figure 1).

High-altitude grasslands are extensively grazed from May to October by sheep, cows and horses. Domestic herbivores have been present in the area since the Neolithic, as numerous prehistoric pastoral remains indicate. Where an appropriate extensive summer grazing exists, grasslands constitute high-diversity communities that are burned every 6-7 years by the bush-to-bush traditional practice to control localized shrub resprouting (San Emeterio et al., 2016). However, in the last decades, the number of grazers has sharply declined due to socio-economical changes experienced in the valleys, and the use of repeated winter burnings to control the build-up of ungrazed biomass has increased (Durán et al., 2020). Nowadays, many areas are burned recurrently, every 1-2 years (Canals et al., 2017). Due to the frequent burnings and the lack of grazing, the grasslands are far less diverse, favored by the expansion and dominance of *B. rupestre*, which grows in dense and tall clumps. Consequently, the current open landscape is a combination of grassland communities with different degrees of cover by *B. rupestre*, which accurately reflects the level of herbivory and the burning recurrence.

Based on the cartographic information provided by the management plan of the area (Ferrer and Canals, 2008), we selected six grassland communities where *B. rupestre* was present at different coverages: three areas had above 75% cover and three areas under 25% cover (Table 1 and Figure 1). The detailed floristic communities of the area are available in Durán et al. (2020). At each location, 40 turfs (400 cm^3) of *B. rupestre* (including shoots, rhizomes, roots and rhizosphere) were collected in summer 2018. We established a distance of ca 30 m among collected plants to avoid clonal individuals (Bąba et al., 2012). In the three areas of diverse grasslands, 40 turfs of *Festuca rubra* and 40 turfs of *Agrostis capillaris* were also collected. Sampling points were georeferenced and the sampling grid covered ca 2.5 ha per location (Supplementary Figure 1). In total, 480 turfs were collected, placed in seedbed trays and transported to the UPNA laboratory. The plants in the turfs were processed for the isolation of fungi from shoots, rhizomes and roots in the same week.

Table 1. Characteristics of the six locations selected

Location		Fire recurrence	Grazing level	***B. rupestre* cover (%)**	Plant diversity
1	Arpea	High: 1-2 years	Low to nonexistent	> 75%	Very low
2	Errozate				
3	Armorieta				
4	Urkulu	Low: 6-7 years	Moderate	< 25%	High
5	Zalbetea				
6	Azalegi				

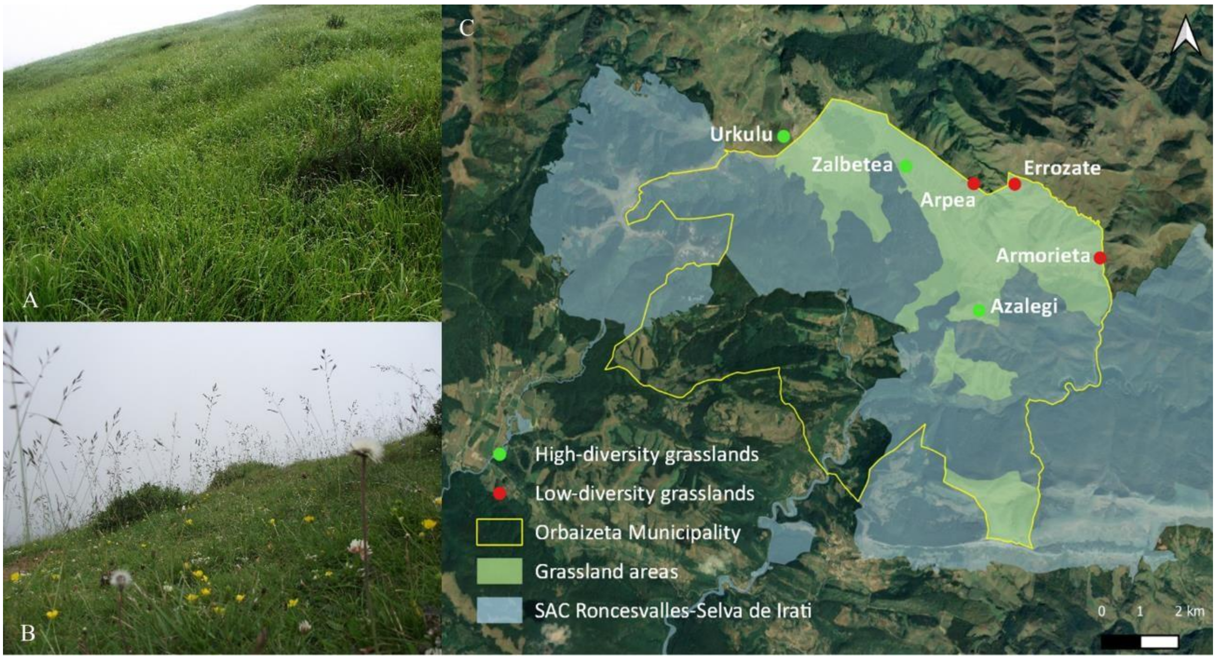


Figure 1. Locations selected for collecting plants. Red points represent low-diversity grasslands and green points high-diversity grasslands (C). Pictures of the *Brachypodium rupestre* invaded (A) and non-invaded (B) areas.

2.2. Isolation and identification of fungi

We isolated fungi from shoots, rhizomes and roots of *B. rupestre* and from shoots of *F. rubra* and *A. capillaris*. Leaf sheaths and stems were cut into fragments of ca 5 mm, and surface-disinfected by immersion in a solution of 20% commercial bleach (1% active chlorine) containing 0.02% Tween 80 (v:v) for 10 min, and finally rinsed with sterile water. The fragments of rhizomes and roots were surface-sterilized with the same bleach-Tween 80 solution, but then treated with an aqueous solution of 70% ethanol for 30 s, and a final rinse with sterile water. About 10-12 tissue fragments of the same individual were plated in a Petri dish with potato dextrose agar (PDA) containing 200 mg/L of chloramphenicol to avoid the growth of endophytic bacteria. Petri dishes, kept at room temperature and ambient light, were checked daily for mycelium growth during 5 weeks. When mycelium emerged from a tissue fragment, a small amount was transferred to a new Petri dish to obtain a culture.

Isolated fungi were grouped into morphotypes according to their morphological characteristics (colony color, exudates, growth type, and mycelium appearance). One or more isolates of each morphotype were genotyped for taxonomic purposes. To do this, a small amount of mycelium was scratched from the isolate culture and its DNA extracted using the Phire Plant Direct PCR Kit (Thermo Fisher Scientific). The ITS1, 5.8s and ITS2 regions were amplified using ITS4 and ITS5 primers (White et al., 1990). PCR amplification was done at 98°C for 5 min, followed by 35 cycles of 95°C for 5 s, 54°C for 5 s, 72°C for 20 s, and a final phase of 72°C for 1 min. Amplicons were purified (Favor PrepTM Plant Genomic DNA Extraction Mini Kit, Favorgen) and sequenced by the Sanger method at an external sequencing service (STABvida).

Because the range of intraspecific variation in ITS sequences is unknown for most fungal species (Taylor et al., 2000), DNA sequences were clustered using the CD_HIT program (Li and Godzik, 2006; Huang et al., 2010) and those with 97% or more similarity were considered to belong to the same taxon. A representative sequence of each cluster was selected and used to search the database of the ITS region from fungi type and reference material (Schoch et al., 2014) at the National Center for

Biotechnology Information (NCBI) using the BLAST algorithm.

In addition, the UNITE database of fungal nucleotide sequences was used as a complement for sequences without type specimens in NCBI. We also used the database FUNGuild (Nguyen et al., 2016) to get information on the ecological guild of each taxon, and to estimate for their possible functional roles.

2.3. Data analysis

To evaluate the efficiency of our sampling effort for measuring species richness, species accumulation curves were estimated with all the species, and also excluding singletons, species that appeared only once (Vegan package, Oksanen et al., 2017). We calculated the incidence of all endophyte species within each type of tissue (shoots, rhizomes and roots), grassland type (low and high-diversity) and host plant species, and determined richness and Shannon and Simpson diversity indexes (Vegan package, Oksanen et al., 2017). Differences in species richness and diversity indexes of *B. rupestre* endophytes were analyzed using two-way ANOVA with tissue and grassland type as fixed factors, and permutation tests was used to compare diversity indexes of *B. rupestre*, *F. rubra,* and *A. capillaris* shoots (Coin package, Hothorn et al., 2008).

Venn diagrams were used to represent the taxa shared among grassland types, plant tissues and grass species (Euler package, Chen and Boutros, 2011). Frequencies were calculated from the matrix of presence/absence of isolates and permutational analyses of variance (PERMANOVA) were used to evaluate the variability of the fungal endophyte assemblages of *B. rupestre* between grassland types and among plant tissues. For that purpose, Adonis function was used (Vegan package, Oksanen et al., 2017). Distances were calculated using Bray-Curtis dissimilarities, set the number of permutations to 9999 and constrained the permutations within each location. Since PERMANOVA analyses are very sensitive to heterogeneity of multivariate dispersions, homogeneous dispersion between treatment groups was tested using the betadisper function.

To identify which species were characteristic of a particular tissue and grassland type, the indicator species tests in the Labsdv package was used (Roberts, 2019). The indicator value of as species (indval) measures the fidelity and relative abundance of the species in a particular situation (Dufrêne and Legendre, 1997). General Linear Mixed Models (GLMM) was used to determine whether the indicator species for a particular grassland type presented differences in the probability of incidence among grassland types. The grassland type was included as the fixed factor, the location as the random factor, using a binomial distribution. In addition, GLMM was used to analyze whether the probability of incidence of some mycobiome core species from the aboveground tissues presented differences among host plant species. Host plant species was included as the fixed factor, location as the random factor, using a binomial distribution. GLMM's were done using the lme4 package (Bates et al., 2015).

3. RESULTS

3.1. Isolation and identification of fungal endophytes

We plated ca 10000 tissue fragments of *B. rupestre* (shoots, rhizomes and roots), *F. rubra* (shoots) and *A. capillaris* (shoots) in 960 culture media plates (10-12 fragments per plate). We obtained

1151 isolates (ca 190 isolates per location) which were classified into a total of 95 morphotypes. One or more isolates of each morphotype were sequenced, obtaining 116 sequences. After the sequence clustering process, 61 different sequence types remained, which were identified into 53 different taxa using the NCBI and UNITE databases. Twenty-two of them were classified to species rank, 20 to genus, 6 to family, 3 to order, and the remaining two to class rank (Supplementary Table 1 contains the complete list of taxa identified and their accession number).

3.2. Diversity of the *B. rupestre* mycobiome

Fungi were isolated from 58% of the *B. rupestre* plants in low-diversity grasslands and from 43% in high-diversity grasslands (Table 2). Endophyte incidence varied among tissues: 37% of shoots, 52% of rhizomes and 64% of roots harbored fungi. Forty-five different taxa were identified in *B. rupestre*. Endophyte species richness in *B. rupestre* shoots, rhizomes and roots were 9, 25, and 26 taxa, respectively. Endophyte species richness in *B. rupestre* growing in low-diversity grasslands ranged from 9 to 22, and from 12 to 18 taxa in high-diversity grasslands (Table 2). Roots and rhizomes shared 23.9% of the species, whereas shoots with rhizomes and with roots shared 8.7 and 6.5%, respectively, all tissues had only 6.5% of all species in common (Figure 2). *B. rupestre* plants in low and high-diversity grasslands shared 39.1% of the species.

The most frequent species were *Albotricha* sp., *Lachnum* sp. B, *Omnidemptus graminis,* and Mollisiaceae sp. (Table 3). *Omnidemptus graminis* was more frequent in shoots than in rhizomes and absent in roots, whereas *Albotricha* sp., *Lachnum* sp. B, and Mollisiaceae sp. were more frequent in belowground tissues than in shoots. In addition to the Mollisiaceae sp. taxon, other dark septate endophytes (DSE) such as *Cadophora* sp., *Microdochium bolleyi*, *Microdochium neoqueenslandicum,* and *Periconia* sp. were found, although with a low incidence. Members of the Clavicipitaceae family, such as *Metapochonia bulbillosa*, *Metarhizium carneum,* and *Epichlöe typhina* in shoots and rhizomes also occurred. The frequencies of the remaining species were under 2% (Supplementary Table 2). Three trophic types (pathotroph, saprotroph, and symbiotroph) were found in the mycobiome of *B. rupestre*. The most common guilds were plant pathogens, endophytes and undefined saprotrophs. However, a remarkable number of taxa were animal pathogens (*Clonostachys rosea*, *Tolypocladium album,* and *Trichoderma koningii* are described as entomopathogens, *Metapochonia bulbillosa* and *Sarocladium strictum* as nematophagous). Fungi with antimicrobial activity (*Fusarium circinatum*, *Glarea* sp., *Nemania* sp., and *Penicillium ortum*) and species with a high source of bioactive compounds (*Acremonium* sp., *Gaeumannomycella* sp., and *Lachnum* sp.) also occurred (Supplementary Table 1).

The species-accumulation curves including all the endophyte taxa were non-asymptotic (Figure 3), but when singletons were excluded the curves reached a plateau, suggesting that an increase in the sampling effort would reveal few common taxa, and mostly rare species. Therefore, a sampling effort of 100 plants is adequate to flatten the curves of the different tissues when excluding the singletons (Figures 3B-D). A greater sampling effort would be needed to include all the tissues (Figure 3A), although low-diversity grasslands need more plants than high-diversity grasslands to characterize their endophyte richness (Figures 3E,F).

Species richness and diversity indexes were significantly different among tissues ($F = 10.319$, $p < 0.001$; $F = 18.336$, $p < 0.001$; $F = 24.114$, $p < 0.001$; for richness, Shannon and Simpson, respectively). Shoots showed consistently lower diversity values than rhizomes or roots in both types of grasslands (Figure 4). No significant differences in diversity were found between low and high-diversity grasslands ($F = 0.210$, $p > 0.05$; $F = 0.180$, $p > 0.05$; $F = 0.311$, $p > 0.05$; for richness, Shannon and Simpson, respectively).

Table 2. Number of plants collected, final plates (taking into account the contaminations), fungal endophyte incidence (% plants), and number of fungal species in *Brachypodium rupestre*, all of that according to tissue and location.

	Tissue	**Number of plants**	**Number of plates**	**Endophyte incidence (% plants)**	**Number of fungal species**
	Shoot	240	231	37	9
	Rhizome	240	213	52	25
	Root	240	228	64	26
	Total	240	672		45
Cover	**Location**				
Low-diversity grasslands	Arpea	40	107	59	21
	Errozate	40	112	60	9
	Armorieta	40	110	56	22
	Total	120	329		32
High-diversity grasslands	Urkulu	40	114	42	12
	Zalbetea	40	114	49	18
	Azalegi	40	115	39	16
	Total	120	343		30

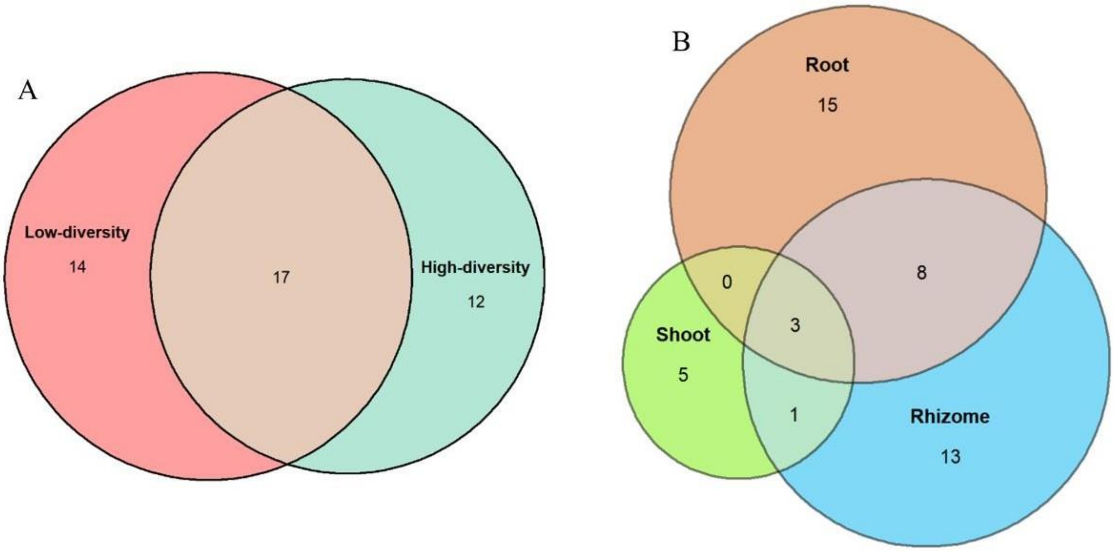


Figure 2. Venn's diagrams indicating the number of shared fungal species across grassland types (A) and tissues (B).

Table 3. Incidence in *Brachypodium rupestre* plants of the most abundant fungal endophyte taxa, differentiated by tissues and number of locations where they were present.

		Low-diverse grasslands		**High-diverse grasslands**	
Tissue	**Fungal endophyte**	**Incidence (%)**	**Number of locations**	**Incidence (%)**	**Number of locations**
Shoots	*Omnidemptus graminis*	46.1	3	23.3	3
	Epichloë typhina	2.6	1	3.4	2
	Sarocladium strictum	3.5	2	0	0
Rhizomes	*Lachnum* sp.B	13.9	3	8.9	2
	Mollisiaceae sp.	14.9	2	7.1	2
	Albotricha sp.	2	1	16.1	2
	Omnidemptus graminis	5.9	1	5.4	2
	Metapochonia bulbillosa	3	1	2.7	3
	Fusarium circinatum	3	3	1.8	1
	Penicillium ortum	2	2	2.7	1
	Tolypocladium album	3	1	0.9	1
	Clonostachys rosea	2	1	1.8	2
	Ilyonectria robusta	2	2	0.9	1
	Microdochium bolleyi	0	0	2.7	2
Roots	*Lachnum* sp. B	35.4	3	16.5	3
	Mollisiaceae sp.	14.2	3	14.8	3
	Albotricha sp.	9.7	3	19.1	2
	Lachnum sp. A	3.5	2	2.6	2
	Ilyonectria robusta	0.9	1	5.2	2
	Glarea sp.	1.8	2	3.5	2
	Dictyochaeta sp.	3.5	2	0	0
	Acremonium sp.	1.8	2	1.7	1
	Fusarium circinatum	0	0	3.5	2
	Mollisia sp.	2.7	1	0	0

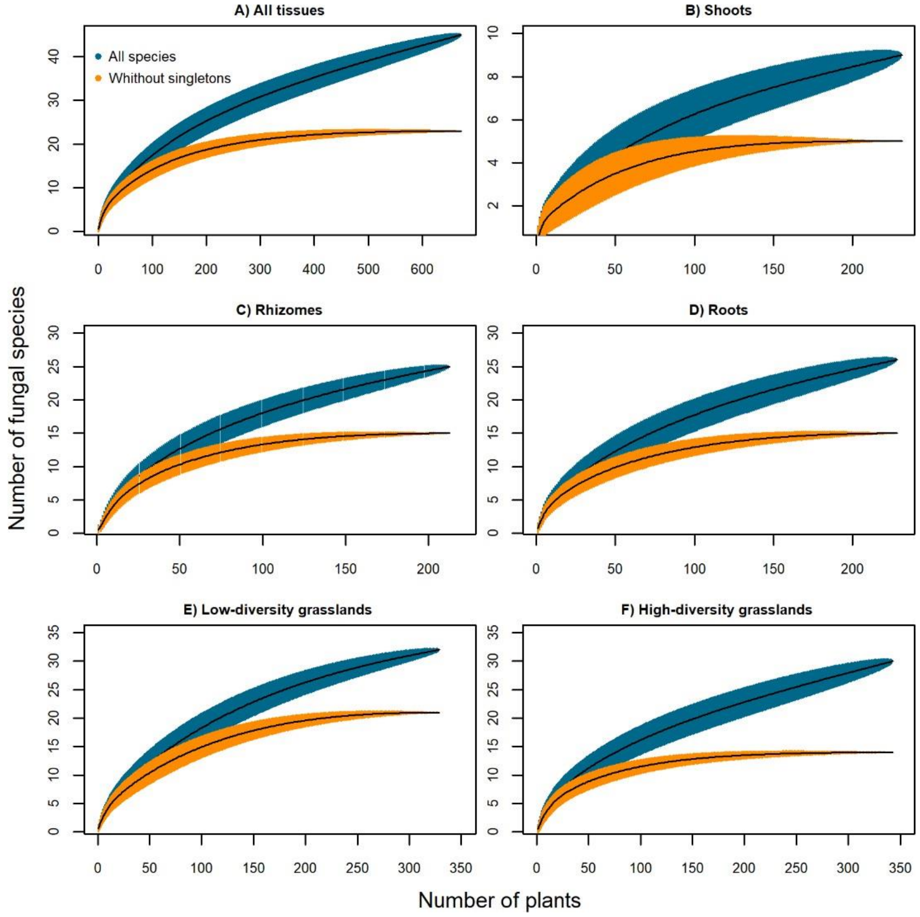


Figure 3. Species-accumulation curves of the mycobiota isolated from *Brachypodium rupestre* according to plant tissue and grassland type. Black line, based estimator of the total number of species; Shaded zone, standard deviation; Dark blue, all species included; orange, excluding singleton species.

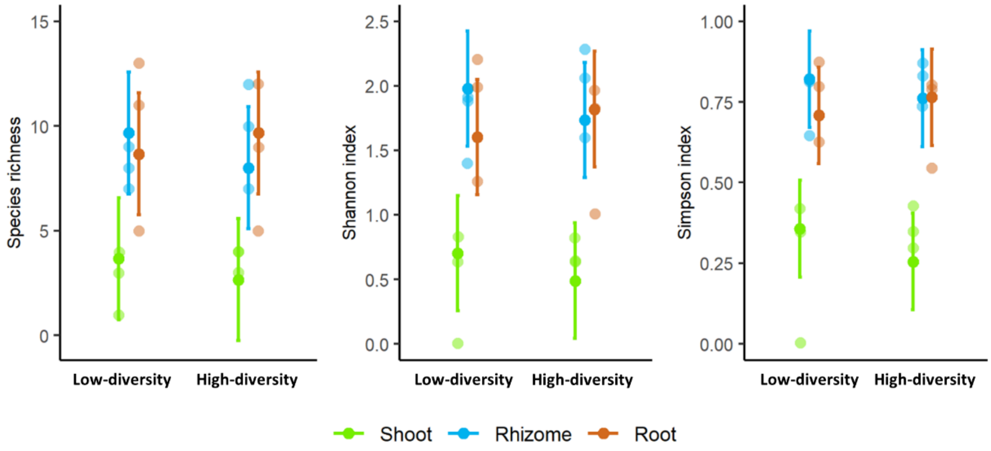


Figure 4. Species richness and diversity indexes (Shannon and Simpson) of the mycobiome of shoots, rhizomes and roots of *Brachypodium rupestre* in low and high-diversity grasslands.

3.3. Distribution patterns of the *B. rupestre* mycobiome

A homogeneous dispersion was found within different tissues (Beta-disper; F2,15 = 2.195, p = 0.146) and grassland types (Beta-disper; F1,16 = 0.028, p = 0.870), contrasting with the significant effects on community assemblages. PERMANOVA analysis showed that tissue and grassland type had a significant effect on the endophytic community of *B. rupestre* plants (both $p < 0.001$). Tissue explained ca 50% of the variance of the model ($r^2 = 0.508$), whereas grassland type explained ca 5% ($r^2 = 0.052$). The NMDS (Non-Metric Multidimensional Scaling) plot discriminated the assemblages according to the tissues and included the indicator species (Figure 5). *Omnidemptus graminis* (p = 0.001) and *Sarocladium strictum* (p = 0.032) were indicators of shoots. *Metapochonia bulbillosa* (p = 0.003), *Clonostachys rosea* (p = 0.011) and *Tolypocladium album* (p = 0.009) were indicators of rhizomes. And *Lachnum* sp. B (p = 0.001), *Albotricha* sp. (p = 0.001), Mollisiaceae sp. (p = 0.001), *Lachnum* sp. A (p = 0.008), *Acremonium* sp. (p = 0.021) and *Dictyochaeta* sp. (p = 0.024) were indicators of roots. Regarding the grassland type, *Albotricha* sp. (p = 0.001) was an indicator of high-diversity grasslands and *Lachnum* sp. B (p = 0.004) and *Omnidemptus graminis* (p = 0.003) of low-diversity grasslands.

GLMM's showed that *B. rupestre* plants from low-diversity grasslands had a greater probability of having their roots infected by *Lachnum* sp. B (LRT = 4.5719, p = 0.032) and their shoots by *Omnidemptus graminis* (LRT = 4.679, p = 0.030) than plants from high-diversity grasslands (Figure 6). Regarding *Albotricha* sp., the patterns were not so clear and its probability of incidence in *B. rupestre* roots was not significantly different between grassland types (LRT = 0.2849, p = 0.594, Figure 6).

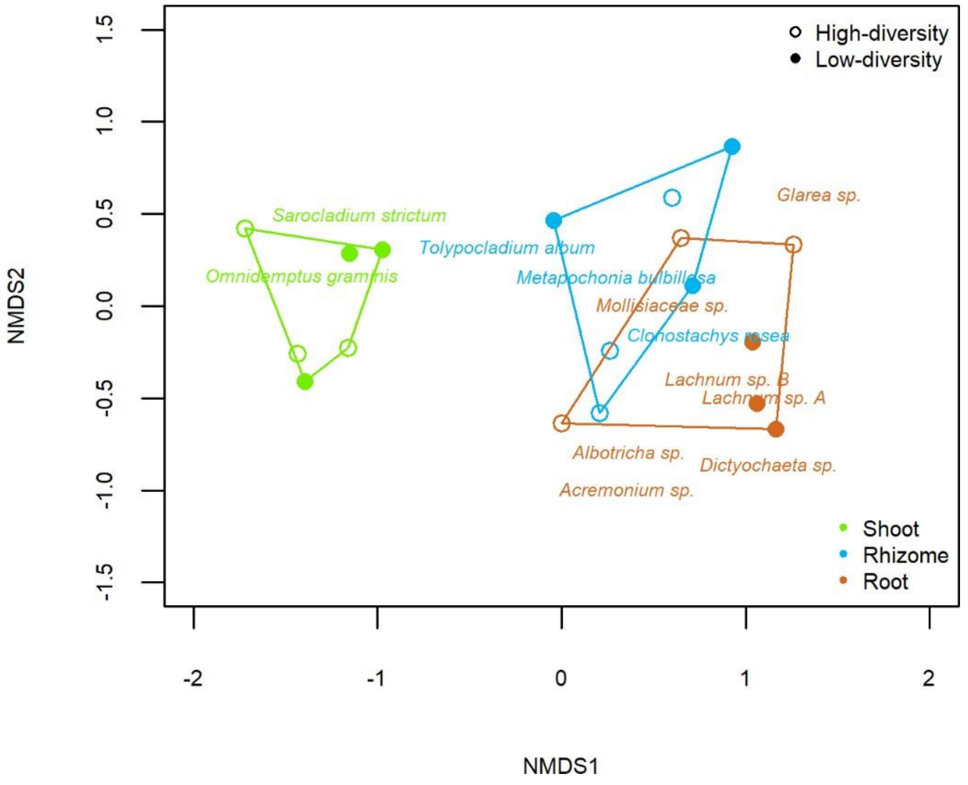


Figure 5. Non-metric multidimensional scaling (NMDS) of the fungal endophyte community composition of tissues of *Brachypodium rupestre* from low and high-diversity grasslands.

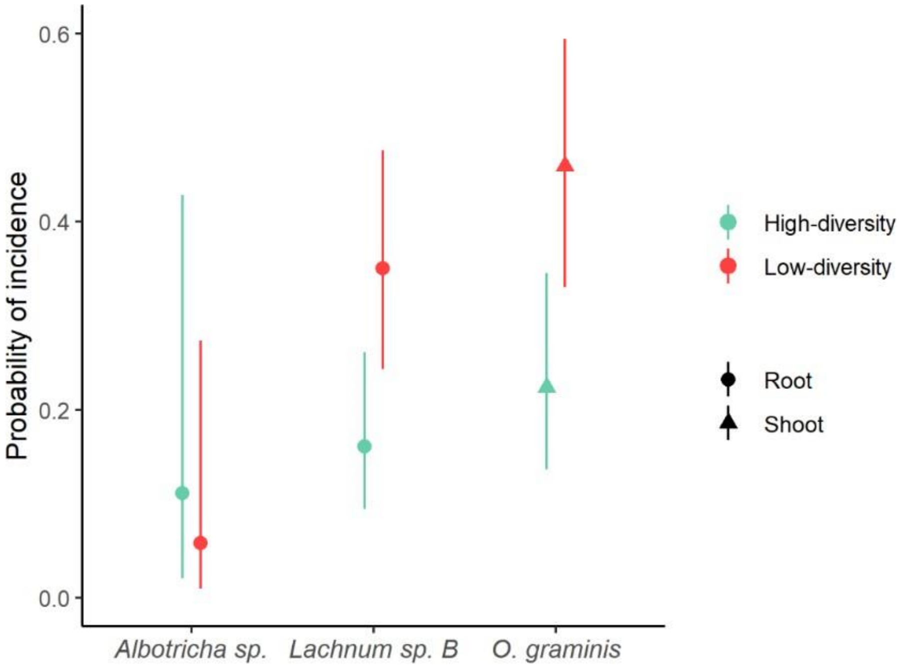


Figure 6. Probability of incidence of fungal indicator species of *Brachypodium rupestre* calculated with GLMM.

3.4. Aboveground endophyte assemblages of the most common grasses in high-diversity grasslands

The incidence of endophytes varied among the shoots of the three grass species, ranging from 25% in *B. rupestre* and *A. capillaris* to 38% in *F. rubra*. For each plant species, a strong variability of endophyte incidence among locations was detected (Table 4). Eight taxa not observed in the *B. rupestre* survey were isolated from *F. rubra* or *A. capillaris* (Supplementary material, table S1).

A total of 23 taxa were isolated from the shoots of *B. rupestre*, *F. rubra* and *A. capillaris* (Supplementary material, table S3). Endophyte species richness in *B. rupestre, F. rubra* and *A. capillaris* shoots were 5, 10 and 16, respectively (Table 4). *F. rubra* and *A. capillaris* shared more endophyte species between them than *B. rupestre* with either (Figure 7). The three grasses had two endophyte species in common, *Lachnum* sp. B and *Omnidemptus graminis*. The most frequent endophyte species in *B. rupestre* shoots were *Omnidemptus graminis* and *Epichloë typhina,* in *F. rubra Epichlöe festucae* and Mollisiaceae sp*.,* and in *A. capillaris Lachnum sp.* B, *Epichloë baconii* and Mollisiaceae *sp.* (Table 5).

When including all endophyte species, the species-accumulation curves of the three host grasses were non-asymptotic (Figure 8), but when removing singletons the curves flattened with a sampling effort of 50 plants for *B. rupestre* and 60 plants for *F. rubra* and *A. capillaris* in high-diversity grasslands (Figure 8).

The mycobiome of *A. capillaris* appeared to be more rich and diverse (averaged per site richness$_{AC}$ = 7.0, Shannon$_{AC}$ = 1.85, Simpson$_{AC}$ = 0.83) than that of *F. rubra* and *B. rupestre* (averaged richness$_{FR}$ = 4.3, Shannon$_{FR}$ = 0.76, Simpson$_{FR}$ = 0.36; averaged richness$_{BR}$ = 2.7, Shannon$_{BR}$ = 0.49, Simpson$_{BR}$ = 0.26). However, the high variability among locations and the size of the sample (Figure 9) did not allow to detect significant differences in richness and diversity indexes among grasses in the permutations tests (richness, t = 1.7876, p = 0.1737; Shannon, t = 2.0697, p = 0.09614; Simpson, t = 2.0193, p = 0.1076).

Omnidemptus graminis and *Epichloë typhina* were species indicators of *B. rupestre* (p = 0.001; p = 0.008), whereas *Epichloë festucae* was a species indicator of *F. rubra* (p = 0.001) and *Epichloë baconii* of *A. capillaris* (p = 0.009). The GLMM showed that the probability of incidence of *Omnidemptus graminis* significantly varied among species (LRT = 38.194, p < 0.001), and had a higher probability of incidence in *B. rupestre*, despite its presence in the other grasses (Figure 10). On the contrary, *Epichloë* species were specific of each grass (*E. typhina* in *B. rupestre*, *E. festucae* in *F. rubra* and *E. baconii* in *A. capillaris*), but the probability of *Epichloë* infection was higher in *F. rubra* than in the rest of grasses (LRT = 34.581, p < 0.001; Figure 10).

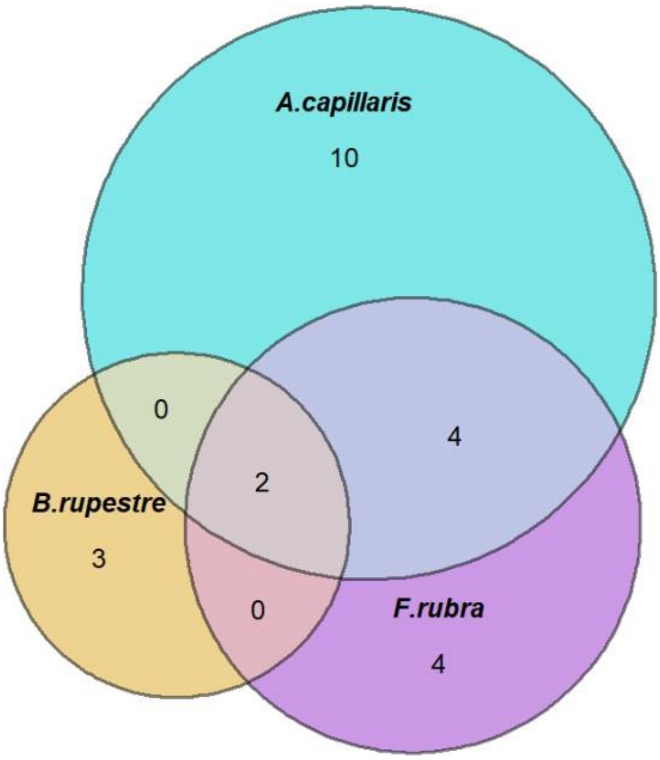


Figure 7. Venn's diagrams indicating the number of shared fungal endophyte species among grass species.

Table 4. Number of plants collected, number of final plates (taking into account the contaminations), fungal endophyte incidence (% plants), and number of fungal taxa per location for each grass species from high-diversity grasslands in aboveground tissues.

Grass species	Location	Number of plants	Number of plates	Fungal endophyte incidence (%)	Number of fungal species
B. rupestre	*Urkulu*	40	39	26	4
	Zalbetea	40	39	41	3
	Azalegi	40	38	8	1
	Total	120	116	25	5
F. rubra	*Urkulu*	40	39	51	7
	Zalbetea	40	38	8	1
	Azalegi	40	40	55	5
	Total	120	117	38	10
A. capillaris	*Urkulu*	40	40	38	7
	Zalbetea	40	40	20	8
	Azalegi	40	39	18	6
	Total	120	119	25	16

Table 5. Percentage of incidence of dominant fungal endophyte species in shoots of three representative grasses of high-diversity grasslands.

Grass species	Fungal endophyte	Urkulu	Zalbetea	Azalegi
B. rupestre	*Omnidemptus graminis*	23.1	38.5	7.9
	Epichloë typhina	2.6	7.7	0
F. rubra	*Epichloë festucae*	23.1	0	50
	Mollisiaceae sp.	20.5	0	2.5
	Albotricha sp.	0	7.9	2.5
	Alfaria dandenongensis	7.7	0	0
	Omnidemptus graminis	0	0	5
A. capillaris	*Lachnum* sp. B	15	0	0
	Epichloë baconii	7.5	0	5.1
	Mollisiaceae sp.	12.5	0	0
	Alfaria dandenongensis	5	2.5	0
	Ilyonectria robusta	2.5	0	2.6
	Albotricha sp.	5	0	0

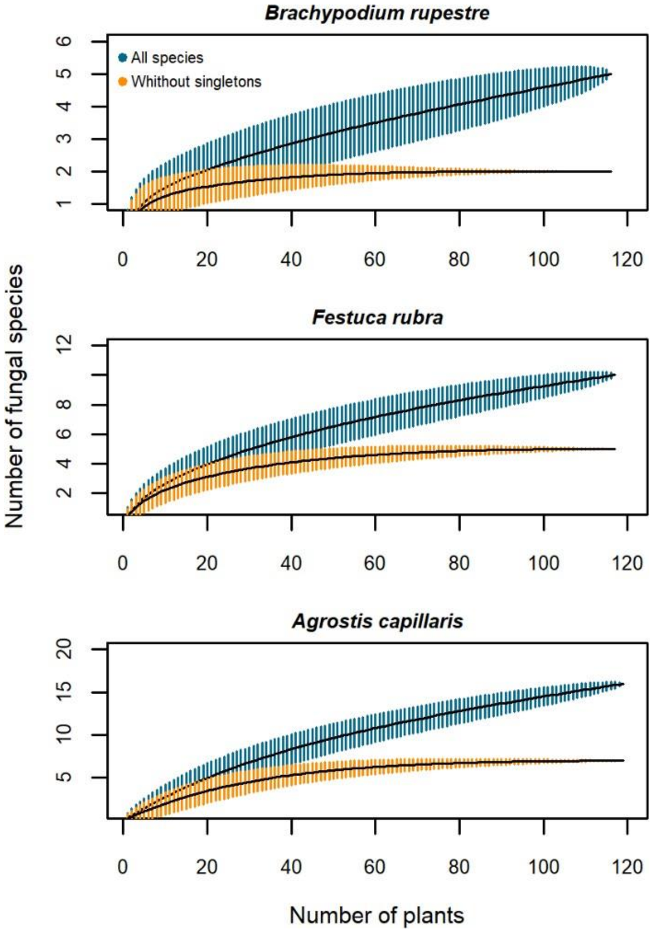


Figure 8. Species-accumulation curves of fungal endophytes from *Brachypodium rupestre*, *Festuca rubra* and *Agrostis capillaris* shoots from high-diversity grasslands. Black line, based estimator of the total number of species; Shaded zone, standard deviation; Dark blue, all species included; orange, excluding singleton species.

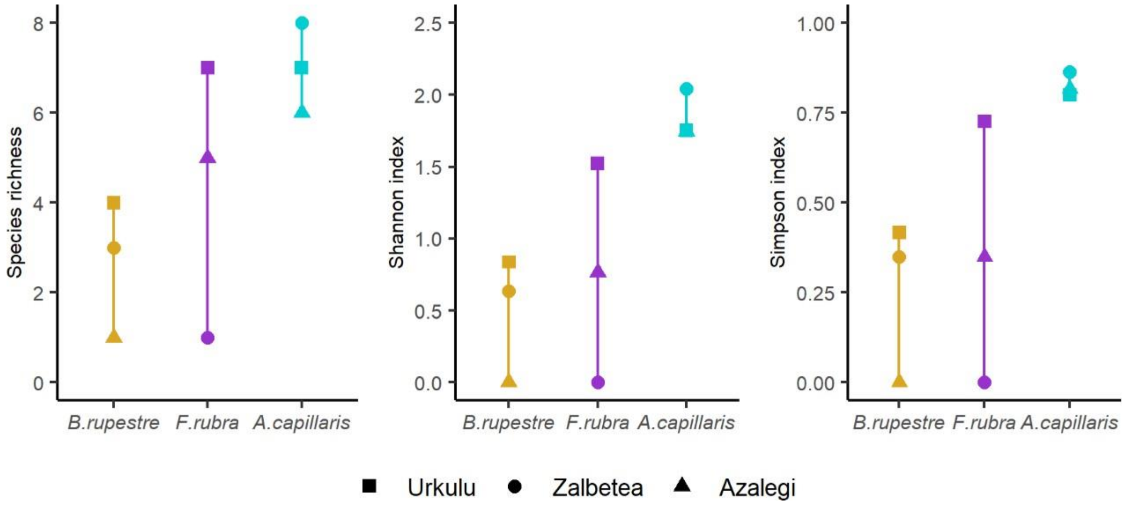


Figure 9. Species richness and diversity indexes (Shannon, Simpson) of the mycobiome of shoots from three representative grasses of high-diversity grasslands from three locations.

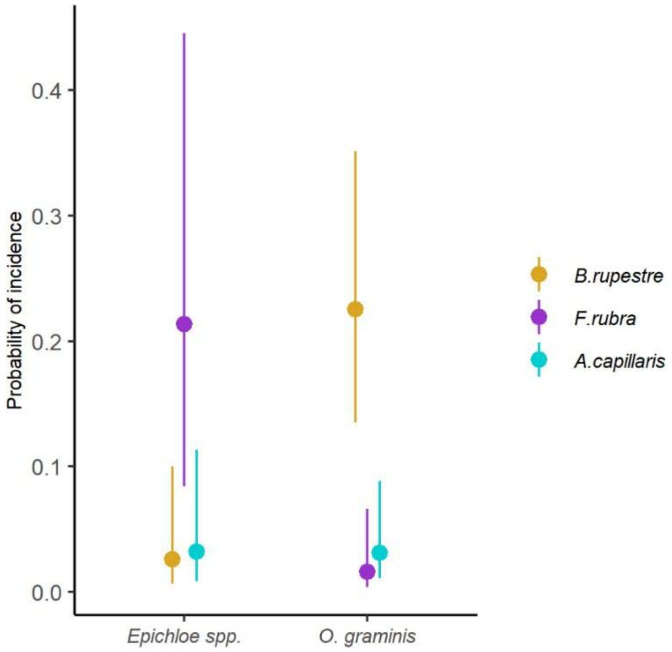


Figure 10. Probability of incidence of *Epichloë* spp. and *Omnidemptus graminis* in the aboveground tissues of the most common grasses of high-diversity grasslands.

4. DISCUSSION

4.1. Shoot fungal endophyte assemblages of the most common grasses

The richness and diversity of the mycobiomes from this survey was relatively low compared to other studies in grasses (Sánchez-Márquez et al., 2008, 2010; Pereira et al., 2019). The study of fungal endophytes is highly method-dependent (Hyde and Soytong, 2008). In this research, we applied a conservative protocol, with along sterilization process -i.e. 10 minutes *vs* 6 minutes in Pereira et al., (2019)-, and discarding the mycelia growing in the first day, which might influence the final counts of species (Burgdorf, 2014).

Despite the low richness reported, interesting patterns emerged from the shoot assemblages of the grasses. *B. rupestre* hosted the poorest mycobiome, half of that present in *F. rubra,* and one third of that of *A. capillaris.* Only two of 23 endophyte species identified were common to all grasses (*Omnidemptus graminis* and *Lachnum* sp. B). Recent studies indicate that plant identity influences foliar fungal assemblages more than expected from previous literature (Kivlin et al., 2019). Since the three grasses are sympatric and share an identical abiotic environment (i.e. climate, soil, location), other factors might define their mycobiomes. Plant abundance and capacity for carbon provision (*B. rupestre* develops more biomass but it is generally less abundant in diverse grasslands than the other grasses), potential interactions among fungal species (i.e. *Omnidemptus graminis*, which is abundant in *B. rupestre,* can induce host defence against other fungi (Schulz and Boyle, 2005; Constantine et al., 2020) and the different degree of herbivory (*B. rupestre* is less palatable to livestock than *A. capillaris* and *F. rubra*), may play a definite role in the structure of fungal assemblages.

Regarding systemic endophytes, the three grasses were infected by species of the genus

Epichloë. *E. typhina, E. baconii and E. festucae* are highly specific for *B. rupestre*, *A. capillaris* and *F. rubra,* respectively (Saikkonen et al*.,* 2016; Leutchmann et al*.,* 2014). *Epichloë* species are known to produce long-term, systemic infections and have a narrow range of hosts, limited to a genus or to related genera (Schardl et al., 2009; Schirrmann et al., 2015). In the area of study, the incidence of *Epichloë* was variable depending on species and locations, and symbioses with *E. festucae* were by far the most abundant (up to 50% incidence in one location) compared to *E. typhina* and *E. baconii* (less than 8% incidence in all locations). This is in accordance with previous research showing that the incidence of *Epichloë*-grass infections change depending on the associations (Leuchtmann and Schardl, 1998). *E. festucae* mainly reproduces asexually by vertical transmission to seeds, while in *E. typhina* and *E. baconii* seed transmission is absent (Leutchmann et al., 2014). *B. sylvaticum*, a species close to *B. rupestre,* is very common in forest undergrowth, and displays high levels of infection by *E. sylvatica*, a seed-transmitted species (Meijer and Leuchtmann, 1999; Brem and Leuchtmann, 2001). The results suggest that the capacity of systemic endophytes to infect host seeds and transmit vertically, which is the most common reproductive mechanism in *Epichloë* species, leads to higher levels of incidence than in sexual, horizontally-transmitted *Epichloë* species. This makes sense because in vertically transmitted endophytes reproductive fitness is intimately tied to that of their hosts (Saikkonen et al., 2002). As a consequence, mutualisms are expected to be more successful and intense in these situations. Of the three *Epichloë* species identified, *E. festucae* has been extensively studied for its capacity to establish successful symbioses in the most extreme environments (Pereira et al., 2019; Vázquez-de-Aldana et al., 2013a; Zabalgogeazcoa et al., 2013, Leinonen et al., 2019), and for the variety of advantages that confers to the host plant (i.e. tolerance to stress, resistance to herbivores, plant fitness) (Malinowski et al., 2000; Zaurov et al., 2001)

4.2. Fungal tissue assemblages and core mycobiome of *B. rupestre*

Roots and rhizomes of *B. rupestre* displayed different endophyte assemblages than aboveground tissues, had the greatest percentages of fungal infection (Figure 5), and a significantly high richness and diversity of taxa. Previous studies have shown that belowground tissues have greater endophyte diversity than aerial tissues, and this could be related to the level of tissue exposition to fungal inoculum (Sánchez-Márquez et al., 2010; Harrison and Griffin, 2020). In *B. rupestre*, as in many perennial grasses, most aboveground tissues die and renew annually, while belowground tissues such as rhizomes, survive and consequently have a larger time frame for fungal reinfection.

As in other grass species, the mycobiome of *B. rupestre* was constituted by a few core species and many rare species (Comby et al., 2016; Ofek-Lalzar et al., 2016; Pereira et al., 2019; Sun et al., 2020). Only two species were found in more than 20% of the *B. rupestre* plants and in most locations, *Omnidemptus graminis* in shoots and rhizomes and *Lachnum* sp. B in roots and rhizomes. *O. graminis* is a member of the Magnaporthaceae that has been described recently (Hernández-Restrepo et al., 2019). Although some Magnaporthaceae strains have shown plant-growth promoting activity (Yuan et al., 2010; Changyeol et al., 2017), the best known members of this family are pathogens of grasses, associated to roots (*Gaeumannomyces graminis* and *Magnaporthe poae*) and shoots (*Magnaporthe oryzae*) (Illana et al., 2013). *M. oryzae* is a hemibiotrophic fungus that causes the rice blast disease. This species switches from a biotrophic growth phase -it feeds from the host plant without killing its cells- in early infection to a necrotrophic stage (Kankanala et al., 2007). According to Talbot et al. (1997), the switch between phases may be triggered by the lack of nutrients within the host cell. In this survey, *O. graminis* was present in asymptomatic plants, suggesting at least the occurrence of a biotrophic phase of unknown duration. As many other fungal endophytes, this species may have a latent saprobic lifestyle (Vázquez-de-Aldana et al., 2013b).

Lachnum is a large genus within the Hyaloscyphaeceae family with more than 250 species described and distributed in a wide range of habitats and host species (Wu and Su, 2007; Nagao, 2008). Most *Lachnum* species are latent saprophytes, that may grow as endophytes in roots of perennial grasses (Sánchez-Márquez et al., 2010; Pereira et al., 2019) and form ericoid mycorrhiza coils in some Ericaceae (Walker et al., 2011). Many *Lachnum* sp. are bioactive, producing a wide range of biologically active compounds (Shan et al., 1997; Ondeyka et al., 2009; McMullin et al., 2017; Zong et al., 2017).

In addition to the core species *Omnidemptus graminis* and *Lachnum* sp., the culturable mycobiota of *B. rupestre* was characterized by a remarkable number of taxa with recognized defensive activities, such as entomopathogenic fungi (*Tolypocladium album* and *Sarocladium strictum*) (Gera Hol et al., 2007; Quesada-Moraga et al., 2014; El-Sayed et al., 2020), nematophagous fungi (*Metapochonia bulbillosa* and *Clonostachys rosea*) (Sankaranarayanan et al., 1997; Ownley et al., 2010; Manzanilla-López and Lopez-Llorca, 2017), and fungi with antifungal activity (*Fusarium circinatum*, *Penicillium ortum*, *Lachnum sp., Glarea* sp., *Trichoderma* sp., *Trichoderma koningii* and *Nemania* sp.) (Ondeyka et al., 2009, Youssar et al., 2011; Zhang et al., 2014, Mousa et al., 2015, Kornsakulkarn et al., 2017). The known defensive functions of this group of microorganisms may confer a decisive advantage to *B. rupestre*, which may use them for its own protection, as predators of its pathogens and pests, according to the bodyguard hypothesis (Elliot et al., 2000). To what extent this particular defensive assemblage plays a key role in the success and expansion of *B. rupestre* with regard to other grasses is a matter of interest that needs further research.

4.3. Fungal assemblages in *B. rupestre* associated to specific grasslands

Low and high-diversity grasslands, generated by a distinctive disturbance regime, had similar fungal richness and diversity (Figure 4), but different endophyte assemblages (Figure 2 and PERMANOVA analysis). Differences were mostly due to the different probability of incidence of the core taxa (Figure 6). *B. rupestre* in low-diversity grasslands had a greater probability of incidence of *Omnidemptus graminis* and *Lachnum* sp. B than in high-diversity grasslands, and the two grassland types only shared 36% of the total fungal taxa identified. Considering that we sampled 120 turfs per grassland type, and that we did not reach the asymptote in the species accumulation curves (Figure 3), a more exhaustive sampling effort would be necessary to draw conclusions on a specific cohort of rare taxa per grassland type. With regard to the systemic endophyte *E. typhina*, no significant differences in the probability of incidence were found between grassland types. In consequence, the highest degree of incidence of the two core taxa, *O. graminis* (in shoots and rhizomes) and *Lachnum* sp. B (in roots and rhizomes), in low-diversity, recurrently burned grasslands was the most sound result of this part of the study.

From the spectrum of core species infecting *B. rupestre, O. graminis* was only found in shoots and rhizomes, which indicates its affinity for both tissues, which are anatomically similar (de Kroon and Knops, 1990), and different from roots (Table 3). When isolated and cultured in plates, we observed rapid mycelial growth of *O. graminis* compared to the rest of species. We hypothesize that fires destroy the aerial mycelium of *O. graminis,* together with the aboveground biomass of the plant, but the fungus remains in the rhizome reservoir. Since fire is applied in winter time in moist soils, the temperatures reached in the top soil are low (at 1 cm deep, soil temperature rise 9-10 °C and no change is measured at 5 cm depth - data unpublished), and the function of rhizomes remains unaffected. Consequently, recolonization of the aboveground tissues by the rhizome mycelium of *O. graminis* can occur rapidly, paralleling the regrowth of the plant, and conferring a decisive initial advantage to this fungal species.

Regarding *Lachnum* sp. B, the increased incidence of this fungal species in frequently-burnt areas paralleled a consistent decrease of *Albotricha* sp. in roots and rhizomes (Table 3). These two endophytic taxa constitute a large part of the belowground mycobiome of *B. rupestre* and belong to the same family, Hyaloscyphaceae. The close phylogeny of both genera and the polyphyly of the *Lachnum* group has been demonstrated in genetic studies (Ye and Zhuang, 2003; Hosoya et al., 2010). The pattern of increased *Lachnum* sp. and decreased *Albotricha* sp. infection in the most burned sites, may indicate a negative interaction between both species, or a contrasting response among them to the fire disturbance. The capacity of *Lachnum sp.* to cope with environmental stress, by producing sclerotia and adopting latent forms, and its growth in belowground structures, which are less affected by fire than aboveground structures, may help to explain its success. In particular, in temperate shrublands close to the region of study, ascocarps of *Lachnum pygmaeum* have been observed on charred wood and roots of *Ulex europaeus* (unpublished results). It is known that the thermal shock produced by fire may enhance fungal fecundity and activate the development of sexual structures in some pyrophilous fungi (Raudabaugh et al., 2020). Also, fungal biomass may be increased by fires, as demonstrated for the pyrophilous fungi *Morchella* sp. infecting the grass *Bromus tectorum* (Baynes et al., 2012). To what extent the particular *Lachnum* sp. B identified in the area of study is a pyrophilous fungus merits additional study.

Although the incidences of *O. graminis* and *Lachnum* sp. B are favoured in such anthropized fire-prone habitats, the extent to which these endophytes confer particular advantages to its host, *B. rupestre*, is unknown. Further experimental research is needed to evaluate whether *O. graminis* and/or *Lachnum* sp. B infected plants perform better after fires than non-infected plants and whether the relationship in such a disrupted environment relies in a mutualistic relationship. In this research, the systemic *E. typhina* did not display significant differences in incidence among grasslands, and its percentage of infection was low at all sites. Previous research on *Epichloë* endophytes of grasses did not find evidence of a mutualistic relationship associated to fire (Faeth et al., 2002; Hall et al., 2014), contrary to grazing intensities that have been positively related to the abundance of vertically-transmitted *Epichloë* producers of toxic metabolites (Hume et al., 2020; Vázquez-de-Aldana et al., 2010).

5. CONCLUSIONS

The perennial tall-grass *B. rupestre* had a moderately diverse endophytic mycobiome consisting of a few core species and many rare species in assemblages that differed between aerial and belowground tissues. Recurrent grassland burnings, which eliminate the aerial biomass of the grass every 1-2 years, did not affect the richness and the diversity of the fungal community in *B. rupestre*, but the percentages of infection of two core taxa, *Omnidemptus graminis* and *Lachnum* sp. B, were significantly modified. The results indicate that although in frequently-burnt areas the same core species of diverse grasslands subsist, *Omnidemptus graminis* and *Lachnum* sp. B are singularly benefitted due to the proposed following mechanisms: 1) the capacity to survive belowground in rhizomes during the winter, and to spread rapidly to the shoots when the plant starts its spring growth (*O. graminis*), and 2) the location in belowground tissues (*Lachnum* sp. B) and the higher resistance to stress than other core root fungi, such as the related-taxon *Albotricha* sp. Following steps should address whether these two core taxa benefit the expansive success of *B. rupestre* in these antrophized, fire-prone environments, as well as to determine whether the cohort of less abundant fungi with well-defined defensive functions play a role too.

REFERENCES

Aizpuru, I., Aseginolaza, C., Uribe-Echebarría, P.M., Urrutia, P., Zorrakin, I., 1999. Claves ilustradas de la flora del País Vasco y territorios limítrofes. Vitoria-Gasteiz: Servicio central de publicaciones del Gobierno Vasco. doi:10.1016/0022-4375(87)90074-0

Alcañiz, M., Outeiro, L., Francos, M., Úbeda, X., 2018. Effects of prescribed fires on soil properties: a review. Science of the Total Environment 61, 944-957. doi:10.1016/j. scitotenv.2017.09.144

Anderson, P.M.L., Hoffman, M.T., 2007. The impacts of sustained heavy grazing on plant diversity and composition in lowland and upland habitats across the Kamiesberg mountain range in the Succulent Karoo, South Africa. Journal of Arid Environments 70, 686-700. doi:10.1016/j.jaridenv.2006.05.017

Archibald, J.K., Mort, M.E., Crawford, D.J., Kelly, J.K., 2005. Life history affects the evolution of reproductive isolation among species of *Coreopsis* (Asteraceae). Evolution 59, 2362-2369. doi:10.1111/j.0014-3820.2005.tb00 946.x

Armas-Herrera, C.M., Martí, C., Badía, D., Ortiz-Perpiñá, O., Girona-García, A., Porta, J., 2016. Immediate effects of prescribed burning in the central Pyrenees on the amount and stability of topsoil organic matter. Catena 147, 238-244. doi:10.1016/j.catena.2016.07.016

Artz, R.R.E., Reid, E., Anderson, I.C., Campbell, C.D., Cairney, J.W.G., 2009. Long term repeated prescribed burning increases evenness in the basidiomycete laccase gene pool in forest soils. Fems Microbiology Ecology 67, 397-410. doi:10.1111/j.1574-6941.2009.00650.x

Augustine, D J., McNaughton, S J., Frank, D A., 2003. Feedbacks between soil nutrients and large herbivores in a managed savanna ecosystem. Ecological Applications 13, 1325-1337. doi:10.1890/02-5283

Bąba, W., Kurowska, M., Kompała-Bąba, A., Wilczek, A., Dlugosz, J., Szarejko,I., 2012. Genetic diversity of populations of *Brachypodium pinnatum* (L.) P. Beauv.: expansive grass in a fragmented landscape. Polish Journal of Ecology 60, 31-40.

Bardgett, R.D., Wardle, D.A., 2003. Herbivore-mediated linkages between aboveground and belowground communities. Ecology 84, 2258-2268. doi:10.1890/02-0274

Bates, D., Mächler, M., Bolker, B.M., Walker, S.C., 2015. Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67, 1-48. doi:10.18637/jss. v067.i01

Baynes, M., Newcombe, G., Dixon, L., Castlebury, L., O'Donnell, K., 2012. A novel plant-fungal mutualism associated with fire. Fungal Biology 116, 133-144. doi:10.1016/j.funbio.2011.10.008

Bellgard, S.E., Whelan, R.J., Muston, R.M., 1994. The impact of wildfire on vesicular-arbuscular mycorrhizal fungi and their potential to influence the re-establishment of post-fire plant communities. Mycorrhiza 4, 139-146. doi:10.1007/BF00203532

Bråthen, K.A., Jahiri, X., Jusdado, J.G.H., Soininen, E.M., Jensen, J.B., 2015. Fungal endophyte diversity in tundra grasses increases by grazing. Fungal Ecology 17, 41-51. doi:10.1016/j.funeco.2015.05.002

Brem, D., Leuchtmann, A. 2001. *Epichloë* grass endophytes increase herbivore resistance in the woodland grass *Brachypodium sylvaticum*. Oecologia 126, 522-530. doi:10.1007/s004420000551

Buckland, S.M., Thompson, K., Hodgson, J.G., Grime, J.P., 2001. Grassland invasions: effects of manipulations of climate and management. Journal of Applied Ecology 38, 301-309. doi:10.1046/j.1365-2664.2001.00603.x

Burgdorf, R.J., Laing, M.D., Morris, C.D., Jamal-Ally, S.F., 2014. A procedure to evaluate the efficiency of surface sterilization methods in culture-independent fungal endophyte studies. Brazilian Journal of Microbiology 45, 977-983. doi:10.1590/S1517-83822014000300030

Canals, R.M., 2019. Landscape in motion: Revisiting the role of key disturbances in the preservation of mountain ecosystems. Geographical Research Letters 45, 515-531. doi:10.18172/cig.3634

Canals, R.M., Pedro, J., Rupérez, E., San-Emeterio, L., 2014. Nutrient pulses after prescribed winter fires and preferential patterns of N uptake may contribute to the expansion of *Brachypodium pinnatum* (L.) P. Beauv. in highland grasslands. Applied Vegetation Science 17, 419-428. doi:10.1111/avsc.12088

Canals, R.M., San Emeterio, L., Durán, M., Múgica, L., 2017. Plant-herbivory feedbacks and selective allocation of a toxic metal are behind the stability of degraded covers dominated by *Brachypodium pinnatum* in acidic soils. Plant and Soil 415, 373-386. doi:10.1007/s11104-016-3153-1

Canals, R.M., Sebastià, M.T., 2000. Soil nutrient fluxes and vegetation changes on molehills. Journal of Vegetation Science 11, 23-30. doi:10.2307/3236771

Changyeol, L., Kim, S., Li, W., Bang, S., Lee, H., Lee, H., et al., 2017. Bioactive secondary metabolites produced by an endophytic fungus *Gaeumannomyces* sp. JS0464 from a maritime halophyte *Phragmites communis*. Journal of Antibiotics 70, 737-742. doi:10.1038/ja.2017.39

Chen, H., Boutros, P.C., 2011. VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. BMC Bioinformatics 12:35. doi:10.1186/1471-2105-12-35

Comby, M., Lacoste, S., Baillieul, F., Profizi, C., Dupont, J., 2016. Spatial and temporal variation of cultivable communities of co-occurring endophytes and pathogens in wheat. Frontiers in Microbiology 7:403. doi:10.3389/fmicb.2016. 00403

Constantin, M. E., Vlieger, B. V., Takken, F. I., Rep, M., 2020. Diminished pathogen and enhanced endophyte colonization upon coinoculation of endophytic and pathogenic *Fusarium* strains. Microorganisms 8:544. doi:10. 3390/microorganisms8040544

Dawson, L.A., Mayes, R.W., Elston, D.A., and Smart, T.S., 2000. Root hydrocarbons as potential markers for determining species composition. Plant, Cell and Environment 23, 743-750. doi:10.1046/j.1365-3040.2000.00592.x

de Kroon, H., Knops, J., 1990. Habitat exploration through morphological plasticity in two chalk grassland perennials. Oikos 59, 39-49. doi:10.2307/ 3545120

Dufrêne, M., Legendre, P., 1997. Species assemblages and indicator species: the need for a flexible asymmetrical approach. Ecological Monographs 67, 345-366. doi:10.2307/2963459

Durán, M., Canals, R.M., Sáez, J.L., Ferrer, V., Lera-López, F., 2020. Disruption of traditional land use regimes causes an economic loss of provisioning services in high-mountain grasslands. Ecosystem Services 46:101200. doi:10.1016/j.ecoser.2020.101200

Egidi, E., McMullan-Fisher, S., Morgan, J. W., May, T., Zeeman, B., Franks, A.E., 2016. Fire regime, not time-since-fire, affects soil fungal community diversity and composition in temperate grasslands. FEMS Microbiology Letters 363, 1-11. doi:10.1093/femsle/fnw196

Eldridge, D.J., Delgado-Baquerizo, M., 2018. Functional groups of soil fungi decline under grazing. Plant and Soil 426, 51-60. doi:10.1007/s11104-018-3617-6

Elliot, S.L., Sabelis, M.W., Janssen, A., van der Geest, L.P.S., Beerling, E.A.M., Fransen, J., 2000. Can plants use entomopathogens as bodyguards? Ecology Letters 3, 228-235. doi:10.1046/j.1461-0248.2000.00137.x

El-Sayed, A.S.A., Moustafa, A.H., Hussein, H.A., El-Sheikh, A.A., El-Shafey, S.N., Fathy, N.A.M., et al., 2020. Potential insecticidial activity of *Sarocladium strictum*, an endophyte of *Cynanchum acutum*, against *Spodoptera littorales*, a polyphagous insect pest. Biocatalysis and Agricultural Biotechnology 24:101524. doi:10.1016/j. bcab.2020.101524

Eom, A.H., Hartnett, D.C., Wilson, G.W.T., Figge, D.A.H. 1999. The effect of fire, mowing and fertilizer amendment on arbuscular mycorrhizas in tallgrass prairie. American Midland Naturalist 142, 55-70. doi:10.1674/0003-0031(1999)142[0055:TEOFMA]2.0.CO;2

Faeth, S.H., Haase, S.M., Sackett, S.S., Sullivan, T.J., Remington, R.K., Hamilton, C.E., 2002. Does fire maintain symbiotic, fungal endophyte infections in native grasses? Symbiosis 32, 211-228.

Ferrer, V., Canals, R.M., 2008. Proyecto de ordenación de los recursos pascícolas forestales del monte Aezkoa n◦ 1 del CUP. Pamplona: Consultoría Belardi & Universidad Pública de Navarra.

Frank, D. A., 2005. The interactive effects of grazing ungulates and aboveground production on grassland diversity. Oecologia 143, 629-634. doi:10.1007/ s00442-005-0019-2

Gera Hol, W.H., de la Peña, E., Moens, M., Cook, R., 2007. Interaction between a fungal endophyte and root herbivores of *Ammophila arenaria*. Basic and Applied Ecology 8, 500-509. doi:10.1016/j.baae.2006. 09.013

Giauque, H., Hawkes, C.V., 2016. Historical and current climate drive spatial and temporal patterns in fungal endophyte diversity. Fungal Ecology 20, 108-114. doi:10.1016/j.funeco.2015.12.005

Glynou, K., Ali, T., Buch, A.K., Kia, S.H., Ploch, S., Xia, X., et al., 2016. The local environment determines the assembly of root endophytic fungi at a continental scale. Environmental microbiology 18, 2418-2434. doi:10.1111/1462-2920. 13112

Hall, S.L., McCulley, R.L., Barney, R.J., Phillips, T.D., 2014. Does fungal endophyte infection improve tall fescue's growth response to fire and water limitation? Plos One 9:e0086904. doi:10.1371/journal.pone.0086904

Hamilton, E.W., Frank, D.A., 2001. Can plants stimulate soil microbes and their own nutrient supply? Evidence from a grazing tolerant grass. Ecology 82, 2397-2402.

Harrison, J.G., Griffin, E.A., 2020. The diversity and distribution of endophytes across biomes, plant phylogeny and host tissues: how far have we come and where do we go from here? Environmental Microbiology 22, 2107-2123. doi:10.1111/1462-2920.14968

Hernández-Restrepo, M., Bezerra, J.D.P., Tan, Y.P., Wiederhold, N., Crous, P.W., Guarro, J., et al., 2019. Re-evaluation of *Mycoleptodiscus* species and morphologically similar fungi. Persoonia 42, 205-227. doi:10.3767/persoonia. 2019.42.08

Higgins, K.L., Arnold, A.E., Coley, P.D., Kursar, T. A., 2014. Communities of fungal endophytes in tropical forest grasses: highly diverse host- and habitat generalists characterized by strong spatial structure. Fungal Ecology 8, 1-11. doi:10.1016/j.funeco.2013.12.005

Hiiesalu, I., Bahram, M., Tedersoo, L., 2017. Plant species richness and productivity determine the diversity of soil fungal guilds in temperate coniferous forest and bog habitats. Molecular Ecology 26, 4846-4858. doi:10.1111/mec. 14246

Hill, N.S., Stringer, W.C., Rottinghaus, G.E., Belesky, D.P., Parrott, W.A., Pope, D.D., 1989. Influence of endophyte and water regime upon tall fescue accessions. I. Growth characteristics. Annals of

Botany 63, 495-503. doi:10.1093/ oxfordjournals.aob.a087775

Holub, P., Tuma, I., Fiala, K., 2012. The effect of nitrogen addition on biomass production and competition in three expansive tall grasses. Environmental Pollution 170, 211-216. doi:10.1016/j.envpol.2012.07.007

Hosoya, T., Sasagawa, R., Hosaka, K., Gi-Ho, S., Hirayama, Y., Yamaguchi, K., et al., 2010. Molecular phylogenetic studies of *Lachnum* and its allies based on the Japanese material. Mycoscience 51, 170-181. doi:10.1007/s10267-009-0023-1

Hothorn, T., Van De Wiel, M.A., Hornik, K., Zeileis, A., 2008. Implementing a class of permutation tests: the coin package. Journal of Statistical Software 28, 1-23. doi:10.18637/jss.v028.i08

Huang, Y.L., Devan, M.M.N., U'Ren, J.M., Furr, S.H., Arnold, A.E., 2016. Pervasive effects of wildfire on foliar endophyte communities in montane forest trees. Microbial Ecology 71, 452-468. doi:10.1007/s00248-015-0664-x

Huang, Y.L., Niu, B.F., Gao, Y., Fu, L.M., Li, W.Z., 2010. CD-HIT Suite: a web server for clustering and comparing biological sequences. Bioinformatics 26, 680-682. doi:10.1093/bioinformatics/btq003

Hume, D.E., Stewart, A.V., Simpson, W.R., Johnson, R.D., 2020. *Epichloë* fungal endophytes play a fundamental role in New Zealand grasslands. Journal of the Royal Society of New Zealand 20, 279-298. doi:10.1080/03036758.2020.1726415

Hyde, K.D., Soytong, K., 2008. The fungal endophyte dilemma. Fungal Diversity 33, 133-173. doi:10.1016/S0006-3207(98)00089-5

Illana A., Rodriguez-Romero J., Sesma A. 2013. Major plant pathogens of the Magnaporthaceae family. In: Horwitz B., Mukherjee P., Mukherjee M., Kubicek C. (eds) Genomics of soil- and plant-associated fungi. Soil Biology, vol 36. Springer, Berlin, Heidelberg. doi:10.1007/978-3-642-39339-6_4

Jing, Z., Cheng, J., Su, J., Bai, Y., Jin, J., 2014. Changes in plant community composition and soil properties under 3-decade grazing exclusion in semiarid grassland.Ecological Engineering 64, 171-178. doi:10.1016/j.ecoleng.2013.12.023

Kankanala, P., Czymmek, K., Valent, B., 2007. Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. Plant Cell 19, 706-724. doi:10.1105/tpc.106.046300

Kivlin, S.N., Kazenel, M.R., Lynn, J.S., Lee Taylor, D., Rudgers, J.A., 2019. Plant identity influences foliar fungal symbionts more than elevation in the colorado rocky mountains. Microbial Ecology 78, 688-698. doi:10.1007/s00248-019-01336-4

Köhler, B., Gigon, A., Edwards, P. J., Krüsi, B., Langenauer, R., Lüscher, A., et al., 2005. Changes in the species composition and conservation value of limestone grasslands in Northern Switzerland after 22 years of contrasting managements. Perspectives in Plant Ecology, Evolution and Systematics 7, 51-67. doi:10.1016/j.ppees.2004. 11.003.

Koide, R.T., Ricks, K.D., Davis, E.R., 2017. Climate and dispersal influence the structure of leaf fungal endophyte communities of *Quercus gambelii* in the eastern Great Basin. USA. Fungal Ecology 30, 19-28. doi:10.1016/j.funeco.2017. 08.002

Komac, B., Alados, C.L., Bueno, C.G., Gomez, D., 2013. Spatial patterns of species distributions in grazed subalpine grasslands. Plant Ecology 212, 519-529. doi:10.1007/s11258-010-9842-6

Kornsakulkarn, J., Saepua, S., Suvannakad, R., Supothina, S., Boonyuen, N., Isaka, M., et al., 2017.

Cytotoxic tropolones from the fungus *Nemania* sp. BCC30850. Tetrahedron 73, 3505-3512. doi:10.1016/j.tet.2017.05.030

Kotilínek, M., Hiiesalu, I., Košnar, J., Šmilauerová, M., Šmilauer, P., Altman, J., et al., 2017. Fungal root symbionts of high-altitude vascular plants in the Himalayas.Scientific Reports 7, 1-14. doi:10.1038/s41598-017-06938-x

Lasanta-Martínez, T., Vicente-Serrano, S.M., Cuadrat-Prats, J.M., 2005. Mountain mediterranean landscape evolution caused by the abandonment of traditional primary activities: a study of the spanish central pyrenees. Applied Geography 25, 47-65. doi:10.1016/j.apgeog.2004.11.001

Lau, M.K., Arnold, A.E., Johnson, N.C., 2013. Factors influencing communities of foliar fungal endophytes in riparian woody plants. Fungal Ecology 6, 365-378. doi:10.1016/j.funeco.2013.06.003

Leinonen, P.H., Helander, M., Vázquez-de-Aldana, B.R., Zabalgogeazcoa, I., Saikkonen, K., 2019. Local adaptation in natural European host grass populations with asymmetric symbiosis. PLoS One 14:e0215510. doi:10.1371/ journal.pone.0215510

Leuchtmann, A., Bacon, C.W., Schardl, L., White, J.F., Tadych, M., 2014. Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. Mycologia 106, 202-215. doi:10.3852/13-251

Leuchtmann, A., Schardl, C.L., 1998. Mating compatibility and phylogenetic relationships among two new species of *Epichloë* and other congeneric European species. Mycological Research 102, 1169-1182. doi:10.1017/S0953756298006236

Li, H.Y., Shen, M., Zhou, Z.P., Li, T., Wei, Y.L., Lin, L.B., 2012. Diversity and cold adaptation of endophytic fungi from five dominant plant species collected from the Baima Snow Mountain. Southwest China. Fungal Diversity 54, 79-86. doi:10.1007/s13225-012-0153-1

Li, W., Godzik, A., 2006. Cd-hit: a fast program for cluestering and comparing large sets of protein or nucleotide sequences. Bioinformatics Applications Note 22, 1658-1659. doi:10.1093/bioinformatics/btl158

Liu, C., Wang, L., Song, X., Chang, Q., Frank, D.A., Wang, D., et al., 2017. Towards a mechanistic understanding of the effect that different species of large grazers have on grassland soil N availability. Journal of Ecology 106, 357-366. doi:10.1111/1365-2745.12809

Malinowski, D., Belesky, P., 2000. Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Science 40, 923-940. doi:10.2135/cropsci2004.0523

Manzanilla-López, R., López-Llorca, L.V., 2017. Perspectives in sustainable nematode management through *Pochonia chlamydosporia* applications for root and rhizosphere health. In: Sustainability in plant crop protection, eds R. Peshin and A. Dhawan (Berlin: Springer), doi:10.1007/978-3-319-99768-1

Mataix-Solera, J., Navarro-Pedreno, J., Guerrero, C., Gomez, I., Marco, B., Mataix, J., 2002. Effects of an experimental fire on soil microbial populations in a Mediterranean environment. In: Man and soil at the third millennium. Proceedings international congress of the European society for soil conservation, Vol. 2, eds J. L. Rubio, R. P. C. Morgan, S. Asins, and V. Andreu (Valencia), 1607-1613. Geoforma Ediciones.

McMullin, D.R., Green, B.D., Prince, N.C., Tanney, J.B., Miller, D.J., 2017. Natural products of *Picea* endophytes from the acadian forest. Journal of Natural Products 80, 1475-1483. doi:10.1021/acs.jnatprod.6b01157

Meijer, G., Leuchtmann, A., 1999. Multistrain infections of the grass *Brachypodium sylvaticum* by its fungal endophyte *Epichloë sylvatica*. New Phytologist 141, 355-368. doi:10.1046/j.1469-8137.1999.00332.x

Milchunas, D.G., Sala, O.E., Lauenroth, W.K., 1988. A generalized model of the effects of grazing by large herbivores on grassland community structure. American Naturalist 132, 87-106. doi:10.1086/284839

Mousa, W.K., Schwan, A., Davidson, J., Strange, P., Liu, H., Zhou, T., et al., 2015. An endophytic fungus isolated from finger millet (*Eleusine coracana*) produces anti-fungal natural products. Frontiers in Microbiology 6:1157. doi:10.3389/fmicb.2015. 01157

Múgica, L., Canals, R.M., San Emeterio, L., 2018. Changes in soil nitrogen dynamics caused by prescribed fires in dense gorse lands in SW Pyrenees. Science of the Total Environment 639, 175-185. doi:10.1016/j.scitotenv.2018.05.139

Nagao, H., 2008. Discomycetes on decayed tree fern. *Lachnum lanariceps* and *Lachnum oncospermatum* new to Japan. Mycoscience 49, 403-406. doi:10.1007/ S10267-008-0438-0

Nguyen, N.H., Song, Z., Bates, S.T., Branco, S., Tedersoo, L., Menke, J., et al., 2016. FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecology 20, 241-248. doi:10.1016/j.funeco. 2015.06.006

Ofek-Lalzar, M., Gur, Y., Ben-Moshe, S., Sharon, O., Kosman, E., Mochli, E., et al., 2016. Diversity of fungal endophytes in recent and ancient wheat ancestors *Triticum dicoccoides* and *Aegilops sharonensis*. FEMS Microbiology Ecology 92:fiw152. doi:10.1093/femsec/fiw152

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al., 2017. Vegan: Community ecology package. R Package Version 2.4-3. Available online at: https://CRAN.R-project.org/package=vegan (accessed April 10, 2019).

Ondeyka, J., Harris, G., Zink, D., Basilio, A., Vicente, F., Bills, G., et al., 2009. Isolation, structure elucidation, and biological activity of virgineone from *Lachnum virgineum* using the genome-wide *Candida albicans* fitness test. Journal of Natural Products 72, 136-141. doi:10.1021/np800511r

Ownley, B. H., Gwinn, K. D., Vega, F. E., 2010. Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. BioControl 55, 113-128. doi:10.1007/s10526-009-9241-x

Pauchard, A., Kueffer, C., Dietz, H., Daehler, C.C., Alexander, J., Edwards, P.J., et al., 2009. Ain't no mountain high enough: plant invasions reaching new elevations. Frontiers in Ecology and the Environment 7:479-486. doi:10.1890/08 0072

Pellegrini, A.F.A., McLauchlan, K.K., Hobbie, S.E., Mack, M.C., Marcotte, A.L., Nelson, D.M., et al., 2020. Frequent burning causes large losses of carbon from deep soil layers in a temperate savanna. Journal Ecology. 108, 1426-1441. doi:10.1111/1365-2745.13351

Pereira, E., Vázquez-de-Aldana, B.R., San Emeterio, L., Zabalgogeazcoa, I., 2019. A survey of culturable fungal endophytes from *Festuca rubra* subsp. *pruinosa*, a grass from marine cliffs, reveals a core microbiomes. Frontiers in Microbiology 9, 1-14. doi:10.3389/fmicb.2018.03321

Poosakkannu, A., Nissinen, R., Kytöviita, M.M., 2014. Culturable endophytic microbial communities in the circumpolar grass, *Deschampsia flexuosa* in a sub- Arctic inland primary succession are habitat and growth stage specific. Environmental Microbiology Reports 7, 111-122. doi:10.1111/1758-2229.12195

Quesada-Moraga, E., Herrero, N., Zabalgogeazcoa, I., 2014. Entomopathogenic and nematophagous fungal endophytes. In: Advances in endophytic research, eds V. Verma and A. Gange (New Delhi: Springer), doi:10.1007/978-81-322-1575-2_4

Ranelli, L.B., Hendricks, W.Q., Lynn, J.S., Kivlin, S.N., Rudgers, J.A., 2015. Biotic and abiotic predictors of fungal colonization in grasses of the Colorado Rockies.Diversity and Distributions 21, 962-976. doi:10.1111/ddi.12310

Raudabaugh, D.B., Matheny, P.B., Hughes, K.W., Iturriaga, T., Sargent, M., Miller, A.N., 2020. Where are they hiding? Testing the body snatchers hypothesis in pyrophilous fungi. Fungal Ecology 43:100870. doi:10.1016/j.funeco. 2019.100870

Roberts, D.W., 2019. labdsv: Ordination and multivariate analysis for ecology. R Package Version 1.8-0.

Rudgers, J.A., Kivlin, S.N., Whitney, K.D., Price, M.V., Waser, N.M., Harte, J., 2014. Responses of high-altitude graminoids and soil fungi to 20 years of experimental warming. Ecology 95, 1918-1928. doi:10.1890/13-1454.1

Saikkonen, K., Ion, D., Gyllenberg, M., 2002. The persistence of vertically transmitted fungi in grass metapopulations. Proceedings of the Royal Society: Biological Sciences 269, 1397-1403. doi:10.1098/rspb.2002.2006

Saikkonen, K., Young, C.A., Helander, M., Schardl, C.L., 2016. Endophytic *Epichloë* species and their grass hosts: from evolution to applications. Plant Molecular Biology 90, 665-675. doi:10.1007/s11103-015-0399-6

San Emeterio, L., Múgica, L., Ugarte, M.D., Goicoa, T., Canals, R.M., 2016. Sustainability of traditional pastoral fires in highlands under global change: Effects on soil function and nutrient cycling. Agriculture, Ecosystems and Environment 235, 155-163. doi:10.1016/j.agee.2016.10.009

Sánchez-Márquez, S., Bills, G.F., Acuña, L.D., Zabalgogeazcoa, I., 2010. Endophytic mycobiota of leaves and roots of the grass *Holcus lanatus*. Fungal Diversity 41, 115-123. doi:10.1007/s13225-009-0015-7

Sánchez-Márquez, S., Bills, G.F., Zabalgogeazcoa, I., 2008. Diversity and structure of the fungal endophytic assemblages from two sympatric coastal grasses. Fungal Diversity 33, 87-100.

Sankaranarayanan, C., Hussaini, S.S., Kumar, P.S., Prasad, R.D., 1997. Nematicidal effect of fungal filtrates against root-knot nematodes. Journal of Biological Control 11, 37-41. doi:10.18311/jbc/1997/7575

Schardl, C.L., Balestrini, R., Florea, S., Zhang, D., Scott, B., 2009. *Epichloë* endophytes: clavicipitaceous symbionts of grasses. In: Plant relationships. The mycota (a comprehensive treatise on fungi as experimental systems for basic and applied research), Vol. 5, ed. H. B. Deising (Berlin: Springer Berlin Heidelberg), 275-306. doi:10.1007/978-3-540-87407-2_15

Schippmann, U., 1991. Revision der europäischen arten der gattung *Brachypodium* palisot de beauvois (Poaceae). Boissiera 45, 1-250.

Schippmann, U., Jarvis, C.E., 1988. Typification of three Linnean names associated with the genus *Brachypodium* (Poaceae). Taxon 37, 158-164. doi:10.2307/1220950

Schirrmann, M.K., Zoller, S., Fior, S., Leuchtmann, A., 2015. Genetic evidence for reproductive isolation among sympatric *Epichloë* endophytes as inferred from newly developed microsatellite markers. Microbial Ecology 70, 51-60. doi:10.1007/s00248-014-0556-5

Schoch, C.L., Robbertse, B., Robert, V., Vu, D., Cardinali, G., Iriny, L., et al., 2014. Finding needles in haystacks: linking scientific names, reference specimens and molecular data for Fungi. Database (Oxford) 2014:bau061. doi:10.1093/ database/bau061

Schulz, B., Boyle, C., 2005. The endophytic continuum. Mycological Research 109, 661-686. doi:10.1017/S095375620500273X

Semenova-Nelsen, T.A., Platt, W.J., Patterson, T.R., Huffman, J., Sikes, B.A., 2019. Frequent fire reorganizes fungal communities and slows decomposition across a heterogeneous pine savanna landscape. New Phytologist 224, 916-927. doi:10.1111/nph.16096

Shan, R., Stadler, M., Anke, H., Sterner, O., 1997. Naphthalenone and phthalide metabolites from *Lachnum papyraceum*. Journal Natural Products 60, 804-805. doi:10.1021/np970145s

Shaw, E.A., Denef, K., de Tomasel, C., Cotrufo, M.F., Wall, D.H., 2016. Fire affects root decomposition, soil food web structure, and carbon flow in tallgrass prairie. Soil 2, 199-210. doi:10.5194/soil-2-199-2016

Slaughter, L.C., Nelson, J.A., Carlisle, E., Bourguignon, M., Dinkind, R.D., Phillips, T.D., et al., 2018. Climate change and *Epichloë coenophiala* association modify belowground fungal symbioses of tall fescue host. Fungal Ecology 31, 37-46. doi:10.1007/s00248-016-0828-3

Soong, J.L., Cotrufo, M.F., 2015. Annual burning of a tallgrass prairie inhibits C and N cycling in soil, increasing recalcitrant pyrogenic organic matter storage while reducing N availability. Global Change Biology 21, 2321-2333. doi:10.1111/gcb.12832

Sun, X., Kosman, E., Sharon, O., Ezrati, S., Sharon, A., 2020. Significant host- and environment-dependent differentiation among highly sporadic fungal endophyte communities in cereal crops-related wild grasses. Environmental Microbiology 22, 3357-3374. doi:10.1111/1462-2920.15107

Talbot, N.J., McCafferty, H.R.K., Ma, M., Moore, K., Hamer, J.E., 1997. Nitrogen starvation of the rice blast fungus *Magnaporthe grisea* may act as an environmental cue for disease symptom expression. Physiological and Molecular Plant Pathology. 50, 179-195. doi:10.1006/pmpp.1997.0081

Tardella, F.M., Malatesta, L., Goia, I.G., Catorci, A., 2018. Effects of long-term mowing on coenological composition and recovery routes of a *Brachypodium rupestre*-invaded community: insight into the restoration of sub-Mediterranean productive grasslands. Rendiconti Lincei. Scienze Fisiche e Naturali 29, 329-341. doi:10.1007/s12210-018-0711-x

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., et al., 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31, 21-32. doi:10.1006/fgbi.2000.1228

Uys, R.G., Bond, W.J., Everson, T.M., 2004. The effect of different fire regimes on plant diversity in southern African grasslands. Biological Conservation 118, 489-499. doi:10.1016/j.biocon.2003.09.024

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., Dufresne, A., 2015. The importance of the microbiome of the plant holobiont. New Phytologist 206, 1196-1206. doi:10.1111/nph.13312

Vannier, N., Bittebiere, A.K., Mony, C., Vandenkoornhuyse, P., 2020. Root endophytic fungi impact host plant biomass and respond to plant composition at varying spatio-temporal scales. Fungal Ecology 44:100907. doi:10.1016/j.funeco. 2019.100907

Vázquez-de-Aldana, B.R., Bills, G., Zabalgogeazcoa, I., 2013a. Are endophytes an important link between airborne spores and allergen exposure? Fungal Diversity 60, 33-42. doi:10.1007/s13225-013-0223-z

Vázquez-de-Aldana, B.R., García-Ciudad, A., García-Criado, B., Vicente-Tavera, S., Zabalgogeazcoa, I., 2013b. Fungal endophyte (*Epichloë festucae*) alters the nutrient content of *Festuca rubra* regardless of water availability. Plos One 8:e84539. doi:10.1371/journal.pone.0084539

Vázquez-de-Aldana, B.R., Zabalgogeazcoa, I., Rubio de Casas, R., García-Ciudad, A., García-Criado, B., 2010. Relationships between the genetic distance of *Epichloë festucae* isolates and the ergovaline and peramine contents of their *Festuca rubra* hosts. Annals of Applied Biology 156, 51-61. doi:10.1111/j.1744-7348.2009. 00360.x

Walker, J.F., Aldrich-Wolfe, L., Riffel, A., Barbare, H., Simpson, N.B., Trowbridge, J., et al., 2011. Diverse Helotiales associated with the roots of three species of arctic ericaceae provide no evidence for host specificity. New Phytologist 191, 515-527. doi:10.1111/j.1469- 8137.2011. 03703.x

Wang, Y., Liu, L., Yang, J., Duan, Y., Luo, Y., Taherzadeh, M.J., et al., 2020. The diversity of microbial community and function varied in response to different agricultural residues composting. Science of the Total Environment 715:136983. doi:10.1016/ j.scitotenv.2020.136983

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplificacition and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A guide to methods and applications, ed. M. A. Innis (San Diego, CA: Academic Press), 315-322.

Wu, M., Su, Y., 2007. *Lachnum alnifolium*,a foliicolous discomycetes new to Taiwan. Fungi Science 22, 85-89.

Ye, M., Zhuang, W.Y., 2003. New taxa of *Lachnum* (Helotiales. Hyaloscyphaceae) from temperate China. Nova Hedwigia 76, 443-450. doi:10.1127/0029-5035/2003/0076-0443

Youssar, L., Grüning, B.A., Erxleben, A., Günther, S., Hüttel, W., 2011. Genome sequence of the fungus *Glarea lozoyensis*: the first genome sequence of a species from the Helotiaceae family. Eukaryotic Cell 11:250. doi:10.1128/ EC.00142-12

Yuan, Z., Lin, F., Zhang, C., Kubicek, C.P., 2010. A new species of *Harpophora* (Magnaporthaceae) recovered from healthy wild rice (*Oryza granulata*) roots, representing a novel member of a beneficial dark septate endophyte. FEMS Microbiology Letters 307, 94-101. doi:10.1111/j.1574-6968.2010.01963.x

Zabalgogeazcoa, I., Gundel, P.E., Helander, M., Saikkonen, K., 2013. Non-systemic fungal endophytes in *Festuca rubra* plants infected by *Epichloë festucae* in subarctic habitats. Fungal diversity 60: 25-32. doi:10.1007/s13225-013-0233-x

Zaurov, D.E., Bonos, S., Murphy, J.A., Richardson, M., Belanger, F.C., 2001. Endophyte infection can contribute to aluminum tolerance in fine fescues. Crop Science 41, 1981-1984. doi:10.2135/cropsci2001.1981

Zhang, Q., Zhang, J., Yang, L., Zhang, L., Jiang, D., Chen, W., et al., 2014. Diversity and biocontrol potential of endophytic fungi in *Brassica napus*. Biological Control 72, 98-108. doi:10.1016/j.biocontrol.2014.02.018

Zong, S., Ji, J., Li, J., Yang, Q. H., Ye, M., 2017. Physicochemical properties and anticoagulant activity of polyphenols derived from *Lachnum singerianum*. Journal of Food and Drug Analysis 25, 837-844. doi:10.1016/j.jfda.2016.08.011

Supplementary material table S1. The proposed identification of the isolated taxa based on the information provided by the matches in the databases.

GenBank accession number	**Greatest sequence identity (%)**	**Proposed taxon**	**Guild**	**Supplementary references**
MW528548	*Acremonium furcatum* (98.07)	*Acremonium* sp.	Animal Pathogen Endophyte Fungal Parasite Plant Pathogen Wood Saprotroph	Tian *et al.,* 2017 An *et al.,* 1993
MW528549	*Albotricha* spp. (98.46)	*Albotricha* sp.	Undefined Saprotroph	Wu M-L., 2003
MW528550	*Alfaria thymi* (100)	*Alfaria thymi*	Undefined	Lombard *et al.,* 2016
MW528551	*Funiliomyces biseptatus* (92.31)	Amphisphaeriaceae sp.	Undefined Saprotroph	Kang *et al.,* 1999
MW528552	*Bullanockia australis* (99.59)	*Bullanockia australis*	Undefined Saprotroph	Crous *et al.,* 2016
MW528553	*Cadophora obovata* (99.47)	*Cadophora* sp.	Endophyte	Newsham K.K., 2010
MW528554	*Chaetosphaeria ciliata* (98)	*Chaetosphaeria* sp.	Undefined Saprotroph	Réblová *et al.,* 1999
MW528555	Chaetosphaeriales (100)	Chaetosphaeriales sp.	Undefined	Maharachchikumbura *et al.*, 2016
MW528556	*Clonostachys rosea* (100)	*Clonostachys rosea*	Plant Saprotroph Wood Saprotroph Animal Pathogen	Rodríguez *et al.,* 2011 Lahlali and Peng, 2014 Fournier *et al.,* 2020
MW528557	Cucurbitariaceae spp. (94.76)	Cucurbitariaceae sp.	Plant Pathogen Wood Saprotroph	Doilom *et al.,* 2013
MW528558	*Dictyochaeta lithocarpi* (98)	*Dictyochaeta* sp.	Undefined Saprotroph	Réblová *et al.,* 1999
MW528559	*Dinemasporium morbidum* (100)	*Dinemasporium morbidum*	Undefined Saprotroph	Krohn *et al.,* 2008 Crous *et al.,* 2012
MW528560	*Epichloë typhina* (100)	*Epichloë typhina*	Endophyte Plant pathogen	White J.F., 1988 Zabalgogeazcoa *et al.,* 2007

				Clay and Schardl, 2002
MW528561	*Fusarium circinatum* (100)	*Fusarium circinatum*	Animal Pathogen Endophyte Lichen Parasite Plant Pathogen Soil Saprotroph Wood Saprotroph	Hanada *et. al.*, 2010 Martínez-Álvarez *et al.*, 2016
MW528562	*Gaeumannomycella cariciola* (96.59)	*Gaeumannomycella* sp.	Plant Pathogen	Hernández-Restrepo *et al.*, 2016a
MW528563	*Glarea lozoyensis* (96.59)	*Glarea* sp.	Undefined Saprotroph	Youssar *et. al.*, 2011
MW528564	Hypocreales spp. (94.54)	Hypocreales sp.		
MW528565	*Ilyonectria robusta* (100)	*Ilyonectria robusta*	Plant Pathogen	Martínez-Diz *et al.*, 2018 Liu *et al.*, 2019
MW528566	*Lachnum pygmaeum* (99.01)	*Lachnum* sp. A	Undefined Saprotroph	Pereira *et al.*, 2019 McMullin *et al.*, 2017 Xu *et al.*, 2017 Hou et al., 2019
MW528567	*Lachnum* spp. (99.04)	*Lachnum* sp. B	Undefined Saprotroph	
MW528568	*Metapochonia bulbillosa* (99.16)	*Metapochonia bulbillosa*	Animal Pathogen	Manzanilla-López and Lopez-Llorca, 2017
MW528569	*Metarhizium carneum* (98.7)	*Metarhizium carneum*	Animal Pathogen	Lomer *et al.*, 2001 Inyang *et al.*, 1998
MW528570	*Microdochium bolleyi* (99.57)	*Microdochium bolleyi*	Plant Pathogen	Ernst *et al.*, 2011 Shadmani *et al.* , 2020 Zhang *et al.*, 2008
MW528571	*Microdochium neoqueenslandicum* (99.77)	*Microdochium neoqueenslandicum*	Plant Pathogen	Hernández-Restrepo *et al.*, 2016b
MW528572	*Mollisia* spp. (97.88)	*Mollisia* sp.	Endophyte Plant Pathogen	Tanney and Seifert, 2020 Fan *et al., 2016*
MW528573	*Loramyces* spp. (99.40)	Mollisiaceae sp.	Endophyte Plant Pathogen	Tanney and Seifert, 2020
MW528574	*Mycena fulgoris* (96)	*Mycena* sp.	Leaf Saprotroph Plant Pathogen Undefined Saprotroph Wood Saprotroph	Tejesvi *et al.*, 2010 Ogura-Tsujite *et. al.*, 2009

MW528575	*Myrmecridium phragmitis* (95.89)	*Myrmecridium* sp.	Endophyte	Jie *et al.*, 2013
MW528576	*Myrothecium* spp. (100)	*Myrothecium* sp.	Undefined Saprotroph	Clarke *et al.*, 2007 Hoagland *et al.*, 2007 Bezerra *et al.*, 2015
MW528577	*Nemania* spp. (99.79)	*Nemania* sp.	Undefined Saprotroph	Kornsakulkarn *et al.*, 2017
MW528578	*Omnidemptus graminis* (99.54)	*Omnidemptus graminis*	Plant Pathogen	Hernández-Restrepo *et al.*, 2019 Ortega *et al.*, 2013
MW528579	*Ophiosphaerella korrae* (100)	*Ophiosphaerella* sp.	Plant Pathogen	Flores *et al.*, 2015
MW528580	*Parasola crataegi* (96.27)	*Parasola* sp.	Undefined Saprotroph	Szarkándi *et al.*, 2017
MW528581	*Penicillium ortum* (100)	*Penicillium ortum*	Wood Saprotroph	Dastogeer *et al.*, 2018 Mousa *et al.*, 2015 Visagie *et al.*, 2015
MW528582	*Vararia calami* (84.06)	Peniophoraceae sp.	Plant Pathogen Wood Saprotroph	Miller *et al.*, 2006
MW528583	*Periconia* spp. (99.8)	*Periconia* sp.	Endophyte Plant Pathogen Wood Saprotroph	*Liu et al.* , 2017
MW528584	*Petrakia* spp. (99.19)	*Petrakia* sp.	Undefined Saprotroph	Jaklitsch and Voglmayr, 2017 Gross *et al.* , 2017
MW528585	*Pezicula rhizophila* (100)	*Pezicula rhizophila*	Undefined Saprotroph	Chen *et al.*, 2016 Lynch *et al.*, 2013
MW528586	Pleosporales spp. (99.68)	Pleosporales sp.		
MW528587	*Pyrenochaetopsis leptospora* (100)	*Pyrenochaetopsis leptospora*	Endophyte Lichen Parasite Undefined Saprotroph	Doilom *et al.*, 2013 Chi *et al.*, 2019
MW528588	*Sarocladium strictum* (98.96)	*Sarocladium strictum*	Undefined Saprotroph Animal pathogen	El-Sayed *et al.*, 2020
MW528589	Fusidium spp. (96.06)	Sordariomycete sp. A		Maharachchikumbura *et al.*, 2016
MW528590	*Myrothecium chiangmaiense* (100)	Sordariomycete sp. B		

MW528591	*Tolypocladium album* (100)	*Tolypocladium album*	Animal Pathogen Endophyte Fungal Parasite	Hanada *et al.,* 2010 Gazis *et al.,* 2014 Fukuda *et al.,* 2015
MW528592	*Trichoderma koningii* (99.63)	*Trichoderma koningii*	Endophyte Plant Pathogen Fungal parasite	Sankaranarayanan *et al.,* 1997 Xiao-Yan *et al.,* 2006 Worasatit *et al.,* 1994 Taha *et al.,* 2021
Species not present in *B. rupestre*				
MW528593	*Alfaria dandenongensis* (100)	*Alfaria dandenongensis*	Undefined	Lombard *et al.,* 2016
MW528594	*Dilophospora alopecuri* (99.76)	*Dilophospora* sp.	Plant Pathogen	Riley *et al.*, 1998 Barbetti *et al.*, 2006
MW528595	*Epichloë baconii* (100)	*Epichloë baconii*	Endophyte Plant pathogen	Romo-Vaquero *et al.*, 2003
MW528596	*Epichloë festucae* (99.79)	*Epichloë festucae*	Endophyte Fungal parasite	Vázquez.de-Aldana *et al.*, 2013 Zabalgogeazcoa et al., 2013
MW528597	*Paracremonium* sp. (88.59)	Nectriaceae sp.	Animal Pathogen Endophyte Fungal Parasite Lichen Parasite Plant Pathogen Wood Saprotroph	Lombard *et al.*, 2015 Jumpponen *et al.*, 2010 Zheng and Gong, 2019
MW528598	*Oculimacula anguioides* (97.29)	*Oculimacula* sp.	Plant Pathogen	Parnell *et al.*, 2008 Vera and Murray, 2016
MW528599	*Scytalidium album* (99.78)	*Scytalidium album*	Wood Saprotroph	El-Elimat et al., 2015
MW528600	Tricholomataceae sp. (100)	Tricholomataceae sp.	Ectomycorrhizal Fungal Parasite	Campoamor and Molina, 2001

Supplementary references

An, Z., Siegel, M.R., Hollin, W., Tsai, H., Schmidt, D., Schardl, C.L., 1993. Relationships among Non-*Acremonium* sp. fungal endophytes in five grass species. Applied and Environmental Microbiology 59, 1540-1548. doi:10.1128/AEM.59.5.1540-1548.1993

Barbetti, M.J., Riley, I.T., 2006. Field application of *Dilophospora alopecuri* to manage annual ryegrass toxicity caused by *Rathayibacter toxicus*. Plant Disease 90:229-232. doi:10.1094/PD-90-0229

Bezerra, J.D.P., Nascimento, C.C.F., Barbosa, R. do N., Da Silva, D.C.V., Svedese, V.M., Silva-Nogueira, E.B., Gomes, B.S., Paiva, L.M., Souza-Motta, C.M., 2015. Endophytic fungi from medicinal plant *Bauhinia forficata*: Diversity and biotechnological potential. Brazilian Journal of Microbiology 46, 49-57. doi:10.1590/S1517-838246120130657

Campoamor, J.N., Molina, J.A., 2001. Diversity of Tricholomataceae along a mediterranean altitudinal gradient. Cryptogamie mycologie 22 (3) 175-184. doi:10.1016/S0181-1584(01)01068-5

Chen, C., Verkley, G.J.M., Sun, G., Groenewald, J.Z., Crous, P.W., 2016. Redefining common endophytes and plant pathogens in Neofabraea, *Pezicula*, and related genera. Fungal Biology 120, 1291-1322. doi:10.1016/j.funbio.2015.09.013

Chi, W.C., Chen, W., He, C.C., Guo, S.Y., Cha, H.J., Tsang, L.M., Ho, T.W., Pang, K.L., 2019. A highly diverse fungal community associated with leaves of the mangrove plant *Acanthus ilicifolius* var. *xiamenensis* revealed by isolation and metabarcoding analyses. PeerJ. doi:10.7717/peerj.7293

Clarke, T.C., Shetty, K.G., Jayachandran, K., Norland, M.R., 2007. *Myrothecium verrucaria* - A potential biological control agent for the invasive “old world climbing fern” (*Lygodium microphyllum*). BioControl 52, 399-411. doi:10.1007/s10526-006-9035-3

Clay, K., Schardl, C., 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. The American Naturalist 160, 99-127. doi:10.1086/342161

Crous, P.W., Verkley, G.J.M., Christensen, M., Castañeda-Ruiz, R.F., Groenewald, J.Z., 2012. How important are conidial appendages? Persoonia: Molecular Phylogeny and Evolution of Fungi 28, 126-137. doi:10.3767/003158512X652624

Crous, P.W., Wingfield, M.J., Burgess, T.I., Hardy, G.E.S.J., Crane, C., Barrett, S., Roux, J.J. Le, Thangavel, R., Guarro, J., Stchigel, A.M., et al., 2016. Fungal Planet description sheets : 469-557. Persoonia 37, 218-403.

Dastogeer, K.M.G., Li, H., Sivasithamparam, K., Jones, M.G.K., Wylie, S.J., 2018. Host Specificity of endophytic mycobiota of wild nicotiana plants from arid regions of northern Australia. Microbial Ecology 75, 74-87. doi:10.1007/s00248-017-1020-0

Doilom, M., Liu, J-K., Jaklitsch, W.M., Ariyawansa, H., Wijayawardene, N.N., Chukeatirote, E., Zhang, M., McKenzie, E.H.C., Geml, J., Voglmayr, H., Hyde, K.D., 2013. An outline of the family Cucurbitariaceae. Sydowia 65, 164-192.

El-Elimat, T., Raja, H., Figueroa, M., Swanson, S.M., Falkinham, J.O., Lucas, D.M., Grever, M.R., Wani, M.C., Pearce, C.J., Oberlies, N.H., 2015. Sorbicillinoid analogs with cytotoxic and selective anti-*Aspergillus* activities from *Scytalidium album*. The Journal of Antibiotics 68, 191-196. doi:10.1038/ja.2014.125

El-Sayed, A.S.A., Moustafa, A.H., Hussein, H.A., El-Sheikh, A.A., El-Shafey, S.N., Fathy, N.A.M., Enan, G.A., 2020. Potential insecticidal activity of *Sarocladium strictum*, an endophyte of *Cynanchum acutum*, against *Spodoptera littoralis*, a polyphagous insect pest. Biocatalysis and Agricultural

Biotechnology 24, 101524. doi:10.1016/j.bcab.2020.101524

Ernst, M., Neubert, K., Mendgen, K.W., Wirsel, S.G., 2011. Niche differentiation of two sympatric species of *Microdochium* colonizing the roots of common reed. BMC Microbiology 11. doi:10.1186/1471-2180-11-242

Fan, N.W., Chang, H.S., Cheng, M.J., Chan, H.Y., Hsieh, S.Y., Liu, T.W., Chen, S.W., Yuan, G.F., Chen, I.S., 2016. New metabolites from the endophytic fungus *Mollisia* sp. Chemistry of Natural Compounds 52 (4). doi:10.1007/s106000-016-1718-0

Flores, F.J., Marek, S.M., Anderson, J.A., Mitchell, T.K., Walker, N.R., 2015. Infection and colonization of several bermudagrasses by *Ophiosphaerella korrae*. Phytopathology 105, 656-661. doi:10.1094/PHYTO-07-14-0205-R

Fournier, B., Pereira Dos Santos, S., Gustavsen, J.A., Imfeld, G., Lamy, F., Mitchell, E.A.D., Mota, M., Noll, D., Planchamp, C., Heger, T.J., 2020. Impact of a synthetic fungicide (fosetyl-Al and propamocarb-hydrochloride) and a biopesticide (*Clonostachys rosea*) on soil bacterial, fungal, and protist communities. Science of the Total Environment 738, 1-10. doi:10.1016/j.scitotenv.2020.139635

Fukuda, T., Sudoh, Y., Tsuchiya, Y., Okuda, T., Matsuura, N., Motojima, A., Oikawa, T., Igarashi, Y., 2015. Tolypoalbin, a new tetramic acid from *Tolypocladium album* TAMA 479. Journal of Antibiotics 68, 399-402. doi:10.1038/ja.2014.165

Jumpponen, A., Jones, K.L., Blair, J., 2010. Vertical distribution of fungal communities in tallgrass prairie soil. Mycologia 102 (5) 1027-1041. doi:10.3852/09-316

Gazis, R., Skaltsas, D., Chaverri, P., 2014. Novel endophytic lineages of *Tolypocladium* provide new insights into the ecology and evolution of *Cordyceps*-like fungi. Mycologia 106, 1090-1105. doi:10.3852/13-346

Gross, A., Beenken, L., Dubach, V., Queloz, V., Tanaka, K., Hashimoto, A., Holdenrieder, O., 2017. *Pseudodidymella fagi* and *Petrakia deviata*: Two closely related tree pathogens new to central Europe. Forest Pathology 47, 1-15. doi:10.1111/efp.12351

Hanada, R.E., Pomella, A.W. V., Costa, H.S., Bezerra, J.L., Loguercio, L.L., Pereira, J.O., 2010. Endophytic fungal diversity in *Theobroma cacao* (cacao) and *T. grandiflorum* (cupuaçu) trees and their potential for growth promotion and biocontrol of black-pod disease. Fungal Biology 114, 901-910. doi:10.1016/j.funbio.2010.08.006

Hernández-Restrepo, M., Bezerra, J.D.P., Tan, Y.P., Wiederhold, N., Crous, P.W., Guarro, J., Gené, J., 2019. Re-evaluation of *Mycoleptodiscus* species and morphologically similar fungi. Persoonia: Molecular Phylogeny and Evolution of Fungi 42, 205-227. doi:10.3767/persoonia.2019.42.08

Hernández-Restrepo, M., Groenewald, J.Z., Elliott, M.L., Canning, G., McMillan, V.E., Crous, P.W., 2016a. Take-all or nothing. Studies in Mycology 83, 19-48. doi:10.1016/j.simyco.2016.06.002

Hernández-Restrepo, M., Groenewald, J.Z., Crous, P.W., 2016b. Taxonomic and phylogenetic re-evaluation of *Microdochium*, *Monographella* and *Idriella*. Persoonia 36, 57-82. doi:10.3767/003158516X688676

Hoagland, R.E., Boyette, C.D., Abbas, H.K., 2007. *Myrothecium verrucaria* isolates and formulations as bioherbicide agents for kudzu. Biocontrol Science and Technology 17, 721-731. doi:10.1080/09583150701527268

Hou, G., Chen, X., Li, J., Ye, Z., Zong, S., Ye, M., 2019. Physicochemical properties, immunostimulatory activity of the *Lachnum polysaccharide* and polysaccharide-dipeptide conjugates. Carbohydrate

Polymers 206: 446-454. doi:0.1016/j.carbpol.2018.09.067

Inyang, E.N., Butt, T.M., Ibrahim, L., Clark, S.J., Pye, B.J., Beckett, A., Archer, S., 1998. The effect of plant growth and topography on the acquisition of conidia of the insect pathogen *Metarhizium anisopliae* by larvae of *Phaedon cochleariae*. Mycological Research 102, 1365-1374. doi:10.1017/S095375629800673X

Jaklitsch, W.M., Voglmayr, H., 2017. Three former taxa of Cucurbitaria and considerations on *Petrakia* in the Melanommataceae. Sydowia 69, 81-95. doi:10.12905/0380.sydowia69-2017-0081

Jie, C., Zhou, Q., Zhao, W., Jiang, Y., 2013. A new *Myrmecridium* species from Guizhou, China. Mycotaxon 124, 1-8. doi:10.5248/124.1

Kang, J.C., Hyde, K.D., Kong, R.Y.C., 1999. Studies on Amphisphaeriales: The Amphisphaeriaceae (*sensu stricto*). Mycological Research 103, 53-64. doi:10.1017/S0953756298006650

Kornsakulkarn, J., Saepua, S., Suvannakad, R., Supothina, S., Boonyuen, N., Isaka, M., Prabpai, S., Kongsaeree, P., Thongpanchang, C., 2017. Cytotoxic tropolones from the fungus *Nemania* sp. BCC 30850. Tetrahedron 73, 3505-3512. doi:10.1016/j.tet.2017.05.030

Krohn, K., Sohrab, M.H., Van Ree, T., Draeger, S., Schulz, B., Antus, S., Kurtán, T., 2008. Dinemasones A, B and C - New bioactive metabolites from the endophytic fungus *Dinemasporium strigosum*. European Journal of Organic Chemistry 5, 5638-5646. doi:10.1002/ejoc.200800688

Lahlali, R., Peng, G., 2014. Suppression of clubroot by *Clonostachys rosea* via antibiosis and induced host resistance. Plant Pathology 63, 447-455. doi:10.1111/ppa.12112

Liu, D., Sun, H., Ma, H., 2019. Deciphering microbiome related to rusty roots of *Panax ginseng* and evaluation of antagonists against pathogenic *Ilyonectria*. Frontiers in Microbiology 10, 1-12. doi:10.3389/fmicb.2019.01350

Liu, J.M., Zhang, D.W., Du, W.Y., Zhang, M., Zhao, J.L., Chen, R.D., Xie, K.B., Dai, J.G., 2017. Four new monoterpenoids from an endophytic fungus *Periconia* sp. F-31. Journal of Asian Natural Products Research 19, 541-549. doi:10.1080/10286020.2017.1313241

Lombard, L., Houbraken, J., Decock, C., Samson, R.A., Meijer, M., Réblová, M., Groenewald, J.Z., Crous, P.W., 2016. Generic hyper-diversity in Stachybotriaceae. Persoonia: Molecular Phylogeny and Evolution of Fungi 36, 156-246. doi:10.3767/003158516X691582

Lombard, L., van der Merwe, N.A., Groenewald, J.Z., Crous, P.W., 2015. Generic concepts in Nectriaceae. Studies in mycology 80: 189-245. doi:10.1016/j.simyco.2014.12.002

Lomer, C.J., Bateman, R.P., Johnson, D.L., Langewald, J., Thomas, M., 2001. Biological control of locusts and grasshoppers. Annual Review of Entomology 46, 667-702. doi:10.1146/annurev.ento.46.1.667

Lynch, S.C., Zambino, P.J., Mayorquin, J.S., Wang, D.H., Eskalen, A., 2013. Identification of new fungal pathogens of coast live oak in California. Plant Disease 97, 1025-1036. doi:10.1094/PDIS-11-12-1055-RE

Maharachchikumbura, S.S.N., Hyde, K.D., Jones, E.B.G., et al., 2016. Families of Sordariomycetes, Fungal Diversity. doi:10.1007/s13225-016-0369-6

Manzanilla-López, R., Lopez-Llorca, L.V., 2017. Perspectives in sustainable nematode management through *Pochonia chlamydosporia* applications for root and rhizosphere health. In: Peshin, R., Dhawan, A. (eds) Sustainability in plant crop protection. Springer. doi:10.1007/978-3-319-99768-1

Martínez-Álvarez, P., Fernández-González, R.A., Sanz-Ros, A.V., Pando, V., Diez, J.J., 2016. Two fungal endophytes reduce the severity of pitch canker disease in *Pinus radiata* seedlings. Biological Control 94, 1-10. doi:10.1016/j.biocontrol.2015.11.011

Martínez-Diz, M.P., Díaz-Losada, E., Armengol, J., León, M., Berlanas, C., Andrés-Sodupe, M., Gramaje, D., 2018. First report of *Ilyonectria robusta* causing black foot disease of grapevine in Spain. Plant Disease 102, 2381. doi:10.1094/PDIS-05-18-0730-PDN

McMullin, D.R., Green, B.D., Prince, N.C., Tanney, J.B., Miller, J.D., 2017. Natural products of *Picea* endophytes from the Acadian forest. Journal of Natural Products 80, 1475-1483. doi:10.1021/acs.jnatprod.6b01157

Miller, S.L., Larsson, E., Larsson, K.-H., Verbeken, A., Nuytinck, J., 2006. Perspectives in the new Russulales. Mycologia 98, 960-970. doi:10.1080/15572536.2006.11832625

Mousa, W.K., Schwan, A., Davidson, J., Strange, P., Liu, H., Zhou, T., Auzanneau, F.I., Raizada, M.N., 2015. An endophytic fungus isolated from finger millet (*Eleusine coracana*) produces anti-fungal natural products. Frontiers in Microbiology 6:1154 doi:10.3389/fmicb.2015.01157

Newsham, K.K., 2011. A meta-analysis of plant responses to dark septate root endophytes. New Phytologist 190, 783-793. doi:10.1111/j.1469-8137.2010.03611.x

Ogura-Tsujita, Y., Gebauer, G., Hashimoto, T., Umata, H., Yukawa, T., 2009. Evidence for novel and specialized mycorrhizal parasitism: The orchid *Gastrodia confusa* gains carbon from saprotrophic *Mycena*. Proceedings of the Royal Society: Biological Sciences 276, 761-767. doi:10.1098/rspb.2008.1225

Ortega, H.E., Graupner, P.R., Asai, Y., TenDyke, K., Qiu, D., Shen, Y.Y., Rios, I., Arnold, A.E., Coley, P.D., Kursar, T.O., Gerwick, W.H., Cubilla-Rios, L., 2013. Mycoleptodiscins A and B, cytotoxic alkaloids from the endophytic fungus *Mycoleptodiscus* sp. F0194. Journal of Naturals Products 76, 741-744. doi:10.1021/np300792t

Parnell, S., Gilligan, C.A., Bock, C.H., van den Bosch, F., 2008. Changes in fungicide sensitivity and relative species abundance in *Oculimacula yallundae* and *O. acuformis* populations (eyespot disease of cereals) in Western Europe. Plant pathology 57, 509-517. doi:10.1111/j.1365-3059.2007.01786.x

Pereira, E., Vázquez-de-Aldana, B.R., San Emeterio, L., Zabalgogeazcoa, I., 2019. A survey of culturable fungal endophytes from *Festuca rubra* subsp. *pruinosa*, a grass from marine cliffs, reveals a core microbiomes. Frontiers in Microbiology 9, 1-14. doi:10.3389/fmicb.2018.03321

Réblová, M., Barr, M.E., Samuels, G.J., 1999. Chaetosphaeriaceae, a new family for *Chaetosphaeria* and its relatives. Sydowia 51, 49-70.

Riley, I.T., Reardon, T., Bertozzi, T., 1998. Allozyme analysis of Australian isolates of *Dilophospora alopecuri.* Mycological research 102 (3), 301-307. doi:10.1017/S095375629700498X

Rodriguez, M.A., Cabrera, G., Gozzo, F.C., Eberlin, M.N., Godeas, A., 2011. *Clonostachys rosea* BAFC3874 as a *Sclerotinia sclerotiorum* antagonist: Mechanisms involved and potential as a biocontrol agent. Journal of Applied Microbiology 110, 1177-1186. doi:10.1111/j.1365-2672.2011.04970.x

Romo-Vaquero, M., Vázquez-de-Aldana, B.R., García-Ciudad, A., García-Criado, B., Zalbalgogeazcoa, I., 2007. First report of choke disease caused by *Epichloë baconii* in the grass *Agrostis castellana*. Plant disease 90 (2) 314-314. doi:10.1094/PDIS.2003.87.3.314B

Sankaranarayanan, C., Hussaini, S.S., 1997. Nematicidal effect of fungal filtrates against root-knot nematodes. Journal of Biological Control 11, 37-41. doi:10.18311/jbc/1997/7575

Shadmani, L., Jamali, S., Fatemi, A., 2020. Effects of root endophytic fungus, *Microdochium bolleyi* on cadmium uptake, translocation and tolerance by *Hordeum vulgare* L. Biologia. doi:10.2478/s11756-020-00598-5

Szarkándi, J.G., Schmidt-Stohn, G., Dima, B., Hussain, S., Kocsubé, S., Papp, T., Vágvölgyi, C., Nagy, L.G., 2017. The genus *Parasola*: phylogeny and the description of three new species. Mycologia 109, 620-629. doi:10.1080/00275514.2017.1386526

Taha, M.A., Ismaiel, A.A., Ahmed, R.M., 2021. 6-pentyl-α-pyrone from *Trichoderma koningii* induces systemic resistance in tobacco against tobacco mosaic virus. European Journal of Plant Pathology 159, 81-93. doi:10.1007/s10658-020-02142-2

Tanney, J.B., Seifert, K.A., 2020. Mollisiaceae: An overlooked lineage of diverse endophytes. Studies in Mycology 95, 293-380. doi:10.1016/j.simyco.2020.02.005

Tejesvi, M. V., Ruotsalainen, A.L., Markkola, A.M., Pirttilä, A.M., 2010. Root endophytes along a primary succession gradient in northern Finland. Fungal Diversity 41, 125-134. doi:10.1007/s13225-009-0016-6

Tian, J., Lai, D., Zhou, L., 2017. Secondary metabolites from *Acremonium* fungi: Diverse structures and bioactivities. Mini. Rev. Med. Chem. 17, 603-632. doi:10.2174/1389557516666160914194134

Vázquez-de-Aldana, B.R., Zabalgogeazcoa, I., García-Ciudad, A., García-Criado, B., 2013. An *Epichloë* endophyte affects the competitive ability of *Festuca rubra* against other grassland species. Plant soil 362: 201-213. doi:10.1007/s11104-012-1283-7

Vera, D.I., Murray, T.D., 2016. Occurrence and survival of apothecia of the eyespot pathogens *Oculimacula acuformis* and *O. Yallundae* on wheat stubble in the U.S. Pacific Northwest. Plant Disease. doi:10.1094/PDIS-09-15-1056-RE

Visagie, C.M., Houbraken, J., Seifert, K.A., Samson, R.A., Jacobs, K., 2015. Four new *Penicillium* species isolated from the fynbos biome in South Africa, including a multigene phylogeny of section Lanata-Divaricata. Mycological Progress 14:96 doi:10.1007/s11557-015-1118-z

White, J.F.J., 1988. Endophyte-host associations in forage grasses. XI. A proposal concerning origin and evolution. Mycologya 80, 442-446. doi:10.1080/00275514.1988.12025565

Worasatit, N., Sivasithamparam, K., Ghisalberti, E.L., Rowland, C., 1994. Variation in pyrone production, lytic enzymes and control of *Rhizoctonia* root rot of wheat among single-spore isolates of *Trichoderma koningii*. Mycological Research 98 (12), 1357-1363. doi:10.1016/S0953-7562(09)81063-0

Wu, M.L., 2003. A new species of *Albotricha* from Taiwan. Mycotaxon 88: 387-392.

Xiao-Yan, S., Qing-Tao, S., Shu-Tao, X., Xiu-Lan, C., Cai-Yun, S., Yu-Zhong, Z., 2006. Broad-spectrum antimicrobial activity and high stability of Trichokonins from *Trichoderma koningii* SMF2 against plant pathogens. FEMS Microbiology Letters 260, 119-125. doi:10.1111/j.1574-6968.2006.00316.x

Xu, C., Li, J., Yang, Liuqing, Shi, F., Yang, Liu, Ye, M., 2017. Antibacterial activity and a membrane damage mechanism of *Lachnum* YM30 melanin against *Vibrio parahaemolyticus* and *Staphylococcus aureus*. Food Control 73, 1445-1451. doi:10.1016/j.foodcont.2016.10.048

Youssar, L., Grüning, B.A., Erxleben, A., Günther, S., Hüttel, W., 2012. Genome sequence of the fungus *Glarea lozoyensis*: The first genome sequence of a species from the helotiaceae family. Eukaryotic Cell 11, 250. doi:10.1128/EC.05302-11

Zabalgogeazcoa, I., García-Ciudad, A., Leuchtmann, A., Vázquez-de-Aldana, B.R., García-Criado, B., 2008. Effects of choke disease in the grass *Brachypodium phoenicoides*. Plant Pathology 57, 467-472. doi:10.1111/j.1365-3059.2007.01784.x

Zabalgogeazcoa, I., Gundel, P.E., Helander, M., Saikkonen, K., 2013. Non-systemic fungal endophytes in *Festuca rubra* plants infected by *Epichloë festucae* in subarctic habitats. Fungal diversity 60: 25-32. doi:10.1007/s13225-013-0233-x

Zhang, W., Krohn, K., Draeger, S., Schulz, B., 2008. Bioactive isocoumarins isolated from the endophytic fungus *Microdochium bolleyi*. Journal Natural Products 71: 1078-1081. doi:10.1021/np800095g

Zheng, Y., Gong, X., 2019. Niche differentiation rather than biogeography shapes the diversity and composition of microbiome of *Cycas panzhihuaensis*. Microbiome 7:152. doi:10.1186/s40168-019-0770-y

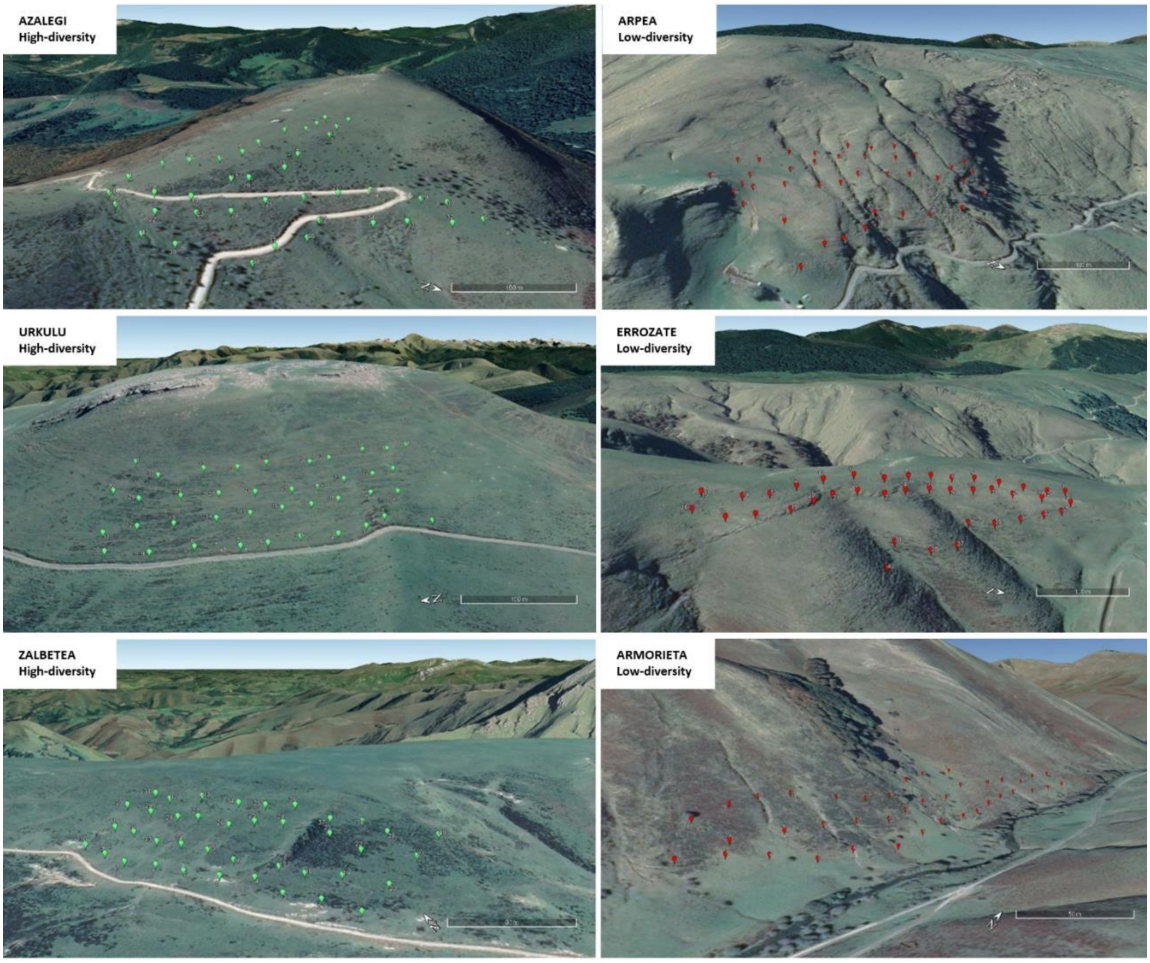
Supplementary material table S2. The presence/absence data of the *B. rupestre* identified taxa.

Fungal endophyte	***B.rupestre* tissue**			**Low-diversity grasslands**			**High-diversity grasslands**		
	Shoot	**Rhizome**	**Root**	**Arpea**	**Errozate**	**Armorieta**	**Urkulu**	**Zalbetea**	**Azalegi**
Acremonium sp.	0	0	4	1	0	1	2	0	0
Albotricha sp.	0	20	33	1	3	9	23	17	0
Alfaria thymi	0	1	0	0	0	0	0	1	0
Amphisphaeriaceae sp.	0	0	1	0	0	1	0	0	0
Bullanockia australis	0	0	1	0	0	1	0	0	0
Cadophora sp.	0	0	1	0	0	0	0	0	1
Chaetosphaeria sp.	0	0	2	1	0	1	0	0	0
Chaetosphaeriales sp.	0	0	1	0	0	0	0	0	1
Clonostachys rosea	0	4	0	0	2	0	1	1	0
Cucurbitariaceae sp.	0	2	1	2	0	0	0	1	0
Dictyochaeta sp.	0	0	4	2	0	2	0	0	0
Dinemasporium morbidum	1	0	0	0	0	0	1	0	0
Epichloe typhina	7	2	2	0	0	4	3	4	0
Fusarium circinatum	0	5	4	1	1	1	0	2	4
Gaeumannomycella sp.	0	2	0	2	0	0	0	0	0
Glarea sp.	0	2	6	1	0	2	0	4	1
Hypocreales sp.	0	1	0	0	0	1	0	0	0
Ilyonectria robusta	0	3	7	0	2	1	0	5	2
Lachnum sp. A	0	1	9	4	1	1	0	3	1
Lachnum sp. B	1	24	59	14	26	14	9	4	17
Metapochonia bulbillosa	0	6	0	3	0	0	1	1	1
Metarhizium carneum	3	0	0	2	0	1	0	0	0
Microdochium bolleyi	0	3	2	1	0	0	0	1	3
Microdochium neoqueenslandicum	0	0	2	0	0	1	0	0	1
Mollisia sp.	0	0	3	2	0	1	0	0	0
Mollisiaceae sp.	3	23	33	10	18	6	8	7	10
Mycena sp.	0	1	0	0	0	0	1	0	0
Myrmecridium sp.	1	0	0	0	0	0	0	1	0
Myrothechium sp.	0	1	0	0	0	1	0	0	0
Nemania sp.	0	1	0	1	0	0	0	0	0
Omnidemptus graminis	80	12	0	19	20	20	12	15	6
Ophiospaerella korrae	0	0	1	0	0	0	0	0	1
Parasola sp.	0	1	0	1	0	0	0	0	0
Penicillium ortum	0	5	2	0	1	1	0	0	5

Peniophoraceae sp.	1	0	0	0	0	1	0	0	0
Periconia sp.	0	1	0	1	0	0	0	0	0
Petrakia sp.	0	0	1	0	0	0	0	0	1
Pezicula rhizophila	0	0	1	0	0	0	0	1	0
Pleosporales sp.	0	0	1	0	0	0	0	0	1
Pyrenochaetopsis leptospora	0	0	1	0	0	0	0	1	0
Sarocladium strictum	4	0	0	1	3	0	0	0	0
Sordariomycete sp. A	0	0	1	0	0	0	0	1	0
Sordariomycete sp. B	0	1	0	0	0	0	1	0	0
Tolypocladium album	0	4	0	0	0	3	1	0	0
Trichoderma koningii	0	1	0	1	0	0	0	0	0

Supplementary material table S3. The presence/absence data of the aerial tissues in three representative grasses of high-diversity grasslands.

Fungal endophyte	**Grass species**			**High-diversity grasslands**		
	B. rupestre	***F. rubra***	***A. capillaris***	**Urkulu**	**Zalbetea**	**Azalegi**
Albotricha sp.	0	4	2	2	3	1
Alfaria dandenongensis	0	3	3	5	1	0
Cadophora sp.	0	1	0	0	0	1
Clonostachys rosea	0	1	0	1	0	0
Dilophospora sp.	0	0	1	0	1	0
Dinemasporium morbidum	1	0	0	1	0	0
Epichloe baconii	0	0	5	3	0	2
Epichloe festucae	0	29	0	9	0	20
Epichloe typhina	4	0	0	1	3	0
Fusarium circinatum	0	0	1	0	0	1
Glarea sp.	0	0	1	0	1	0
Ilyonectria robusta	0	0	2	1	0	1
Lachnum sp. B	1	1	6	8	0	0
Microdochium neoqueenslandicum	0	1	1	1	0	1
Mollisiaceae sp.	0	9	5	13	0	1
Myrmecridium sp.	1	0	0	0	1	0
Nectriaceae sp.	0	0	1	0	1	0
Oculimacula sp.	0	0	1	0	1	0
Omnidemptus graminis	27	2	4	10	17	6
Pyrenochaetopsis leptospora	0	0	1	0	0	1
Scytalidium album	0	0	1	0	1	0
Sordariomycete sp. B	0	1	0	1	0	0
Tricholomataceae sp.	0	0	1	0	1	0



Supplementary Figure S1. Georeferenced sampling points in each location.

CHAPTER 3

3513

Universidad Pública de Navarra
Nafarroako Unibertsitate Publikoa

Comparison of culturing and metabarcoding methods to describe the fungal endophytic assemblage of *Brachypodium rupestre* growing in a range of anthropized disturbance regimes

ABSTRACT

Fungal endophytes develop inside plants without visible external signs and they may confer adaptive advantages to their hosts. Culturing methods have been traditionally used to recognize the fungal endophytic assemblage, but novel metabarcoding techniques are being increasingly applied. This study aims to characterize the fungal endophytic assemblage in shoots, rhizomes and roots of the tall-grass *Brachypodium rupestre* growing in a large area of natural grasslands with a continuum of anthropized disturbance regimes. Seven out of 88 taxa identified by metabarcoding accounted for 81.2% of the reads (Helotiaceae, *Lachnum* sp. A, *Albotricha* sp. A, Helotiales A, Agaricales A, *Mycena* sp. and Mollisiaceae C), revealing a small group of abundant endophytes and a large group of rare species. Although both methods detected the same trends in richness and fungal diversity among the tissues (root > rhizome > shoot) and grasslands (low-diversity > high-diversity grasslands), the metabarcoding tool identified 5.8 times more taxa than the traditional culturing method (15 taxa) but, surprisingly, failed to sequence the most isolated endophyte on plates, *Omnidemptus graminis*. Since both methods are still subject to important constraints, both are required to obtain a complete characterization of the fungal endophytic assemblage of the plant species.

1. INTRODUCTION

The study of microorganisms in their natural environment is a recent branch of research compared to microbial investigations undertaken in disciplines such as medicine and agronomy, with high impact on human health and development (Bud, 1991; United Nations, 1992). Nowadays, microbial ecology, i.e., their diversity in nature, their response to prevailing and future environmental conditions, the associations they establish with plants and the complex network of interactions and functions they are involved in, are gaining ground in ecological research (Tikhonovich and Provorov, 2011; Guerrero et al., 2013; Carthey et al., 2019).

One example involves examining the associations that endophytic fungi establish with plants. These associations were first studied in agronomic grasses (Saikkonen et al., 2006, 1998; Hume and Sewell, 2014) and the research has extended to natural plant communities in recent decades (Rodriguez et al., 2009; Binet et al., 2013; Hardoim et al., 2015). Scientific literature has shown that these hidden associations are ubiquitous in nature, and that all plants harbor an endophyte assemblage that delivers different functions and constitutes a collective and complex holobiont (Vandenkoornhuyse et al., 2015).

Nowadays, two techniques, culturing and metabarcoding, are used for the determination of fungal endophyte assemblages (Hyde and Soytong, 2008). The protocols of culturable techniques have a longer record and have been implemented in many laboratories (Verma and Gange, 2014). In this method, important constrains include the possibility that some fungal species are unculturable on artificial medium, and the accumulation of inaccuracies and errors due to different sterilization times, diverse species growth rates and the presence of surface contaminants (Chi et al., 2019). The metabarcoding techniques (culture-independent) (Nilsson et al., 2019a), despite appearing very promising, still remain costly and lack a complete repository of sequences with taxonomic identification, a task which is under way (Ebach et al., 2011; Nilsson et al., 2019b). In the latter, the potential for providing quantitative data based on the proportion of read sequences makes it a very powerful ecological tool (Li, 2009; Sun and Guo, 2012).

The genus *Brachypodium* encompasses several perennial tall-grasses, native to european calcareous grasslands, that are expanding aggressively in the last decades due to the global change conditions (*B. pinnatum*, *B. genuense* and *B. rupestre)* (Bobbink and Willems, 1987; Buckland et al., 2001; Catorci et al., 2011; Bąba et al., 2012; Bricca et al., 2020). This tall-grass expansion causes a decline of the biodiversity of the natural grasslands and also has an impact on the ecosystem service of provisioning (Durán et al., 2020). The competitive strategies of this group of species that explain the expansive process is a matter of interest (Catorci et al., 2014; Canals et al., 2014, 2017; Bąba et al., 2016; Tardella et al., 2018; Múgica et al., 2021; San Emeterio et al., 2021) as it is the study of the mycobiome that may help to understand these advantages. To date, the research in the *Brachypodium* genus has focused on the systemic fungi of the Clavicipitaceae family hosted by *B. sylvaticum* (Meijer and Leuchtmann, 1999; Miwa et al., 2017), *B. phoenicoides* (Vazquez-de-Aldana et al., 2003; Zabalgogeazcoa et al., 2008) and *B. pinnatum* (Leuchtmann and Schardl, 1998). Only a previous study of our research team has characterised the systemic and non-systemic mycobiome of *Brachypodium rupestre* under a gradient of grazing and fire disturbances using culturable techniques (Durán et al., 2021).

The aim of this research is to provide a characterization of the endophytic mycobiome of the tall-grass species *Brachypodium rupestre* and to compare culture and metabarcoding techniques applied to conditions with restricted sampling effort due to the high cost of the novel technique. The comparison includes the aboveground (shoot) and the underground (rhizome and root) component of a set of *B. rupestre* individuals growing in the same region but subjected to different levels of anthropic disturbance (grasslands with different regimes of grazing and prescribed burning and, consequently, encompassing a different plant community composition). Through this range of regional variation, and considering different tissues and different environmental drivers, we are interested in determining the capacity of the two methods to identify and characterize the fungal endophyte assemblage of *B. rupestre*.

2. MATERIALS AND METHODS

2.1. The study area

The Aezkoa valley (Navarra county, Spain) is the westernmost valley of the southern Pyrenees (42.53' - 43.3'N, 1.8' - 1.17'W) (Figure 1d). The climate is snowy and cold in winter, and mild and foggy in summer. The annual temperature averages 9.3°C and the accumulated precipitation reaches 1856 mm per year (Irabia climatic station, http://meteo.navarra.es). The landscape is a mosaic of forests (e.g. *Fagus sylvatica*, *Abies alba*), shrubland communities (e.g. *Erica* spp., *Ulex gallii*) and grasslands. The area of study is part of the Special Area of Conservation (SAC) Roncesvalles-Selva de Irati (code ES0000126; Figure 1f) and is located in the north of the valley. High-altitude grasslands (800 - 1400 m.a.s.l.) comprise diverse communities of perennial grasses (*Festuca* gr. *rubra*, *Agrostis capillaris*, *Brachypodium rupestre*, *Danthonia decumbens*), forbs (*Achillea millefolium*, *Potentilla erecta*, *Gallium saxatile*) and legumes (*Trifolium repens*, *Lotus corniculatus*). Sandstones and calcareous clays dominate the substrate, upon which develop acidic, deep and organic soils, with clay-loamy and loamy textures.

Depending on the grazing pressure of the livestock during the summer months, farmers schedule different types of burnings to control the build-up of litter and resprouting of woody species. As a result, traditional (bush-to-bush) burnings applied every 6-7 years coexist with more intense fire regimes, applied across the whole surface every 1-2 years in the less grazed areas. The regional plant community composition reflects the dominant grazing/burning regime, which leads to a mosaic of high-diversity grasslands (more grazed, less burned) and low-diversity grasslands highly dominated by *B. rupestre* (less grazed, more burned). Based on previous floristic surveys undertaken in the area (Durán et al., 2020), we selected two representative locations according to the percentage of *B. rupestre* cover. A low-diversity grassland (LD) in Arpea, with a dominant cover of *B. rupestre* up to 80%, and a high-diversity grassland (HD) located in Urkulu, with a *B. rupestre* cover lower than 25% (Table 1).

2.2. Plant sampling

In summer 2018, a total of 10 turfs of *B. rupestre* were collected (turfs included shoots, rhizomes and roots surrounded by soil) from the two locations (Figure 1f). The distance between turf samples was ca 150 m to avoid collecting clonal individuals. Turfs were transported to the UPNA laboratory and processed in the following days.

One *B. rupestre* plant with high biomass was selected from each turf. Tissues were separated (shoots, rhizomes and roots) and cut into fragments of ca 2 cm, surface-disinfected by immersion in a solution of 20% commercial bleach (1% active chlorine) containing 0.02% Tween 80 (v:v) for 10 min and finally, rinsed with sterile water. The rhizome and root fragments were also treated with an aqueous solution of 70% ethanol for 30 s. Thirty fragments (10 shoots, 10 rhizomes and 10 roots) assigned to the metabarcoding method were ground using a pestle with liquid nitrogen and preserved at -20°C until shipment.

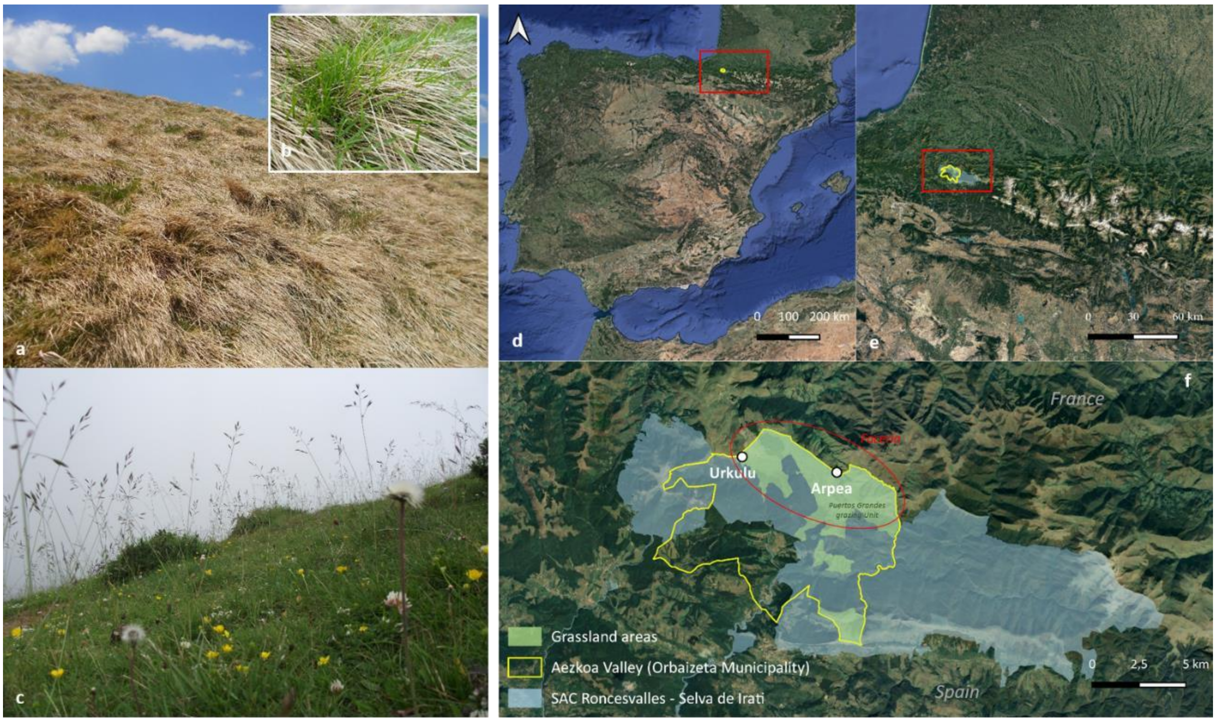


Figure 1. The appearance of low (a, b) and high (c) diversity grasslands. Location of the Aezkoa Valley in Spain (d) and within the western Pyrenees (e). The two locations (Arpea and Urkulu) where the samples were collected in the Roncesvalles-Selva de Irati SAC (f).

Table 1. General description of the study sites.

	Study site	**ARPEA**	**URKULU**
	Type of grassland	**LD = Low diversity**	**HD = High diversity**
General description	Location	-1° 10’ 57’’ W 43° 2’ 12’’ N	-1° 14’ 38’’ W 43° 2’ 49’’ N
	Soil classification (WRB)	Cambic Umbrisol	Dystric Cambisol
	Altitude (m.a.s.l.)	893	1256
	Slope (%)	40	45
Management	Burning recurrence	High 1-2 years	Low 6-7 years
	Type of burning	Large grassland areas	Bush-to-bush
	Grazing level	Low to nonexistent	Moderate to high
	B. rupestre cover (%)	> 80%	< 25%

2.3. Isolation and identification of fungi by the culturing method

We plated 300 tissue fragments of *B. rupestre* onto 30 culture media plates (10 fragments/tissue/plate, 90 mm diameter), containing PDA medium (potato dextrose agar) with chloramphenicol (200 mg/L). Dishes with tissue fragments were kept at room temperature and ambient light, and checked daily for 4 weeks. Any emerging mycelium was transferred and individually isolated to a new mini petri dish (60 mm diameter). Isolates with the same morphological characteristics (colony color, exudates, growth type and general appearance) were grouped into morphotypes, and at least one of them was genotyped for taxonomic analysis.

A small amount of mycelium was collected and its DNA extracted using a Phire Plant Direct PCR Kit (Thermo Fisher Scientific). The complete ITS region (ITS1-5.8S-ITS2) was amplified using ITS4 and ITS5 primers (White et al., 1990). The amplification cycles followed were: 98°C for 5 min, 95°C for 5 s (35 repeated cycles), 54°C for 5 s, 72°C for 20 s and a final phase of 72°C for 1 min. PCR amplicons were purified (Favor PrepTM Plant Genomic DNA Extraction Mini Kit, Favorgen) and sequenced by the Sanger method, copying single-stranded DNA, at STABVIDA enterprise. The returned DNA sequences were grouped using the CD_HIT program at 97% identity threshold (Li and Godzik, 2006; Huang et al., 2010), considering the clustered sequences to represent the same taxon. A representative sequence of each cluster was selected and contrasted to the closest match of the ITS region from fungal types at the National Centre for Biotechnology Information (NCBI) using the BLAST algorithm (Schoch et al., 2014). The database UNITE was also interrogated for sequences.

2.4. Metabarcoding analysis and taxonomic assignment

A total of 30 samples was sent to AllGenetics services for metabarcoding analysis. The DNA of samples was isolated using the Dneasy PowerSoil DNA isolation kit (Qiagen, Hilden, Germany) and the complete ITS2 region was amplified using the primers ITS86F and ITS4 (White et al., 1990; Turenne et al., 1999), to which the Illumina sequencing primer sequences were attached to their 5’ ends. The PCR cycle consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 49°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 10 minutes. The index sequences required for multiplexing libraries were attached in a second PCR with the same conditions but only 5 cycles and 60°C as the annealing temperature. Libraries were purified using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek, Norcross, USA), pooled in equimolar amounts and sequenced in a MiSeq PE300 run (Illumina, San Diego, USA).

The Illumina raw files R1 (forward) and R2 (reverse) reads were trimmed and checked using the software FastQC (www.bioinformatics.babraham.ac.uk). FLASH2 was used to merge reads and CUTADAPT software 1.3 to remove sequences that did not contain the PCR primers and those shorter than 100 nucleotides (Magoč and Salzberg, 2011; Martin, 2011). The sequences were filtered by quality using Qiime v1.9.1. and the FASTA file was processed using VSEARCH (Caporaso, 2011). Sequences were dereplicated, sorted and clustered at a similarity threshold of 100%. Artefacts were detected and filtered using the UCHIME algorithm implemented in VSEARCH (Edgar et al., 2011). Sequences were then assigned to OTUs and those occurring at a frequency below 0.005% in the whole dataset were removed. In the same way as the sequences obtained from the culture method, sequences were grouped using the CD_HIT program at 97% identity threshold (Li and Godzik, 2006; Huang et al., 2010); we considered that the clustered OTUs were the same taxon. A representative OTU of each cluster was selected and compared with the NCBI and UNITE data using the BLAST algorithm (Schoch et al., 2014).

2.5. Data analysis

For the metabarcoding data, we estimated accumulation curves with and without singletons (OTUs and taxa that were only present in one sample) to evaluate the sampling effort and to compare the importance of rare taxa/OTUs between grasslands and tissue types. We calculated the OTU richness and Shannon and Simpson diversity indexes and we analyzed the effects of tissue and grassland type on fungal endophyte richness and diversity using two-way ANOVAs (Oksanen et al., 2017). We calculated the relative abundance at the taxonomic level of phyla, orders, families and OTUs grouped into taxa using read sequences, within each tissue (shoot, rhizome and root) and each grassland type (LD and HD). We evaluated the effects of tissue and grassland type on fungal endophyte assemblages of *B. rupestre* using nonmetric multidimensional scaling (NMDS) with a Bray-Curtis dissimilarity index matrix, and we identified the distinctive fungal endophytes of a specific tissue and grassland type using indicator species tests (Roberts, 2019), measuring the fidelity of the taxa to a particular situation (Dufrêne and Legendre, 1997).

3. RESULTS

3.1. Comparison of *B. rupestre* mycobiome obtained by culturing and metabarcoding methods

For the culture method, we obtained 28 isolates which were classified into a total of 19 morphotypes. Their corresponding sequences were matched in databases, a total of 15 taxa were obtained and classified to species (2), genus (9), family (3) and order (1) rank (Table 2). Ten taxa were isolated in plants collected in the LD grassland (66.6%), while eight were from the HD grassland (53.3%). We identified 2, 5 and 11 taxa from shoots, rhizomes and roots, respectively (Table 3).

The thirty samples of *B. rupestre* analyzed by the metabarcoding method produced 1,622,980 reads from 1,822 OTUs before filtering, and 513,671 reads from 352 OTUs after the filtering process. We obtained 316 OTUs from the LD grassland (61.1%) and 246 OTUs from the HD grassland (38.9%). There were 19,197 and 340 OTUs from shoots, rhizomes and roots, respectively. The OTU clustering process returned a total of 88 taxa: 38 assigned to genus, 16 to family, 19 to order, 9 to class and the remaining 6 to phylum or still unidentified (Appendix A). According to grassland type, 75 taxa were identified in the LD grassland (85.2%) and 52 in the HD grassland (59.1%). According to tissue type, 15, 37 and 82 taxa were identified in shoots, rhizomes and roots, respectively (Table 3).

The culturing method isolated 13 taxa out of 88 sequenced by metabarcoding. Since we used a conservative approach in the process of identification, it is likely that we arrived at different taxonomic levels of identification depending on the methodology, for example, *Codinaea* sp. (culturing) *vs*. Chaetosphaeriaceae (metabarcoding), Didymosphaeriaceae (culturing) *vs. Paracamarosporium* sp. (metabarcoding) and *Mollisia* sp. and *Phialocephala* sp. (culturing) *vs*. Mollisiaceae (metabarcoding). The rest of the isolated taxa did match at the taxonomic level assigned (*Albotricha* sp., *Drechslera* sp., *Falciphora* sp., Helotiaceae, *Lachnum* sp., *Microdochium phragmitis* and *Neoascochyta* sp.). Table 4 shows the complete information obtained from both methods for each sample, as well as the samples where the same taxon was isolated by the culturing method and also sequenced by the metabarcoding analysis. The two taxa isolated by culturing but not sequenced by metabarcoding were *Ilyonectria* sp. and *Omnidemptus graminis*. The latter was the most isolated fungal endophyte from shoots of *B. rupestre* by the culturing method.

Despite the remarkable differences in the number of sequences obtained by the two methods (28 isolates *vs.* 513,671 reads), the pattern of fungal endophyte richness and diversity among grassland and tissue types followed a similar trend, with the highest values in the root tissue and plants collected from the LD grassland.

Table 2. Fungal endophytes isolated from *B. rupestre* by the culturing method, their greatest percentage identity in both databases (NCBI and UNITE), the proposed taxon and the available accession number in GenBank.

	Match taxon (NCBI)			**Match taxon (UNITE)**				
		Accession number	**Greatest percentage identity (%)**		**Accession number**	**Greatest percentage identity (%)**	**Taxon proposed**	**GenBank accession number**
1	*Lachnellula hyalina*	NR_165202	90.11	*Albotricha* sp.	HM136666	98.22	*Albotricha* sp.	MW789554
2	*Codinaea paniculata*	NR_166297	99.74	*Codinaea* sp.	MT118230	99.74	*Codinaea* sp.	MW789567
3	*Paracamarosporium* sp.	NR_154318	94.28	*Paracamarosporium* sp.	MT882131	97.6	Didymosphaeriaceae	MW789559
4	*Drechslera* sp.	NR_153992	94.43	*Drechslera* sp.	UDB0174425	100	*Drechslera* sp.	MW789560
5	*Falciphora oryzae*	NR_153972	96.69	*Falciphora* sp.	UDB0162916	99.76	*Falciphora* sp.	MW789558
6	*Glarea lozoyensis*	NR_137138	96.18	*Glarea* sp.	KF617491	99.58	Helotiaceae	MW789565
7	*Ilyonectria leucospermi*	NR_152889	99.36	*Ilyonectria crassa*	MT294410	100	*Ilyonectria* sp.	MW789566
8	*Lachnellula hualina*	NR_165202	88.89	*Lachnum virgineum*	MT133783	98.15	*Lachnum* sp.	MW789564
9	*Microdochium phragmitis*	NR_132916	100	*Microdochium phragmitis*	MH861162	100	*Microdochium phragmitis*	MW789562
10	*Mollisia asteliae*	NR_173037	96.44	*Mollisia* sp.	KJ188683	98.69	*Mollisia* sp.	MW789555
11	*Phialocephala spaheroides*	NR_121302	95.71	*Loramyces* sp.	KF618060	99.36	Mollisiaceae	MW789556
12	*Neoascochyta dactylidis*	NR_170041	100	*Neoascochyta* sp.	MT185527	100	*Neoascochyta* sp.	MW789561
13	*Omnidemptus graminis*	NR_164058	100	*Omnidemptus graminis*	MK487758	100	*Omnidemptus graminis*	MW789553
14	*Phialocephala sphaeroides*	NR_121302	89	*Phialocephala* sp.	JN995646	98.87	*Phialocephala* sp.	MW789563
15	*Paraphaeosphaeria michotii*	NR_155640	91.41	Pleosporales	MN450621	100	Pleosporales	MW789557

Table 3. Total number of reads, OTUs and taxa associated with *B. rupestre* tissues and the type of grassland where plants were collected (LD: Low-diversity grassland, HD: High-diversity grassland).

		Type of grassland		Tissue		
				Shoot	**Rhizome**	**Root**
Metabarcoding method	**Reads**	**LD**	313621	4680	47268	261673
		HD	200050	3204	29692	167154
		Total	513671	7884	76960	428827
	OTUs	**LD**	316	12	165	305
		HD	246	11	58	236
		Total	352	19	197	340
	Taxa	**LD**	75	10	27	69
		HD	52	10	23	45
		Total	88	15	37	82
Culture method	**Taxa**	**LD**	10	2	3	6
		HD	8	1	3	5
		Total	15	2	5	11

3.2. The mycobiome of *B. rupestre* according to the metabarcoding method

3.2.1. Fungal endophytic richness and diversity

The quantitative data from metabarcoding, based on read sequences, allowed an exhaustive characterization of the endophytic diversity of *B. rupestre*. Both the OTUs (352) and the clustering of OTUs into taxa (88) produced non-asymptotic species accumulation curves (Figure 2). However, 23 out of 88 taxa and 71 out of 352 OTUs were sequenced in only one sample (designated as singletons). Additional curves were constructed without singletons, suggesting that an increase in sampling effort would increase the number of rare taxa/OTUs but not the more common ones (Figure 2a and d). Accumulation curves comparing tissues and grassland types did not approach horizontal asymptotes (Figure 2b, c, e and f), therefore greater sampling effort is required for reliable richness estimates.

The two factor ANOVA showed a significant effect of plant tissue (F = 19.9, $p < 0.001$) but not of grassland type (F = 2.5, p = 0.126) on OTU richness, whereas Shannon and Simpson indexes showed significant differences between grassland types (F = 5.1, p = 0.033 and F= 4.4, p = 0.046, respectively) and tissues (F = 32.7, $p < 0.001$ and F= 9.5, $p < 0.001$, respectively) (Figure3).

Table 4. Culturing and metabarcoding comparison. Total number of reads, OTUs and taxa and match identification for each sample.

Sample		Culture method	Match methods	Metabarcoding		
		Isolated taxa		Taxa	OTUs	Reads
Shoot LD	1	*Neoascochyta* sp.	✓	5	5	233
	2	*Omnidemptus graminis*	✗	5	6	1639
	3	*Omnidemptus graminis*	✗	7	9	1619
	4	*Omnidemptus graminis*	✗	3	3	207
	5		✗	4	4	982
Shoot HD	6		✗	2	2	229
	7	*Omnidemptus graminis*	✗	2	2	37
	8	*Omnidemptus graminis*	✗	5	5	644
	9	*Omnidemptus graminis*	✗	1	1	13
	10		✗	3	3	2281
Rhizome LD	1		✗	8	102	12546
	2		✗	11	21	24377
	3	Didymosphaeriaceae Helotiaceae	✗ ✓	4	6	1312
	4		✗	5	7	831
	5	Mollisiaceae	✓	12	41	8202
Rhizome HD	6	Helotiaceae	✓	6	22	5621
	7	Helotiaceae	✓	3	4	267
	8	*Phialocephala* sp.	✗	11	17	15380
	9		✗	4	5	2035
	10	*Microdochium phragmitis*	✗	11	18	6389
Root LD	1	Didymosphaeriaceae	✗	15	180	49606
	2	*Falciphora* sp. *Codinaea* sp.	✓ ✗	29	53	40482
	3	Didymosphaeriaceae	✗	27	184	70132
	4	*Mollisia* sp.	✗	34	73	52335
	5	Pleosporales Didymosphaeriaceae *Lachnum* sp.	✗ ✗ ✓	31	95	49118
Root HD	6	Helotiaceae	✓	18	141	62044
	7	Mollisiaceae	✓	20	116	23703
	8	*Albotricha* sp.	✓	16	32	12379
	9	*Albotricha* sp.	✓	20	55	47814
	10	*Drechslera* sp. *Ilyonectria* sp.	✓ ✗	17	59	21214

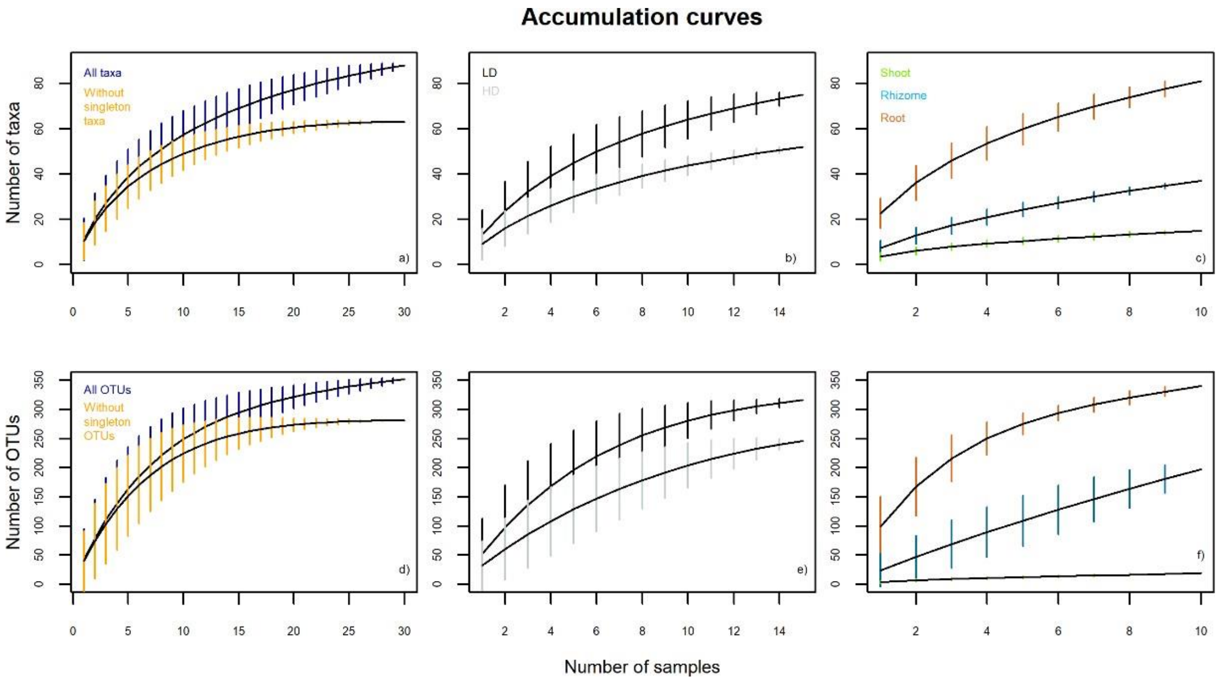


Figure 2. Taxon and OTU accumulation curves for the endophytic community of *B. rupestre* from metabarcoding (LD: Low-diversity grassland , HD: High-diversity grassland). Black line shows the total number of taxa/OTUs, and vertical colored lines indicate the standard deviation.

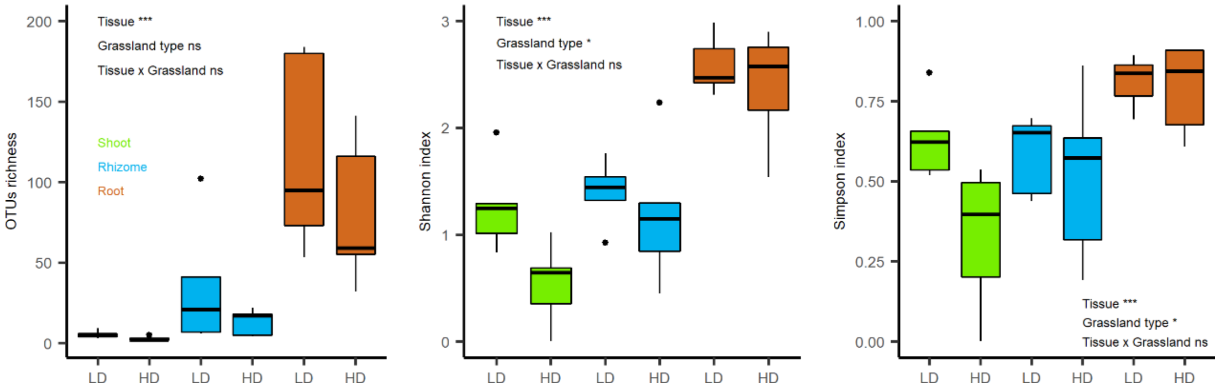


Figure 3. OTU richness and diversity indexes (Shannon and Simpson) for the endophytic community of *B. rupestre* from different tissues and grasslands (LD: Low-diversity grassland, HD: High-diversity grassland). *** p-value < 0.001; ** p-value < 0.01; * p-value < 0.05 and ns = no significance. Black points represent outliers.

3.2.2. Taxonomic assemblages for grassland types and tissues

The relative abundance of phyla, orders and families was estimated from the read sequences. Most taxa were included in the phyla Ascomycota (71.21%) and Basidiomycota (21.21%). Figure 4 shows the relative abundance of orders and families according to tissue and grassland type.

Pleosporales dominated in shoots of plants from both grassland types (52.59% LD and 65.39% HD), followed by Phyllachorales (20.49%) and Pucciniales (16.05%) in the LD grassland, and

Hypocreales (17.45%) and Capnodiales (12.73%) in the HD grassland. The other orders did not exceed 4%, except Xylariales in the LD grassland (5.58 %). Helotiales dominated in the belowground tissues of both grassland types ranging from 85.81% (Rhizome-HD) to 77.47% (Rhizome-LD), followed by Agaricales ranging from 20.93% (Rhizome-LD) to 7.17% (Root-LD). The other orders did not exceed 2.5% except Pleosporales in roots from the HD grassland (6.74%) (Figure 4, left).

Phaeosphaeriaceae dominated in shoots from both grassland types (63.48% HD and 34.08% LD), followed by unidentified family A (22.28%), Didymellaceae (16.94%) and Pucciniaceae (16.05%) in the LD grassland and unidentified family A (17.45%) and Mycosphaerellaceae (12.73%) in the HD grassland. The rest of the families did not exceed 5.5%. Hyaloscyphaceae dominated in belowground tissues ranging from 54.47% (Rhizome-HD) to 32.72% (Root-HD), followed by Helotiaceae ranging from 35.05% (Root-HD) to 13.16% (Rhizome-HD). Other families with relatively high abundance were Tricholomataceae and unidentified family A, ranging from 20.93% (Rhizome-LD) to 5.18% (Root-LD) and from 24.21% (Root-LD) to 8.18% (Root-HD), respectively. The other families did not exceed 4% (Figure 4, right).

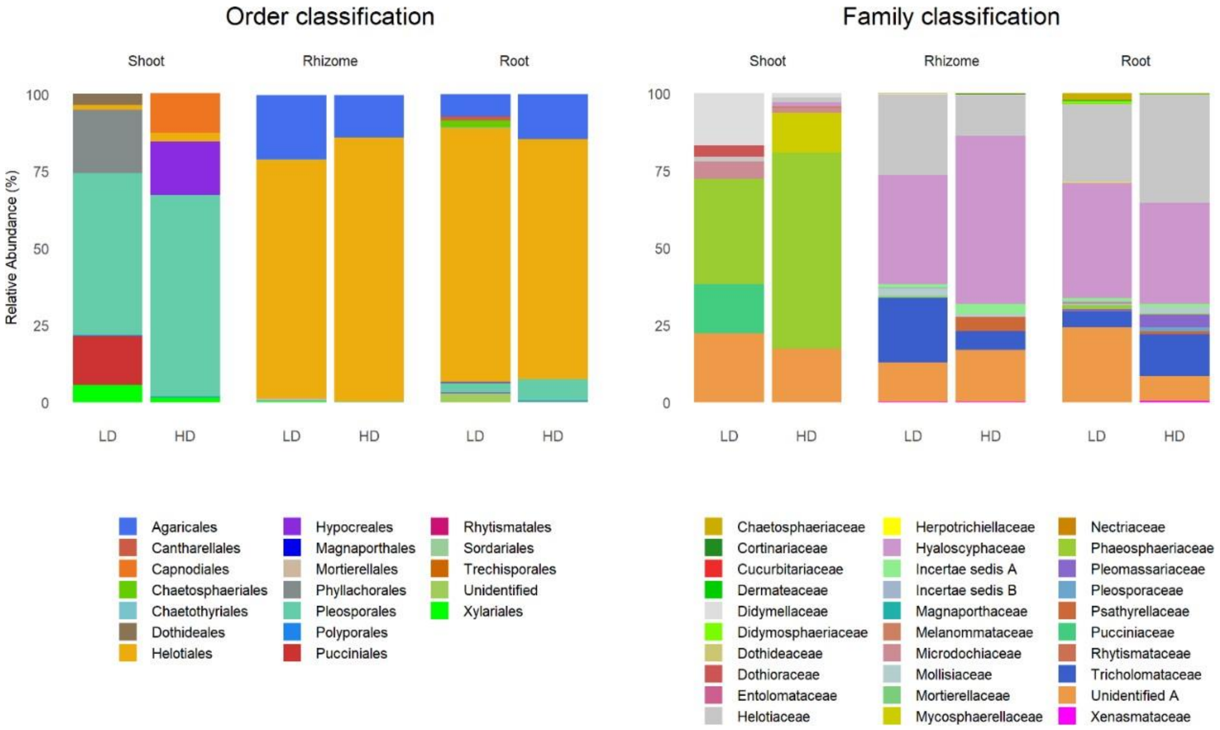


Figure 4. Taxonomic structure (orders, left and families, right) of fungal endophytes in *B. rupestre* tissues (shoot,rhizome and root) in the different grassland types (LD: Low-diversity grassland , HD: High-diversity grassland).

The relative abundance of endophytic taxa after the OTU clustering process according to their high genetic similarity (97% threshold), was estimated from the read sequences. The most abundant read sequences were located in the root tissue and were reached by Helotiaceae (22.60%), *Lachnum* sp. A (21.94%), Helotiales A (8.29%) and *Albotricha* sp. A (7.00%). All of these were more abundant in plants collected in the LD grassland, with the exception of *Albotricha* sp. A.

In the roots, taxa with abundances higher than 5% were *Lachnum* sp. A (35.08%), Helotiaceae

(24.51%) and Helotiales A (12.43%) in LD grassland plants, and Helotiaceae (31.09%), *Albotricha* sp A (18.83%), *Lachnum* sp. A (12.50%), Agaricales A (9.65%) and Helotiales A (6.03%) in HD grassland plants (Table 5).

The dominant taxon in the shoots was Phaeosphaeriaceae (34.08% LD and 58.80% HD). In LD grasslands, it was accompanied by Phyllachorales (20.49%), *Puccinia* sp. (16.05%), *Neoascochyta* sp. A (14.94%) and *Microdochium* sp. (5.58%), and in HD grasslands by Sordariomycete A (17.45%), Mycosphaerellaceae (12.73%) and *Ophiosphaerella* sp. (4.68%). The remaining taxa did not exceed 4% (Table 6).

The dominant taxa in the rhizomes of the LD grassland were *Lachnum* sp A. (29.13%), Helotiaceae (25.95%), *Mycena* sp. A (20.88%), Helotiales A (10.98%) and *Albotricha* sp. A (5.23%) and in the rhizomes of the HD grassland *Albotricha* sp. A (50.58%), Helotiaceae (16.49%), *Lachnum* sp. A (9.01%), *Parasola* sp. (5.81%) and Tricholomataceae (4.21%) (Table 5).

Table 6. List of taxa in the *B. rupestre* shoots and their relative abundance based on number of reads, number of OTUs and infected plants (out of five).

SHOOT

Endophyte taxon	**Relative abundance (%)**		**Reads**		**OTUs**		**Infected plants**	
	LD	**HD**	**LD**	**HD**	**LD**	**HD**	**LD**	**HD**
Phaeosphaeriaceae	34.08	58.80	1595	1884	2	1	4	2
Phyllachorales	20.49	0	959	0	1	0	4	0
Puccinia sp.	16.05	0	751	0	3	0	1	0
Neoascochyta sp. A	14.94	0.53	699	17	1	1	4	1
Sordariomycete A	0	17.45	0	559	0	1	0	1
Mycosphaerellaceae	0	12.73	0	408	1	0	0	1
Microdochium sp.	5.58	1.59	261	51	1	1	4	1
Dothideales	3.65	0	171	0	1	0	2	0
Ophiosphaerella sp.	0	4.68	0	150	0	1	0	1
Epicoccum sp.	2.01	0.75	94	24	1	1	1	1
Helotiaceae	1.41	1.56	66	50	1	2	2	3
Periconia sp.	1.56	0	73	0	1	0	1	0
Lachnum sp. A	0	1.28	0	41	0	1	0	1
Phragmocephala sp. B	0	0.63	0	20	1	0	0	1
Unidentified C	0.23	0	11	0	1	0	1	0
	100	100	4680	3204				

Table 5. List of the most abundant taxa in *B. rupestre* underground tissues. The relative abundance based on number of reads, number of OTUs and infected plants (out of five). Shaded taxa were sequenced in both underground tissues. The complete table is available in Appendix B.

	ROOT								RHIZOME							
Endophyte taxon	**Relative abundance (%)**		**Reads**		**OTUs**		**Infected samples**		**Relative abundance (%)**		**Reads**		**OTUs**		**Infected samples**	
	LD	**HD**	**LD**	**HD**	**LD**	**HD**	**LD**	**HD**	**LD**	**HD**	**LD**	**HD**	**LD**	**HD**	**LD**	**HD**
Helotiaceaea	24.51	31.09	64132	51968	114	116	2	4	25.95	16.49	12267	4897	94	18	2	4
***Lachnum* sp. A**	35.08	12.5	91790	20889	36	20	5	5	29.13	9.01	13771	2676	5	7	4	4
***Albotricha* sp. A**	1.71	18.83	4465	31473	7	6	3	3	5.23	50.58	2473	15018	4	6	3	3
Helotiales A	12.43	6.03	32534	10072	40	28	5	5	10.98	2.77	5188	823	25	1	2	3
Agaricales A	3.55	9.65	9281	16124	3	3	2	4	0.05	0	24	0	2	0	1	0
***Mycena* sp. A**	2.03	0.17	5323	289	10	2	4	1	20.88	0	9870	0	1	0	1	0
Mollisiaceae C	4.17	0.45	10913	745	2	1	4	1	0.42	0.04	198	13	2	1	1	1
Pleosporales A	0.56	4.04	1476	6751	2	4	3	5	0	0.04	0	12	0	1	0	1
***Glarea* sp.**	0.41	3.94	1060	6589	2	1	2	1	0	0.29	0	86	0	1	0	1
Mollisiaceae B	0.43	1.89	1118	3161	1	3	2	3	2.35	1.44	1111	429	1	3	2	2
Mollisiaceae D	0.89	1.07	2330	1782	1	2	2	1	0.78	3.56	369	1056	1	2	2	2
Chaetosphaeriaceae	1.76	0	4608	0	4	0	1	0	0.07	0	33	0	1	0	1	0
***Mycena* sp. B**	0	2.08	0	3479	0	3	0	1	0	3.34	0	993	0	1	0	1
Tricholomataceae B	0	1.48	0	2474	0	1	0	2	0	4.21	0	1251	0	1	0	1
***Lachnum* sp. B**	0.38	1.27	1007	2119	11	7	4	4	0.87	0.14	411	43	1	1	1	1
Cantharellales	1.3	0	3397	0	2	0	1	0								
***Parasola* sp.**	0	0.9	0	1503	0	1	0	1	0	5.81	0	1725	0	3	0	1
Unidentified A	1.21	0.01	3174	19	2	1	1	1								
***Ophiosphaerella* sp.**	0.96	0.32	2513	535	2	1	2	1	0.11	0.06	50	17	1	1	1	1
Mollisiaceae A	0.88	0.4	2309	666	4	3	4	5	0.03	0.28	13	83	1	1	1	1
***Drechslera* sp.**	0.03	1.43	87	2388	2	2	2	5								

3.2.3 Indicator species of the fungal assemblages

NMDS analysis showed that the fungal endophyte assemblage from above and belowground tissues of *B. rupestre* was clearly different (Figure 5a). In addition, fungal assemblages from root tissues (Figure 5d), unlike shoots (Figure 5b) and rhizomes (Figure 5c), displayed significant differences between grassland types.

The indicator species for shoot tissues were Phaeosphaeriaceae (p = 0.002), *Neoascochyta* sp. A (p = 0.005) and Phyllachorales (p = 0.029), and for root tissues were Helotiales A (p = 0.001), *Lachnum* sp. A (p = 0.001), Mollisiaceae A (p = 0.001), Pleosporales A (p = 0.001), *Drechslera* sp. (p = 0.001), *Lachnum* sp. B (p = 0.002), Agaricales A (p = 0.001), *Cladophialophora* sp. (p = 0.003), *Leohumicola* sp. (p = 0.003), *Pseudolachnella* sp. A (p = 0.007), Mollisiaceae C (p = 0.011), *Pseudolachnella* sp. B (p = 0.023), Unidentified B (p = 0.041), *Phragmocephala* sp. A (0.019) and *Paracamarosporium* sp. (p = 0.036). No species were indicative of rhizome tissues.

The indicator species for grassland type were Phyllachorales (p = 0.044) and *Neoascochyta* sp. A (p = 0.035) in shoots collected in the LD grassland (Figure 5b). *Drechslera* sp. (p = 0.012) and Pleosporales A (p = 0.036) were indicators in roots from the HD grassland, while *Lachnum* sp. A (p = 0.007), *Phragmocephala* sp. A (p = 0. 042), *Paracamarosporium* sp. (p = 0.037) and *Pseudolachnella* sp. A (p = 0.047) were indicators in roots from the LD grassland (Figure 5d). No species in the rhizome tissues were indicative of plant community (Figure 5d).

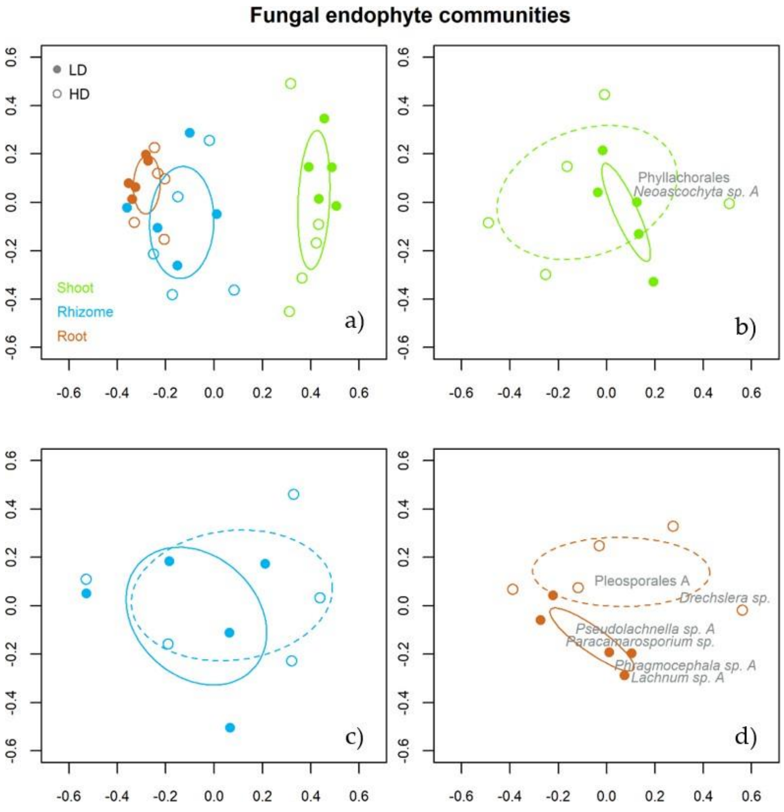


Figure 5. Non-metric multidimensional scaling analysis (NMDS) for the endophytic community of *B. rupestre* according to the effect of tissue (a) and plant diversity (shoot 1b, rhizome 1c & roots 1d). The ellipse formed by the solid line encompasses the fungal composition of *B. rupestre* tissues (a). The ellipses formed by broken and solid lines encompass the fungal composition of the low and high-diversity grassland, respectively (Figure b, c & d). The taxon names in the graphs for shoots (b) and roots (d) are the indicator species for the effect of plant diversity.

4. DISCUSSION

4.1. The mycobiome of *B. rupestre* according to the metabarcoding data

The results of the metabarcoding showed that 88 taxa constituted the mycobiome of *B. rupestre* and that only seven taxa sequenced from the belowground tissues accounted for 81.2% of the total reads (Helotiaceae, *Lachnum* sp. A, *Albotricha* sp. A, Helotiales A, Agaricales A, *Mycena* sp. A and Mollisiaceae C), while the other 81 taxa were responsible for the remaining 18.8%, and 25 of them were only sequenced in a single sample. Therefore, a restricted sampling effort using the metabarcoding method was able to identify a small group of abundant fungal endophytes and a large group of rare species. The accumulation curves also supported the idea that extension of the sampling effort would enrich the group of rare species but not the most common species. This pattern of fungal endophyte distribution seems common to grasses (Sun et al., 2020) and indicates that a limited sampling effort is enough to provide good characterization of the dominant fungal species in plants, which is important considering the high cost of metabarcoding. However, when addressing studies on fungal richness and diversity, more extended sampling appears necessary to avoid an underestimation of the values.

The results of the study also highlight the importance of sampling the different tissues of plants to obtain a reliable characterization of its mycobiome (Harrison and Griffin, 2020; Wearn et al., 2012). Aboveground fungal assemblages were much poorer in species, less diverse, and taxonomically different from those of rhizomes and roots, and this pattern was consistent between the grassland types, as observed by other authors in different plant species and different habitats (Arnold and Lutzoni, 2007; Herrera et al., 2010; Rudgers et al., 2014). The soil rhizosphere is the main route of fungal transmission to plants (Compant et al., 2016; Frank et al., 2017) and the high biomass of rhizome and roots developed by *B. rupestre* offers a large surface in contact with the soil microbiome. The majority of taxa identified were specific to a tissue, or exhibited a strong preference for it, and only five taxa appeared in all tissues (Helotiaceae, *Lachnum* sp. A, *Ophiosphaerella* sp., *Microdochium* sp. and *Epicoccum* sp.). As expected, the relative abundances of taxonomic orders and families also varied between tissues, with Pleosporales and Phaeosphaeriaceae more abundant in shoots, and Helotiales and Hyaloscyphaceae more abundant in rhizomes and roots.

When comparing these results with previous characterizations of fungal endophyte assemblages in perennial temperate grasses based on culture techniques and extensive surveys, we realize the power of the metabarcoding tool, which is capable of identifying a large set of taxa with much less sampling effort. In *Dactylis glomerata*, 22 and 48 taxa were identified by culturing methods from the leaves and the roots of 120 samples (Sánchez-Márquez et al., 2007) and in *Holcus lanatus* 77 and 79 in the same tissues of 77 samples (Sánchez-Márquez et al., 2010). The results of our survey of the leaves and roots of *B. rupestre* (2 and 11 taxa identified by the culturing method and 12 and 82 taxa identified by metabarcoding) obtained from a small number of samples in a regional sampling suggest that the real diversity and richness of the endophytic fungal assemblages of the previously studied grass species have probably been underestimated and would increase greatly if the novel metabarcoding techniques were used.

4.2. Culturing *vs.* metabarcoding methods

The modern massive sequencing techniques are gaining ground to traditional culturing methods due to the quantitative power of data that are able to generate. With equal sampling effort, metabarcoding identified 13, 32 and 71 more taxa than culturing methods in shoots, rhizomes and roots, respectively, which means around x 5.8 times more species identified by the novel technique consistently in the three tissues. In similar studies comparing both methods, the metabarcoding identified x 5.2 and x 4.3 times more OTUs in roots of *Elymus repens* and *Deschampsia flexuosa* respectively than the culturing technique (Tejesvi et al., 2010; Høyer and Hodkinson, 2021). A parallel study using 240 plants of *B. rupestre* recognized 45 fungal endophytic taxa using the culturing method (Durán et al., 2021), in contrast to the 88 taxa sequenced by metabarcoding from 10 plants in the current survey. In this parallel study, the singletons isolated accounted for 48.9% of the taxa identified by culturing methods and 28.4% of the taxa identified by metabarcoding (with OTUs clustered with a 97% of similarity threshold).

Regarding belowground tissues, four fungal species with high incidence in root tissues were identified by both methodologies: *Albotricha* sp., Helotiaceae, *Lachnum* sp. and Mollisiaceae. In shoots, surprisingly, the most frequent shoot endophyte identified by the culturing method, *Omnidemptus graminis*, was not identified by the metabarcoding technique. *O. graminis* is a recently described taxon, included in a family associated with ongoing taxonomic changes due to molecular advances (Hernández-Restrepo et al., 2019, 2016). Its fast mycelial growth observed on culture plates may suggest the encrypting of other endophytes, but how *O. graminis* escaped the sequencing process of the metabarcoding is a matter that needs further study.

At this point, some issues need to be discussed when comparing the technical procedures of sequencing in both techniques. The ITS region is a universal and commonly used DNA barcode marker for fungi (Schoch et al., 2012) and, in the metabarcoding study undertaken by an external company, only the ITS2 region was amplified to identify the fungal sequences (Mbareche et al., 2020; Zhang et al., 2020). In the culturing method undertaken in the UPNA's lab, the fungal mycelium was collected and the complete ITS region was amplified (ITS1-5.8S-ITS2), generating longer DNA sequences. We suggest that, since the ITS2 region is more restrictive, taxonomic inconsistencies may occur when short sequences are compared in the databases, thus affecting taxon identification (Ebach et al., 2011). The percentages of taxa identified for the metabarcoding were in the range 78.1-100%, and 97.6-100% for the culture sequencing, evidencing this restriction and indicating the value of sequencing the complete ITS region to achieve better fungal taxa identification. As a particular example, the taxon proposed as *Codinaea* sp. reached a match of 99.74% with the complete ITS region sequenced, while this percentage decreased to 97.52% when considering only the ITS2 region. As a consequence, the species was identified as Chaetosphaeriaceae in the metabarcoding, following a more conservative approach, although it was probably the same taxon. Similar situations may occur in other closely related taxa, when there is no reference specimen in the database (Arnold et al., 2007; Schoch et al., 2014). Taxa identified as Mollisiaceae in our study probably belong to the genera *Mollisia* and/or *Phialocephala* (Tanney et al., 2016; Tanney and Seifert, 2020), and family Helotiaceae to the genera *Glarea* and/or *Hymenoscyphus* (Bills et al., 1999). Both families were abundant in our samples. Other highly inclusive taxa, such as Pleosporales, raised similar doubts in the identification due to the still high uncertainty in the genetic characterization of the type specimens.

Despite the remarkable differences between the quantitative data generated by the two methods, the characteristics of the fungal assemblages in the different plant communities and tissues types are consistent between methods. Root tissues display the most diverse and rich fungal assemblages, and the endophytic community in plants collected in more disrupted, LD grasslands had the highest diversity and richness. Similar patterns have been reported in previous research in the area, conducted with a much greater sampling effort and using the culturing method (Durán et al., 2021), that analyzed the fungal assemblages in terms of the ecological mechanisms favored by the different disturbance regimes.

5. CONCLUSIONS

The endophytic mycobiome of *B. rupestre* is composed of a few abundant and many rare species, the identification of which depends on the sampling effort. Despite the restricted sampling effort, the two methodologies produced consistent results and detected the same trends in endophytic richness and diversity among tissues (roots > rhizomes > shoot) and grassland types (low-diversity > high-diversity). Comparatively, the metabarcoding method allowed the identification of a much larger number of taxa than the culturing method and revealed differences in richness and diversity that were not apparent with the culturing method (even when a larger number of samples was collected; Durán et al., 2021).

Despite the promising results of the metabarcoding technique, the data indicate that a combination of the two methodologies is the best current option to obtain an adequate characterization of the plant fungal assemblage. In this study, metabarcoding did not identify *Omnidemptus graminis*, the most abundant fungal endophyte isolated in shoots by culturing; this recently described species is included in a family where there have been repeated taxonomic restructurings as a result of molecular advances (Hernández-Restrepo et al., 2019).

Appendix A. Table with the 88 identified taxa from the metabarcoding method

	Match taxon (NCBI)			**Match taxon (UNITE)**				
		Accession number	**Greatest percentage identity (%)**		**Accession number**	**Greatest percentage identity (%)**	**Taxon proposed**	**Accession number**
1	*Gerronema* sp.	NR_166278	82.37	*Delicatula integrella*	UDB034203	99.77	Agaricales A	OK430888
2	*Gerronema* sp.	NR_166278	78.9	*Mycena* sp.	KT224934	89	Agaricales B	OK430889
3	*Ramariopsis flavescens*	NR_119913	85.06	Agaricales	JX456916	95.2	Agaricales C	OK430890
4	*Gerronema indigoticum*	NR_166278	78.06	Mycenaceae	KT224934	94.29	Agaricales D	OK430891
5	*Laccaria aurantia*	NR_154113	78.57	*Mycena floridula*	MH856660	99.35	Agaricales E	OK430892
6	*Radulotubus resupinatus*	NR_153458	84.66	Agaricomycete	LR864837	99.32	Agaricomycete	OK430893
7	*Lachnellula hyalina*	NR_165202	93.02	*Albotricha* sp.	JN995639	100	*Albotricha* sp. A	OK430894
8	*Lachnellula hyalina*	NR_165202	91.59	*Albotricha* sp.	JN995639	98.71	*Albotricha* sp. B	OK430895
9	*Lachnellula hyalina*	NR_165202	91.59	*Albotricha* sp.	HM136666	100	*Albotricha* sp. C	OK430896
10	*Funiliomyces biseptatus*	NR_159862	96.39	*Acremonium* sp.	MT911439	100	Ascomycota A	OK430897
11	*Tricladium terrestre*	NR_160144	93.67	Ascomycota sp.	KR266584	93.67	Ascomycota B	OK430898
12	*Auricularia scissa*	NR_125807	80.48	*Oliveonia* sp.	MT235652	97.16	Auriculariales	OK430899
13	*Hydnum albidum*	NR_164025	78.7	*Sistotrema* sp.	KC965692	93.87	Cantharellales	OK430900
14	*Codinaeae* sp.	NR_168799	97.52	*Codinaea* sp.	MT626587	98.35	Chaetosphaeriaceae	OK430901
15	*Chalara hyalocuspica*	NR_137568	91.25	*Chalara* sp.	MK965778	98.33	*Chalara* sp.	OK430902
16	*Cladophialophora tengchongensis*	NR_172399	90.07	*Cladophialophora* sp.	KP889848	100	*Cladophialophora* sp.	OK430903
17	*Coccomyces pinicola*	NR_158295	83.54	*Coccomyces dentatus*	KU986782	93.82	*Coccomyces* sp.	OK430904
18	*Conlarium duplumascospora*	NR_138382	94.9	*Conlarium* sp.	MK164654	96.85	*Conlarium* sp.	OK430905
19	*Laburnicola centaurear*	NR_154131	93.6	*Laburnicola* sp.	MK018553	97.95	Didymosphaeriaceae	OK430906
20	*Pseudoseptoria collariana*	NR_156560	97.63	*Pseudoseptoria donacis*	MH859141	99.6	Dothideales	OK430907
21	*Roussoella thailandica*	NR_155717	80.56	Dothideomycete	KJ827952	95	Dothideomycete A	OK430908

22	*Pirozynskiella laurisilvica*	NR_153488	91	Capnodiales	KX403688	91	Dothideomycete B	OK430909
23	*Drechslera* sp.	NR_164466	92.89	*Drechslera* sp.	MT816433	99.6	*Drechslera* sp.	OK430910
24	*Entoloma luteofuscum*	NR_152900	95.24	*Entoloma conferendum*	MT741744	100	*Entoloma* sp.	OK430911
25	*Epicoccum phragmospora*	NR_165920	99.19	*Epicoccum* sp.	MW054426	100	*Epicoccum* sp.	OK430912
26	*Falciphora oryzae*	NR_153972	98.86	*Falciphora oryzae*	MH201898	99.23	*Falciphora* sp.	OK430913
27	*Glarea lozoyensis*	NR_137138	98.48	*Glarea* sp.	KT268823	100	*Glarea* sp.	OK430914
28	*Glarea lozoyensis*	NR_137138	95.96	*Glarea* sp.	KF617491	100	Helotiaceae	OK430915
29	*Loramyces macrosporus*	NR_138379	89.8	*Loramyces* sp.	KF618060	99.58	Helotiales A	OK430916
30	*Loramyces macrosporus*	NR_138379	89.07	*Mollisia sp.*	UDB0778890	99.59	Helotiales B	OK430917
31	*Triposporium cycadicola*	NR_156587	89.71	*Hymenoscyphus* sp.	HQ625461	99.58	Helotiales C	OK430918
32	*Bisporella shangrilana*	NR_153628	97.02	Helotiales	LR863043	99.58	Helotiales D	OK430919
33	*Hyaloscypha finlandica*	NR_121279	92.27	*Hyaloscypha vraolstadiae*	KC876248	96.23	Hyaloscyphaceae	OK430920
34	*Lachnellula hyalina*	NR_165202	91.12	*Lachnum* sp.	MT913626	96.61	*Lachnum* sp. A	OK430921
35	*Lachnum fusiforme*	NR_154122	89.91	*Lachnum* sp.	MK808968	97.45	*Lachnum* sp. B	OK430922
36	*Proliferodiscus* sp.	NR_164304	86.67	*Lachnum* sp.	MH628228	99.57	*Lachnum* sp. C	OK430923
37	*Leohumicola minima*	NR_121307	100	*Leohumicola* sp.	FM999596	100	*Leohumicola* sp.	OK430924
38	*Variabilispora flava*	NR_165906	86.83	Helotiales	AY969994	95.65	Leotiomycete	OK430925
39	*Menispora ciliata*	NR_171740	99.5	*Menispora ciliata*	MH860017	99.12	*Menispora* sp.	OK430926
40	*Microdochium phragmitis*	NR_132916	100	*Microdochium phragmitis*	MN077456	100	*Microdochium* sp.	OK430927
41	*Phialocephala* sp.	NR_119482	90.38	*Phialocephala* sp.	MG066460	97.88	Mollisiaceae A	OK430928
42	*Mollisia scopiformis*	NR_119460	93.22	*Phialocephala* sp.	MK808244	98.72	Mollisiaceae B	OK430929
43	*Mollisia monilioides*	NR_171261	96.22	*Phialocephala* sp.	MT911435	100	Mollisiaceae C	OK430930
44	*Mollisia prismatica*	NR_171258	91.9	*Phialocephala* sp.	MK965789	99.57	Mollisiaceae D	OK430931
45	*Mollisia asteliae*	NR_173037	95.15	*Mollisia* sp.	MH633925	100	Mollisiaceae E	OK430932
46	*Mollisia diesbachiana*	NR_171259	96.77	*Mollisia* sp.	MT179560	100	Mollisiaceae F	OK430933
47	*Mortierella gemmifera*	NR_111559	94.81	Mortierellaceae	LR863033	99.43	*Mortierella* sp.	OK430934
48	*Podila horticola*	NR_111572	99.09	*Mortierella* sp.	DQ388818	99.7	Mortierellaceae	OK430935
49	*Mycena fulgoris*	NR_163300	93.29	*Mycena* sp.	JF519186	98.4	*Mycena* sp. A	OK430936
50	*Mycena fulgoris*	NR_163300	93.29	*Mycena* sp.	MK961197	99.67	*Mycena* sp. B	OK430937

51	*Mycena fulgoris*	NR_163300	93.31	*Mycena arcangeliana*	JF908402	99.35	*Mycena* sp. C	OK430938
52	*Mycena fulgoris*	NR_163300	87.99	*Mycena* sp.	UDB020406	100	*Mycena* sp. D	OK430939
53	*Mycena fulgoris*	NR_163300	89.64	*Mycena* sp.	HQ625481	99.32	*Mycena* sp. E	OK430940
54	*Cercospora coniogrammes*	NR_147260	97.89	*Cercospora* sp.	MN970528	97.89	Mycosphaerellaceae	OK430941
55	*Myrmecridium spartii*	NR_155376	96.25	*Myrmecridium* sp.	MW133876	98.32	*Myrmecridium* sp.	OK430942
56	*Pseudomassariella vexata*	NR_164217	87.78	*Fusidium* sp.	HG936132	100	Nectriaceae	OK430943
57	*Neoascochyta europaea*	NR_136131	97.03	*Neoascochyta europaea*	MK190674	97.17	*Neoascochyta* sp. A	OK430944
58	*Neoascochyta soli*	NR_158269	100	*Neoascochyta paspali*	MT373264	100	*Neoascochyta* sp. B	OK430945
59	*Ophiosphaerella aquatica*	NR_154352	89.96	*Ophiosphaerella* sp.	MH063799	98.38	*Ophiosphaerella* sp.	OK430946
60	*Paracamarosporium fagi*	NR_154318	99.18	*Paracamarosporium fagi*	MN244221	99.18	*Paracamarosporium* sp.	OK430947
61	*Parasola parvula*	NR_160509	94.43	*Parasola schroeteri*	UDB024639	99.67	*Parasola* sp.	OK430948
62	*Periconia epilithographicola*	NR_157477	94.55	*Periconia* sp.	MG543950	100	*Periconia* sp.	OK430949
63	*Pezicula rhizophila*	NR_155659	100	*Pezicula* sp.	MN385513	100	*Pezicula* sp.	OK430950
64	*Parastagonospora poagena*	NR_168147	97.94	*Parastagonospora nodorum*	MN313349	99.17	Phaeosphaeriaceae	OK430951
65	*Phragmocephala garethjonessi*	NR_147636	92.21	*Phragmocephala garethjonessi*	MN660752	92.21	*Phragmocephala* sp. A	OK430952
66	*Phragmocephala garethjonessi*	NR_147636	90.2	*Phragmocephala atra*	MN660752	90.61	*Phragmocephala* sp. B	OK430953
67	*Phyllachora* sp.	NR_156611	85	*Phyllachora graminis*	AF257111	96.68	Phyllachorales	OK430954
68	*Pleotrichocladium opacum*	NR_155696	94.21	Pleosporales	KY228531	99.58	Pleosporales A	OK430955
69	*Camposporium multiseptatum*	NR_171863	100	*Camposporium* sp.	MN758889	100	Pleosporales B	OK430956
70	*Anteaglonium rubescens*	NR_164489	89.92	*Lophiostoma* sp.	EU977287	93.17	Pleosporales C	OK430957
71	*Pseudolachnella fusiformis*	NR_154280	94.24	*Pseudolachnella fusiformis*	AB934080	94.24	*Pseudolachnella* sp. A	OK430958
72	*Pseudolachnella fusiformis*	NR_154280	93.78	*Pseudolachnella fusiformis*	AB934080	93.77	*Pseudolachnella* sp. B	OK430959
73	*Puccinia aizazii*	NR_158929	99.2	*Puccinia brachypodii*	GQ457303	100	*Puccinia* sp.	OK430960
74	*Plectosphaerella niemeijerarum*	NR_156677	88.24	Plectosphaerellaceae	MK762215	88.23	Sordariomycete A	OK430961
75	*Phaeoacrenonium cinereum*	NR_ 132066	80.62	Sordaryomycete	KP050604	80.62	Sordariomycete B	OK430962
76	*Cordana pauciseptata*	NR_154771	88.98	Sordariales	UDB067041	96.69	Sordariomycete C	OK430963
77	*Neomyrmecridium guizhouense*	NR_170024	82.45	Sordariomycete	LR865231	100	Sordariomycete D	OK430964
78	*Atractospora verruculosa*	NR_153542	89.53	Sordariales	EU754966	100	Sordariomycete E	OK430965
79	*Subulicistidium oberwinkleri*	NR_159060	86.42	Trechisporales	JF519283	100	Trechisporales A	OK430966

80	*Subulicystidium oberwinkleri*	NR_159060	80.53	Trechisporales	UDB020436	83.77	Trechisporales B	OK430967
81	*Trichoderma hispanicum*	NR_138451	99.25	*Trichoderma koningii*	MT781958	99.24	*Trichoderma* sp.	OK430968
82	*Corinarius hadrocroceus*	NR_131854	79.62	Tricholomataceae	KX115676	100	Tricholomataceae A	OK430969
83	*Mycena seminau*	NR_154170	88.82	Tricholomataceae	MH016642	99.67	Tricholomataceae B	OK430970
84	*Phialocephala humicola*	NR_103570	87.7	Chaetosphaeriales	HM136627	100	Unidentified A	OK430971
85	*Rhodosporidiobolus fluvialis*	NR_077089	93.65	Agaricomycete	UDB0327559	100	Unidentified B	OK430972
86	*Mycosymbioces mycenaphila*	NR_137807	85.06	Helotiales	UDB0779249	100	Unidentified C	OK430973
87	*Mollisia monilioides*	NR_171261	90.34	Helotiales	KT203037	96.61	Unidentified D	OK430974
88	*Linteromyces quintiniae*	NR_171989	86.25	Xylariales	MN218782	99.62	Xylariales	OK430975

Appendix B. Complete table with all identified taxa in underground tissues of the *B. rupestre* by metabarcoding method. The relative abundance based on number of reads, number of OTUs and infected plants (out of five). Shaded taxa were sequenced in both underground tissues.

Endophyte taxon	ROOT								RHIZOME							
	Relative abundance (%)		Reads		OTUs		Infected plants		Relative abundance (%)		Reads		OTUs		Infected plants	
	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD
Helotiaceae	24.51	31.09	64132	51968	114	116	2	4	25.95	16.49	12267	4897	94	18	2	4
***Lachnum* sp. A**	35.08	12.5	91790	20889	36	20	5	5	29.13	9.01	13771	2676	5	7	4	4
***Albotricha* sp. A**	1.71	18.83	4465	31473	7	6	3	3	5.23	50.58	2473	15018	4	6	3	3
Helotiales A	12.43	6.03	32534	10072	40	28	5	5	10.98	2.77	5188	823	25	1	2	3
Agaricales A	3.55	9.65	9281	16124	3	3	2	4	0.05	0	24	0	2	0	1	0
***Mycena* sp. A**	2.03	0.17	5323	289	10	2	4	1	20.88	0	9870	0	1	0	1	0
Mollisiaceae C	4.17	0.45	10913	745	2	1	4	1	0.42	0.04	198	13	2	1	1	1
Pleosporales A	0.56	4.04	1476	6751	2	4	3	5	0	0.04	0	12	0	1	0	1
***Glarea* sp.**	0.41	3.94	1060	6589	2	1	2	1	0	0.29	0	86	0	1	0	1
Mollisiaceae B	0.43	1.89	1118	3161	1	3	2	3	2.35	1.44	1111	429	1	3	2	2
Mollisiaceae D	0.89	1.07	2330	1782	1	2	2	1	0.78	3.56	369	1056	1	2	2	2
Chaetosphaeriaceae	1.76	0	4608	0	4	0	1	0	0.07	0	33	0	1	0	1	0
***Mycena* sp. B**	0	2.08	0	3479	0	3	0	1	0	3.34	0	993	0	1	0	1
Tricholomataceae B	0	1.48	0	2474	0	1	0	2	0	4.21	0	1251	0	1	0	1
***Lachnum* sp. B**	0.38	1.27	1007	2119	11	7	4	4	0.87	0.14	411	43	1	1	1	1
Cantharellales	1.3	0	3397	0	2	0	1	0								
***Parasola* sp.**	0	0.9	0	1503	0	1	0	1	0	5.81	0	1725	0	3	0	1
Unidentified A	1.21	0.01	3174	19	2	1	1	1								
***Ophiosphaerella* sp.**	0.96	0.32	2513	535	2	1	2	1	0.11	0.06	50	17	1	1	1	1
Mollisiaceae A	0.88	0.4	2309	666	4	3	4	5	0.03	0.28	13	83	1	1	1	1
***Drechslera* sp.**	0.03	1.43	87	2388	2	2	2	5								
***Paracamarosporium* sp.**	0.92	0	2419	0	1	0	4	0	0.06	0	28	0	1	0	2	0
Agaricales C	0.58	0.07	1514	114	1	1	2	1								

Auriculariales	0.5	0	1308	0	2	0	1	0								
Tricholomataceae A	0.48	0	1266	0	1	0	1	0								
Unidentified B	0.13	0.43	340	718	1	1	3	2	0.19	0.29	90	86	1	1	1	1
***Pseudolachnella* sp. B**	0.42	0.04	1097	72	1	1	3	2	0.11	0	51	0	1	0	1	0
***Trichoderma* sp.**	0.41	0.01	1076	15	1	1	2	1								
Didymosphaeriaceae	0	0.58	0	963	0	2	0	1								
***Conlarium* sp.**	0.35	0	925	0	1	0	3	0	0.07	0	35	0	1	0	1	0
Helotiales C	0.34	0	900	0	1	0	1	0								
***Phragmocephala* sp. A**	0.28	0	734	0	2	0	4	0								
Agaricales B	0.26	0.02	675	32	2	1	1	1								
***Menispora* sp.**	0.27	0	702	0	1	0	2	0								
***Cladophialophora* sp.**	0.23	0.04	604	74	1	1	3	3	0.04	0	18	0	1	0	1	0
Pleosporales B	0.11	0.2	299	341	1	2	1	2								
***Pseudolachnella* sp. A**	0.23	0.01	606	11	2	1	4	1								
Mortierellaceae	0.11	0	283	0	2	0	1	0	0.43	0	201	0	3	0	1	0
Mollisiaceae F									1.01	0	476	0	1	0	1	0
***Chalara* sp.**	0.06	0.03	168	57	2	2	3	2	0.4	0.09	187	26	1	1	1	1
Sordariomycete D	0.17	0	435	0	1	0	1	0								
Helotiales B	0.03	0	87	0	1	0	1	0	0.21	0.73	99	218	1	1	1	1
Agaricomycete	0.12	0	325	0	1	0	1	0								
Ascomycota B	0.1	0	266	0	1	0	1	0	0.09	0	41	0	1	0	1	0
Mollisiaceae E	0	0.13	0	216	0	1	0	1	0	0.31	0	91	0	1	0	1
***Microdochium* sp.**	0.07	0	187	0	1	0	3	0	0.25	0	119	0	1	0	1	0
***Mortierella* sp.**	0.12	0	305	0	2	0	1	0								
Sordariomycete B	0.11	0	297	0	1	0	1	0								
***Pezicula* sp.**	0	0.16	0	272	0	1	0	2								
***Coccomyces* sp.**	0.09	0.02	227	31	1	1	2	1								
***Albotricha* sp. B**	0	0.13	0	217	0	2	0	1	0	0.06	0	19	0	1	0	1
***Leohumicola* sp.**	0.06	0.03	166	57	1	1	4	2	0.03	0	13	0	1	0	1	0
***Lachnum* sp. C**	0.09	0	228	0	1	0	1	0								

Pleosporales C	0	0.13	0	213	0	1	0	3								
Nectriaceae	0.08	0	212	0	1	0	1	0								
***Phragmocephala* sp. B**	0.06	0.03	147	45	1	1	1	2								
Sordariomycete C	0	0.11	0	188	0	1	0	2								
Ascomycota A	0.06	0	168	0	1	0	3	0								
Sordariomycete E	0.06	0	167	0	1	0	1	0								
Trechisporales B	0	0.09	12	151	1	1	1	1								
Trechisporales A	0.03	0	76	0	1	0	2	0	0	0.28	0	82	0	1	0	1
Agaricales D	0.06	0	153	0	1	0	1	0								
***Myrmecridium* sp.**	0.06	0	149	0	1	0	2	0								
Dothideomycete A	0.04	0	117	0	1	0	1	0	0.06	0	30	0	1	0	1	0
***Entoloma* sp.**	0.06	0	144	0	1	0	1	0								
***Falciphora* sp.**	0.05	0	141	0	1	0	1	0								
Leotiomycete	0.05	0	139	0	1	0	1	0								
***Mycena* sp. D**	0.05	0.01	120	14	1	1	1	1								
***Mycena* sp. E**	0	0.08	0	133	0	1	0	1								
Unidentified D	0.05	0	132	0	1	0	1	0								
Agaricales E	0.05	0	128	0	1	0	1	0								
Dothideomycete B	0	0.08	0	127	0	1	0	1								
Unidentified C	0.05	0	124	0	1	0	1	0								
***Albotricha* sp. C**	0.05	0	123	0	1	0	1	0								
Helotiales D	0.05	0	121	0	1	0	1	0								
***Neoascochyta* sp. B**	0.01	0	17	0	1	0	1	0	0.22	0	102	0	1	0	3	0
***Mycena* sp. C**	0.03	0	86	0	1	0	1	0	0	0.07	0	22	0	1	0	1
Hyaloscyphaceae	0.03	0.02	73	27	1	1	2	1								
***Periconia* sp.**	0.02	0	51	0	1	0	2	0								
***Epicoccum* sp.**	0	0	12	0	1	0	1	0	0	0.09	0	26	0	1	0	1
Phaeosphaeriaceae	0	0.02	0	28	0	1	0	1								
Xylariales sp.	0.04	0.01	105	12	1	1	2	1								
	100	100	261673	167154					100	100	47268	29692				

3978

References

Arnold, A.E., Henk, D.A., Eells, R.L., Lutzoni, F., Vilgalys, R., 2007. Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. Mycologia 99, 185–206. doi:10.1080/15572536.2007.11832578

Arnold, A.E., Lutzoni, F., 2007. Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? Ecology 88, 541-549. doi:10.1890/05-1459

Bąba, W., Kalaji, H.M., Kompała-Bąba, A., Goltsev, V., 2016. Acclimatization of photosynthetic apparatus of tor grass (*Brachypodium pinnatum*) during expansion. Plos One 11, 1-27. doi:10.1371/journal.pone.0156201

Bąba, W., Kurowska, M., Kompała-Bąba, A., Wilczek, A., Długosz, J., Szarejko, I., 2012. Genetic diversity of the expansive grass *Brachypodium pinnatum* in a changing landscape: Effect of habitat age. Flora 207, 346-353. doi:10.1016/j.flora.2012.01.011

Bills, G.F., Platas, G., Peláez, F., Masurekar, P., 1999. Reclassification of a pneumocandin-producing anamorph, *Glarea lozoyensis* gen. et sp. nov., previously identified as *Zalerion arboricola*. Mycological Research 103, 179-192. doi:10.1017/S095375629800687X

Binet, M.N., Sage, L., Malan, C., Ement, J.C.C.L., Redecker, D., Wipf, D., Geremia, R.A., Lavorel, S., Mouhamadou, B., 2013. Effects of mowing on fungal endophytes and arbuscular mycorrhizal fungi in subalpine grasslands. Fungal Ecology 6, 248-255. doi:10.1016/j.funeco.2013.04.001

Bobbink, R., Willems, J.H., 1987. Increasing dominance of *Brachypodium pinnatum* (L.) Beauv. in chalk grasslands: A threat to a species-rich ecosystem. Biological Conservation 40, 301-314. doi:10.1016/0006-3207(87)90122-4

Bricca, A., Tardella, F.M., Tolu, F., Goia, I., Ferrara, A., Catorci, A., 2020. Disentangling the effects of disturbances from those of dominant tall grass features in driving the functional variation of restored grassland in a sub-mediterranean context. Diversity 12, 1-12. doi:10.3390/d12010011

Buckland, S.M., Thompson, K., Hodgson, J.G., Grime, J.P., 2001. Grassland invasions: effects of manipulations of climate and management. Journal of Applied Ecology 38, 301-309. doi:10.1046/j.1365-2664.2001.00603.x

Bud, R., 1991. Biotechnology in the Twentieth Century. Social Studies of Science 21, 415-457. doi:10.1177/030631291021003002

Canals, R.M., Pedro, J., Rupérez, E., San-Emeterio, L., 2014. Nutrient pulses after prescribed winter fires and preferential patterns of N uptake may contribute to the expansion of *Brachypodium pinnatum* (L.) P. Beauv. in highland grasslands. Applied Vegetation Science 17, 419-428. doi:10.1111/avsc.12088

Canals, R.M., San Emeterio, L., Durán, M., Múgica, L., 2017. Plant-herbivory feedbacks and selective allocation of a toxic metal are behind the stability of degraded covers dominated by *Brachypodium pinnatum* in acidic soils. Plant and Soil 415, 373-386. doi:10.1007/s11104-016-3153-1

Caporaso, J.G., 2011. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7, 335-336. doi:10.1038/nmeth.f.303.QIIME

Carthey, A.J.R., Blumstein, D.T., Tetu, S.G., Gillings, M.R., Gallagher, R.V, 2019. Conserving the holobiont. Functional Ecology 34, 764-776. doi:10.1111/1365-2435.13504

Catorci, A., Cesaretti, S., Gatti, R., Ottaviani, G., 2011. Abiotic and biotic changes due to spread of *Brachypodium genuense* (DC.) Roem. & Schult. in sub-Mediterranean meadows. Community Ecology 12, 117-125. doi:10.1556/ComEc.12.2011.1.14

Catorci, A., Cesaretti, S., Tardella, F.M., 2014. Effect of tall-grass invasion on the flowering-related functional pattern of submediterranean hay-meadows. Plant Biosystems 148, 1127-1137. doi:10.1080/11263504.2014.980353

Chi, W.C., Chen, W., He, C.C., Guo, S.Y., Cha, H.J., Tsang, L.M., Ho, T.W., Pang, K.L., 2019. A highly diverse fungal community associated with leaves of the mangrove plant *Acanthus ilicifolius* var. *xiamenensis* revealed by isolation and metabarcoding analyses. PeerJ. doi:10.7717/peerj.7293

Compant, S., Saikkonen, K., Mitter, B., Campisano, A., Mercado-Blanco, J., 2016. Editorial special issue: soil, plants and endophytes. Plant and Soil 405, 1-11. doi:10.1007/s11104-016-2927-9

Dufrêne, M., Legendre, P., 1997. Species assemblages and indicator species: The need for a flexible asymmetrical approach. Ecological Monographs 67, 345-366. doi:10.2307/2963459

Durán, M., Canals, R.M., Sáez, J.L., Ferrer, V., Lera-López, F., 2020. Disruption of traditional land use regimes causes an economic loss of provisioning services in high-mountain grasslands. Ecosystem Services 46.101200. doi:10.1016/j.ecoser.2020.101200

Durán, M., San Emeterio, L., Múgica, L., Zabalgogeazcoa, I., Vázquez-de-Aldana, B.R., Canals, R.M., 2021. Disruption of traditional grazing and fire regimes shape the fungal endophyte assemblages of the tall-grass *Brachypodrium rupestre*. Frontiers in Microbiology 12:679729. doi:10.3389/fmicb.2021.679729

Ebach, M.C., Valdecasas, A.G., Wheeler, Q.D., 2011. Impediments to taxonomy and users of taxonomy: accessibility and impact evaluation. Cladistics 27, 550-557. doi:10.1111/j.1096-0031.2011.00348.x

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194-2200. doi:10.1093/bioinformatics/btr381

Frank, A.C., Paola, J., Guzm, S., Shay, J.E., 2017. Transmission of bacterial endophytes. Microorganisms 5, 1-21. doi:10.3390/microorganisms5040070

Guerrero, R., Margulis, L., Berlanga, M., 2013. Symbiogenesis: The holobiont as a unit of evolution. International Microbiology 16, 133-143. doi:10.2436/20.1501.01.188

Hardoim, P.R., van Overbeek, L.S., Berg, G., Pirttilä, A.M., Compant, S., Campisano, A., Döring, M., Sessitsch, A., 2015. The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiology and Molecular Biology Reviews 79, 293-320. doi:10.1128/mmbr.00050-14

Harrison, J.G., Griffin, E.A., 2020. The diversity and distribution of endophytes across biomes, plant phylogeny and host tissues: how far have we come and where do we go from here? Environmental Microbiology 00. doi:10.1111/1462-2920.14968

Hernández-Restrepo, M., Bezerra, J.D.P., Tan, Y.P., Wiederhold, N., Crous, P.W., Guarro, J., Gené, J., 2019. Re-evaluation of *Mycoleptodiscus* species and morphologically similar fungi. Persoonia: Molecular Phylogeny and Evolution of Fungi 42, 205-227. doi:10.3767/persoonia.2019.42.08

Hernández-Restrepo, M., Groenewald, J.Z., Elliott, M.L., Canning, G., McMillan, V.E., Crous, P.W., 2016. Take-all or nothing. Studies in Mycology 83, 19-48. doi:10.1016/j.simyco.2016.06.002

Herrera, J., Khidir, H.H., Eudy, D.M., Porras-Alfaro, A., Natvig, D.O., Sinsabaugh, R.L., 2010. Shifting fungal endophyte communities colonize *Bouteloua gracilis*: Effect of host tissue and geographical distribution. Mycologia 102, 1012-1026. doi:10.3852/09-264

Høyer, A.K., Hodkinson, T.R., 2021. Hidden fungi: Combining culture-dependent and -independent DNA barcoding reveals inter-plant variation in species richness of endophytic root fungi in *Elymus repens*. Journal of Fungi 7, 466. doi:10.3390/jof7060466

Huang, Y., Niu, B., Gao, Y., Fu, L., Li, W., 2010. CD-HIT Suite: A web server for clustering and comparing biological sequences. Bioinformatics 26, 680-682. doi:10.1093/bioinformatics/btq003

Hume, D.E., Sewell, J.C., 2014. Agronomic advantages conferred by endophyte infection of perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinacea* Schreb.) in Australia. Crop and Pasture Science 65, 747-757. doi:10.1071/CP13383

Hyde, K.D., Soytong, K., 2008. The fungal endophyte dilemma. Fungal Diversity 33, 133-173. doi:10.1016/S0006-3207(98)00089-5

Leuchtmann, A., Schardl, C.L., 1998. Mating compatibility and phylogenetic relationships among two new species of *Epichloë* and other congeneric European species. Mycological Research 102, 1169-1182. doi:10.1017/S0953756298006236

Li, W., 2009. Analysis and comparison of very large metagenomes with fast clustering and functional annotation. BMC Bioinformatics 10, 1-9. doi:10.1186/1471-2105-10-359

Li, W., Godzik, A., 2006. Cd-hit: A fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 22, 1658-1659. doi:10.1093/bioinformatics/btl158

Magoč, T., Salzberg, S.L., 2011. FLASH: Fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27, 2957-2963. doi:10.1093/bioinformatics/btr507

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.Journal 17, 10-12. doi:10.14806/ej.17.1.200

Mbareche, H., Veillette, M., Bilodeau, G., Duchaine, C., 2020. Comparison of the performance of ITS1 and ITS2 as barcodes in amplicon-based sequencing of bioaerosols. PeerJ 2020, 1-36. doi:10.7717/peerj.8523

Meijer, G., Leuchtmann, A., 1999. Multistrain infections of the grass *Brachypodium sylvaticum* by its fungal endophyte *Epichloe sylvatica*. New Phytologist 141, 355-368. doi:10.1046/j.1469-8137.1999.00332.x

Miwa, E., Okane, I., Ishiga, Y., Sugawara, K., Yamaoka, Y., 2017. Confirmation of taxonomic status of an *Epichloë* species on *Brachypodium sylvaticum* in Japan. Mycoscience 58, 147-153. doi:10.1016/j.myc.2016.12.001

Múgica, L., Canals, R.M., San Emeterio, L., Peralta, J., 2021. Decoupling of traditional burnings and grazing regimes alters plant diversity and dominant species competition in high-mountain grasslands. Science of the Total Environment 790, 147917. doi:10.1016/j.scitotenv.2021.147917

Nilsson, R.H., Anslan, S., Bahram, M., Wurzbacher, C., Baldrian, P., Tedersoo, L., 2019. Mycobiome diversity: high-throughput sequencing and identification of fungi. Nature Reviews Microbiology 17, 95-109. doi:10.1038/s41579-018-0116-y

Nilsson, R.H., Larsson, K.H., Taylor, A.F.S., Bengtsson-Palme, J., Jeppesen, T.S., Schigel, D., Kennedy, P., Picard, K., Glöckner, F.O., Tedersoo, L., Saar, I., Kõljalg, U., Abarenkov, K., 2019. The UNITE database for molecular identification of fungi: Handling dark taxa and parallel taxonomic classifications. Nucleic Acids Research 47, D259-D264. doi:10.1093/nar/gky1022

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al., 2017. Vegan:

Community ecology package. R Package Version 2.4-3. Available online at: https://CRAN.R-project.org/package=vegan (accessed April 10, 2020).

Roberts, D.W., 2019. labdsv: Ordination and multivariate analysis for ecology. R Package Version 1.8-0.

Rodriguez, R.J., White, J.F., Arnold, A.E., Redman, R.S., 2009. Fungal endophytes: Diversity and functional roles. New Phytologist 182, 314-330. doi:10.1111/j.1469-8137.2009.02773.x

Rudgers, J.A., Kivlin, S.N., Whitney, K.D., Price, M. V., Waser, N.M., Harte, J., 2014. Responses of high-altitude graminoids and soil fungi to 20 years of experimental warming. Ecology 95, 1918-1928. doi:10.1890/13-1454.1

Saikkonen, K., Faeth, S.H., Helander, M., Sullivan, T.J., 1998. Fungal endophytes: A continuum of interactions with host plants. Annual Review of Ecology and Systematics 29, 319-43. doi:10.1146/annurev.ecolsys.29.1.319

Saikkonen, K., Lehtonen, P., Helander, M., Koricheva, J., Faeth, S.H., 2006. Model systems in ecology: dissecting the endophyte-grass literature. Trends in Plant Science 11, 428-433. doi:10.1016/j.tplants.2006.07.001

San Emeterio, L., Durán, M., Múgica, L., Jiménez, J.J., Canals, R.M., 2021. Relating the spatial distribution of a tall-grass to fertility islands in a temperate mountain grassland. Soil Biology and Biochemistry 163:108455. doi:10.1016/j.soilbio.2021.108455

Sánchez-Márquez, S., Bills, G.F., Acuña, L.D., Zabalgogeazcoa, I., 2010. Endophytic mycobiota of leaves and roots of the grass *Holcus lanatus*. Fungal Diversity 41, 115-123. doi:10.1007/s13225-009-0015-7

Sánchez-Márquez, S., Bills, G.F., Zabalgogeazcoa, I., 2007. The endophytic mycobiota of the grass *Dactylis glomerata*. Fungal Diversity 27 I, 171-195.

Schoch, C.L., Robbertse, B., Robert, V., Vu, D., Cardinali, G., Irinyi, L., Meyer, W., Nilsson, R.H., Hughes, K., Miller, A.N., et al., 2014. Finding needles in haystacks: Linking scientific names, reference specimens and molecular data for Fungi. Database 2014. doi:10.1093/database/bau061

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America 109, 6241-6246. doi:10.1073/pnas.1117018109

Sun, X., Guo, L.D., 2012. Endophytic fungal diversity: Review of traditional and molecular techniques. Mycology 3, 65-76. doi:10.1080/21501203.2012.656724

Sun, X., Kosman, E., Sharon, O., Ezrati, S., Sharon, A., 2020. Significant host- and environment-dependent differentiation among highly sporadic fungal endophyte communities in cereal crops-related wild grasses. Environmental Microbiology 22, 3357-3374. doi:10.1111/1462-2920.15107

Tanney, J.B., Douglas, B., Seifert, K.A., 2016. Sexual and asexual states of some endophytic *Phialocephala* species of *Picea*. Mycologia 108, 255-280. doi:10.3852/15-136

Tanney, J.B., Seifert, K.A., 2020. Mollisiaceae: An overlooked lineage of diverse endophytes. Studies in Mycology 95, 293-380. doi:10.1016/j.simyco.2020.02.005

Tardella, F.M., Malatesta, L., Goia, I.G., Catorci, A., 2018. Effects of long-term mowing on coenological composition and recovery routes of a *Brachypodium rupestre*-invaded community: insight into the restoration of sub-Mediterranean productive grasslands. Rendiconti Lincei. Scienze Fisiche e Naturali 29, 329-341. doi:10.1007/s12210-018-0711-x

Tejesvi, M. V., Ruotsalainen, A.L., Markkola, A.M., Pirttilä, A.M., 2010. Root endophytes along a primary succession gradient in northern Finland. Fungal Diversity 41, 125-134. doi:10.1007/s13225-009-0016-6

Tikhonovich, I.A., Provorov, N.A., 2011. Microbiology is the basis of sustainable agriculture: An opinion. Annals of Applied Biology 159, 155-168. doi:10.1111/j.1744-7348.2011.00489.x

Turenne, C.Y., Sanche, S.E., Hoban, D.J., Karlowsky, J.A., Kabani, A.M., 1999. Rapid identification of fungi by using the ITS2 genetic region and automated fluorescent capillary electrophoresis system. Journal of Clinical Microbiology 1846-1851. doi:10.1080/13693780310001600435

United Nations, 1992. Convention on Biological Diversity, In: United Nations.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., Dufresne, A., 2015. The importance of the microbiome of the plant holobiont. New Phytologist 206, 1196-1206. doi:10.1111/nph.13312

Vazquez-de-Aldana, B.R., Zabalgogeazcoa, I., Garcia-Ciudad, A., Garcia-Criado, B., 2003. Ergovaline occurrence in grasses infected by fungal endophytes of semi-arid pastures in Spain. Journal of the Science of Food and Agriculture 83, 347-353. doi:10.1002/jsfa.1319

Verma, V.C., Gange, A.C., 2014. Advances in endophytic research, Advances in Endophytic Research. doi:10.1007/978-81-322-1575-2

Wearn, J.A., Sutton, B.C., Morley, N.J., Gange, A.C., 2012. Species and organ specificity of fungal endophytes in herbaceous grassland plants. Journal of Ecology 100, 1085-1092. doi:10.1111/j.1365-2745.2012.01997.x

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A guide to methods and applications, Ed M.A. Innis (San Diego, CA: Academic Press). pp. 315-322.

Zabalgogeazcoa, I., García-Ciudad, A., Leuchtmann, A., Vázquez-de-Aldana, B.R., García-Criado, B., 2008. Effects of choke disease in the grass *Brachypodium phoenicoides*. Plant Pathology 57, 467-472. doi:10.1111/j.1365-3059.2007.01784.x

Zhang, W., Tian, W., Gao, Z., Wang, G., Zhao, H., 2020. Phylogenetic utility of rRNA ITS2 sequence-structure under functional constraint. International Journal of Molecular Sciences 21, 1-13. doi:10.3390/ijms21176395

upna
Universidad Pública de Navarra
Nafarroako Unibertsitate Publikoa

4181

DISCUSIÓN GENERAL

Universidad Pública de Navarra
Nafarroako Unibertsitate Publikoa

1. El coste económico de la pérdida del valor de aprovisionamiento en pastos degradados

La pérdida de biodiversidad de los pastos de montaña de Aezkoa debido a la expansión de *B. rupestre* ha encontrado, en el primer capítulo de esta memoria, un modo de ser cuantificada económicamente. En un planeta globalizado y movido por intereses económicos, otorgar un valor monetario a los recursos naturales puede ser una forma de valorizarlo y fomentar su conservación (de Groot y col., 2012). Valorizar la pérdida de biodiversidad florística y relacionarlo con la pérdida de abastecimiento de alimento de calidad para los herbívoros domésticos, puede ser un primer paso para fomentar un cambio de hábitos de la gestión del pastoreo y el fuego en la región, además de en el mundo científico.

La expansión de *B. rupestre* en Aezkoa no sólo va ligada a una grave pérdida de diversidad florística sino que su escasa palatabilidad también implica un fuerte impacto en la pérdida del valor forrajero de las cubiertas que invade. El rechazo que los animales tienen hacia su consumo encuentra relación con su rápido y temprano desarrollo primaveral cuando los animales todavía no han sido trasladados a las zonas altas. Su rápido agostamiento disminuye su calidad forrajera por lo que el valor del índice de calidad otorgado a la especie en la bibliografía (Jouglet, 1999) no corresponde con la calidad real que tiene en el momento de ser consumida por los animales.

La posibilidad de comparar zonas que aún mantienen una alta calidad forrajera y diversidad florística frente a otras totalmente degradadas por la dominancia de *B. rupestre*, permitió realizar esta valoración económica en base a inventarios florísticos en ambas cubiertas. El cálculo final arrojó un coste económico de 21.146€ para la temporada de pastoreo del año 2018, lo que supone un coste de 107€/ha. Un valor importante a considerar para una región pequeña y tan económicamente ligada a la ganadería extensiva.

Los problemas que puede acarrear no disponer de pastos de calidad y depender exclusivamente de insumos externos también han sido expuestos en este trabajo: el incremento de la demanda y la competencia por el uso de las zonas bajas de los valles, las fuertes fluctuaciones de precios y volatilidad de los mercados exteriores (de Roest y col., 2018; Dronne, 2018; Schirpke y col., 2019), o el empeoramiento de las condiciones de vida de los animales reflejado *a posteriori* en la calidad de sus productos (Cabiddu y col., 2019), pueden ser algunos de los principales inconvenientes.

Los esfuerzos por recuperar las zonas degradadas, deberían ir encaminados a un pastoreo temprano y dirigido hacia estas áreas concretas, de modo que los animales se viesen forzados a consumirlo. Junto al consumo de biomasa, se sumaría el efecto que el aporte de nutrientes y el pisoteo de los animales ejerce en la vegetación, propiciando el establecimiento de otras nuevas especies acompañantes. Sin embargo, la expansión de *B. rupestre* por zonas con fuertes pendientes, dificulta el movimiento de los animales por ellas, incluso del ganado ovino de la zona, algo que también fue tomado en cuenta en la investigación, determinando un modelo de cabaña ganadera en función de los censos y la proporción de especies ganaderas que aprovechan la zona en la actualidad.

Respecto al uso del fuego que los ganaderos acometen recurrentemente sobre los lastonares de Aezkoa, el objetivo de eliminar la biomasa acumulada y aprovechar su rebrote primaveral, carece de sentido en el actual escenario, donde tras las quemas, los animales no son conducidos hacia estas áreas concretas. El actual número de cabezas de la cabaña ganadera española y francesa dispone de suficiente superficie de pastos de buena calidad, por lo que desplazar a los animales a otras zonas menos apreciadas no es una opción para los ganaderos implicados. Sin embargo, el pago que los ganaderos franceses realizan anualmente a Aezkoa desde hace siglos pone en evidencia el interés que estos muestran por disponer de estos pastos de altura de tan buena calidad (figura 1a). En los censos de animales de ambas partes del Pirineo, principalmente del ganado ovino, se observa el paulatino descenso de animales aezkoanos frente al incremento de la cabaña ganadera francesa (figura 1b,c,d). El interés de los ganaderos franceses por aumentar el número de animales en la zona facera, e incluso en una hipotética zona más extensa, podría ser utilizado como herramienta de mejora ambiental además de económica. El beneficio ambiental y económico que supondría la recuperación de la calidad de los pastos degradados, bajo una gestión sostenible, podría revertir el problema de la pérdida de diversidad florística en muchas zonas del valle viables de ser recuperadas lo que podría reflejarse en un progresivo incremento del número de animales pastantes.

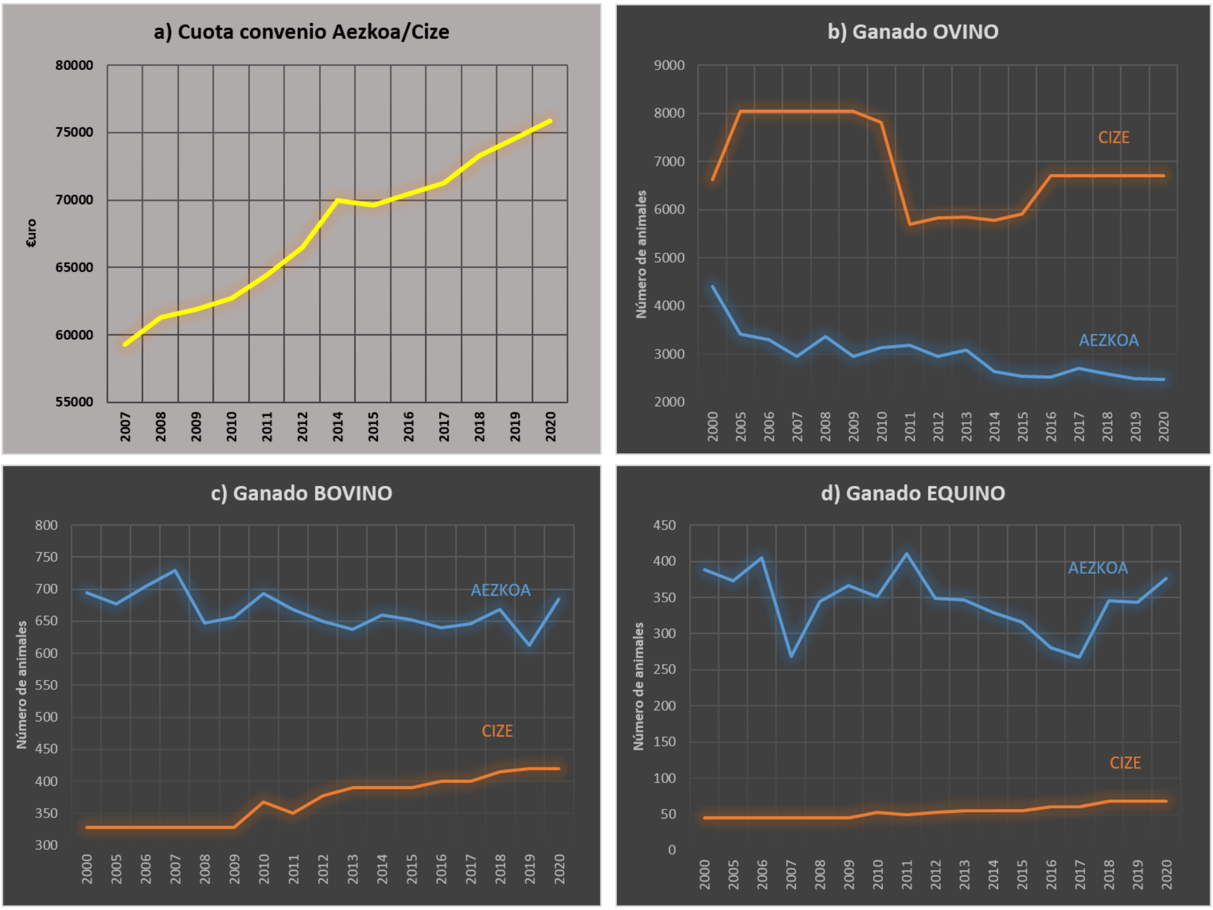


Figura 1. Evolución de la cuota pagada por los ganaderos del valle del Cize al valle de Aezkoa (a). Evolución de los censos de ganado español (Aezkoa) y francés (Cize) en los últimos 20 años (b,c,d).

2. El micobioma de *Brachypodium rupestre*

Ampliar los conocimientos de biodiversidad y apreciar posibles alteraciones en un ecosistema de clima extremo puede ayudarnos a predecir la resiliencia del mismo al cambio global (Vandenkoornhuyse y col., 2015). Estudiar la riqueza de hongos endófitos en *B. rupestre* nos ha permitido, además de conocer el conjunto de especies que forma su hasta ahora desconocido micobioma, determinar los efectos de regímenes de perturbación ligados al uso del fuego recurrente.

El micobioma de *B. rupestre* en los pastos de Aezkoa está compuesto por un pequeño grupo de especies abundantes y un numeroso grupo de especies raras o poco frecuentes. Esta distribución es común en numerosas comunidades ecológicas (Magurran y Henderson, 2003), y ha sido observada en otras gramíneas (Arnold y Lutzoni, 2007; Comby y col., 2016).

Caracterizar por separado el tejido aéreo (vainas) y subterráneo (rizomas y raíces) nos ha permitido conocer lo diferentes que son las comunidades fúngicas que alberga cada uno de ellos. La especie con la mayor incidencia en las vainas de la gramínea resultó ser *Omnidemptus graminis* siempre que se empleó el método de cultivo, sin embargo, la técnica de *metabarcoding* no fue capaz de identificar su presencia, y el taxón más abundante con esta última técnica fue el propuesto como Phaeosphaeriaceae. La caracterización de la comunidad endofítica del tejido aéreo resultó por tanto altamente dependiente del método utilizado, ya que no se obtuvo ningún taxón en común. Esta dependencia no se vio reflejada en la caracterización de los tejidos subterráneos.

Los principales taxones de los tejidos subterráneos de *B. rupestre* pertenecen a las familias Hyaloscyphaceae, Mollisiaceae y Helotiaceae, y los géneros más representativos fueron *Lachnum* sp., *Albotricha* sp., *Mollisia* sp., *Phialocephala* sp. y *Glarea* sp. La agrupación de estos alcanzó el 80% de las lecturas de *metabarcoding* y casi el 60% de los aislados por el método del cultivo tradicional. Todos ellos fueron detectados con las dos técnicas de identificación aplicadas (Figura 2). Otros taxones en común pero menos frecuentes fueron: *Drechslera* sp., Didymosphaeriaceae, *Falciphora* sp., *Microdochium* sp., *Mycena* sp., *Myrmecridium* sp., *Ophiosphaerella* sp., *Parasola* sp., *Periconia* sp., *Pezicula* sp. y *Trichoderma* sp.

Figura 2. Placas con micelio aislado de los taxones más abundantes del micobioma de las raíces de *B. rupestre*.

La diversidad y riqueza de especies del tejido aéreo de *B. rupestre* fue mucho menor que la albergada en los tejidos subterráneos. El contacto directo de estos con el suelo, una gran fuente de inóculos microbianos (Tedersoo y col., 2014), puede estar detrás de estas notables diferencias de diversidad (Harrison y Griffin, 2020). Sin embargo, rizoma y raíz, pese a desarrollarse en el mismo ambiente subterráneo, no albergan comunidades exactamente iguales, lo que puede indicar la influencia de los rasgos funcionales particulares de cada uno de ellos frente al proceso de inoculación de microorganismos desde el suelo a la planta (Compant y col., 2016). También resulta importante remarcar el mayor tiempo de exposición al inóculo fúngico de los tejidos subterráneos (Sánchez-Márquez y col., 2010; Harrison y Griffin, 2020). En la perenne *B. rupestre*, el tejido aéreo muere y se renueva anualmente, sin embargo, los subterráneos sobreviven, ampliando temporalmente las oportunidades de ser infectados.

3. El efecto de las perturbaciones de fuego y pastoreo

La vegetación del valle de Aezkoa cuenta con el intenso efecto de las perturbaciones del fuego y el pastoreo en el desarrollo de la vegetación y por tanto también en su micobioma. Inesperadamente, se detectó que las plantas de *B. rupestre* que se desarrollan en cubiertas con mayor diversidad florística tienden a albergan una menor diversidad endofítica en sus tejidos, es decir, no se observó una correlación positiva entre ambas diversidades con ninguno de los métodos de identificación aplicados. Aunque perceptibles, estas diferencias no fueron significativas para el conjunto de la comunidad. Sin embargo, los análisis de especies indicadoras nos permitieron estudiar las tendencias de distribución de cada uno de los taxones por separado. Saber las razones que promueven las preferencias de cada hongo por un tejido o cubierta particular no fue objeto de estudio, pero sin duda, dos especies despertaron gran interés.

La parte aérea de las cubiertas vegetales de Aezkoa cuentan con el intenso y directo efecto de las perturbaciones del fuego y el pastoreo. Siempre que se aplicó el método tradicional de cultivo, se observó una gran incidencia del hongo *Omnidemptus graminis* en las vainas de *B. rupestre*, y concretamente en el capítulo 2, por la intensificación del muestreo (240 plantas *vs.* 10 plantas), su incidencia fue significativamente mayor en las plantas recolectadas en las zonas degradadas. La mayor y más cercana cantidad y densidad de biomasa disponible en los lastonares puede estar contribuyendo a la dispersión de esta especie, de forma similar a la transmisión de fitopatógenos en monocultivos agrícolas de especies emparentadas (Kankanala y col., 2007). Además, su presencia en el tejido del rizoma, unido a la rápida tasa de crecimiento miceliar observado durante su cultivo en placa petri, podría explicar esta facilidad y rapidez de colonización, sobre todo tras la aplicación de una quema. Por qué esta especie está tan presente en las vainas de *B. rupestre*, si el shock termal tras la quema la favorece (Uys y col., 2004) y si planta/hongo pueden haber desarrollado algún tipo de interacción positiva, o más bien casual, deberá ser objeto de futuros trabajos.

Respecto a los efectos de las perturbaciones en los tejidos subterráneos, sólo la técnica de *metabarcoding* fue capaz de hallar disimilitudes entre la comunidad endofítica de las raíces recolectadas en plantas de cubiertas de alta y baja diversidad florística. Sin embargo, ambas técnicas detectaron la significativa y mayor incidencia del taxón *Lachnum* sp. en raíces procedentes de las cubiertas de baja diversidad (*Lachnum* sp. B en el capítulo 2 y *Lachnum* sp. A en el capítulo 3). La conocida bioactividad de algunas especies de este género (Ondeyka y col., 2009; Xu y col., 2017; Hou y col., 2019) podría suponer una base para futuros trabajos que determinen si su presencia puede estar jugando un rol positivo en el proceso de expansión de la gramínea. Al igual que ocurre con *O. graminis* en las vainas de *B. rupestre*, conocer las razones que motivan este aumento en su incidencia necesitará ser más profundamente estudiado. Otro interesante resultado a analizar sería saber por qué el incremento de *Lachnum* sp. implica la reducción de la incidencia del cercano género *Albotricha* sp., un hecho observado con ambos métodos.

4. La comparativa de técnicas de identificación de hongos endófitos

El método de cultivo, tradicionalmente usado para el estudio de las comunidades fúngicas endófitas, parece haberse quedado relegado frente a las novedosas técnicas de secuenciación masiva. Sin embargo, en este trabajo queda de manifiesto que ambas metodologías se complementan y pueden ser buenas aliadas a la hora de caracterizar este tipo de comunidades endófitas.

En la actualidad, los principales inconvenientes del método de cultivo tradicional radican en el complejo y lento protocolo de laboratorio a seguir ligado a un proceso exhaustivo y minucioso de esterilización del material vegetal para evitar la contaminación de las placas por la proliferación de microorganismos externos (bacterias u otros hongos). Además, se sabe que no todos los hongos son capaces de crecer en medios de cultivo artificiales (hongos no cultivables) y que hongos con diferentes tasas de crecimiento pueden llegar a encriptar a otras especies por su desarrollo más lento (Hyde y Soytong, 2008; Verma y Gange, 2014; Chi y col., 2019).

En el caso del *metabarcoding*, los altos costes de este tipo de técnicas suelen repercutir en el tamaño muestral de los estudios y de las regiones de ADN a secuenciar. Su poder cuantitativo requiere de una compleja metodología bioinformática y el continuo aporte de secuencias genéticas desconocidas, no vinculadas a un taxón o altamente inclusivas conlleva en numerosas situaciones incongruencias genéticas o constantes modificaciones taxonómicas (Ebach y col., 2011). El modo de asignación de los taxones, contrastando las secuencias obtenidas con las almacenadas en las bases de datos (NCBI y UNITE), se siguió de forma muy conservadora con el objetivo de evitar propuestas de taxón menos inclusivas pero inexactas. La región ITS2, utilizada en las secuencias de *metabarcoding*, originó porcentajes de similitud menores que los alcanzados por la región ITS completa del método de cultivo, lo que propició asignaciones de taxones altamente inclusivos (a nivel de órdenes, clases y filos).

El número de taxones identificados por la técnica de *metabarcoding* fue de 88, a partir de 10 plantas, mientras que el método del cultivo identificó 52, a partir de 250 plantas unificando los datos del capítulo 2 y 3 (Figura 3). Este hecho pone de manifiesto la relevancia de adecuar el tamaño muestral al método que se aplique. Las curvas de acumulación de especies indican que ampliar el tamaño muestral incrementa la identificación de endófitos poco frecuentes. Es precisamente en los tejidos subterráneos de *B. rupestre,* donde este conjunto de especies con escasa incidencia presenta las principales diferencias entre metodologías. Sin embargo, como se indicó en el apartado 2, el poco diverso micobioma del tejido de la vaina resultó ser altamente dependiente del método aplicado. La incertidumbre generada por los resultados del tejido aéreo pone de manifiesto la importancia de poder aplicar ambas técnicas en este tipo de caracterización (Chi y col., 2019). Las respuestas a este conflicto deberán ser más profundamente estudiadas, para llegar a saber cómo y por qué *O. graminis* escapó del análisis de secuenciación masiva, pese a ser aislada en las mismas plantas y tener su secuencia tipo ya consolidada en las bases de datos actuales (Hernández-Restrepo y col., 2019).

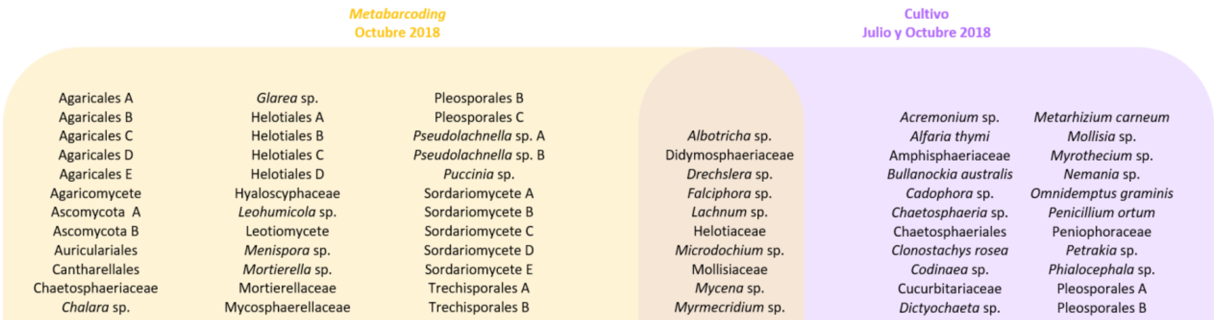


Figura 3. Conjunto de taxones del micobioma de *B. rupestre* identificados a partir del método de cultivo (derecha), de *metabarcoding* (izquierda) y por ambas técnicas (intersección).

BIBLIOGRAFÍA

Arnold, A.E., Lutzoni, F., 2007. Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? Ecology 88, 541-549. doi:10.1890/05-1459

Cabiddu, A., Delgadillo-Puga, C., Decandia, M., Molle, G., 2019. Extensive ruminant production systems and milk quality with emphasis on unsaturated fatty acids, volatile compounds, antioxidant protection degree and phenol content. Animals 9. doi:10.3390/ani9100771

Chi, W.C., Chen, W., He, C.C., Guo, S.Y., Cha, H.J., Tsang, L.M., Ho, T.W., Pang, K.L., 2019. A highly diverse fungal community associated with leaves of the mangrove plant *Acanthus ilicifolius* var. *xiamenensis* revealed by isolation and metabarcoding analyses. PeerJ. doi:10.7717/peerj.7293

Comby, M., Lacoste, S., Baillieul, F., Profizi, C., Dupont, J., 2016. Spatial and temporal variation of cultivable communities of co-occurring endophytes and pathogens in wheat. Frontiers in Microbiology 7. doi:10.3389/fmicb.2016.00403

Compant, S., Saikkonen, K., Mitter, B., Campisano, A., Mercado-Blanco, J., 2016. Editorial special issue: soil, plants and endophytes. Plant and Soil 405, 1-11. doi:10.1007/s11104-016-2927-9

de Groot, R., Brander, L., van der Ploeg, S., Costanza, R., Bernard, F., Braat, L., Christie, M., Crossman, N., Ghermandi, A., Hein, L., et al., 2012. Global estimates of the value of ecosystems and their services in monetary units. Ecosystem Services 1, 50-61. doi:10.1016/j.ecoser.2012.07.005

de Roest, K., Ferrari, P., Knickel, K., 2018. Specialisation and economies of scale or diversification and economies of scope? Assessing different agricultural development pathways. Journal of Rural Studies 59, 222-231. doi:10.1016/j.jrurstud.2017.04.013

Dronne, Y., 2018. Agricultural raw materials for food and feed: the world. Inra Prod. Animal. doi:10.20870/productions-animales.2018.31.3.2345.

Ebach, M.C., Valdecasas, A.G., Wheeler, Q.D., 2011. Impediments to taxonomy and users of taxonomy: accessibility and impact evaluation. Cladistics 27, 550-557. doi:10.1111/j.1096-0031.2011.00348.x

Harrison, J.G., Griffin, E.A., 2020. The diversity and distribution of endophytes across biomes, plant phylogeny and host tissues: how far have we come and where do we go from here? Environmental Microbiology 00. doi:10.1111/1462-2920.14968

Hernández-Restrepo, M., Bezerra, J.D.P., Tan, Y.P., Wiederhold, N., Crous, P.W., Guarro, J., Gené, J., 2019. Re-evaluation of *Mycoleptodiscus* species and morphologically similar fungi. Persoonia: Molecular Phylogeny and Evolution of Fungi 42, 205-227. doi:10.3767/persoonia.2019.42.08

Hou, G., Chen, X., Li, J., Ye, Z., Zong, S., Ye, M., 2019. Physicochemical properties, immunostimulatory activity of the *Lachnum polysaccharide* and polysaccharide-dipeptide conjugates. Carbohydrate Polymers 206, 446-454. doi:10.1016/j.carbpol.2018.09.067

Hyde, K.D., Soytong, K., 2008. The fungal endophyte dilemma. Fungal Diversity 33, 133-173. doi:10.1016/S0006-3207(98)00089-5

Jouglet, J., 1999. Les végétations des alpages des Alpes françaises du sud. Guide technique pour la reconnaissance et la gestion des milieux pásturés d’altitude. Cemagref et Aten, France.

Kankanala, P., Czymmek, K., Valent, B., 2007. Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. The Plant Cell 19, 706-724. doi:10.1105/tpc.106.046300

Magurran, A.E., Henderson, P.A., 2003. Explaining the excess of rare species in natural species abundance distributions. Nature 422, 714-716. doi:10.1038/nature01547

Ondeyka, J., Harris, G., Zink, D., Basilio, A., Vicente, F., Bills, G., Platas, G., Collado, J., González, A., De La Cruz, M., et al., 2009. Isolation, structure elucidation, and biological activity of virgineone from *Lachnum virgineum* using the genome-wide *Candida albicans* fitness test. Journal of Natural Products 72, 136-141. doi:10.1021/np800511r

Sánchez-Márquez, S., Bills, G.F., Acuña, L.D., Zabalgogeazcoa, I., 2010. Endophytic mycobiota of leaves and roots of the grass *Holcus lanatus*. Fungal Diversity 41, 115-123. doi:10.1007/s13225-009-0015-7

Schirpke, U., Tappeiner, U., Tasser, E., 2019. A transnational perspective of global and regional ecosystem service flows from and to mountain regions. Scientific Reports 9, 1-11. doi:10.1038/s41598-019-43229-z

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N.S., Wijesundera, R., Villarreal-Ruiz, L., Vasco-Palacios, A.M., Quang Thu, P., Suija, A., et al., 2014. Global diversity and geography of soil fungi. Science 346. doi:10.1126/science.aaa1185

Uys, R.G., Bond, W.J., Everson, T.M., 2004. The effect of different fire regimes on plant diversity in southern African grasslands. Biological Conservation 118, 489-499. doi:10.1016/j.biocon.2003.09.024

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., Dufresne, A., 2015. The importance of the microbiome of the plant holobiont. New Phytologist 206, 1196-1206. doi:10.1111/nph.13312

Verma, V.C., Gange, A.C., 2014. Advances in endophytic research, Advances in Endophytic Research. doi:10.1007/978-81-322-1575-2

Xu, C., Li, J., Yang, Liuqing, Shi, F., Yang, Liu, Ye, M., 2017. Antibacterial activity and a membrane damage mechanism of *Lachnum* YM30 melanin against *Vibrio parahaemolyticus* and *Staphylococcus aureus*. Food Control 73, 1445-1451. doi:10.1016/j.foodcont.2016.10.048

4454

CONCLUSIONES

CAPÍTULO 1

1. En aquellos pastos naturales en los que la pérdida de diversidad florística está asociada a la merma de palatablidad de la comunidad vegetal y de su valor energético, es posible evaluar la pérdida del servicio ecosistémico de abastecimiento mediante el método de sustitución económica, realizando inventarios florísticos que permitan estimar la pérdida de raciones alimentarias que deberían ser sustituidas en establo. Este enfoque permite evaluar económicamente un problema ambiental y concienciar a la población más directamente afectada.

2. Los pastos de puerto de Aezkoa ocupan una superficie de 2147 ha, de las cuales 287,76 ha están degradadas o en proceso de degradación. La superficie potencial de recuperación mediante prácticas de pastoreo dirigido se estima en 200,05 ha, lo que representa el 9,3% de la superficie total de pastos. La no recuperación de estas superficies supone una pérdida económica de entre 10925€ y 33399€ anuales, dependiendo del valor del índice de calidad aplicado a *B. rupestre* (entre 1 y 0) y de los costes de las raciones alimenticias de sustitución. Considerando valores promedio, la pérdida de valor de aprovisionamiento para el ganado se estima en 107€/ha al año.

CAPÍTULO 2 Y 3

3. La previamente desconocida comunidad endofítica de *B. rupestre* está constituida por un escaso número de especies abundantes y un numeroso grupo de especies raras o poco frecuentes. Los taxones con mayor incidencia en los tejidos subterráneos son Mollisiaceae (*Mollisia* sp. y *Phialocephala* sp.), Helotiaceae (*Glarea* sp.) y Hyaloscyphaceae (*Albotricha* sp. y *Lachnum* sp.). En el tejido aéreo es *Omnidemptus graminis* cuando se aplica el método de cultivo, y Phaeosphaeriaceae cuando se utiliza la técnica de *metabarcoding*.

4. Las plantas de *B. rupestre* recolectadas en las comunidades de menor diversidad florística (lastonares), tienden a albergar una mayor riqueza y diversidad de hongos endófitos que las de las cubiertas de mayor diversidad florística. Los dos métodos de identificación (cultivo y *metabarcoding*) muestran esta tendencia, pero sólo es significativa en el caso de los endófitos de la raíz identificados por *metabarcoding*.

5. El análisis de especies indicadoras permitió detectar tendencias de distribución y fidelidad de los taxones según tipo de tejido (vaina, rizoma o raíz) y tipo de cubierta vegetal (de alta o baja diversidad florística). La incidencia de *O. graminis* en las vainas y de *Lachnum* sp. en las raíces es significativamente mayor en las plantas muestreadas en las cubiertas de baja diversidad. Se precisa profundizar en las interacciones que establecen estas especies con el vegetal para determinar si alguna de ellas está aportando ventajas a la gramínea en su proceso de expansión.

6. La comparación del método de cultivo tradicional frente a la novedosa técnica de *metabarcoding* resaltó la importancia de utilizar ambos métodos para este tipo de caracterización endofítica, debido a los inconvenientes que ambas técnicas siguen teniendo en la actualidad. Con el mismo esfuerzo de muestreo, la técnica de *metabarcoding* identificó 5.8 veces más taxones que el método de cultivo, sin embargo, no fue capaz de detectar la especie más aislada del tejido de la vaina, *O. graminis*. Ambas técnicas sí fueron capaces de detectar el efecto de la diversidad endofítica de cada tejido (raíz > rizoma > vaina) y entre el tipo de cubiertas analizadas (baja diversidad > alta diversidad florística).

CHAPTER 1

1. In natural grasslands where the loss of floristic diversity is related to a decrease in the palatability of the plant community and its energy value, it is possible to evaluate the loss of the ecosystem service of supply applying the economic substitution method, using floristic inventories and estimating the loss of food ration that should be substituted in barns. This approach evaluates economically an environmental problem and may be an effective tool to raise awareness among affected population.

2. The high-mountain grasslands of Aezkoa occupy 2147 ha, which 287.76 ha are degraded or in process of degradation. The potential recovery area is estimated at 200.05 ha, 9.3% of the total surface of grasslands. The non-recovery of these areas entails an economic loss between 10925€ and 33399€ per year, depending on the value of the quality index applied to *B. rupestre* (from 1 to 0) and the costs of replacement food rations. Considering average values, the loss of provisioning value is estimated at 107€/ha per year.

CHAPTER 2 AND 3

3. The unknown fungal endophytic community of *B. rupestre* consists of a few core species and many rare species. The taxa with the highest incidence in the underground tissues are Mollisiaceae (*Mollisia* sp. and *Phialocephala* sp.), Helotiaceae (*Glarea* sp.) and Hyaloscyphaceae (*Albotricha* sp. and *Lachnum* sp.). In the aboveground tissue, the most abundant species is *Omnidemptus graminis* by the culturing method, and Phaeosphaeriaceae by the metabarcoding technique.

4. *B. rupestre* plants collected from low-diversity grasslands tend to harbour a higher fungal species richness and diversity than plants collected from high-diversity grasslands. This trend is detected in both methods (culturing and metabarcoding), but is statistically significant for root endophytes identified by the metabarcoding.

5. The indicator species analysis detected the fidelity of each taxon to a specific plant tissue (shoot, rhizome or root) and plant community type (high or low floristic diversity). The incidence of *O. graminis* in shoots and *Lachnum* sp. in roots is higher in plants collected in low-diversity grasslands. These specific fungus/plant interactions need further research, in order to determine whether the fungal species provide advantages to *B. rupestre* in its expansion process.

6. The comparison of culturing and metabarcoding techniques highlighted the importance of applying both methods for fungal endophytic characterisation, due to the current disadvantages of both. With the same sampling effort, the metabarcoding technique identified 5.8 times more taxa than the culturing method, however, metabarcoding was not able to detect the most isolated species from shoots by culturing, *O. graminis*. Both methods were able to capture the effect of endophytic diversity among tissues (root > rhizome > shoot) and grasslands types (low-diversity > high-diversity grasslands).