Host generalists dominate fungal communities associated with alpine knotweed roots: a study of Sebacinales

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ABSTRACT

Bistorta vivipara is a widespread herbaceous perennial plant with a discontinuous pattern of distribution in arctic, alpine, subalpine and boreal habitats across the northern Hemisphere. Studies of the fungi associated with the roots of *B. vivipara* have mainly been conducted in arctic and alpine ecosystems. This study examined the fungal diversity and specificity from root tips of *B. vivipara* in two local mountain ecosystems as well as on a global scale. Sequences were generated by Sanger sequencing of the internal transcribed spacer (ITS) region followed by an analysis of accurately annotated nuclear segments including ITS1-5.8S-ITS2 sequences available from public databases. In total, 181 different UNITE species hypotheses (SHs) were detected to be fungi associated with *B. vivipara*, 73 of which occurred in the Bavarian Alps and nine in the Swabian Alps–with one SH shared among both mountains. In both sites as well as in additional public data, individuals of *B. vivipara* were found to contain phylogenetically diverse fungi, with the Basidiomycota, represented by the Thelephorales and Sebacinales, being the most dominant. A comparative analysis of the diversity of the Sebacinales associated with *B. vivipara* and other co-occurring plant genera showed that the highest number of sebacinoid SHs were associated with *Quercus* and *Pinus*, followed by *Bistorta*. A comparison of *B. vivipara* with plant families such as Ericaceae, Fagaceae, Orchidaceae, and Pinaceae showed a clear trend: Only a few species were specific to *B. vivipara* and a large number of SHs were shared with other co-occurring non-*B. vivipara* plant species. In Sebacinales, the majority of SHs associated with *B. vivipara* belonged to the ectomycorrhiza (ECM)-forming Sebacinaceae, with fewer SHs belonging to the Serendipitaceae encompassing diverse ericoid–orchid–ECM–endophytic associations. The large proportion of non-host-specific fungi able to form a symbiosis with other non-*B. vivipara* plants could suggest that the high fungal diversity in *B. vivipara* comes from an active recruitment of their associates from the co-occurring vegetation. The non-host-specificity suggests that this strategy may offer ecological advantages; specifically, linkages with generalist rather than specialist fungi. Proximity to co-occurring non-*B. vivipara* plants can maximise the fitness of *B. vivipara*, allowing more rapid and easy colonisation of the available habitats.

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INTRODUCTION

Fungi play a critical role in a wide range of ecosystems either as decomposers allowing nutrient recycling, parasites or commensals, or by establishing mutualist interactions with plants. Within the mutualist interactions, a highly diverse and widely distributed group of fungi form symbioses known as ectomycorrhizae (ECM) with the roots of various plant species in boreal, temperate and arctic regions. Investigations exploring and investigating the mycorrhizal status in plants and its occurrence in terrestrial ecosystems have revealed that ECMs are common in some herbaceous plant genera belonging to the Cyperaceae, Rosaceae, and Polygonaceae (*Haselwandter & Read, 1980*; *Massicotte et al., 1998*; *Schadt & Schmidt, 2001*; *Mühlmann & Peintner, 2008a*; *Gao & Yang, 2016*). Out of these, *Bistorta vivipara* (L.) Delarbre (syn.: *Polygonum viviparum*; Polygonaceae) with a disjoint arctic and alpine distribution across Europe and Northern America, and temperate and tropical Asia (*Marr et al., 2013*) has the ability to establish an ECM interaction with a wide range of fungi, enabling it to function as a pioneer plant in stressful habitats (*e.g.*, *Brevik et al., 2010*). Several studies, including those of *Harley & Harley (1987)*, *Massicotte et al. (1998)*, *Cripps & Eddington (2005)*, *Mühlmann, Bacher & Peintner (2008)* and *Kauserud et al. (2012)* have reviewed the earlier literature on morphological studies of these symbiotic interactions.

In the last 20 years, molecular approaches have detected that the belowground diversity of fungi associated with *B. vivipara* is much greater than that observed only by sporocarps. Using Sanger sequencing, *Mühlmann, Bacher & Peintner (2008)* detected a high heterogeneity in the ECM community of an alpine primary successional glacier forefront, and *Brevik et al. (2010)* found that the ECM diversity was higher in stabilised vegetation than in pioneer vegetation, whereas *Thoen et al. (2019)* detected spatial structuring of the root-associated fungi within the root system. On the other hand, studies using next-generation sequencing have found high patchiness in fungal communities along a primary succession gradient (*Blaalid et al., 2012*). Other observations have shown that a turnover in fungal community composition along an alpine ridge-to-snow gradient was not linked with increasing or decreasing EMC species richness (*Yao et al., 2013*), and that there was a decrease in per-plot plant molecular operational taxonomic unit richness with increasing latitude and a non-spatial autocorrelation between sites (*Blaalid et al., 2014*). *Botnen et al. (2019)* found that *B. vivipara* root-associated fungal communities exhibited strong biogeographical structuring, and both compositional variation and fungal species richness correlated with annual temperature and precipitation. Furthermore, the primary succession of *B. vivipara* root-associated fungi was reported to reflect that of the co-occurring vegetation (*Davey et al., 2015*). At a fine scale, the root-associated communities are driven by factors of the neighbouring ECM plants (*Mundra et al., 2015b*) and differences between summer and winter months (*Mundra et al., 2015a*). *Botnen et al.*

(2014) revealed that *B. vivipara* and other widely distributed arctic plant species share several similar root-associated fungi.

Fungi belonging to the phyla Glomeromycota (Eriksen, Bjurek & Dhillio, 2002), Ascomycota (*e.g.*, Pezizales and *Cenococcum geophilum*), and Basidiomycota (Agaricales, Sebacinales and Thelephorales) have been reported to be commonly associated with *B. vivipara* (see *e.g.*, Mühlmann, Bacher & Peintner, 2008; Davey et al., 2015; Mundra et al., 2015a, 2015b). Because members of the Sebacinales are widely distributed and common in a variety of habitats with different functional roles role ranging from ECM and orchid mycorrhiza to endophytic interactions with leafy liverworts and a wide group of other plants, they are an attractive group to explore fungal diversity, composition, and host-plant specificity at different spatial scales (Garnica et al., 2013; Riess et al., 2014; Mundra et al., 2015a; Garnica et al., 2016a; Arraiano-Castilho et al., 2021). We have extensive knowledge of the occurrence of Sebacinales either from fruiting bodies or from fungal sequencing from roots of various plant groups in the Bavarian Alps (Garnica et al., 2013; Riess et al., 2014; Arraiano-Castilho et al., 2021). However, despite these important contributions, there are still significant unanswered questions concerning the fungal communities associated with the roots of *B. vivipara*. Since this plant species has a moderately long lifespan—according to Callaghan & Collins (1981), individuals of *B. vivipara* can reach an age of 27+ years—one important question is if the co-occurring vegetation (especially trees that can live some hundreds of years) may serve as a reservoir of ECM fungi. According to this perspective and based on the active symbiont recruitment from neighbouring plants reported by Thoen et al. (2019), we hypothesise that *B. vivipara* should have the same fungi as the plants present in its surroundings and we therefore would expect a similar associated ECM diversity and composition. To test this hypothesis, we generated new Sanger sequences data for fungi associated with the roots of populations of *B. vivipara* from a site in the Bavarian Alps and also a site in the Swabian Alps following the natural range of distribution of this plant species in Germany (Fig. 1A) and analysed them together with sequences from public databases. We first determined and compared the ECM diversity associated with roots of *B. vivipara* at local, regional, and global scales. We then compared the diversity and composition of root-associated fungi between *B. vivipara* and other plant species in a global dataset of Sebacinales. In addition, we examined how much of the Sebacinales diversity associated with *B. vivipara* is shared with other host plant species in a global dataset encompassing many co-occurring plant families.

MATERIALS AND METHODS

Collection sites

Root-associated fungi were surveyed from *B. vivipara* plants collected in the Bavarian Alps along a short (500 m) altitudinal gradient on the northern and southern slopes of Mt. Iseler (1,876 m) in the region of Bad Hindelang-Oberjoch in Germany (47°29′57″N, 10°25′5′E) (Fig. 1B). The soil layer consists predominantly of intense lithoidal calcareous brown soils (secondary podzols and chromic luvisols) with an average thickness of >60 cm (Bräker, 2000), whereas the deeper soil layers are mainly characterised by basic dolomite, a sedimentary carbonate rock consisting of the mineral calcium magnesium carbonate

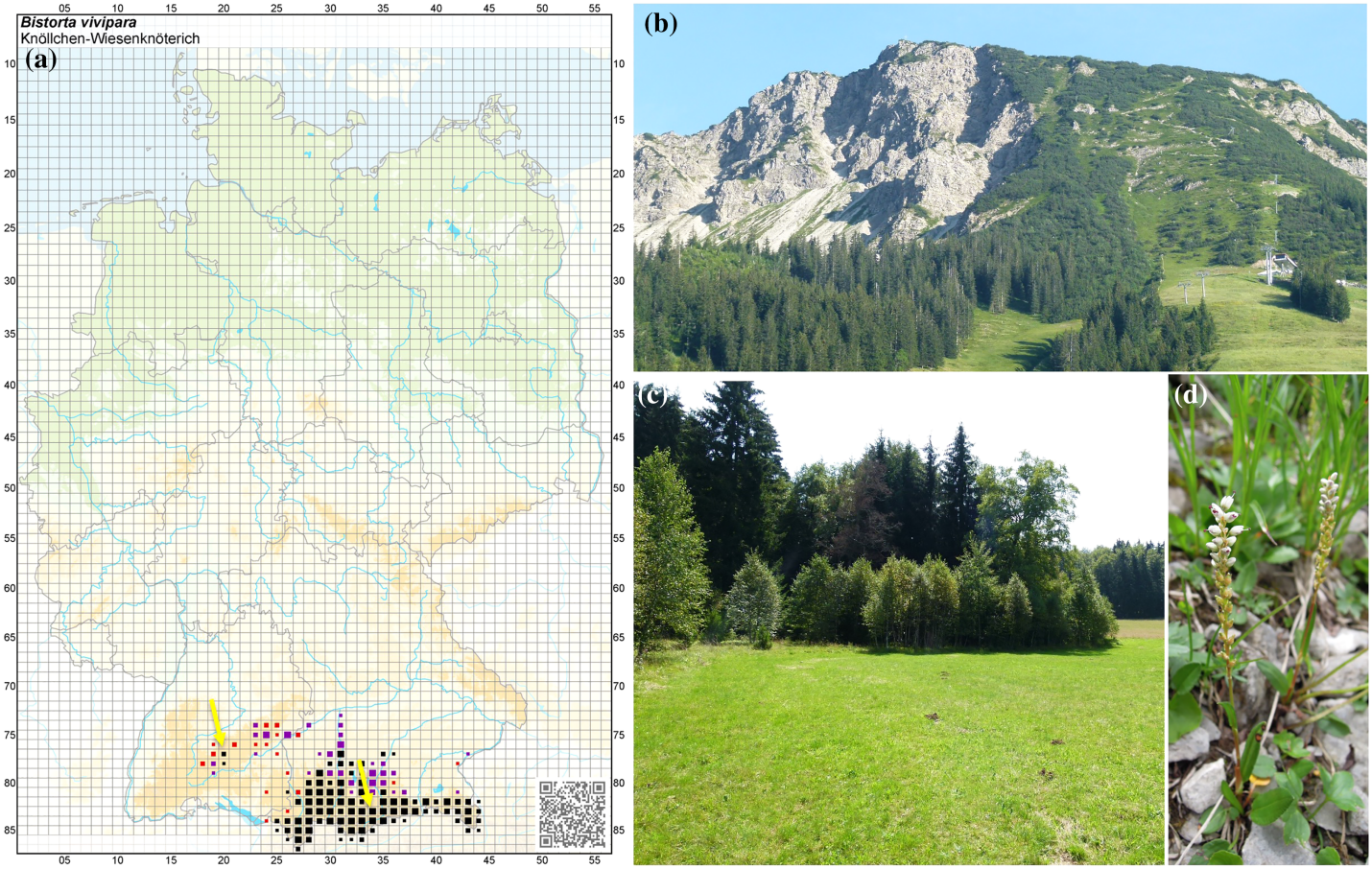

Figure 1 Natural occurrence of *Bistorta vivipara* in southern Germany. (A) Map showing the natural occurrence sites in Germany of the populations of *B. vivipara*. Image credit: Phytodiversität Deutschland & Bundesamt für Naturschutz (Hrsg.) 2013. Verbreitungsatlas der Farn- und Blütenpflanzen Deutschlands, Landwirtschaftsverlag, Münster (ISBN 978-3-7843-5319-7). Records before 1950 (red) and after 1980 (black). (B) Collection site (timberline) in the Bavarian Alps (Mt. Isler) (right arrow). (C) Collection site in the Swabian Alps (left arrow). (D) Individual *B. vivipara* specimen with flowers.
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(*Freudenberger & Schwerd, 1996*). Mt. Iseler's northern and southern slopes are subject to seasonal pasture grazing by cattle. At disturbed sites, *B. vivipara* grows especially well in cattle hoof prints and narrow trails. The total annual rainfall is 1,799 mm (1961–1990), with maximum levels from May to August (228.1 mm/month) and minimum levels from September to October (98.9 mm/month). The annual average air temperature is 5.9 °C at an altitude of 1,136 m, with a maximum of 14.4 °C during July and a minimum of –1.8 °C in January. The average annual snow cover is around 150 days from October to mid-April.

Furthermore, individuals of *B. vivipara* were also collected from the Swabian Alps located in the Irndorfer Hardt Nature Reserve (Baden-Württemberg) near Tuttlingen, Germany (8°94′50.14″N, 48°09′00.28″E) (Fig. 1C). The nature reserve is located between 855 and 880 m above sea level and has a surface area of 103 ha (*Döler & Detzel, 2008*). Although calcareous soils are found on the hills and on the slopes of Irndorfer Hardt, the shallow portion of the valley is covered with deep and decalcified clay soil. The annual average temperature is 6 °C, with extreme temperatures reaching –30 °C in winter

(*Wilmanns, 2005*). In general, the reserve is characterised by having a long period of snow cover in winter. Hoarfrost or frost is likely to occur throughout the year. These low temperatures are the result of a low-pressure trough that hinders the flow of cool air away from the area. The Authority for Unit Environment from the Government of Freiburg (Germany) granted permission to conduct sampling at the Natural Reserve Irndorfer Hardt.

Sample collection and processing of root samples

Samples were collected from Mt. Iseler in the Bavarian Alps at the end of July 2012. At the two lowest collection sites, *B. vivipara* occurred in disturbed meadows (which are used by cattle farmers), whereas at the highest sites it occurred in pristine mountain habitats. Along the altitudinal transect, we randomly sampled 80 individuals of *B. vivipara* at different stages of development (rooted bulbils to fully flowered plants) from 16 patches.

In the Swabian Alps, we were, with permission from the local nature reserve authorities, only allowed to collect 10 individuals of *B. vivipara* (August 2011). The reserve harbours a small relict population under strong protection. Plants were sampled from three sites at a distance of approx. 300 m to 800 m from each other.

Plants with intact root systems and a portion of soil were carefully excavated, placed in plastic bags, and stored in a cooler for transport to the laboratory. In the laboratory, plants were placed in buckets containing tap water for a few hours before roots were rinsed with additional tap water to remove soil particles and foreign roots. Subsequently, *B. vivipara* roots were rinsed with sterile ddH_2O at least five times. From each plant collected in Bavarian Alps, ten ECM root tips were randomly selected and cut from each plant with a sterile razor blade and separated from the others with forceps. As some plants from the Swabian Alps had a small root system, a smaller number of root tips per individual were taken. ECM root tips were then classified primarily on the basis of their morphology, *i.e.*, ramification type, size, colour, presence of emanating hyphae and rhizomorphs (*Agerer, 1987–1997*). To analyse the widest range of ECM diversity, we selected morphotypes covering the complete spectrum of forms and colours. In addition, for those dominant morphotypes were arbitrarily chosen two to five ECM root tips from each morphotype. Individual ECM tips were placed in a sterile 1.5-ml Eppendorf tube, and air dried at 50 °C over night.

DNA extraction, PCR, cloning, DNA sequencing and sequence editing

In total, we processed 800 root tips of *B. vivipara* (5 plant individuals × 16 patches × 10 morphotypes) from the Bavarian Alps and a total of 57 root tips from the Swabian Alps (10 plant individuals × 2 patches, as some plants had a root system that was too small, we were unable to collect 10 root tips). Thus, genomic DNA was extracted from 857 ECM root tips with the innuPREP Plant DNA Kit (Analytik Jena AG, Jena, Germany), according to the manufacturer's instructions. Dried samples were placed in liquid nitrogen and ground to a fine powder with sterile pestles. PCR reactions and thermal profiles were performed as described in *Garnica et al. (2016b)*. Negative controls lacking DNA were used in all PCRs. For the detection of *B. vivipara* mycobionts, we amplified the internal transcribed spacers

(ITS1 and ITS2) and 5.8S rDNA with the primers ITS1F (*Gardes & Bruns, 1993*) and ITS4 (*White et al., 1990*).

PCR products were cleaned with a diluted 1:20 ExoSAP-IT purification kit (USB Corporation, Cleveland, OH, USA), following the manufacturer's instructions. In the case of multiple ITS amplicons or where the PCR products could not be sequenced directly, they were cloned according to *Garnica et al. (2016a)*. DNA amplifications of selected colonies were used directly as templates for PCR with MangoTaq DNA-polymerase (Bioline, London, England), and the M13 forward and reverse primers (Invitrogen, Waltham, MA, USA). From each DNA extraction, eight bacterial colonies were used for PCR reactions. Amplicons containing cloning inserts were diluted 1:20 before purification.

Both DNA strands were cycle-sequenced with the same PCR primer combination used in the PCR amplifications. In cases where the sequence quality was too low, we additionally sequenced the PCR products with the highly universal DNA barcoding primers ITS2 (*White et al., 1990*) and 5.8SR (*Vilgalys & Hester, 1990*) and ITS3Seb for Sebacinales (Berbee, in *Setaro et al., 2006*). Cycle sequencing was carried out with 1 µL of the sequencing primer in combination with 4 µL of a dye terminator sequencing kit (BigDye 3.1; Applied Biosystems, Foster City, CA, USA) diluted 1:6 and 5 µL of the purified DNA template. Cycling products were precipitated with isopropanol (75%), purified with ethanol (80%), and dried in a vacuum centrifuge.

DNA strands were sequenced on an ABI Prism 3130*xl* automated sequencer (Applied Biosystems, Foster City, CA, USA). Forward and reverse sequence chromatograms were assembled and manually edited with Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA). Plant specimens yielding DNA of ECM fungi were deposited in the Herbarium Tubingense.

All sequences forming ECM generated in this study are available in GenBank under accession numbers KF000466–KF000687 and KC986236–KC986286.

Fungal diversity and composition associated with *Bistorta vivipara*

Well-annotated public fungal rDNA ITS sequences associated with *B. vivipara* as a host were downloaded *via* the PlutoF platform (https://plutof.ut.ee) on 05-02-2022.
All sequences available from the International Nucleotide Sequence Databases (http://www.insdc.org/) and the UNITE database were included. These data come from different locations, but are restricted to European sites, mainly from Norway, Germany and Estonia (Table S1). We performed quality control and data cleaning steps as well as the extraction of the full ITS1-5.8S-ITS2 region with ITSx (*Bengtsson-Palme et al., 2013*) for further data analysis. The sequences were then subjected to hierarchical clustering with the 1.0% distance threshold as used by UNITE (*Kõljalg et al., 2013*) and implemented in the species hypothesis (SH) matching analysis tool available on the PlutoF workbench. This dataset also includes all full-length ITS sequences obtained in this study.

Using this dataset, we counted the number of SHs that were observed in either one of the two sites sampled here (Swabian Alps, Bavarian Alps) or in any other location, as well as the overlaps between these three groups. Furthermore, we compared the relative

abundance of fungal orders for these three groups in order to identify common dominant fungal partners of *B. vivipara* over all of its sampled natural range.

Sebacinales diversity associated with *Bistorta vivipara* and other plants

Similarly to the above described workflow, we downloaded well-annotated public rDNA ITS sequences of Sebacinales *via* the PlutoF platform (https://plutof.ut.ee) on 29-09-2020. These included sequences from the studies by *Brevik et al. (2010)* from Norway (Svalbard archipelago), by *Mühlmann & Peintner (2008b)* from Austria (Tyrolean Alps) and *Garnica et al. (2013)* from Germany (Bavarian Alps), and those from other papers, available from the International Nucleotide Sequence Databases (http://www.insdc.org/) and the UNITE database (*Kõljalg et al., 2005*; https://unite.ut.ee/). The PlutoF platform (*Abarenkov et al., 2010*) provides third-party annotation possibilities for DNA sequences available in International Nucleotide Sequence Databases and UNITE (*Nilsson et al., 2019*) and includes metadata on the country of origin and interacting taxa, which are often missing in the original data sources. Quality control, ITS extraction and hierarchical clustering were performed as described above.

We then focused on the Sebacinales fungi associated with *B. vivipara* and other plant hosts. For this, we used the data generated here, our own data from previous studies, and the public data described above. We counted the number of SHs and sequences per genus of plant host that co-occurs with *B. vivipara*. To show any potentially co-occurring plants, we were permissive in this definition, including all plant genera that occur in the northern hemisphere. We further normalised the number of SHs with the number of sequences per plant genus in order to reduce the bias of uneven sampling across plant genera.

For the interactive Tree Of Life (iTOL) ITS tree (*Kozlov et al., 2019*; *Letunic & Bork, 2021*), all Sebacinales sequences were subjected to three-step clustering (90%, 95%, and 97% sequence similarity) *via* vsearch v2.15.0 (*Rognes et al., 2016*). In all clustering steps, for clusters that contained at least one sequence from *B. vivipara*, all sequences were kept, whereas for clusters without any sequence from *B. vivipara*, only one representative per cluster was retained. Because of the high number of originating countries and the great diversity of host taxa, we decided to colour-code the continent of the country of origin and the plant host class, marking sequences from *B. vivipara* with a distinct colour.

Finally, we counted the amount of SHs associated with *B. vivipara* that were shared between two plant host families and visualised these numbers as a circular network, with the width of the connecting edges representing the number of pairwise shared SHs.

RESULTS

Diversity and composition of fungi associated with the roots of *B. vivipara* in the Bavarian Alps, the Swabian Alps, and other European sites

In total, 332 non-chimerical fungal ITS sequences, clustered into 90 different SHs, were detected as associated with *B. vivipara* from the two study sites in the Bavarian Alps and the Swabian Alps (Table S1).

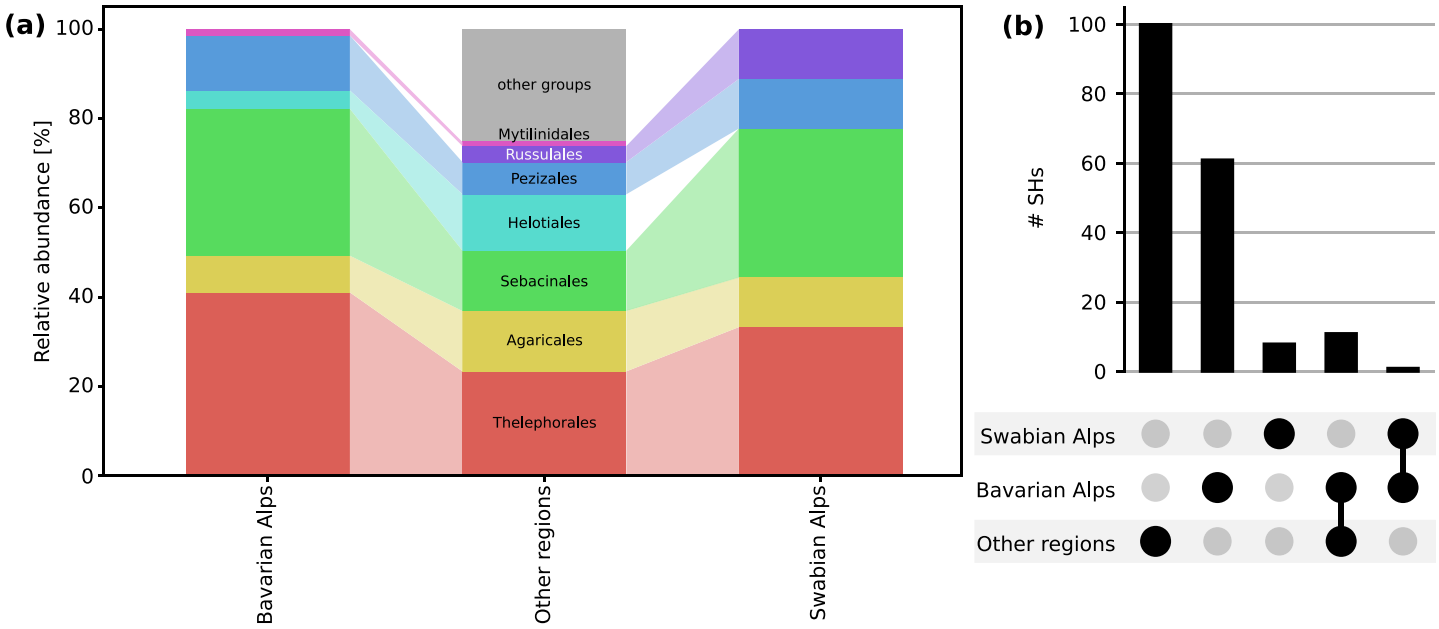

Figure 2 Fungal diversity and composition associated with *Bistorta vivipara*. (A) Relative abundance of fungal orders in the Bavarian Alps, the Swabian Alps and other sites globally. Only the orders that are present at the sites studied here are shown for clarity. (B) Number of fungal species hypotheses (SHs) associated with *B. vivipara* in two mountain sites located in the Bavarian Alps and the Swabian Alps (Germany), as well as in other sites globally (combined). The number of SHs that are shared between these three groups (Bavarian Alps-Other and Bavarian Alps-Swabian Alps) is also shown.
Full-size DOI: 10.7717/peerj.14047/fig-2

From Mt. Iseler in the Bavarian Alps, 264 ITS sequences representing 73 SHs were obtained from *B. vivipara* roots. Members of the Basidiomycota were represented as follows: Thelephorales, 30 SHs (94 sequences); Sebacinales, 24 SHs (114 sequences); Agaricales, six SHs (20 sequences). In the Ascomycota, the Pezizales were represented by nine SHs (30 sequences); Helotiales by three SHs (four sequences), and Mytilinidales (*Cenococcum*) by a single SH (two sequences) (Fig. 2A).

In the Swabian Alps, 68 ITS sequences were obtained from *B. vivipara* roots which clustered into nine SHs. The Basidiomycota were represented as follows: Sebacinales, three SHs (11 sequences); Thelephorales, three SHs (44 sequences); Russulales, one SH (two sequences), and Agaricales, one SH (one sequence). The Ascomycota were represented by Pezizales with one SH (10 sequences) (Fig. 2A).

From other publicly available data sources, covering a wide range of European distribution of *B. vivipara*, we identified 123 fungal ITS sequences distributed over 111 SHs. The most frequently identified fungal orders were, in the Basidiomycota, the Thelephorales (26 SHs, 30 sequences), the Sebacinales (15 SHs, 16 sequences), and the Agaricales (15 SHs, 15 sequences). Ascomycota were represented by Helotiales (14 SHs, 15 sequences) and Pezizales (8 SHs, nine sequences).

In terms of overlaps between sites, most SHs were only detected in a single location, but one SH was shared between the Bavarian Alps and the Swabian Alps (Sebacinales SH 1800251). A total of 11 SHs were shared between the Bavarian Alps and other sites (six

Thelephorales SHs, three Sebacinales SHs, one Helotiales, and one Mytilinidales SH) (Fig. 2B).

Diversity of Sebacinales associated with *Bistorta* and other plants

In total, 5,650 Sebacinales ITS sequences clustered in 1,520 SHs with a cutoff of 99% (Table S2). For 4,500 ITS sequences, a match to 1,350 known SHs from the UNITE database was found, whereas 1,150 ITS sequences had no match to a known SH (170 SHs in total). Of the ITS sequences generated in this study, 36 ITS sequences had no known SHs. The Sebacinales sequences were associated with the roots of 240 different plant genera (Fig. 3 and Table S3). The plant genera with the highest number of associated fungal SHs were *Quercus* (149 SHs with 214 sequences), *Pinus* (102 SHs with 139 sequences), and *Bistorta* (54 SHs, whereof 49 with *B. vivipara* and 5 with *B. officinalis*, with 176 sequences).

A very diverse group of Sebacinales SHs from the Sebacinaceae family with 467 SHs was found to be associated as ECM with *B. vivipara*; a smaller number of SHs came from the Serendipitaceae family involved in cavendishioid, ericoid, orchid, and ECM interactions (52 SHs, see Fig. S1 and Table S4).

Co-occurrence of sebacinoid SHs with *B. vivipara* and other plant hosts

Of the 49 sebacinoid SHs associated with *B. vivipara*, with members of Fagaceae were shared 22 SHs, with Pinaceae 16 SHs, with Rosaceae 15 SHs, with Salicaceae 12 SHs, with Betulaceae 11 SHs, and with Orchidaceae 10 SHs (Fig. 4). One SH (SH1723664) was associated with 14 different plant host families, followed by two SHs (SH1731002 and SH1731004) that were associated with 12 and 11 plant hosts, respectively.

DISCUSSION

Harsh environments promote generalist fungal communities more adapted to these conditions rather than plant-specific specialists (*Kernaghan & Harper, 2001*; *Ryberg, Andreasen & Björk, 2011*; *Botnen et al., 2014*; *Brunner et al., 2017*). Several common fungal lineages in these environments are widely distributed and can occur in several plant communities (*Ryberg, Andreasen & Björk, 2011*). Thus, the high proportion of non-host-specific fungi is advantageous for *B. vivipara* plants specifically because they can recruit all the available fungi and thus ensure their survival under unfavourable conditions (*Kernaghan & Harper, 2001*). In this context, because most studies have focused on the diversity of fungal communities associated with *B. vivipara* from artic, subarctic, and mountainous regions, we expand on this issue here by studying two mountain ecosystems as well as a more global distribution by comparing *B. vivipara* with other plant hosts and focus on a phylogenetically diverse and widely distributed fungal lineage, the Sebacinales.

Fungal communities associated with *B. vivipara* are diverse but stable over large geographic distances

Both mountainous study sites, the Bavarian Alps and the Swabian Alps, harbour populations of *B. vivipara* that are characterised by a phylogenetically diverse array of

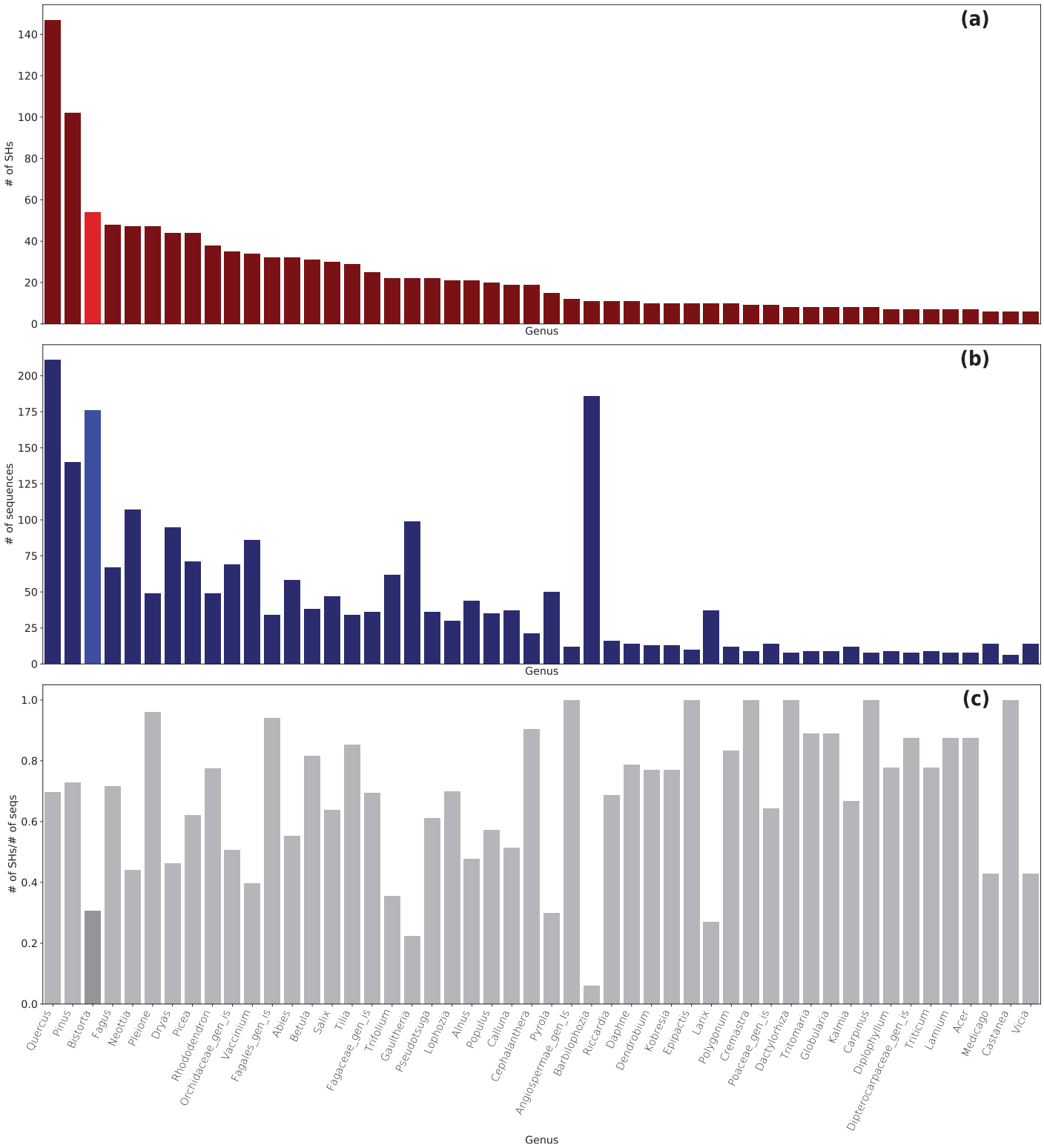

Figure 3 **Sebacinales diversity associated with *B. vivipara* and other plants co-occurring with *B. vivipara*.** (A) Number of species hypotheses (SHs) per plant genus. (B) Number of Sebacinales sequences per plant genus. (C) Number of SHs *vs* number of Sebacinales sequences (seqs) per plant genus.
Full-size DOI: 10.7717/peerj.14047/fig-3

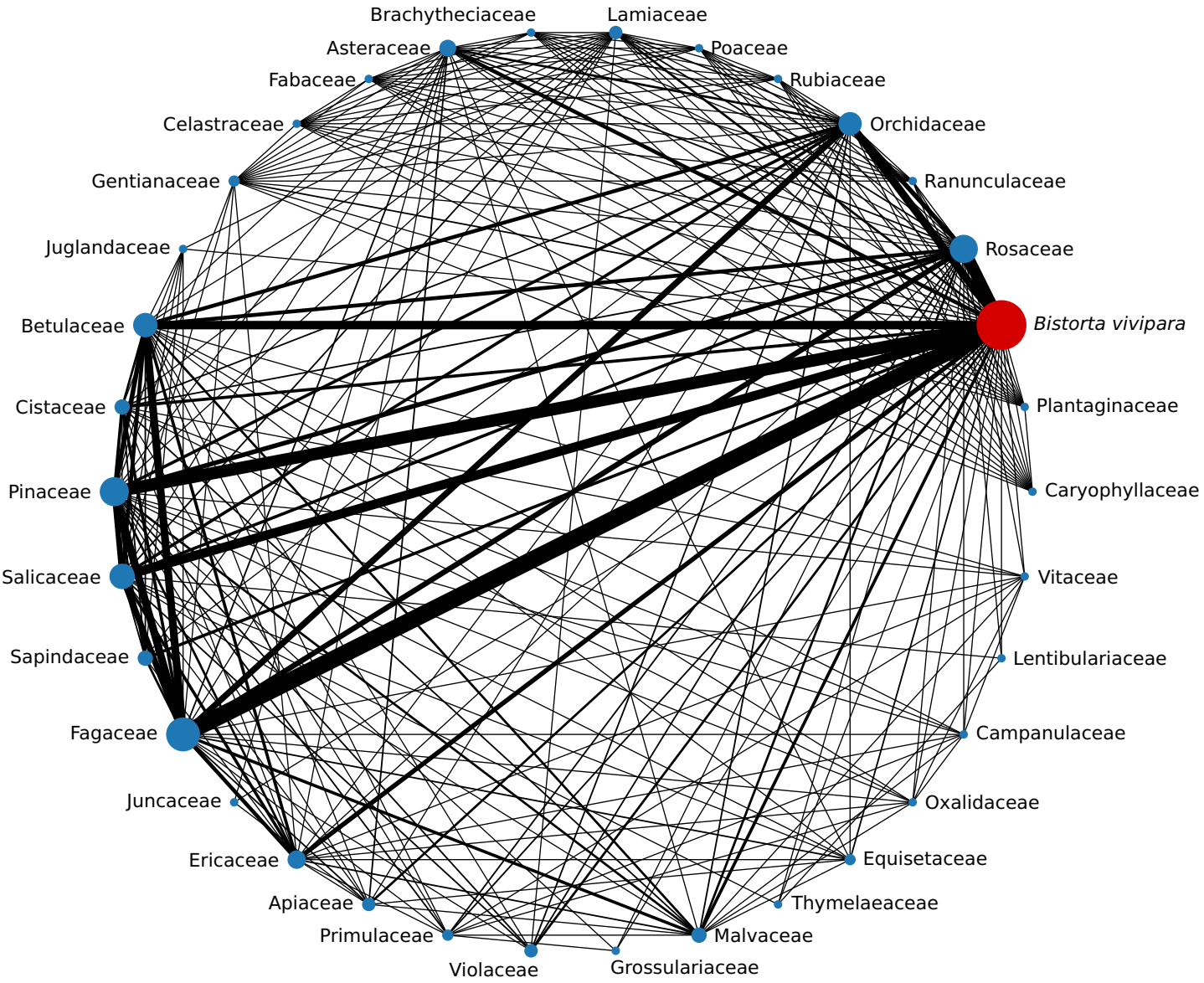

Figure 4 Number of Sebacinales SHs shared between *Bistorta vivipara* and other host plant families. The width of each edge is proportional to the number of SHs shared between the two plant family nodes it connects. The size of the nodes is proportional to the number of SHs associated with *B. vivipara* that has been observed with that plant family. Full-size DOI: 10.7717/peerj.14047/fig-4

symbionts, mainly from the group of ECM-forming fungi, in line with previous morphological studies (*e.g.*, *Harley & Harley, 1987*; *Massicotte et al., 1998*). Our results confirm the previously suggested dominance of Agaricales, Sebacinales and Thelephorales (all Basidiomycota), Pezizales and *Cenococcum geophilum* (Ascomycota) in the ECM communities of various plant species (*Mühlmann & Peintner, 2008b*; *Mühlmann, Bacher & Peintner, 2008*; *Bjorbækmo et al., 2010*; *Peintner & Kuhnert, 2010*; *Blaalid et al., 2012*). Representatives of the Thelephorales were the most frequent group of ECM fungi associated with *B. vivipara*. Their relevance and importance as ECM-forming fungi was long underestimated (*Koljalg et al., 2000*). Sebacinoid fungi formed the second largest

group of ECM fungi, both in terms of the number of SHs and of sequences. Previous research reported an abundance of Pezizales ECM with *B. vivipara* (*Tedersoo et al., 2003*; *Izzo, Agbowo & Bruns, 2005*), which was also confirmed here. Most of the identified Pezizales sequences matched sequences from sporocarp tissue of the genera *Genea*, *Peziza*, *Sarcosphaera*, *Tarzetta*, and *Trichophaea*, which are already known as major representatives of the Pezizales from northern temperate forests (*Tedersoo et al., 2006*). In spite of the high ECM diversity detected, the non-asymptotic shape of the species accumulation curve suggests that many fungal species remained undetected (Fig. S2).

When comparing the dominant fungal groups of the two studied sites with data from *B. vivipara* available from public data sources, a strikingly similar pattern is observable. In this dataset, Thelephorales, Sebacinales, and Agaricales are also the most abundant representatives of the Basidiomycota, while Ascomycota are most often represented by Pezizales or Helotiales. While these fungal groups generally dominate ECM communities of many plants, this could also indicate active selection by *B. vivipara*. However, since the public data only covers (Central and Northern) Europe, we cannot make any inferences about the diversity and composition of fungal communities associated with *B. vivipara* across its whole natural distribution.

It has been hypothesised that *B. vivipara* originated in Asia, followed by relatively recent expansions to North America and Europe before the Last Glacial Maximum (*Marr et al., 2013*). Prior to and during the Last Glacial Maximum, habitat modification affected the dispersal patterns of many plant species (*Hewitt, 2000*; *Birks, 2008*). In Europe, macrofossils (bulbils) of *B. vivipara* in sediments collected in Southern Norway were dated to around 11,000 years ago (*Birks & Van Dinter, 2010*). In this study, our two study sites (Swabian Alps and the Bavarian Alps) spanned the natural distribution range of two haplotypes of *B. vivipara* (*Marr et al., 2013*). Here we found evidence of several shared SHs among the two studied populations of *B. vivipara* and between the Bavarian alps and other European locations. Furthermore, the composition of associated fungi in terms of the major groups closely resembled each other in all three groups. This could indicate the co-migration of host plants and some of their associated fungi throughout Europe. Moreover, it seems possible that more intense sampling and advanced sequencing techniques could reveal additional SHs shared between these regions.

Sebacinales are a diverse group associated with *B. vivipara* and other plants

Our analyses reveal that Sebacinales form a major part of the fungal diversity associated with *B. vivipara*, and that the diversity of Sebacinales associated with it is comparable to that observed for several tree species. While most of the sebacinoid associations seem to be ECM, other types of interactions between Sebacinales and *B. vivipara* have also been reported. Previously, *Riess et al. (2014)* and *Garnica et al. (2013)* detected the presence of Sebacinales belonging to the Serendipitaceae associated with *B. vivipara*. In this study, the presence of Sebacinales specifically belonging to the family Serendipitaceae was also detected for *B. vivipara* (Fig. S1). This family encompasses endophytic, ericoid, orchid, and ectomycorrhizal fungi (*Oberwinkler et al., 2013*). Future studies integrating data from both

full-length ITS and ITS1/ITS2 (*i.e.*, also taking into account short-read data from *e.g.*, *Blaalid et al., 2012*, *2014*; *Botnen et al., 2014*; *Davey et al., 2015*; *Mundra et al., 2015a*, *2015b*; *Arraiano-Castilho et al., 2021*) are likely to give more insights into the different types of associations between Sebacinales and *B. vivipara*.

Sebacinoid fungi associated with *B. vivipara* are likely recruited from co-occurring plants

Similar to observations in other plants (*Bahram et al., 2012*; *DeBellis et al., 2006*; *Bogar & Kennedy, 2013*), our study indicates that the diversity of sebacinoid fungi is greatly influenced by the presence of co-occurring plant species, mainly ECM trees, suggesting that unspecific mycobionts are able to colonise *B. vivipara* when it grows near other ECM-forming plants. In general, an enormous diversity of symbionts is involved in ECM associations with *B. vivipara*, as supported by the large number of SHs from the Sebacinaceae and Serendipitaceae revealed in this study. The unspecific nature of many of these interactions, combined with stochastic processes of colonisation mediated by the bulb dispersion, for example through birds (*Moss & Parkinson, 1975*), and/or the patchiness of fungal distributions, might be responsible for the very heterogeneous ECM communities detected in this study.

In accordance with a previous study by *Botnen et al. (2014)* that revealed the low host specificity of ECM communities associated with *B. vivipara*, we detected many SHs that were shared between *B. vivipara* and both ECM-forming and non-ECM plants in the Sebacinales. In fact, only 4 non-singleton SHs were found to be exclusive to *B. vivipara*. However, while SHs found with *B. vivipara* are mostly shared with other plants, our data suggests that ECM-forming trees associate mostly with fungal partners not shared with other plants. This could be ascribed to a higher fraction of specific fungal partners of these plants, or to sampling bias and/or the molecular approach applied. For example, considering the enormous root systems of *P. abies*, it is difficult to detect the complete ECM diversity from a few samples. In *B. vivipara*, in contrast, its small size makes it easier to investigate whole root systems with relatively little sampling effort. Our results also confirm those of *Riess et al. (2013)*, indicating high cryptic diversity in Sebacinales.

CONCLUSIONS

Our findings at the local and continental levels reinforce previous observations in *B. vivipara* and other plant groups, that the presence of surrounding vegetation may serve as reservoirs of fungal symbionts. Members of the order Sebacinales represent a frequent and dominant group of fungi associated with *B. vivipara*. Most Sebacinales species form ECM associations, but some, specifically those from the Serendipitaceae family, can also form endophytic and different mycorrhizal interactions. Few sebacinoids are associated specifically with *B. vivipara*, whereas most are shared with co-occurring plants. Here, we also present the first fungal ITS sequences from *B. vivipara* from the Swabian Alps. The data from this site, though obtained by limited sampling, reveals similar taxonomic

groups of fungi associated with *B. vivipara* and reflects the high amount of shared fungal partners observed in more densely sampled sites. However, a more complete sampling of *B. vivipara* across its natural distribution range, with a locally finer scale and more intense sampling, and the application of high-throughput studies such as metabarcoding could further elucidate major patterns of fungal diversity and composition at both the local and global scale.

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The authors declare that they have no competing interests.

Author Contributions

- Max Emil Schön conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Kessy Abarenkov performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Sigisfredo Garnica conceived and designed the experiments, performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

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The data is available at GenBank: KF000466–KF000687 and KC986236–KC986286.

Data Availability

The following information was supplied regarding data availability:

The raw data from the phylogenetic tree are available in the Supplemental File. Custom scripts are available at https://github.com/maxemil/bistorta-vivipara.

Supplemental Information

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