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Partial ecological niche partitioning between *Beauveria brongniartii* and *Beauveria pseudobassiana* entomopathogens at *Melolontha melolontha* infested sites

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ABSTRACT

Beauveria brongniartii specifically infects the insect pest *Melolontha melolontha* and is commonly isolated from soil and larvae. Since 1990, it has been used commercially as a biological control agent (BCA) against the larvae. Recent research revealed that *B. pseudobassiana* was the most frequently isolated pathogen from adult beetles collected aboveground across 35 alpine sites. This led us to hypothesize that *B. pseudobassiana* primarily infects adults aboveground, while *B. brongniartii* mainly targets larvae in soil. To test these hypotheses, we investigated the occurrence and distribution of both species at two *M. melolontha*-infested sites in Switzerland using a combination of phylogenetic analyses and microsatellite markers. Species-specific microsatellite markers were developed for *B. pseudobassiana*, and existing markers were applied to *B. brongniartii*. From 399 *Beauveria* spp. isolates collected from adult beetles, soil, grassland plants, and tree leaves, 362 were identified as *B. brongniartii*, which was present in all sources except tree leaves, and 37 as *B. pseudobassiana*, found in all sources including tree leaves, except soil. The most common *B. brongniartii* multilocus genotypes (MLGs) were those of the BCAs BIPESCO 2 and BIPESCO 4, perhaps reflecting previous BCA applications at these locations. One *B. pseudobassiana* microsatellite-based MLG was isolated from both tree leaves and *M. melolontha* in the tree canopy. Additionally, *B. pseudobassiana* isolated from the phylloplane exhibited pathogenicity toward *M. melolontha* adults, suggesting the possibility of acquiring infections aboveground. To detail *B. pseudobassiana* intraspecific diversity, a sequence dataset was constructed including 13 unique haplotypes of the nuclear intergenic region Bloc sequenced in this study, 58 Bloc sequences downloaded from GenBank, and sphyngomyelin phosphodiesterase, glycosyl hydrolase, and AAA-ATPase midasin I sequences from 18 isolates illustrative of *B. pseudobassiana* genome diversity accessioned in GenBank. Phylogenetic analysis of this data reveals the species to comprise a cryptic complex with distinct genetic clusters that group isolates independently of their geographic origin and isolation substrate. Overall, this study shows *B. brongniartii* predominates in soil, while *B. pseudobassiana* occurs in the phylloplane, suggesting its potential as a complementary biological control for adult beetles aboveground.

1. Introduction

The interaction between the fungal entomopathogen *Beauveria brongniartii* and the European cockchafer *Melolontha melolontha* L., (Coleoptera, Scarabaeidae), an insect pest in Europe, has been monitored and studied for decades (Zimmermann, 2007). *B. brongniartii* is considered the primary antagonist of *M. melolontha* (Büchi et al., 1986), and the potential of the fungus to control *M. melolontha* was first recognized and implemented in the late 20th century (Dolci et al., 2006; Keller et al., 2003; Tereba & Niemczyk, 2017; Wagenhoff et al., 2014).

M. melolontha is a polyphagous species, causing damage in agriculture and horticulture throughout Europe (Pedrazzini et al., 2024). Adult insects preferentially feed on fresh young leaves and blossoms of trees and shrubs, e.g., *Betula pendula*, *Acer pseudoplatanus*, and *Corylus*

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avellana (Büchi et al., 1986; Wagenhoff et al., 2014). However, the most injurious plant damage is inflicted by the soil-dwelling white grubs, which feed on roots of tree and grassland species, including *Malus domestica*, *Taraxacum officinale*, *Trifolium pratense*, and cultivated crops, e.g., potato (Büchi et al., 1986; Laengle et al., 2005; Poženel, 2005; Sukovata et al., 2015; Woreta, 2015). *M. melolontha* completes its life cycle in three to four years (Faber, 1951; Wagenhoff et al., 2014). Infested areas are typically populated by a single, temporally synchronized population of *M. melolontha*, with individuals at similar developmental stages (Wagenhoff et al., 2014). In April-May of their third/fourth year, *M. melolontha* adults emerge from soil and aggregate along forest borders, at distances up to 2–3 km from their sites of emergence (Büchi et al., 1986; Wagenhoff et al., 2014). Following mating, females remigrate and oviposit in the fields from which they originally emerged and lay groups of 10–36 eggs (Wagenhoff et al., 2014).

As *B. brongniartii* predominantly infects *M. melolontha* in Europe, it has been used to develop a BCA. Since the 1990s, the control of *M. melolontha* is mainly based on commercial products based on sterile barley kernels colonised by *B. brongniartii* that target the larvae in soil (Kessler et al., 2003). The application of "Fungus Colonized Barley Kernels" (FCBK) on infested soil has proven highly efficient in reducing *M. melolontha* infestations (Kessler et al., 2004; Sukovata et al., 2015). BCA products are frequently applied in Switzerland, Italy and Austria, i.e., Beauveria–Schweizer® (E. Schweizer Seeds, Switzerland), strain BIPESCO 4, and Melocont® Pilzgerste (Agri-futur, Italy), strain BIPESCO 2 (Enkerli et al., 2007; Mayerhofer et al., 2015; Zimmermann, 2007). In Switzerland, for instance, it is estimated that approximately 300 ha are annually subjected to treatments against *M. melolontha* (Giselher Grabenweger, Agroscope; personal communication).

Despite the perception that *B. brongniartii* is the primary fungal pathogen associated with *M. melolontha*, recent study results challenge this decades-long assumption. The study, which was conducted at 35 sites across Switzerland, Austria, and Italy (Pedrazzini et al., 2024), revealed that *B. pseudobassiana* is instead the predominant pathogen of *M. melolontha* adults.

B. pseudobassiana, a recently described species (Rehner et al., 2011), occurs in soil and, in contrast to European *B. brongniartii*, has a wide host range (Imoulan et al., 2019; Kovač et al., 2021; Wang et al., 2020). In recent years, it has been demonstrated that *B. pseudobassiana* infects various insect pests in Eurasia, such as the pollen beetle *Meligethes aeneus* F. (Coleoptera: Nitidulidae; Meyling et al., 2012), the European Pine Wilt Disease (PWD) vector *Monochamus galloprovincialis* Olivier (Coleoptera: Curculionidae; Álvarez-Baz et al., 2015), the bark beetle *Dendroctonus micans* Kugelann (Coleoptera: Curculionidae; Kocacevik et al., 2015; Tanyeli et al., 2010) and the oak lace bug *Corythucha arcuata* Say (Hemiptera: Tingidae; Kovač et al., 2020). However, there are no commercialized products based on *B. pseudobassiana* yet available. Recently, *B. pseudobassiana* has also been detected in the phylloplane of several plant species (Garrido-Jurado et al., 2015; Howe et al., 2016). Howe et al. (2016) detected *B. pseudobassiana* in the phylloplane of lime trees and on the beetle *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) collected from a single site in a park area, suggesting that *H. axyridis* encounters *B. pseudobassiana* in arboreal habitats.

Although *B. pseudobassiana* has been consistently detected in soils infested by *Melolontha* spp. (Mayerhofer et al., 2015; Niemczyk et al., 2019), its relevance as a fungal pathogen of *M. melolontha* adults or larvae, has not been considered. Previous investigations emphasized *B. brongniartii* as the most frequently isolated pathogen from *M. melolontha* larvae, biasing research and white grub control strategies exclusively towards this fungal species (Ciornei et al., 2006; Dolci et al., 2006; Keller et al., 2002). This far, no comprehensive survey of *Beauveria*-infected *M. melolontha* larvae using molecular identification tools has been conducted to validate the presumed status of *B. brongniartii* as the predominant fungal pathogen of *M. melolontha* larvae.

The recently discovered prevalence of *B. pseudobassiana* isolated from *M. melolontha* adults collected aboveground (Pedrazzini et al., 2024) and its detection in the phylloplane (Garrido-Jurado et al., 2015; Howe et al., 2016), suggests a partial niche separation between these two fungal species and their hosts. However, it may be plausible that adult beetles become infected with *B. pseudobassiana* in the soil as well as aboveground through contact with, or consumption of, plant material (leaves from grassland plant species and trees) contaminated by this species. Our hypothesis posits that *B. brongniartii* and *B. pseudobassiana* exhibit partial niche differentiation, which might result in differential exposure of *M. melolontha* to *Beauveria* spp. at different developmental stages and locations.

The aim of this project was to elucidate the ecological niches occupied by *B. brongniartii* and *B. pseudobassiana* within *M. melolontha* infested sites, by discerning the specific locations where microsatellite-based genotypes of these fungal species predominantly occur. Two sites in the alpine region of Switzerland, heavily infested with *M. melolontha* adults in 2021, were selected for this study due to their particularly high insect flight activity (Supplementary Fig. 1). One of these sites, Bristen, is adjacent to a location previously studied by Pedrazzini et al. (2024). This investigation aimed to provide preliminary insights into the origins of *M. melolontha* adult infections by the two *Beauveria* species. Specifically, the objectives of this study were to: (1) Isolate *Beauveria* species from different sources at two sites infested with *M. melolontha*, including adults emerging from soil or swarming in tree canopies, soil, grassland plants with which beetles get in contact when emerging and tree leaves consumed by swarming beetles. (2) Identify the species and analyze the phylogenetic diversity of the *Beauveria* isolates within the broader context of the species genetic variation. (3) Develop species-specific microsatellite markers for *B. pseudobassiana* and use these, along with existing markers, to genotype *B. pseudobassiana* and *B. brongniartii* isolates to assess their occurrence and distribution. (4) Test the infection ability of *B. pseudobassiana* isolates from *M. melolontha* and plant surfaces on adult beetles.

2. Material and methods

2.1. Field sites and sample collection

In 2021, collections of *Beauveria* spp. were obtained from two grassland sites infested with *M. melolontha* adults in Switzerland, i.e., Laax (Coordinates WGS84 46.7381/9.25) and Bristen (Coordinates WGS84 46.76/8.71; Table 1, Supplementary Fig. 1). The two sites had previously received regular treatments with commercial BCA formulations using either strain BIPESCO 2 (Melocont® Pilzgerste) and BIPESCO 4 (Beauveria–Schweizer®) during the past 10–20 years (Christian Schweizer, Agroscope; personal communication). Four rectangular open-bottom insect tents of 2 m x 3 m x 2.5 m (WxLxH; Howitec Netting BC, Joure, The Netherlands), were placed (separated by up to 50 m) at each site before emergence of *M. melolontha* adults in April 2021. Two days after adult emergence from soil, three different sample types were collected from each tent, denoted as *Mm*_soil, Soil and Grass_pl, that is: (1) all the *M. melolontha* adults (31–69) that emerged from the soil (*Mm*_soil), (2) three soil samples (Soil) separated by 1.5–2.5 m each consisting of two adjacent soil cores of 20 cm depth and 2.5 cm diameter, and (3) three plant samples, including aboveground plant material, and each including different grassland plant species (Grass_pl) belonging to different families (e.g., *Ranunculaceae*, *Poaceae* and *Adoxaceae*), from the same position the soil samples were obtained. Collected beetles were stored individually in peat-filled cylindrical plastic containers (diameter 4.5 cm, height 6 cm) and transported immediately to the laboratory for incubation. Soil samples were placed in sterile plastic bags, while plant material was stored in clean plastic boxes. All samples were transported to the laboratory at room temperature within a few hours of collection. Upon arrival, they were stored at 4 °C and further processed on the following day. To explore the possibility that *M. melolontha* adults acquire infections by contact with tree leaves, one week following collection of the beetles within the tents, 54–169 *M. melolontha* adults

Table 1
Source of origin and number of samples (N) of *Beauveria* spp. collections, number of *Beauveria brongniartii* and *B. pseudobassiana* isolates, number of MLGs and Stoddard and Taylor diversity index per sampling site. Abbreviation of source material are given for each category of sources. *Mm*_soil: *M. melolontha* adults collected within the tents, *Mm*_tree: *M. melolontha* adults collected on trees, Soil: composite soil samples, Grass_pl: grassland plant species, Tree_lv: leaves of tree plants. N represents the number of samples collected from each source material.

Site	Source of isolation	Abbreviation	*B. brongniartii*			*B. pseudobassiana*		
			Total isolates	Number MLG	S. T. d.[a]	Total isolates	Number MLG	S. T. d.
Laax (LX21)	*M. melolontha* emerged from soil (N = 131)	*Mm*_soil	44	7	5.1	0	0	–
	M. melolontha on trees (N = 131)	*Mm*_tree	16	7	3.3	0	0	–
	Soil (N = 12)	Soil	36	9	5.7	0	0	–
	Grassland plants (N = 12)	Grass_pl	13	8	3.9	0	0	–
	Tree leaves (N = 12)	Tree_lv	0	0	0	19	10	3.3
	Total		**109**	**16**	–	**19**	**10**	–
Bristen (B21)	*M. melolontha* emerged from soil (N = 220)	*Mm*_soil	102	16	7.8	5	5	5
	M. melolontha on trees (N = 311)	*Mm*_tree	105	29	7.4	5	5	5
	Soil (N = 12)	Soil	34	6	4.3	0	0	–
	Grassland plants (N = 12)	Grass_pl	12	6	5.1	3	3	3
	Tree leaves (N = 12)	Tree_lv	0	0	–	5	5	5
	Total		**253**	**37**	–	**18**	**14**	–

[a] Stoddard Taylor diversity index.

were collected from two infested trees per site and located up to 60 m from the tents, denoted as *Mm*_tree. At both sites, beetles were collected from the two most heavily infested trees, that is sycamore maple (*Acer pseudoplatanus*,two trees) in Laax and common hazel (*Corylus avellana*) and silver birch (*Betula pendula*) in Bristen. Leaves on which beetles were feeding were collected from the same trees at two vertical strata, approximately 1.5 m and 2.5 m aboveground, and are referred to as Tree_lv. Three samples per strata each consisting of approximately 10–15 leaves were collected, put in plastic bags and transported as described above.

2.2. *Beauveria spp. isolation, cultivation and DNA extraction*

Beauveria spp. were isolated from different sources (described in section 2.1.), including *M. melolontha* adults, soil samples, grassland plant phyllosphere, and tree leaves.

To isolate *Beauveria* spp. from *M. melolontha* adults, individual beetles were incubated at 80 % relative humidity and 22 °C in peat-filled cylindrical plastic containers with a 4 cm diameter and 6 cm height until beetle death and emergence and conidiation of *Beauveria* spp. Isolates were transferred from mycosed cadavers to 90 mm Petri dishes containing a semi-selective medium (SSM; Strasser et al., 1996) using a sterile needle. Single-colony subcultures of each isolate were established and maintained on SSM. All cultures were grown at 80 % relative humidity and 22 °C.

For soil samples, 5 g of each homogenized sample were suspended in 25 mL sterile 0.1 % Tween 80 solution in 100 mL Erlenmeyer flasks. Flasks were placed on a rotatory shaker at 120 rpm at room temperature for 3 h. After sedimentation for 20 s, 100 μL and 200 μL aliquots of the suspensions were spread onto SSM. Each soil sample and dilution were plated in triplicate, and the number of *Beauveria* spp. colony forming units (CFU) per g (dry weight) of soil was determined for each plate after incubation at 22 °C for three weeks. Means were calculated per tent. From each plate, three sporulating *Beauveria* spp. single colonies were randomly selected and subcultured on SSM at 22 °C.

Isolation of *Beauveria* spp. from grassland plant and tree leaf surfaces was conducted using 5 g of plant material, evenly selected to ensure representation of all plant species present. Plant material was transferred to extraction bags (Bioreba, Reinach, Switzerland), along with 20 mL of 0.1 % sterile Tween 80 solution. Plant material was homogenized using an electronic homogenizer (Bioreba) and 100 μL aliquots of the homogenate were plated on SSM in duplicates. All fungal colonies that emerged from plant material were included for subsequent analyses and were subcultured on SSM at 22 °C.

To produce mycelium for DNA extraction, single-colony subcultures of each isolate were grown on sterile filter paper placed on 3 % PDA for seven days. The mycelium was harvested, lyophilized for six hours at −4°C using a CentriVap benchtop centrifugal vacuum concentrator (LabConco, Kansas City, MO, USA) and homogenized using a FastPrep-24™ 5G Grinder (Thermo Fisher Scientific, Waltham, MA, USA) at 6 m/s for 25 s with 2 glass beads of 3 mm and 0.15 g of 1 mm diameter. DNA extractions were conducted using the LGC sbeadex Plant Kit (LGC, Berlin, Germany) automated with the KingFisher Sample Purification System (Thermo Fisher Scientific, Waltham, MA, USA). DNA quality was assessed by electrophoresis in 1 %-agarose gels and quantification was performed with PicoGreen® fluorescent nucleic acid stain (Invitrogen, Carlsbad, CA, USA) with the Cary Elipse fluorescence spectrophotometer (Varian, Palo Alto, CA, USA).

2.3. *Species identification*

Species identification and diversity of *Beauveria* spp. isolates was inferred by phylogenetic analysis of the nuclear intergenic region Bloc (Rehner et al., 2006) from an alignment matrix that included Bloc sequences of 25 *Beauveria* spp. reference strains. Isolates that were not *Beauveria* spp. (e.g., Bloc amplification failures) were instead analyzed by sequencing the Internal Transcribed Spacer (ITS) region and performing BLAST similarity searches (Altschul et al., 1990) in the non-redundant nucleotide database of GenBank (National Center for Biotechnology Information, Bethesda, MD, USA). The Bloc region was amplified with forward B5.1F (5′-CGACCCGGCCAACTACTTTGA-3′) and reverse B3.1R (5′-GTCTTCCAGTACCACTACGCC-3′) primers, and the ITS region amplified with forward ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and reverse ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; Rehner et al., 2011; White et al., 1990) primers. PCR amplification was performed in 20 μL reactions that included 15 ng template DNA, 0.2 μM of each primer, 0.2 mM dNTP, 3 % DMSO, 1x Phusion HF Buffer and 0.4 U Phusion Hot Start II High Fidelity DNA Polymerase (ThermoScientific, MA, USA). PCR cycling conditions consisted of 30 s of initial denaturation at 98 °C and 36 cycles of 5 s at 98 °C, 20 s at 60 °C and 1 min at 72 °C, concluding with 10 min at 72 °C. Product quality was assessed visually by 1.5 %-agarose gel electrophoresis, and PCR products were purified with the Nucleospin® Gel and PCR clean-up kit (Macherey & Nagel, Germany). For Bloc sequencing, an internal region of the purified PCR product was sequenced with primers B22U (5′-GTCGCAGCCAGAGCAACT-3′, *B. brongniartii*) and B22U2 (5′-GTCGGAGCCAAAACAACT-3′, *B. pseudobassiana*) and reverse B822Ldg2 primer (3′-AGATTCGCAACGTCMACTTT-5′). Primers ITS5 and ITS4 were used for sequencing the ITS region. Sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit and sequencing products were analyzed with an 3500xL Genetic Analyzer (Applied Biosystems, CA, USA) equipped with 50 cm capillaries and the POP-7 polymer. Bloc

sequences were assembled and aligned with 25 reference *Beauveria* species sequences obtained from the GenBank database using the software MAFFT (Katoh & Standley, 2013; Rehner et al., 2011). A phylogenetic tree was inferred using maximum likelihood bootstrapping (1.000) using the Kimura 2-parameter (K2P) correction model in MEGA11 (Kimura, 1980; Tamura et al., 2021). Sequences were deposited in GenBank as accessions PQ406678-PQ406714 and PQ459198-PQ459247.

2.4. Microsatellite marker development for Beauveria pseudobassiana

Perfect tri-, tetra-, and pentanucleotide microsatellite repeats were identified in the *B. pseudobassiana* KACC 47484 draft genome assembly ASM326790v1 by analyzing the output of the microsatellite prediction tool MISA (Thiel et al., 2003) using the online MISA-web utility (Beier et al., 2017; https://misaweb.ipk-gatersleben.de/). Blast searches with candidate loci consisting minimally of four repeat units, plus 100 bp 5′ and 3′ flanking single copy sequences, were conducted against additional *B. pseudobassiana* genomes including RGM 2184 (ASM2298505v1) and ARSEF 3405 (Rehner, unpubl.). Searches were limited to the 14 largest contigs (0.83–4.09 × 10^6 kb) of the ASM326790v1 assembly to bias marker selection toward physically distant (>100 kb in individual contigs) or unlinked loci. Multisequence alignments (MSA) for each locus were performed using the MAFFT (Katoh & Standley, 2013) module of Geneious Prime 2023.1.1 (BioMatters Ltd.) and only loci whose length varied by at least one perfect unit repeat in at least two of the three examined genomes were retained. PCR primers of the 18 selected loci were designed using the Primer 3 module in Geneious Prime (Supplementary Table 1).

2.5. Phylogenetic diversity of B. pseudobassiana isolates

This study aimed to explore the phylogenetic diversity of *B. pseudobassiana* isolates obtained from the current study in the broader molecular diversity of the species. To achieve this, we constructed a reference dataset based on two genomic loci, enabling a comprehensive and robust framework for phylogenetic analysis. The first locus, approximately 5 kb in length, encompasses the complete coding sequences of two adjacent genes, sphingomyelin phosphodiesterase (SMase) and glycosyl hydrolase 92 (GH92), along with their five introns and the short intergenic region between them. This region also includes the canonical Bloc (~1500 bp), which comprises partial 3′ exons of SMase and GH92, as well as their intergenic sequence (Supplementary Fig. 2). The second locus is AAA-ATPase midasin I (MDN1) and consists of a single open reading frame of 14.727 kb. To construct the reference dataset, we extracted the SMase, GH92, and MDN1 sequences from 87 *B. pseudobassiana* genome assemblies available in GenBank (Supplementary Table 2). A maximum likelihood (ML) phylogenetic tree was then constructed using these sequences, with *B. bassiana* ARSEF 2860 as an outgroup (Supplementary Fig. 3). From the tree, 18 sequences were selected to represent the overall phylogenetic diversity, indicated by asterisks in Supplementary Fig. 3. Since GenBank does not allow third-party annotations, the reference SMase + GH92 and MDN1 sequences extracted from these genomes are provided in FASTA format (Supplementary Data 1, 2) to facilitate their accessibility. Additionally, we retrieved 177 partial or complete *B. pseudobassiana* Bloc sequences from GenBank (Supplementary Table 3). These sequences were aligned and used to construct a separate ML tree alongside the *B. pseudobassiana* Bloc sequences obtained from this study (Supplementary Fig. 4). From the 177 sequences, 58 were selected to represent the overall phylogenetic diversity based on the Bloc locus, indicated by asterisks in Supplementary Fig. 4. The final reference dataset combined the 18 genome-derived sequences, the 58 GenBank Bloc sequences, and the 13 unique Bloc haplotypes identified from the 37 isolates of *B. pseudobassiana* collected in this study. Nucleotide sequences for each locus were aligned with MAFFT 1.5.0 (Katoh & Standley, 2013) and concatenated in Geneious Prime 2024.0.7 (BioMatters, Ltd.), yielding an alignment of 19.741 bp that included 653 parsimony-informative sites and 1.170 singleton sites. The data were initially partitioned by gene, codon position, and noncoding introns and intergenic region, with models of sequence evolution estimated using ModelFinder, and the merge partition function was implemented to define 4 partitions consisting of a single partition for SMase, GH92 and Bloc sequence (TPM3u + F + I + G4) and separate partitions for each codon position at MDN1 (1st pos. = GTR + F + I + G4; 2nd pos. = TPM3 + F + I; 3rd pos. = GTR + F + I + G4; Chernomor et al., 2016; Kalyaanamoorthy et al., 2017). A phylogeny was inferred under the maximum likelihood (ML) criterion with IQ-TREE (Minh et al., 2020; Nguyen et al., 2015) with branch support estimates obtained with 1000 bootstrap pseudoreplicates.

2.6. Microsatellite marker analyses

B. brongniartii and *B. pseudobassiana* isolates from Laax and Bristen were genotyped at six (Enkerli et al., 2001) and 18 microsatellite loci (see above), respectively. Target loci for *B. brongniartii* and *B. pseudobassiana* were amplified in two and six multiplexed PCR reactions, each including a set of three primer pairs (*B. brongniartii*: Set-1: Bb1F4/Bb5F4/Bb8D6, Set-2: Bb2A3/Bb2F8/Bb4h9, *B. pseudobassiana*: Set-1: BpsSSR01/BpsSSR02/BpsSSR03, Set-2: BpsSSR04/BpsSSR05/BpsSSR06, Set-3: BpsSSR07/BpsSSR08/BpsSSR09, Set-4: BpsSSR10/BpsSSR011/BpsSSR12, Set-5: BpsSSR13/BpsSSR14/BpsSSR15, Set-6: BpsSSR16/BpsSSR17/BpsSSR18; Supplementary Table 1), with forward primers labelled with ATO, HEX or FAM (Microsynth, Balgach, CH). PCR reactions for *B. brongniartii* were performed in 20 μL reactions including 10 ng template DNA, 0.2 μM of each primer, 0.2 mM dNTP, 3 mM $MgCl_2$, 1x GoTaq® Flexi Reaction Buffer and 0.25 U GoTaq G2 Flexi DNA Polymerase (Promega, WI, USA). PCR cycling conditions for *B. brongniartii* consisted of 2 min of initial denaturation at 94 °C, 12 cycles of 30 s at 94 °C, 60 s at 72 °C to 60 °C, and 40 s at 72 °C, followed by 22 cycles of 30 s at 94 °C, 30 s at 60 °C and 40 s at 72 °C, and finalized with a 15 min incubation at 72 °C. PCR reactions for *B. pseudobassiana* were performed in 20 μL reactions including 10 ng template DNA, 0.2 μM of each primer, 1x QIAGEN Multiplex PCR Master Mix (QIAGEN, Aarhus, Denmark). PCR cycling conditions for *B. pseudobassiana* consisted of 5 min of initial denaturation at 95 °C, 35 cycles of 30 s at 95 °C, 1:30 min at 58 °C, and 30 s at 72 °C, and finalized with a 10 min incubation at 68 °C. Amplicon sizing was performed on a 3500xL Genetic Analyzer (Applied Biosystems, CA, USA) with 50 cm capillaries and POP-7 polymer and fragment sizes estimated with GeneMarker® software (SoftGenetics, PA, USA). Analyses were performed including isolates BIPESCO 4 and ARSEF 3405 as amplicon size standards for *B. brongniartii* and *B. pseudobassiana*, respectively.

2.7. Pathogenicity test and analyses

Pathogenicity tests were conducted with adult individuals of *M. melolontha* collected from a population in Meiringen, Switzerland, which is independent from the population in which the experiments were performed (Coordinates WGS84 46.73/8.17, Supplementary Fig. 1). Beetles were collected on May 9, 2022, transported to the laboratory and submitted to pathogenicity test on the following day. Tests included 25 individuals for each treatment and were replicated three times. *M. melolontha* adults were dipped individually twice for three seconds in conidia suspensions of 10^7 conidia/ml in 0.1 % sterile Tween 80 solution. Treatments included a negative control with 0.1 % sterile Tween 80, a positive control consisting of a conidia suspension of *B. brongniartii* BIPESCO 2, and two treatments with mixed conidia suspensions of *B. pseudobassiana*, each including three genotypically distinguishable microsatellite multilocus genotypes (MLG) obtained from Laax and Bristen, to represent the genetic and ecological diversity of *B. pseudobassiana* observed at the sampling sites. One *B. pseudobassiana* suspension consisted of three MLGs obtained from

infected *M. melolontha* collected in Bristen (i.e., *Mm*_soil-1: original isolate ID B21_A16, *Mm*_soil-2: original isolate ID B21_B26 and *Mm*_soil-3: original isolate ID B21_C30; Fig. 2, Supplementary Table 5, Supplementary Fig. 8). The second *B. pseudobassiana* suspension consisted of three MLGs isolated from tree leaves in Laax or Bristen (i.e., Tree_lv-1: original isolate ID LX21_Q3, Tree_lv-2: original isolate ID LX21_R3 and Tree_lv-3: original isolate ID B21_P1; Fig. 2, Supplementary Table 5, Supplementary Fig. 8). For each *B. pseudobassiana* treatment, the conidia concentration of the three isolates was first determined using a hemocytometer, and volumes were adjusted to achieve equal representation of each isolate in the final 10^7 conidia/mL suspension.

Treated *M. melolontha* adults were incubated in individual peat-filled cylindrical plastic containers as described in section 2.2. The beetles were checked daily for 21 days and provided with fresh *Corylus avellana* leaves every three days. Deceased *M. melolontha* adults were surface sterilized in 1 % Sodium hypochlorite (NaClO) for 1 min, rinsed two times in sterile distilled water for 1 min and placed on a sterilized filter paper, moistened with sterile distilled water, in 90 mm Petri dishes. Emergence of *Beauveria* spp. was monitored daily for three weeks after *M. melolontha* death. Additionally, randomly selected leaves (*Corylus avellana*) used as food for *M. melolontha* were assessed for the presence of *Beauveria* spp. by plating leaf homogenates on SSM plates as described in section 2.2.

All *Beauveria* spp. isolates that emerged from *M. melolontha* adults were transferred to SSM plates and incubated at 80 % relative humidity and 22 °C. Single colony subcultures and DNA extractions were performed as described in section 2.2. *Beauveria* spp. isolates were genotyped using six microsatellite markers for *B. brongniartii* (Set-1: Bb1F4/Bb5F4/Bb8D6, Set-2: Bb2A3/Bb2F8/Bb4h9) and *B. pseudobassiana* (Set-1: BpsSSR01/BpsSSR02/BpsSSR03, Set-4: BpsSSR10/BpsSSR011/BpsSSR12) that enabled discrimination of the six MLGs used in the test. Reactions were performed as described above. For isolates that were not identified as *B. brongniartii* and *B. pseudobassiana*, the ITS region was sequenced and analyzed as outlined in section 2.3.

2.8. Data analysis and statistics

Analysis of Variance (ANOVA) and Tukey's post-hoc test were

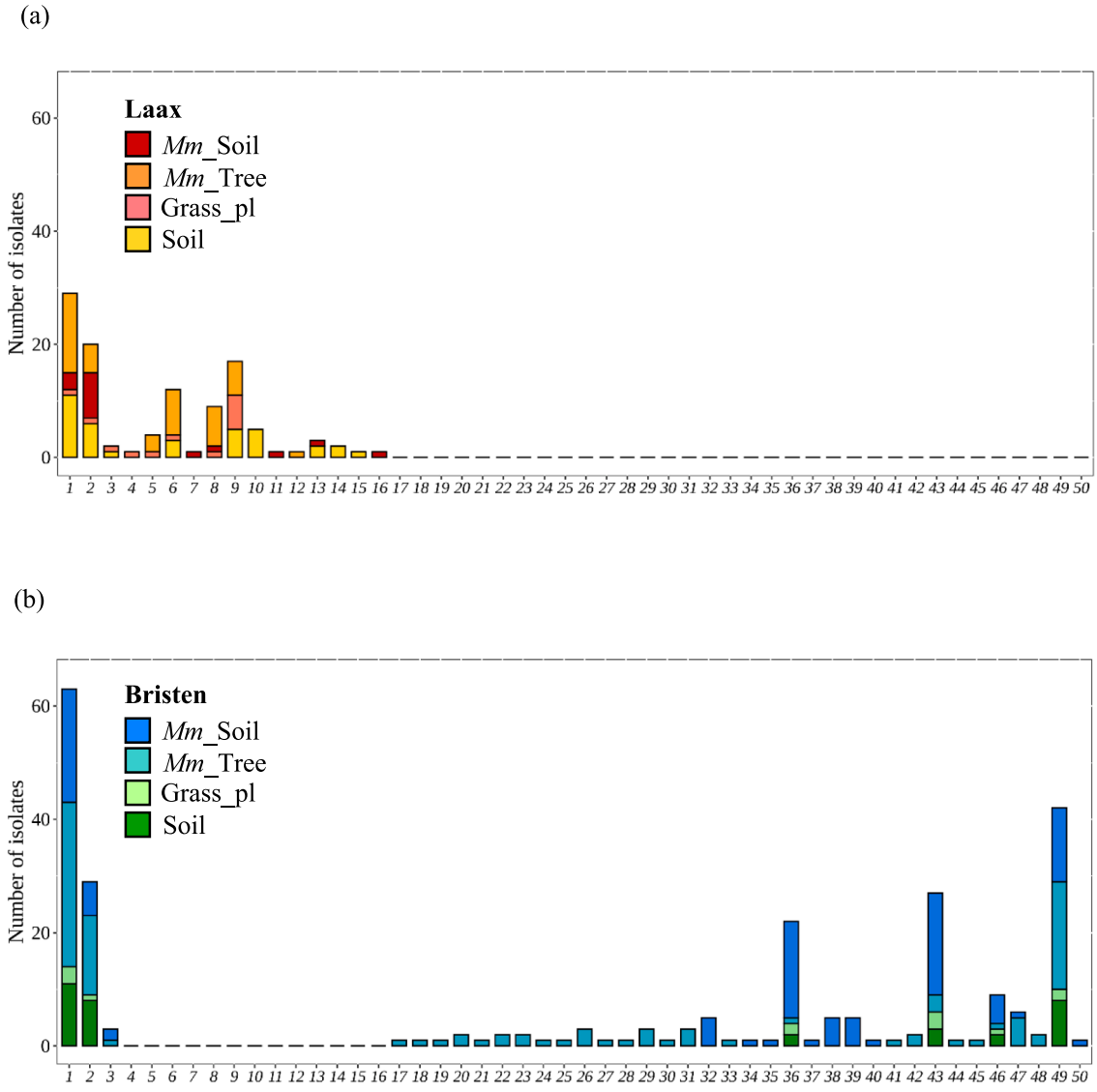

Fig. 1. *Beauveria brongniartii* MLGs and their abundance in samples of the different sources in Laax (**a**) and Bristen (**b**). The x-axis represents the number of individual multilocus genotypes (MLGs) shown in the y-axis, with each number corresponding to a unique genotype. Colors indicate source material of *B. brongniartii* isolation, including fungal isolates established from *Melolontha melolontha* adults (emerging from soil or on trees), soil and grassland plants. MLG 1 and MLG 2 correspond to the MLGs of BIPESCO 2 and BIPESCO 4.

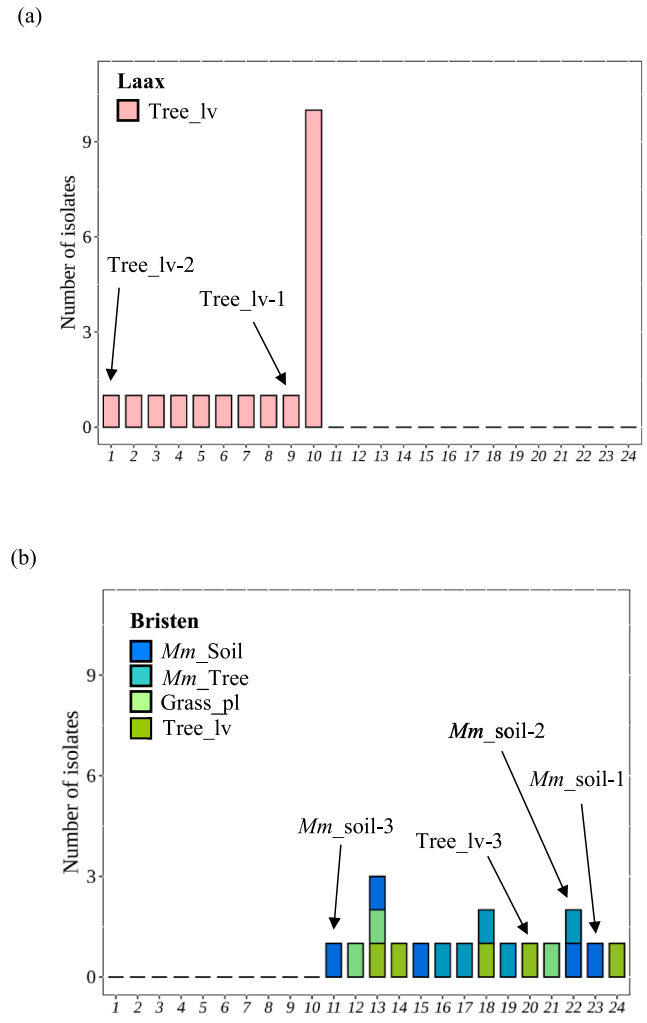

Fig. 2. *Beauveria pseudobassiana* MLGs and their abundance in the different source materials in Laax (**a**) and Bristen (**b**). The x-axis represents the number of individual multilocus genotypes (MLGs) presented in the y-axis, with each number corresponding to a unique genotype. Colors indicate source material of *B. pseudobassiana* isolation, including fungal isolates established from *Melolontha melolontha* adults (emerging from soil or on trees), grassland plants and tree leaves. Arrows indicate MLGs isolated from *M. melolontha* adults (i.e., *Mm*_soil-1, *Mm*_soil-2 and *Mm*_soil-3) and from tree leaves (i.e., Tree_lv-1, Tree_lv-2 and Tree_lv-3) used for the pathogenicity test.

conducted to determine significant differences among *Beauveria* spp. CFU g^{-1} dry wt soil values originating from different tents using R packages stats 4.3.1 (Team, 2013) and agricolae 1.3–6 (De Mendiburu, 2023), respectively. The results were visually represented with a bar plot constructed with the R package ggplot2 3.3.5 (Wickham, 2009).

Analyses of microsatellite-based multilocus-genotypes (MLGs), and the calculation of diversity estimates, i.e., the Stoddard and Taylor diversity index (S. T. d.), were performed with R package poppr 2.9.3 (Kamvar et al., 2014). Barplots showing identity of MLG and number of isolates per MLG were drawn with the R package ggplot2 3.3.5 (Wickham, 2009). Principal component analyses (PCA) were performed with R packages ade4 1.7–18 (Dray & Dufour, 2007), adegenet 2.1.5 (Jombart, 2008) and factoextra 1.0.7 (Kassambara & Mundt, 2017).

Survival probabilities for a 21-day post-pathogenicity test period were assessed with Kaplan-Meier survival curves using the R package survival 3.5–7 (Therneau & Lumley, 2015). To compare treatment outcomes, both the log-rank test and Cox proportional hazards regression were calculated with the R package survival 3.5–7 (Therneau & Lumley, 2015). MLG analyses of *Beauveria* spp. isolated from dead *M. melolontha* adults were conducted with the R package poppr 2.9.3 (Kamvar et al., 2014).

3. Results

3.1. Number of isolates per species, location and source

A total of 403 fungal isolates were obtained from the two sites, i.e., Laax and Bristen, including samples from *M. melolontha* adults (278), soil (73), grass (28), and tree leaves (24). Of these, 399 isolates were identified as *Beauveria* species, with 128 isolates from Laax and 271 from Bristen (Table 1). All the isolates ontained from plant samples, i.e., grass and tree leaves, and cultivated belonged to the genus *Beauveria*. At the Bristen site, two isolates from Soil and one from Mm_tree were identified as *B. bassiana*, and one soil-derived isolate was classified as belonging to the genus *Keithomyces*, based on BLAST sequence similarity searches in the GenBank non-redundant nucleotide database. The three *B. bassiana* isolates and the *Keithomyces* isolate were excluded from further analyses.

Genotyping of the isolates and species assignment was performed in a combined genetic approach: (1) all isolates were genotyped applying the six *B. brongniartii* microsatellite markers. (2) One isolate for each multilocus genotype (MLG) obtained in step 1 as well as all fungal isolates for which microsatellite amplification failed at one or more loci were subjected to sequencing the Bloc and/or ITS regions for species identification. (3) Fungal isolates identified as *B. pseudobassiana* by Bloc sequencing were genotyped using the *B. pseudobassiana* microsatellite markers developed in this study.

Genetic analyses identified 109 and 253 isolates as *B. brongniartii*, and 19 and 18 as *B. pseudobassiana* in the samples from Laax and Bristen, respectively (Table 1, Supplementary Fig. 5). The prevalence of fungal infection among the collected beetles was 33.6 % in *Mm*_soil and 12.2 % in *Mm*_tree samples in Laax and 48.6 % in *Mm*_soil and 35.4 % in *Mm*_tree samples in Bristen. From the infected *M. melolontha* adults, a total of 60 and 207 isolates of *B. brongniartii* were obtained from Laax and Bristen, respectively. Regarding *B. pseudobassiana*, only 10 isolates were collected in Bristen from adult *M. melolontha*.

B. brongniartii was detected in all 12 soil samples collected at both sampling sites, with CFU densities ranging from 2×10^2 to 8×10^4 CFU g^{-1} dry wt soil and averages per tent ranged from 3×10^2 to 2×10^4 CFU g^{-1} dry wt soil (Supplementary Fig. 6). No consistent significant differences were observed among tents and variable densities were found among soil samples taken within the same tent (Supplementary Fig. 6). In contrast, *B. pseudobassiana* was not detected in any soil sample at either sampling site (Table 1).

B. brongniartii was isolated from grassland plant samples collected from each of the four tents in Laax and Bristen. In Laax, 13 *B. brongniartii* isolates were recovered from nine grassland plant samples, and in Bristen, 12 isolates were recovered from seven samples (Table 1). Three isolates of *B. pseudobassiana* were detected in two grassland plant samples collected from two tents in Bristen (Table 1).

At both locations, *B. brongniartii* was not detected in tree leaf samples, whereas *B. pseudobassiana* was found in tree leaf samples from all trees across all strata (Table 1). In Laax, 19 isolates of *B. pseudobassiana* were obtained from ten tree leaf samples (Table 1), i.e., 13 isolates originated from six tree leaf samples of plant 1 (*Acer pseudoplatanus*) and six isolates originated from four three leaf samples of plant 2 (*Acer pseudoplatanus*). In Bristen, five *B. pseudobassiana* isolates were obtained from four tree leaf samples (Table 1), with two isolates originating from two tree leaf samples of plant 1 (*Betula pendula*), and three isolates from two tree leaf samples of plant 2 (*Corylus avellana*).

3.2. Multilocus microsatellite genotypes of *B. brongniartii* and *B. pseudobassiana*

Microsatellite marker analyses of six loci revealed 50 MLGs in

B. brongniartii and 24 MLGs in *B. pseudobassiana* across the two sampling locations (Figs. 1, 2, Supplementary Table 4, 5). A lower number of *B. brongniartii* MLGs was detected in Laax (16 MLGs) compared to Bristen (37 MLGs), while a comparable number of *B. pseudobassiana* MLGs was detected at both locations (Laax: 10 MLGs; Bristen: 14 MLGs; Table 1).

In Laax, a greater genotypic Stoddard and Taylor diversity of *B. brongniartii* was observed among soil isolates and isolates obtained from *M. melolontha* emerged from soil compared to those established from other sources (Table 1). In contrast, in Bristen, a greater diversity was observed among the isolates of *B. brongniartii* obtained from *M. melolontha*, which displayed all 37 MLGs detected in this study, compared to lower diversity observed among those obtained from other sources (Table 1). One *B. brongniartii* MLG, MLG 3, was detected at both sampling sites from different environmental sources, i.e., *Mm*_soil and *Mm*_tree in Bristen and Grass_pl and Soil in Laax (Fig. 2, Supplementary Table 4). At both sampling sites, the MLGs matching *B. brongniartii* BCA strains BIPESCO 2 (MLG 1) and BIPESCO 4 (MLG 2) were abundant and were detected in all source types, except in tree leaf material (**Figure**, Supplementary Table 4). In Laax, 26 % and 18 % and in Bristen, 24 % and 11 % of the isolates revealed the BIPESCO 2 and BIPESCO 4 MLG, respectively (Fig. 1, Supplementary Table 4). At both sites, certain MLGs of *B. brongniartii* were exclusively identified in isolates from a single source, while others were detected across multiple sources, including *Mm*_soil, *Mm*_tree, Grass_pl, and/or Soil (e.g., Laax: MLG 6, 13 and 5; Bristen: MLG 36 and 43; Fig. 1, Supplementary Table 4). In a Principal Component Analysis (PCA) of the *B. brongniartii* microsatellite data, the first axis explained 28.6 % of the overall variance and separated the isolates with MLG1 (BIPESCO 2) from all other isolates detected in Bristen and Laax (Supplementary Fig. 7).

None of the identified *B. pseudobassiana* MLGs were observed at both locations, i.e., Bristen and Laax (Fig. 2). In Laax, *B. pseudobassiana* was isolated from tree leaves only, with 10 MLGs detected among 19 isolates. One MLG (MLG 10) was isolated 10 times, whereas the remaining nine MLGs were singletons (Table 1, Fig. 2). In Bristen, 14 MLGs were identified, 11 of which were singletons. MLG 13 was detected in *Mm*_soil, Grass_pl and Tree_lv, while MLG 18 was isolated from *Mm*_tree as well as from Tree_lv and MLG 22 from *Mm*_soil and *Mm*_tree (Fig. 2, Supplementary Table 5). In a PCA of the *B. pseudobassiana* microsatellite data, the first axis explained 40 % of data variation and separated the isolates into two main clusters, without distinct clustering patterns according to isolation source (Supplementary Fig. 8).

3.3. Phylogenetic diversity of *Beauveria pseudobassiana*

To assess the phylogenetic diversity of *B. pseudobassiana* isolates from this study in the context of the broader genetic variation within the species, we conducted phylogenetic analyses using publicly available sequences of the loci sphingomyelin phosphodiesterase (SMase) and glycosyl hydrolase 92 (GH92) loci, including the Bloc region separating the two loci, and the AAA-ATPase midasin I (MDN1) locus. Based on these analyses we selected 58 Bloc sequences downloaded from GenBank, and the SMase, GH92, and MDN1 sequences from 18 isolates representing the known and described genetic diversity of *B. pseudobassiana* for subsequent analyses (Supplementary Figs. 3, 4). The selected sequences were incorporated into a *B. pseudobassiana* reference dataset, which included the 13 unique Bloc haplotypes identified among the 37 isolates collected in this study and the 76 sequences downloaded from GenBank. The maximum likelihood analysis of these loci revealed a well-defined phylogenetic structure within the species (Fig. 3). Based on this structure, we identified and informally labeled five prominent clades (A–E) to aid in the discussion of our findings. However, numerous branches lack strong bootstrap support, particularly along the tree backbone, which we attribute in part to the uneven distribution of available genomes representing the various lineages, particularly the lack of genome representing both subclades within Clade A, the basal lineage in Clade B, and for multiple lineages within Clade C. Taken together, additional taxon and gene sampling will be necessary to infer a fully resolved, well-supported intraspecific phylogeny of *B. pseudobassiana*. Of the five clades recognized here, isolates with haplotypes from four clades were recovered from the sampling sites in this study, which, in descending order of unique haplotype diversity, include Clades B (n = 7), C (n = 3), A (n = 2), and E (n = 1). Phylogenetic analyses incorporating the isolates from this study and those retrieved from NCBI revealed no clustering related to the source of isolation, geographic origin, or host species.

3.4. Pathogenicity test

M. melolontha survival following submersion in conidia suspensions of *B. brongniartii* BIPESCO 2, *B. pseudobassiana* isolated from infected *M. melolontha* adults, which just emerged from the soil (*Mm*_soil-1: original isolate ID B21_A16, *Mm*_soil-2: original isolate ID B21_B26 and *Mm*_soil-3: original isolate ID B21_C30) or *B. pseudobassiana* isolated from tree leaves (Tree_lv-1: original isolate ID LX21_Q3, Tree_lv-2: original isolate ID LX21_R3 and Tree_lv-3: original isolate ID B21_P1) was significantly lower compared to the control group ($p < 0.005$; Fig. 4). Twenty days post infection, all beetles receiving a fungal treatment were dead and the Hazard ratios (HR) for treatment with BIPESCO 2, *B. pseudobassiana* isolated from *M. melolontha* and *B. pseudobassiana* isolated from tree leaves were 9.8 (±0.27), 2.5 (±0.25), and 2.6 (±0.25), respectively. *M. melolontha* adults treated with BIPESCO 2 showed higher mortality rates and significantly lower survival probability compared to the other two treatments ($p < 0.005$; Fig. 4). *M. melolontha* individuals treated with BIPESCO 2 showed a lower median survival time (7 d) compared to beetles treated with *B. pseudobassiana* (9 and 10 d) or untreated (13 d; Fig. 4). No overall significant differences in survival probability were detected between treatments with *B. pseudobassiana* isolated from infected beetles or tree leaves ($p > 0.005$; Fig. 4).

After 21 days, all beetles were dead. Of the surface sterilized and incubated cadavers, a total of 123 fungal isolates emerged and were genotyped after cultivation (Supplementary Table 6, 7). From *M. melolontha* treated with BIPESCO 2 (N = 75), 58 isolates of *B. brongniartii* were obtained, with 57 displaying the microsatellite-based MLG of BIPESCO 2, while one isolate displayed a unique *B. brongniartii* MLG, i.e., MLG 51, not detected from other sources in this study (**Supplementary Table 6**, Supplementary Fig. 9). Twenty-three *Beauveria* spp. isolates emerged from *M. melolontha* treated with the fungal suspension of *B. pseudobassiana* isolated from infected beetles (N = 75), with four, 16, and two isolates having MLGs *Mm*_soil-1, *Mm*_soil-2, or *Mm*_soil-3, respectively (Supplementary Table 7, Supplementary Fig. 9). In addition, one isolate displayed a MLG identical to isolate Tree_lv-2 (Supplementary Table 6, 7, Supplementary Fig. 9). A total of 30 *Beauveria* spp. isolates were collected from deceased *M. melolontha* adults that were treated with the three *B. pseudobassiana* isolates obtained from tree leaves (N = 75), of which, two, 23 and four had MLG as Tree_lv-1, Tree_lv-2 and Tree_lv-3, respectively (Supplementary Table 7, Supplementary Fig. 9). One additional isolate was identified as *B. brongniartii* MLG 57. MLG 57 was also detected in a single isolate from the control group (Supplementary Table 6, Supplementary Fig. 9). From the 27 deceased *M. melolontha* in the control group (N = 75), 12 *Beauveria* spp. isolates emerged. Species affiliation based on sequence analyses of the nuclear intergenic region Bloc assigned six of these isolates each to *B. brongniartii* or *B. pseudobassiana*, with six MLGs (MLG52-MLG57) and six MLGs (MLG9 and MLG25-MLG29) detected within the two species, respectively. None of these MLGs were detected among any of the isolates from Laax and Bristen. One isolate of the control group displayed MLG9, which corresponds to the MLG of isolate Tree_lv-1, an isolate obtained from a tree leaf in Laax and included in the virulence assay (Supplementary Table 6, 7, Supplementary Fig. 9). Two *B. pseudobassiana* MLGs (MLG30 and 31), which were not detected from

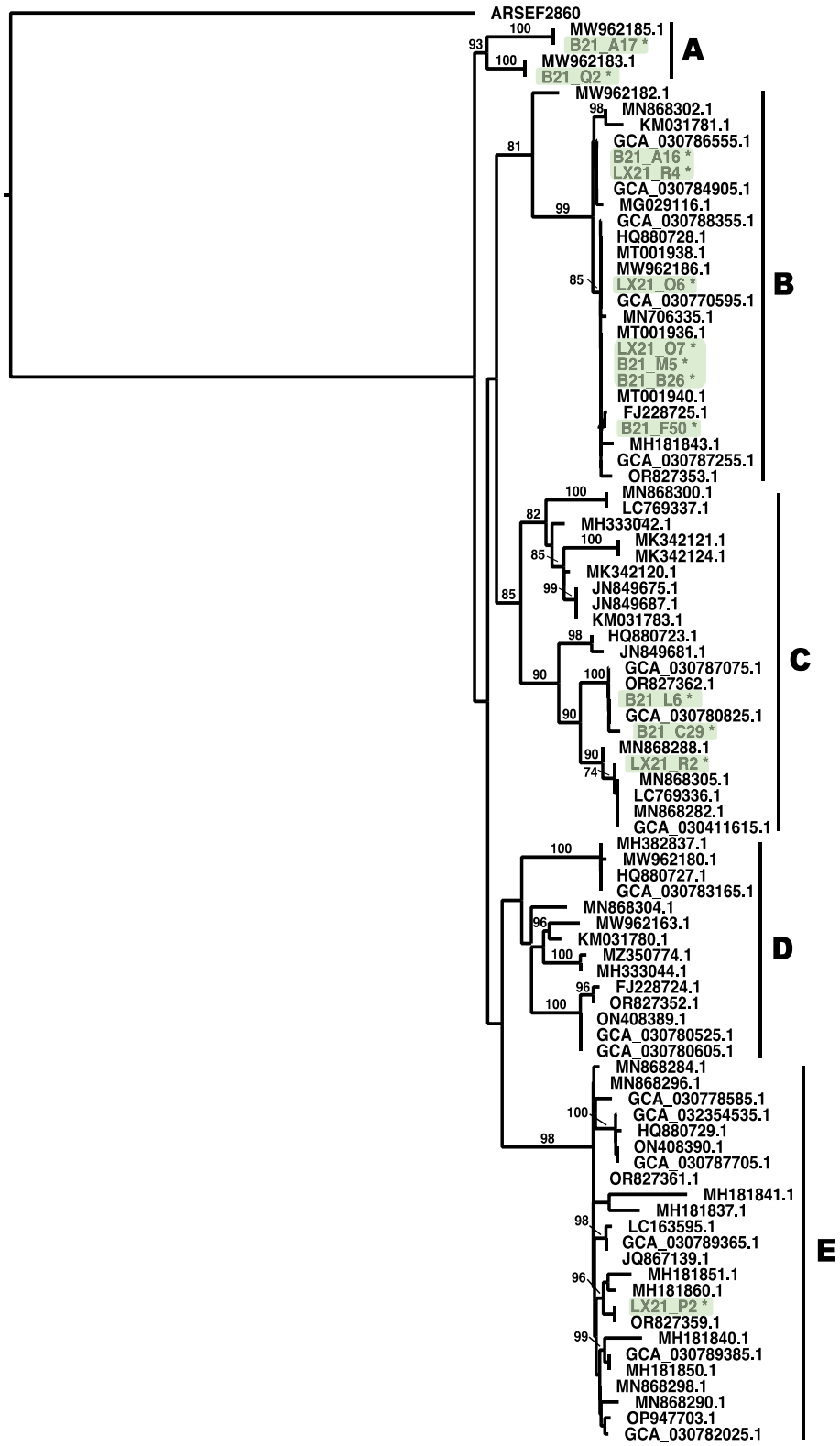

Fig. 3. Phylogenetic tree of *Beauveria pseudobassiana* based on Bloc sequences (from both GenBank and this study) and, for genome data, complete sequences of sphingomyelin phosphodiesterase, glycosyl hydrolase 92, and Midasin I ATPase (MDN1). The tree was inferred using the maximum likelihood (ML) method, with branch support calculated from 1,000 fast bootstrap pseudoreplicates. Isolates corresponding to the 13 Bloc haplotypes identified in this study are marked with asterisks and highlighted in green. All other sequences were obtained from GenBank and are labeled by their GenBank accession numbers, as detailed in Supplementary Tables 2 and 3. The five prominent clades are indicated (A – E).

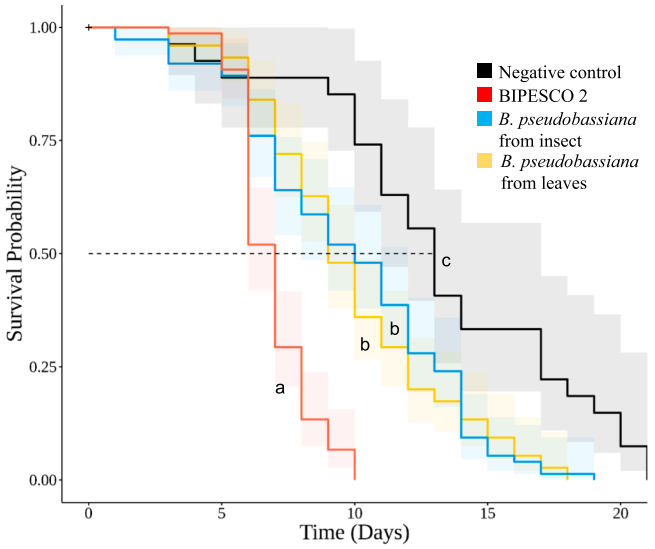

Fig. 4. Kaplan-Meier survival curves for *Melolontha melolontha* adults following immersion in fungal suspensions. Survival probability plotted as a function of time (days) after treatment with fungal suspension of BIPESCO 2 (red), *Beauveria pseudobassiana* isolated from infected *M. melolontha* adults (blue, mixture of isolates *Mm*_soil-1, *Mm*_soil-2 and *Mm*_soil-3) and *B. pseudobassiana* isolated from tree leaves (yellow, mixture of Tree_lv-1, Tree_lv-2 and Tree_lv-3). Shaded areas represent 95 % confidence intervals. Kaplan-Meier curves for each treatment are based on three replicates each consisting of 25 *M. melolontha* adults. Different letters indicate significant differences among treatments.

M. melolontha adults, were isolated from *Corylus avellana* fresh leaves used to feed *M. melolontha* adults during the experiment (Supplementary Table 7).

4. Discussion

This study aimed to explore the occurrence and distribution of the pathogens *B. brongniartii* and *B. pseudobassiana* within different ecological niches at two Swiss sites heavily infested with *M. melolontha*, and to identify potential locations in the environment where adult beetles may acquire *Beauveria* spp. infections. Consistent with previous research (Dolci et al., 2006; Keller et al., 2003), *B. brongniartii* emerged as the predominant pathogen, widely isolated from soil, but absent from tree leaves. In contrast, *B. pseudobassiana* was isolated from all sources except soil and was notably detected on tree leaves. One microsatellite-based MLG of *B. pseudobassiana* was found in both tree leaves and a *M. melolontha* in the tree canopy, suggesting a potential aboveground fungal infection. *B. pseudobassiana* from tree leaves was pathogenic to *M. melolontha*, further suggesting its potential role in infecting adult beetles.

At both locations, *B. brongniartii* was the predominant pathogen found in *M. melolontha* adults, whether obtained from soil or the tree canopy. These results differ from those of Pedrazzini et al. (2024), who found that, on average, *B. pseudobassiana* was the predominant fungus isolated from *M. melolontha* adults collected aboveground on trees across 35 Alpine sites. However, Pedrazzini et al. (2024) also noted high variability in the relative abundance of *B. pseudobassiana* and *B. brongniartii* among sites, with some locations showing a high prevalence of *B. pseudobassiana* and others dominated by *B. brongniartii* (e.g., 100 % *B. pseudobassiana* at 11 sites, 100 % *B. brongniartii* at two sites). Notably, a site at Bristen located 300 m adjacent to site sampled in this study was included in the study of Pedrazzini et al. (2024). At this site beetles were collected in 2018, and a *B. pseudobassiana/B. brongniartii* ratio of 1:2 was reported. In contrast, our findings at Bristen in 2021 showed a ratio of 1:20, suggesting that the abundance ratio between these species can vary over short distances or shift over time, even at sampled locations that are only 300 m apart.

At both sites, *B. brongniartii* was the most frequently isolated *Beauveria* spp. from soil where adult *M. melolontha* beetles emerged, which aligns with findings from previous studies across Europe that identified *B. brongniartii* as the dominant entomopathogenic fungus in *M. melolontha*-infested soils (Mayerhofer et al., 2015). In contrast, *B. pseudobassiana* was not detected in our soil samples, despite being commonly isolated from soil in previous studies (Mayerhofer et al., 2015; Niemczyk et al., 2019). In a recent review, Gielen et al. (2024) emphasized the complexity of natural entomopathogenic fungal infection dynamics, highlighting key factors that may influence infection rates, such as density-dependent processes in regulating natural insect populations or the spatial distribution of both insects and their pathogens in the environment. The absence of *B. pseudobassiana* might indicate that it is less competitive in belowground environments, occurs at lower densities, or may have been undetected due to methodological limitations, such as sampling scope or cultivation biases. A deeper exploration and long-term monitoring of *Beauveria* species, both in soil and among soil-dwelling larvae, a topic that remains underexplored, would help clarify the dynamics of *Beauveria* spp. community structure in soil ecosystems and natural insect populations. In aboveground plant samples of each site, both *B. brongniartii* and *B. pseudobassiana* were detected. However, *B. brongniartii* was isolated exclusively from grassland plants, suggesting its link to adjacent soil and soil-connected habitats. Previous studies have documented *B. brongniartii* in the rhizosphere, colonizing root surfaces and persisting endophytically when inoculated into plant leaves and stems (Jaber & Enkerli, 2017; Matek et al., 2019). It remains unclear from this study whether *B. brongniartii* was present endophytically within grassland plants or epiphytically on their surface. The presence of *B. brongniartii* may result from widespread soil applications of BCA products or dispersal of fungal conidia via air or rain splash from soil or infected *M. melolontha* individuals, which could result in the transient presence of *B. brongniartii* on the surface of grassland plants. Furthermore, conidia might be dispersed from soil to aboveground plant parts by emerging *Melolontha* adults or non-host insects as demonstrated by Meyling et al. (2006), who have reported dispersal of fungal inoculum by *Anthocoris nemorum* (L.) (Heteroptera: Anthocoridae) from soil to the nettle canopy, as well as spread of conidia from infected insect cadavers. In contrast, *B. pseudobassiana* was found on both grassland plants and tree leaves, including those consumed by *M. melolontha* and used in pathogenicity tests, indicating its widespread presence in aboveground ecosystems. Recent studies conducted in Spain and Denmark also reported *B. pseudobassiana* as an epiphyte of various plant species, including *Olea europaea*, *Quercus ilex* and *Tilia* × europaea (Garrido-Jurado et al., 2015; Howe et al., 2016). These findings, along with ours, demonstrate the occurrence of *B. pseudobassiana* aboveground on different plant species across regions with diverse climatic conditions.

The prevalence of *B. pseudobassiana* aboveground from plant material and *B. brongniartii* belowground might suggest a potential niche differentiation, possibly reducing competition and enabling their coexistence (Mujic et al., 2016). Such differentiation may result from varying competitive advantages in different habitats, shaped by factors such as virulence, insect-host susceptibility, specificity, and environmental conditions (Fernández-Bravo et al., 2016; Hare & Andreadis, 1983; Romaña & Fargues, 1992; Valero-Jiménez et al., 2016). For instance, Canfora et al. (2017) demonstrated that the competition between *B. bassiana* and *B. brongniartii* can vary depending on the substrate, with each species exhibiting advantages on different types of carbon sources. Moreover, some insect species possess a defensive microbiome on their cuticles, which can protect them against fungal infections (Hong et al., 2023). Recent observations (Baur, Küng, Pedrazzini & Enkerli, unpublished) showed that *M. melolontha* larvae treated with antibiotics before exposure to *B. brongniartii* had higher mortality rates than untreated larvae, suggesting that surface microorganisms may influence their susceptibility. Changes in the cuticle microbiome across insect life stages

could further affect vulnerability to fungal pathogens. While *B. brongniartii* may have a competitive advantage below- and *B. pseudobassiana* aboveground, unraveling the competitive dynamics between these species within the ecosystem requires further investigation.

The exclusive presence of *B. pseudobassiana* aboveground on plant samples may be attributed to its adaptations and/or resilience to environmental conditions such as UV radiation and temperature. UV light, particularly UV-A and UV-B, can affect the germination and development of fungal conidia, influencing their persistence in exposed habitats (Fernández-Bravo et al., 2016), with fungal species exhibiting varying degrees of UV-B tolerance (Fernandes et al., 2015). While isolation of *B. pseudobassiana* from tree leaves suggests its possible adaptation to aboveground conditions, including UV exposure, Fernández-Bravo et al. (2016) found no discernible differences in UV-B tolerance between *B. bassiana* isolates from soil and those from the phylloplane. Similarly, Couceiro et al. (2021) found no correlation between UV-B tolerance and the latitude from which *Metarhizium* fungal isolates were obtained. Intriguingly, Dias et al. (2021) observed that *B. bassiana* and some *Metarhizium* species developed increased UV tolerance when conidia were produced under white light, possibly activating protective stress-responsive genes. Couceiro et al. (2021) reported that temperature also influences growth and viability of *Metarhizium* conidia. They demonstrated that *Metarhizium* species exhibit optimal growth and tolerance at different temperatures, with *M. brunneum* growing optimally at 25 °C, *M. robertsii* growing fastest at 33 °C and *M. anisopliae* showing the highest tolerance at 40 °C, although intraspecific variability was noted. Thus, the occurrence of *B. pseudobassiana* in aboveground habitats may be driven by a combination of genetic or epigenetic mechanisms that confer adaptation and/or resilience to environmental stressors such as UV radiation, high temperatures, and potentially other abiotic factors including drought and humidity.

Among *B. brongniartii* isolates, the BCA BIPESCO 2 and BIPESCO 4 MLGs were abundant in all sampled soils and were recovered from all sources except tree leaves. The highest prevalence of *M. melolontha* infection with BCA strain BIPESCO 2 was observed in beetles emerging from soil in Bristen. The persistence of BCA strains in treated areas, along with an increase in *M. melolontha* larvae infections within 1–2 years post-treatment (Enkerli et al., 2004), aligns with previous studies showing fungal persistence in soil for up to 15 years (Enkerli et al., 2001; Enkerli et al., 2004; Mayerhofer et al., 2015; Pedrazzini et al., 2024). BCA products were applied in Laax in 2019 and in Bristen in 2016 and 2019, which precedes the sampling of *M. melolontha* in this study by two years (Christian Schweizer, Agroscope; personal communication). The previous treatments with BCA-based products may have contributed to the high infection rates observed in our results. However, despite the prevalent BCA BIPESCO 2 and BIPESCO 4 MLGs, indigenous microsatellite-based MLGs of *B. brongniartii* were also detected at both sites, confirming that indigenous strains can persist and coexist with BCA strains (Enkerli et al., 2004; Mayerhofer et al., 2015; Pedrazzini et al., 2024; Schwarzenbach et al., 2009). However, to ascertain whether BCA strains outcompete indigenous MLGs when applied in high quantities and to assess changes in the frequency of MLGs over time, further longitudinal monitoring is required. Additionally, certain indigenous MLGs of *B. brongniartii* and *B. pseudobassiana* were more prevalent at the two sites than others. To our knowledge, no studies have investigated which factors drive the prevalence of specific MLGs in these species. In our pathogenicity assay, we applied mixtures of three *B. pseudobassiana* isolates (each representing a distinct MLG) at equal spore concentrations. Interestingly, the recovery of genotypes from infected beetles was not evenly distributed, with some MLGs being re-isolated more frequently than others, suggesting a possible fitness advantage during infection or post-infection development. This may include faster colonization, enhanced sporulation, or higher persistence within the host. While we are not aware of prior studies on MLG-specific dominance in *B. pseudobassiana*, similar patterns have been documented in *B. bassiana* and *Metarhizium* spp., where certain genotypes dominate in soil or plant-associated environments (Islam et al., 2023; Steinwender et al., 2015; Wang et al., 2022). Although the mechanisms underlying genotype-specific differences are not clear, variations in factors such as conidial adhesion, germination rate, and secondary metabolite production have been proposed as potential contributors (Islam et al., 2023). These same factors may influence the dominance of specific MLGs at Bristen and Laax, leading to more efficient sporulation and dispersal. A single MLG of *B. pseudobassiana* was identified in both tree leaves consumed by *M. melolontha* adults and a *M. melolontha* adult sampled from the same tree canopy. Similarly, Howe et al. (2016) detected *B. pseudobassiana* in the phylloplane of lime trees and on the beetle *Harmonia axyridis*, suggesting that beetles may acquire fungal infection in arboreal habitats. The detection of a MLG from tree leaves as well as in *M. melolontha* adults could indicate infection through feeding on or contact with *B. pseudobassiana*-colonized leaves. Nevertheless, based on the present results, we cannot exclude that the presence of *B. pseudobassiana* in the phylloplane is a consequence of *M. melolontha* adults transporting fungal propagules from the soil to the aboveground ecosystem. To fully understand the occurrence and abundance of *B. pseudobassiana* in aboveground habitats, further investigation involving a broader range of plant species, plant parts, and insect hosts is necessary.

B. pseudobassiana isolates established from tree leaves were pathogenic to *M. melolontha* adults. In a recent bioassay reported by Barta (2018), larvae of *Cameraria ohredella* placed on *B. pseudobassiana*-colonized leaves showed increased mortality, reduced pupal size, and caused significantly less leaf damages. Fungal outgrowth from dead larvae in this study was limited (i.e., 0.20–0.58 %), suggesting an indirect mechanism of antagonism involving antibiosis and plant-induced resistance (Barta, 2018). With its ability to persist in the phylloplane, *B. pseudobassiana* may have potential use as a BCA to control adult *M. melolontha* beetles swarming in the phylloplane that complements the existing biological control strategy using *B. brongniartii* to target the soil-dwelling larvae.

This study presents the first intraspecific phylogenetic analysis of *B. pseudobassiana*, incorporating isolates collected during this research alongside reference sequences from available databases. While many branches of the inferred phylogenetic tree received equivocal bootstrap support, the phylogenetic analyses based on the SMase, GH92 and MDN1 loci suggests the existence of cryptic diversity within *B. pseudobassiana*. Notably, there were no discernible patterns of geographic clustering, host association, or source of isolation, which suggests that these cryptic lineages share similar ecology. However, it is interesting to note that most reference isolates included in the analysis were isolated from the terrestrial adult phases of multiple insect species, further evidencing the potential for insect infection by *B. pseudobassiana* in aboveground habitats. The existence of cryptic phylogenetic partitions indicates that members of different lineages within *B. pseudobassiana* might possess unique biological and functional traits pertinent to their ecological roles and effectiveness as insect pathogens. Determining whether these partitions should be recognized as distinct phylogenetic species will require expanded taxon and gene sampling, awaits further investigation.

In conclusion, this study revealed that (1) *Beauveria* spp. were established in all sampled sources at both sites. (2) *B. brongniartii* was the prevalent pathogen of *M. melolontha* at both locations, and the most frequently isolated species from soil, while only *B. pseudobassiana* was found in the phylloplane. (3) Microsatellite molecular markers were developed for *B. pseudobassiana* and successfully applied to the sampled isolates. (4) Phylogenetic analyses displayed cryptic phylogenetic divergence within *B. pseudobassiana* and revealed no phylogenetic clustering based on the source of isolation. (5) *B. pseudobassiana* isolated from *M. melolontha* adults and the phyllosphere exhibited pathogenicity against *M. melolontha* adults. Altogether, this study sheds light on the occurrence of *B. brongniartii* and *B. pseudobassiana* at two *M. melolontha* infested sites in Switzerland, demonstrating the consistent presence of

B. pseudobassiana aboveground, that suggest its potential for development as a complementary biological control approach for adult *M. melolontha*.

Data availability statement

Sequences were deposited at GenBank BankIt database as accessions PQ406678-PQ406714 and PQ459198-PQ459247.

CRediT authorship contribution statement

Chiara Pedrazzini: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Stephen A. Rehner:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fiona Stewart-Smith:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Sara Boschi:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Franco Widmer:** Writing – review & editing, Visualization. **Jürg Enkerli:** Writing – review and editing, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization, Supervision.

Declaration of competing interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2025.108356.

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