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BRIEF REPORT

ENVIRONMENTAL MICROBIOLOGY

Clonal genomic population structure of *Beauveria brongniartii* and *Beauveria pseudobassiana*: Pathogens of the common European cockchafer (*Melolontha melolontha* L.)

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Abstract

Beauveria brongniartii is a fungal pathogen that infects the beetle *Melolontha melolontha*, a significant agricultural pest in Europe. While research has primarily focused on the use of *B. brongniartii* for controlling *M. melolontha*, the genomic structure of the *B. brongniartii* population remains unknown. This includes whether its structure is influenced by its interaction with *M. melolontha*, the timing of beetle-swarming flights, geographical factors, or reproductive mode. To address this, we analysed genome-wide SNPs to infer the population genomics of *Beauveria* spp., which were isolated from infected *M. melolontha* adults in an Alpine region. Surprisingly, only one-third of the isolates were identified as *B. brongniartii*, while two-thirds were distributed among cryptic taxa within *B. pseudobassiana*, a fungal species not previously recognized as a pathogen of *M. melolontha*. Given the prevalence of *B. pseudobassiana*, we conducted analyses on both species. We found no spatial or temporal genomic patterns within either species and no correlation with the population structure of *M. melolontha*, suggesting that the dispersal of the fungi is independent of the beetle. Both species exhibited clonal population structures, with *B. brongniartii* fixed for one mating type and *B. pseudobassiana* displaying both mating types. This implies that factors other than mating compatibility limit sexual reproduction. We conclude that the population genomic structure of *Beauveria* spp. is primarily influenced by predominant asexual reproduction and dispersal.

INTRODUCTION

Entomopathogenic fungi (EPF) are important pathogens and antagonists of insect species, including many agricultural and horticultural pests. Consequently, EPF are promising candidates for the development of effective biological control strategies (Duarte et al., 2016; Rombach et al., 1986). Among EPFs, species of the genus *Beauveria* offer potential as biological control agents (BCA) against a wide range of insect species due to their insect pathogenicity and their various ranges of host specificities (Zimmermann, 2007). The genus includes both generalist species, for example, *B. bassiana* and *B. pseudobassiana*, which infect species of many insect orders, and specialist species, for example, *B. brongniartii*, which specifically infects Coleoptera species (Maurer et al., 1997; Piatti et al., 1998; Wang et al., 2020).

While detailed investigations have focused on the potential of EPF as BCA of insect pests, there is a lack of knowledge on how populations of pathogens and their hosts are structured at local and regional scales, representing a limitation for a detailed understanding of the complex interactions between EPFs or BCAs and their insect hosts. With the advent of molecular genomic tools and advanced analytical approaches for population surveillance, a detailed insight into the genomic structure of both pathogens and their hosts can now be achieved. This allows inference of key factors that may affect population genomic structures like host-pathogen interaction and co-evolution, geographic and temporal dynamics as well as life history traits (Allen et al., 2018; Blasco-Costa & Poulin, 2013; Cheng et al., 2022; Mei et al., 2020).

In this study, we investigated the population genomic structure of EPFs of the genus *Beauveria* spp. infecting *Melolontha melolontha* L. (Coleoptera: Scarabaeidae) in an Alpine region in Europe. *M. melolontha*, the European cockchafer, is a widespread pest throughout central Europe, including the Alpine region (Dolci et al., 2006; Keller et al., 1997; Laengle et al., 2005; Pedrazzini et al., 2023). Damage is mostly caused by the larvae (white grubs), which feed on the roots of plant species, for example, potatoes, resulting in significant economic loss in agriculture and horticulture (Laengle et al., 2005; Sukovata et al., 2015; Wagenhoff et al., 2014). *M. melolontha* completes its life cycle in three to 4 years with infested areas being typically inhabited by a temporally synchronized population, that is, individuals at the same developmental stage. Adults can emerge at different sites and perform swarming flights in region-specific years, which results in temporally shifted and isolated region-specific populations of *M. melolontha* (Pedrazzini et al., 2023; Wagenhoff et al., 2014). In a recent study, we detected two main genomic clusters of *M. melolontha* in the same Alpine region reported in the present study, that is, northwest Alpine and South Tyrol, and we demonstrated that geographical separation and temporal isolation affect the population genomic structure of *M. melolontha* (Figure 1; Pedrazzini et al., 2023).

In Europe, the soil-borne insect pathogenic fungus *B. brongniartii* has been considered the most relevant and prevalent pathogen of *M. melolontha*, and its occurrence typically coincides with the presence of the cockchafer (Dolci et al., 2006; Keller et al., 2003). *B. brongniartii*, like other members of the genus *Beauveria*, is haploid and reproduces predominantly by

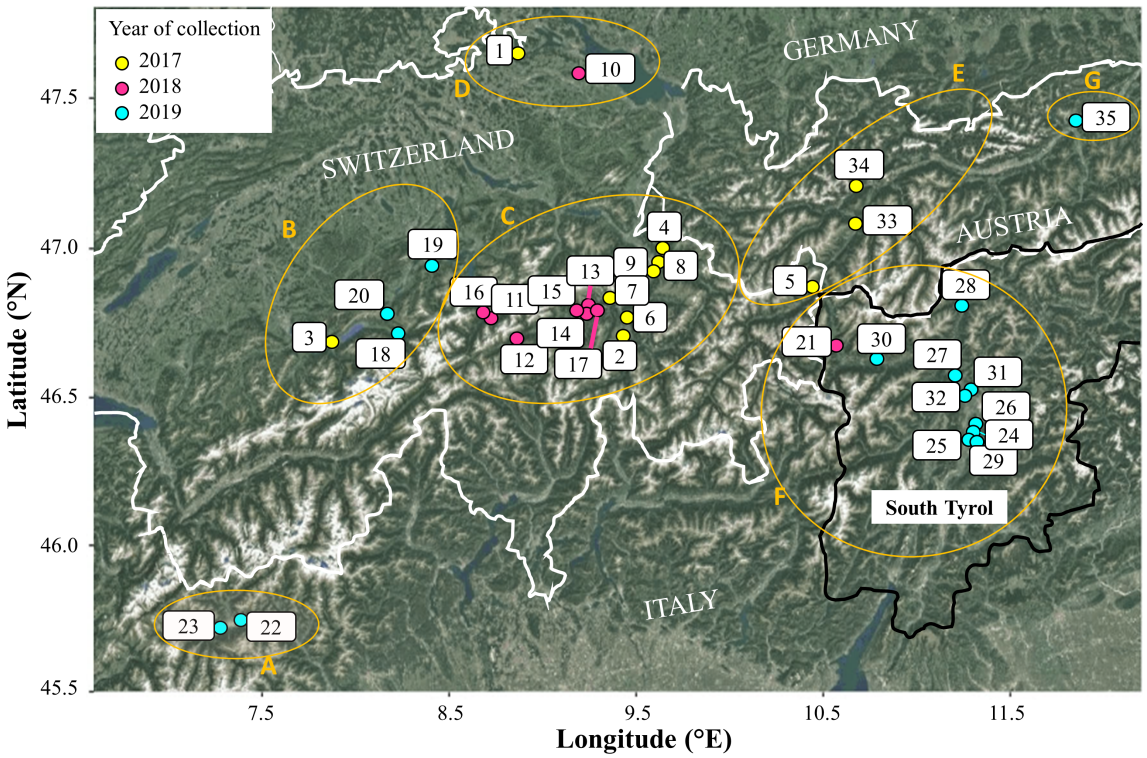

FIGURE 1 Map of collection sites of *Melolontha melolontha* adults (Pedrazzini et al., 2023), from which, after incubation in the laboratory, *Beauveria* spp. were isolated. Individual sites are indicated by colour-filled dots and corresponding numbers that are also given in Table A1. White lines indicate the borders of Switzerland, Italy and Austria. Colour-coding of the dots represents the year of collection of *M. melolontha* and isolation of *Beauveria* spp. Areas encircled in orange represent the defined sub-regions where collection sites were located: A: southwest Alps, B: northwest Alps, C: central Alps, D: north plateau, E: centraleast Alps, F: southeast Alps, G: east Alps. The region of South Tyrol is indicated with a black line.

asexual conidia often produced on mycosed host cadavers, and less frequently sexually, with mating occurring in co-infected hosts and meiosis and ascospore formation produced within *Cordyceps*-like fruiting bodies growing from host cadavers (Rehner et al., 2011; Sasaki et al., 2007). The sexual reproductive mode of *Beauveria* spp. is regulated by mating-type (MAT) genes, whose genomic organization determines whether the mating system is outcrossing or selfing (Bennett & Turgeon, 2016). In *Beauveria* spp., the MAT locus exhibits either a MAT 1–1–1 or MAT 1–2–1 idiomorph, and unequal mating type idiomorphs are required to initiate sexual mating between different *Beauveria* spp. strains. Molecular diagnostic detection of both mating types in *B. brongniartii* has only been demonstrated in two Asian strains (Yokoyama et al., 2006). Sexual morphs (*Cordyceps*-like fruiting bodies) of *B. brongniartii* have been reported from Japan, but their presence in European populations has not been documented and therefore it is still unclear whether sexual reproduction may occur in Europe (Sasaki et al., 2007; Shimazu et al., 1988).

Several studies have examined the use of *B. brongniartii* to control *M. melolontha* larvae, which cause most of the damage in agriculture and horticulture (Laengle et al., 2005; Sukovata et al., 2015; Wagenhoff et al., 2014). BCA-based products have been developed and have been commercially available since 1990, that is, Beauveria–Schweizer® (E. Schweizer Seeds, Switzerland) based on strain BIPESCO 4, and 2000, that is, Melocont® Pilzgerste (Agrifutur, Italy) based on strain BIPESCO 2 (Dolci et al., 2006; Enkerli et al., 2007; Keller et al., 1997; Mayerhofer et al., 2015). Application of these products has resulted in a high abundance of the BCA in soil (1×10^3–1×10^4 CFU g^{-1} dry weight of soil; Keller et al., 2002). Monitoring of applied *B. brongniartii* BCA and discriminating genotypes of indigenous isolates has been performed with microsatellite (simple sequence repeat, SSR) markers developed by Enkerli et al. (2001). Kessler et al. (2004) demonstrated that following application of *B. brongniartii* strain BIPESCO 4, abundance of *B. brongniartii* remains at elevated levels at sites with *M. melolontha* infestation as compared to *M. melolontha*-free sites and decreases as *M. melolontha* populations decline during the epizootic (period of increased *B. brongniartii* disease prevalence), emphasizing a close interaction between the two organisms and the dependence of *B. brongniartii* on *M. melolontha* for its proliferation. Investigations conducted at various treated sites have shown that, despite high BCA concentrations following application, naturally occurring *B. brongniartii* isolates can persist in treated fields and co-occur with the BCA at the same site (Enkerli et al., 2004; Mayerhofer et al., 2015; Schwarzenbach et al., 2009).

Despite extensive monitoring of the interaction between *B. brongniartii* and *M. melolontha*, considerable knowledge gaps persist. For example, while most studies have focused on *M. melolontha* larvae, it remains to be assessed whether *B. brongniartii* is also the main fungal pathogen infecting adults. Furthermore, it remains uncertain whether these two species due to their strict interaction (*B. brongniartii* mainly occurs at *M. melolontha*-infested sites) exhibit similar patterns in their population genomic structures, that is, whether factors such as the mobility and geographical distribution of *M. melolontha* adults, and the timing of *M. melolontha* swarming flights drive dispersal of *B. brongniartii* propagules and thereby influence the genomic structure of *B. brongniartii*. Additionally, whether specific life history traits of *B. brongniartii*, particularly its reproductive mode, influence its population genomic structure remains poorly known. To date, only a single population genetics study employing microsatellite markers has been conducted for *B. brongniartii* isolated from *M. melolontha* grubs and soil in central and southeastern Poland, in which no substantial population differentiation was detected among various sampling sites (Niemczyk et al., 2019). However, the use of single nucleotide polymorphisms (SNPs) for a comprehensive genome-wide investigation of *B. brongniartii* population structure at a comprehensive geographical scale, particularly within the context of its association with *M. melolontha*, has yet to be explored. Studies comparing the resolution of microsatellite and genome-wide SNP molecular markers in other taxa have shown that although both marker types perform well in estimating population genetic structure, SNP data provide higher resolution for multivariate analyses and quantification of the phylogenetic relationships among individuals. It has been shown that SSR markers occasionally fail to detect clear population structures when resolved by genome-wide SNP-based approaches (Ackiss et al., 2020; Lemopoulos et al., 2019; Thrasher et al., 2018).

Therefore, this study aimed to determine (1) whether *B. brongniartii* is the prevalent pathogen of *M. melolontha* adults. (2) Infer within and between the population genomic structure of *Beauveria* spp. isolated from infected *M. melolontha* adults collected from 35 sites in a central European Alpine region, and investigate (3) whether the genomic structure inferred for *Beauveria* spp. populations reflects or differs from the geographic and temporal structuring observed among *M. melolontha* host populations reported by Pedrazzini et al. (2023). (4) Infer the potential for sexual reproduction in *Beauveria* populations isolated from *M. melolontha* adults by performing population-wide PCR assays of mating type and (5) assess the prevailing mode of reproduction in *Beauveria* spp. by performing tests of recombination. (6) Compare the genotypic discrimination achieved with genome-wide SNPs to data obtained with the standard monitoring approach for *B. brongniartii* based on multilocus microsatellite genotyping.

EXPERIMENTAL PROCEDURES

Isolation of *Beauveria* spp. and DNA extraction

Beauveria spp. collections were sampled from 35 European sites infested with *M. melolontha* between 2017 and 2019, including 20 sites in Switzerland, 12 sites in Northern Italy, and three sites in Austria (Figure 1, Table A1). The sampled sites have been regularly or occasionally treated over the last 15–20 years with commercial BCA products based on the *Beauveria brogniartii* strains BIPESCO 2 (BCA product Melocont® Pilzgerste; strain originating from Kramsach, Tyrol, Austria) and BIPESCO 4 (BCA product Beauveria–Schweizer®; strain originating from Buochs, Nidwalden, Switzerland). A map depicting the locations of *Beauveria* spp. collections was produced with the R package ggmap 3.0.0 in R version 4.2.2 (Kahle & Wickham, 2013; Team, 2013). At each sampling site, 100–200 *M. melolontha* adults were collected and incubated in individual peat-filled cylindrical plastic containers of 4 cm diameter at 80% relative humidity and 22°C until beetle death and emergence and conidiation of *Beauveria* spp. Isolates were obtained from mycosed cadavers and cultivated on a semi-selective medium (Strasser et al., 1996). In addition, the 2 *B. brongniartii* BCA strains BIPESCO 2 and BIPESCO 4 were included as genetic references. Single-conidia subcultures were obtained for each isolate and maintained on 3% potato dextrose agar medium (PDA; Merck, Darmstadt, Germany). Mycelia were harvested from 7-day-old solid cultures, lyophilized for 6 h 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 two glass beads of 3 mm and 0.15 g of 1 mm diameter. DNA extractions were performed 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 visually on 1%-agarose gels and quantified with PicoGreen® fluorescent nucleic acid stain (Invitrogen, Carlsbad, CA, USA) in a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA, USA).

ddRADseq library preparation and sequencing

For double-digest restriction site-associated DNA sequencing (ddRADseq), 240 ng of high-quality DNA was prepared from each isolate according to Westergaard et al. (2019). The restriction enzymes *Eco*RI and *Taq*Ia (New England Biolabs, Ipswich, MA, USA) were used to double-digest genomic DNA, and T4 DNA Ligase (New England Biolabs, Ipswich, MA, USA) was applied to ligate digested DNA to biotinylated Illumina barcoded adapters (Table A2). Barcoded DNA samples of fungal isolates were then multiplexed into 16 ddRADseq libraries, each containing DNA of 46 barcoded fungal isolates as well as positive and negative controls. For each library, a 500 bp size selection was performed using 0.57x Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA), to obtain a collection of 400–700 bp fragments. Dynabeads® M-270 Streptavidin (Thermo Fisher Scientific, Waltham, MA, USA) was used to select biotinylated fragments, which were then washed and purified. PCR amplification was performed to enrich and label the libraries using primers with Illumina indexes and the Phusion® High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs, Ipswich, MA, USA; Table A3). Cycling conditions consisted of an initial denaturation of 2 min at 95°C and followed by 11 cycles of 20 s at 98°C, 20 s at 65°C and 30 s at 72°C. The resulting DNA for each Library was quantified with a Qubit 2.0 fluorometer (HS dsDNA kit, Thermo Fisher Scientific, Waltham, MA, USA), and fragment size was assessed on an Agilent 2200 Tape Station. Libraries were sequenced with 0.034–1.5 Mio reads using the NovaSeq 6000 platform with 150 bp paired end reads (Novogene, UK). Raw reads are available at the European Nucleotide Archive (ENA) under accession number PRJEB70245.

Sequence quality control, variant calling, and SNP filtering

Raw sequences were demultiplexed with the *process_radtags* component of stacks 2.55 (Catchen et al., 2013) and high-quality genome-wide SNP markers were detected. Reads were mapped against the reference genome of *B. brongniartii* (accession number: AZHA00000000.1; Shang et al., 2016) using bwa-mem2 2.2 (Vasimuddin et al., 2019) and low-quality mappings (MAP<20) were removed. Samples with low number of reads (<10,000) and mapping rates (<50%) were removed. After that, SNPs were called with freebayes 1.3.7 (Garrison & Marth, 2010) and filtered with vcftools 0.1.16 (Danecek et al., 2011) to satisfy the following criteria: (1) a minimum quality score of 30, (2) a minor allele count of five, (3) a minimum depth of two, (4) a minimum mean depth of five, (5) a minor allele frequency of 1% and (6) successfully genotyped in 50% of individuals. Individuals with more than 50% missing sites were excluded from the analysis. SNP loci with more than 20% missing data per population, excessive coverage (i.e., >45x), complex SNPs, and indels were excluded. Furthermore, loci with more than 5% missing data across the remaining individuals were also removed. Only biallelic sites were retained. For determining the index of association, loci separated by

≥10 bp were retained to avoid the effect of complex regions, for example, complex SNPs. In contrast, for analyzing population genomic structures, loci that were ≥one kilobase apart were retained to minimize issues related to linkage disequilibrium and to prevent biased results of population genomic structure (O'Leary et al., 2018). The software pgd Spider 2.1.1.5 was used for the conversion of the final vcf file to other formats (Lischer & Excoffier, 2012).

Species assignment

Species assignment of 18 *Beauveria* isolates representing each of the clusters resolved in an initial principal component analysis (PCA) based on SNP data was determined by phylogenetic analysis of nuclear intergenic region Bloc sequences (Rehner et al., 2006). The target locus was amplified with forward primer B5.1F (5′-CGACCCGGCCAACTACTTTGA-3′) and reverse primer B3.1R (5′-GTCTTCCAGTACCACTACGCC-3′). PCR was performed in 20 μL reactions, including 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. The PCR was finalized with 10 min at 72°C. Product quality was verified with 1.5%-agarose gel electrophoresis, and PCR products were purified with the Nucleospin® Gel and PCR clean-up kit (Macherey & Nagel, Germany). An internal region of the purified PCR product was sequenced with forward B22U (5′-GTCGCAGCCAGAGCAACT-3′, *B. brongniartii*) and B22U2 (5′-GTCGGAGCCAAAACAACT-3′, *B. pseudobassiana*) and reverse B822Ldg2 primer (5′-AGATTCGCAACGTCMACTTT-3′). Sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kits and a 3500xL Genetic Analyser (Applied Biosystems, CA, USA) equipped with 50 cm capillaries and the POP-7 polymer. Sequences were assembled and aligned with 23 reference *Beauveria* spp. sequences obtained from the GenBank database representing different *Beauveria* species (Rehner et al., 2011) using the software BioEdit® 7.0.9 (Ibis Biosciences, Carlsbad, CA, USA). Phylogenetic trees were inferred under maximum likelihood and Kimura 2-parameter correction model in MEGA11 (Kimura, 1980; Tamura et al., 2021). Sequences were deposited at GenBank BankIt database under the accession numbers OR827340-OR827344, OR827346-OR827349, OR827352, OR827353, OR827357-OR827362, OR827364.

Mating type assignment

To determine mating type idiomorphs of *Beauveria* spp., a MAT PCR-amplification protocol was developed and applied to all isolates of *B. brongniartii* and *B. pseudobassiana*. Amplification primers for the MAT 1-1-1 (MAT-1) and MAT 1-2-1 (MAT-2) idiomorphs were designed using Primer3 in Geneious Prime 2022.2.2 (https://www.geneious.com). Sequences for primer design included the MAT-1 sequence from the genome of *B. brongniartii* RCEF 3172 (AZHA00000000.1; Shang et al., 2016) and for MAT-2 the unpublished genome of *B. asiatica* ARSEF 4834 (SA Rehner, United States Department of Agriculture USDA, Beltsville, unpublished genome). Primers Bbr_Mat1_111F (5′-CGCCAC-CAAGTGTTTCGAAG-3′) and Bbr_Mat1_486R (5′-TTTGCCCATCTCGTCACGAA-3′) were used to amplify a 375 bp fragment of MAT-1, whereas primers Bbr_Mat2_19F (5′-CGGACCAAACTYCAAGACCA-3′) and Bbr_Mat2_408R (5′-GATATGCTTGCGCG-GAAGTG-3′) were used to amplify a 389 bp fragment of MAT-2. A multiplexed PCR reaction was performed in 20 μL reaction volumes, including 15 ng of genomic DNA, 1x GoTaq Flexi buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM (i.e., Bbr_Mat1_111F, Bbr_Mat1_486R, and Bbr_Mat2_408R) or 0.4 μM (i.e., Bbr_Mat2_19F) of all four primers (reverse primers labelled with ATO or FAM) and 0.25 U/μL GoTaq G2 Flexi DNA Polymerase (Promega, WI, USA). Cycling conditions consisted of an initial denaturation of 2 min at 95°C followed by 24 cycles of 30 s at 95°C, 30 s at 59°C and 1 min at 72°C, with a final extension for 7 min at 72°C. Sizes of amplification products were determined with a 3500xL Genetic Analyser (Applied Biosystems, CA, USA) using 50 cm capillaries and POP-7 polymer. GENESCAN™ 400HD [ROX™] was included as an internal size standard.

Microsatellite marker analyses

B. brongniartii isolates from clusters 2 and 3 resolved by k-means clustering, including the reference strains BIPESCO 2 and BIPESCO 4, were genotyped at six microsatellite loci (Enkerli et al., 2001). Target loci were amplified in two multiplexed PCR reactions, each including a set of three primer pairs (Bb1F4, Bb5F4 & Bb8D6 and Bb2A3, Bb2F8 & Bb4h9), with forward primers labelled with ATO, HEX, or FAM (Microsynth, Balgach, CH). PCR reactions 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). Touch-down PCR cycling conditions 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. Amplicon sizing was performed on a 3500xL Genetic Analyser (Applied Biosystems, CA, USA) with 50 cm capillaries and POP-7 polymer, and fragment sizes were estimated with GeneMarker® software (SoftGenetics, PA, USA).

Population genomic structure analyses

PCAs 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). Estimates of genotype diversity, that is, the Shannon Wiener index, were calculated with the R package poppr 2.9.3 (Kamvar et al., 2014). To identify and visualize genomic clusters in the data, a discriminant analysis of principal components (DAPC) was implemented and run with ade4 1.7-18 (Dray & Dufour, 2007) and adegenet 2.1.5 (Jombart, 2008) without a priori group information, with the function *find.cluster*. DAPC does not presume linkage equilibrium or Hardy–Weinberg equilibrium, employs sequential K-means, and relies on the Bayesian information criterion to infer clusters (Jombart et al., 2010). Maps showing the relative membership coefficient to clusters were created with rworldmap 1.3-6 (South, 2011) and marmap 1.0.6 (Pante & Simon-Bouhet, 2013). Neighbour-joining trees were calculated based on Nei's genetic distance with 1000 bootstrap values with the R packages poppr 2.9.3 (Kamvar et al., 2014), ape 5.5 (Paradis & Schliep, 2019) and ggtree 3.0.4 (Yu, 2020). To compare SNP and microsatellite datasets, a Mantel test was performed between the Nei's genetic distance of the SNP data and the Bruvo genetic distance (i.e., model assuming stepwise mutation) among microsatellite data using the R package vegan 2.6-2 (Oksanen et al., 2013) with 1000 permutations.

To investigate the index of association and population differentiation according to subregions and sampling years, *Beauveria* spp. SNP datasets were clone-corrected by including unique contracted genotypes per collection, to prevent bias due to asexual reproduction and to avoid redundancy. Multilocus-genotypes (MLGs) were collapsed into multilocus lineages (MLLs) based on the Nei's genetic distance with a threshold value of 0.08 and 0.03 in *B. brongniartii* and *B. pseudobassiana*, respectively, and clone-corrected with the R package poppr 2.9.3 (Kamvar et al., 2014). Genomic differentiation between geographic areas and years of sampling was estimated with an analysis of molecular variance (AMOVA). For this, data were stratified into groups defined by the year of sampling (i.e., 2017–2019) and geographic area (i.e., A–G) in which they were collected (Figure 1). A total of seven subregions (i.e., A: southwest Alps, B: northwest Alps, C: central Alps, D: north plateau, E: central east Alps, F: southeast Alps, G: east Alps) were defined by grouping collections belonging to proximate biogeographic areas (maximum distance 30 km, Figure 1). Variance quantification among subregions/year of sampling, among collections within subregions/year of sampling, and within collections was performed with hierarchical AMOVA conducted in poppr 2.9.3 (Kamvar et al., 2014). Pairwise Nei's genetic distance among collections of *Beauveria* spp. was calculated using the R package adegenet 2.1.5 (Jombart, 2008), and the Euclidean geographic distance matrix was obtained with the R package reat 3.0.3 (Wieland, 2020). To test for the correlation of genetic and geographic distances, a Mantel test was performed using the R package vegan 2.6-2 (Oksanen et al., 2013) with 1000 permutations between pairwise genetic distance and pairwise geographic distance matrices. For illustration, Nei's genetic distance was regressed against geographic distance and visualized with R package ggplot2 3.3.5 (Wickham, 2009).

The index of association (I_A; Brown, 1975) and the related statistic $\bar{r}$(rd = rbarD = rD; Agapow & Burt, 2001) were calculated with R package poppr 2.9.3 (Kamvar et al., 2014) with 1000 permutations. The I_A recombination test was used to determine the extent of linkage equilibrium by testing the null hypothesis of unlinked loci expected in sexually recombining populations (values of I_A and $\bar{r}$ close to zero imply linkage equilibrium, values significantly different from zero indicate disequilibrium, suggesting a prevalence of clonal reproduction).

Beauveria spp. and *Melolontha melolontha* comparison

In a previous study, the population genomic structure of *M. melolontha* was collected at the same 35 sampling sites as studied here for *Beauveria* spp. (Figure 1, Table A1) was investigated based on genome-wide SNPs (Pedrazzini et al., 2023). To test whether *Beauveria* spp. gene flow is mediated by *M. melolontha* dispersal, two new subsets of *M. melolontha* SNPs were constructed, including only sites with co-occurrence with *B. brongniartii* (24 sites), or with *B. pseudobassiana* (33 sites). This yielded datasets of 9659 SNPs among 311 *M. melolontha* individuals at sites where they co-occurred with *B. brongniartii*, and 9641 SNPs among 446 *M. melolontha* individuals at sites with *B. pseudobassiana* co-occurrence. Nei's genetic distance matrices were calculated for *Beauveria* spp. and *M. melolontha* in the R package poppr 2.9.3 (Kamvar et al., 2014). Genetic distance matrices were compared between *M. melolontha* and the clone-corrected data of *Beauveria* spp. with a Mantel test (1000 permutations) performed with the R package vegan 2.6-2 (Oksanen et al., 2013).

RESULTS

Species assignment and multivariate analyses

The prevalence of *Beauveria* spp. infection of *M. melolontha* adults at the 35 sampling sites ranged from 4.5% to 42.9% (Table A1) and a total of

541 *Beauveria* spp. isolates were obtained, with 6–20 fungal isolates per site (Figure 1, Table A1). No other insect pathogenic fungal species were detected in the 35 collections. Following mapping to the reference genome and SNP filtering, 22 fungal isolates that did not satisfy the quality criteria were excluded from further analyses, resulting in a final dataset of 686 SNPs across 519 isolates, including the two commercial BCA strains BIPESCO 2 and BIPESCO 4. In the first PCA of the ddRADseq SNP data, the first axis separated the 519 isolates into three main clusters, that is, explaining 81.7% of the overall variance, while the second axis, separated 333 fungal isolates, that is, explaining 7.6% of the overall variance (Figure 2). Species determination performed on 18 fungal isolates, representing 2–8 isolates from each of the three groups (defined on first PCA axis), identified the three groups as three *Beauveria* species, that is, *B. brongniartii* (green, $N = 182$ and the BCA strains BIPESCO 2 and BIPESCO 4), *B. pseudobassiana* (blue, $N = 333$), and *B. bassiana* (purple, $N = 2$; Figure 2, Figure A1). Shannon–Wiener genetic diversity (H) was highest among *B. pseudobassiana* isolates ($H = 5.07$) as compared to *B. brongniartii* ($H = 3.44$) and *B. bassiana* ($H = 0.69$; Figure 2). *B. brongniartii* and *B. pseudobassiana* were detected at 24 and 33 of the 35 sampling sites (co-occurrence at 22 sites) and the number of isolates ranged from 3 to 18 isolates per site (Table A1). Due to the low number of isolates available, *B. bassiana* was excluded from subsequent analyses. Two new SNP datasets for population genomic structure and index of association (I_A) analyses were constructed including only isolates of *B. brongniartii* (population genomic structure 96 SNPs, I_A 157 SNPs) or *B. pseudobassiana* (population genomic structure 955 SNPs, I_A 2990 SNPs) to allow separate analyses of the two prevalent species isolated from *M. melolontha*.

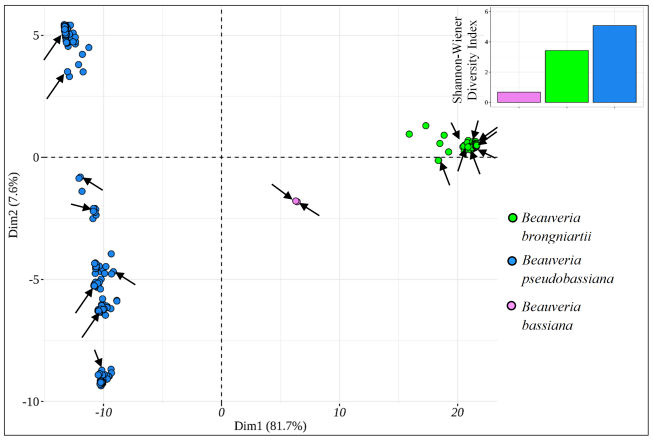

FIGURE 2 Principal components analysis (PCA) based on 686 SNPs including the 517 *Beauveria* isolates from the 35 sampling sites and the BCA strains BIPESCO 2 and BIPESCO 4. *Beauveria brongniartii*, *B. pseudobassiana*, and *B. bassiana* isolates are represented by green, blue, and purple circles, respectively. Arrows indicate fungal isolates selected to determine species affiliation. Shannon–Wiener index (H) of genetic diversity values for the three species are shown in bar plots in the upper right.

Population genomic structure

DAPC resolved three and four clusters in the genomic population structure of *B. brongniartii* and *B. pseudobassiana*, respectively (Figure 3, Figure A2A, B). In *B. brongniartii*, the three clusters, designated Bbr-1, Bbr-2, and Bbr-3 included 101, 43, and 38 isolates, respectively (Table A4). The BCA strain BIPESCO 2 was assigned to Bbr-2, while strain BIPESCO 4 was associated with Bbr-3 (Table A4, Figure 3A). The DAPC scatterplot showed clear genomic differentiation of Bbr-2 from clusters Bbr-1 and Bbr-3. Isolates of Bbr-1 formed a grade of several clades in the neighbour-joining tree, however, few were supported by high bootstrap values, while clusters Bbr-2 and Bbr-3 formed more coherent clades (Figure 3B). Cluster Bbr-1 was the most abundant clade and was detected at 20 of the 24 sampling sites where *B. brongniartii* was found (Figure 4A, Table A4). The simultaneous presence of the three *B. brongniartii* clusters was observed at five sites (9-Zizers, 11-Bristen, 28-Passeier-Sandwirt, 29-Plattl and 34-Schoenwis), while only one cluster was observed at 11 sites (2-Masein, 6-Tomils, 7-Trin Mulin, 10-Andhausen, 12-Disentis, 16-Silenen, 17-Valendas, 18-Aareschlucht, 26-Laimburg, 31-Siebeneich, 32-Unterrain). In South Tyrol, a prevalence of Bbr-2, including strain BIPESCO 2, was observed (Figure 4).

Four clusters comprising 54, 50, 47, and 182 isolates were discriminated in *B. pseudobassiana*, respectively designated as Bps-1, Bps-2, Bps-3, and Bps-4 (Figure 3C, Table A5). Isolates belonging to cluster Bps-2 were separated into several clades in the neighbour-joining tree, whereas isolates of Bps-1, Bps-3, and Bps-4 each formed discrete clades (Figure 3D). Cluster Bps-4 was the most widespread and present at 33 sampling sites (Figure 5). Isolates of Bps-4 were the most abundant in Switzerland (108 out of 192 samples), in Italy, (62 out of 120), and in Austria (12 out of 21). The co-occurrence of the four clusters was observed at five locations (i.e., 10-Andhausen, 15-Siat, 21-Glurns, 35-Muenster, and 30-Schlanders), and at one site (i.e., 11-Bristen) only Bps-4 was observed.

Comparison of molecular markers

Isolates of Bbr-2 and Bbr-3, including the two commercialized biocontrol strains BIPESCO 2 and BIPESCO 4, were further examined with microsatellites to compare their resolution with that of SNPs. Excluding the reference BCAs, in Bbr-2 ($N = 43$), three microsatellite

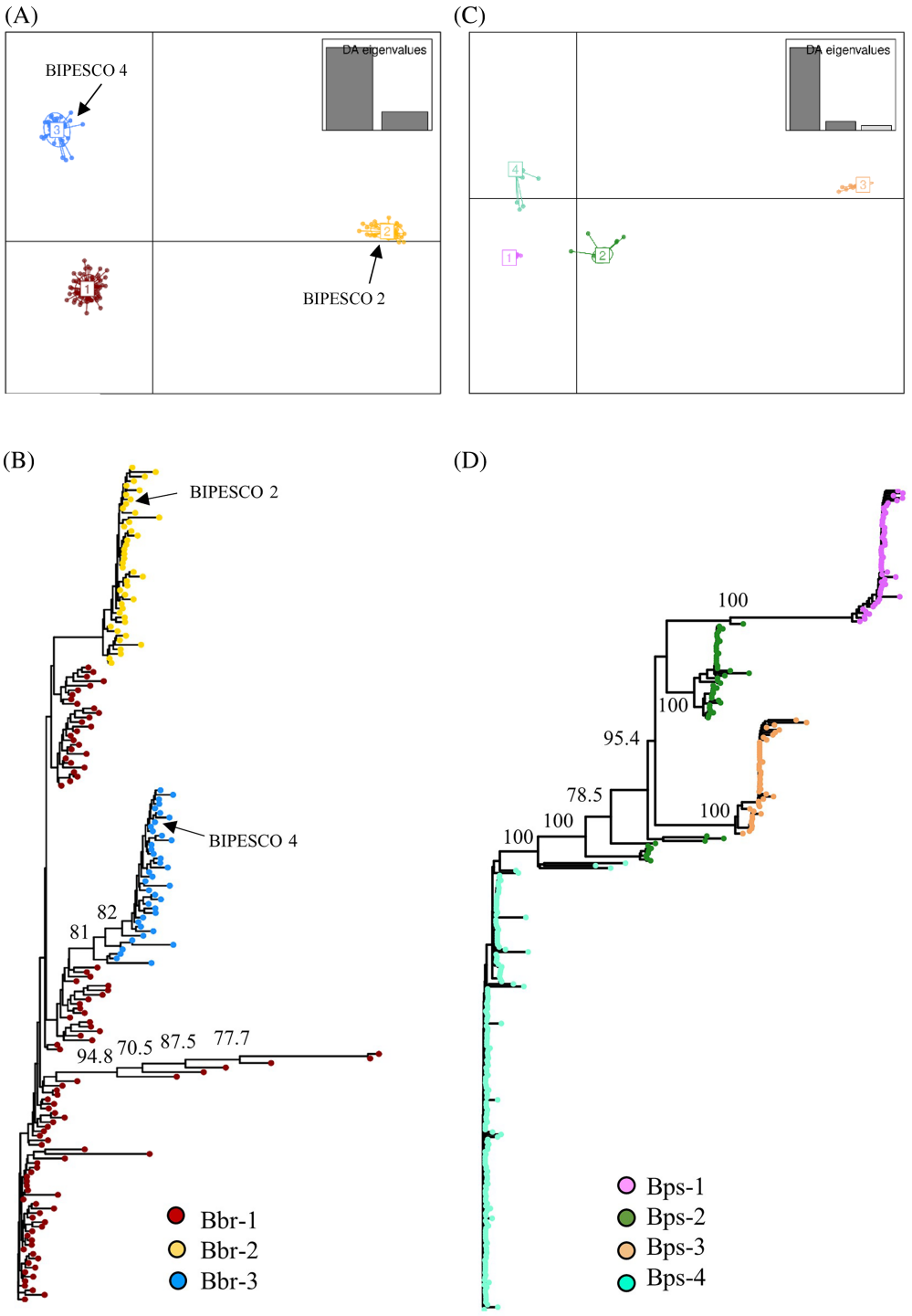

FIGURE 3 Discriminant analysis of principal components (DAPC) scatterplot and neighbour-joining tree (bootstrap values >70% at major phylogenetic clades are labelled at relevant nodes) for *Beauveria brongniartii* and *B. pseudobassiana*. (A) DAPC and (B) neighbour-joining tree for *B. brongniartii* (96 SNPs; $N = 184$) including BIPESCO 2 and BIPESCO 4. (C) DAPC and (D) neighbour-joining tree for *B. pseudobassiana* (955 SNPs; $N = 333$). *B. brongniartii* and *B. pseudobassiana* clusters 1–3 and 1–4 are highlighted in colour, and cluster membership of *B. brongniartii* strains BIPESCO 2 and BIPESCO 4 are indicated by arrows.

MLGs were detected, and the MLG corresponding to the MLG of the commercialized strain BIPESCO 2, accounted for 34 out of 43 isolates. In Bbr-3 ($N = 38$), a total of 19 microsatellite MLGs were identified among 38 isolates, and 13 isolates had an MLG identical to the commercial strain BIPESCO 4. Isolates identified as BIPESCO 2 and BIPESCO 4 by microsatellite markers were observed at 10 sites

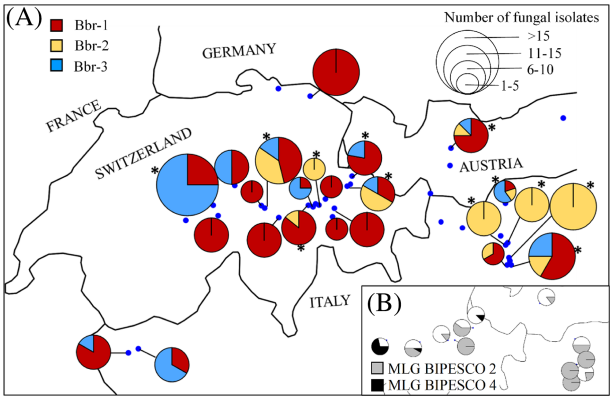

FIGURE 4 (A) Locations of collection sites of *Beauveria brongniartii* and their discriminant analysis of principal components (DAPC) derived affiliation to different clusters at $K = 3$ (96 SNPs, $N = 182$). Blue dots indicate the 35 sampling sites in the European Alpine region and pie charts indicate occurrence and proportions of *B. brongniartii* isolates belonging to clusters inferred by DAPC analyses. Asterisks indicate sampling sites at which microsatellite based MLGs BIPESCO 2 and MLG BIPESCO 4 were detected. The size of the pie charts represents the number of fungal isolates per sampling site, indicating sites with the following number of isolates: 1–5, 6–10, 11–15, and more than 15. (B) Ratio of MLG BIPESCO 2 and BIPESCO 4 detected at different sampling sites are indicated in grey and black, respectively.

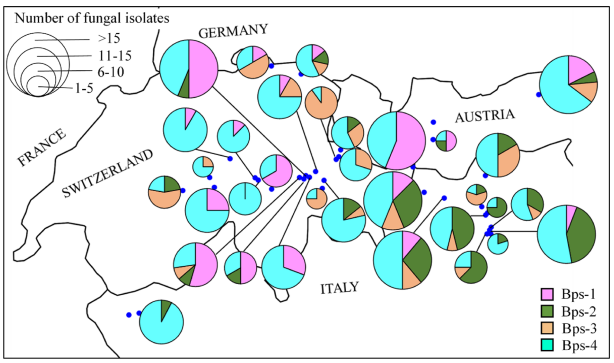

FIGURE 5 Locations of collection sites for *Beauveria pseudobassiana* and their discriminant analysis of principal components (DAPC) derived affiliation to different clusters at $K = 4$ (955 SNPs, $N = 333$). Blue dots indicate the 35 sampling sites in the European Alpine region and pie charts indicate occurrence and proportions of *B. pseudobassiana* isolates belonging to clusters inferred by DAPC analyses. The size of the pie charts represents the number of fungal isolates per sampling site, indicating sites with the following number of isolates: 1–5, 6–10, 11–15, and more than 15.

(9-Zizers, 34-Schoenwies, 11-Bristen, 15-Siat, 17-Valendas, 26-Laimburg, 29-Plattl, 31-Siebeneich, 32-Unterrain, 28-Passeier-Sandwirt) and three sites (4-Seewis, 11-Bristen, 20-Lungern), respectively (Figure 4B). Microsatellite markers failed to detect any population genetic structure within *B. brongniartii*. In contrast, the SNP-based PCA analysis revealed greater population genomic differentiation within *B. brongniartii*, in which the first dimension separated the isolates into two main groups (i.e., 65.1% of the overall variance; Figure A3A, B). However, the corresponding neighbour-joining trees showed roughly congruent patterns of phylogenetic relationships among isolates of Bbr-2 and Bbr-3 (Figure A4A, B). A Mantel test of SNP and microsatellite genetic distance matrices among the 83 fungal isolates including both BCA strains BIPESCO 2 and BIPESCO 4 revealed a significant positive correlation (r: 0.84, p <0.001).

Mating type

The MAT PCR-amplification protocol was applied to the 519 *Beauveria* spp. isolates to determine mating type idiomorphs and infer reproductive strategies of *B. brongniartii* and *B. pseudobassiana*. A single mating type, that is, MAT-1 (MAT 1-1-1), was detected for each of the 184 isolates of *B. brongniartii*, including the two reference BCAs, BIPESCO 2 and BIPESCO 4 (Table A4). In contrast, 213 and 120 isolates of *B. pseudobassiana* displayed MAT-1 or MAT-2 (MAT 1-2-1), respectively (Table A5). The co-occurrence of MAT-1 and MAT-2 idiomorphs in *B. pseudobassiana* was detected at 28 of 33 sampling sites, while at five sampling sites, only MAT-1 was detected (3-Matten, 7-Trin Mulin, 9-Zizers, 14-Ilanz and 33-Prutz; Table A5). The ratio of MAT-1 and MAT-2 in Bps-1, Bps-3, and Bps-4 was similar, that is, 63.2%–79.6% MAT-1 and 20.4%–36.8% MAT-2, but in Bps-2, idiomorph MAT-2 was more abundant (64%) than MAT-1 (36%; Figure A5). Co-occurrence of MAT-1 and MAT-2 within Bps-1, Bps-2, Bps-3, and Bps-4 was detected at 5, 5, 5, and 24 sampling sites, respectively (Figure A5A–D).

Test of recombination

To evaluate the prevalence of sexual or asexual reproduction within *Beauveria* spp. populations, the index of association was calculated across all SNP loci of the I_A datasets. To minimize potential bias resulting from asexual reproduction, only representative unique *Beauveria* spp. genotypes were retained per collection. Therefore, SNP-based genotypes were collapsed into MLLs and clone-corrected, resulting in 83 MLLs among 137 haploid *B. brongniartii* individuals, and 66 MLLs among 207 haploid *B. pseudobassiana* individuals. In both species, tests of recombination using the index of association (I_A) on clone-corrected data revealed a strong association among loci, leading to the rejection of the null hypothesis of linkage equilibrium (p <0.001) in all cases. A test of recombination was also performed separately for the isolates of putative cryptic

taxa Bps-1, Bps-2, Bps-3, and Bps-4, which revealed in all cases a strong linkage among loci (p <0.001). Furthermore, the index of association (I_A) was also analysed in sub-clades of cluster Bps-2 and revealed no evidence of linkage equilibrium (p <0.001).

Population differentiation according to subregions and sampling year

An AMOVA was performed across the established collections to test whether population genomic structure varied in different subregions and years of *M. melolontha* swarming flights. Therefore, only *Beauveria* spp. isolates obtained from the 35 sampling sites were included and the two *B. brongniartii* references BIPESCO 2 and BIPESCO 4 were excluded. SNP-based genotypes of the population genomic structure datasets were collapsed into MLLs and clone-corrected, resulting in 68 MLLs among 124 haploid *B. brongniartii* individuals, and 69 MLLs among 196 haploid *B. pseudobassiana* individuals.

In both species, AMOVA revealed greater within-site variation for both *B. brongniartii* (i.e., 85.38%) and *B. pseudobassiana* (i.e., 94.48%) than among subregions, that is, the geographic area in which isolates were collected (Figure 1, Table 1). Similarly, greater within-site variation in *B. brongniartii* (i.e., 86.53%) and *B. pseudobassiana* (i.e., 94.51%) was observed than among years (Table 1). Isolation by distance plots demonstrated a wide genetic distance range among *B. brongniartii* and *B. pseudobassiana* collections, with mean genetic distances of 0.12 and 0.13, respectively. A weak positive significant correlation was detected between genetic distance and geographic distance in *B. brongniartii* (r: 0.21, p <0.05), whereas no correlation was observed in *B. pseudobassiana* (r: −0.09, p >0.5; Figure A6).

TABLE 1 Analysis of molecular variation (AMOVA) on clone-corrected data for *Beauveria brongniartii* and *B. pseudobassiana* nested by subregion and sampling year.

	Source	%	p
B. brongniartii	Among subregions	5.21	0.016
	Among sites within subregions	9.41	0.002
	Within sites	85.38	0.001
	Among years	0.09	0.271
	Among sites within a year	13.36	0.001
	Within sites	86.53	0.001
B. pseudobassiana	Among subregions	5.94	0.003
	Among sites within subregions	−0.42	0.60
	Within sites	94.48	0.06
	Among years	4.42	0.004
	Among sites within years	1.05	0.330
	Within sites	94.51	0.043

Comparison of *Beauveria* spp. and *Melolontha melolontha* populations

To test whether *Beauveria* spp. and *M. melolontha* population genomic structures exhibit similarities, Mantel tests were performed between the genetic distances of the pathogens and the host. Genetic distances calculated separately among collections from sampling sites ranged from 0.018 to 0.381 for the 24 sites where *B. brongniartii* was detected and from 0.012 to 0.512 for the 33 sites where *B. pseudobassiana* occurred. For *M. melolontha* collections, genetic distance ranged in both datasets from 0.016 to 0.054. No correlation was detected between *M. melolontha* and *B. pseudobassiana* (r: −0.12, p >0.5) and a weak positive correlation was detected with *B. brongniartii* (r: 0.28, p <0.05).

DISCUSSION

In the present study, we inferred the genomic population structures of *Beauveria* spp. isolated from infected *M. melolontha* adults, collected at 35 sites in a European Alpine region representing an area of approximately 30,000 km^2, which included locations in Switzerland, Italy, and Austria. Molecular identification showed only one-third of the isolates to be *B. brongniartii*, whereas two-thirds were determined to be *B. pseudobassiana*, a species previously not recognized as a prevalent pathogen of *M. melolontha*. Analyses indicated that both species displayed a clonal population structure and the presence of cryptic phylogenetic lineages. The population structures of both *Beauveria* spp. sampled in this study showed no patterns related to the sampling year, geographical origin, or population genomic structure of the host, *M. melolontha*.

B. pseudobassiana was identified as the predominant pathogen of *M. melolontha* adults, challenging the prior assumption that *B. brongniartii* is the main fungal pathogen of *M. melolontha* larvae and adults in the studied European region. Previous investigations consistently emphasized *B. brongniartii* as the most frequently isolated pathogen from larvae and as the prevalent *Beauveria* spp. in soils at *M. melolontha*-infested sites (Enkerli et al., 2004; Mayerhofer et al., 2015). The perception that *B. brongniartii* is the most predominant and relevant pathogen of *M. melolontha* may be the result of a previous emphasis on applied research to primarily control white grubs. Consequently, few studies have focused on *M. melolontha* adults, and the importance of *B. pseudobassiana* as a pathogen may have been

insufficiently recognized although it has repeatedly been detected in *Melolontha* spp. infested soils (Mayerhofer et al., 2015; Niemczyk et al., 2019). Recently, *B. pseudobassiana* has been detected in the phylloplane of several plant species (Garrido-Jurado et al., 2015; Howe et al., 2016), and its presence on foliage on which adult beetle feeds has also been documented at *M. melolontha* infested sites (unpubl. data). Howe et al. (2016) detected *B. pseudobassiana* in the phylloplane of lime trees and on the beetle *Harmonia axyridis* collected from the same site in a park area, suggesting that *H. axyridis* encounters *B. pseudobassiana* in the arboreal habitat. These observations, combined with the present results, indicate a potential partial ecological niche differentiation between *B. brongniartii* and *B. pseudobassiana*, with both species occurring in soil but only *B. pseudobassiana* present aboveground. Based on these observations, it might be hypothesized that *M. melolontha* encounters the two *Beauveria* spp. at different developmental stages. Barta (2018) observed reduced leaf damage and higher mean mortality of *Cameraria ohredella* larvae feeding on *B. pseudobassiana* colonized leaves. If the presence of *B. pseudobassiana* increases *M. melolontha* mortality and reduces leaf damage, targeting *M. melolontha* adults with *B. pseudobassiana* could provide a biological control approach complementary to the current soil applications targeting *M. melolontha* white grubs. However, additional investigation is required to elucidate whether the *B. brongniartii* as well as *B. pseudobassiana* infection of *M. melolontha* occurs in soil during the larval stage and is subsequently transmitted to the adult form. Whether *B. brongniartii* is the predominant fungal pathogen in *M. melolontha* larvae necessitate further assessment. To date, no comprehensive study encompassing a large number of *M. melolontha* larvae is available that addresses this research gap.

Both *B. brongniartii* and *B. pseudobassiana* populations exhibited pronounced clonal population structure within all resolved cryptic phylogenetic lineages in the region sampled. We document for the first time the fixation of the MAT-1 mating type in the *B. brongniartii* population. The absence of any report of the sexual morph in the *B. brongniartii* population in Europe might thus be due to the unavailability of compatible mating partners, that is, MAT-2. However, sexual morphs of *B. brongniartii* have been identified in northeast China and Japan, demonstrating the existence of both mating types in other populations (Sasaki et al., 2007; Shimazu et al., 1988). These findings could imply that the European population perhaps originated from a single or a limited number of founding individuals carrying only the MAT-1 mating type. Alternatively, it may be hypothesized that only MAT-1 is pathogenic to *M. melolontha*, as previous studies in other systems, for example, mice fungal pathogen *Mucor irregularis*, have documented that specific virulence can differ and depend on different mating types (Xu et al., 2017). In contrast, *B. pseudobassiana* exhibited both MAT idiomorphs in each of the four identified phylogenetic lineages, suggesting that individuals carrying opposite mating types within each of these clusters have the potential to engage in sexual reproduction and produce sexual morphs, so far not observed in Europe. However, the I_A analyses revealed a prevalence of asexual recombination within these *B. pseudobassiana* cryptic phylogenetic lineages. A recent study conducted by Wang et al. (2020) documented the occurrence of a sexual morph for *B. pseudobassiana* in southeast Asia on larvae and pupae of Lepidoptera. Although both mating types coexist in the sampled region, the present results, as indicated by the association index (I_A), suggest that sexual mating remains infrequent for *B. pseudobassiana*, with a prevailing tendency towards asexual reproduction. From a biosafety point of view, infrequent or even lack of sexual recombination in *B. brongniartii* populations might represent an advantage for BCA use based on *B. brongniartii*, as out-crossing with native isolates is reduced and the genetic stability of the biological control product is maintained.

The population genomic structures of both *Beauveria* species sampled here were not affected by sampling year and geographic origin. Wide geographic distribution of genotypic clusters including genetically similar isolates or lineages has also previously been reported for *Beauveria* spp., for example, *B. bassiana* (Cai et al., 2013; Wang et al., 2003). Prior studies of *B. bassiana* have also reported no correlation between the distribution of genotypes with geographic origin, climate, or host, with high local phylogenetic diversity among geographically close locations (Garrido-Jurado et al., 2011; Meyling et al., 2009). The predominant clonal asexual reproduction in *Beauveria* spp. likely facilitates rapid proliferation and efficient dispersal without genetic recombination, leading to a clonal population structure that might have remained relatively genetically stable over regions and time due to infrequent sexual reproduction, as also suggested for *B. bassiana* (Xiao et al., 2012). In addition, the detection of members of all clusters within *B. brongniartii* and *B. pseudobassiana* across all sampling sites and years likely reflects the long-term persistence of these clonal lineages and their capacity to disperse and establish at new sites via asexual conidia transported by wind, rain, and organisms, for example, insects, including *M. melolontha* (Ortiz-Urquiza & Keyhani, 2016). The absence of a temporal structure in both *Beauveria* spp. may also be attributed to the continuous production and persistence of conidia, including their dispersal extending beyond the swarming flights of *M. melolontha*. The ability of *B. brongniartii* to infect larval and adult stages of *M. melolontha* ensures a constant source of infection throughout the lifecycle of the insect. The generalist nature of *B. pseudobassiana* might allow the infection of multiple insect species resulting in a constant presence of conidia that might readily disperse between adjacent and distant habitats.

In both fungal species, isolation by distance plots revealed a wide range of genetic distances among *Beauveria* spp. collections, both covering values from low to significantly higher values, suggesting intricate dispersal dynamics within *Beauveria* spp. Some collections displayed low genetic distance values, which could indicate either frequent or sporadic gene flow among sampling sites, with an ability of clones to persist in the soil over extended periods. In contrast, other collections exhibited substantially higher genetic distances, perhaps reflecting diverse population interactions and lower rates of gene flow, along with the influence of local drift or mutations. The genetic differentiation among some populations and the broad spectrum of genetic distances might signify that gene flow is not a consistent process within this system and that persistence capacity in the soil might be one of the principal factors shaping the population genomics of *Beauveria* spp. A recent study conducted by Mei et al. (2020) in China, covering the years 1997–2017, revealed that clonal *B. bassiana* isolates often show temporal specificity, suggesting a recurring pattern of strain replacement, which typically occurs at a decadal scale. However, some *B. bassiana* strains released in pine forests for biocontrol of the caterpillar *Dendrolimus punctatus* have persisted for up to two decades. While our study provides insights into the temporal dynamics of *Beauveria* spp. populations at specific sampling locations and time intervals, further investigation is required to fully understand the dynamics of genetic composition over time, particularly in the context of BCA applications and to provide additional information on the persistence and dispersal capacity of *Beauveria* spp. in the studied region.

A lower degree of differentiation was observed among the collections of *Beauveria* spp. compared to *M. melolontha* and weak or no correlation between the genetic distance matrices of *M. melolontha* and *B. brongniartii*, and *B. pseudobassiana*, respectively. For many mutualistic, co-evolving organisms that are strongly dependent on each other, for example, symbiotic or parasitic interactions, significant correlations of genetic distances are often observed, as host dispersal is assumed to drive dispersal of mutualists and thereby shape the mutual genomic population structure (Blasco-Costa & Poulin, 2013; Mazé-Guilmo et al., 2016). For example, Bracewell et al. (2018) identified a significant correlation between the genetic distances of the beetle *Dendroctonus brevicomis* and its obligate fungal mutualists *Ceratocystiopsis brevicomi* and *Entomocorticium* sp., which are involved in beetle development and survival, suggesting that the evolution of the mutualist is likely dependent on the host, including its dispersal. The lack of a correlation between the genetic distance matrices of *M. melolontha* and *Beauveria* spp. suggests that the behaviour of *M. melolontha* does not directly shape the genomic structure of the fungal pathogens. As mentioned above, additional factors besides host dispersal may contribute to the population genomic structure of the *Beauveria* spp. pathogens, such as asexual reproduction, the dispersal mechanism of asexual spores, and the ability to establish and persist in the environment, which could lead to the presence and co-occurrence of independently distributed clusters of *M. melolontha* and *Beauveria* spp. In addition, free-living stages and host specificity are factors known to shape the population structure of pathogens and parasites, and may also affect the population genomic structure of *Beauveria* spp. (Mazé-Guilmo et al., 2016). Pathogenic species with wide host ranges usually show lower levels of population differentiation, as they may be transported over longer geographical distances to more varied habitats, whereas species with a narrower host range generally present higher genomic differentiation among populations (Karlsson et al., 2014; Wacker et al., 2019). Although *B. brongniartii* mainly occurs at *M. melolontha*-infested sites, the fungus has also been detected in soils where *M. melolontha* is absent, suggesting a lack of strict dependence on the *M. melolontha* host despite the apparent specificity (Keller et al., 2003; Lee et al., 2015). In addition, both *B. brongniartii* and *B. pseudobassiana* are facultative pathogens of *M. melolontha*, and based on their saprophytic fungal growth they have the potential to occur in soil independently of *M. melolontha* (Keller et al., 2003).

ddRADseq-based SNP data revealed higher resolution and accuracy when compared to microsatellite-based analyses, thus allowing to group the genetically similar isolates while simultaneously displaying genetic variability between them. However, phylogenetic analyses demonstrated that both methods allow estimation of phylogenetic relationships among individuals, and a significant positive correlation was detected between microsatellite and SNP-marker data. Our results are in agreement with previous studies (Lemopoulos et al., 2019; Thrasher et al., 2018), confirming the effectiveness of both microsatellites and SNP molecular markers in estimating individual relationships, with SNP data showing superior resolution for population genomic structure. Our results also demonstrate that microsatellites, which can be flexibly and consistently applied, remain valuable tools for monitoring the occurrence of specific genotypes as well as for genotyping indigenous *B. brongniartii*.

A large proportion of *B. brongniartii* isolates belonging to Bbr-2 (79.07% of Bbr-2) matched the microsatellite marker-based MLG of BIPESCO 2. These isolates predominantly originated from South Tyrol, where the BIPESCO 2 BCA was first isolated (origin: Kramsach, Tyrol, Austria) and was subsequently frequently applied since the late 1990s (Schweigkofler & Zelger, 2002). Isolates with the microsatellite MLG of BIPESCO 2 were recovered to a lesser extent in Switzerland and Austria, where the strain may occur either naturally or has been applied as BCA. BIPESCO 4 was detected only in Switzerland where it was originally isolated (Canton

Nidwalden) and was subsequently applied as a BCA at various locations (Kessler et al., 2004). The detection of BIPESCO 2 and BIPESCO 4 together with indigenous *Beauveria* spp., are in line with previous pot and field experiments documenting the persistence of the BCA strain and coexistence with indigenous *Beauveria* spp. even at sites receiving extensive BCA applications (Enkerli et al., 2004; Mayerhofer et al., 2015; Schwarzenbach et al., 2009). The occurrence of microsatellite MLGs that correspond to either BIPESCO 2 or BIPESCO 4 at sites where the BCAs have been applied may reflect residual persistence after repeated treatments for up to 20 years.

In conclusion, the results of this study revealed (1) *B. brongniartii* and *B. pseudobassiana*, of which the latter species was hitherto unrecognized as a pathogen of *M. melolontha*, are the main fungal pathogens of *M. melolontha* adults in the sampled region; (2) population genomic structure analyses conducted on both *Beauveria* species indicated persistence and co-occurrence of multiple clonal lineages of both *B. brongniartii* and *B. pseudobassiana* throughout sampling years and sampling sites; and (3) clonal lineages of both fungal species were widely distributed among sites and sampling years, independently of the population structure of *M. melolontha*, indicating that they (partly) disperse independently of their host. Mating type analyses revealed that (4) sampled *B. brongniartii* lacked individuals with the MAT-2 mating type, while all four cryptic taxa of *B. pseudobassiana* exhibited both mating types. (5) Index of association (I_A) analyses revealed predominant asexual reproduction in both *Beauveria* spp., indicating that factors other than co-occurrence of compatible mating partners limit sexual reproduction in *B. pseudobassiana* populations. (6) The positive correlation between microsatellite and genome-wide SNP molecular markers for resolving genomic clusters, including those containing the BCAs BIPESCO 2 and BIPESCO 4, validate the accuracy and sensitivity of microsatellite markers for discriminating BCA genotypes and inferring relationships within *B. brongniartii*. However, multivariate analyses revealed that a genome-wide SNP-based approach is better suited for population genomic structure analyses.

This study provides insights into the population genomic structure of *B. brongniartii*, which is one of the principal fungal pathogens of the insect *M. melolontha* in a European Alpine region and is known for its long-standing interaction with this insect. In addition, relevant information is provided for *B. pseudobassiana*, which was identified as the predominant EPF infecting *M. melolontha* adults in this study, demonstrating its potential as a new BCA for *M. melolontha*.

AUTHOR CONTRIBUTIONS

Chiara Pedrazzini: Investigation; writing – original draft; visualization; validation; methodology; formal analysis; data curation. **Stephen Rehner A:** Investigation; writing – review and editing; visualization; validation; methodology; formal analysis; data curation. **Hermann Strasser:** Conceptualization; investigation; writing – review and editing; methodology; validation; data curation. **Niklaus Zemp:** Conceptualization; investigation; writing – review and editing; methodology; validation; software; formal analysis; data curation. **Rolf Holderegger:** Conceptualization; investigation; writing – review and editing; visualization; methodology; validation; formal analysis; data curation; supervision. **Franco Widmer:** Conceptualization; investigation; writing – review and editing; visualization; methodology; validation; formal analysis; data curation. **Jürg Enkerli:** Conceptualization; investigation; writing – review and editing; visualization; methodology; validation; formal analysis; supervision; data curation.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw sequence data are available in the European Nucleotide Archive (ENA) under BioProject PRJEB 70245: https://www.ebi.ac.uk/ena/browser/view/PRJEB70245. Sequences of the nuclear intergenic region Bloc were deposited in GenBank under accession numbers OR827340-OR827344, OR827346-OR827349, OR827352, OR827353, OR827357-OR827362, OR827364.

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APPENDIX A

TABLE A1 Year and origin of *Beauveria* spp. collections, number of *Beauveria brongniartii* and *B. pseudobassiana* isolates, and prevalence of infection per sampling site. Sampling site locations are given in Figure 1.

Number of the collection	Country	Site	Coordinates latitude WGS84	Coordinates longitude WGS84	Year of collection	*N B. brongniartii*[b]	*N B. pseudobassiana*[b]	Prevalence of infection (%)
1	Switzerland	Eschenz	47.64186	8.85218	2017	0	6	4.5
2	Switzerland	Masein	46.70192	9.42093	2017	8	4	20.1
3	Switzerland	Matten	46.68555	7.86128	2017	0	9	9
4	Switzerland	Seewis	46.99789	9.62616	2017	9	7	19.4
5	Switzerland	Strada	46.86693	10.43487	2017	0	16	19.4
6	Switzerland	Tomils	46.76392	9.4404	2017	4	14	26.1
7	Switzerland	Trin Mulin	46.83169	9.34607	2017	4	12	29.1
8	Switzerland	Valzeina	46.94506	9.60554	2017	0	10	19.4
9	Switzerland	Zizers	46.92481	9.57573	2017	6	10	19.4
10	Switzerland	Andhausen	47.57967	9.18453	2018	13	7	27.3
11	Switzerland	Bristen	46.76556	8.70538	2018	13	6	22.5
12	Switzerland	Disentis	46.96566	8.85452	2018	7	9	31.5
13	Switzerland	Falera	46.79985	9.23692	2018	0	16	17.9
14	Switzerland	Ilanz	46.77473	9.21549	2018	4	6	14.7
15	Switzerland	Siat	46.78566	9.16621	2018	7	11	21.7
16	Switzerland	Silenen	46.78356	8.67031	2018	4	8	12
17	Switzerland	Valendas	46.78745	9.27761	2018	5	13	42.9
18	Switzerland	Aarenschlucht	46.71014	8.21455	2019	7	12	21.6
19	Switzerland	Bueren	46.9389	8.39378	2019	8	12	15.7
20	Switzerland	Lungern	46.77879	8.15541	2019	16	4	14.9
21	Italy (ST[a])	Glurns	46.672067	10.559742	2018	0	16	18.3
22	Italy	Aosta-1	45.743897	7.373035	2019	6	13	23.4
23	Italy	Aosta-2	45.714218	7.268098	2019	9	0	8.2
24	Italy (ST[a])	Branzoll	46.404286	11.308656	2019	0	17	24.1
25	Italy (ST[a])	Kaltern-OG Roen	46.352351	11.262679	2019	3	8	12.6
26	Italy (ST[a])	Laimburg	46.381803	11.291314	2019	11	9	23.6
27	Italy (ST[a])	Nals Prissianer Auen	46.561458	11.203442	2019	0	5	8.3
28	Italy (ST[a])	Passeier-Sandwirt	46.800521	11.244354	2019	5	12	29.3

TABLE A1 (Continued)

Number of the collection	Country	Site	Coordinates latitude WGS84	Coordinates longitude WGS84	Year of collection	*N B. brongniartii*[b]	*N B. pseudobassiana*[b]	Prevalence of infection (%)
29	Italy (ST[a])	Plattl	46.352211	11.305128	2019	12	5	29
30	Italy (ST[a])	Schlanders	46.627335	10.784224	2019	0	18	31.7
31	Italy (ST[a])	Siebeneich	46.513761	11.268657	2019	7	4	20.8
32	Italy (ST[a])	Unterrain	46.497576	11.246578	2019	6	13	26.9
33	Austria	Prutz	47.077031	10.659714	2017	0	4	9
34	Austria	Schoenwies	47.201453	10.670111	2017	8	0	18
35	Austria	Muenster	47.421711	11.840794	2019	0	17	35.6

[a]ST: South Tyrol.
[b]Number of fungal isolates per site after mapping and SNP filtering of the ddRADseq data.

TABLE A2 Barcode sequences used for the ddRADseq protocol to construct libraries from *Beauveria* spp. DNA.

Barcode number	Sequence	Barcode number	Sequence
1	GCATG	25	CTGCG
2	AACCA	26	CTGTC
3	CGATC	27	CTTGG
4	TCGAT	28	GACAC
5	TGCAT	29	GAGAT
6	CAACC	30	GAGTC
7	GGTTG	31	GCCGT
8	AAGGA	32	GCTGA
9	AGCTA	33	GGATA
10	ACACA	34	GGCCA
11	AATTA	35	GGCTC
12	ACGGT	36	GTAGT
13	ACTGG	37	GTCCG
14	ACTTC	38	GTCGA
15	ATACG	39	TACCG
16	ATGAG	40	TACGT
17	ATTAC	41	TAGTA
18	CATAT	42	TATAC
19	CGAAT	43	TCACG
20	CGGCT	44	TCAGT
21	CGGTA	45	TCCGG
22	CGTAC	46	TCTGC
23	CGTCG	47	TGGAA
24	CTGAT	48	TTACC

TABLE A3 Index sequences used for the ddRADseq protocol to construct libraries from *Beauveria* spp. DNA. Forward primer: AATGATACGGCGACCACCGAGATCTACAC-(i5 index)-ACACTCT TTCCCTACACGACG; Reverse primer: CAAGCAGAAGACGGCATA CGAGAT-(i7 index)-GTGACTGGAGTTCAGACGTGTGC.

Library name	i7 index	i5 index
Library 1	GGAACGTT	TGACAAGC
Library 2	TGCATTGC	TGACAAGC
Library 3	TCTCATTC	TGACAAGC
Library 4	GTTGTCCG	TGACAAGC
Library 5	GGAACGTT	TCCGGATT
Library 6	TGCATTGC	TCCGGATT
Library 7	TCTCATTC	TCCGGATT
Library 8	AAGACGTC	TCCGGATT
Library 9	GGAACGTT	GCTCCGAC
Library 10	TGCATTGC	GCTCCGAC
Library 11	TCTCATTC	GCTCCGAC
Library 12	AAGACGTC	GCTCCGAC
Library 13	GGAACGTT	TGCATTGC
Library 14	TGCATTGC	TGCATTGC
Library 15	TCTCATTC	TGCATTGC
Library 16	AAGACGTC	TGCATTGC

TABLE A4 Number of isolates of *Beauveria brongniartii* and the two BCA strains BIPESCO 2 and BIPESCO 4 per cluster (i.e., Bbr-1, Bbr-2, and Bbr-3), as identified by the *find.cluster* function of the R package adegenet 2.1.5 (Jombart, 2008). The proportion of MAT-1 and MAT-2 genotypes is depicted per collection.

Number of the collection	Bbr-1	Bbr-2	Bbr-3	MAT-1	MAT-2
2	8	0	0	8	0
4	7	0	2	9	0
6	4	0	0	4	0
7	4	0	0	4	0
9	2	3	1	6	0
10	13	0	0	13	0
11	6	5	2	13	0
12	7	0	0	7	0
14	1	0	3	4	0
15	6	1	0	7	0
16	4	0	0	4	0
17	0	5	0	5	0
18	7	0	0	7	0
19	4	0	4	8	0
20	4	0	12	16	0
22	5	0	1	6	0
23	3	0	6	9	0
25	2	1	0	3	0
26	0	11	0	11	0
28	1	1	3	5	0
29	7	2	3	12	0
31	0	7	0	7	0
32	0	6	0	6	0
34	6	1	1	8	0
Total isolates	**101**	**43**	**38**	**182**	**0**
Sampling sites	20	11	11	24	0
BIPESCO 2	-	1	-	1	0
BIPESCO 4	-	-	1	1	0

Note: Bold values indicate the total number.

TABLE A5 Number of isolates of *Beauveria pseudobassiana* per cluster (i.e., Bps-1, Bps-2, Bps-3, and Bps-4), as identified by the *find.cluster* function of the R package adegenet 2.1.5 (Jombart, 2008), and distribution of MAT-1 and MAT-2 mating type isolates. The proportion of MAT-1 and MAT-2 genotypes is depicted per collection.

Number of the collection	Bps-1	Bps-2	Bps-3	Bps-4	MAT-1	MAT-2
1	1	0	3	2	4	2
2	0	0	3	1	3	1
3	0	2	5	2	9	0
4	0	1	2	4	5	2
5	9	0	0	7	7	9
6	0	2	1	11	6	8
7	1	0	2	9	12	0
8	0	0	3	7	6	4
9	0	0	9	1	10	0
10	1	1	1	4	5	2
11	0	0	0	6	2	4
12	6	0	0	3	6	3
13	8	1	0	7	11	5
14	3	1	0	2	6	0
15	6	1	1	3	9	2
16	1	0	0	7	2	6
17	4	0	0	9	7	6
18	3	0	0	9	9	3
19	1	0	0	11	8	4
20	0	0	1	3	3	1
21	2	5	2	7	10	6
22	0	1	0	12	6	7
24	1	7	0	9	11	6
25	0	5	1	2	4	4
26	0	3	1	5	7	2
27	0	1	3	1	1	4
28	0	2	4	6	8	4
29	0	1	0	4	3	2
30	2	5	2	9	9	9
31	0	3	0	1	2	2
32	0	6	1	6	5	8
33	2	1	0	1	4	0
35	3	1	2	11	13	4
Total isolates	**54**	**50**	**47**	**182**	**213**	**120**
Sampling sites	17	20	19	33	33	28
MAT-1	43	18	37	115	-	-
MAT-2	11	32	10	67	-	-

Note: Bold values indicate the total number.

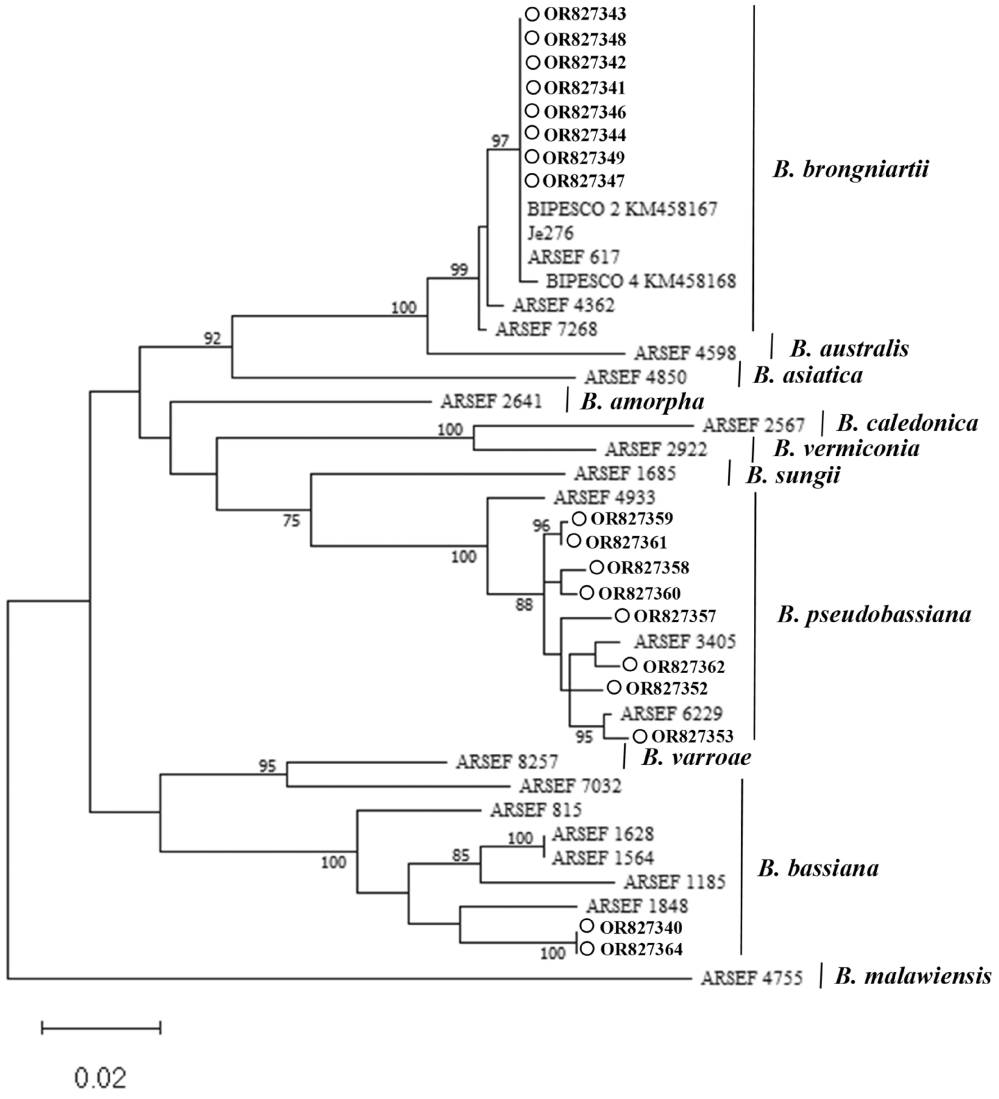

FIGURE A1 Maximum likelihood dendrogram based on Kimura-2-parameter similarities inferred from 18 new sequences of the Bloc intergenic region and 23 *Beauveria* spp. reference sequences (Rehner et al., 2011) including BCA strains BIPESCO 2 and BIPESCO 4. Bootstrap values >70% of 1000 pseudoreplicates are shown at the nodes and the scale bar indicates branch length as the number of substitutions per site. Empty circles symbolize samples sequenced in this study.

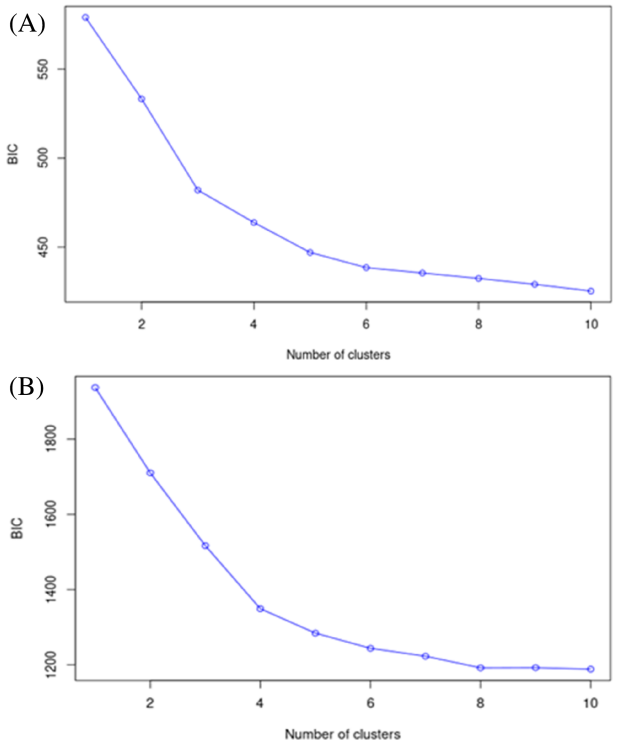

FIGURE A2 Bayesian information criterion (BIC) performed for $K = 1$–10 including 24 collections of *Beauveria brongniartii* as well as the BCA strains BIPESCO 2 and BIPESCO 4 (96 SNPs, $N = 184$; (A) and 33 collections of *B. pseudobassiana* (955 SNPs, $N = 333$, (B)).

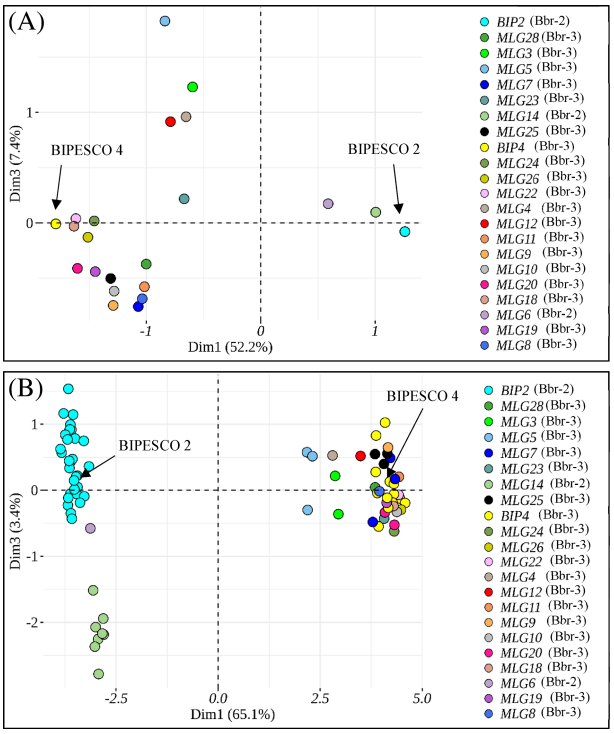

FIGURE A3 Principal component analyses (PCA) constructed with 81 isolates of *Beauveria brongniartii* and the two BCA strains BIPESCO 2 and BIPESCO 4 based on 6 SSRs (A) and on 96 SNPs (B) and labelled as MLG identified by SSR. BIPESCO 2 and 4 are indicated by arrows.

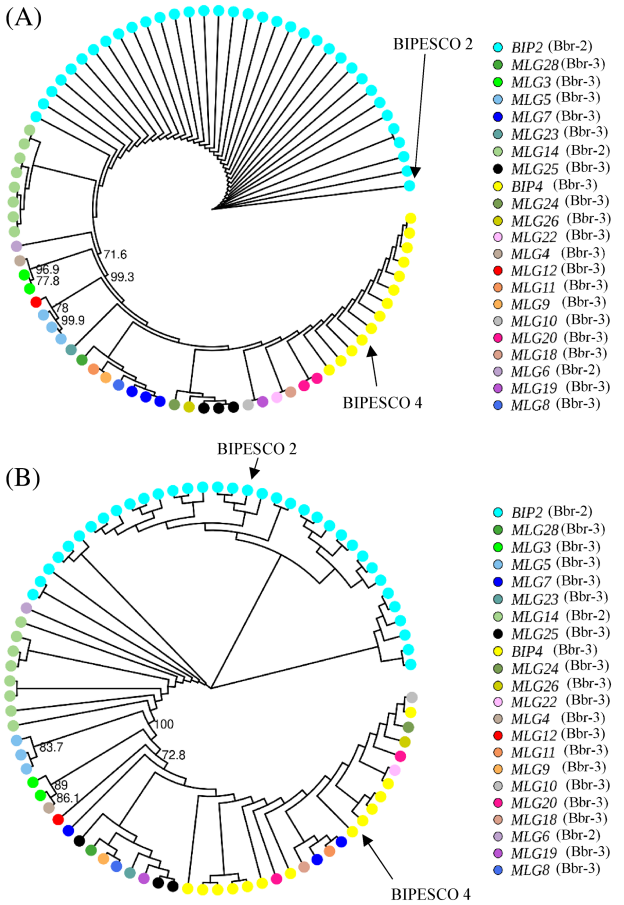

FIGURE A4 Neighbour-joining tree (bootstrap values >70% are labelled at relevant nodes) constructed with 81 isolates of *Beauveria brongniartii* and the two BCA strains BIPESCO 2 and BIPESCO 4 based on 6 SSRs (A) and on 96 SNPs (B) and labelled as MLG identified by SSR. BIPESCO 2 and 4 are indicated by arrows.

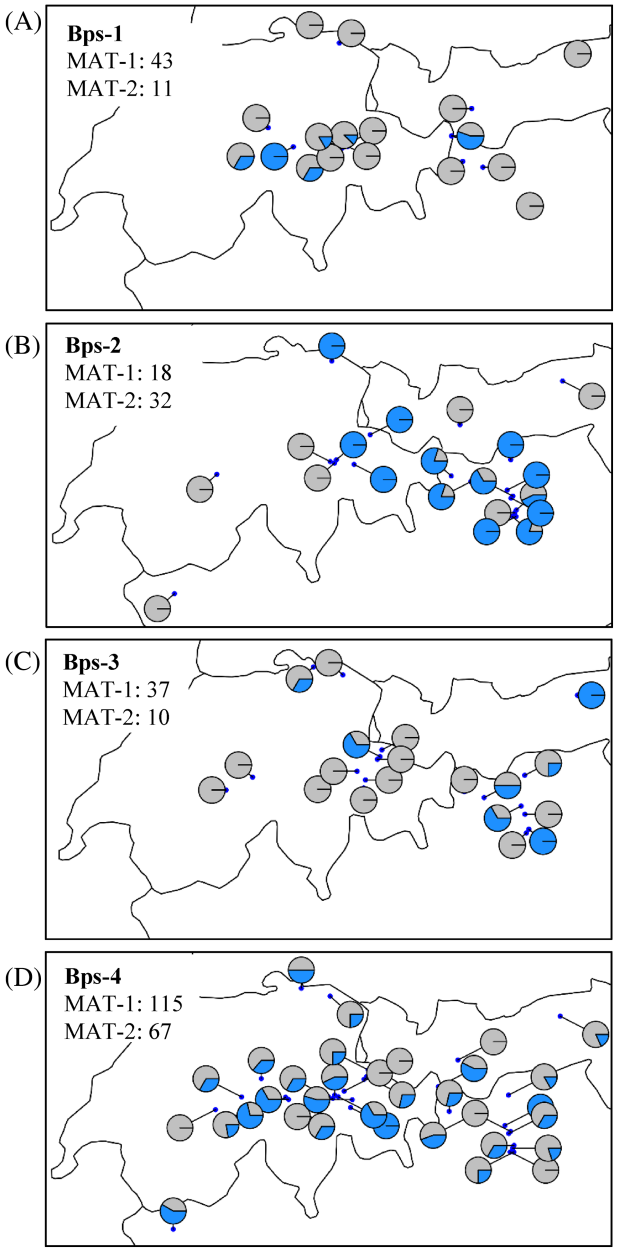

FIGURE A5 Maps of collection sites for *Beauveria pseudobassiana* with pie charts depicting the proportion of MAT-1 and MAT-2 genotypes in Bps-1 (A), Bps-2 (B), Bps-3 (C) and Bps-4 (D).

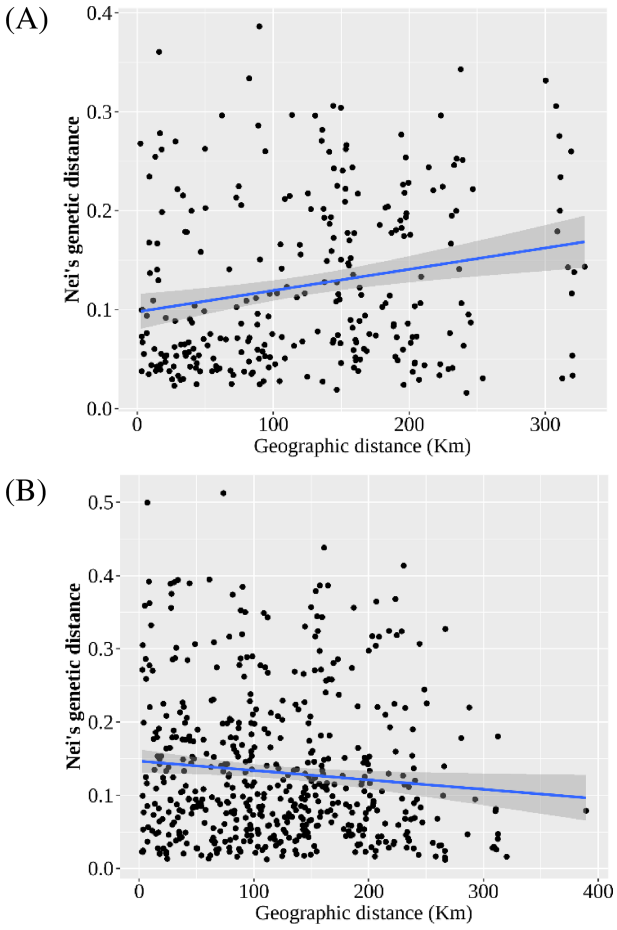

FIGURE A6 Isolation by distance (IBD) plots: Nei's genetic distance regressed (for illustration) against geographic distance (Km) in (A) *Beauveria brongniartii* dataset excluding the BCA strains BIPESCO 2 and BIPESCO 4 (96 SNPs, $N = 182$) and in (B) *B. pseudobassiana* dataset (955 SNPs, $N = 333$).