Biogeographia – The Journal of Integrative Biogeography

Title

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Journal

Biogeographia – The Journal of Integrative Biogeography, 37(1)

ISSN

1594-7629

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Publication Date

2022

DOI

10.21426/B637156644

Biogeographia – The Journal of Integrative Biogeography 37 (2022): a017
https://doi.org/10.21426/B637156644

Preliminary assessment of entomopathogenic fungi and nematodes in hot springs in central Italy with the first record of *Pristionchus uniformis* for the country

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Keywords: Central Italy; Fungi; *Galleria* bait method; geothermal soils; Nematoda; soil samples.

SUMMARY

Geothermal soils in Central Italy are characterized by high temperatures (up to 60°C), low pH values, and sparse vegetation. The biodiversity of entomopathogenic soil fungi and nematodes has never been assessed in these areas. Soil samples (N = 12) separated from each other by at least 100 meters were collected in the areas of Le Biancane Natural Park (Grosseto) and Sasso Pisano (Pisa). The *Galleria* bait method was used to assess the presence of entomopathogenic fungi and nematodes. A total of nine species of fungi and one nematode were isolated and molecularly identified. All these species came from samples collected in the immediate surroundings of geothermal spring, apart from one *Beauveria pseudobassiana* collected at about 16 meters from the nearest spring. Moreover, *Pristionchus uniformis* previously reported only from North America and few European countries is recorded for the first time in Italy.

INTRODUCTION

Natural geothermal fields represent habitats with peculiar environmental conditions, including high temperature (up to 50-60°C), low pH (below 4), and the presence of gases such as H_2S, CO_2, boric acid, and water vapour (Bonini et al., 2005). Geothermal areas host a peculiar biodiversity, which is adapted to thrive at these environmental conditions (Giacomini, 1955; Alderman, 1992; Loppi & Bonini, 2000; Cortini Pedrotti, 2001; Bonini et al., 2005), together with mesophilic species that show high stress levels, e.g. *Calluna vulgaris* (L.) Hull and *Rubus ulmifolius* Schott, 1818 (Pippucci et al., 2015; Costantini & Pagani, 2017). Eukaryotic and prokaryotic communities of thermophilic and mesophilic bacteria in these hot environments remarkably differ from reference microbial communities of similar cold soils (Cuebas et al., 2011; Arif et al., 2021). Regarding the eukaryotic diversity, high abundances of unicellular red algae, primary consumers (Collembola), and Apicomplexa (*Gregarina* spp.) occur in fumaroles of central Italy (Arif et al., 2021).

Moreover, geothermal areas represent still understudied fungal and nematode niches, i.e. habitats where extreme temperature and pH conditions may be expected to prevent the development of well-diversified microbial communities (Redman et al., 2009; Cantrell et al., 2011; De Oliveira et al., 2015; Fraser et al., 2018). Species adapted to grow under these extreme conditions are endowed with heat-tolerant enzymes that are used for biotechnological processes (Redman et al., 2009). Redman et al. (2009) characterized fungal communities in geothermal soils around a sulfurous hot spring in North America where have been isolated only two thermophilic species, whereas Chen et al. (2003) found 12 species in Taiwan, and Zhou et al. (2015) isolated 38 taxa. Particularly, Zhou et al. (2015) showed that endophytic and rhizospheric fungi are an important component of geothermal ecosystems, as potentially playing important roles in improving host-plant thermotolerance. Microbial diversity at geothermal sites have been assessed marked by high level of endemism, as showed by metabarcode sequencing both for bacteria and for fungi (Pang et al., 2019; Bazzicalupo et al., 2021). Studies from North America and the Far East showed that the fungal community of geothermal ecosystems may be composed of several species and that most taxa have adapted to high environmental temperatures (Bunn & Zabinski, 2003; Chen et al., 2003; Salar & Aneja, 2006; Redman et al., 2009; Pan et al., 2010; Cantrell et al., 2011; Zhou et al., 2015). Some species able to resist to high temperatures can also thrive in temperate soils, which implies a great physiological tolerance and plasticity of these taxa (Salar & Aneja, 2006). Furthermore, some species become dominant or endemic to these extreme habitats, improving their ability to grow at high temperatures (Bunn & Zabinski, 2003; Chen et al., 2003; Zhou et al., 2015).

The species richness and the heat-adaptation of fungi and nematodes have never been evaluated in European geothermal areas. Tuscany, in central Italy, includes one of the best known volcanic geothermal areas in Europe (cf. Manzella et al., 2018). In particular, high-temperature steam-dominated reservoirs occur mainly in southern areas of this region (provinces of Grosseto and Pisa). Many thermal springs occur in this area as a result of post-volcanism and of an active geothermal system (Pippucci et al., 2015). Geothermal activity deeply alter the chemical and physical characteristics of the local soil, creating hard ecological conditions, including high temperatures on the ground surface (up to 55 °C: Arif et al., 2021), low pH, low water retention, lack of nutrients, and the occurrence of chemical toxins (Pippucci et al., 2015; Arif et al., 2021). Such a stressful condition and strong selective pressure may be the main cause of the local low richness of eukaryotic species (Bonini et al., 2005; Pippucci et al., 2015, for other taxa).

The main objective of this work was to provide the first assessment of entomopathogenic fungi and nematodes that inhabit geothermal soils around thermal springs in Italy, with specific focus on the geothermal areas of Tuscany.

MATERIALS AND METHODS

Study areas

Research was conducted in Sasso Pisano (“Fumarole and Putizze”) and Le Biancane Natural Park, which include natural geothermal areas in the municipalities of Castelnuovo Val di Cecina and Monterotondo Marittimo, in the provinces of Pisa and Grosseto, respectively (about 50 ha, 43.15°N - 10.85°E WGS84, 485-614 m a.s.l.; Figure 1).

The study area was characterised by natural geothermal spring known in Tuscany as 'Soffioni Boraciferi', similar to the volcani solfataras of southern Italy, the volcanic regions of Iceland, and the Rocky Mountains of the USA (Bonini et al., 2005). About 33% of the study area was covered with bare soil and white rocks with steam, approximately 45% with scrublands (mostly *Calluna vulgaris, Rubus ulmifolius*, and *Erica arborea* L.), and the remaining 22% with mixed deciduous woodland (mostly *Quercus suber* L., with *Castanea sativa* Mill., *Quercus ilex* L., and *Pinus pinea* L.). This area has been known for its natural volcanic activity and hot springs since ancient times, characterised by steam vent emissions, emerging from small fissures on the ground, containing H_2S, CO_2, boric acid, and water vapor, as well as by soil surface often reaching temperatures above 50-60°C (Bonini et al., 2005; Silvestri et al., 2020). Locally, vapor condenses on the ground to form hot water puddles, called ‘Lagoni’ (Duchi et al., 1991). The atmosphere above the ground surface is rich in CO_2, H_2S, NH_3, and CH_4, with some amounts of boric acid derivatives (Bussotti et al., 2003). This peculiar chemical composition is clearly shown by stress responses of plant species (Pippucci et al., 2015; Costantini & Pagani, 2017). The local climate shows submontane characteristics, with an average annual rainfall of 870 mm and an average annual temperature of 14°C, with peaks up to 30 °C in July and August. The main winds are oriented SW-NE (Costantini & Pagani, 2017). This area, characterised by the presence of both natural geothermal springs and power plants, is a site rich in biodiversity, although no published information is available so far on species richness excluding bryophytes (Bonini et al., 2005). During our field surveys (February-June 2021), we uploaded species occurrences to iNaturalist (www.inaturalist.org: Table S1 in the Supplementary Material 1). In particular, the site Le Biancane showed more diverse vegetation than Sasso Pisano, which was mostly covered with bare soil and steam vents. Among plants, the most important one is the Etruscan crocus *Crocus etruscus* Parl., detected both in the Sasso Pisano and Le Biancane sites. This endemic species to Southern Tuscany was never recorded in this area before our surveys.

Soil sample collection

We crossed the dirt road around the hot springs, for a total of three transects (3.0 km each). These transects have been identified to cross all geothermal springs of our study site and surrounding woodlands. Soil samples were collected at five randomly selected points in Sasso Pisano and at seven points in Le Biancane, located at different distances from geothermal springs (see Table 1). The soil temperature was measured at noon at each sampling point at 15 cm depth, using a soil thermometer (©BestPrato, measuring range = from – 25° C to + 100° C).

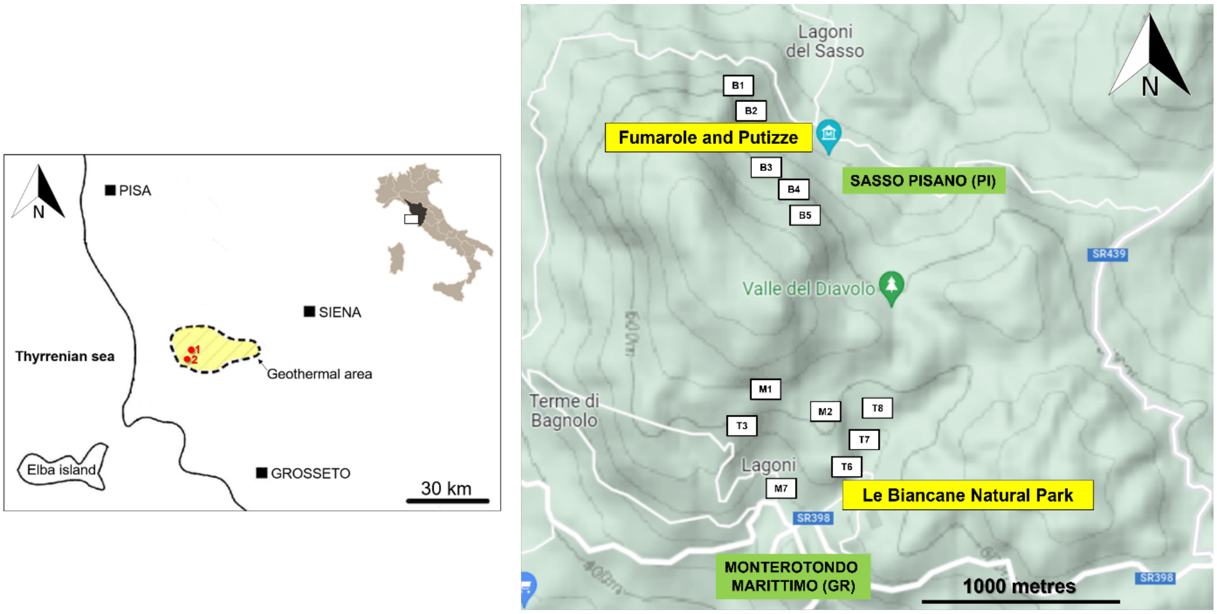

Figure 1. Location of the study area; points of soil collection are also shown (boxes). Sources: Data SIO, NOAA, US Navy, NGA, GEBCO © 2016 TerraMetrics © 2016 Google; Wikimedia Commons, user Norman Einstein, CC-BY-SA-3.0.

Samples of soil (about 2 dm^3) collected in May-June 2021 at each selected sampling point were placed in plastic bags and then transferred to the CREA-Research Center for Plant Protection and Certification Laboratory of Florence to be processed. The pH of each sample was analysed in the laboratory on 10 g of soil from each point following standard procedures (Violante, 2000). Furthermore, to isolate entomopathogenic fungi and nematodes, for each sample, five commercial last instar *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae were placed on the soil and incubated at room temperature (20 ± 3°C), following Bedding & Akhurst (1975) and Zimmerman (1986). Before their use, larvae were kept under observation for one week to assess the absence of pathogens. Mortality was assessed daily for two weeks. Larval mortality during the experiment was not within the aim of this study. We only wanted to ascertain the possible presence of entomopathogenic fungi and nematodes. Dead larvae showing signs of fungal infection were processed by external sterilization in a 1% sodium hypochlorite water solution and three rinses in sterile distilled water (Zimmermann, 1986). Subsequently, the samples were placed separately in Petri dishes lined with sterile moistened filter paper. Positive fungal infection was confirmed by external sporulation of fungi from dead individuals (Llàcer et al., 2013). The emerging fungi were seeded in Rose Bengal chloramphenicol agar (VWR International PBI s.r.l.), purified and seeded again in quarter-strength Sabouraud Dextrose agar (VWR International PBI s.r.l.) plus 0.25% yeast extract (Sigma-Aldrich Chemie GmbH) to obtain pure cultures to be used in further analysis. Larvae showing signs of entomopathogenic nematodes were rinsed in sterile distilled water and individually placed in modified white traps to collect the emerging infective juveniles (Glazer et al., 2000).

We used a chi-squared test to evaluate the effect of distance from geothermal springs on the occurrence of entomopathogenic species in soil samples.

Molecular identification

Aliquots of mycelium and conidia (about 10 mg maximum) from purified colonies were frozen at -80°C and pulverized with Precellys 24. Genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. The amplification and sequencing of the ITS locus was performed using ITS4 and ITS5 primers according to White et al. (1990). As the only isolates interesting from the entomopathogenic point of view were those belonging to the genus *Beauveria*, only these ones were further characterized by sequencing loci EF1α and Bloc according to Rehner et al. (2011). The identification of fungal species was based on a homology search in the GenBank database using the ITS sequence as a query. Entomopathogenic fungi belonging to the genus *Beauveria* were further characterized through a Neighbor-Joining (NJ) phylogenetic tree; orthologue sequences were mined from GenBank considering only fully characterized strains (ARSEF or Type material) (see Table S2 in Supplementary Material 1). Tree was constructed with MEGA-X software (Kumar et al., 2018) on BLOC and EF1α concatenated loci considering an alignment 2488 positions long including gaps. The resulting tree was tested with 1000 pseudo-replicates (Supplementary Material 2).

DNA extraction from 6 individual nematodes isolated from white traps was performed according to Torrini et al. (2019). Three loci were selected for amplification: ITS locus was amplified with 18S and 26S primers (Vrain et al., 1992); SSU gene fragment with Nem1 and Nem2 primers (Foucher & Wilson, 2002); and the D2/D3 region of the LSU gene with D2F and 536 (Molina-Ochoa et al., 2009). The identification of nematode species was based on the homology search in the GenBank database using the ITS sequence as a query. For both fungi and nematodes, amplicons were sequenced in both directions at Bio-Fab Research Company (Rome, Italy) and assembled and aligned with Geneious 2021 software. The resulting sequences were deposited on GenBank (see Table 1).

RESULTS

The molecular identification of entomopathogenic fungi isolated from the sampled areas allowed a total of nine species to be detected: *Beauveria pseudobassiana* S.A. Rehner & Humber, *Fusarium oxysporum* Schltdl., *Fusarium proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg, *Penicillium chrysogenum* species complex Thom, *Penicillium camemberti* species complex Thom, *Penicillium* sp., *Aspergillus flavus* Link, *Gloetinia* sp., and *Cunninghamella bertholletiae* Lendn. As reported in Table 1, all fungi came from the immediate surroundings of geothermal vents, apart from the sample M2 of *Beauveria pseudobassiana* collected under an individual of *Quercus suber* about 16 meters from the nearest spring. Species found in more than one sampling site share almost the same sequence in the ITS locus, i.e. they differ only in one or two nucleotide substitutions. The only exceptions referred to the two *Beauveria pseudobassiana* isolates that were found to be quite different from each other. In particular, the isolate collected at point M2 appears to be more phylogenetically close to most *B. pseudobassiana* strains than the one collected in M7, as shown by the Neighbor-Joining (NJ) phylogenetic tree (Figure 2).

The six nematodes analysed were extracted from the same *Galleria* larva (sample T8). Their ITS, LSU, and SSU DNA fragment sequences were identical and were sufficient to assess the identification to *Pristionchus uniformis* Fedorko & Stanuszek, 1971.

Most positive samples for nematodes and fungi came from the immediate surroundings of the geothermal spring ($\chi^2 = 21.37$, df = 2, $P < 0.001$).

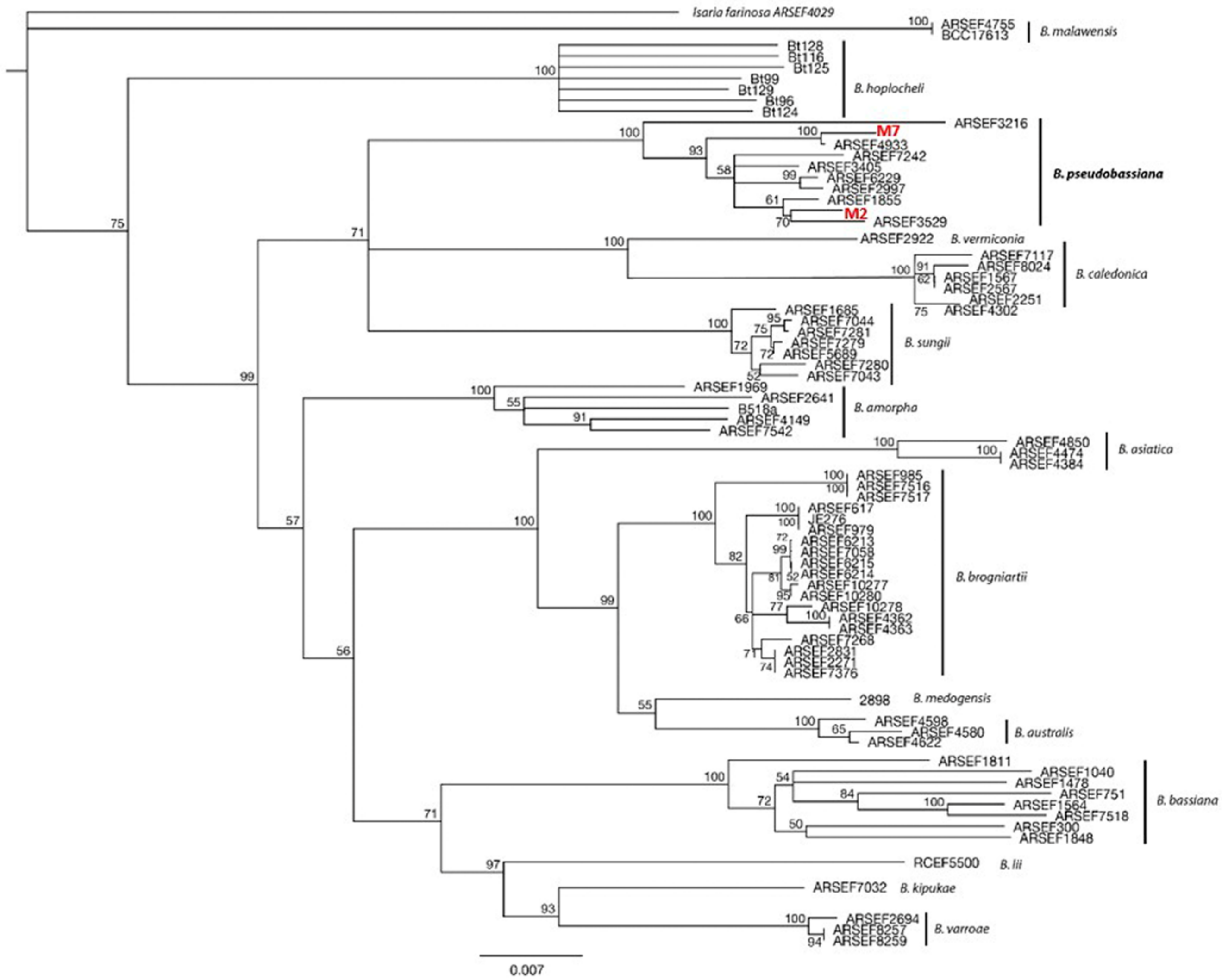

Figure 2. Neighbor-Joining phylogenetic tree of *Beauveria* sequences. Numbers at nodes indicate bootstrap values. Sequences in bold are those detected in our sample. The number of substitutions per site is shown in the unit of measure.

DISCUSSION

We showed for the first time the presence of nine species of entomopathogenic fungi and one nematode in the acid soils from geothermal areas in central Italy. Particularly, the only nematode found, *Pristionchus uniformis* is reported in necromenic association with beetle hosts (as well as in some soils and rotten plants), which explains its occurrence in dead *Galleria* larvae (D'Anna & Sommer, 2011). The genus *Pristionchus* is present in all soils, although it is usually rare. In particular, the detected species is new for Italy, as it is reported only in North America and a few European countries (i.e. France, Serbia, Montenegro, Bulgaria, Germany, and Poland: D'Anna & Sommer, 2011), and also new for geothermal areas. Other species of this genus were recorded in Italy using the same method (Landi et al., 2017). In particular, in our work, *P. uniformis* was detected in the immediate nearby of the hot spring, at a low pH (around 5) and at over 50°C suggesting its ability to survive at high soil temperatures.

Table 1. Entomopathogenic fungi and nematodes detected in soil samples collected and determined through genetic analyses. NA, not available; georeferenced data of points of soil collection, surface soil temperature, pH, and plant species detected at each point are also shown.

Point	Latitude (°N)	Longitude (°E)	Surface soil T (° C)	pH	Distance from the nearest geothermal spring (m)	Nematode species (GenBank Accession number)	Fungal species (GenBank Accession number)
T3	43.1524	10.8491	38	4.45	31	NA	NA
T6	43.1528	10.8533	47	2.56	< 10	NA	*Fusarium oxysporum* (OK663512; OL348440) [ID=99.9% Cov=100%]
T7	43.1535	10.8543	56	4.08	< 10	NA	NA
T8	43.1540	10.8551	54	5.39	< 10	*Pristionchus uniformis* (OL348453 [ID=100% Cov=100%]; OL348440; OL348232)	NA NA
M1	43.1548	10.8516	28	5.67	36	NA NA	NA NA
M2	43.1539	10.8527	26	4.99	16	NA	*Beauveria pseudobassiana* (OK663510; ON408389 [ID=99 Cov=98.06%]; ON408391)
M7	43.1512	10.8508	43	5.56	< 10	NA	*Beauveria pseudobassiana* (OK663516; ON408390 [ID=99 Cov=99.49%]; ON408392)
B1	43.1642	10.8595	44	2.5	< 10	NA	*Gloeotinia* sp. (OK663505) [ID=100% Cov=100%]
B2	43.1648	10.8591	43	2.16	< 10	NA	*Cunninghamella bertholletiae* (OK663511) [ID=96.3% Cov=100%] *Fusarium oxysporum* (OK663504) [ID=99.9% Cov=100%] *Penicillium chrysogenum* species complex (OK663508) [ID=99 Cov=100%] *Aspergillus flavus* (OK663514) [ID=100% Cov=100%]
B3	43.1665	10.8582	52	2.83	< 10	NA	NA
B4	43.1668	10.8579	58	0.87	< 10	NA	*Aspergillus flavus* (OK663515) [ID=100% Cov=100%] *Penicillium camemberti* species complex (OK663506) *Penicillium* sp. (OK663513) [ID=100% Cov=100%] *Fusarium sp.* (OK663509) [ID=99.8% Cov=100%]
B5	43.1674	10.8584	53	1.97	< 10	NA	*Aspergillus flavus* (OK663507) [ID=100% Cov=100%] *Fusarium oxysporum* (OK663512) [ID=99.9% Cov=100%]

Before our study, the composition of microbial communities around hot geothermal springs in Italy only included a prokaryotic community analysis (Arif et al., 2021), which showed that highly adaptable thermophilic and mesophilic species occur.

Surprisingly, all fungi and nematode species detected in our samples come from the surrounding geothermal springs, suggesting that these species are capable of effectively tolerate the extreme environmental conditions of the study areas, including low pH and high temperatures. This result is in line with the scientific literature on these species. *Penicillium* spp. and *Aspergillus* spp. are generally reported to be able to resist to high temperatures (30-37°C as a range of optimal growth), and commonly recorded at high metal content and low pH (Redman et al., 2009; Arif et al., 2021). The presence of these fungi confirms the possibility of adaptation to the extreme conditions of these microorganisms. *Fusarium* species can grow from 7 to 9 pH, showing a lower growth at pH 4, as a potential adaptation to acid environments (as our study areas). These species may also resist to high temperatures (Bazzicalupo et al., 2021). *Fusarium oxysporum* generally grows slowly over 40 °C (Bazzicalupo et al., 2021). Regarding *Gloeotinia*, this genus is poorly studied and includes necrotrophic, opportunistic plant pathogenic fungi, which degrade tissues of a wide variety of plants and generally overwinter as soil-borne sclerotia (Boldù et al., 2014). However, the detection of this genus in high-temperature soils is very interesting, as previous literature reported 30 °C as the extreme temperature above which the species could not grow (Alderman, 1992). *Cunninghamella bertholletiae* is widely reported as a thermophilic species and can represent a human pathogen (Kobayashi et al., 2004; Bragulat et al., 2017). It grows very fast when incubated at 30-40 °C, while showing limited growth at 45 °C (Bragulat et al., 2017). Furthermore, *C. bertholletiae* is a species that shows great tolerance to heavy metals (Bello & Abdullahi, 2016; Ren et al., 2018), so it can also survive in geothermal soils (cf. Costantini & Pagani, 2017). Furthermore, it is considered a good candidate for bioremediation of polluted environments due to its ability to remove heavy metals (Bello & Abdullahi, 2016). The two collected strains of *B. pseudobassiana* were classified as entomopathogenic fungi. They localize in different subclades of *B. pseudobassiana* species (Fig. 2), this lead to hypothesize a possible independent adaptations history of the two different isolates. Moreover, the strain collected at point M2 was found in soil with a temperature of 26 °C which falls within the typical temperature ranges of this mesophilic species (6-35 °C, with optimal at 20-30 °C: Fargues et al., 1997). On the other hand, *B. pseudobassiana* found at point M7 located near the geothermal spring deserves particular attention, since at this point a surface temperature of 43 °C was measured.

To conclude, adaptive strategies of this peculiar community might have included the development of physical and physiological structures to tolerate prolonged exposure to high temperatures and low pH values (Redman et al., 2009). Thermotolerant fungi and nematodes use are currently increasing in biotechnological applications, particularly in this period of global warming and climatic change, thus requiring a great research effort on their detection and taxonomic classification (Cantrell et al., 2011; De Oliveira et al., 2015). Therefore, more interdisciplinary projects involving botanists, geologists, applied researchers in molecular science, and taxonomy would be strongly needed to fulfil this need.

ACKNOWLEDGEMENTS

The authors would like to sincerely thank Paola Ferri (MUBIA, Monterotondo Marittimo, province of Grosseto), who provided us with maps and informative material of our study area, and Dr. Luca Nerva for recommendations on an early draft. Alessandro Tondo, Maria Giuseppa Fiele, and Marco Celotto participated in the field work. Two anonymous reviewers kindly took the time to improve our MS with valuable comments. To conclude, we deeply thank the Departments of Biology of the Universities of Siena and Florence for the promised help. A.V. and E.M. were supported by MUR-FOE-Project Capitale Naturale-Task “Biodiversità”.

Data Availability Statement: All data are shown in the text and in the Supplementary Material.

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Submitted: 3 April 2022

First decision: 20 April 2022

Accepted: 5 May 2022

Edited by Diego Fontaneto