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Molecular detection of beneficial hyphal soil-born microorganisms in different soil types in areas infested with parasitic broomrapes - *Orobanche cumana* Wallr. and *Phelipanche ramosa* L. in Bulgaria

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ABSTRACT
Molecular markers and classic microbiological approaches for assessment of the population density and biodiversity of beneficial hyphal soil-born microorganisms (species from order Actinomicetales and arbuscular mycorrhizal fungi from Glomeromycota). Seven different soil types infested by parasitic broomrapes – *Orobanche cumana* Wallr. and *Phelipanche ramosa* L. were studied. The soil samples were collected from seventeen regions in Northern and Southern Bulgaria. Soil types were defined as: Chromic Cambisols; Chromic Luvisols; Calcic Vertisols; Mollic Fluvisols; Rendzic Leptosols; Chernozems – Calcic and Haplic, based on agrochemical characteristics. We found that the Chromic Luvisols soils have the highest population density of beneficial microorganisms and a high degree of colonization with arbuscular mycorrhizal fungi, followed by Mollic Fluvisols soils. Two main species of arbuscular mycorrhizal fungi were identified by molecular markers: *Glomus intraradices* and *G. mosseae*. The highest density of beneficial streptomycetes was found Chromic Luvisols soils. Based on molecular markers five species of Streptomycetes were identified namely: *Streptomyces ambofaciens*, *S. aureocirculatus*, *S. carnosus*, *S. fasiculatus*, *S. griseorubens*. The number of other hyphal fungi was also recorded from genera *Aspergillus, Penicillium, Trichoderma, Fusarium, Mucor*. Molecular identification of the strains of those fungi, however, will require a metagenomics approach. The results obtained will allow us to develop a new strategy for using beneficial hyphal microorganisms to control parasitic weeds from Orobanchaceae.

Key words: Actinomicetales, arbuscular mycorrhizal fungi, broomrapes, molecular markers, soil types, *Streptomyces.*

Introduction

The microbiological communities in the soil are extremely heterogeneous in a number of aspects like species composition, population density, performance, and environmental relevance. As a result of the activity of these communities, the global biogeochemical circle is maintained. This role of soil microorganisms is related to the processes of their metabolism – energetic and constructive (Anderson, 2003; Büchs, 2003; Karlen, 2010; Prasad et al., 2017, Conway & Bagyaraj, 2018). The trophic groups of microscopic fungi, actinomycetes (mainly streptomycetes) and arbuscular mycorrhizal fungi (Barnett & Hunter, 1998; Bagyaraj, 2018) can be referred to as hyphal soil-born microorganisms (Hall, 2018).

Probably the most widespread symbiosis in nature is between the roots of plants and the arbuscular mycorrhizal fungi (AMF) (Fitter, 2005; Prasad et al., 2017). Over 90% of land plant species form associations with AMF. The monophyletic Glomeromycota have a very wide range of plant symbionts, while other fungi have strict symbiont selectivity (Schübler et al., 2001; Hibbett et al., 2007). AMF intimately connects plants to the hyphal network of the fungi, which can exceed 100 meters of hyphae per cubic centimeter of soil and can connect a number of plant species (Miller et al., 1995). AMF hyphal networks provide plants with mineral nutrient (predominantly phosphate, but also nitrates and microelements) and water (Finlay, 2008; Bona et al., 2017). In return, AM fungi receive carbohydrates from plants (Solaiman & Saito, 2001; Rich et al., 2017). Therefore, AMF symbiosis contributes significantly to global phosphate and carbon

cycling and influences ecological services in soil ecosystems (Fitter et al., 2005). During this long co-evolution, plant and symbiotic micro-fungi established an elegant subterranean signaling system based on emission into the soil of soluble chemical compounds promoting the establishment of symbiosis. Many of these plant-derived signals are strigolactones (SL) (Akiyama et al., 2005; Cheng et al., 2017; Guillotin et al., 2017). Strigolactones stimulate not only spore germination in some symbiotic fungi, but also germination of the seeds of parasitic weeds such as broomrapes, parasitizing on plant roots. One of the recent breakthroughs in broomrape research is the understanding that upon their evolution, broomrapes took advantage of the ancient conservative signaling system between symbiotic fungi and their host plants and started to exploit it to recognize "chemical signature" of prospective hosts (Akiyama et al., 2005; Akiyama & Hayashi, 2006; Bouwmeester et al., 2007; Lopez-Raez et al., 2008, Kohlen et al., 2011).

Broomrapes are obligate root holoparasites from the family Orobanchaceae. They totally abandoned the autotrophic way of life and rely entirely on other "host" plants to provide them with water, mineral elements and carbohydrates (Kuijt, 1969; Fernández-Aparicio et al., 2011; Samejima & Sugimoto, 2018). Broomrape seeds are capable of persisting for over a decade in the soil. To start germination, they require not only favorite abiotic conditions but also an exposure of above-described signals exuded by roots of the host plants (Butler, 1995; Yoder, 2001; Bouwmeester et al., 2007; Lopez-Raez et al., 2009; Kohlen et al., 2011). After germination, the broomrape radicle is using a chemical gradient of the signals to protrude to the host root and to form haustorial connections with the hosts' vascular system. Colonization of roots by symbiotic microfungi can significantly suppress the parasite attack (Fernández-Aparicio et al., 2014). This effect could be a consequence of direct suppression of parasite germination by symbiotic fungi, but also as a protective mechanism of the host, since the mycorrhiza enhances the defense against root pathogens. Vierheilig (2004) proposed that once the plant is colonized by AMF fungi it starts to exude different signals that suppress further AMF colonization. Collaterally, this effect represses soil pathogens attracted by the same signal molecules that are active for AMF (Vierheilig, 2004). Using tomato as a model plant, Lopes-Raez et al. (2011) have shown that AMF symbiosis induces changes in transcriptional and hormonal profiles, including a significant reduction of strigolactone production.

Recently was reported that Actinomycetales species in particularly from genus Streptomycetes can also affect interactions host plants and pathogens (Krishna et al., 2018; Schlatter et al., 2017).

The aim of the present study is to apply classical and molecular biologic instruments in order to evaluate the biodiversity of hyphal soil-born microorganisms (in particularly species Actinomicetales and AMF) in different soil types collected from fields in Bulgaria infected with parasitic plants – *Orobanche cumana* Wallr. and *Phelipanche ramosa* L.

Materials and Methods

Soil samples for analysis

Soil samples were collected between May and July during 2017 and 2018 vegetative seasons from farmland used for growing of sunflower (host *Orobanche cumana*) and tobacco (host of *Phelipanche ramosa*). Samples were collected from 17 locations (Table 1) of Northern and South Bulgaria, where the degree of infestation with seeds of parasitic plants was high (Stoyanov, 2005; Shindrova, 2006). From each field average sample of the cultivated humus horizon was taken at a depth of 0-30 cm.

The determination of the soil types was done by Koinov et al. (1998). Seven main types and subtypes of soils were determined (Table 1). Each soil sample was analyzed for: total humus content (%) by Tyurin method; total nitrogen (%) in Kjeldahl; mobile forms P_2O_5 according to Olsen; mobile forms of K_2O – in 2N HCl and soil pH (as H_2O extract) – potentiometric according to ISO 14870:2001.

Classical microbiological analysis

- Quantitative microbiological analysis

The quantities of soil-born microorganisms from the group of microscopic fungi and Actinomycetes were determined. For this purpose soil samples were diluted whit sterile water in different proportions according to the Koch method. Suspensions were plated on Chapek agar for microscopic fungi and on starch-ammonium agar for Actinomycetes. Analyzes are made in three replicates. Amounts were determined after cultivation at 28°C for 7 days. The numbers were calculated as the number of colonies formed from 1 g dry soil (CFU / g a.d.s.) at confidence level $P < 0.05$ (Grudeva et al., 2006).

- Isolation of pure cultures

Individual and well-defined colonies developed on primary cultures were re-plated on Chapek Agar – for microscopic fungi and on Gause1 agar - for Actinomycetes. Cultivation was performed at 28°C for 72 h. The purity of the cultures was checked by surface plating on depleting agar according to the Drigalski method (Grudeva et al., 2006).

The choice of pure cultures was made using the macromorphological characteristics of colonies and microscopy for microscopic fungi (Barnett & Hunter, 1998). Actinomycetes cultures were analyzed based on the color of the color of the air and substrate micelles of the colonies, pigment release in the nutrient medium and probably belonging to Streptomycetes. The color of the air and substrate micelles were determined on 14-day cultures, on a Tresner-

Backus color scale using the Nonomuras' key (Nonomura at al., 1974; Locci, 1989).

-Further strains cultivations

Inoculums of the selected strains were prepared by the following procedure: Spores and micelles were transferred into 5 ml of sterile physiological solution until cloudy suspensions were formed. The suspension than were transferred into introduced into Erlenmeyer flasks containing 200 ml of liquid Gaussian medium for Streptomycetes or 200 ml liquid Chapek medium for other microscopic fungi. Each sample was cultivated for 14 days at 28°C. The inoculums than were used for molecular detection.

Classical analyses of AMF

AMF cannot be cultivated in vitro; we used trap crops (*Triticum sp.*) instead. Three hundred grams of each soil sample were laced in vessels in 3 replicas. After growing in a cultivation facility for 60 days, the plant roots are removed from the soil, washed and the degree of colonization was analyzed by the method of Heinman (Koske & Gemma, 1989).

The rest of the roots were cleaned very well from soil and debris. The samples were next placed in a blender and blended at high speed for approximately five seconds to release spores. The blended material was immediately poured through three sieves. The top sieve had 500 µm openings and captured roots, large debris. The two other sieves with 250 µm and 100 µm openings captured the majority of spores. The solution with spores was distributed in centrifuge tubes filled with 60% sucrose solution and centrifuged for 2 minutes at 1800 rpm, supernatant sucrose was poured through a 100 µm sieve and washed rapidly with water to remove the sucrose from mycorrhizal spores. The materials were then transferred in test tubes for observation, morphological determination (McGonigle et al., 1990; Muthukumar et al., 2009) and DNA extraction.

DNA extraction

Cultivated Streptomycetes were pelleted from 200 ml of a liquid medium of Gauze by centrifugation at 10 000 RPM for 5 min. The pellet was processed with QIAamp UCP Miniprep Kit (Qiagen) following the original protocol. The isolated DNAs were dissolved in 50 mL elution buffer.

Ten to fifteen milligrams of AMF spores were frozen with liquid nitrogen in microcentrifuge tubes and ground with Teflon pestle to a fine powder. Innu PREP DNA Mini Kit (Analytic Jena AG) was used for extraction of DNA following the enclosed protocol for processing of fungi.

The absorption at 260 nm was used to determine the concentrations of the isolated DNA in samples.

Primers

We used primers designed from the isolation of 16 S ribosomal RNA (for Streptomycetes) and 18S rRNA-ITS1-5,6S rRNA-ITS2- 26S rRNA for AMF. The primers were designed by the University of British Columbia, Nucleic Acid-Protein Service Unit, NAPS Unit, www.michaelsmith.ubc.ca/services/ NAPS/ Ribosomal Primers Sets). Because the production of this primer sets was discontinued by the UBC–NAPS Unit, the primers were ordered from Metabion International AG (Martinsried, Germany) and upon arrival were dissolved in DNase-free water to 100 mmol and stored at -20° C.

Isolation of ribosomal regions for molecular taxonomic studies

Six independent PCR reactions were performed for each sample to isolate rRNA coding regions respectively 16S rRNA for Streptomycetes and partial 18S rRNA-ITS1-5,6S rRNA-ITS2- partial 26S rRNA for AMF. For this reason, about 150 ng DNA template was taken for each reaction and mixed with 1 mL of corresponding forward and reverse primers (10 mmol/L), 25 mL PCR master mix (Fermentas/Thermo Fisher Scientific) and 22 mL DNase-free water. The PCR reactions were carried out using the following program: initial DNA denaturation at 940 C for 5 min; next 35 cycles of 94° C – 1 min; 54/56° C – 1 min; 72° C – 2 min and 30 s. The PCR products next were mixed with 6.5 mL of loading dye, loaded onto 1% agarose gel containing 0.5 mg/ mL ethidium bromide and 0.5X Tris–borate–EDTA buffer and separated by 150 V electrical currency for 45 min. The PCR products were visualized by ultraviolet light and the products with expected size were isolated from the gel, purified using QIAquick Gel Extraction Kit (Qiagen) and sent for sequencing in GATC / Eurofins Ltd – Germany.

Data analyses

Data from quantitative microbiological analyses and the level of colonization were statistically processed by ANOVA. P value<0.05 was considered as a statistically significant difference. Online nucleotide BLAST (Basic Local Alignment Search Tool) analyses in the NCBI database were performed using the nblast algorithm of Altschul et al. (1997). The multiple alignments of obtained sequences, phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7 (Kumar et al., 2016).

Results

Based on the data about agrochemical parameters as well as the current geographic distribution of the main soil types and subtypes in Bulgaria, the analyzed soil samples were assigned to the following soil types: Chromic Cambisols; Chromic Luvisols; Calcic Vertisols; Mollic Fluvisols; Rendzic Leptosols; Calcic Chernozems and Haplic Chernozems (Table 1).

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Table 1. *List of analyzed soil samples, soil types and major agrochemical parameters.*

Sample №	Location	Soil Type	Potential crops	Agrochemical parameters of soils				
				Total humus (%)	Total N (%)	Available forms, mg kg^{-1}		pH (in H_2O)
						P_2O_5	K_2O	
1.	Topolovgrad (Haskovo)	Chromic Cambisols	tobacco	3.08	0.166	18.30	583.40	7.81
2.	Harmanli (Haskovo|	Chromic Cambisols	tobacco	2.07	0.113	176.2	304.40	7.11
3.	Christiyanovo (Stara Zagora)	Chromic Cambisols	sunflower	2.67	0.158	1.20	326.30	8.03
4.	Podvis (Burgas)	Chromic Luvisols	sunflower	2.03	0.119	12.00	373.30	6.02
5.	Prilep (Burgas)	Chromic Luvisols	tobacco	1.85	0.103	19.60	415.70	6.47
6.	Hadzhidimitrovo (Yambol)	Chromic Luvisols	sunflower	2.07	0.124	20.60	336.20	6.40
7.	Karnobat (Burgas)	Calcic Vertisols	sunflower	3.60	0.205	8.60	528.50	7.19
8.	Lesovo (Yambol)	Calcic Vertisols	sunflower	2.64	0.145	15.10	640.50	7.67
9.	Parvomay (Plovdiv)	Calcic Vertisols	tobacco	2.55	0.155	57.10	654.80	7.29
10.	Stroevo (Plovdiv)	Mollic Fluvisols	sunflower	2.95	0.162	150.10	777.40	6.77
11.	Biser (Haskovo)	Mollic Fluvisols	tobacco	1.85	0.113	78.50	76.80	7.58
12.	Markovo (Plovdiv)	Rendzic Leptosols	tobacco	2.99	0.167	34.00	723.30	7.79
13.	Kuklen (Plovdiv)	Rendzic Leptosols	sunflower	1.55	0.123	36.40	940.09	8.13
14.	Zlokuchene (Shumen)	Rendzic Leptosols	sunflower	2.99	0.164	20.00	467.70	7.93
15.	Bezvoditsa (Dobrich)	Calcic Chernozems	sunflower	3.25	0.185	1.50	378.30	6.86
16.	L. Karavelovo (Aksacovo)	Calcic Chernozems	sunflower	3.38	0.179	98.60	679.00	7.67
17.	L. Karavelovo (Varna)	Haplic Chernozems	sunflower	2.51	0.146	34.20	322.30	6.46

Quantitative microbiological analyzes

The average population density of Actinomycetales was at a magnitude of tens of millions CFU/g.a.d.s. The highest densities were found in samples containing Calcic Vertisols type of soils followed by samples containing Chromic Cambisols type of soils: ranging up to several million CFU/g.a.d.s. The lowest population density was found in the Calcic Chernozems type of soils (Figure 1).

As expected the predominant part of Actinomycetales representatives were determined as *Streptomyces* species. For molecular analyses of *Streptomyces* single colonies with all macromorphological characteristic of Streptomycetes were selected.

As for other microscopic fungi the results showed the highest population density can be observed in Chromic Cambisols soils, followed by Mollic Fluvisols soil samples. The lowest population density was observed in samples containing Calcic Vertisols soils (Figure 2). There is a tendency of increase of the amounts of microscopic fungi in acidification in soils. The macromorphological characteristics of the colonies and their microscopic determination indicated that in all soil samples studied, can be found mainly representatives of genera *Aspergillus, Penicillium, Trichoderma, Fusarium* and *Mucor*.

The results of pots experiments with wheat trap cultures showed that the soils studied, were rich in arbuscular mycorrhizal fungi. A high degree of colonization was found in Chromic Luvisols, Mollic Fluvisols and Rendzic Leptosols soil types (Figure 3).

Molecular taxonomic studies

Two sets of forward/reverse primers were used to isolate 16S rRNA from *Streptomyces* and ITS regions from the ribosomal gene cluster of AMF. The external sets were used for isolation by PCR the ribosomal markers in three replicas

per sample, while the internal sets were used for sequencing. PCR products were sequenced in both directions. Reverse sequences first were converted by reverse complementation (MEGA 7) and next to the two sequences of each product were overlapped to obtain a consensus sequence. In cases where differences between sequences were encountered both sequencing reactions were repeated.

The nblast algorithm was used to compare isolated by us sequences of *Streptomyces spp.* with those annotated in the NCBI Genbank database. The results showed between 100% and 99% identity with e-value 0 with sequences deposited from five species: *Streptomyces ambofaciens*, *S. aureocirculatus*, *S. carnosus*, *S. fasiculatus* and *S. griseorubens*.

The same algorithm was used to study ITS sequences isolated from representatives of genus Glomus. Investigations indicated that was have isolated DNA from spores of two AMF species: *Glomus intraradices* and *G. mosseae*.

Next, the isolated by us sequences were subjected to phylogenic analyses together with the most similar sequences deposited in NCBI Genebank. In order to simplify phylogenic trees not all 17 sequences per species were incorporated in the tree. Depending on how many identical sequences were isolated they were represented by one or a few sequences, while those containing unique SNPs were all subjected to phylogenic analyses. The phylogenic tree containing 16S rRNA sequences isolated from five Streptomyces species is presented in Figure 4. The grouping by species confirmed nblast and macromorphological analyses.

Following the same logic, the isolated by us IST1/2 sequences were used together with similar sequences deposited in NCBI Genebank to build a phylogenic tree (Figure 5). The tree confirmed morphological and nblast results about species determination as well.

Discussion

The majority of the analyzed soils had medium and high humus content (Chernozems, Chromic Cambisols, and Calcic Vertisols). Only the Chromic Luvisols soils had relatively lower humus content. The content of humus in Mollic Fluvisols and Rendzic Leptosols soils was low to medium. Soils of all types and subtypes had medium average enrichment of total nitrogen and very high enrichment with mobile potassium, while enrichment of mobile phosphorus in a prevailing number of soils was low. Soils with a neutral and slightly alkaline pH dominated among studied samples. Slightly acidic pH was registered in the samples of Chromic Luvisols soils.

Correlational between the agrochemical and microbiological data obtained in this study had not been found. There are tendencies of increasing the number of microscopic

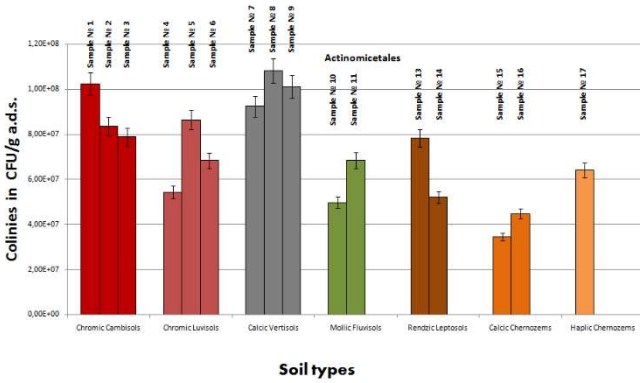

Figure 1. *Population density of Actinomycetales. The number of colonies formed from 1 g dry soil (CFU / g a.d.s.) was determined for each soil type at confidence level P < 0.05. Numbers of samples on the figure corresponds with numbers presented in Table 1.*

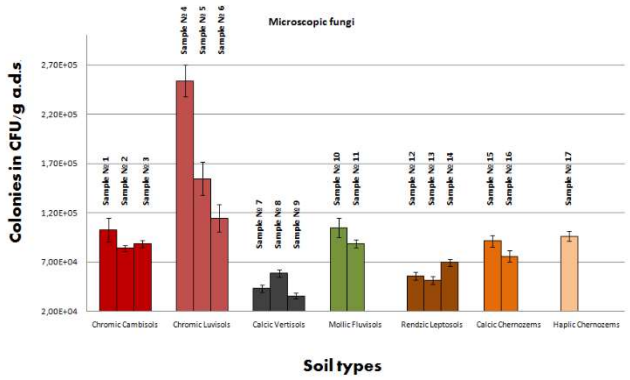

Figure 2. *Population density of other microscopic fungi. Total number of colonies was determined and presented as CFU / g a.d.s. for each soil type. Confidence level P < 0.05. Numbers of samples on the figure corresponds with numbers presented in Table 1.*

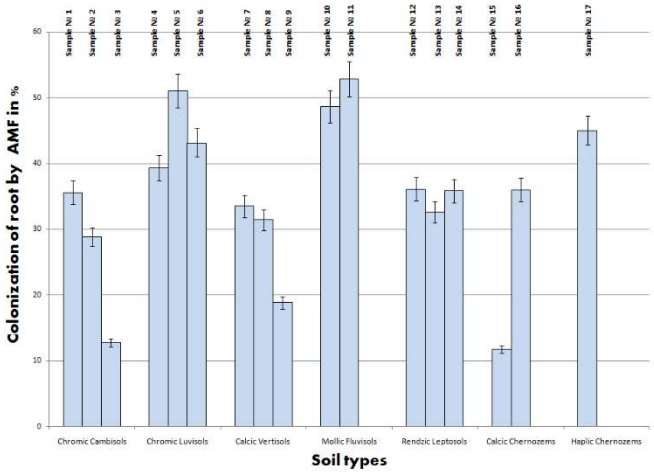

Figure 3. *Colonization of roots trap-culture Triticum sp. by AMF. Level of colonization was determined for each soil type as percentage. Confidence level P < 0.05. Numbers of samples on the figure corresponds with numbers presented in Table 1.*

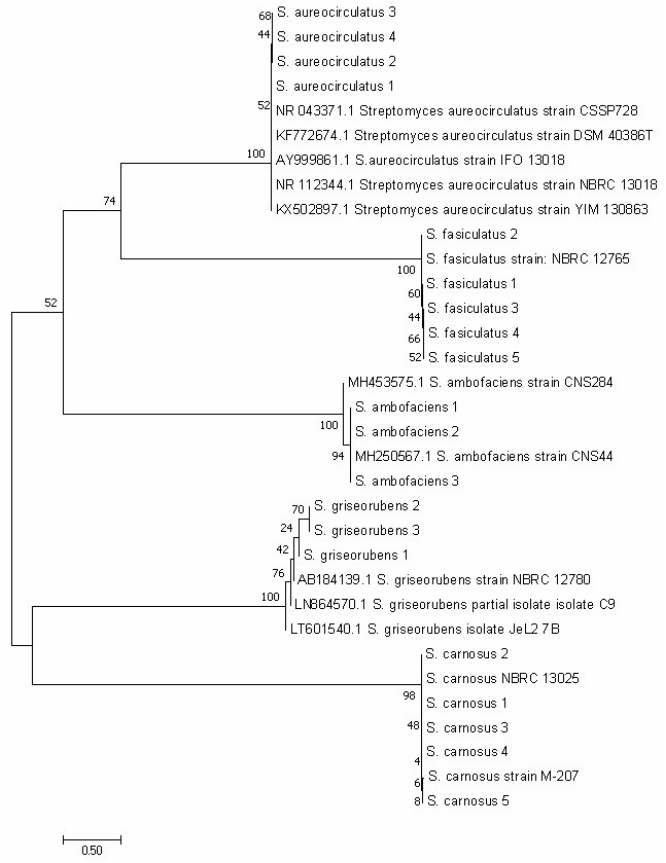

Figure 4. *Phylogenetic tree built by processing of 16 S rRNA sequences of Streptomyces ambofaciens, S. aureocirculatus, S. carnosus, S. fasiculatus, S. griseorubens annotated in NCBI and the isolated sequences from Bulgarian samples. Maximum likelihood was used, applying general time reversal model and uniform rate of substitution. Phylogeny test- bootstrap method by 500 replications. Details about soil samples are presented in Table 1.*

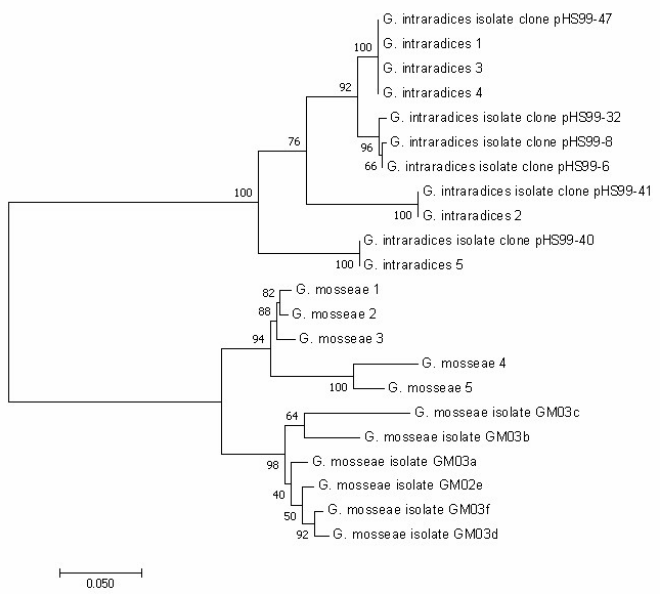

Figure 5. *Phylogenetic tree built by processing of ITS1/2 sequences of G. intraradices and G. mosseae species annotated in NCBI and the isolated sequences from Bulgarian representatives. Maximum likelihood was used, applying general time reversal model and uniform rate of substitution. Phylogeny test- bootstrap method by 500 replications. Details about soil samples are presented in Table 1.*

microfungi and the colonization of roots by AMF in soils with neutral and slightly acidic pH, while Actinomycetes (*Streptomyces*) were better presents in soils with higher humus content.

Actinomycetes and microscopic fungi are characteristic inhabitants of soil biocenoses and determine soil productivity because their activity participate in the final stages of utilization of poorly degradable organic compounds in the soil. At the same time, they are among the most active producers of a variety of biologically active substances that can affect both plants and the composition and structure of microbial communities.

AMF, apart from symbiotic relationships with plants, also affect the rest of the inhabitants of the soil microbiome. There are quite little studies on the processes determining exact mechanisms, but during the last few years, molecular genomics and metabolic studies started to reveal new mechanisms and pathways of interaction.

Molecular analyses confirmed that pure cultures from five *Streptomyces* species were isolated. The obtained sequences allowed us to confirm independently from macromorphological and microscopic data the species. On the other hand, molecular data allowed us to study the intraspecific structure and variability of Streptomyces representatives. According to the phylogenic tree of Streptomycetes and sequence analyses, the lower internal variability can be observed in *S. ambofaciens*. That is why only three types of sequences were incorporated in the phylogenic tree. Seq. type № 1 was completely identical to the one annotated n NCBI as *S. ambofaciens* strain CNS44. Seq. № 2 differed from № 1 with one SNP: transversion at position 583 (G => C) and № 3 with transversion at position 848 (G => T). A similar situation was observed in *S. carnosus* sequences: one transition at position 129 (A => G) and one transversion at position 137 (G=> T). Seq. types № 1, 2 and 3 were 100% identical to *S. carnosus* isolate NBRC 13025, while Seq. type №5 was identical to *S. carnosus* strain M-207. Seq. type № 4 took intermediate place with only one transition at position 129. In both cases correlations between type of soils and location of samples were not found.

S. aureocirculatus and S. *fasiculatus* sequences displayed five and six SNPs respectively. While in *S. aureocirculatus* only transitions were registered: C => T at positions 115 and 139, and G => A at positions 121. 197 and 510 *in S. fasiculatus*

both type of SNPs were found. The relative positions of SNPs were: T => C transitions at positions 135, 141, 153, 757, A => G transition at position 161 and A => C transversion at position 142. While in case with *S. aureocirculatus* no correlation with soil types and location was found, in a case with *S. fasiculatus* some correlations exist. Seq. types № 3, 4, 5 were isolated from Chromic Luvisols soils (soil samples 4, 5, 6). The sequences type 1 and 2, which were nearly identical with *S. fasiculatus* strain NBRC 12765 displayed no correlation with soil type.

The highest internal variability was observed in *S. griseorubens* – nine SNPs: C => transition at positions 102, 107, 116; A => C transversion at position 120; A => T transversion at positions 123, 168, 604 and G => T transversions at positions 656 and 784. In fact, the three sequence types of Bulgarian differed from the sequences deposited at NCBI Genebank (Figure 4). Seq. types № 2 and 3 possessed all eight out of nine SNPs, while Seq. type № 1 displayed seven SNPs. No correlations between soil types and SNPs were found. Nevertheless, the results indicated that *S. griseorubens* microflora is distinguished for Bulgaria as far as it concerns the level of variability in 16S rRNA.

Investigations of AMF based on variability in ITS1/2 region revealed that in the studies soil they are represented by two species: *Glomus intraradices* and *G. mosseae*. They formed two distinctive branches on the phylogenic tree. No correlation between types of soil and needed for isolation of pure cultures. qRT-PCR with such primers can speed-up investigation.

On the other hand, the results can be used for preparation to bioecological field practices for sustainable agriculture, including biological agents for control of plant pathogens.

Several SNPs were found in isolated sequences, which also is clearly visible on the phylogenic tree (Figure 5). *Glomus intraradices* sequences were different by eight SNPs: six C/T transitions at positions 32, 119, 263, 274, 486 and 552; one A/G transition at position 506 and one SNPs where A. G or T can be found. Sequence types № 1, 3, 4 were identical with G. intraradices isolate clone pHS99-47. Sequence type № 2 was identical with *G. intraradices* isolate clone pHS99-41, while sequence type № 5 was identical with *G. intraradices* isolate clone pHS99-40.

Unlike *G. intraradices*, sequences isolated from Bulgarian representatives of *G. mosseae* where different from those deposited in NCBI Genebank. As it is clearly seen in Figure 5, all five sequence types formed separate sub-branch. It is not surprising because the difference between our sequences and those annotated in NCBI varies between 18 and 20 SNPs (Table 2). Therefore, we can assume that the isolated representatives of *G. mosseae*, judged on ITS1 / 2 sequences differ from AMF studied from other authors.

In general, our data provide a good base for further investigation of biodiversity of hyphal soil-born microorganisms at different soil types in Bulgaria.

The discovered SNPs will serve in our pending research for the design of new highly specific primers for identified strain/isolates. They can be used for rapid determination of population density of certain strain/isolate of Streptomyces and AMF without long and labor consuming processes.

Acknowledgement

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Table 2. *Comparative list of unique SNPs in ITS1/2 sequences deposited at NCBI Genebank and our five sequence types for G. mosseae.*

Name	Relative position of SNP																			
	40	48	61	70	75	91	92	96	101	162	176	179	231	233	273	292	300	343	478	530
G. mosseae (NCBI)	G/A	C	A	T	G	T	T	T	T	G/T/C	G	G/A/T	T	T/C	T	C	A	C	C	A/C
Seq. type № 1	G	T	A	C	A	C	A	C	G	G	G	T	C	C	C	T	A	T	C	C
Seq. type № 2	C	T	A/G	T	T	C	A	C	G	C	T	T	T	C	C	T	A	T	C	C
Seq. type № 3	G	T	G	T	T	C	A	C	G	G	G	G	T	C	C	T	A	T	C	C
Seq. type № 4	G	T	A	T	T	C	A	T	G	G	G	G	T	C	C	C	T	C	G	A
Seq. type № 5	G	T	A	T	T	C	A	T	G	C	G	G	T	C	C	C	-	T	T	C

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