NGS TECHNOLOGIES FOR A COMPARATIVE CHARACTERIZATION OF ENDOPHYTIC BACTERIA IN THE ROOTS OF CONSTANTINOPLE CHICKPEAS (*CICER MONTBRETII* JAUB. & SPACH) ASSOCIATED WITH CINNAMON FOREST AND YELLOW EARTH PODZOLIC SOILS

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Abstract

Previous studies have analyzed bacterial taxa associated with rhizospheric cinnamon forest soil. *Cicer montbretii* Jaub. & Spach (*C. montbretii*), an endemic species for the region of Strandzha, was analyzed using a metagenomic approach. The diversity of endophytic microorganisms is essential for ecology, as it makes it possible to understand in detail the plant-microbial interactions. The objectives of this study were to isolate and identify endophytic bacteria from the roots of *C. montbretii* associated with cinnamon forest (A1) and yellow earth podzolic soils (A2). A study on endophytic bacteria in the roots of *C. montbretii* legume plant, done by assessing its PCR amplicon of 16S rDNA with the Illumina metagenomics technique, used to generate a total of 81,782 reads from the samples. The analyses revealed that *Proteobacteria* were similar in cinnamon forest and in yellow earth podzolic soils. *Bacteriodota* prevailed in yellow earth podzolic soils. Firmicutes and Actinobacteria were the common genera in cinnamon forest soil. The roots of the plants grown in A1 soil had the largest composition with Proteobacteria 78%, which consisted of the genus— *Erwinia* 13%, *Pseudomonas 11%*, *Shingomonas* 11%, *Rhizobium* 5%, and *Bradirhyzobium* 4%. In the A2 soil prevailed Proteobacteria 76% and Bacterioidota 15%. The most abandoned genus in A2 was *Orchobactrum* 20%, *Stenotrophomonas* 15%, *Sphingobacterium* 12%, *Serratia* 4%, and *Flavobactrerium* 4%. The α-diversity analysis indicated the richness and inverse Simpson diversity index of the bacterial endophyte communities for the root tissues to be 6.551 for A1 and 4,692 for A2 soil, respectively. The endophytic bacterial communities differed among the two soil samples, indicating that the soil and the climatic conditions considerably affected the entire endophytic community.

Keywords: NGS, Endophytes, *Cicer montbretii*, Strandzha Nature Park

INTRODUCTION

The soils in Strandzha were formed under the influence of a unique combination of climate, forest tree vegetation, the enormous variety of roots and soil-forming rocks, low-mountain relief with a great fragmentation, a densely located hydrographic network with short slopes and a dominant southern exposure.

In Strandzha, the cinnamon forest soils predominate, followed by the mountain-specific yellow soils. They are associated with *C. montbretii* and greater moisture retention, both from atmospheric precipitation and subsoil runoff and from fogs that linger in deep valleys and maintain high air humidity. The cinnamon-brown forest soils are spread only in one massif between the villages of Kosti and Bulgari and are surrounded by yellow-earth podzolic soils which are spread in Strandzha. The development of yellow soils under the influence of South Euxine forest vegetation distinguishes

them from the soils of Mediterranean Europe and reveals their natural connections with the low valleys of the Caucasus. Typical for Bulgaria and Europe is the weathering of the soil-forming rocks to alumino-silicate minerals. In the conditions of greater humidification and increased temperature, the organic matter is rapidly decomposed and deep washing of bases and silicates from the soil profile takes place. For this reason, yellow soils are strongly acidified and enriched with aluminium and iron oxides, giving them their characteristic yellow color.

Wild relatives of cultivated plants are an important element of plant biodiversity. Bulgaria ranks sixth among the ten countries in the world with the highest concentration per unit area. This group of plants has sources of valuable qualitative indicators of resistance: to extreme environmental conditions (drought, temperature inversions, very low winter temperatures), tolerance to diseases and pests, specific qualities such as food sources, etc. (Iriondo et al. 2008; Maxted et al., 2003; Angelova et al., 2018; Angelova et al., 2023). The flora of the Strandzha Mountain is characterized by a large number of relict species (Uzunova and Uzunov, 2008). More than 120 species are listed in the Red Book of Bulgaria, including *C. montbretii*. This paper reports the results obtained from an expedition survey of several localities of *C. monbretii* in Strandzha Nature Park. According to Uzunova and Uzunov (2008), Constantinople chickpeas can be found near the villages of Stoilovo, Kondolovo, and Bulgari, as well as in the protected areas of Veleka River and Silistar. The use of wild species is especially relevant today when under the influence of various anthropogenic factors, climate changes, etc. the disappearance or narrowing of the range of their distribution is observed. In 2001, Miho Mihov registered a small locality of *C. monbretii*, along the road from the village of Gramatikovo to the town of Malko Tarnovo (Petkova et al. 2023). Later, this deposit was confirmed during our expeditions. On the Ahtopol - Brodilovo forest road in 2011, two new small habitats were discovered and marked. They were distributed in small groups of plants near the road. During two expeditions under international projects, in 2012 and 2014, larger deposits of *C. monbretii* were identified in the localities "Mishkova Niva" and "Indipasha" in oak forest communities, on leached cinnamon-forest soil (Angelova et al. 2023, 2018; Petrova et al. 2013).

Microorganisms play a major part in ecosystem functions, making them unique due to their diverse microbial composition. They are directly related to plant species and their abundance. They are dominant components of the plant microbiota and can influence plant growth and responses to stresses according to Alibrandi et al. 2020. Quite a few studies in the last decade focused on the structure and functions of the plant microbiota, with the aim to link them to plant dissemination and productivity (Bulgarelli et al. 2013; Compant et al. 2019; Tian et al. 2020; Alibrandi et al. 2020). The study of microbial ecology is essential to ecology because it enables a detailed understanding of both microbial interactions (Kent and Triplett, 2002). The association between plants and microorganisms can be influenced by several factors, and some of the them include the symbiont's genetic diversity; how they are acquired from the environment and an individual's ability to colonize hosts (De Tender, 2017).

The current research focused on NGS analyses of endophytes bacteria living within Constantinople chickpeas (for at least part of their life cycle) in intercellular spaces, tissue cavities, or conducting vessels without causing diseases. They are ubiquitous and found in all types of plants. Endophytes can help host plants in preventing colonization by pathogenic or parasitic organisms. They can colonize plant roots and assemble their communities, thereby being involved in different interactions. The majority of these interactions is not limited to

microorganisms and plant alone, but also involves associations among other microorganisms (Agler et al. 2016). This gives a strong impetus to the study of endophytic microorganisms, thus resulting in knowledge of how their functions can be changed or modulated in the direction of improving their quality. Since only 0.1 - 1% of the microorganisms in the natural ecosystems can be cultivated in a laboratory, traditional methods for microbiological analyses do not allow for revealing the actual species diversity in them and the dynamics in mixed cultures associated with relevant biochemical changes in soils. The relationship between the species diversity of microorganisms and the geographical origin of leguminous plants cannot be reliably established. In 2018 Frąc et al. claimed that the adaptation to environmental changes determined by processes of natural selection is unique. The molecular analysis of the entire soil DNA component can effectively combine the benefits of microbiology, molecular biology, genetics, ecology and botany, and answer the question about the influence of soil microorganisms on the specific and very often restricted distribution of wild relatives (Barea, 2005; López-García and Moreira, 2008).

MATERIALS AND METHODS

Expedition survey

The expeditions aimed to establish the natural habitats of *C. monbretii* were held annually from 2010 until 2023. These trips were carried out during the flowering and ripening phases of *C. monbretii* (Angelova et al., 2023; Petkova et al.2023). One of the habiatat of *C. monbretii* was associated with the cinnamon forest soil (A1) in the area of Mishkova Niva and the second habitat with the yellow earth podzolic soils (A2) around the village of Brodilovo. Mishkova Niva is located on the rounded ridge of a low peak with a direct view to Bosna, Papia and Gradishte peaks of the

Strandzha Mountain (Latitude. 41° 96' 53" N and Longitude 41° 57' 55") (Figure 1). The village of Brodilovo is located in the easternmost branches of the Strandzha Mountain - about 5 km southwest of the nearest part of the Black Sea on the left (northern) bank of the Veleka River (Latitude: 42° 04' 60.00" N Longitude: 27° 50' 59.99") (Figure 1).The village of Brodilovo is located in the easternmost branches of the Strandzha Mountain - about 5 km southwest of the nearest part of the Black Sea on the left (northern) bank of the Veleka River (Latitude: 42° 04' 60.00" N Longitude: 27° 50' 59.99") (Figure 1).

Figure 1. The map of the Strandzha Mountain, Bulgaria with the location of *C. monbretii* habitats around Mishkova Niva and Brodilovo village.

C. monbretii sampling and surface-sterilization

Samples of *C. monbretii* plants from the two different habitats were harvested during flowering in the Strandzha mountain (Bulgaria), stored on ice in sterile containers, transported to the laboratory and kept at 4°C before being processed (within 2 days). Plant tissues were surface–sterilized by stepwise immersion in 3% sodium hypochlorite for 2 min and finally in 70% ethanol for 1 min, followed by three rinses in sterile distilled water (Petkova et al. 2020).

DNA extraction from *C. monbretii* roots

The complete genome of DNA was extracted using the HiPurA soil DNA isolation kit (Himedia, India), as defined by the method of Siddhapura et al. (2010) and modified by Nair et al. (2014). At A260/280 nm the isolated DNA was quantified by utilization of a Quantus fluorometer (Promega) and stored at -20°C before being processed. For NGS (Illumina HiSeq), these quantified DNA samples were sent to Novogene (Cambridge, UK).

Metagenomic sequencing

Purified DNA was used as a template for amplification of V5-V7 region of the 16S rRNA gene was amplified with polymerase chain reaction (PCR) using primers V5 (AACMGGATTAGATACCCKG) and V7 primers (ACGTCATCCCCACCTTCC) (Fuks et al. 2018; Turner et al. 1999). Metagenomic sequencing was done at Novogene UK. The library preparation was done with Nextera DNA Flex kit (Illumina) following a standard procedure. The exact amount of PCR products from each sample were pooled, A-tailed, and further ligated with Illumina adapters. The amplicon was sequenced on Illumina paired-end platform to generate 377 bp paired-end raw reads (Raw PE) and then merged and pretreated to obtain clean tags. The data were processed by utilization of QIIME software, v.1.8.0 (http://qiime.org/) as published by Caporaso et al. 2010. The first stage of the 16S rRNA gene analysis involved quality control of the sequences to exclude from the analysis those with a length of fewer than 200 nucleotides, with a quality score of less than 25, with misread sequences of primers and multiplex identifiers, extensive homopolymer repeats (more than 8 nucleotides) and unidentified nucleotides. The chimeric sequences in clean tags were removed to obtain effective tags, which were used for subsequent analysis (Martin, 2011; Haas et al. 2011; Edgar, 2013). The OTUs were selected at more than 97% similarity. The estimation of

ACE and Chao indices and Shannon and Simpson diversity indices were calculated using the Mothur program (Abell et al. 2005). Alpha diversity was analyzed by utilization of six indices, including Observed-species, ACE, Shannon, Simpson, Chao1, and Good-coverage, and calculated using QIIME (Version 1.7.0, http://qiime.org/1.7.0/) and displayed with R software (Version 2.15.3) (Chopkova et al. 2023; Caporaso et al. 2011; Zhang et al. 2019). OTU comparisons were performed using the Venn diagram package (Zhang et al. 2019). All significance tests were two-sided, and p values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Expedition survey

The expeditions were carried out during the flowering and ripening phases of *C. monbretii* (see Petkova et al. 2023). The structure, size of populations and their vulnerability are described, as well as the accompanying plant species. The morphological description of the plants is done in the flowering phase, and in the ripening phase, the characteristics of the beans and seeds. The differences between the species in the localities are mainly in the height and positioning of the plants. An important moment here is the registration of the main phonological phases (mass flowering and ripening), as well as the possibilities for collecting seeds. In the habitats themselves, the ripening phase is uneven and depends on the exposure of the terrain. The species is represented by 5, 10, and 20 plants, in groups scattered on the slope between the road and the oak forest, as well as in open places near motorways and tourist routes. The flowers are grouped from 2 to 5, rarely single, in loose racemes in the axils of the leaves. The bean is smooth, wide, oblong, brown, and 3 - 4 seeded. The seeds are globose, brown or black. The deposits are located in an oak forest, on leached cinnamon-forest soils and yellow earth podzolic soils. Depending on the

exposure of the habitat, the height of the plants varies within different limits. In some literary sources, it is indicated that it is from 20 to 40 cm (Uzunova and Uzunov, 2008; Petkova et al. 2023), and in our survey it varies from 50 to 70 cm. The deposits located near the road have a size of 5-10 m^2, and those on the slopes near the oak forests are about 15-20 m^2 and have an altitude of 56 to 368 m. The main accompanying species are from the family *Poaceae* and the family *Vicia*. The most common species are *Trifolium campestre* Schreb., *Stellaria holostea* L.; *Crataegus monogina* Jacq. In previous studies (see Angelova et al., 2023, 2018; Petrova and Angelova, 2013), seeds from the species *C. monbretii* were collected at physiological maturity (Figure 2) and unsuccessful attempts were made using classical methods to establish germination and maintain the species outside the natural environment (ex-situ). Keeping in an ex-situ collection allows for propagating and obtaining seeds for storage in a gene bank, and for reintroduction in natural habitats.

OTU identification and taxonomic annotation

A total of raw tags were generated from the Illumina Miseq sequencing of both samples A1 124,004 and A2-126,168 (Tabl. 1). After quality control, a total of clean tags remained for A1-112,668 and for A2 115,033. Then, after the removal of the chimaeras a total of 227,701 effective tags were obtained, ranging from 93,368 for A1 to 70,198 for A2 OTU generation. The Q20 values for the four samples ranged

from 98,52 for A1 to 98.55 for A2, indicating the high quality of the Illumina sequencing.

Figure 2. *C. monbretii* at the stage of physiological maturity.

The rarefaction curve and the rank abundance curve evaluating the OTU richness per sample were shown in Figures 3 A and B, indicating the difference in the bacterial communities of endophytic bacteria from the roots of *C. montbretii*, associated with the cinnamon forest (A1) and the yellow earth podzolic soils (A2). The endophytic bacteria from the roots of *C. montbretii* associated with the cinnamon forest (A1) had the highest abundance of bacterial species, compared to the endophytic bacteria from the roots of *C. montbretii* grown on the yellow earth podzolic soils (A2) (Figure 3).

Table 1. OTUs number of each sample (endophytic bacterial OTUs (A1) *C. montbretii* associated with the cinnamon forest (A2) and the yellow earth podzolic soils).

| Sample Name	Raw PE(#)	Raw Tags(#)	Clean Tags(#)	Effective Tags(#)	Base(nt)	AvgLen (nt)	Q20	Q30	GC%	Effective %
A1	124,004	113,282	112,668	93,368	35,237,433	377	98.52	95.35	54.99	75.29
A2	126,168	115,647	115,033	70,198	26,428,431	376	98.55	95.49	53.73	55.64

A)

B)
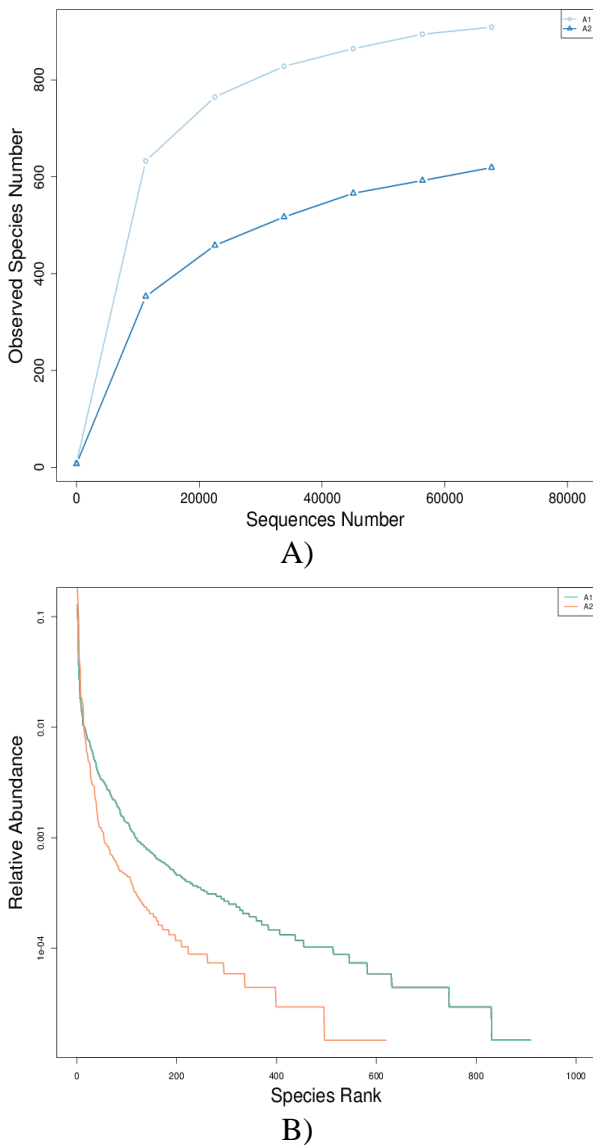
Figure 3. Rarefaction curve (A) and rank abundance curve (B) of endophytic bacterial 16S rDNA clone library of roots *C. montbretii* associated with the cinnamon forest (A1) and the yellow earth podzolic soils (A2).

Phylum level distribution of endophytic bacteria in *C. montbretii* roots

The metagenomic analysis done by using the Subsystem database revealed a total of 10 phyla in all the samples, but they were more abundant in samples in (A1) *C. montbretii* roots associated with the cinnamon forest, compared to the endophytes in the plants grown on the yellow earth podzolic soils (A2). The major

phyla observed were the following: Proteobacteria, Bacteroidota, Firmicutes, Actinobacteria, Acidobacteria, Verrucomicrobia, Myxococcota, Gemmtimonadota, Patescibacteria, and Chloroflexi (Figure 4). The current data are in accordance with the findings of Žifčáková (2016) and Lladó (2017) who claimed that the acidic soils of coniferous forests harbour mainly Proteobacteria, Acidobacteria, and Actinobacteria. The yellow earth podzolic soils developed under coniferous or mixed forests in warm-temperate moist climates are composed of thin organic and organic-material layers resting on a greyish-yellow leached layer that in turn rests on a yellow layer. This soil is poor with many limitations, including low soil pH, low clay content, low aggregate stability, and low nutrient content. The process of podzolization is a complex soil-forming process in which iron and aluminium ions are released by the weathering of various minerals from the organo-mineral complexes and are removed from the upper parts of the soil profile. It is deposited in the deeper layers of the soil and the eluvial horizon of the podzol is bleached and acquires an ash-grey colour. The bacterial diversity in temperate deciduous forests, and litter bacterial communities seem to be especially enriched with Proteobacteria and Bacteroidetes in accordance with López-Mondéjar et al., 2015 and Purahong et al., 2014.

As shown in Figure 4, at the phylum level, Proteobacteria was the dominant endophyte that coexisted with Actinobacteria and Bacteroidota, in different proportions, in each of the different samples of *C. montbretii* roots. Similarly to Wang et al., 2021, the current results indicate that the abundance distribution of bacterial endophytes had some discrepancies among the different *C. montbretii* root samples, which was in line with the results obtained in Figure 5. Proteobacteria was the main dominant phyla in both *C. montbretii* roots samples and in the soil samples analyzed in previous research on the soil metagenomics (Petkova et al., 2023).
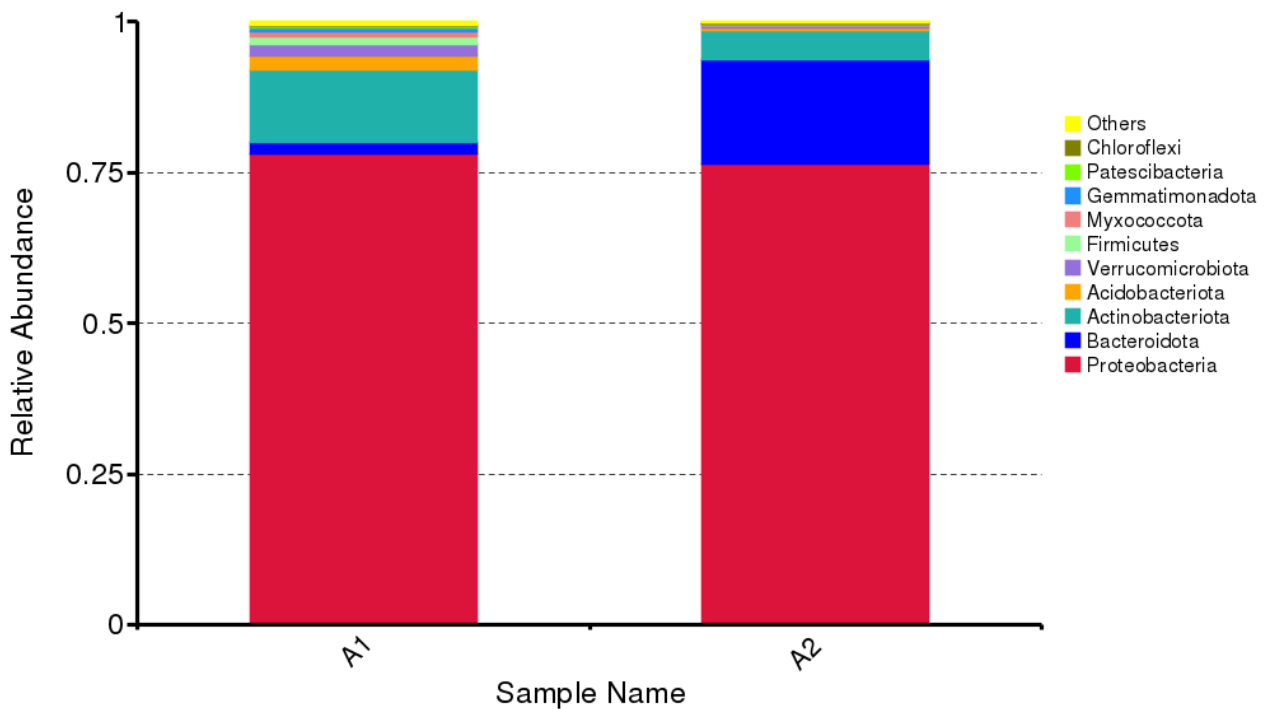
Figure 4. Relative abundance of an endophytic bacterial phylum of endophytic bacteria taxa in the roots of *C. montbretii* roots associated with the cinnamon forest and the yellow earth podzolic soils (A2). Data are presented as relative abundance in per cent.

The abundance at the phylum level of endophytic bacteria from the roots of *C. montbretii* associated with the cinnamon forest soil (A1) was 78% Proteobacteria, 12 % Actinobacteria, 2% Acidobacteria and 1% of Bactereroidota, and 1% Firmicutes. The endophytes in the roots of plants often penetrate the site at which the lateral roots emerge and help in colonizing the epidermis, in the root cracks and below the root hair zone (Zakria et al., 2007; Fadiji and Babalola, 2020). Colonizers of this nature can effectively establish populations both intracellularly and intercellularly (Zakria et al., 2007).

The roots of plants grown in the cinnamon forest soil had the largest composition with Proteobacteria 78%, which consisted of the genus - *Erwinia* 13%, *Pseudomonas* 11%, *Shingomonas* 11%, *Rhizobium* 5%, and *Bradirhyzobium* 4% (Figure 5).

In the yellow earth podzolic soils (A2) Proteobacteria 76% prevailed. The difference with the cinnamon forest soil is that in the

yellow earth podzolic soil Bacterioidota had a very high abundance of 15%. The most abandoned genus was *Orchobactrum* 20%, followed by *Stenotrophomonas* 15%, *Sphingobacterium* 12%, *Serratia* 4%, and *Flavobactrerium* 4% (Figure 5). Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, and Firmicutes appear to be abundant in most soils and plants (Lauber, 2009). The most important drivers of the bacterial community composition in soils seem to be pH, organic matter content, nutrient availability, climate conditions, and biotic interactions (especially the effect of vegetation) affect the composition of bacterial communities (Fierer, 2006). The current result for the endophytic bacteria colonizing *C. montbretii* roots is in agreement with the previous metagenomic study of rhizospheric soil. For the cinnamon forest soil associated with *C. montbretii* from Miskova Niva it was reported that at the class level, the amount of Gammaproteobacteria was 87%, and Alphaproteobacteria was only 2% (Petkova et

al., 2023). Similar to the cinnamon forest soil microbiome, the Alphaproteobacteria were signified by the genus *Bradyrhizobium* (12%), *Rhizobium* (4%), *Podomicrobium* (4%), and *Phenylobacterium* (3%) (Petkova et al., 2023).

Those results infer that the soil samples from the soil and the rock have a substantial effect on the formation of endophytic bacteria in wild species of legumes.
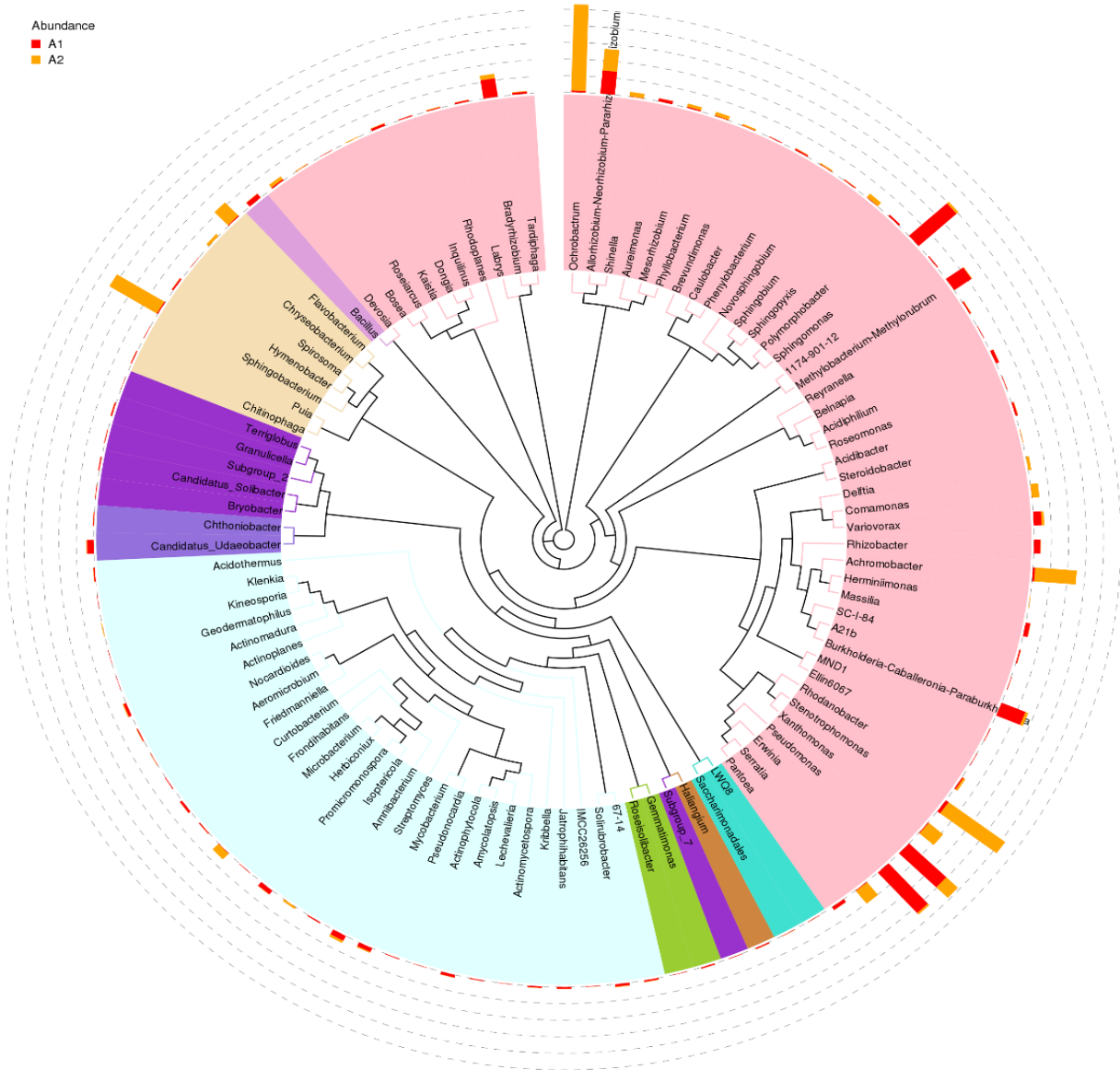
Figure 5. The result of taxonomic annotation of endophytic bacteria in *C. montbretii* roots by Krona. A1 (red color) represent endophytic bacteria from the roots of *C. montbretii* associated with the cinnamon forest and A2 the (yellow color) yellow earth podzolic soils. Bars show

Alpha diversity of bacterial communities associated with *C. montbretii* roots

The main focus is on the alpha diversity of forest vegetation of *C. montbretii* on two different soil types. Thus we expect potential

differences between their alpha-diversity patterns and related soil factors. Diversity at the habitat level is the most widely used factor in the characterization of communities as reported by Thukral (2017). Species richness indices also known as variety indices are higher for species-

rich communities (Prober et al., 2015). The alpha diversity is displayed in Table 2. The OTU coverage estimator values ranged from 99.8% to 99.9%, suggesting that the sequence numbers per sample were high enough to capture the majority of the 16S rRNA gene sequences to show bacterial diversity. The highest richness value was measured in the A1 plant sample (909), while the lowest was in the A2 plant sample (619). One of the most widely used

diversity indices is the Shannon-Wiener index proposed by Shannon in 1948. The highest Shannon index value was in A2 (4.692), while the A1 sample showed the highest (6.551). Another widely used index for a community analysis is Simpson's index proposed by Simpson (1949). The Simpson indices parameters ranged from 0.961 for A1 and 0.907 for A2.

Table 2. Alpha diversity indices of endophytic bacteria from the roots of *C. montbretii* associated with the cinnamon forest (A1) and the yellow earth podzolic soils (A2).

Sample name	Observed species	shannon	simpson	chao1	ACE	Goods coverage	PD whole tree
A1	909	6.551	0.961	944.826	944.987	0.999	63.418
A2	619	4.692	0.907	696.816	705.572	0.998	47.119
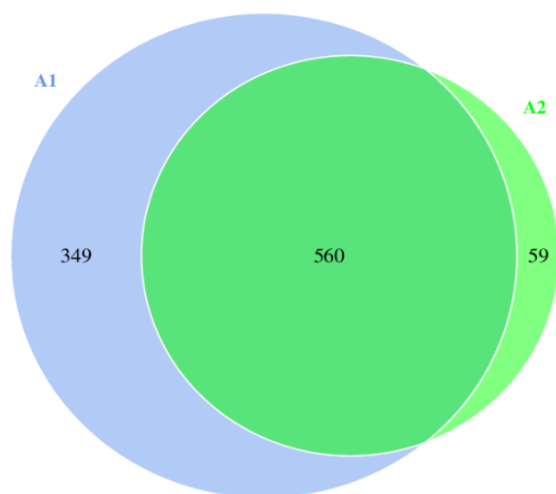
Figure 6. Venn diagram, constructed based on the number of operational taxonomic units (OTUs) shared and unique among *C. monbretii* associated with the cinnamon forest (A1) and the yellow earth podzolic soils (A2).

Figure 6 shows the result of the Venn diagram, constructed based on the number of operational taxonomic units (OTUs) shared and unique among *C. monbretii* plants drowning in two different ecological habitats in Strandzha Park. The traditional approach was used to cluster sequences into operational taxonomic units that reflect the phylogenetic boundaries of

distinct bacterial species. The most OTUs (349) were obtained for endophytic bacteria in the roots of *C. montbretii* associated with the cinnamon forest soils (A1), which had the highest number of unique OTUs (Figure 1). The number of unique OTUs in the plants associated with the yellow earth podzolic soils (A2) was 59. The cross-section of the two sets of endophytic bacterial 16S rDNA clone library of roots *C. montbretii* associated included 560 OTUs common for the cinnamon forest (A1) and the yellow earth podzolic (A2) soils.

CONCLUSION

In conclusion, the relative abundance of Proteobacteria, Bacteroides and Firmicutes in the rhizosphere soil was established, and these bacteria colonized the endophyticaly root tissues of *C. montbretii*. Proteobacteria were found to be the most predominant phylum and may be associated with lignin digestion as well as with the catabolism of various components. Strandzha is one of the five protected territories in Central and Eastern Europe. The total number of the habitat types is 121, and according to this indicator, the park holds the first place among the other protected areas in Europe. New source

information in the project will provide identification of new deposits and establishment of their status. The inclusion of microbiological studies is due to the fact that the habitats of the studied species have different sizes and abundances depending on the places and the altitude at which they are located. Some of these species are found only in Strandzha. This research was provoked by the interest in searching for the relationship between the soil microflora and the habitat of the wild relatives of legumes like *C. monbretii* in Strandzha.

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