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The bacterial community structure of rhizosphere soil associated with Cicer montbretii Jaub. & Spach endemic to Strandzha Mountain

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Abstract. *Cicer montbretii* Jaub. & Spach (*C. montbretii*) is a species of plant in the Fabaceae family. It is a perennial herbaceous plant, a Tertiary relict found in the Strandzha Nature Park on brown oak soils. This protected species is of increasing interest for leguminous breeding programs. In the present study, the diversity of the bacterial community in *C. montbretii* roots was analyzed using a metagenomic approach. Soil testing has focused on soil chemical-nutrient contents, generally the macronutrients nitrogen (N), phosphorus (P) and potassium (K). The diversity of bacterial taxonomy was evaluated at different Operational Taxonomic Unit (OTU) levels using QIIME and MG-RAST. At the phylum level, Proteobacteria (89%) was the most dominant group followed by Bacteroidia (5%), Firmicutes (2%) and Actinobacteria (0.9%). Class level analysis revealed that the abundance of Gammaproteobacteria was 87%, and Alphaproteobacteria was only 2%. In the rhizosphere soil, the most abundant genera from the Gammaproteobacteria were *Pseudomonas* (24%), *Pantoea* (21%), and *Stenotrophomonas* (6%). The Alphaproteobacteria was represented by genus *Bradyrhizobium* (12%), *Rhizobium* (4%), *Podomicrobium* (4%), and *Phenylobacterium* (3%). The phylum Firmicutes consisted of genus *Bacillus* (61%), *Paenibacillus* (21%), and *Sporocarcina* (13%). The study discovered that 17% of bacterial sequences in the soil microbiome were unclassified OTUs. This result emphasizes the significance of metagenomics in assessing the diversity of microflora in the rhizosphere of these wild legumes.

Key words: Metagenomic analyses, Rhizosphere, *Cicer montbretii*, Strandzha Nature Park.

Introduction

The Strandzha Mountain is an anticline with core layers formed by igneous and metamorphic rocks with the Paleozoic origin and surface strata covered with Mesozoic sediments dating from the Triassic, Jurassic and Cretaceous periods. The Strandzha National Park ranks first among the protected areas in Europe, with a consistent total number of habitats totaling 121 types. There are unique plant species (about 1665 species), dozens of which are relict and

endemic plants. *Cicer montbretii* falls within protected areas of the European ecological network NATURA 2000 in Bulgaria according to Heywood & Dulloo (2005). The geographical crossroads where Strandzha Mountain is located, the proximity to large water basins, the diverse relief and microclimatic features, the mild and humid climate, and the lack of glaciation in the Quaternary, create floral elements, the combination of which is unique.

Leguminous species constitute a large group of plants included in a wide family named Fabaceae or Leguminosae (Kumar et al., 2017). The benefit of the use of legumes in rotation or intercropping schemes is their low dependence of nitrogen supply due to their ability to obtain it directly from the atmosphere (Remigi et al., 2016). The species *C. montbretii* is distributed in Bulgaria on the territory of Strandzha Mountain and its Black Sea coast (Kaiser et al., 1998). *C. montbretii* is a protected species, morphologically resembling cultivated chickpeas - *Cicer arietinum* (Angelova et al., 2018).

The first steps in the study of the possibilities for protection of the wild relatives of the cultural species through in situ conservation began in 1990 - 1992 in the Institute of Plant Genetic Resources (IPGR) - Sadovo. In 2001, Miho Mihov registered a small locality of *C. montbretii* on the road from the village of Gramatikovo to the town of Malko Tarnovo (Mihov et al., 2001). Later, this locality was confirmed during some expeditions in 2009. In 2011, two new habitats discovered and marked along the Ahtopol forest road - the village of Brodilovo, are small groups of plants near the road. During two expeditions on international projects, in 2012 and 2014, larger localities of *C. monbretii* were identified in the areas "Mishkova niva" and "Indipaskha" in oak forest communities, on old cinnamon-forest soil (Petrova & Angelova, 2013; Angelova et al., 2018). The scientific team involved in this study conducted three expeditions in 2021 and confirmed the location of the described sites. The new initial information in the project was the identification of new localities from *C. monbretii* and the establishment of their condition. Attempts to sow directly in pots and sow in the field were unsuccessful. The research of IPGR - Sadovo includes conducting numerous expeditions to collect wild plant species, create an ex-situ collection and store them in the gene bank (Mihov et al., 2001; Petrova & Angelova, 2013; Angelova et al., 2018).

Many wild plants may become extinct in the near future. Faced with this overwhelming prospect, plant conservationists must take advantage of every technique available (Falk & Holsinger, 1991). The aim of the present study is to reliably establish the relationships between microbial diversity and the geographical origin of wild legumes.

The diversity of microorganisms in the rhizosphere is essential for ecology, as it makes it possible to understand in detail the plant-microbial interactions. Plants are inhabited by a great number of microorganisms. which includes bacteria, archaea, fungi, viruses and oomycetes (Brader et al., 2017). Microorganisms play a major role in the functions of ecosystems, which makes them unique due to their different microbial composition and is directly related to plant species and their abundance. This gives a strong impetus to the study of microorganisms and leads to knowledge of how their functions can be changed or modulated in the direction of improving their quality (Maxted et al., 2003; Iriondo et al., 2008). Prominent among organisms involved in beneficial association with the plant is the endophyte (Maxted et al., 2008). Many plants have different microbes specific for their colonization, either mutualistic or beneficial for plant growth improvement and health (Verma et al., 2018). The study of microbial ecology is essential as it makes it possible to understand in detail both plant-microbial interactions (Kent et al., 2002). Among the leguminous plants of the family Fabaceae, the prominent soil bacteria of Rhizobiaceae family are restricted to the root nodules only (Olivares et al., 2013). Within these root nodules, rhizobia efficiently and effectually perform biological nitrogen fixation (Teotia et al., 2017). This activity is performed through the acceptable control of the

existence of oxygen air, which is an inhibitor of enzyme nitrogenase functioning (Galloway et al., 2008; Bru et al., 2011; Hagemann et al., 2016). The significance of nitrogen-fixing bacteria has also been the main objective of the findings in non-leguminous plants such as sugarcane (*Saccharum officinarum* L.) (Thaweenut et al., 2011). The production of soybean crop (*Glycine max* L.) is an exceptional example of the effectiveness of nitrogen fixation through the application of diverse strains of *Bradyrhizobium* sp., such as *B. elkanii* and *B. japonicum* (Alves et al., 2003). The legume nodule microbiome can be analyzed by culture-dependent and by metagenomics techniques, which to date have been mostly used to detect rhizobia in nodules for which they were not isolated (Muresu et al., 2008) or to confirm the ability of rhizobia to nodulate a legume that is not its common partner, as occurs in the case of *R. legumino-sarum symbiovar trifolii*, which is able to nodulate *Cicer canariense* (Martínez-Hidalgo et al., 2015).

Different ecological niches contain numerous microorganisms. Plants modify soil biotic properties and those changes in turn influence plant growth, survival or reproduction (Brader et al., 2017). These feedback effects are not well understood as mechanisms for invasive plant species (Buerdsell et al., 2021). Since a very limited number of microorganisms in natural ecosystems can be cultivated in the laboratory, traditional methods of microbiological analysis do not allow the disclosure of the actual species diversity in them and the dynamics of mixed crops associated with relevant biochemical changes in soils. For the purposes of the present study, the soils of the marked habitats of the endangered species *C. montbretii*, which are of interest for legumes breeding, were studied. Advances in molecular biology have led to the development of omics techniques, which recently gained prominence in the diversity and abundance study of microbes (Fadiji et al., 2020). The word 'metagenomics' was first introduced in the year 1998, and was defined as the evaluation of all the genetic materials isolated directly from environmental samples (Handelsman et al., 2005; Alawiye & Baba-lola, 2019). Adaptations to changes in the environment driven by natural selection processes are unique. Molecular analysis can effectively link the benefits of microbiology, molecular biology, genetics, ecology and botany, and answer the question of the impact of endophytic microorganisms on the specific and very limited distribution of wild relatives in Strandzha Mountain.

Materials and Methods

Experimental design and expeditionary survey by route method

An expeditionary survey was carried out during the flowering and ripening phases of *C. montbretii* - the period from 14-18 May 2021 and from 18-30 June 2021 in the following Strandzha Mountain localities: "Mishkova niva" and DMS 42°0′45″N, 27°36′31″E; UTM 35T 550394 4651343. This region has brown mountain-forest soils which have a high level of stability. They have formed over silicate soil-forming rocks and have acidic properties. The Black Sea and the Mediterranean Sea influence the specific climate of the Strandzha region. The average annual temperatures are high at 12.8°C and precipitation is 800 - 100 mm. *C. monbretii* grows on soils covered with beech and mixed beech-oak forests and minimal quantities of *C. montbretii* roots and seeds have been collected.

Collecting soil material of C. monbretii

A locality of *C. montbretii* was found near the "Mishkova niva" plot. Five samples were taken from the entire block using a standard 10 cm diameter soil probe to a depth of 20 cm (6.0 inches). The samples were kept on ice until stored in a cooler at 4°C between use for testing and analysis. Soils were homogenized and sieved to 2 mm before all assays.

Analyses of physical and chemical properties of the soil, soil respiration (SR), and soil-induced respiration (SIR)

Soil pH and electrical conductivity were determined in 1:1 (v/w) water: bulk soil suspension using a pH meter and a conductivity meter, respectively, according to Deribeeva's method (1986, ISO 10390:2005). Other basic physicochemical characteristics of the soil

(total nitrogen, NH_4, NO_3, total phosphorus, K_2O, and water content) were determined by conventional analyzes performed in the Department of Agrochemistry and Soil Science of the Agricultural University-Plovdiv. The determination of the organic matter was done indirectly, starting from the determination of the organic carbon content in the soil. The determination of the total phosphorus was made spectrophotometrically and the total nitrogen was determined following the Kjeldahl Method (Amin & Flowers, 2004).

In soil ecosystems, microorganisms are the largest part of the biomass. In order to determine the influence of soil microorganisms on the development of rare wild legumes, some quantitative and functional characteristics were analyzed using the soil respiration evaluation method. Respiration shows the current state of the soil by determining the amount of CO_2 released and indirectly serves to determine the microbial biomass. It is possible that the microorganisms present at the given time were not active; therefore, induction of microbial activity by the addition of glucose was used.

The experiment was conducted immediately after sampling the habitats of the wild species. Sample of 50 g of air-dry soil was weighted, sieved through a 2 mm sieve, in order to better clean it from plant material, and placed in a glass jar with a lid. A flask with 20 ml of 0.05 M NaOH was placed at the bottom of the glass vessel. The soil was incubated for 6 hours in a thermostat at 25-27^{o}C. After the specified time, the soil was removed from the bank and immediately 5 ml of 0.05M $BaCl_2$, was added to precipitate the carbamates (Petkova et al., 2020). Glucose was added for induction. Titration with 0.1% HCl under phenolphthalein indicator was made until a white color was obtained. A sample with only NaOH as a control was also prepared.

Measurement of Fungi-bacteria ratio in the soil by MicroBIOMETER®

Soil microbes secrete exopolysaccharides and other metabolites that bind non-living soil particles to each other and to themselves. In the microBIOMETER® assay (Prolific Earth Sciences, Inc., Montgomery, NY, USA), a small soil sample is placed in a test tube with a reagent salt and blended with a whisker. This releases microbes bound to or within soil particles and suspends them in solution, while soil particles settle to the bottom of the tube. The microbes that remain suspended in the salt solution are sampled by placing drops of the solution on a test card, which is then scanned using a smartphone camera application. The application measures the color intensity of the spot where sampled drops were placed, and the resulting color is compared to a color background surrounding the sample area on the test card. The color generated by the sampled drops is thought to measure the density of microbial cells in the sample by virtue of the chroma taken on by the cells themselves when living in the soil (Prolific Earth Sciences, Inc., 2020).

Extraction of soil genome DNA

Total genomic DNA was extracted from the rhizosphere soil using CTAB/SDS method in the stage of mass flowering of the peas plants (Fatima et al., 2014). DNA concentration and purity were monitored on 1% agarose gel. DNA was diluted to 1 ng/μL using sterile water according to the concentration required and mixed with the same volume of 1 × loading buffer (containning SYB green) with PCR products and separated by electrophoresis on 2% agarose gel for detection. The samples were sent for analysis to Novogene (Cambridge, UK) where they were used to generate libraries of amplicons with primers designed to identify prokaryotic microorganisms. Sequencing was performed on the Illumina MiSeq PE 300 platform. Raw FASTQ sequencing data of about 100,000 sample sequences were obtained. The bioinformatics analysis of the sequenced amplicons was performed on the MG-RAST and QIIME platforms (Caporaso et al., 2010; Keegan et al., 2016).

Library preparation protocol and Metagenomic Sequencing

Metagenomics analysis of the total sequences within the sample to uncover the

microbial community composition within that particular sample through a targeted approach via targeted metagenomics was performed with the Illumina MiSeq PE 300 platform in Novogene (Cambridge, United Kingdom). In the approach, a certain sub-group within the microbial community was targeted by first PCR amplifying sequences within the target group via a barcode gene unique to that sub-population, prior to sequencing those amplicons. Primers for the amplification of the V3–V4 hypervariable region of the 16S rDNA gene of Eubacteria and Archaea have been used.

The FASTQ sequences were filtered to remove chimeric sequences and singletons to obtain preprocessed reads, which were then clustered to obtain Operational Taxonomic Unit (OTU). Further taxonomic annotation of the prokaryotic OTUs obtained was done using QIIME and MG-RAST tools (Caporaso, 2010; Keegan et al., 2016).

Bioinformatics analyses

Data Filtration

Quality filtering on the raw reads was performed under specific filtering conditions to obtain the high-quality clean reads according to the Cutadapt (Martin, 2011) (V1.9.1, http://cutadapt.readthedocs.io/en/stable/) quality controlled process.

Data are available at MG-RAST platform (ID ba82560e0b6d676d343937373032342e33).

Chimera removal

The reads were compared with the reference database (Silva database, https://www.arb-silva.de/) (Quast et al., 2013) using UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) (Edgar et al., 2011) to detect chimera sequences, and the chimera sequences were removed (Haas, 2011). Then the Clean Reads were finally obtained.

OTU cluster and Species annotation

OTU Production

Sequences analyses were performed by Uparse software (Uparse v 7.0.1001, http://drive5.com/uparse/). Sequences with ≥ 97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation.

Species annotation

For each representative sequence, the Silva Database (https://www.arb-silva.de/) was used based on Mothur algorithm to annotate taxonomic information.

Phylogenetic relationship

In order to study phylogenetic relationship of different OTUs, and the difference of the dominant species in different samples (groups), multiple sequence alignment was conducted using the MUSCLE software (Version 3.8.31, available at http://www.drive5.com/muscle/) (Edgar, 2004, 2011, 2013).

Data Normalization

OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed basing on this output normalized data.

Alpha diversity

Alpha diversity is applied in analyzing complexity of species diversity for a sample through 6 indices, including Observed-species, Chao1, Shannon, Simpson, ACE, Good-coverage. All indices in our samples were calculated with QIIME (Version1.7.0) and displayed with MG-RAST software. Two indices were selected to identify community richness and http://www.mothur.org/ was applied in analyzing complexity of species diversity for a sample through all 6 indices (López-García et al., 2018).

Results and Discussion

An expeditionary survey

The species *Cicer montbretii* Jaub. & Spach is distributed in Bulgaria on the territory of Strandzha Mountain and its Black Sea coast. It is a perennial herbaceous plant, a Tertiary relict, and a protected species. Morphologically, it resembles cultivated chickpeas - *Cicer arietinum* (Angelova et al., 2018).

Expeditionary surveys in 2021 established the condition of the plants that form the habitat and the threats that exist. An important point of the work is the registration

of the main phenological phases (mass flowering – from 12-14 of May 2021 and ripening – from 28-30 of June 2021), as well as the possibilities for collecting seeds. In the habitats themselves, the maturation phase is uneven and depends on exposure of 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 bike paths and tourist routes. The flowers are from 2 to 5, rarely single, in loosely clustered inflorescences in the axils of the leaves. The bean is smooth, broad, oblong, brown, with 3 to 4 seeds. The seeds are globose, brown or black. The localities are situated in an oak forest, on leached cinnamon-forest soil. Depending on the exposure of the habitat, the height of the plants varies (Fig. 1).

Fig. 1. Taking soil samples from the site of *C. montbretii* in the phenological phases of full ripening from the "Mishkova Niva" site, Strandzha Mountain.

In some literature sources, it is stated that the height is from 20 to 40 cm (Uzunova & Uzunov, 2008), but in our survey it varies from 50 to 70 cm. The localities near the road are 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 a.s.l. The main accompanying plants are from the family Poaceae, genus Vicia and the species *Trifolium campestre* Schreb., *Stellaria holostea* L.; *Crataegus monogina* Jacq (Fig. 1).

During the last expedition in June 2021, it was established that the identified sites have been preserved, the species were well developed, and there is no violation of habitat, except where they are located along the road - Gramatikovo - Malko Tarnovo and "Mishkova niva". They are quite vulnerable as a result of rehabilitation, widening and repair of roads and road traffic.

In the case of habitats located in forests and other areas, the dangers come from sanitary felling and logging. In previous studies (Maxted et al., 2008; Petrova & Angelova, 2013; Angelova et al., 2018), seeds of the species *C. montbretii* were collected at physiological maturity and attempts were made to maintain them outside the natural environment – ex-situ, but all were unsuccessful.

Maintaining an ex-situ collection allows the propagation and production of seeds for storage in a gene bank, as well as their use in various fields.

Physicochemical characteristics of rhizosphere soil of C. montbretii

Some physicochemical parameters of soils such as pH and electrical conductivity were investigated. The result shows that the cinnamon forest soil from the southern slope of "Mishkova niva" has an average acidic pH, which is most likely due to forest litter from broadleaved plantations (mainly oak) or to the washing of basic cations from the profile (Table 1).

pH is important not only for the physiology of microbial cells but also for nutrient availability. Most microorganisms grow in relatively wide ranges of pH, but their enzymatic activity is highest in a neutral environment. In a neutral pH, the activity of enzymes is activated, on the processes of entry of substances into the cell, etc. In this sense, the studied soils from the habitats of rare wild leguminous plants have a close to neutral reaction and a better representation of the main groups of microorganisms can be expected.

The measurement of soil electrical conductivity shows us the content of soluble salts in the soil. This is an easy way to track the movement of available forms of nutrients in the soil profile and their spatial availability

to the plant root system. The data from the present study show that forest cinnamon soil from "Mishkova niva" has a very low electrical conductivity of 77 µS/cm.

From the agrochemical analysis of the soil samples, it was found that total nitrogen content is on average high in cinnamon forest soils with values of 59.3 mg/kg soil. Ammonium nitrogen was in low concentration 39.1 mg/kg soil, and nitrate nitrogen was 20.2 mg/kg soil. The rhizosphere soil of *C. montbretii* is characterized by a low content of phosphorus, measured as P_2O_5 mg/kg and increased values of available forms of K_2O in values of 60.7 mg/kg soil.

Table 1. Physicochemical characteristics of rhizosphere soil of *C. montbretii*

N	Physicochemical characteristics	Values
1	pH	6.04
2	Electrical conductivity, µS/cm	77
3	SR, mg CO_2 /g soil	6.35
4	SIR, mg CO_2 /g soil	10.25
5	NH_4, mg/ 1000 g	39.1
6	NO_3, mg/ 1000 g	20.2
7	Total N, mg/ 1000 g	59.3
8	P_2O_5, mg/ 1000 g	3.5
9	K_2O, mg/ 1000 g	60.7
10	Biomass, µg C/g	363
11	Fungi:Bacteria ratio	0.7:1 (41%: 59%)

The biomass estimated of cinnamon soil associated with *C. montbretii* by the micro-BIOMETER® was good with a value of 363 µg C/g soil and was higher than the soil respiration measurement of 6.35 mg CO_2/g soil. Fungi (41%)-to-bacteria (59%) ratio was 0.7:1. Generally, the ratio between fungi and bacteria varies in different types of soil and in different plant communities. Forest soils have a higher percentage of fungi while grassland and agricultural soil have a higher quantity of bacteria (Wang et al., 2019). This has led to the idea that fungal to bacterial ratio is important for the plants to grow and best when is matched to their needs (Blagodatskaya & Anderson, 1998).

Soil health testing should be considered more frequently for monitoring agronomic and ecosystem analyses. Nutrient and soil health management is a great need and is evaluated by soil respiration in a laboratory. Micro-BIOMETER® mobile soil health test was marketed as an affordable and easy-to-use test specifically for measuring soil microbial biomass carbon on the field (Bongiorno et al., 2019).

Metagenomic analysis of bacterial community

Total DNA was isolated from soil and the presence of the 16S rRNA gene was confirmed by amplification with universal primers. Total raw sequencing reads (paired-end) of 196,595 with an average sequence length of 151 bp each were obtained from Illumina MiSeq™ sequencer.

A total of 58677 raw tags were generated from the Illumina MiSeq sequencing of the sample. After quality control, a total of 53423 taxon tags remained. Then, after the removal of the chimaeras, 686 effective tags were obtained for OTU generation (Fig. 2).

The rarefaction curve is shown in Figure 3A and B and represents the sequencing depth that generally covered most species in the sample, which could better reflect the bacterial community structure and diversity. Rarefaction curves (Fig. 3A) and rank abundance curves (Fig. 3B) are widely used for indicating the biodiversity of the samples. Both curves approached a plateau, which suggested that the number of OTUs was sufficient to reveal the authentic bacterial community. Furthermore, rank abundance curves (Fig. 3 A) were used to analyze the community diversity, which indicated both the evenness and abundance of species in the samples.

Rank abundance curves have a wider span and reflect a higher relative species abundance, and the smoother curve on the y-axis reveals a higher evenness of bacterial species in the *C. montbretii* rhizosphere soil.

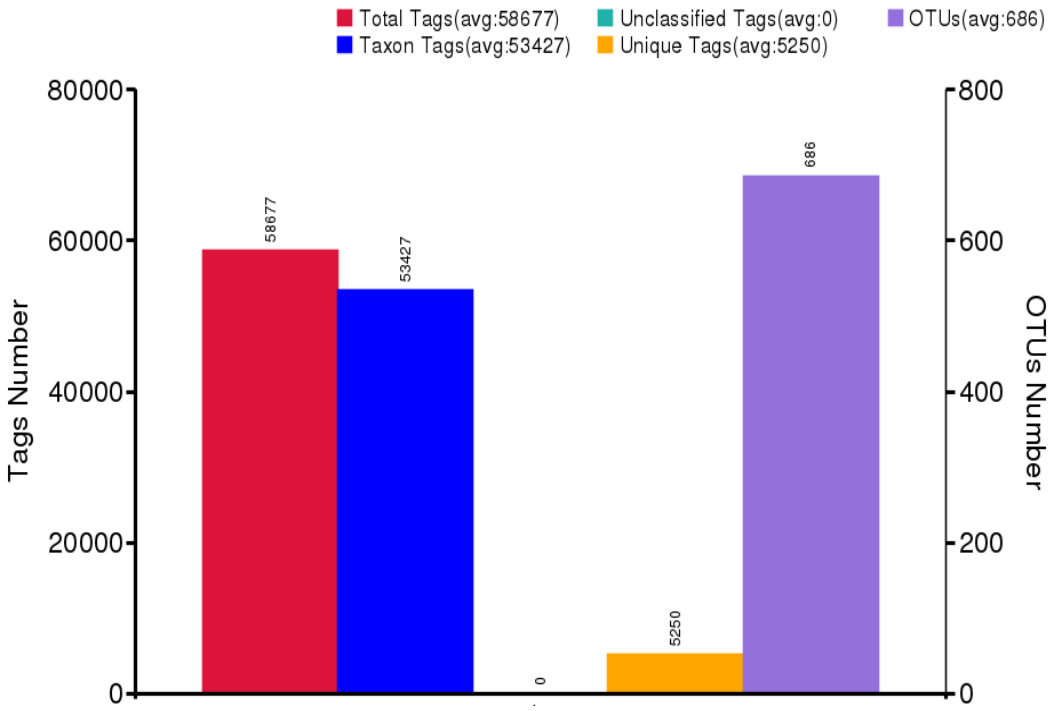

Fig. 2. Summarization of the tags and OTUs number of each sample.
Notes (Left to Right): The Y1-axis entitled "Total tags" (Red bars) means the number of effective tags; "Taxon Tags" (Blue bars) means the number of annotated tags; "Unclassified Tags" (Green bars) means the number of unannotated tags; "Unique Tags" (Orange bars) means the number of tags with a frequency of 1 and only occurs in one sample. The Y2-axis entitled "OTUs Numbers" means the number of OTUs which displayed as "OTUs" (Purple bars) in the above picture to identify the numbers of OTUs in different samples.

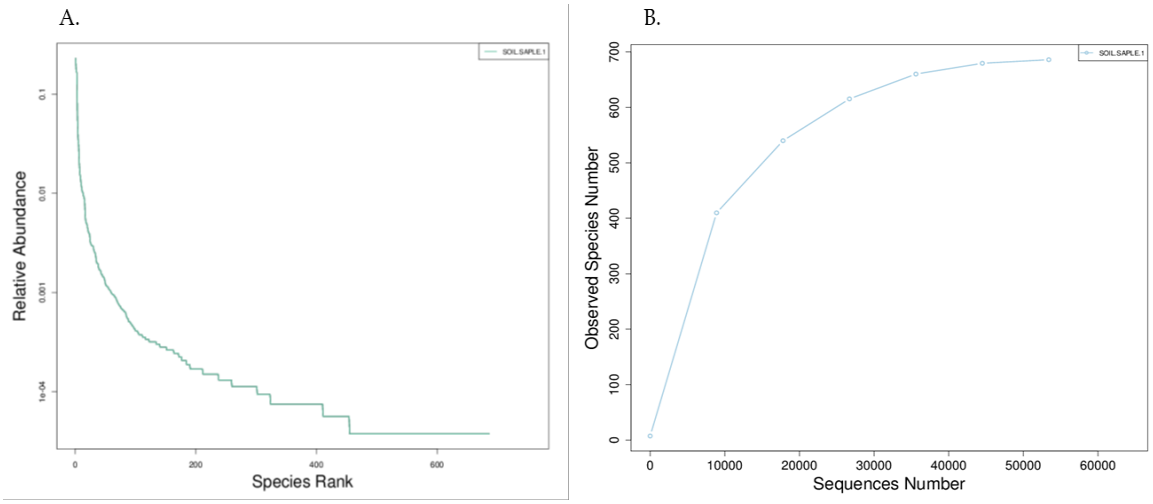

Fig. 3. Rank abundance curves (A) and Rarefaction curves (B) of *C. montbretii* rhizosphere soil.

The abundance of major bacterial groups in each taxonomic category is presented in Figure 4A. Altogether, 36 bacterial phyla were detected and among these ten different phyla were predominant. Proteobacteria (89%) was the most dominant group followed by Bacteroidia (5%), and Firmicutes (2.14%). Reads belonging to

Acidobacteria were found to be 1%, Actinobacteria 0.9% (Fig 4A). The higher abundance of Proteobacteria (89%) in the roots of wild legumes suggests that members of this species are particularly well adapted to colonize inner plant tissues and establish as root endophytes. The phylum Proteobacteria comprises several classes that promote plant growth and act as biological control agents for different diseases (Bulgarelli et al., 2013). This result is in accordance with Lin et al. (2011) who *published that in the natural hardwood* forest soils, Proteobacteria predominated and are the major factors to control the metabolic processes. Firmicutes were found to be metabolically the most versatile group with the production of multiple enzyme activities (Babalola, 2010). In the cinnamon forest soil Bacilli and which belongs to Firmicutes, were the other main component (Fig. 4B).

Three major bacterial classes were identified and among them, Gammaproteobacteria was the most dominant group (87%), Alphaproteobacteria (2%), Bacilli (2%), and Acidobacteria (1%) (Fig 4 B). Class Gammaproteobacteria includes 24% of *Pseudomonas*, 21% *Pantoea*, 16% of unclassified species, 6% of *Stenotrophomonas*, and 2% Oxalobacteria (Fig 4C). In previous research on the microbiome of forest soils published that the majority of the rhizospheric soil isolates belonged to Proteobacteria, and *Pseudomonas* spp. constituting the most dominant species. The endophytic bacterial community, on other hand, consisted almost exclusively of Firmicutes (Dokic et al., 2010; Kumar et al., 2012). The current investigation discovered that 61% of Firmicutes in the rhizosphere soil of *C. montbretii* belonged to *Bacillus*, 21% to *Penibacillus* and 13% to *Sporosarcina*. *Pseudomonas* genus contains many endophytic bacterial strains that benefit hosts by producing indole-3 acetic acid (IAA), producing biocontrol lipopeptides (Berry, 2010) and solubilizing phosphate (Otieno et al., 2015). O'Sullivan & O'Gara (1992) reported that *Pseudomonas* species increase nutrient absorption, as N, P, and K, and in addition, act as biocontrol agents of phytopathogenic fungi and produce phytohormones in the rhizosphere, which promote plant growth. *Pseudomonas putida* strains have been cited as phosphate solubilizers (Kumar & Singh, 2001). *Pantoea* species promote plant growth and tolerance to environmental stresses (Chen et al., 2017).

The most abandoned genera in the class Alphaproteobacteria are *Bradyrhizobium* (12%), *Sphingomonas* (5%), *Rhizobium* (4%), *Pedomicrobium* (4%), and *Phenylobacterium* (3%) (Fig. 4C). *Sphingomonas* is an alphaproteobacterial genus containing strains that produce IAA and provide nutrients to hosts (Ruiza et al., 2011). *Bradyrhizobium* spp. were abundant in all nodules analyzed, despite differences in compost, amendment, preceding crop, or any of the differences in the growth media, suggesting strong selection by the host plant specifically for *Bradyrhizobium*. This result is entirely consistent with previous findings that *Bradyrhizobium* is the dominant endophyte of soybean under acidic conditions. Liu (2021) proposed several microbes - *Bradyrhizobium, Sphingomonas, Mesorhizobium, Nocardioides, Acidobacterium*, and *Phenylobacterium*, as candidates to reflect the soil fertility and plant health.

In the forest cinnamon rhizosphere soil from Strandzha Mountain the representtatives of Actinobacteria were around 0.9% (Fig. 4A). They play specific roles, for instance, protecting the host plants against insects and diseases, especially by the production of bioactive compounds including antibiotics, antimicrobial, anticancer, antitumor, enzyme, enzyme inhibitors and immunosuppressive agents (Lee et al., 2014). Actinobacteria are found to be common soil inhabitants and have a high proportion of total microbial biomass in forest soil in China (Qin et al., 2009).

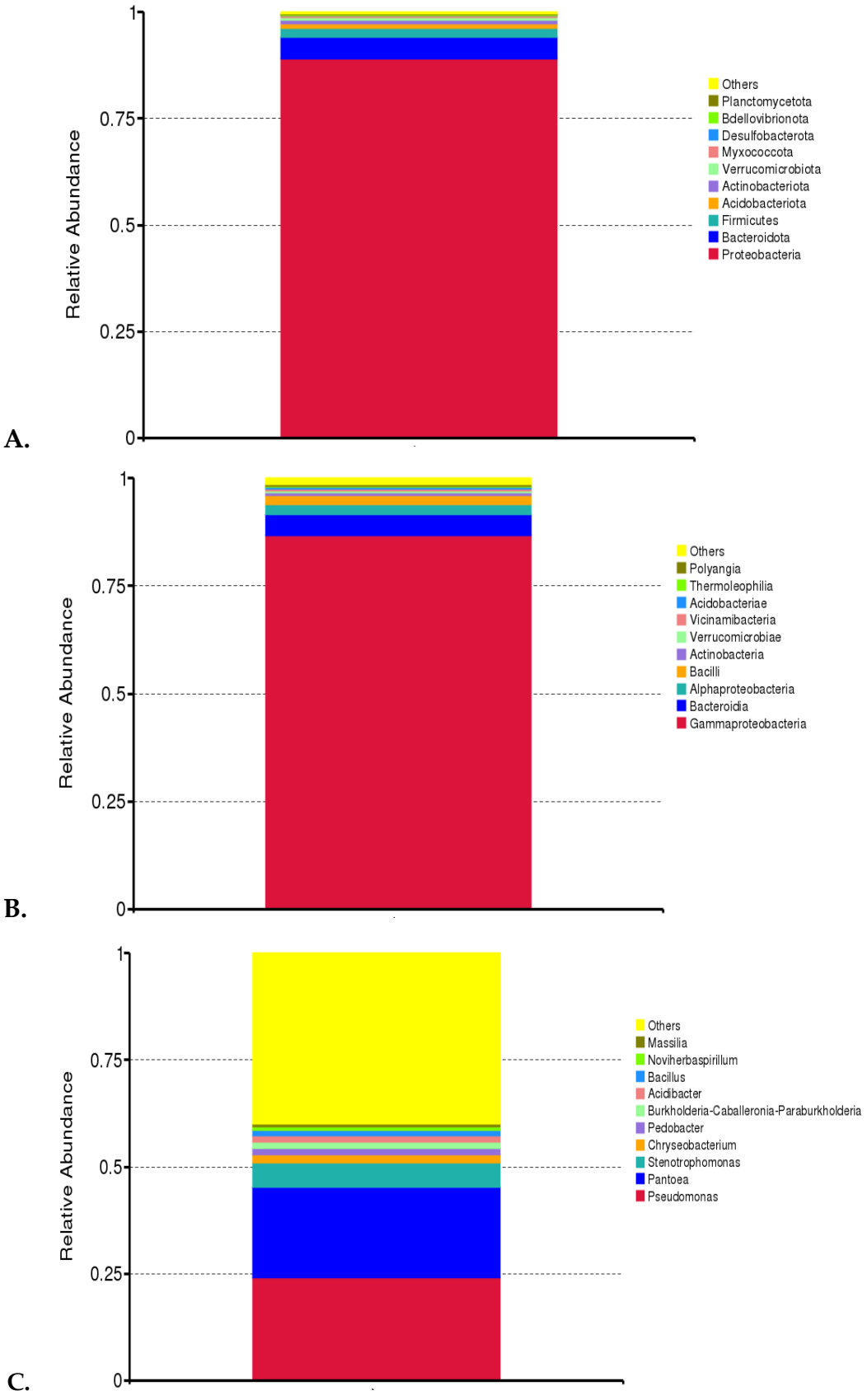

Fig. 4. Relative abundance of taxonomic annotation at (A) phylum level, (B) class level, and (C) genus level.

a-Diversity Analysis of Bacterial Community in Rhizosphere Soil

The alpha diversity was described based on the Chao1, ACE, Shannon, and Simpson indices. Species richness was measured using the Chao1 and ACE indices, and species diversity was measured through the Shannon and Simpson indices (Table 2). Alpha diversity metrics summarize the structure of an ecological community with respect to its richness (number of taxonomic groups), evenness (distribution of abundances of the groups), or both. The alpha-diversity value obtained by MG-RAST and QIIME2 analysis indicated that the rhizosphere soil of *C. montbretii* had a high microbial diversity (Table 2). The cumulative explanatory degree of soil, climate, and geographical factors to rhizosphere bacteria diversity was 70.20%. Moreover, soil factors (N, P, K, and N/P) and annual average precipitation were the main factors, which significantly affected alpha-diversity indices.

In conclusion, the relative abundance of Proteobacteria, Bacteroidia, and Firmicutes in the *C. montbretii* rhizosphere soil was high. Proteobacteria were found to be the most predominant phylum and may be related to lignin digestion as well as the catabolizing of various components. Those bacteria are generally known to produce various bioactive compounds like antibiotics which are of both pharmaceutical and industrial relevance (Lee et al., 2014; Babalola, 2010). In addition, 2% of analyzed bacteria in the soil have been unclasified. Hence it is important to further study these different bacteria found in the rhizosphere soil of wild legumes in Strandzha mountain.

Table 1. Alpha diversity indices. Statistical indices of alpha diversity (number of reads chosen for normalization 53427).

Sample name	Observed species	Shannon	Simpson	Chao 1	ACE	goods_ coverage	PD_ whole_ tree
Rhizosphere soil from Strandza Mountain	686	4.614	0.884	686	686	1.000	56.246

Conclusions

The distribution of the soil microbial community on a large scale has been widely investigated (Delgado-Baquerizo et al., 2016). However, the bacterial community in the rhizosphere soil from the Strandzha mountains is still poorly characterized. This study shows the spatial distribution of the rhizosphere bacteria of forest cinnamon soil from "Mishkova niva". Proteobacteria, Acidobacteria, Actino-bacteria, and Firmicutes were widespread in the rhizosphere soil of *C. montbretii*. The purpose of the metagenomic analysis is to answer the question of whether there was a connection between the soil microflora and the limited distribution of the studied wild species only in certain areas of Strandzha Mountain. The results carried out the first functional diversity study of endophytic microbiomes in *C. montbretii* plant using metagenomics sequencing analysis. This study extends the knowledge of the composition and diversity in the *C. montbretii* microbial populations. Moreover, most of the microorganisms observed in the present study are perhaps good producers of bioactive compounds, which can promote plant growth. Annual monitoring of *C. montbretii* in Strandzha National Park will determine the methods for its most effective maintenance and storage outside its habitat.

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