

Taxonomical Structure of Black Soil Bacterial Community on the Level of Phyla

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Abstract—Soil microbial communities perform a number of important functions ensuring fertility. They depend on physical and chemical composition of soil and applied agricultural technology. To control the state of the soil, it is necessary to use methods that allow to quickly assess the dynamics of the structure of microbial communities. To develop convenient method for analysis of microbiota the set of taxon-specific primers for RT PCR, recognizing 16S rRNA genes of domain *Bacteria*, six bacterial phyla and one class was chosen. Calculation of the percentages of lower-order taxa in the upper-order taxon was carried out based on the number of amplification cycles required to achieve the threshold value of fluorescence intensity taking into account the values of amplification factors. Taxonomic structure of the bacterial component of the soil microbial community was analyzed using as an example a sample of the surface layer of arable black soil enriched with regular application of organic fertilizers. The compiled analysis protocol made it possible to obtain data on the percentages of phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, and of class *Gammaproteobacteria* belonging to phylum *Proteobacteria*.

Keywords—soils, black soil, microbiome, RT-PCR

I. INTRODUCTION

One of the most important factors in ensuring soil fertility are the microbial communities functioning in it. Its functions include decomposition of complex organic substances, fixation of atmospheric nitrogen, conversion of phosphates to a soluble form, production of bioactive substances, suppression of phytopathogenic organisms and others. Their analysis is important for determining the functional state of the soil, the prospects for its agricultural use and the list of measures necessary to increase its fertility.

The methods used to analyze soil microflora include cultivation of microorganisms on differential media, real-time taxon-specific PCR (RT PCR), metagenomic

sequencing of evolutionary conserved sequences, transcriptomic, proteomic and other methods [1, 2]. The strengths of taxon-specific RT PCR include rapidity and relatively low cost of analysis, the simplicity of sample preparation, processing and interpretation of the results. This makes the method suitable for routine analysis of soil communities to compare the results with expected indicators.

In this study taxon-specific primer pairs specific for prokaryotic domains and phyla [3-5] and about usage of such ones [6-8] were selected and representative primer set was formed. Taxonomic analysis of microflora of black soil enriched by regular application of organic fertilizers was carried out.

Loss of the soil fertility due to different factors is an important problem of nowadays. A rather serious factor in the crop production is soil compaction [9]. Soil damage of this nature occurs when excessive use of heavy machinery in conditions of insufficient measures for deep loosening. This problem is especially typical for heavy soils with small average size of mineral particles.

Also important causes of loss of soil fertility are wind, water and biological erosion [10]. Biological erosion means the loss of stocks of soil organic matter as a result of the mineralization or “combustion” of humus. These reasons are manifested in the peculiarities of the wind regime, the nature of the relief and precipitation, and other phenomena influencing the cultivated soils.

Soil humus stocks consist of its active and conservative parts. The first, due to mineralization, is a gratuitous food supplier for plants [10]. Currently most of arable soils, only the conservative part of humus remains. It practically does not lend itself to mineralization; the sources of nutrition for it are fertilizers and nutrients that are formed during the mineralization of plant debris.

These and many other factors alter physical, chemical and biological properties of arable soils. The aim of this research is to elaborate a method for determination of taxonomical structure of soil bacterial communities based on RT PCR for routine practical use. On this way, we were aimed to choose a set of taxon-specific primers capable to recognize evolutionary conserved sequences identically for the major of the representatives of high rank taxa such as phyla and classes. Also, we had check applicability of chosen amplification conditions. and of available reagents of budget price segment. Using samples of the black soil rich with organics as an example, we also were to collect information about microbiota of this valuable soil type.

So, the sense of this study was to select and verify a real-time polymerase chain reaction protocol using a set of taxon-specific primers for the analysis of microflora of black soil.

II. EXPERIMENTAL

For the analysis, we used a set of taxon-specific primers for phyla and certain classes of bacteria proposed by Young *et al.* 2015 [3]. In our study, primers complementary to 16S rRNA gene sequences were chosen from this source, including pairs specific for 6 phyla and one class of bacteria, as well as for the *Bacteria* domain as a whole (table I).

Young and co-authors [3] validated all pairs using plasmid vector DNA bearing 16S rRNA genes of different taxa. The single PCR thermal cycler program, which included melting at a single temperature of 60 °C was applied. The program of the thermal cycler included 40 cycles with three stages lasting 20 s each: melting at 95 °C, annealing the primers at 60 °C and DNA elongation at 72°C.

TABLE I. TAXON-SPECIFIC PRIMERS USED IN THIS STUDY

Target taxon	Primer	Sequence	Amplification, bp	t _m , °C
<i>Bacteroidetes</i>	Bac960F	GTTTAATTCGATGATACGCGAG	122	60
	Bac1100R	TTAASCCGACACCTCACGG		
<i>Firmicutes</i>	Firm934F	GGAGYATGTGGTTTAA TTCGAAGCA	126	60
	Firm1060R	AGCTGACGACAACCATGCAC		
<i>Actinobacteria</i>	Act664F	TGTAGCGGTGGAATGC GC	277	60
	Act941R	AATTAAGCCACATGCTCCGCT		
<i>Deferribacteres</i>	Defer1115F	CTATTTCAGTTGCTAACGG	150	60
	Defer1265R	GAGHTGCTTCCCTCTGATTATG		
<i>Verrucomicrobia</i>	Ver1165F	TCAKGTGAGTATGGCCCTTAT	97	60
	Ver1263R	CAGTTTTYAGGATTCCTCCGCC		
<i>Tenericutes</i>	Ten662F	ATGTGTAGCGGTAATAATGCGTAA	200	60
	Ten862R	CMTACTTGGTACGTACTACT		
<i>Gammaproteobacteria</i>	Gamma877F	GCTAACGCATTAAGTRYCCCG	189	60
	Gamma1066R	GCCATGCRGCACCTGTCT		
<i>Bacteria</i>	926F	AAACTCAAAGAATTGACGG	136	60
	1062R	CTCACRRCACGAGCTGAC		

Unlike the original method [3], we used a mixture of reagents from Syntol (Moscow, Russia), that also provided us with the synthesis of primers. In the reaction mixture, the final concentration of magnesium ions was 2.5 mM, 0.3 µM primers, 25 and 2.5 pg/µl DNA templates.

For practical verification of the applicability of the compiled methodology, a soil sample was taken from depth of about 5 cm shortly after thawing in March and was kept for three weeks at room temperature under conditions of abundant moistening to activate microbiota. The preparation of the total soil DNA was isolated from a sample of arable black earth soil using diaGene kit by Dia-M (Moscow, Russia). The DNA concentration in preparation, determined by measuring the optical absorption at a wavelength of 260 nm, was 9.75 µg, the total amount of DNA was 39 µg.

Reactions were carried out using recombinant SynTaq DNA polymerase at pH 8.8, in the presence of 2.5 mM MgCl₂, with fluorescence detection of the SYBR Green I dye. Mixtures of 25 µl each contained 10 µl of 2.5-fold reaction mixture, 7.5 µl of primer solutions added to final concentration of 0.33 pmol/µl each, and 7.5 µl of the DNA template preparation added to final concentration of 25 and 2.5 pg/µl. Both dilutions of the DNA template were taken in triplicate. The reaction was carried out in a real-time thermal cycler CFX96 Touch by Bio-Rad (Hercules, USA). The program contained the step of melting DNA and activating DNA polymerase at a temperature of 96 °C for 5 minutes. It was followed by 40 cycles of amplification, which included three stages lasting 20 seconds: melting at 96 °C, annealing of primers at 60 °C, and elongation of the growing chain at 72 °C.

The C_q values obtained for three parallels with each pair of primers and each concentration of the DNA template were averaged and the standard error of the mean was calculated. The calculation of the amplification factor for each pair of primers was carried out according to the equation 1:

$$A = 10^{\frac{1}{Cq(25\frac{pg}{\mu l}) - Cq(2,5\frac{pg}{\mu l})}} \quad (1)$$

Determination of taxa percentage in the bacterial component of the community was carried out according to the equation 2 [2]:

$$X = \frac{A_{Cq(Bac)}}{A_{Cq(X)}} * 100 \% \quad (2)$$

where X - the percentage of DNA of a particular bacterial taxon among the entire bacterial DNA, A is the amplification factors for a universal pair of primers (Bac) and a pair of primers specific to the DNA of this taxon (X), C_q - the number of amplification cycles required to reach

the threshold value, installed by the thermal cycler software in each case (or installed manually).

III. RESULTS AND DISCUSSION

According to the data obtained (table II, fig. 1), the *Firmicutes* and *Bacteroidetes* phyla dominated in the studied sample. The *Actinobacteria* phylum and the *Gammaproteobacteria* class were represented in a smaller number, and the *Verrucomicrobia* phylum in a small number. The phyla *Deferribacteres* and *Tenericutes* have not been detected.

The choice of taxon-specific pairs of primers for analysis was based on published data indicating the presence of selected taxa in the microflora of soils [11, 12]. A significant proportion of bacteria belonging to the phyla *Bacteroidetes*, *Actinobacteria* and class *Gammaproteobacteria* is consistent with the data presented in these sources. A high percentage of representatives of the phyla *Firmicutes* can be associated with the introduction of manure fertilizers, since microorganisms belonging to this taxon dominate the intestines of farm animals.

We had to compile a methodology for analyzing the taxonomic composition of soil bacterial communities at the level of phyla and certain classes of bacteria, and to confirm the effectiveness of its use. The analysis of literature allowed us to choose a set of primers that allows us to analyze some of wide-spread bacterial taxa by PCR.

TABLE II. INITIAL AND CALCULATED DATA ON THE ANALYSIS OF PERCENTAGES OF LOWER ORDER TAXA OF THE BACTERIA DOMAIN IN THE BACTERIAL COMMUNITY OF BLACK SOIL BY RT PCR

Target group	Cq (25 ng/μl)	Cq (2,5 ng/μl)	Difference	Amp-lification factor	%
<i>Bacteria</i>	23,07±0,26	25,67±0,06	2,60	2,42	100
<i>Bacteroidetes</i>	26,64±0,12	29,45±0,08	2,81	2,27	23,45
<i>Firmicutes</i>	26,15±0,13	28,39±0,12	2,90	2,24	49,62
<i>Actinobacteria</i>	28,79±0,18	31,60±0,24	2,81	2,27	4,02
<i>Verrucomicrobia</i>	40,21±0,67	43,36±0,15	3,18	2,06	0,017
<i>Gammaproteobacteria</i>	29,29±0,16	32,92±0,31	3,63	1,89	2,31
<i>Deferribacteres</i>	-	-	-	-	-
<i>Tenericutes</i>	-	-	-	-	-

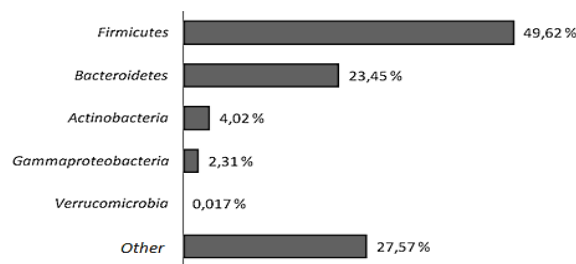


Fig. 1. Percentages of phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia* and class *Gammaproteobacteria* in a black soil sample.

The applied method met the requirement of applicability as a commercial rapid analysis, since it did not require use of different programs of the thermal cycler with reaction mixtures containing various pairs of primers. This feature reduces the time of analysis, allowing all the necessary reactions during the single run of a single PCR amplifier program. Thus, taking into account the duration of sample preparation, all procedures related to the analysis can be performed within one working day. So, the approach used makes it possible to achieve high performance of the method using conventional laboratory equipment.

To test the practical applicability of this technique, a preparation of total soil DNA concentration of 1,95 μg/ml in a volume of 40 μl was obtained. To isolate this preparation, a sample was selected in which the intensive development of microorganisms was expected due to high initial concentration of organic substances (which is typical for black soils), as well as due to the annual introduction of manure and compost for many years. The experiment was aimed to obtain data for one specific sample taken under known conditions to confirm the applicability of the method first of all.

The results of the analysis can be assessed as realistic because of their consistency with the literature data [11-15]. A significant proportion of bacteria belonging to the phyla *Bacteroidetes*, *Actinobacteria* and class *Gammaproteobacteria* is consistent with the data presented in these sources. A high percentage of representatives of the phylum *Firmicutes* can be associated with the introduction of manure fertilizers, since microorganisms belonging to this taxon dominate the intestines of farm animals [16].

IV. CONCLUSION

As the result of the work, the methodology was selected for analysis of soil communities at the level of phyla and some classes. The list of analyzed taxa can be further expanded by the introduction of new taxon-specific primer pairs. The compiled methodology allows analysis within one day from sampling to obtaining results. Moreover, during its development it was necessary to solve a number of experimental problems, such as ensuring an acceptable purity of the extracted preparations of total soil DNA using the diaGene (Dia-M, Russia) reagent kit, as well as preventing the influence of trace amounts of bacterial DNA observed as impurities in commercial 2.5-fold reaction mixture for PCR RT produced by Synthol CJSC. Thus, the

technique we used is not an exact repetition of one described in the original work [3].

The soil sample used to test the effectiveness of the compiled methodology was taken during a period of low activity of microorganisms, however, it was incubated after selection under conditions favorable for increasing their numbers. According to the results of the analysis, it contained taxa, which were previously found in various soils in significant quantities. The unexpectedly high percentage of the phylum *Firmicutes* can be explained by the annual application of manure compost as fertilizer, which is consistent with its microbiota structure [16].

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