TAXONOMIC STRUCTURE OF MICROBIAL ASSOCIATION IN DIFFERENT SOILS INVESTIGATED BY HIGH-THROUGHPUT SEQUENCING OF 16S-rRNA GENE LIBRARY

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Summary

The features of soil microbiome may be an universal and very sensitive indicator of soil state used for optimization and biologization of agriculture systems. However, this approach to the matter requires a preliminary analysis of microbiomes composition in different types of soils. An analogical taxonomic investigations presented difficult task formerly and took considerable material and time expenditures. The introduction to molecular ecology of the new progeny methods of sequencing permits to increase both a number of revealed microorganism species and analyzed ecotops. The authors made the primary analysis of microbial associations with the use of pyrosequencing of soil metagenome. For the study, the collection of soils from different regions of Russia (19 samples) and also from the Crimea (Ukraine, 1 sample) was created. The bacteria from phyla of *Proteobacteria* (up 59.3 %), *Actinobacteria* (up 55.4 %), *Acidobacteria* (up 26.5 %), *Verrucomicrobia* (up 13.6 %), *Bacteroidetes* (up 10.5 %), *Firmicutes* (up 8.2 %), *Gemmatimonadetes* (up 6.9 %), *Chloroflexi* (up 5.7 %) and archaea from *Crenarchaeota* phyla were dominating in microbial associations. The comparison of taxonomic structure of microbial associations indicates that physiochemical factors (acidity and moisture of soil) have a more influence on prokaryote biodiversity than other factors (for example, type of soil or sampling point). So the soils from south regions with lesser moisture contain more the actinobacteria, when the moister north soils contain mainly the proteobacteria. The soils with low pH are characterized by a raise of acidobacteria percent.

Keywords: soil, amplicon library, 16S rRNA, microbiome, taxonomy.

Taxonomic description of microbial communities for a long time was an intractable problem with significant material and time costs. The complexity of cloning procedure and further sequencing of nucleotide chains impose limitations on number of identified species and investigated habitats (1). A notable advance was achieved by the new generation methods introduced in molecular ecology, e.g. pyrosequencing (2, 3). These approaches have improved the performance of sequencing from hundreds (Sanger's method) to thousands of nucleotide sequences, which now allows the most accurate identification of complex multi-component systems such as soil microbial communities. Currently, several international projects are being working on characterization of the global microbial community. Thus, Earth Microbiome Project (<http://www.earthmicrobio-me.org/>) has already collected the data on taxonomic structure of microbiomes of different ecological niches obtained with the use of novel sequencers. Within the framework of this project it has been already investigated more than 9.000 samples, i.e. over 800 million nucleotide sequences. However, for Russia, this database contains just about a dozen of samples related to permafrost. The presented work is aimed at filling the existing gap. This task is particularly important in Russia, whose territory exhibits almost all variety of soil types being the most promising experimental area of this kind in the planet. Along with it, studying soil microbial communities by modern methods of sequencing is a practically important step to creating new optimized agricultural technologies. Such approaches can be developed based on features of the soil microbiome as universal and very sensitive indicator of condition of the soil.

A starting point to implementation of such projects is a survey analysis of soil microbiomes in different soil types, which task was set in the presented research.

Technique. A collection of samples of the most common soil types (in total 20 samples) were derived from different regions of Russia and Ukraine in September 2011. The samples were transported to a laboratory and stored there at – 70 °C.

DNA was extracted from 0.5 g of frozen soil after grinding it with glass beads; the sample was grinded for 1 min on the device FastPrep 24 ("MP Medicals", USA) at maximum power. Composition of the extraction buffer: 350 ul of A solution (sodium phosphate buffer - 200 mM, guanidine isothiocyanate - 240 mM; pH 7.0), 350 ul of B solution (Tris-HCl – 500 mM; SDS - 1% by weight to volume; pH 7.0), and 400 ul of phenol-chloroform mixture (1:1). The resulting preparation was centrifuged at maximum speed for 5 min, then the aqueous phase was collected and re-extracted with chloroform. DNA was precipitated by adding an equal volume of isopropyl alcohol. After centrifugation, the precipitate was washed with 70 % ethanol and dissolved in water at 65 °C for 5-10 min. The DNA was purified by electrophoresis in 1% agarose gel and then extracted from the gel by adsorption on silicon oxide (4, 5).

The construction and sequencing of amplicon libraries were performed using the purified DNA preparation (10-15 ng) as template in PCR (temperature profile: 95 °C — 30 s, 50 °C — 30 s, 72 °C — 30 s; total 30 cycles) with the addition of polymerase Encyclo ("Evrogen", Russia) and universal primers for the variable region of V4 gene of 16S-rRNA – F515 (GTGCCAGCMGCCGCGGTAA) and R806 (GGACT-ACVSGGGTATCTAAT) (6). Into the primers were introduced oligonucleotide identifiers for each sample (20 identifiers) and service sequences necessary for pyrosequencing protocol "Roche" (Switzerland). Sample preparation and sequencing were performed on the device GS Junior ("Roche", Switzerland) according to the manufacturer's guidelines.

Taxonomic analysis of nucleotide sequences for the amplicon libraries was performed using QIIME program (7). Data analysis included the following steps: segregation of libraries by the identifiers; verifying the correctness of sequencing and filtering the nucleotide sequences; clustering the sequences in operational taxonomic units (OTU) with 97 % similarity threshold; alignment of the sequences by Uclust method; constructing the matrix of genetic distances and phylogenetic tree with Fasttree method. Taxonomic identification of OTU was carried out using a databank RDP (<http://rdp.cme.msu.edu/>). The occurrence of OTU in the samples was

considered in calculations of biodiversity indices – Shannon's index and ChaoI: $H = -\sum p_i \ln p_i$ (p_i — proportion of the i^{th} species in a community), $S_{\text{est}}(\text{ChaoI}) = S_{\text{obs}} + a^2/2b$ (S_{est} — estimated number of OTU, S_{obs} — observed number of OTU, a — number of singly-identified OTU, b — number of doubly-identified OTU). Along with it, rarefaction test was performed to assess the dynamics of accumulation of OTU depending on the number of sequenced chains, as well as UPGMA cluster analysis allowing to estimate the significance of clusters with Bootstrap algorithm.

Results. Characteristics of soil samples of different origin are shown in Table 1.

1. Collection description of soil samples derived from different geographic regions (Russia, Ukraine, collected in September, 2011)

Institution	Origin of a sample	No samples	Designation	Soil type	Biogeocenosis	GPS location
Prikaspiysky Research and Development Institute of Arid Agriculture, Astrakhan	Astrakhan Oblast	19	BURSOL19	Brown solonets	Virgin land	N 47°88'7.49" E 46°12'2.94"
Prikaspiysky Research and Development Institute of Arid Agriculture, Astrakhan	Astrakhan Oblast	37	SOLONC37	Fine solonchak-like solonets	Virgin land	N 47°88'5.07" E 46°12'6.01"
Voronezh Research and Development Institute of Agriculture	Voronezh Oblast	59	CHERNZ59	Chernozem	Hay meadow lea land	N 51°09'16.24" E 40°47'09.08"
Voronezh Research and Development Institute of Agriculture	Voronezh Oblast	86	CHERNZ86	Meadow Chernozem	Lea land since 1892	N 51°09'16.24" E 40°47'09.08"
V.V. Dokuchaev Central Museum of Soil Science, St. Petersburg	Leningrad Oblast	108	PODZOL108	Podzol	Lea land	N 60°32'33.45" E 33°9'6.75"
All-Russia Research and Development Institute of Agricultural Use of the Improved Land, Tver, agrolandscape experimental station "Gubino"	Tver Oblast	124	PODZOL124	Sod-podzol	Meadow near a forest	N 56°46'25.38" E 36°04'50.61"
Stavropol Research and Development Institute of Agriculture, Mikhaylovsk	Stavropol Krai	187	CHERNZ187	Chernozem	Virgin land	N 56°25'24.40" E 59°06'40.25"
Tatarstan Research and Development Institute of Agriculture, Kazan	Republic of Tatarstan	195	SERLES195	Gray wood soil	Lea land	N 55°40'15" E 52°08'28"
Bogdo Research Land and Forest Improvement Station of All-Russia Research and Development Institute of Land and Forest Improvement, Kharabali	Astrakhan Oblast	202	POJMEN202	Riverside layered alluvial soil	Virgin land	N 47°24'20.38" E 47°14'50.47"
Novosilsk Zonal Station for Land and Forest Improvement of All-Russia Research and Development Institute of Land and Forest Improvement, Mtsensk	Orel Oblast	235	SERLES235	Gray wood soil	Meadow	N 53°17' E 33°33'
All-Russia Research and Development, Production and Technology Institute of Organic Fertilizers and Peat, Sudogoda	Vladimir Oblast	253	PODZOL253	Sod high-podzolic soil	Mixed forest	N 56°03'7" E 40°29'52"
All-Russia Research and Development Institute of Agriculture and Soil Erosion Prevention, Kursk	Kursk Oblast	279	CHERNZ279	Chernozem	V.V. Alekhin Central-Chernozem Biosphere State Reserve	N 51°34'27.8" E 36°05'67.2"
Kletskaya Experimental Station of All-Russia Research and Development Institute of Land and Forest Improvement	Volgograd Oblast	294	KASHTM294	Dark-brown soil	Virgin land	N 49°13'29" E 42°56'32"
All-Russia Research and Development Institute of Land and Forest Improvement, Volgograd	Volgograd Oblast	312	KASHSV312	Light-brown soil	Lea land	N 48°38'49" E 44°22'57"
Kalmykiya Research and Development Forest Experimental Station of All-Russia Research and Development Institute of Land and Forest Improvement	Republic of Kalmykia	326	KASHSV326	Light-brown solonets	Virgin land	N 46°17'09" E 44°15'11"
State Agricultural Enterprise "Kotlasskoye"	Arkhangelsk Oblast	345	SUGLSV345	Sod loamy soil	Lea land	N 61°8'50" E 46°32'55"
State Agricultural Enterprise "Kholmogorskoye"	Arkhangelsk Oblast	357	ALLDER357	Sod alluvial soil	Bottomland hay meadow	N 63°45'17" E 41°56'19"
State Agricultural Enterprise "Arkhangelskoye"	Arkhangelsk Oblast	384	ALLDER384	Sod alluvial soil	Central bottomland lea hay meadow	N 64°30'13.0" E 40°26'45.1"
Institute of Agriculture of the Crimea, Klepinino	Crimea	399	KRASZM399	Southern chernozem	Virgin land	N 45°32'21.74" E 34°12'6.24"
Orel State Agrarian University	Orel Oblast	404	SERLES404	Dark-gray wood soil	Lea land	N 52°59'34.94" E 36°02'29.92"

Analysis of nucleotide sequences. Primers used in this work were designed based on 16S-rRNA sequence of both *Bacteria* and *Archaea*, which allows using them in a complex analysis of Prokaryote communities.

2. Results of sequencing of 16S-rRNA gene, operational taxonomic units (OTU), and calculated diversity indices of Shannon and ChaoI in the soil samples derived from different geographic regions (Russia, Ukraine)

Sample	Shannon index (H)	ChaoI index	Number of OTU	Number of sequences
BURSOL19	8.48	1841	740.8	3857
SOLONC37	8.70	2068	824.2	4435
CHERNZ59	8.61	2365	830.0	4594
CHERNZ86	8.37	1927	759.2	4433
PODZOL108	7.45	1193	513.6	4101
PODZOL124	8.87	1926	857.9	2011
CHERNZ187	8.28	1662	697.3	2181
SERLES195	8.50	1775	735.9	3952
POJMEN202	8.09	1735	681.1	3877
SERLES235	8.84	2289	844.6	4113
PODZOL253	8.15	1681	693.6	4113
CHERNZ279	8.12	1696	733.6	5010
KASHTM294	8.29	1954	725.7	4209
KASHSV312	6.42	848	469.6	5029
KASHSV326	8.25	2034	729.6	3935
SUGLSV345	9.18	3422	1010.3	5704

ALLDER357	8.68	2210	815.4	4124
ALLDER384	7.77	1139	565.5	2783
KRASZM399	7.47	1370	535.9	3218
SERLES404	8.62	2287	837.7	5089

Note. Number of OTU per 2011 sequences.

In total there were sequenced 100 589 sequences; their quality was assessed (QUALITY> 25), and after filtering there were selected for further analysis 62 977 sequences with the length of 270 nucleotides. The number of sequences in each sample ranged from 2011 to 5704 (mean 4059.5) (Table 2). Total number of OTU (sequences of 16S-rRNA gene with 97 % similarity, which nearly corresponds to the taxonomic level of species) in the samples was 10 891. They were clustered in 42 phyla (Fig. 1, A; Table 2) and 350 families (Fig. 1, B; Table 2). A significant number of sequences couldn't be attributed to nor any species or genus, neither to higher taxonomic levels such as phylum. Such non-attributable sequences are a common fact of metagenomic research resulting from the incompleteness of available databases.

At the level of domains, *Bacteria* were predominant (93.3-99.9 %) over *Archaea* (from 0.01 % in sample PODZOL253 to 6.7 % in KASHSV326). Several samples contained nucleotide sequences non-attributable at the level of domain. Qualitative composition of bacterial and archaeal phyla was similar in all investigated samples. Among *Bacteria*, the dominant groups were representatives of the phyla *Proteobacteria* (up to 59.3%), *Actinobacteria* (up to 26,5%), *Verrucomicrobia* (up to 13.6%), *Bacteroidetes* (up to 10.5%), *Firmicutes* (up to 8.2 %), *Gemmatimonadetes* (up to 6.9 %), *Chloroflexi* (up to 5.7 %).

Fig. 1. Abundance of phyla (A) and families (B) of microorganisms (based on data of sequencing of 16S-rRNA gene) **in the soil samples from different geographic regions** (Russia, Ukraine; entries show dominant phyla and families).

Archaea were represented by the phylum *Crenarchaeota*. Such distribution is quite typical for soil microbiomes (8, 9). The major role in taxonomic assessment of soil microbial communities belongs to bacterial phyla. Thus, the phylum *Actinobacteria* was quite abundant in samples KRASZM399, SOLONC37, KASHSV326, POJMEN202, CHERNZ86, KASHTM294, and KASHSV326. These samples were derived from the south of Russia and the Crimea (Table 1, Fig. 2) – the regions with a long dry period, which explains the dominance of *Actinobacteria* who are very resistant to low moisture content in the medium. At the same time, in two other “southern” samples – KASHSV312 and CHERNZ279 (Table 1, Fig. 2) population of *Actinobacteria* significantly yielded to the phylum *Proteobacteria* (Fig. 1, A).

3. Abundance of phyla and families of microorganisms in the total metagenome (based on data of sequencing of 16S-rRNA gene) of soil samples derived from different geographic regions (Russia, Ukraine)

Taxonomic group	Percentage, %	Taxonomic group	Percentage, %
Phyla			
<i>Actinobacteria</i>	39.0	<i>Euryarchaeota</i>	0.0
<i>Proteobacteria</i>	27.8	<i>Chlorobi</i>	0.0
<i>Acidobacteria</i>	7.6	<i>Fusobacteria</i>	0.0
<i>Verrucomicrobia</i>	3.8	<i>Spirochaetes</i>	0.0
<i>Chloroflexi</i>	3.6	<i>Tenericutes</i>	0.0
<i>Gemmatimonadetes</i>	3.6	<i>Thermi</i>	0.0
<i>Firmicutes</i>	3.2	ZB2	0.0
Non-identified phylum	2.9	<i>Deferribacteres</i>	0.0
<i>Bacteroidetes</i>	2.8	<i>Caldiserica</i>	0.0
<i>Crenarchaeota</i>	2.4	<i>Spirochaetes</i>	0.0
<i>Planctomycetes</i>	1.8	<i>Chrysiogenetes</i>	0.0
<i>Nitrospirae</i>	0.2	<i>Thermotogae</i>	0.0
SPAM	0.2	<i>Aquificae</i>	0.0
Non-identified domain	0.1	<i>Synergistetes</i>	0.0
AD3	0.1	<i>Dictyoglomi</i>	0.0
<i>Armatimonadetes</i>	0.1	<i>Elusimicrobia</i>	0.0
CCM11b	0.1	OP11	0.0
<i>Chlamydiae</i>	0.1	<i>Fibrobacteres</i>	0.0
<i>Cyanobacteria</i>	0.1	<i>Lentisphaerae</i>	0.0
<i>Elusimicrobia</i>	0.1	<i>Thermodesulfobacteria</i>	0.0
WPS2	0.1	SR1	0.0
WS3	0.1		
Families			
<i>Hyphomicrobiaceae</i>	3.3	<i>Koribacteraceae</i>	0.6
<i>Spartobacteriaceae</i>	2.9	<i>Geodermatophilaceae</i>	0.6
<i>Enterobacteriaceae</i>	2.8	<i>Acetobacteraceae</i>	0.6
<i>Nitrososphaeraceae</i>	2.4	AKIW874	0.5
<i>Solirubrobacteraceae</i>	2.2	<i>Haliangiaceae</i>	0.5
<i>Sphingomonadaceae</i>	2.1	<i>Intrasporangiaceae</i>	0.4
<i>Solibacteraceae</i>	1.9	<i>Microbacteriaceae</i>	0.4
<i>Gemmatimonadaceae</i>	1.9	<i>Micromonosporaceae</i>	0.4
<i>Bradyrhizobiaceae</i>	1.8	<i>Mycobacteriaceae</i>	0.4
<i>Nocardioideaceae</i>	1.7	<i>Pseudonocardaceae</i>	0.4
<i>Pseudomonadaceae</i>	1.6	<i>Flexibacteraceae</i>	0.4
<i>Rubrobacteraceae</i>	1.5	<i>Bacillaceae</i>	0.4
<i>Patulibacteraceae</i>	1.2	<i>Caulobacteraceae</i>	0.4
<i>Rhodospirillaceae</i>	1.1	<i>Oxalobacteraceae</i>	0.4
EB1017	1.0	CL500-29	0.3
<i>Syntrophobacteraceae</i>	1.0	<i>Lamiaceae</i>	0.3
<i>Sinobacteraceae</i>	0.9	<i>Paenibacillaceae</i>	0.3
<i>Acidobacteriaceae</i>	0.8	<i>Burkholderiaceae</i>	0.3
<i>Streptomyetaceae</i>	0.8	<i>Comamonadaceae</i>	0.3
<i>Xanthomonadaceae</i>	0.8	<i>Frankiaceae</i>	0.2
<i>Micrococcaceae</i>	0.7	FFCH4570	0.2
<i>Gemmataceae</i>	0.7		

Except these two samples, taxonomic structure of the soil microbiota had a trend to predominance of the phylum *Actinobacteria* in southern regions and *Proteobacteria* – in northern regions. It should be also noted that southern soils contained more abundant populations of the phylum *Firmicutes* (KASHSV326 and POJMEN202). There was a pronounced variation in population size of the phylum *Acidobacteria* found mainly in sod-podzolic soils (PODZOL108, PODZOL124, ALLDER384, PODZOL253; Fig. 1, A). Such distribution most likely resulted from low pH of podzolic soils, because highly acid medium provides optimal conditions for development of *Acidobacteria* (10). The authors' findings perfectly illustrate and complement a recent report about the spread of bacterial phyla depending on physico-chemical environmental factors, the most significant of which are moisture and pH level (11).

At the level of families, the bacterial community had a very complex structure without any distinct dominant groups. Such groups were found just in a few samples: e.g., in the sample 312 KASHSV with very abundant population of *Enterobacteriaceae*, *Pseudomonadaceae* (respectively, 26.43 % and 15.11%), in samples PODZOL124 and ALLDER384 with increased proportion of *Acidobacteria* non-attributable at the level of families, and in KRASZM399 with significant amount of non-attributable *Actinobacteria* (Fig. 1, B). However, many bacterial families were fairly evenly represented in all types of soil – primarily, non-attributable to families members of the order MS47 (*Actinobacteria*), families *Solirubrobacteriaceae*, *Patulibacteraceae* from the order *Solirubrobacteriales* (*Actinobacteria*), various families of *Proteobacteria* from the orders *Rhizobiales* and *Sphingomonadales*, as well as families SOGA31 (*Chloroflexi*) and *Gemmatimonadaceae* (*Gemmatimonadetes*) (Fig. 1, B).

The similar set of bacterial families was earlier found by the authors in saline soils of Kazakhstan (12); some of the abovementioned bacterial families were observed in different soils by other researchers as well (8).

Archaea also had a trend: the family *Nitrososphaeraceae* was dominant in all soils. These microorganisms are spread worldwide due to their active participation in early stages of nitrification (13). Such bacterial and archaeal cosmopolites indicate the presence in soil of core and accessory components of the microbiome (similar to core and accessory components of microbial genomes). The presence of the core component also indicates high ecological plasticity of the soil community associated with adaptive capacities of individual microorganisms and “genetic potential” of the soil itself resulting from, particularly, a huge pool of extracellular DNA maintained in the soil (14).

Fig. 2. Location of sampling points in different geographic regions (Russia, Ukraine).

Fig. 3. Alfa-diversity of microorganisms (based on data of sequencing of 16S-rRNA gene) **in the soil samples derived from different geographic regions** (Russia, Ukraine)

Analysis of Alfa-diversity. All the studied samples were divided into three groups (Fig. 3). The first group was represented by the only sample SUGLSV345 having a highest diversity for all criteria; the second group included almost all other samples with all gray wooded soils (mainly non-cultivated lands, Shannon index 8.08-8.87) (Table 2); the third group – samples ALLDER384, KRASZM399, PODZOL108, and KASHSV312 with biodiversity shifted towards one of the phyla.

Fig. 4. Beta-diversity of microorganisms (based on data of sequencing of 16S-rRNA gene) (A – dendrogram of relationships, 1 – cluster 1; B – two-dimensional chart) **in the soil samples from different geographic regions** (Russia, Ukraine)

Predominance of *Acidobacteria* in samples ALLDER384 and PODZOL108 was most likely associated with high acidity of the soil. The maximum number of *Actinobacteria* was found in the sample KRASZM399 (70.48 % of total bacterial population). The sample KASHSV312 had the lowest biodiversity with absolute predominance of the phylum *Proteobacteria* (Fig. 1, A). It is believed that *Proteobacteria* dominate in disturbed soil habitats (15).

Analysis of Beta-diversity. Clustering by soil type was observed in several samples (PODZOL and SERLES) (Fig. 4, B), as well as a strong trend to clustering by physico-chemical parameters of the soil, such as pH (Fig. 4, A). In this regard it should be mentioned the group CHERNZ, in which two samples with close geographical location (¹ 59 and ¹ 86) had different structure of microbiome (members of different clusters), while two other samples derived from, respectively, southern and western parts of the country (¹ 59 and ¹ 187) belong to one cluster (Fig. 2, Fig. 4, A). Such patterns in the structure of microbiome may be caused by pH level – similar (for samples ¹ 59 and ¹ 187 – pH, respectively, 7.64 and 7.34) or different (for samples ¹ 59 and ¹ 86 – pH, respectively, 7.64 and 6.22). These facts allow concluding that the structure of soil microbiome depends not only (and not so much) on soil type, but rather on physico-chemical characteristics of the medium, in particular, its acidity / alkalinity. This conclusion is supported by results of several studies showing that pH of the medium is the most powerful, and sometimes the only factor determining taxonomic structure of the microbiome (16).

Thus, studying microbial communities by high-throughput sequencing has provided a great advance in knowledge about their natural genetic diversity, and also caused a number of fundamentally new scientific problems of soil microbiology. Results of the presented study reveal association between abiotic factors (pH and moisture regime) and taxonomic structure of the soil microbiome. Of course, only investigating hundreds of samples is sufficient to confirm the discovered patterns and cover the full spectrum of environmental factors. However, conventional ecological approaches can hardly be applied to the soil microbiome including hundreds or even thousands of species, and almost inappropriate for so many tested samples. Therefore, high-throughput sequencing of large samples necessitates the new methods of data analysis allowing to pass from descriptive ecology to functional ecology. This task can be solved by fundamentally new integrated approaches considering a microbial community as a functional unit completely dependent on the environment and being the major factor of its formation.

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