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EXPERIMENTAL ARTICLES

Molecular Analysis of the Diversity of Nitrifying Bacteria in the Soils of the Forest and Steppe Zones of European Russia

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Abstract—Nitrifying bacteria play a key role in the global nitrogen cycle due to their ability to convert reduced nitrogen compounds (ammonium) to oxidized ones (nitrite and nitrate). Recent investigations based on the methods of molecular ecology revealed that bacteria are responsible for nitrification in natural ecosystems. At the same time, data on the species composition of the nitrifiers in soil microbial communities are scarce. Soil samples collected in the forest and steppe areas of European Russia and the enrichment cultures of nitrifying bacteria isolated from these samples were used for molecular studies of the diversity of the *amoA* gene encoding the synthesis of the key enzyme of autotrophic ammonium oxidation. The nitrifying bacteria of the genera *Nitrosospira* and *Nitrosovibrio* were found in all the studied soils from natural biocenoses and agrocenoses.

Keywords: nitrification, ammonium-oxidizing bacteria, enrichment cultures, *amoA*.

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Nitrification plays a critical role in the global nitrogen cycle, closely associated with the processes of ammonification and denitrification. Oxidation of ammonium to nitrate results in nitrogen depletion of soils, since nitrates are easily washed out. Moreover, production of nitrate induces denitrification, which results in formation of gaseous nitrogen compounds. At low oxygen concentrations, ammonium-oxidizing bacteria are also capable of denitrification, producing nitrous oxide (N_2O) [1, 2]. An increase in the N_2O concentration may lead to disruption of the Earth's insolation balance due to increasing levels of UV radiation caused by destruction of the ozonosphere due to photochemical reactions of ozone (O_3) with N_2O and enhance the greenhouse effect [3]. N_2O production and its flux to the atmosphere depend on the amount of incoming and contained nitrogen in soil, as well as on factors that control the processes of nitrogen transformation in soil (nitrification, denitrification, and immobilization). Among the key factors are the contents of NH_4^+ and NO_3^-, the balance between aerobic and anaerobic conditions, and the availability of organic carbon [4].

All autotrophic ammonium-oxidizing bacteria (AOB) belong to β- and γ-*proteobacteria* [5]. At the first stage, ammonia is oxidized to hydroxylamine by the enzyme ammonium monooxygenase (AMO). The substrate specificity of AMO is rather low, and, apart from ammonia, it catalyzes the oxidation of carbon monoxide, methane, and other compounds [6]. The genes encoding AMO proteins, *amoA, amoB*, and *amoC*, are specific for ammonium-oxidizing bacteria. The *amoA* gene encoding the protein of the active site of ammonium monooxygenase is most frequently used as a molecular marker for nitrification. The phylogeny of the *amoA* genes mostly correlates with that of the genes encoding the 16S rRNA of autotrophic ammonium-oxidizing bacteria [5, 6]. This makes it possible to determine the taxonomic position of the studied AOB at the genus, and sometimes at the species, level on the basis of phylogenetic analysis of their *amoA* gene sequences [1,6].

Autotrophic nitrifying bacteria are slow-growing microorganisms with a generation time of up to several days even under laboratory conditions [7]. For this reason, traditional methods based on the use of selective nutrient media are very labor-consuming. At present, molecular biological techniques are widely used for studying AOB in natural samples and enrichment cultures. For instance, the FISH method based on in situ hybridization of intact cells with fluorescently labeled oligonucleotide probes is used for quantitative assessment of the population composition of nitrifiers [8]. Using the immunochemical method based adsorption of strain-specific antibodies labeled with a fluorescent dye on the cell surface, makes it possible to carry out a direct count under a fluorescence microscope [9]. The methods of molecular analysis by polymerase chain reaction (PCR) for detection of the 16S rRNA genes [10] and the frag-

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Some properties of the studied soil samples

Designations for the soil samples (enrichment cultures)	Sampling sites, coordinate position	Sampling time, depth	Soil, soil-forming material	Vegetation	pH_{water}	C_{total}, %
podzol-forest	Experimental forest, Timiryazev Russian State Agrarian University–Moscow Agricultural Academy, Moscow 55°49.10′ N 37°32.24′ E	July 2008, 5–10 cm	Thick low- and midpodzolic on moraine sand and sandy loam	Mixed forest	4.6	2.1
podzol-agro (CHE-2)	Field station, Timiryazev Russian State Agrarian University–Moscow Agricultural Academy, Moscow, 55°50.39′ N 37°33.44′ E		Thick midpodzolic on moraine sand and sandy loam	Continuous spring barley since 1912	5.3	1.2
grey-forest(CHE-5)	Field station, Institute of Physicochemical and Biological Problems of Soil Science, Russian Academy of Sciences, Pushchino, Moscow oblast, 54°50′ N 37°37′ E	November 2008, 7–10 cm	Gray forest midloamy on loess loam	Secondary broad-leaved forest	6.5	2.7
grey-prary(CHE-6)				Unmowed grass meadow	5.4	2.2
grey-agro				Wheat, fertilizer-free field experiment	6.0	1.1
chernozem(CHE-3)	Voronezh oblast, Talovsk raion 51°07′ N 40°43′ E	June 2008, 5–10 cm	Typical chernozem on carbonate loess loam	Feather grass–motley grass steppe	6.7	7.2
chestnut 1	Volgograd oblast, Ol'khovskii raion, Zenzevatka settlement, 49°49′04.4″ N 44°32′28.2″ E	July 2008, 5–10 cm	Meadow-chestnut on light loam	Abandoned meadow	6.9	4.1
chestnut 4(CHE-4)	49°46′53.0″ N 44°31′08.7″ E		Chestnut soil on light loam	Forest belt	5.5	3.4

ments of the functional genes of nitrifiers [1, 6, 8, 11] are widely used.

The application of molecular techniques made it possible to expand the range of data on the compositions of nitrifying communities in the soils from both natural [12] and anthropogenic [7, 9, 13, 14] environments. For example, it was established that nitrifying bacteria belonging to the classes γ- and β-*proteobacteria* prevail in sewage and soils, respectively [15, 16]. Members of the genus *Nitrosospira* were mainly detected in undisturbed soils, whereas representatives of the genus *Nitrosomonas* are usually detected in arable soils with high ammonium content [17, 18].

The goal of the present work was to assess the diversity of autotrophic nitrifying bacteria in some soils of European Russia by analysis of the *amoA* gene fragments.

MATERIALS AND METHODS

Objects of study. Freshly collected samples of undisturbed forest (sod-podzolic and gray forest) and steppe (chernozem and chestnut) soils were the subjects of this study. Samples of the former two soil types collected in agrocenoses were studied as well. The properties of the studied soils are listed in the table.

Isolation and maintenance of enrichment cultures of the nitrifying bacteria. To obtain enrichment cultures, aliquots (0.1 g) of soil were placed in 200-ml glass flasks with 50 ml of mineral medium containing the following (g/l): $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 0.5; NaCl, 2.0; $MgSO_4 \times 7H_2O$, 0.2; $FeSO_4 \times 7H_2O$, 0.05; $CaCO_3$, 6.0; trace element solution [19], 10 ml/100 ml; and pH 7.6 [20]. The flasks were incubated under static conditions at 25°C for 2–4 weeks. Every week, samples were collected to determine pH, as well as to carry out qualitative reactions with the Nessler's

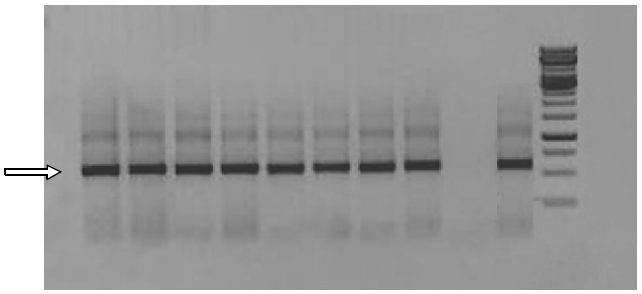

Fig. 1. Results of amplification of the 16S rRNA gene fragments for the DNA extracted from soil samples. The numerals indicate: sod-podzolic soil, forest, and agrocenosis (*1*, *2*); gray forest soil, forest, agrocenosis, and meadow (*3*–*5*); chestnut soil (*6*); chernozem (*7*); *Nitrosomonas* sp. (*8*); control in the absence of the DNA template (*9*); *E. coli* (*10*); and DNA molecular weight marker (1000 bp) (*11*). The arrow points to the target fragment.

reagent (for ammonium), and Griess reagent (for nitrites) [21]. Subsequent transfers of the cultures were carried out every 2 weeks; the inoculum dose was 5% vol/vol.

DNA extraction and amplification of the 16S rRNA and *amoA* gene fragments. DNA was extracted from a soil sample (0.5 g) using the Ultra Clean Soil DNA Isolation Kit (MoBio, United States) according to the manufacturer's protocol.

PCR amplification of the 16S rRNA gene fragments was performed using the primers 341F (5'-GGNGACTGGGACTTCTGG-3') and 907R (5'-GAASGCNGAGAAGAASGC-3') [22]. The reaction mixture (25 μl) contained the following: 5×PCR buffer ($(NH_4)SO_4$, 17 mM; Tris-HCl, 67 mM; $MgCl_2$), 2 mM; pH 8.8), 2 μl of dNTP, 15 pmol of each primer, and 2.0 U of *Taq* DNA polymerase (Helicon Co., Russia). The PCR cycle parameters were as follows: initial denaturation at 95°C for 3 min; 35 cycles: 95°C for 1 min, 50°C for 1 min, 72°C for 1 min; and final elongation for 3 min at 72°C.

The *amoA* gene fragments were amplified using the oligonucleotide primers *amoA*-1F (5'-GGGGTTTC-TACTGGTGGT-3') and *amoA*-2R (5'-CCCCTCKGSAAAGCCTTCTTC-3') [5]. The reaction mixture (25 μl) contained the following: 1× PCR buffer ($(NH_4)SO_4$, 17 mM; Tris-HCl, 67 mM; $MgCl_2$), 4 mM; pH 8.8), 4 μl of dNTP, 30 pmol of each primer, and 1.0 U of *Taq* DNA polymerase (Helicon Co., Russia). The PCR cycle parameters were as follows: 94°C for 5 min; 35 cycles: 94°C for 45 s, 57°C for 30 s, 72°C for 1 min; and final elongation for 7 min at 72°C [22]. Amplification was performed on a MyCycler thermal cycler (BioRad, United States).

Analysis of the PCR products was carried out by electrophoresis in 1.2% agarose gel stained with ethidium bromide (1 μg/ml). PCR fragments were isolated and purified using the Wizard PCR Preps kit (Promega, United States) according to the manufacturer's recommendations.

Cloning and sequencing of PCR products. The obtained PCR products were purified and transformed into competent *E. coli* DH10B cells using the pGEM-T Easy vector system (Promega, United States). For the clone library, 50 colonies exhibiting a positive reaction were randomly chosen. Then, a PCR reaction was carried out for each colony using the universal plasmid primers M13F and M13R. To determine the colonies containing an insert, agarose gel electrophoresis was carried out.

The PCR product was purified using the Ultra Clean PCR Clean-Up (MoBio, United States) according to the manufacturer's recommendations. Then, 1 μl of the purified PCR product was treated with *Taq*I restriction endonuclease (1 μl per 50 μl of the reaction mixture) and incubated at 65°C for 1 h. The mixture was assayed by electrophoresis in 3% agarose gel and, on the basis of analysis of the restriction profiles, the obtained clones were combined into several groups. From each group, three clones were randomly chosen for sequencing.

Sequencing of the PCR products was performed in the service laboratory using the Big Dye Terminator v.3 sequencing kit (Applied Biosystems Inc., United States). Clones containing *amoA* gene fragments were sequenced using the universal primers P338 and P518. Nucleotide sequence analysis was conducted in an ABI Prism 3100 automatic sequencer (Applied Biosystems Inc., United States).

Analysis of nucleotide and protein sequences. Analysis of the obtained nucleotide sequences was performed using the NCBI BLAST software package [http://www.ncbi.nlm.nih.gov/BLAST]. The nucleotide sequences of the studied genes were edited and aligned with the appropriate sequences from the closest relatives using the BioEdit software package [http://jwbrown.mbio.ncsu.edu/BioEdit.html]. The dendrogram showing the similarity level between the studied and reference sequences of the *amoA* gene was constructed on the basis of deduced sequences by the methods implemented in the TREECONW software

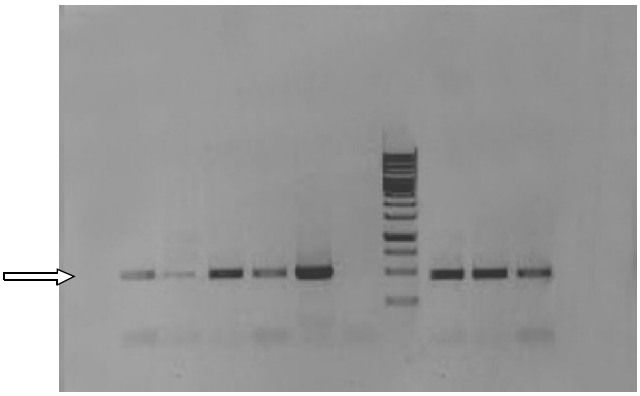

Fig. 2. Results of amplification of the *amoA* gene fragments for the DNA extracted from different soils using the modified technique. The numerals indicate: gray forest soil, forest, agrocenosis, and meadow (*1*, *2*, *4*); chestnut soil (*3*); sod-podzolic soil, forest, and agrocenosis (*8*, *9*); chernozem (*10*); *Nitrosomonas* sp. (positive control) (*5*); *E. coli* (negative control) (*6*); and DNA molecular weight marker (1000 bp) (*7*). The arrow points to the target fragment.

package [http://bioc-www.uia.ac.be/ u/yudp/treeconw.html].

Deposition of the nucleotide sequences. The obtained nucleotide sequences of the *amoA* genes were deposited in GenBank under the accession numbers JF 496679–JF 496692.

RESULTS AND DISCUSSION

PCR analysis of the *amoA* gene fragments of the soil samples. The DNA obtained from soil samples was initially used for amplification of fragments of bacterial 16S rRNA genes (Fig. 1). The presence of specific bands in the agarose gel slabs indicated that the DNA obtained from all the studied soil samples was usable for PCR analysis. However, successful amplification of the *amoA* gene fragments required a special procedure for selection of appropriate conditions. This was probably due to the scarcity of copies of the target genes, as well as to the abundance of other genetic components, which possibly resulted in formation of chimeric sequences. To select the most favorable conditions for amplification of the *amoA* gene fragments, a series of experiments with the DNA extracted from the samples of sod-podzolic soil was carried out; in the course of the experiments, some changes in the standard procedure [23] were made. For instance, the annealing temperature was decreased from 95 to 94°C; the duration of annealing was decreased from 1 min [23] to 45 s. In addition, the elongation temperature was significantly increased from 50 to 57°C, whereas the time interval remained unchanged (30 s) [23]. The number of amplification cycles was increased from 30 to 35. Application of the modified protocol made it possible to obtain *amoA* gene fragments from DNA extracted from the samples of gray forest (forest, meadow), chestnut, and sod-podzolic (forest, agrocenosis) soils and chernozem (Fig. 2). Importantly, the obtained bands differed in their intensity. For example, in the case of gray forest soil, the amplification signal for the samples from the forest biocenosis was much more pronounced than for the soil samples from the agrocenosis, in which the product concentration was at the detection limit (Fig. 2). At the same time, the samples of sod-podzolic soils from both the forest biocenosis and agrocenosis showed almost the same signal intensity. These results are probably due to the differences in AOB numbers in the soils of the ecotopes with different anthropogenic impacts. In the fertilizer-free gray forest soil of the agrocenosis, nitrifying bacteria are probably very scarce, their number being below the PCR detection limit.

Thus, the current conditions of DNA extraction from soil samples and subsequent amplification of the key gene involved in nitrification (*amoA*) may be effectively used for detection of autotrophic AOB in soil samples without using traditional cultivation techniques. The sod-podzolic soils of the agrocenosis are constantly supplemented with fertilizers, which results in an increase in the rate of microbial transformation of mineral nitrogen, as well as in higher numbers of nitrifying microorganisms. On the other hand, the number of nitrifying bacteria in the gray forest soil of the agrocenosis decreases. In the samples of steppe soils (chestnut and chernozem), nitrifying bacteria were detected in all samples, which correlates with the previous data on high rates of nitrification in these soils [23].

Isolation and analysis of enrichment cultures of the nitrifying bacteria. As a result of numerous subculturings (24 transfers over 1.5 years), five stable enrichment cultures of nitrifying bacteria were obtained. The rates and intensity of nitrification differed for the primary enrichment cultures obtained from different soil types. For example, in the culture obtained from chernozem (CHE-3), high levels of nitrite accumulation

(over 20 mg l^{-1}) were detected 2 weeks after the enrichment culture was supplemented with soil. The enrichment cultures obtained from the samples of sod-podzolic (CHE-2) and chestnut (CHE-4) soil were characterized by a longer period of nitrite accumulation, which started after 3 weeks; in the case of the enrichment cultures (CHE-5 and CHE-6) obtained from the samples of gray forest soil, nitrite accumulation was detected only after 5–6 weeks of growth. These pronounced differences in the duration of the lag phase in the enrichments obtained from different soil samples can be attributed to the differences in the initial amount of nitrifiers in the soil samples, as well as to the proportion between the dormant and metabolically active cells. The obtained growth pattern for the enrichment cultures of nitrifying bacteria confirms the results of amplification of the functional *amoA* genes from the soil samples. Since we failed to obtain a distinct PCR product from the fertilizer-free gray forest soil of the meadow and agrocenosis, our assumption that the amount of nitrifying bacteria was too low was confirmed by low rates of nitrite accumulation in the enrichment cultures. On the other hand, the distinct and stable amplification products of the functional *amoA* genes of the nitrifying bacteria obtained from the sod-podzolic and chestnut soils and chernozem samples indicate that the amount of the extracted DNA of nitrifying bacteria was relatively high, which was confirmed by the progressive accumulation of nitrite in the enrichment cultures obtained from these soils.

Sequence and phylogenetic analysis of the clone library of the *amoA* fragments obtained from the soil samples and enrichment cultures. To assess the diversity of nitrifying bacteria in soils, six and five clone libraries of *amoA* gene fragments were constructed for the soil samples and enrichment cultures, respectively. All nucleotide sequences showed high similarity to the *amoA* gene sequences of the nitrifying representatives of the β-*proteobacteria* (95–99%). The results of sequence analysis revealed that the sequences clustered into one group according to the results of restriction analysis were identical and henceforth were regarded as a single phylotype. The highest diversity of AOB (4 phylotypes) was observed in the samples of sod-podzolic soil (forest and agrocenosis). The samples of chernozem, gray forest soil (forest), and chestnut meadow soil contained three phylotypes each; the chestnut soil samples contained two phylotypes. For all enrichment cultures, all sequences were identical, which indicates the presence of the same nitrifying bacterium.

Figure 3 shows the results of phylogenetic analysis of the deduced *amoA* sequences of the clones obtained from soil samples and enrichment cultures. In the dendrogram, the obtained sequences formed three clusters. Cluster 1 included both phylotypes of the nitrifiers isolated from the chestnut 4 soil sample and the enrichment culture CHE-4 obtained from this soil sample, as well as one of the phylotypes from the sample of sod-podzolic soil (podzol-forest-3) from the forest biocenosis. This cluster, in addition to uncultured nitrifying bacteria, includes *Nitrosospira multiformis, N. tenius*, and several *Nitrosospira* sp. isolates.

Cluster 2 includes the sequences of the clones obtained from the samples of chernozem and the sod-podzolic soil of the forest biocenosis, as well as from the enrichment cultures isolated from the samples of chernozem (CHE-3) and the gray forest soil of the forest biocenosis (CHE-5). In addition to the sequences of bacteria of the genus *Nitrosospira*, this cluster includes *Nitrosomonas* and environmental clones of *Nitrosomonadales*.

The majority of the newly obtained sequences formed a compact cluster (Cluster 3), together with the sequences of environmental AOB clones from the soil samples collected in Germany, Japan, and China; however, these sequences did not exhibit any similarity with the known nitrifying bacteria.

Interestingly, analysis of the clone libraries from the enrichment cultures made it possible to gain more detailed insight into the species composition of nitrifiers, probably owing to identification of minor components. It is especially important when studying soil samples with a low AOB content. For instance, we failed to obtain a clone library for the samples of gray forest soil of the meadow; however, a stable enrichment culture was obtained from these samples, which contained a microorganism exhibiting high similarity with *Nitrosospira multiformis*. The enrichment culture isolated from the gray forest soil of the forest cenosis contained a microorganism phylogenetically related to the nitrifying bacteria of Cluster 2, whereas only Cluster 3 sequences were found among the soil clones.

Thus, analysis of the *amoA* gene fragment encoding the synthesis of the key enzyme of the first stage of autotrophic nitrification made it possible to study the species composition of the nitrifying bacterial communities of zonal soils. The sequence analysis and the subsequent phylogenetic analysis of deduced nucleotide sequences revealed that nitrifiers of the genera *Nitrosospira* and *Nitrosovibrio* prevailed in the samples of chernozem, as well as of chestnut, sod-podzolic, and gray forest soils. Despite significant differences in the structure of vegetation, pH values, and the humus content, all the nitrifying bacteria prevailing in the soils of natural biocenosis and agrocenosis belong to the cluster *Nitrosospira–Nitrosovibrio*. The results obtained are in agreement with the data on the community composition of nitrifying bacteria in the meadow soils of the Netherlands [24], where members of the genus *Nitrosospira* were predominant. In the samples of gray forest soil of the forest biocenosis (podzol-forest-4), nitrifying bacteria of the genus *Nitrosomonas* were detected; in other soils, these microorganisms were not detected. The predominance of bacteria of the genera *Nitrosomonas* and

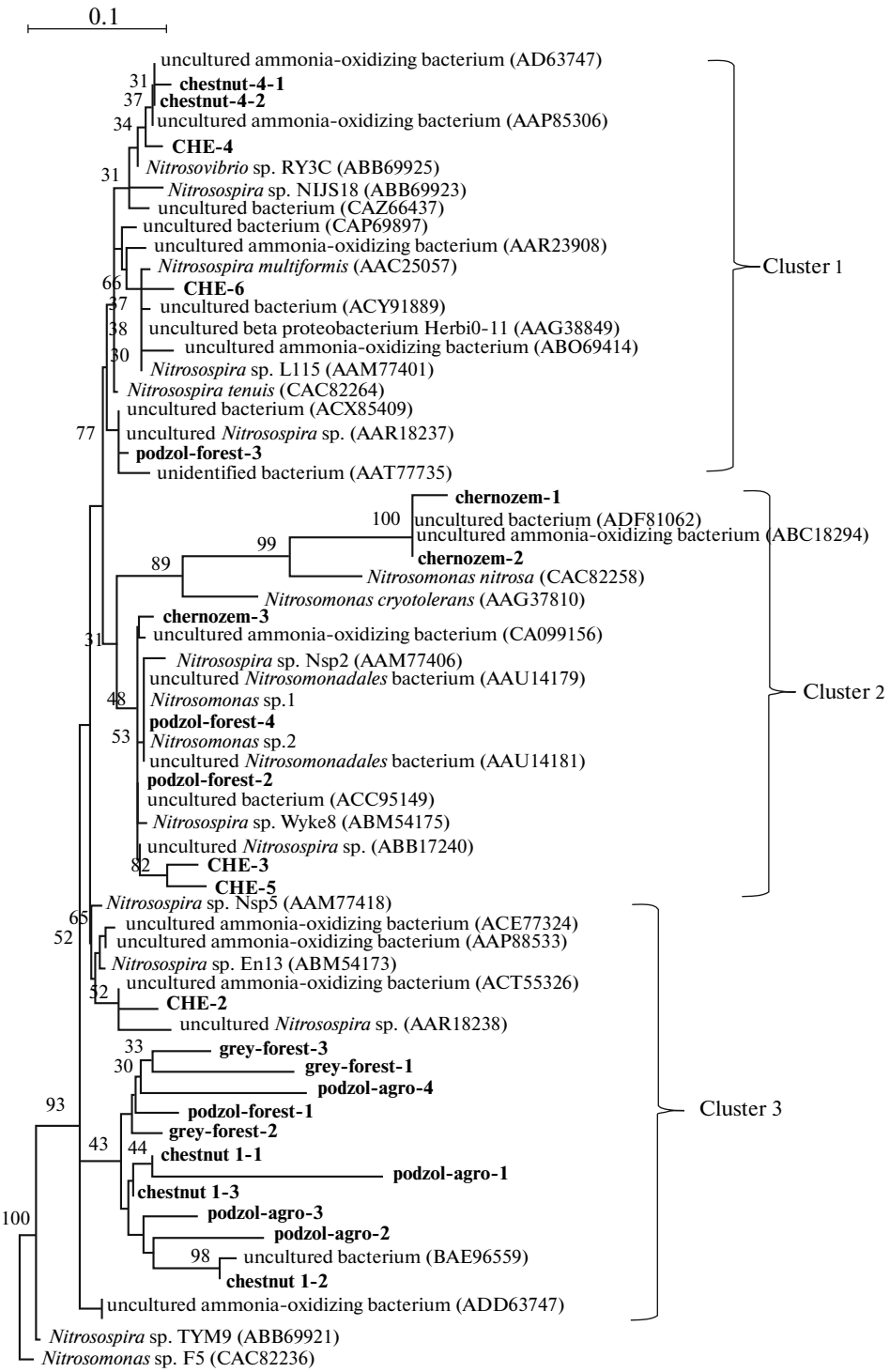

Fig. 3. Phylogenetic tree constructed on the basis of the deduced amino acid sequences of the *amoA* gene fragment. The sequences determined in this work are in boldface. The GenBank accession numbers of the gene fragment sequences are given in parentheses. Scale bar, ten amino acid substitutions per 100 amino acid residues. The numerals at the branching points show the significance of the branching order as determined by bootstrap analysis of 500 alternative trees (only bootstrap values above 50% were considered significant).

Nitrosolobus is typical of the soils with the high ammonium content and neutral pH [25].

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