forests

MDPI

Article

Recovery of Soil-Denitrifying Community along a Chronosequence of Sand-Fixation Forest in a Semi-Arid Desertified Grassland

Chengyou Cao *, Ying Zhang, Zhenbo Cui, Hailong Li, Tingting Wang and Qing Ren

College of Life and Health Sciences, Northeastern University, Shenyang 110169, China; zhangying@mail.neu.edu.cn (Y.Z.); cuizhenbo@mail.neu.edu.cn (Z.C.); lihailong1166@gmail.com (H.L.); wangtingting828@berryoncology.com (T.W.); renqing@genecompany.com (Q.R.)

* Correspondence: caochengyou@mail.neu.edu.cn

Abstract: Revegetation on moving sand dunes is a widely used approach for restoring the degraded sandy land in northeastern China. The development of sand-fixation forest might improve the structures of soil microbial communities and affect soil N cycle. In the present study, the diversities of nitrite (*nirS* and *nirK*) and nitrous oxide (*nosZ*) reductase genes were investigated under a chronosequence of *Caragana microphylla* sand-fixation shrub forest (9- and 19-year), adjacent non-vegetated shifting sand-dune, and a natural forest dominated by *C. microphylla*. The dominant compositions and gene abundance were analyzed by a clone library technique and quantitative polymerase chain reaction, respectively. The compositions and dominant taxa of *nirK*, *nirS*, and *nosZ* communities under forest soil were all similar to those in the shifting sand-dune. However, the three gene abundances all linearly increased across forest age. Clones associated with known denitrifiers carrying *nosZ*, *nirK*, or *nirS* genes, such as members of *Pseudomonas*, *Mesorhizobium*, *Rhizobium*, *Rhodopseudomonas*, *Azospirillum*, and *Cupriavidus*, were detected. These denitrifiers were found to be abundant in soil and dominant in soil denitrification. Soil pH, total N, and available N affected the denitrifying communities by altering the relative abundance of dominant taxa. Overall, although soil attributes and forest age had no significant effects on the dominant constituents of *nirK*, *nirS*, and *nosZ* communities, revegetation on shifting sand-dunes facilitated the quantitative restoration of soil denitrifiers due to the increase in soil nutrients.

Keywords: shrub plantation; soil microbe; denitrifying bacteria; revegetation; moving sand dune

Citation: Cao, C.; Zhang, Y.; Cui, Z.; Li, H.; Wang, T.; Ren, Q. Recovery of Soil-Denitrifying Community along a Chronosequence of Sand-Fixation Forest in a Semi-Arid Desertified Grassland. *Forests* **2021**, *12*, 354. https://doi.org/10.3390/f12030354

Academic Editor: Corina Carranca

Received: 18 February 2021
Accepted: 16 March 2021
Published: 17 March 2021

1. Introduction

Revegetation is widely adopted as useful approach in the restoration of degraded ecosystems in desertified regions [1]. The Horqin Sandy Land, located in the semi-arid zone in north China, has suffered from serious desertification in recent decades due to excessive reclamation, overgrazing, and heavy firewood collection caused by the increasing resident population pressure [2,3]. Revegetation via planting indigenous shrubs on moving or semi-moving sand dunes has been universally recognized as an effective measure for controlling the desertification of an area. Among the planted shrubs, *Caragana microphylla* is the most commonly used pioneer shrub for stabilizing shifting sand dunes because of its strong endurance in arid environments, high resistance to wind erosion of sand burial, fast growth, large root system, and easy propagation [4]. Information on the variations in soil attributes after revegetation on shifting sand dunes is needed to assess the ecological effects and further understand the process of soil restoration along plantation development. Many studies, including the previous works of the authors, have confirmed that the artificial establishment of *C. microphylla* forest improves the microclimate and soil organic matter as well [4–6]. These favorable effects increase with plantation age [7]. The development of plantations could also significantly change the soil microbial community [8,9]. However, whether and how the soil microbial community under secondary barren land (shifting sand dune) can be restored via the establishment of plantations is still unknown. Although

several studies have utilized molecular tools or high-throughput sequencing techniques to investigate soil bacterial community under sand-fixation forests in this region [9–13], the response of soil-specific functional microbial groups, especially N-cycling microbes to the revegetation on shifting sand-dunes, was underestimated.

Some previous studies indicated that soil N content increased with forest age and significantly differed with forest type [1,7,14]. Therefore, close attention should be paid to the microbes associated to soil N dynamics during the recovery of degraded ecosystems [8,15]. It is possible that soil microbial functional communities involved in N transformation would be significantly altered by the development of sand-fixation forest and restored to its original state. However, the diversities and structures of these microbial communities in shifting sand land and vegetation-covered areas of Horqin Sandy Land have not been widely investigated, and limited information is available on the composition of soil microbial communities. Microbial denitrification, one of important process of N transformation, comprises four continuous reaction processes in which nitrate N is reduced into a molecular gaseous form (N_2) [16]. The respiratory nitrate reduction process (i.e., NO_3^- to NO_2^-) is catalyzed by either a membrane-bound or a periplasmic nitrate reductase [17,18]. The reduction reaction of NO_2^- into NO is catalyzed by two types of nitrite reductase, that is, a cytochrome cd1 nitrite reductase and a copper nitrite reductase, which are encoded by *nirK* and *nirS* genes, respectively [16,19]. The reduction of NO to N_2O is catalyzed by NO oxide reductase. The last step in the denitrification process is the reduction of N_2O into N_2, which is mainly governed by the *nosZ* gene (encode N_2O reductase) [16,20]. Denitrification can be regulated by a diversely phylogenetic group, including about 50 genera, mostly from Proteobacteria. Many denitrifiers are N-fixers (e.g., Rhizobiaceae and *Bradyrhizobium*) or nitrifiers (e.g., *Nitrosospira*), and they also can regulate the N cycle process under certain environment [21]. In general, denitrification is one of the key process of the N biogeochemical cycle and is a determinant of N level in soil especially in arid or semi-arid regions. Because all the steps of soil denitrification are driven by different denitrifying bacteria, the information of diversity, composition, and structure of the denitrifying community as well as their dynamics is needed for further understanding the process and the mechanism of the soil N cycle. Meanwhile, denitrifying community may be used as an indicator for ecological restoration and soil quality assessment in degraded ecosystems, because the recovery of soil denitrifying communities also implies the improvement of the N transformation rate, including fixation and nitrification.

In our recent study, we compared the denitrifying microbial communities among six different sand-fixation forests and found that forest type slightly influenced the dominant taxa in soil-denitrifying communities [22]. In this study, we selected a chronosequence native shrub (*C. microphylla*) plantation to study the temporal variations of denitrifying communities by clone library and quantitative polymerase chain reaction (qPCR) techniques. The aims of this study were (1) to study the recovery process of denitrifying community from secondary barren land (shifting sand dune) by planting native shrub plantation and (2) to determine the response of dominant taxa to plantation development. These communities at the early stage of revegetation on shifting sand dunes are assumed to differ from those in the developed substrates.

2. Materials and Methods

2.1. Study Location and Site Description

This study was conducted at the Wulanaodu Desertification Control Station (43°02′ N, 119°39′ E; altitude: 480 m) under the Chinese Academy of Sciences. This station is located in the western Horqin Sandy Land in north China. The geomorphology is characterized by the alternative distribution of shifting sand dunes, fixed/semi-fixed sand dunes, and interdunal lowlands. This region is under the temperate continental semi-arid monsoon climate zone. The annual mean temperature, precipitation, and pan evaporation are 6.3 °C, 340.5 mm, and 2500 mm. The soils are classified as cambic arenosols [23]. The typical plant species included: *Agropyron cristatum*, *Aneurolepidium chinense*, *Astragalus adsurgens*,

Stipa grandis, and *Lespedeza davurica*. However, the original vegetation has been severely destroyed by long-term heavy grazing and overcutting over the past decades. At present, the sandy land vegetation is generally dominated by psammophytes, shrubs, and semishrubs, including *Agriophyllum squarrosum*, *Salsola collina*, *Aristida adscensionis*, *Pennisetum flaecidum*, *C. microphylla*, *Atraphaxis manshurica*, and *Artemisia frigida*.

A large area of *C. microphylla* was planted around the Wulanaodu Region in the 1980s with the aid of sand-protecting barriers to fix shifting or semi-shifting sand dunes and improve the local eco-environment. The experimental site was enclosed after seeding. The sand dunes can be fixed at 3–5 years after planting, and the stabilized shrubby grass vegetation gradually formed as sand binders. This phenomenon improved the soil properties and colonized short grasses and forbs.

2.2. Experimental Design and Soil Sampling

Representative *C. microphylla* sand-fixation forests (9 and 19 years old, designated as CM-9 and CM-19, respectively), adjacent non-vegetated shifting sand dunes (0-year, SSD), and a natural *C. microphylla* forest (approximately 50-year, NC) were selected as the experimental sites in September 2016. The morphological traits of the different sites are provided in Table 1. Three 30 m × 30 m plots were set up in each site, and in each plot 15 subsamples at 0–10 cm soil depth were randomly collected using a plastic shovel at 50 cm away from the center of different plant clumps and mixed into one sample. Half of each sample was air-dried and stored at room temperature, and the other half was immediately frozen at −80 °C.

Table 1. Morphological traits, soil pH, electrical conductivity (EC), and nutrients of *C. microphylla* sand-fixation forest.

Items	SSD	CM-9	CM-19	NC	ANOVA in Response to Age	
					$F_{regression}$	p
Vegetation coverage (%)	<5	55	70	75	–	–
Crown diameter (cm × cm)	–	70 × 75	80 × 85	140 × 155	–	–
Mean height (cm)	–	81.21	90.52	130.21	–	–
Shoot number (N)	–	18.55	19.62	40.54	–	–
pH	6.70 ± 0.05	6.75 ± 0.05	6.63 ± 0.02	6.89 ± 0.07	9.196	0.013
Electrical conductivity ($\mu s \cdot cm^{-1}$)	23.90 ± 4.36	40.78 ± 10.0	40.10 ± 3.11	58.52 ± 2.06	34.247	<0.001
Organic matter ($g \cdot kg^{-1}$)	0.05 ± 0.002	0.26 ± 0.015	0.29 ± 0.08	0.74 ± 0.034	24.109	0.001
Total N ($g \cdot kg^{-1}$)	0.08 ± 0.004	0.62 ± 0.004	0.71 ± 0.005	0.79 ± 0.006	12.243	0.006
Total P ($g \cdot kg^{-1}$)	0.32 ± 0.021	0.59 ± 0.020	0.64 ± 0.103	0.99 ± 0.198	29.767	<0.001
Total K (%)	1.46 ± 0.05	1.49 ± 0.28	1.66 ± 0.11	2.22 ± 0.47	16.614	0.002
Available N ($mg \cdot kg^{-1}$)	1.41 ± 0.22	3.49 ± 0.34	2.80 ± 0.39	1.86 ± 0.57	5.346	0.030
Available P ($mg \cdot kg^{-1}$)	5.09 ± 0.93	6.83 ± 2.12	7.30 ± 0.88	9.01 ± 1.21	12.165	0.006
Available K ($mg \cdot kg^{-1}$)	425.3 ± 2.98	444.5 ± 2.99	447.9 ± 5.24	459.6 ± 4.50	30.817	<0.001

Values are means + SD (n = 3). The response of the content of soil available N to age was evaluated by quadratic regression model, and those of the other indicators were evaluated by linear regression model. SSD: shifting sand dune; CM-9 and CM-19: 9-yr and 19-yr *C. microphylla* forests; NC: natural *C. microphylla* forest.

2.3. Soil Property Analysis

Soil pH and electrical conductivity (EC) were measured in soil–water suspensions at 1:2.5 and 1:5 soil–water ratios, respectively. A portion of the air-dried and sieved samples was ground and passed through a 0.25 mm screen for the soil organic matter (SOM) and total N (TN) analyses. SOM was measured using the $K_2Cr_2O_7$–H_2SO_4 oxidation method described by Nelson and Sommers [24], and TN was determined via an automatic Kjeldahl apparatus. Soil available N (Av. N) was extracted using 1 M KCl solution, and determined via an automated discrete analyzer (CleverChem 380, DeChem-Tech, Germany). Soil total P (TP) and available P (Av. P) were determined with the Olsen and Dean method [25]. Soil total K (TK) and available K (Av. K) were measured via the method of atomic absorption spectroscopy [25].

2.4. Clone Library Construction

Genomic DNA was extracted and purified separately from each soil sample (0.7 g wet soil) with three replicates by using the Soil DNA Quick Extraction Kit (Bioteke, China). The purified DNA was amplified with forward/reverse primer pairs *nirK*-F1aCu/*nirK*-R3Cu, *nosZ*-1F/*nosZ*-2R, and *nirS*-cd3aF/*nirS*-R3cd, which respectively targeted the *nirK*, *nosZ*, and *nirS* denitrification genes [26,27]. The three gene clone libraries were made from 0-, 9-, and 19-year *C. microphylla* forest sites and the NC site. Qualified clones were commercially sequenced according to the standard protocols. The obtained raw nucleotide sequences of the three genes were aligned and trimmed using the BioEdit software [28]. Discrepancies during alignment were manually verified and then compared against the genes in GenBank by using BLASTN on the NCBI's homepage and clustered into operational taxonomic units (OTUs) with a cutoff of 97% similarity [29]. A phylogenetic tree was constructed using the representative OTUs by using the neighbor-joining method on the MEGA 4.0 software [30].

2.5. Quantifications of nosZ, nirK, and nirS Genes

The abundance of *nosZ*, *nirK*, and *nirS* genes was determined by using a real-time qPCR System (StepOne^{TM}, Applied Biosystems, Foster City, CA, USA) in a 20 µL reaction mixture containing the following: 1.0 µL of each primer for *nosZ*, *nirK*, or *nirS*, 10 µL of Gotaq qPCR Master Mix (Promega, France), 1.0 µL of DNA template, 2.5 µL of bovine serum albumin (BSA), and nuclease-free water. qPCRs of the three genes were performed with the above-mentioned primers [31]. Two independent real-time PCR assays were conducted for the three genes and each soil replicate [32].

2.6. Data Analysis

The responses of these indicators to forest development were analyzed by using the linear or quadratic regression model. All statistical analyses were performed using the SPSS 13.0 software, and a difference at the $p < 0.05$ level was considered statistically significant. OTU richness analysis was conducted using the Mothur 1.21.1 software The alpha diversity indexes, including the Shannon–Wiener index, Simpson index, abundance-based coverage estimator (ACE), and Chao's species richness estimator (Chao), were calculated via the Mothur software [33]. Canonical correspondence analysis (CCA) was performed using CANOCO 4.5 to identify which soil factor most significantly affected the variations in the soil dominant denitrifiers.

The accession numbers for *nosZ* were KX695575-KX695638, KX695438-KX695524, KX695296-KX695374, KX695375-KX695437, and KX695639-KX695722. The accession numbers for *nirS* were KU309672-KU309708, KX581498-KX581585, KU309709- KU309734, KU310213-KU310263, and KU309769-KU309798. The accession numbers for *nirK* were KU310122-KU310159, KX555006-KX555070, KU310007-KU310042, KU310213-KU310263, and KU310264- KU310302.

3. Results

3.1. Soil Properties

All parameters in vegetation-cover soils were significantly higher than those in SSD, especially in SOM and TN concentrations. Significantly linear regression relationships were detected between these parameters and plantation age (Table 1, $p < 0.05$), and all the parameters displayed an increasing trend with forest age.

3.2. Diversities of nirK, nirS, and nosZ Gene Libraries

A total of 425 *nirK*, 419 *nirS*, and 417 *nosZ*, clones were obtained in this study. Approximately 94–125 clones for each gene were obtained from the SSD, CM-9, CM-19, and NC samples (Table 2). Among the three genes, *nirK* had the highest OTU number and diversity indexes (ACE, Chao, Shannon–Weiner, and Simpson). This finding indicates that the *nirK* bacteria had a richer species diversity than the two other genes in the sandy soil. Moreover, the OTU numbers and diversity indexes of most samples from vegetation-covered sites

were higher than those from SSD. The OTU number of the three gene library increased with forest age. In the NC site, the diversity of the denitrifying community was lower than that of the plantation sites, except for the *nirK* gene.

Table 2. Diversity indices of different gene clone libraries.

	Site	Sequencing Results		Diversity Estimates				
		Total Sequences	Total OTUs	ACE	Chao	Shannon	Simpson	Coverage
nosZ	SSD	109	15	17.33	16.90	2.15	0.17	0.96
	CM-9	105	15	48.94	32.24	0.87	0.69	0.89
	CM-19	108	19	30.28	31.09	1.78	0.31	0.90
	NC	95	8	11.32	7.61	0.65	0.68	0.97
nirK	SSD	102	25	50.26	32.00	2.48	0.12	0.90
	CM-9	109	29	48.43	44.75	3.26	0.04	0.88
	CM-19	116	61	100.67	111.67	3.40	0.07	0.82
	NC	98	61	151.99	139.09	3.52	0.06	0.86
nirS	SSD	102	9	16.64	14.00	0.70	0.73	0.95
	CM-9	125	19	35.13	32.75	1.29	0.54	0.91
	CM-19	94	24	164.24	48.00	2.09	0.25	0.83
	NC	98	13	59.93	27.00	1.42	0.36	0.92

SSD: shifting sand dune; CM-9 and CM-19: 9-yr and 19-year *C. microphylla* forests; NC: natural *C. microphylla* forest.

3.3. Abundance of the nirK, nirS, and nosZ Genes

qPCR assays were performed to determine the copies of the three genes in different soil samples. The number of bacterial *nosZ* gene ranged from 1.01×10^5 to 7.05×10^6 copies/g dry soil, whereas that of the *nirK* and *nirS* genes ranged from 1.27×10^5 to 5.65×10^7 copies/g dry soil and from 5.23×10^4 to 9.14×10^7 copies/g dry soil, respectively (Figure 1). The lowest and highest values of gene copies were observed in SSD and NC, respectively. Linear regression relationships were observed between the log-transformed gene abundance and forest age (Figure 1, $p < 0.05$). Overall, the copies of the three genes all consistently increased with plantation age. This trend is similar to the variations in soil nutrients with forest development.

3.4. Detection of Denitrifying Bacterial Taxa

The phylogenetic trees of the representative OTUs of the three genes were obtained by using the neighbor-joining method (Figures 2–4). The obtained *nosZ*, *nirK*, and *nirS* gene clones were spread throughout the respective trees. According to the phylogenetic trees, the *nirK* library can be divided into six clusters: *Rhizobium*, *Achromobacter*, *Mesorhizobium*, *Rhodopseudomonas*, *Azospirillum*, and *Nitrosospira*; the *nirS* library can be divided into five clusters: *Cupriavidus*, *Azospirillum*, *Rubrivivax*, *Ralstonia*, and *Achromobacter*, and the *nosZ* library can be classified into four clusters: *Mesorhizobium*, *Chelatococcus*, *Achromobacter*, and *Pseudomonas*.

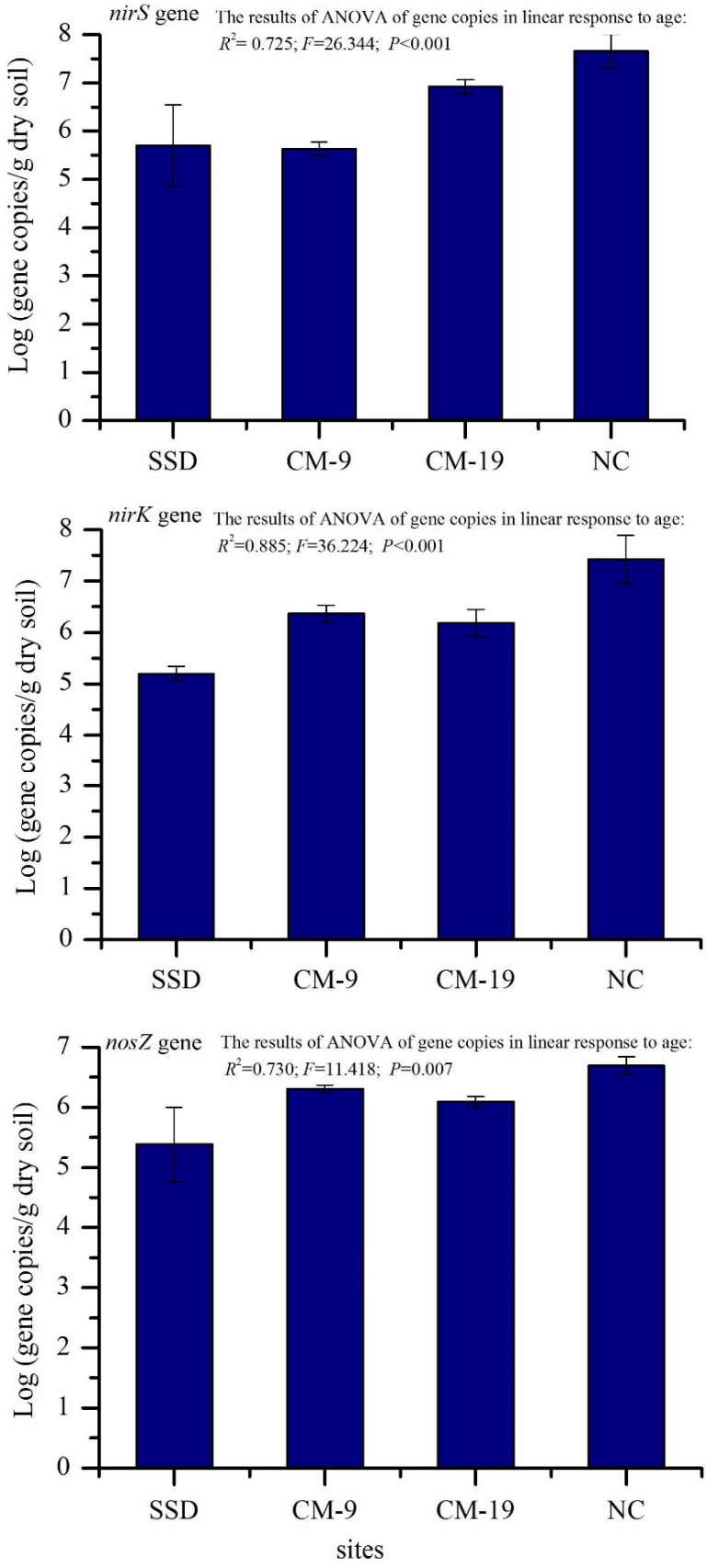

Figure 1. Quantifications of denitrifying genes and the regressions between the logarithm of the gene copies and forest age. SSD: shifting sand dune; CM-9: 9-year *C. microphylla* forest; CM-19: 19-year *C. microphylla* forest; NC: natural *C. microphylla* forest.

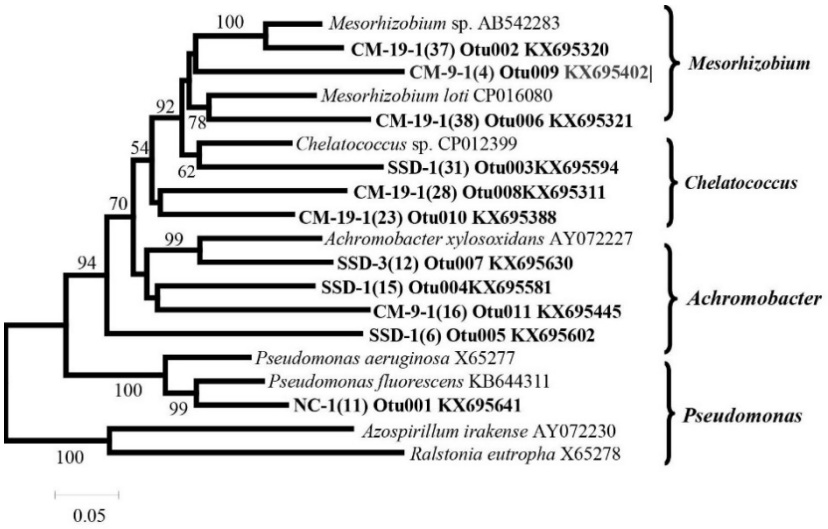

Figure 2. Phylogenetic distribution of 11 representative OTUs of the *nosZ* gene. Tree was constructed by the sequences obtained from sand dune sampling sites and additional their closely matched sequences from GenBank.

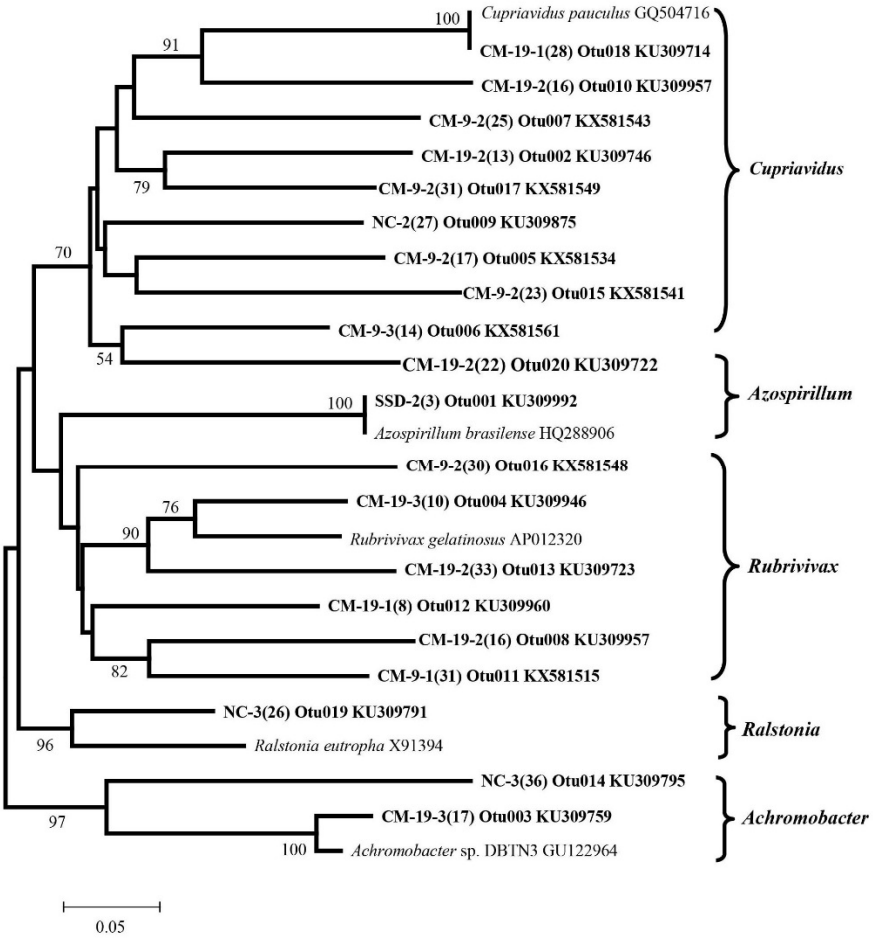

Figure 3. Phylogenetic distribution of 24 representative OTUs of the *nirS* gene. Tree was constructed by the sequences obtained from sand dune sampling sites and additional their closely matched sequences from GenBank.

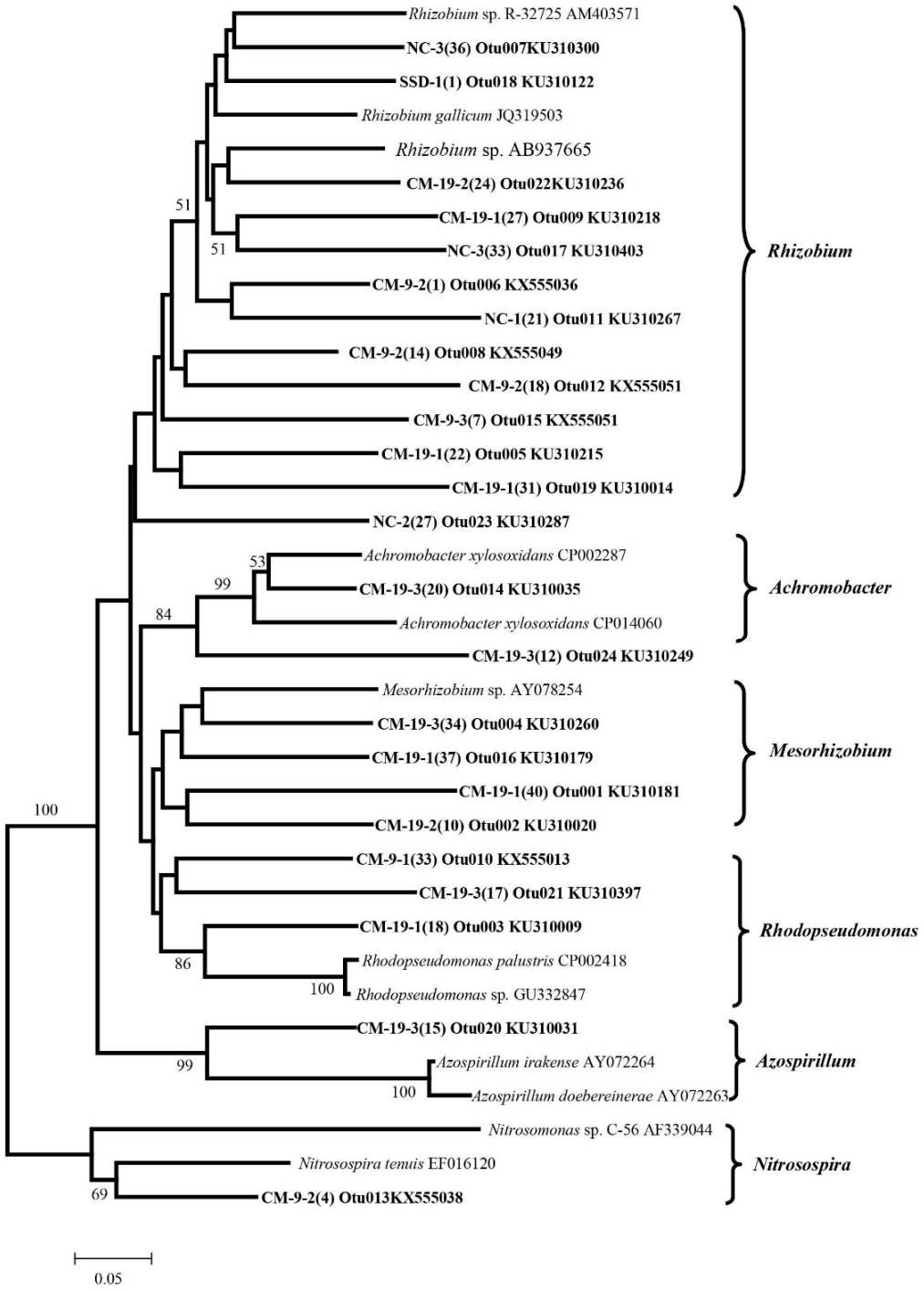

Figure 4. Phylogenetic distribution of 24 representative OTUs of the *nirK* gene. Tree was constructed by the sequences obtained from sand dune sampling sites and additional their closely matched sequences from GenBank.

The *nosZ* bacteria displayed a low diversity in sandy land. *Pseudomonas* was the dominant group of *nosZ* bacteria and the relative abundance in SSD, CM-9, CM-19, and NC were 56.91%, 94.60%, 92.00%, and 100%, respectively (Figure 5). Another dominant group in SSD was *Mesorhizobium*, with a relative abundance of 36.66%. While the relative abundance of *Mesorhizobium* in the plantation samples was all less than 5.48%. In the NC site, only *Pseudomonas* was observed, and the other *nosZ* taxa were absent. Several sequences related to *Achromobacter* in CM and *Chelatococcus* in SSD were also observed. Revegetation on shifting sand-dune significantly increased *nosZ*-carrying *Pseudomonas*

and significantly decreased *Mesorhizobium*. The relative abundance of *Pseudomonas* in CM-9 was similar to that in NC. Six taxa were found in the *nirK* bacterial community, of which *Mesorhizobium* and *Rhizobium* were the dominant groups with relative abundance of 58.62%–87.13% and 9.9%–18.75%, respectively. *Rhodopseudomonas*, *Nitrosospira*, *Achromobacter*, and *Azospirillum* were absent in the SSD sample, and their relative abundance was fairly low in the vegetation-covered samples. Similar to *nosZ*, revegetation on shifting sand-dune induced decreases in the relative abundance of the *Mesorhizobium*, with 86.29%, 62.04%, 58.45%, and 63.38% in SSD, CM-9, CM-19, and NC, respectively. While *Rhizobium* increased in the vegetation-covered samples, and the relative abundance were 12.73%, 23.91%, 15.50%, and 20.99%, respectively. *Rhodopseudomonas* was not detected in SSD, and its abundance was similar to that of *Rhizobium* in vegetation-covered samples. *Azospirillum* was the dominant *nirS* bacteria in all the samples, particularly in SSD, with a relative abundance of 50.85–91.62%. Similarly, *Cupriavidus* in CMs and *Achromobacter* displayed high relative abundance. *Ralstonia* had the lowest relative abundance and was detected only in the NC sample.

Overall, vegetation-covered soils tended to have more diverse denitrifier, and revegetation on shifting sand dunes varied the relative abundance of dominant taxa. Only *Mesorhizobium* carrying the *nirK* and *nosZ* genes decreased after revegetation, while the other groups including *Pseudomonas*, *Rhizobium*, *Rhodopseudomonas*, *Azospirillum*, *Cupriavidus*, and *Achromobacter* showed increasing tendencies. The relative abundance of *Rhizobium*, *Pseudomonas*, *Rhodopseudomonas* and *Mesorhizobium* in CM-9 was already very close to that in NC.

3.5. Relationship between the Composition of the Denitrifying Community and Soil Properties

CCA was carried out to examine the correlation between the dominant compositions of the *nosZ*, *nirK*, and *nirS* denitrifying communities and the selected variables of the soil samples. The results showed that CCA axis 1 and CCA axis 2 could explain 93.0 %, 86.9%, and 83.8% of the total variations in the structures of soil *nosZ*, *nirK*, and *nirS* communities, respectively (Figure 6). Soil pH, TN, and Av. N were most close to CCA axis 1, and correlated to the decrease in *Mesorhizobium* and increases in *Rhizobium*, *Rhodopseudomonas*, and *Achromobacter* in the *nirK* bacterial community. For *nirS*, soil pH and TN were two important variables determining the relative abundance of the *nirS*-carried genera. In the *nosZ* bacterial community, *Pseudomonas* was affected by all selected soil properties. On the whole, the relative abundance of the dominant *nirK*-, *nirS*-, and *nosZ*-carried taxa at the genera level were all affected by soil pH, TN, and Av. N.

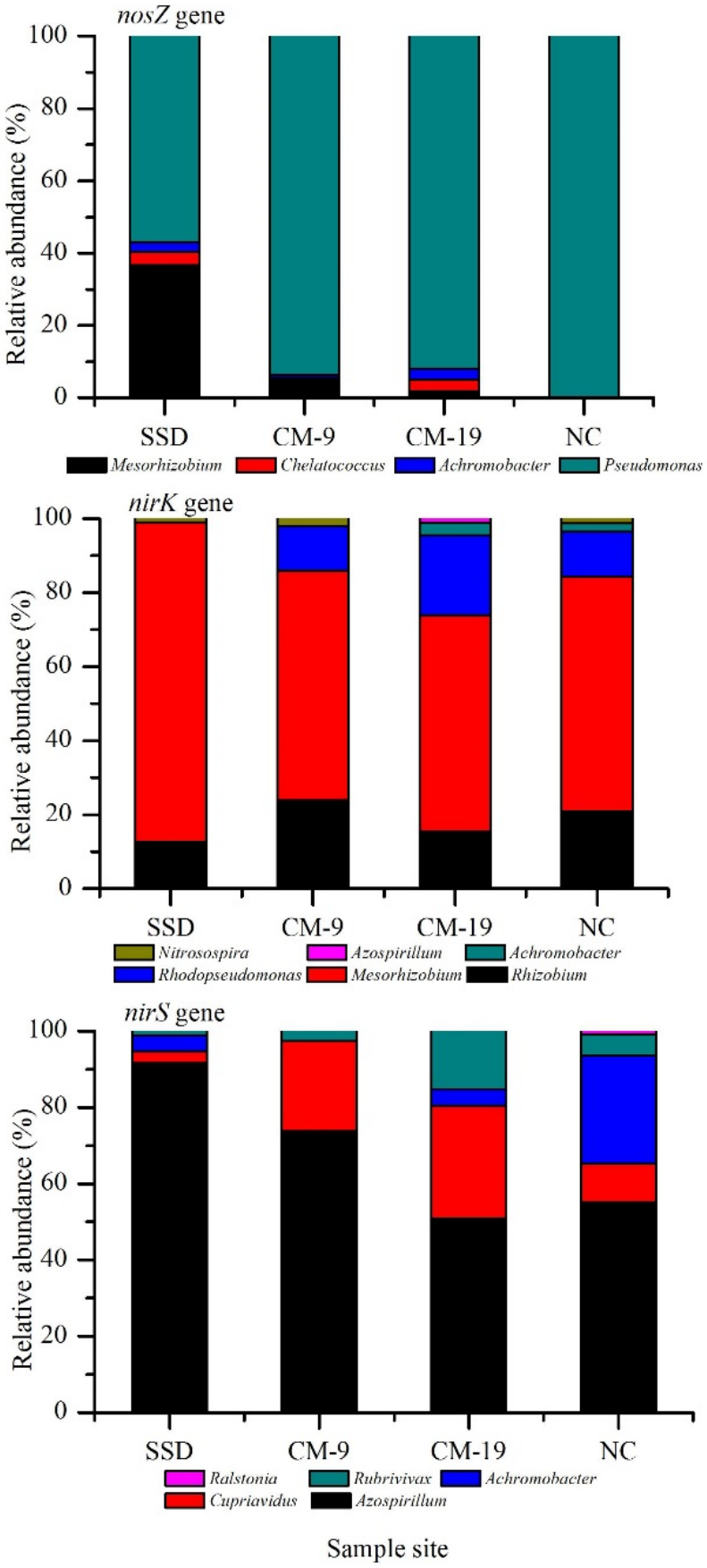

Figure 5. Relative abundance of different denitrifying genes. SSD: shifting sand dune; CM-9: 9-yr *C. microphylla* forest; CM-19: 19-yr *C. microphylla* forest; NC: natural *C. microphylla* forest.

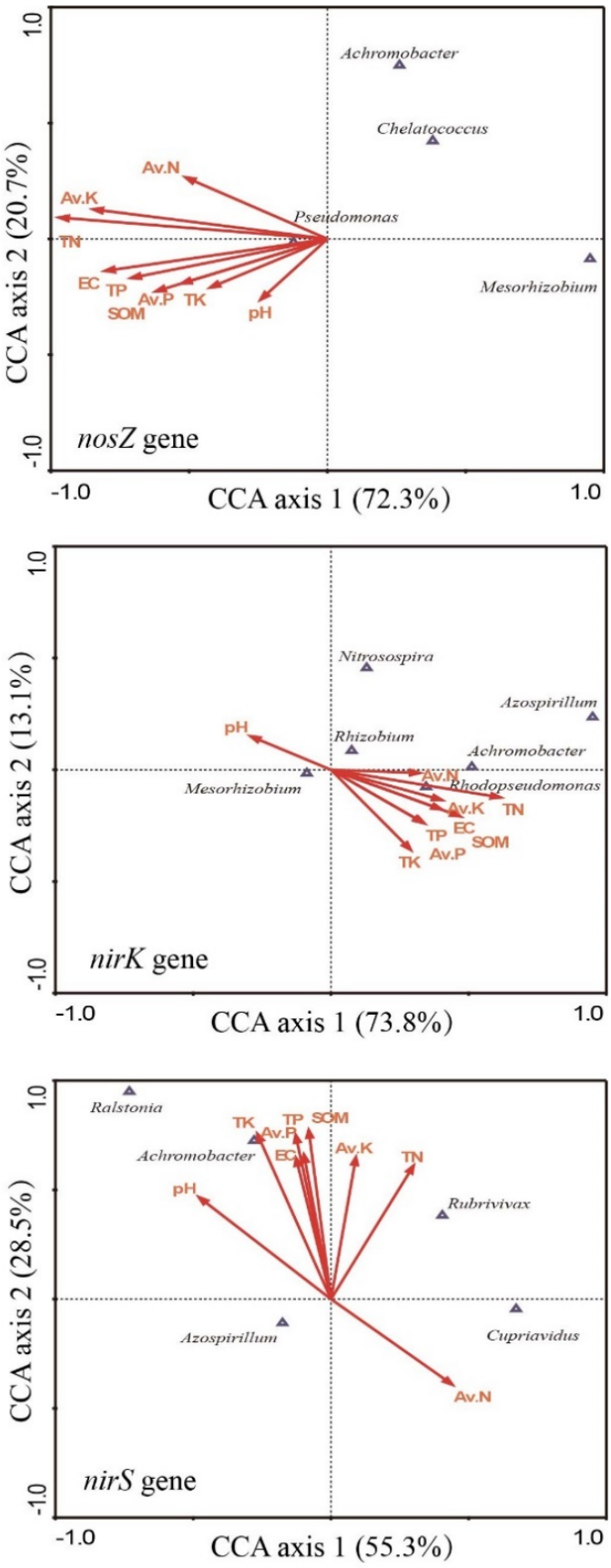

Figure 6. Canonical correspondence analysis (CCA) of dominant genera of *nosZ*, *nirK*, and *nirS* communities and soil properties. SOM: soil organic matter; TP: total P; Av. P: available P; TN: Total N; Av. N: available N; TK: total K; Av. K: available K; EC: electrical conductivity.

4. Discussion

4.1. Improvement of Sand-Fixation Forest on Soil Nutrients

The restoration of soil nutrients via the establishment of sand-fixation plantation is a complicated ecological process that is simultaneously affected by many factors. The establishment of the plantation on shifting sand dunes can decrease the surface albedo and increase soil surface roughness, thereby affecting water-heat balance and wind regimes [34]. Surface soil improvement primarily resulted from the remarkable function of plantation against wind erosion [10]. The established *C. microphylla* forest can significantly reduce wind speed and intercept and deposit fine soil particles [1,10]. This phenomenon facilitates the accumulation of soil fine particles and increases soil nutrients. The biomass and litter of *C. microphylla* all increased with the plantation development; meanwhile, the rapid growth and death of herbs in the plantation increased the input of C, N, and other nutrients. N release via litter decomposition and microbial nitrogen fixation resulted in an increase in soil N. The improvement in SOM was mainly dependent on the increased litter and dead root inputs, reduced soil erosion, and low mineralization rate of soil organic carbon [1].

4.2. Effect of Revegetation on Soil-Denitrifying Communities

Different processes of soil N cycling are closely related to some microbial genes, which simultaneously regulate N cycles [33,35]. The denitrification is driven by different soil denitrifying communities. *NirK*, *nirS* and *nosZ* genes have been used as the molecular markers of denitrifying microbes [32,36], and their abundance shifts can reflect the dynamics of the soil denitrification activity. Molecular-scale investigations revealed that the denitrifying bacteria in shifting sand dune had lower species richness than that in natural community and *C. microphylla* plantations. This finding indicates that soil desertification decreased the diversity of the denitrifying bacteria, whereas revegetation can restore denitrifying communities. However, the dominant groups of denitrifying communities did not shift with the vegetation degradation and the development of *C. microphylla* plantations. The result suggests that the core components of denitrifying communities are relatively stable and are slightly affected by the vegetation and soil amelioration. However, the amount of soil bacteria significantly varied with vegetation cover, land-use change, and plantation age [9,37–41]. In this study, the three gene copies linearly increased with plantation age (Figure 2) probably due to the gradual improvement of soil nutrients after revegetation. The improved soil environment facilitated the growth and propagation of soil denitrifiers and increased the size of the denitrifying community.

The *nirK* genes had relatively higher diversities than that of the other two genes, which is consistent with the results of Yoshida et al. [42]. Most of the *nosZ*, *nirK*, and *nirS* clones were found to be related to known sequences. Many clones were closely related to *nosZ*-carrying *Pseudomonas* bacteria, *nirK*-carrying *Mesorhizobium*, *Rhizobium*, and *Rhodopseudomonas* bacteria, and *nirS*-carrying *Azospirillum* and *Cupriavidus* bacteria, which have been detected in different environments [27,42,43]. These results indicate that the denitrifiers harboring previously uncharacterized *nosZ*, *nirK*, and *nirS* genes were abundant in soil. In line with this finding, the three gene sequences were all observed in arable soil, sea sediment, forests, and wetland soils [27,36,43,44].

Compared with previous studies [27,42], the present study observed considerably fewer sequences and OTUs of the three genes, and only a number of dominant taxa can be detected in the community. For example, all *nosZ* sequences in the natural community sample belonged to *Pseudomonas* and no other taxa were observed. This phenomenon indicates that the denitrifying microbial community in arid sandy soil was simple in composition, prominent in dominant species, and relatively stable in structure. Barren sandy land is unsuitable for microbial survival due to its extremely poor nutrient and the severely arid soil status [10]. Thus, fewer members of soil denitrifiers would be expected. Although an arid environment is unfavorable for microbial existence, species with strong capability to utilize limited resources for growth can survive [10,45]. This phenomenon could lead to the predominance of some microbial populations and/or the disappearance

of others because of interspecific competition [9]. *Pseudomonas* was abundant in sandy soil, which was consistent with the results of some studies [9,22]. This consistent finding is possibly because *Pseudomonas* can metabolize refractory detritus or xenobiotics as C, N, S, and P sources from sandy soil [46]. Additionally, the composition and the relative abundance of dominant taxa of the soil denitrifying community in 9-year-old plantation were already very close to that in the natural community sample; however, the plant diversity under the plantation was much lower than that in the natural community [5], which suggests the restorations of plant community and soil denitrifying community are asymmetric and the constituent recovery of the soil denitrifying community is faster than vegetation restoration, although a long time is still needed to restore the size of soil denitrifying community [47].

Soil environmental variation can significantly change the structure of a microbial community. Sun et al. [48] reported that the *nirK* bacterial community was influenced by nitrate, while pH and soil moisture were the key factors attributed to the shift of the *nosZ* bacterial community. Vegetation type indirectly affects soil denitrifying bacterial communities depending on the differences in microenvironment, quantity and quality of litter, root exudates, and the interaction with symbiotic bacteria [49]. Graham et al. [50] reported that pH was one of determinant of the all N-cycle. This study also suggested that the change in soil pH induced by revegetation is one of determinants for triggering the restoration of the denitrifying community. Meanwhile, the increased in TN and Av. N promoted the recovery of the soil denitrifying community, because N is limited nutrient for plant and microbial growth in arid and semi-arid soil. In addition, our results indicate that the soil properties influenced the *nirK* bacterial community more significantly than the other two communities, which is consistent with the report of Yoshida et al. [42].

Overall, the dominant taxa of *nosZ*, *nirK*, or *nirS* communities in forest samples remained almost unchanged compared with those in shifting sand dunes, suggesting the effects of revegetation on denitrifiers mainly depending on the quantitative change in dominant taxa. This phenomenon also indicated that the basic composition of the denitrifying community was mainly attributed to soil type or/and local climate rather than vegetation [38]. However, the quantities of denitrifying microbes in *C. microphylla* forests was much higher than that in shifting sand dunes. Moreover, the absolute copies of these genes linearly increased with the plantation age because improved soil nutrient and microenvironment could increase the quantity of soil microbes, including denitrifiers. Hence, the soil-denitrifying community structure was quantitatively influenced by the establishment of plantations.

5. Conclusions

The present study indicated that soil *nirK*, *nirS*, and *nosZ* denitrifying communities in shifting sand dunes can be restored by the establishment of native shrub plantation, and the recovery rate is faster than vegetation restoration. The copies of *nosZ*, *nirK*, and *nirS* genes all increased along forest development. Although revegetation had no significant influence on the dominant taxa of the denitrifying community, it facilitated the quantitative restoration because of the increase in soil nutrient. Soil pH, total N, and available N affected the structures of the denitrifying communities by altering the relative abundance of dominant genera.

Author Contributions: Conceptualization: C.C.; Methodology: C.C. and Y.Z.; investigation: Z.C.; Data curation: H.L., T.W. and Q.R.; Writing: C.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (grand No. 41877536, No. 41371505, and No. 41403063).

Data Availability Statement: Datasets can be obtained from the corresponding author.

Acknowledgments: The authors thank the Wulanaodu Desertification Control Station under the Chinese Academy of Sciences for field work.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Cao, C.Y.; Jiang, D.M.; Teng, X.H.; Jiang, Y.; Liang, W.J.; Cui, Z.B. Soil chemical and microbiological properties along a chronosequence of *Caragana microphylla* Lam. plantations in the Horqin Sandy Land of Northeast China. *Appl. Soil Ecol.* **2008**, *40*, 78–85. [CrossRef]
2. Liu, X.M.; Zhao, H.L. *Comprehensive Strategy for Eco-Environmental Control in Horqin Sand Land*; Gansu Science and Technology Publishing House: Lanzhou, China, 1993.
3. Jiang, F.Q.; Cao, C.Y.; Zeng, D.H. *Degradation and Restoration of Ecosystems on Horqin Sand Land*; Chinese Forestry Publishing House: Beijing, China, 2002.
4. Cao, C.Y.; Jiang, S.Y.; Zhang, Y.; Zhang, F.X.; Han, X.S. Spatial variability of soil nutrients and microbiological properties after the establishment of leguminous shrub *Caragana microphylla* Lam. plantation on sand dune in the Horqin Sandy Land of Northeast China. *Ecol. Eng.* **2011**, *37*, 1467–1475. [CrossRef]
5. Cao, C.Y.; Jiang, D.M.; Alamsa; Luo, Y.M.; Kou, Z.W. Ecological processes of vegetation restoration of *Caragana mirophylla* in the sand fixing area. *Chin. J. Appl. Ecol.* **2000**, *11*, 349–354.
6. Guan, P.T.; Zhang, X.K.; Yu, J.; Ma, N.N.; Liang, W.J. Variation of soil nematode community composition with increasing sand-fixation year of *Caragana microphylla*: Bioindication for desertification restoration. *Ecol. Eng.* **2018**, *81*, 93–101. [CrossRef]
7. Su, Y.Z.; Zhao, H.F. Soil properties and plant species in an age sequence of *Caragana microphylla* plantations in the Horqin Sandy Land, north China. *Ecol. Eng.* **2003**, *20*, 223–235. [CrossRef]
8. Wang, L.; Zhao, G.L.; Sun, Y.J.; Yang, Y.; Zhou, H.J. Effects of Thinning on microbial community structure in the organic horizon of Chinese pine plantations in Badaling, Beijing, China. *Forests* **2019**, *10*, 828. [CrossRef]
9. Cao, C.Y.; Zhang, Y.; Cui, Z.B.; Feng, S.W.; Wang, T.T.; Ren, Q. Soil bacterial community responses to revegetation of moving sand dune in semi-arid grassland. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 6217–6228. [CrossRef] [PubMed]
10. Jiang, D.M.; Cao, C.Y.; Zhang, Y.; Cui, Z.B.; Han, X.S. Plantations of native shrub species restore soil microbial diversity in the Horqin Sandy Land, northeastern China. *J. Arid Land* **2014**, *6*, 445–453. [CrossRef]
11. Zhang, Y.; Cao, C.Y.; Peng, M.; Xu, X.J.; Zhang, P.; Yu, Q.J.; Sun, T. Diversity of nitrogen-fixing, ammonia-oxidizing, and denitrifying bacteria in biological soil crusts of a revegetation area in Horqin Sandy Land, Northeast China. *Ecol. Eng.* **2014**, *41*, 71–79. [CrossRef]
12. Liu, L.C.; Liu, Y.B.; Hui, R.; Xie, M. Recovery of microbial community structure of biological soil crusts in successional stages of Shapotou desert revegetation, northwest China. *Soil Biol. Biochem.* **2017**, *107*, 125–128. [CrossRef]
13. Zhelezova, A.; Chernov, T.; Tkhakakhova, A.; Xenofontova, N.; Semenov, M.; Kutovaya, O. Prokaryotic community shifts during soil formation on sands in the tundra zone. *PLoS ONE* **2019**, *14*, e0206777. [CrossRef] [PubMed]
14. Zuo, X.A.; Zhao, H.L.; Zhao, X.Y.; Zhang, T.H.; Guo, Y.R.; Wang, S.K.; Drake, S. Spatial pattern and heterogeneity of soil properties in sand dunes under grazing and restoration in Horqin Sandy Land, Northern China. *Soil Tillage Res.* **2008**, *99*, 202–212. [CrossRef]
15. Che, R.; Qin, J.; Tahmasbian, I.; Wang, F.; Zhou, S.; Xu, Z.; Cui, X.Y. Litter amendment rather than phosphorus can dramatically change inorganic nitrogen pools in a degraded grassland soil by affecting nitrogen-cycling microbes. *Soil Biol. Biochem.* **2018**, *120*, 145–152. [CrossRef]
16. Zumft, W.G. Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 533–616. [CrossRef] [PubMed]
17. Philippot, L.; Hojberg, O. Dissimilatory nitrate reductases in bacteria. *Biochim. Et Biophys. Acta-Gene Struct. Expressopn* **1999**, *1446*, 1–23. [CrossRef]
18. Bru, D.; Sarr, A.; Philippot, L. Relative abundances of proteobacterial membrane-bound and periplasmic nitrate reductases in selected environments. *Appl. Environ. Microbiol.* **2007**, *73*, 5971–5974. [CrossRef] [PubMed]
19. Henry, S.; Baudoin, E.; Lopez-Gutierrez, J.C.; Martin-Laurent, F.; Brauman, A.; Philippot, L. Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR. *J. Microbiol. Methods* **2004**, *59*, 327–335. [CrossRef] [PubMed]
20. Horn, M.A.; Drake, H.L.; Schramm, A. Nitrous oxide reductase genes (*nosZ*) of denitrifying microbial populations in soil and the earthworm gut are phylogenetically similar. *Appl. Environ. Microbiol.* **2006**, *72*, 1019–1026. [CrossRef] [PubMed]
21. Sanchez, C.; Tortosa, G.; Granados, A.; Delgado, A.; Bedmar, E.J.; Delgado, M.J. Involvement of Bradyrhizobium japonicum denitrification in symbiotic nitrogen fixation by soybean plants subjected to flooding. *Soil Biol. Biochem.* **2011**, *43*, 212–217. [CrossRef]
22. Li, H.L.; Zhang, Y.; Wang, T.T.; Feng, S.W.; Ren, Q.; Cui, Z.B.; Cao, C.Y. Responses of soil denitrifying bacterial communities carrying *nirS*, *nirK*, and *nosZ* genes to revegetation of moving sand dunes. *Ecol. Indictors* **2019**, *107*, 105541. [CrossRef]
23. FAO. FAO/ IUSS Working Group WRB, World reference base for soil resources. In *World Soil Resources Reports103*; FAO: Rome, Italy, 2006.
24. Nelson, D.W.; Sommers, L.E. Total Carbon, Organic Carbon and Organic Matter, Part 2. In *Methods of Soil Analysis*, 2nd ed.; Page, A.L., Miller, R.H., Keeney, D.R., Eds.; American Society of Agronomy: Madison, WI, USA, 1982; Volume 9, pp. 539–577.
25. Institute of Soil Science, Chinese Academy of Sciences (ISSCAS). *Physical and Chemical Analysis Methods of Soils*; Shanghai Science Technology Press: Shanghai, China, 1978; pp. 7–59.
26. Rich, J.J.; Heichen, R.S.; Bottomley, P.J.; Cromack, K.; Myrold, D.D. Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. *Appl. Environ. Microbiol.* **2003**, *69*, 5974–5982. [CrossRef]

27. Throbäck, I.N.; Enwall, K.; Jarvis, A.; Hallin, S. Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiol. Ecol.* **2004**, *49*, 401–417. [CrossRef]
28. Tippmann, H.F. Analysis for free: Comparing programs for sequence analysis. *Brief. Bioinform.* **2004**, *5*, 82–87. [CrossRef]
29. Han, M.Q.; Li, Z.Y.; Zhang, F.L. The ammonia oxidizing and denitrifying prokaryotes associated with sponges from different sea areas. *Microb. Ecol.* **2013**, *66*, 427–436. [CrossRef] [PubMed]
30. Kumar, S.; Tamura, K.; Nei, M. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* **2004**, *5*, 150–163. [CrossRef]
31. Henry, S.; Bru, D.; Stres, B.; Hallet, S.; Philippot, L. Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG*, *nirK*, and *nosZ* genes in soils. *Appl. Environ. Microbiol.* **2006**, *72*, 5181–5189. [CrossRef]
32. Kandeler, E.; Deiglmayr, K.; Tscherko, D.; Bru, D.; Philippot, L. Abundance of *narG*, *nirS*, *nirK*, and *nosZ* genes of denitrifying bacteria during primary successions of a glacier foreland. *Appl. Environ. Microbiol.* **2006**, *72*, 5957–5962. [CrossRef] [PubMed]
33. Schloss, P.D.; Westcott, S.L.; Ryabin, T.; Hall, J.R.; Hartmann, M.; Hollister, E.B.; Lesniewski, R.A.; Oakley, B.B.; Parks, D.H.; Robinson, C.J. Introducing mothur: Open-source, platform independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **2009**, *75*, 7537–7541. [CrossRef] [PubMed]
34. Li, S.G.; Harazono, Y.; Zhao, H.L.; He, Z.Y.; Chang, X.L.; Zhao, X.Y.; Zhang, T.H.; Oikawa, T. Micrometeorological changes following establishment of artificially established *Artemisia* vegetation on desertified sandy land in the Horqin sandy land, China and their implication on regional environmental change. *J. Arid Environ.* **2002**, *52*, 101–119. [CrossRef]
35. Wang, H.L.; Deng, N.; Wu, D.Y.; Hu, S.; Kou, M. Long-term net transformation and quantitative molecular mechanisms of soil nitrogen during natural vegetation recovery of abandoned farmland on the Loess Plateau of China. *Sci. Total Environ.* **2017**, *607*, 152–159. [CrossRef] [PubMed]
36. Ligi, T.; Truu, M.; Truu, J.; Nolvak, H.; Kaasik, A.; Mitsch, W.J.; Mander, U. Effects of soil chemical characteristics and water regime on denitrification genes (*nirS*, *nirK*, and *nosZ*) abundances in a created riverine wetland complex. *Ecol. Eng.* **2014**, *42*, 47–55. [CrossRef]
37. Kara, O.; Bolat, I.; Cakiroglu, K.; Ozturk, M. Plant canopy effects on litter accumulation and soil microbial biomass in two temperate forests. *Biol. Fertil. Soils* **2008**, *45*, 193–198. [CrossRef]
38. Jangid, K.; Williams, M.A.; Franzluebbers, A.J.; Schmidt, T.M.; Coleman, D.C.; Whitman, W.B. Land-use history has a stronger impact on soil microbial community composition than aboveground vegetation and soil properties. *Soil Biol. Biochem.* **2011**, *43*, 2184–2193. [CrossRef]
39. Prescott, C.E.; Grayston, S.J. Tree species influence on microbial communities in litter and soil: Current knowledge and research needs. *For. Ecol. Manag.* **2013**, *309*, 19–27. [CrossRef]
40. Liu, J.L.; Dang, P.; Gao, Y.; Zhu, H.L.; Zhu, H.N.; Zhao, F.; Zhao, Z. Effects of tree species and soil properties on the composition and diversity of the soil bacterial community following afforestation. *For. Ecol. Manag.* **2018**, *427*, 342–349. [CrossRef]
41. Jurksiene, G.; Janusauskaite, D.; Baliuckas, V. Microbial community analysis of Nnative *Pinus sylvestris* L. and alien *Pinus mugo* L. on dune sands as determined by Ecoplates. *Forests* **2020**, *11*, 1202. [CrossRef]
42. Yoshida, M.; Ishii, S.; Otsuka, S.; Senoo, K. Temporal shifts in diversity and quantity of *nirS* and *nirK* in a rice paddy field soil. *Soil Biol. Biochem.* **2009**, *41*, 2044–2051. [CrossRef]
43. Katsuyama, C.; Kondo, N.; Suwa, Y.; Yamagishi, T.; Itoh, M.; Ohte, N.; Kimura, H.; Nagaosa, K.; Kato, K. Denitrification activity and relevant bacteria revealed by nitrite reductase gene fragments in soil of temperate mixed forest. *Microbes Environ.* **2008**, *23*, 337–345. [CrossRef] [PubMed]
44. Priemé, A.; Braker, G.; Tiedje, J.M. Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetland soils. *Appl. Environ. Microbiol.* **2002**, *68*, 1893–1900. [CrossRef] [PubMed]
45. Zhang, Y.; Cao, C.Y.; Han, X.S.; Jiang, S.Y. Soil nutrient and microbiological property recoveries via native shrub and semi-shrub plantations on moving sand dunes in Northeast China. *Ecol. Eng.* **2013**, *53*, 1–5. [CrossRef]
46. Molina, L.; Ramos, C.; Duque, E.; Ronchel, M.C.; Garcia, J.M.; Wyke, L.; Ramos, J.L. Survival of Pseudomonas putida KT2440 in soil and in the rhizosphere of plants under greenhouse and environmental conditions. *Soil Biol. Biochem.* **2000**, *32*, 315–321. [CrossRef]
47. Zhang, Y.; Cao, C.Y.; Cui, Z.B.; Qian, W.; Liang, C.P.; Wang, C.M. Soil bacterial community restoration along a chronosequence of sand-fixing plantations on moving sand dunes in the Horqin sandy land in northeast China. *J. Arid Environ.* **2019**, *165*, 81–87. [CrossRef]
48. Sun, Y.F.; Shen, J.P.; Zhang, C.J.; Zhang, L.M.; Bai, W.M.; Fang, Y.; He, J.Z. Responses of soil microbial community to nitrogen fertilizer and precipitation regimes in a semi-arid steppe. *J. Soils Sediments* **2018**, *18*, 762–774. [CrossRef]
49. Urbanova, M.; Snajdr, J.; Baldrian, P. Composition of fungal and bacterial communities in forest litter and soil is largely determined by dominant trees. *Soil Biol. Biochem.* **2015**, *84*, 53–64. [CrossRef]
50. Graham, E.B.; Wieder, W.R.; Leff, J.W.; Weintraub, S.R.; Townsend, A.R.; Cleveland, C.C.; Philippot, L.; Nemergut, D.R. Do we need to understand microbial communities to predict ecosystem function? A comparison of statistical models of nitrogen cycling processes. *Soil Biol. Biochem.* **2014**, *68*, 279–282. [CrossRef]