Article

Molecular Diversity of Arbuscular Mycorrhizal Fungi Associated with the Rhizosphere of *Vachellia seyal* Del. from Selected Saline Soils in Senegal

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Abstract: Drought and salinity are major environmental constraints that severely limit crop production, particularly in arid and semi-arid zones. We investigated the genetic diversity of arbuscular mycorrhizal fungi isolated from the rhizosphere of *Vachellia seyal* in three different soils from Senegal with varying levels of salinity. Soil and root samples were collected from under *V. seyal* and in the vicinity of the trees. After DNA extraction, nested PCR, and sequencing of the large subunit region of the rRNA gene, different phylotypes from rhizospheric soils, roots, and spores were compared by phylogeny in order to investigate the role of salinity in arbuscular fungal diversity. This study revealed several unidentified arbuscular fungi and a particularly high host specificity in *V. seyal* roots. The vast majority of operational taxonomic units (OTUs) isolated in this study had no homologous sequences in the databases.

Keywords: arbuscular mycorrhizal fungi; *Vachellia seyal*; salinity; phylogeny

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1. Introduction

Soil salinity is a serious agro-ecological problem in West Africa. In Senegal, more than 6% of the land, known as "tanns", is affected by salinity, resulting in a loss of biodiversity and arable land that needs to be restored to its initial state. There is increasing evidence that trees can play a key role in the rehabilitation or restoration of ecosystems, and numerous studies have demonstrated that the presence of trees provides a range of ecological benefits by increasing soil organic matter content, enhancing biodiversity conservation, and improving soil microbial activity and nutrient cycling rates. *Vachellia seyal* Del. (syn. *Acacia seyal*) is a moderately salt-tolerant tree species growing in saline and non-saline soils in Senegal. It is a multipurpose tree belonging to the former genus Acacia, one of the largest genera of leguminous trees and shrubs, with a wide distribution throughout the world. Due to its high plasticity, *V. seyal* is adapted to harsh environments and plays a key role in ecosystem functioning. A highly gregarious Sahelian tree species, it grows well on

periodically flooded heavy clays and plays a major role in fuel and fodder production in countries at the southern edge of the Sahara Desert [1]. Gum (gum talha) is collected from the tree, some of which is traded internationally [2]. In addition, *V. seyal* is a nitrogen-fixing tree associated with rhizobia [3], making it ideally suited for silvo-pastoral systems [4] and for soil fertility restoration. Leaves and shoots provide precious fodder, as do the fruits. Branches are used for fencing and are often cut by pastoralists when herbaceous fodder becomes scarce during the dry season. The wood is particularly valued for firewood and for charcoal production. *V. seyal* requires 250 to 1000 mm of annual rainfall and is adapted to clay soils, tolerating temporary flooding. *V. seyal* is also found on rocky soils, along water courses, on alluvial soils, and in shallow depressions. Despite the agro-ecological importance of *V. seyal*, available data on its interactions with soil microbiota are scarce in West Africa [5,6]. These soil microbiota need to be identified in order to better assess their potential role in the restoration of damaged ecosystems.

Plant–microbe associations have been identified as an important strategy for ensuring plant growth and survival, and the effect is more pronounced under stressful conditions, such as saline soils. Soil microorganisms are known to play a key role in ecosystems, mediating many ecological processes essential for ecosystem functioning, including nutrient acquisition, nitrogen and carbon cycling, soil formation, decomposition processes, and the regulation and maintenance of plant biodiversity. Plants adapted to harsh environments, such as saline soils, and their associated soil microorganisms in these habitats make both partners highly competitive and adaptive. Arbuscular mycorrhizal fungi (AMF) are among these soil-dwelling microorganisms that form mutualistic relationships with over 80% of all vascular plants, affecting plant fitness and competitive interactions. They are known to help host plants with phosphorus uptake [7] but can also provide other benefits, including protection against pathogens and assistance with the uptake of water and other nutrients, such as nitrogen [8]. AMF hyphae also play a role in the formation and structural stability of soil aggregates and contribute to the composition of plant community structures. In return, AMF receive photosynthetic products from the host plant. Although this is an important relationship, information on the diversity of colonizing AMF species is still lacking for *V. seyal* tree species. This knowledge gap is surprising given the functional importance of the AMF communities in regulating ecosystem processes. The growing evidence of improved plant survival and growth enhancement through specific mycorrhizal inoculation requires knowledge of the diversity of colonizing AMF species in specific natural ecosystems of the plant species of interest [9]. As a result, better ways of utilizing these species could be considered, such as tree plot restoration strategies and bioremediation.

Traditionally, AMF have been identified by morphological characteristics, especially those of the spores [10]. This process is both laborious and problematic due to the limited variation in spore morphotypes between species and the propensity of some species to produce spores with different morphotypes [10]. With the development of molecular tools, it is now possible to study the composition of the AMF community in an environment [11]. We have used a molecular approach to generate baseline data on the diversity of indigenous AM fungal communities living in the rhizosphere of *V. seyal* in three semi-arid soils differing in salt stress.

2. Materials and Methods

2.1. Study Sites

The study sites (in the west–central region of Senegal known as the "Peanut Basin") were mainly located in the low valleys of the Sine and Saloum Rivers (Figure 1), specifically at Ngane (14°11′ N; 16°05′ W) and Ndiafate (14°04′ N; 16°10′ W), representing areas of moderate and high salinity, respectively, and Bambey (14°42′ N; 16°28′ W) as a low-salinity

zone (Tables 1 and S1). Annual rainfall varied between 500 and 1000 mm, with a dry season from November to June. Average temperatures ranged from 20 °C to 40 °C. The soils were undisturbed in Ngane and Ndiafate, whereas in Bambey, they were disturbed and cultivated.

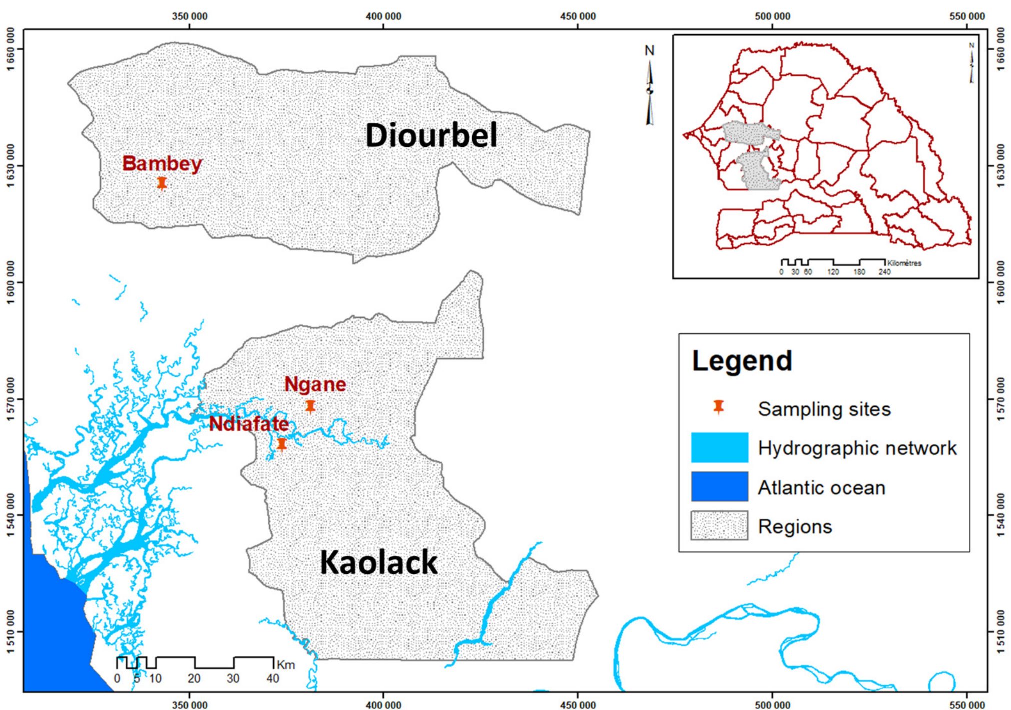

Figure 1. Location of the sampling sites in Senegal.

Table 1. Physico-chemical properties of soil samples from Ngane, Ndiafate, and Bambey.

	Ngane	Ndiafate	Bambey
EC (mmho/cm)	28.3	100.8	0.49
pH water	5.6	3.6	5.5
pH KCl	4.9	4.2	5.2
CEC	2.42	4.26	1.78
C/N	11	20	10
Assimilable Olsen P_2O_2 (ppm)	13.7	16.0	11.5
Clay (%)	4.95	5.67	3.86
Coarse silt 20–50 (%)	7.88	8.57	5.52
Coarse sand > 200 (%)	32.1	21.8	24.9
Texture	sandy	sandy	sandy

EC: electrical conductivity, CEC: cation exchange capacity, C/N: carbon-to-nitrogen ratio, P_2O_2: available phosphorus.

2.2. Soil and Root Sampling

Soil and root samples were collected from under 20 *V. seyal* plants and in the vicinity of the trees. For each plant, rhizosphere soil samples were taken from the root zone (0–20 cm depth). Thirty samples were randomly collected from each site. Approximately 100 g samples of rhizosphere soil and roots were collected and stored in polyethylene bags at 5 °C prior to processing.

The collected samples were pooled, mixed, and analyzed after mixing all subsamples from one site (composite samples). As spores can be heterogeneously distributed [12,13], composite samples provided a more accurate survey than individual samples.

2.3. AMF Trap Cultures and Spore Extraction

Pot cultures were established to trap indigenous spores from *V. seyal* roots and rhizosphere harvested from each site. Pots filled with 2 kg of autoclaved sand (sterilized in

two cycles at 120 °C, each lasting one hour) as a substrate were inoculated with *V. seyal* as host plants. Twenty grams of root fragments (collected from the vicinity of *V. seyal* at the three study sites) were added as starter inoculum. Five replicates were performed per site. Pots were regularly watered up to field capacity and fertilized twice a month with a Long Ashton solution [14] for a period of five months. Plants were maintained in daylight and temperature conditions with an average day/night temperature of 28 °C, 75% relative humidity, and a photoperiod of about 14 h. After harvest, AMF spores were extracted from the substrate by wet-sieving and decanting according to the method of Gerdemann and Nicolson [15].

2.4. DNA Extraction from Soils

Soil DNA was extracted using the method of Martin-Laurent et al. [16] with minor modifications. DNA extractions were performed from the original composite soil from the three different sites. Each soil sample was oven-dried (70 °C for 1 h) and mixed in a porcelain mortar to homogenize it and break up soil clumps. Nucleic acids were extracted from four 250 mg aliquots of soil. A 1 mL solution containing 100 mM Tris-HCl pH 8, 100 mM ethylenediaminetetraacetic acid (EDTA) pH 8, 100 mM NaCl, 1% (wt/vol) polyvinylpyrrolidone, and 2% sodium dodecyl sulfate was added to 250 mg of soil in a 2 mL Eppendorf tube containing 0.2 g of 106 μm diameter glass beads and two 2 mm diameter glass beads. The samples were shaken in a bead-beater cell disruptor (Mixer Mill MM 301, Retsch, Haan, Germany) for 45 s at 1600 rpm. After incubation at 70 °C for 10 min, the samples were centrifuged at 14,000× *g* for 5 min. One volume of isopropanol (cooled to −20 °C) was added to the supernatant to precipitate the nucleic acids. After incubation at −20 °C for 15 min and centrifugation at 13,000 rpm for 30 min, the nucleic acids were washed with 70% ethanol and purified with Sepharose 4B and polyvinyl polypyrrolidone (PVPP). Nucleic acids were electrophoresed on 1.4% agarose gels to check the quality and the fragment size of soil DNA.

2.5. DNA Extraction from Roots

Root DNA extractions were performed from the roots sampled in the rhizosphere of the three different sites. Root samples (20 mg) were placed in Eppendorf tubes (Eppendorf AG, Hamburg, Germany) to which some activated charcoal and a glass bead were added to facilitate milling. The tubes containing root fragments were cooled in liquid nitrogen and then milled in two 1 min phases at 30 pulses per second. After grinding, 500 μL of extraction buffer containing 20% (10 mM Tris-HCl, pH 8; 1 mM EDTA, pH 8) and 80% (10 mM Tris-HCl, pH 8; 0.1 mM EDTA, pH 8) of solution was added to each tube. The tubes were placed in a water bath at 100 °C for 10 min and then centrifuged at 11,000 rpm for 10 min at 4 °C. The supernatant was removed and used as template DNA for nested polymerase chain reaction (PCR).

2.6. DNA Extraction from Spores

DNA extractions from spores were performed using AMF trap cultures. Spores were placed in an Eppendorf tube with 0.5 mL of water before being crushed using a Pasteur pipette with a tip rounded by flame heating. The tubes were heated to 95 °C for about 5 min after the addition of 30 μL of Tris-EDTA. Aliquots (5 μL) of DNA were taken for nested PCR.

2.7. Nested PCR Amplification

DNA amplification was performed on DNA extracted from rhizosphere soils and roots sampled from the three sites, as well as from spores isolated after trap cultures. A

two-step nested PCR procedure [17] was used for specificity testing and amplification of DNA samples. The crude soil DNA suspension was used for the first set of amplifications.

The 25S LSU region of the nuclear rDNA was used as the PCR target region. An approximately 750 bp fragment of the large subunit ribosomal RNA gene (LSU rDNA) was amplified by PCR. The first PCR was performed using the two eukaryotic-specific primers LR1 [5′-GCA TAT CAA TAA GCG GAG GA-3′] [18] and NDL22 [5′-TGG TCC GTT GTT TCA AGA CG-3′] [18].

Reactions were carried out in a final volume of 25 μL containing 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg ml^{-1} BSA (Appligène, Strasbourg, France), 200 mM dNTP, 500 nM of each primer, 1U per 100 μL of Taq DNA polymerase (Appligene, France), and 5 μL DNA extract. Amplifications were performed in an MJ Research thermal cycler PTC-100 (Bio-Rad, Ile-de-France, France) programmed as follows: initial denaturation cycle at 95 °C for 3 min, followed by 30 cycles of denaturation at 93 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. The last cycle was followed by a final extension at 72 °C for 5 min. The second step was carried out using combinations of Glomales-specific primers and universal primers.

The primary PCR products (5 μL) were diluted 1000-fold prior to nested amplification. These dilutions were then used as templates for the second nested PCR step using the universal eukaryotic primer LR1 in combination with a fungal-specific primer FLR4 [5′-TAC GTC AAC ATC CTT AAC GAA-3′] [19]. Amplification conditions were the same as described above. Amplification products (3 μL) were separated by electrophoresis in TAE buffer containing 40 mM Tris (pH 7.8), 20 mM acetic acid, and 2 mM EDTA on a 1.4% agarose gel, stained with ethidium bromide (0.5 μg ml^{-1}) and photographed under UV light. The sizes of the nested PCR products were then analyzed prior to cloning.

2.8. Cloning and Sequencing

Nested PCR fragments were cloned directly into the pCR 2.1-TOPO vector system (Invitrogen Corporation, Carlsbad, CA, USA). Following the manufacturer's recommendations, the vectors were used to transform competent *Escherichia coli* cells using a TOPO TA cloning kit (Invitrogen, Villebon-sur-Yvette, France). Bacteria were transformed and plated on a selective medium following the manufacturer's instructions. Individual, transformed white colonies were selected from the bacterial colonies using a toothpick. A rapid colony screening procedure based on a PCR with the LR1 forward and FLR4 reverse primers was then used to select clones with inserts of the correct specificity and size. Thirty positive clones were screened and checked in 1.4% agarose gel. After quality control, reconfirmed cloning products containing plasmids were isolated and purified using the NucleoSpin® Plasmid protocol (Macherey-Nagel, Hoerdt, France) and then sequenced. Several batches consisting of twenty samples of plasmid DNA randomly taken from clones meeting these criteria were sequenced, using the Dye Chain Terminator method (MWG-Biotech, Ebersberg, Germany). Sequences were submitted to the EMBL database under accession numbers AM397810-AM397831 [20] and KX907020-KX907072.

2.9. Phylogenetic Analysis

Forward and reverse strands were assembled using the Sequencher 3.0 TM software (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were manually aligned using Se-Al Sequence Alignment Editor 3.0 [21]. DNA sequence analyses were performed and screened for possible chimeric origin, and similarity comparisons were performed using the BLAST software sequence similarity search tool [22] provided by GenBank. In addition, the sequences of thirty-three glomalean and one outgroup taxa (*Mortierella* sp.) were obtained from Genbank and BEG databases.

Sequences were aligned with other published glomalean sequences using the CLUSTALW program (Dublin, Ireland) [23] and manually corrected at some positions to optimize the homology of the nucleotide sites with Se-Al 2.0 software (University of Oxford, UK). Sequences of the PCR products were always analyzed without the primers. A phylogenetic analysis was carried out to estimate the phylogenetic relationships between the sequences obtained in this study and those identified as AMF from GenBank. Distance matrices were computed using the two-parameter model of Kimura [24] and using *Mortierella polycephala* as an outgroup. A molecular phylogenetic tree was constructed using PHYLOWIN [25] with the neighbor-joining method [26].

2.10. Diversity Indices Analysis

For this analysis, OTU abundances were calculated based on the sequencing data of arbuscular mycorrhizal fungi collected at each study site. The diversity indices (Shannon–Wiener, Simpson, and Pielou's evenness) were calculated to evaluate the richness, dominance, and evenness of the OTU distribution. These indices were then compared between sites to detect significant variations in AMF community structure, which may relate to environmental or ecological factors at each site.

The Shannon–Wiener index diversity H' [27] provides insight into both the richness and evenness of the AMF OTU distribution at each location and is calculated with the formula:

$$H' = -\Sigma\, p_n \ln(p_n)$$

where p_n is the proportion of individuals in the n-th species (OTU). Higher values indicate greater diversity.

The Simpson diversity index D [28], used to capture dominance among OTUs, reflecting the likelihood of randomly selecting individuals from different OTUs within each sample, is calculated with the formula:

$$D = 1 - \Sigma\, {p_n}^2$$

where p_n is the proportion of individuals in the n-th species (OTU). Lower values indicate higher diversity.

Pielou's evenness E [29] provided a standardized measure of evenness for a community by adjusting the Shannon–Wiener Index relative to the total number of OTUs and was assessed with the formula:

$$J' = H'/\ln(S)$$

where H' is the Shannon diversity index and S is the total number of species (OTUs). Values close to 1 indicate a more even distribution of individuals across species.

The OTUs specific or common to the three study sites were placed in a Venn diagram.

2.11. Rarefaction Indices

Rarefaction indices were calculated to compare species richness among rhizosphere samples from Bambey, Ngane, and Ndiafate. The expected number of species in a random sample of a collection taken from a total population was calculated using the Analytic Rarefaction program 1.3 (Steven M. Holland; http://www.uga.edu/strata/software/ accessed on 25 January 2024).

2.12. Statistical Analysis

The statistical analysis evaluated the influence of various factors—such as site, OTU, and salinity (electrical conductivity)—on the abundance and diversity of AMF. Normality and homogeneity tests were conducted to verify that the data satisfied parametric assump-

tions. The Shapiro–Wilk test was used to assess normality, with a *p*-value greater than 0.05 indicating a normal distribution. Levene's or Bartlett's tests, chosen based on the dataset, were used to confirm the homogeneity of variances across groups. A two-way analysis of variance (ANOVA) was primarily used to evaluate the effects of site (Bambey, Ngane, and Ndiafate), OTUs, and additional variable like salinity (electrical conductivity) on AMF abundance. For datasets that did not meet ANOVA assumptions, nonparametric tests such as the Kruskal–Wallis test were employed. Tukey's post hoc test was used for mean separation when treatment effects were significant at $\alpha = 0.05$. Pearson's correlation analysis further examined the relationships between OTUs and environmental variables, particularly to determine dependencies on salinity.

All statistical and diversity analyses were executed using R-4.4.2, vegan package, and Python 3.12, with the significance threshold consistently set at $p < 0.05$.

3. Results

3.1. Molecular Diversity in the Three Sites

Four DNA extractions were performed on each soil sample. These extractions yielded high-quality DNA, which was checked after electrophoresis in an agarose gel (1.4%) (Figure 2A).

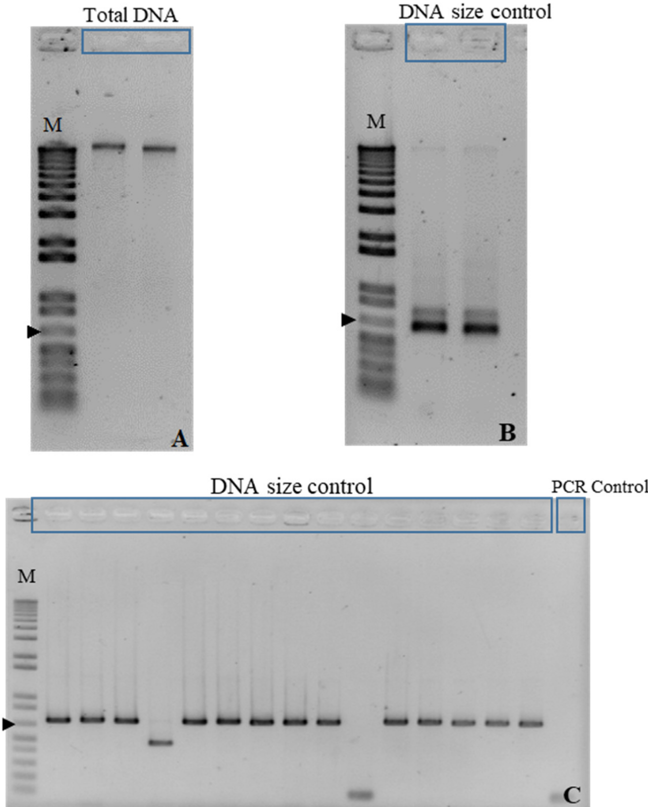

Figure 2. DNA extraction control (**A**), amplification with primers LR1/NDL22 (**B**) and primers LR1/FLR4 (**C**). M: molecular marker (1 Kb Plus DNA Ladder). The arrow indicates the 650 base pair position on each gel.

The nested PCR followed by cloning yielded several clones containing fungal DNA sequences from the D1 and D2 domains of the LSU rDNA. Then, a control regarding the size of the amplified DNA was performed after the nested PCR (Figure 2B,C). Over 1000 clones (recombinant or not) were obtained from bacteria cultured in Petri dishes for each type of soil, root, and spore, and 75 were successfully sequenced. Sequence alignment

was conducted with sequences for the rhizosphere soil from Ndiafate (23), Ngane (18), and Bambey (16), for *V. seyal* roots from Bambey (6) and Ndiafate (4), and for spores (8).

The obtained sequences were aligned with the closely related sequences belonging to members of the Glomeromycota, retrieved from the available AMF sequences in the National Center for Biotechnology Information (NCBI) Gene-Bank database. A phylogenetic tree was built from the multiple alignments of sequences with 593 bp from the D1 and D2 domains of the 25S LSU rDNA.

3.2. Phylogenetic Tree Analysis

After aligning the sequences, all 75 sequences obtained from the three soils were identified as members of the Glomeromycota. A phylogenetic tree was inferred from the sequence alignments, grouping the sequences according to their similarities (Figure 3). The sequence divergence delineated three main operational taxonomic unit (OTU) groups.

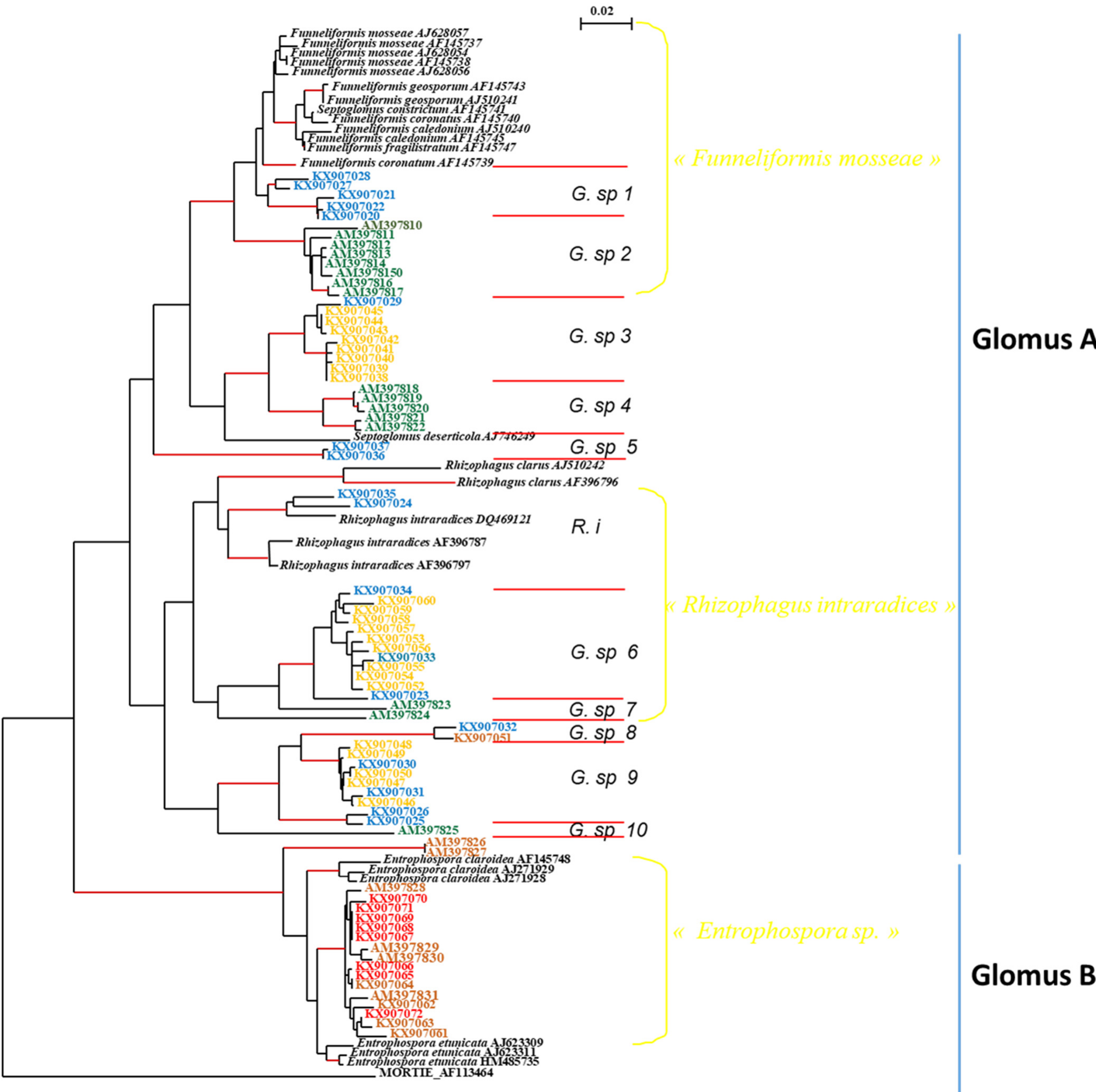

Figure 3. Neighbor-joining tree of arbuscular mycorrhizal (AM) fungi (*Glomeromycota taxa*) based on alignment of 25S rDNA sequence fragments from the rhizosphere of *V. seyal* for Bambey (green), Ngane (blue), and Ndiafate (orange), roots (brown), and spores (red) aligned with known sequences. *Mortierella* sp. was used as relevant outgroup species.

The number of species represented per site showed more OTUs in the moderately saline soils (seven OTUs) compared to the low- and high-salinity soils (four species for each).

The OTUs belonging to the genus *Glomus* sp. 6, 8, and 9 have sequences from moderately saline (Ngane) and high-saline (Ndiafate) soils. On the other hand, OTUs from the low-saline soil (Bambey) did not exhibit homology with sequences from high- and medium-saline soils, as in the case of the genus G. sp. 7 and G. sp. 10. These sequences are placed in a separate branch between sequences from moderately saline soils and those from high-saline soils.

The phylogenetic tree based on the D1 and D2 regions of the 25S LSU rDNA region and the comparison of sequences from the three soils with those available in Genbank allowed the subdivision of the phylogenetic tree into three groups according to their ribotypic homology.

Funneliformis mosseae cluster

This cluster contains 13 sequences from two soils (Ngane and Bambey). The different lineages within this group are assembled into OTUs according to their affinity with known species, such as *Funneliformis mosseae*, *F. geosporum*, *F. coronatum*, *F. fragilistratum*, and *Septoglomus constrictum*. The distribution of these sequences in the phylogenetic tree has allowed the identification of the following:

- Some OTUs whose lineages are in G. sp. 1. These lineages correspond to five clones belonging to the moderately saline soil of Ngane.
- Some OTUs whose lineages belong to G. sp. 2. These lineages correspond to eight clones close to G. sp. 1 from the low-salinity soil (Bambey).

Rhizophagus intraradices cluster

This group consists of sequences from 16 clones of DNA fragments from the three soils studied. Two clone sequences had lineages closely related to *R. intraradices* (R.i), originating from samples from the moderately saline soil of Ngane. The other sequences (G. sp. 6 and G. sp. 7) were also grouped in this cluster according to their homology.

The unspecified cluster

Two unspecified clusters were identified in the phylogenetic tree:

- OTUs whose lineages were more distant from G. sp. 1 and G. sp. 2 and belonged to G. sp. 3, 4, and 5 were grouped in the first unspecified cluster. The sequences included in G. sp. 3, 4, and 5 were composed of OTUs belonging to 16 clones from the three soils studied. These OTUs were unequally distributed in the phylogenetic tree and showed no homologies with the sequences available in databases. Within G. sp. 3, the majority of lineages belonged mainly to the Ndiafate soil, with only one sequence from the Ngane soil. In contrast, G. sp. 4 and G. sp. 5 have 100% sequences from Bambey and Ngane soils, respectively.
- The lineages of the second unspecified group were more distant from G. sp. 6 and 7. This group of 12 sequences consisted of G. sp. 8, 9, and 10.

DNA sequences from roots and spores

Spore propagules were isolated from the various sampling sites. However, DNA extraction and sequencing of these spores showed that they belonged to the same species (Figure 3). The OTUs obtained from the roots of *V. seyal* collected in situ and those of the spores were very closely related, forming a group close to the species *Entrophospora claroidea* and *E. etunicata*.

3.3. AMF Phylotype Diversity

The number of OTUs (Figure 4) was highest in the Ngane soil (seven OTUs), followed by Bambey (four OTUs) and Ndiafate (four OTUs).

The distribution of OTUs across the three zones (Figure 5) showed that all three zones shared 50 common OTUs. Some OTUs were present in Ngane and Ndiafate soils (G. sp. 3, 6, 8, and 9), which are moderately and high-saline soils, respectively. On the other hand, all OTUs from the low-salinity soil of Bambey (G. sp. 2, 4, 7, and 10) had no phylogenetic similarities with those from the other two soils, as showed in Figure 5. Three specific lineages were recovered only from the moderately saline soil of Ngane (G. sp. 1–5 and *R. intraradices*).

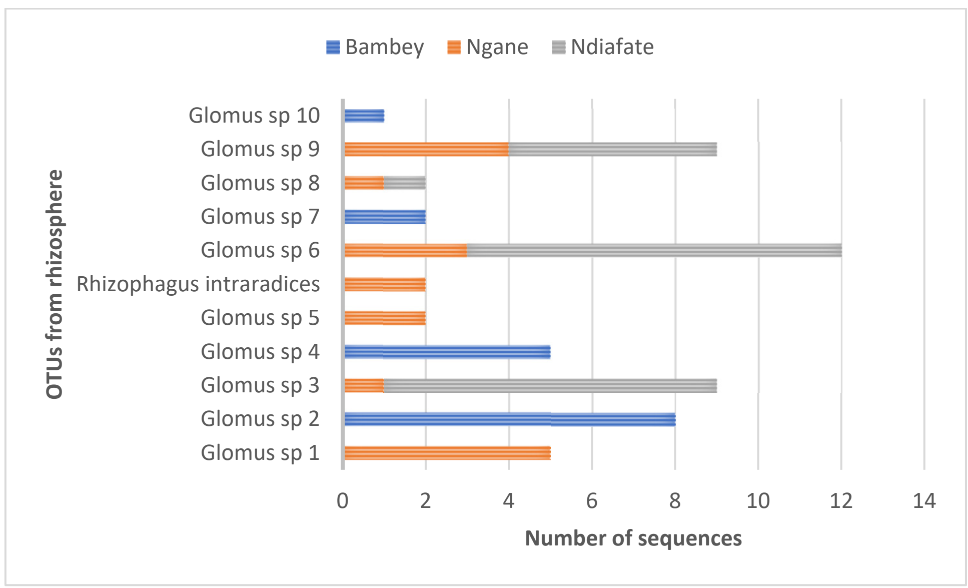

Figure 4. Number of sequences from soils and roots compared to the number of lineages obtained after alignment of the sequences from soils.

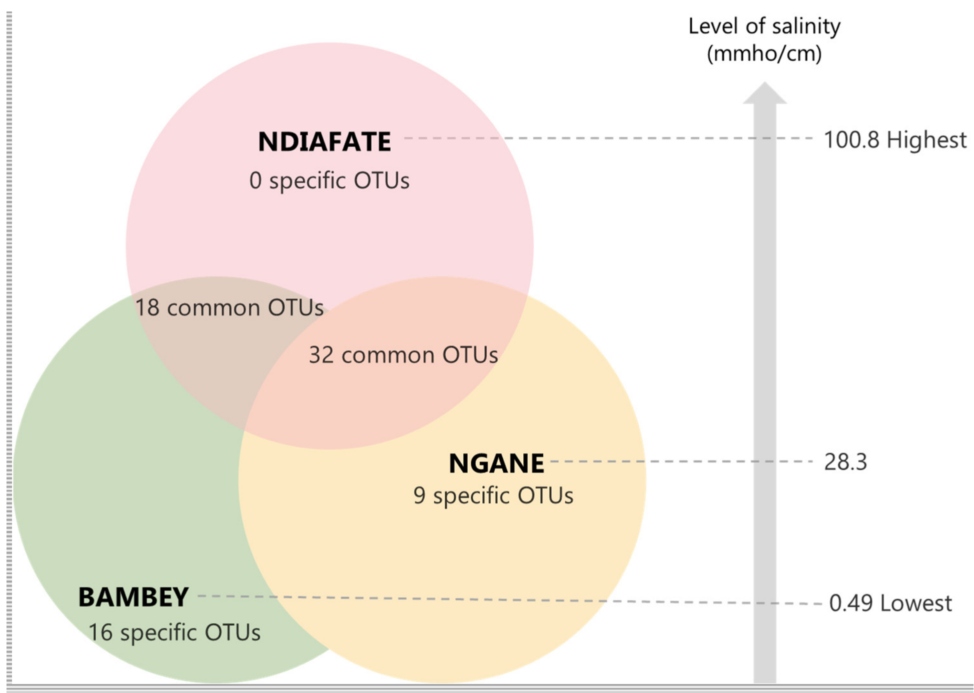

Figure 5. Venn diagram with specific or common OTUs recovered from Bambey, Ngane, and Ndiafate. Overlaps concern common OTUs.

3.4. Relationships Between OTUs and Diversity Indices

The Shannon–Wiener index (H′) measures species diversity, considering both richness (number of OTUs) and evenness. Despite having fewer OTUs, Ngane has the highest Shannon–Wiener index (1.798). This indicates a more even distribution of AMF species compared to other sites. The higher Shannon index for Ngane reflects greater diversity and richness in OTU distribution. Bambey and Ndiafate have comparable Shannon–Wiener indices (1.417 and 1.433, respectively), despite the higher number of OTUs in Ndiafate. This suggests that AMF species distribution in Ndiafate might be less even compared to Bambey. Regarding the ecological implications, AMF diversity and richness appear to respond to local salinity levels differently across the sites. High salinity might support a higher number of AMF OTUs but reduce their evenness, as observed in Ndiafate. Ngane presents an optimal salinity level for both richness and evenness, suggesting that moderate salinity levels could promote a balanced AMF community.

Simpson's index (D) was lower for Ngane (0.137) compared to Bambey (0.234) and Ndiafate (0.235), suggesting that Ngane has greater diversity. This indicates a higher likelihood that two randomly chosen individuals from Ngane would belong to different OTUs. Ngane also had the highest Pielou's evenness index (0.924), indicating that OTUs are more evenly distributed there. Bambey and Ndiafate have slightly lower evenness (0.881 and 0.890, respectively), suggesting a moderate variation in the relative abundance of OTUs (Table 2).

Table 2. Total isolated number of OTUs, Shannon–Wiener and Simpson diversity indices, and Pielou's evenness index for arbuscular mycorrhizal fungi OTUs.

Location	OTUs	H′	D	J′
Bambey	22	1.417	0.234	0.881
Ngane	18	1.798	0.137	0.924
Ndiafate	35	1.433	0.235	0.890

H′, Shannon–Wiener diversity index; D, Simpson diversity index; J′, Pielou's evenness index.

3.5. Rarefaction Index

Rarefaction curves (Figure 6) were used to compare species richness between the three soil samples. The diversity indices of OTUs compared with extrapolation of the expected number of species in rarefaction curves showed that around 90 sequences for Ngane, 70 sequences for Ndiafate, and 100 sequences for Bambey would have to be aligned from clones to account for the total diversity present in each sample.

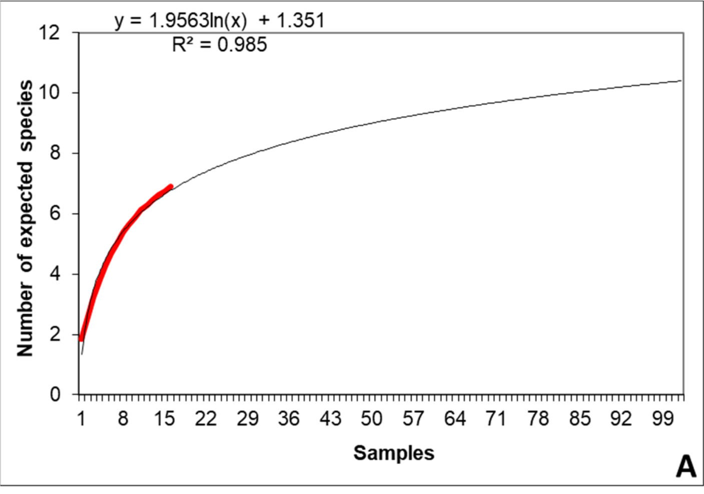

Figure 6. *Cont.*

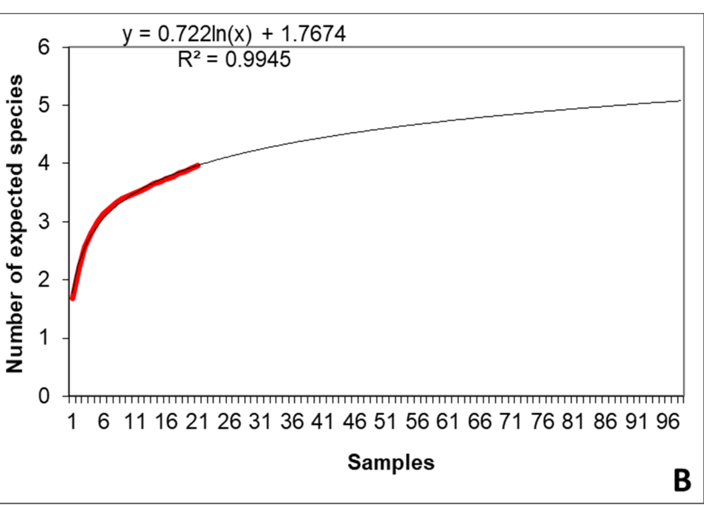

Figure 6. Rarefaction curves of the occurrence of AM sequences from the soils of Ngane (**A**) and Ndiafate (**B**).

3.6. Statistical Analysis Results

The Shapiro–Wilk test produced a *p*-value of 0.056, indicating that the data followed a normal distribution ($p > 0.05$). Levene's test also returned a non-significant *p*-value (0.253), confirming the homogeneity of variances across the three sites.

The two-way ANOVA results indicated that neither site ($p = 0.118$), species ($p = 0.423$), nor electrical conductivity ($p = 0.232$) had a statistically significant effect on the AMF count. This suggests that variations in species counts across sites and levels of electrical conductivity were not large enough to reach statistical significance.

4. Discussion

It is becoming increasingly important to better understand mycorrhizal diversity and, in particular, how it is organized in the field [30]. The ability to directly detect endomycorrhizal fungi in soils will increase the capacity to monitor and evaluate mycorrhizal function and dynamics [31]. However, the current understanding of the geographical distribution of AMF is hindered by the sparsity of available molecular data from certain regions, including West Africa [32]. The present study provides information on the molecular characterization of AMF communities associated with *V. seyal* tree species in saline soils of Senegalese croplands.

The direct assessment of AMF in field samples is challenging due to low concentrations of target DNA and difficulties associated with cell lysis [33]. The results obtained in our study have demonstrated that this method of direct DNA extraction from bulk soil samples can be efficiently used with nested PCR to identify the presence of AMF.

A high concentration of host DNA compared to fungal DNA in roots [34] or in soil samples can limit fungal detection. This problem can be addressed by precipitating host DNA prior to fungal-specific PCR [30], by directly amplifying fungal DNA [35], or by using fungal-specific primers in a nested PCR [18]. The specificity of PCR-based detection methods is determined by the existence of suitable primer sites and the resolution by the degree of polymorphism of the amplified target sequence [36]. In the present study, we used selective primers in the nested PCR, which should limit the sequences obtained to AMF. This was performed to focus the study on this lineage of Glomeromycetes but this approach may underestimate the diversity within the group. The AMF-specific primer (FLR4) used in this study was selected to provide optimal specificity for a wide range of glomeralean, excluding amplification of other groups of eukaryotic DNA present in soil [19]. Only members of the genus *Glomus* were detected by targeting the three soil

samples. Other AMF, such as *Gigaspora*, *Scutellospora*, or *Acaulospora*, were not found. More OTUs belonging to the genera *Glomus*, *Sclerocystis*, *Rhizophagus*, *Scutellospora*, *Gigaspora*, *Racocetra*, *Acaulospora*, and *Redeckera* were found in the rhizosphere of cowpea plants in Senegal by Diop et al. [37] using molecular tools.

The composition of the AM fungal communities in the rhizosphere of *V. seyal* at the three sites differed significantly among the three sites, indicating that the colonizing AMF community is influenced by site factors which are known to influence AMF spore distribution [38].

Several distinct lineages did not match sequences from known species. Species designations of the sequence groups without known matches must await further sequencing of named isolates for comparison. It has been suggested [39] that AMF spores in field-collected soil can be difficult to identify at the species level. As a result, published works on the genetic diversity and population biology of natural communities of AMF are limited, particularly for West Africa [37,40–45]. Furthermore, all DNA-based studies suggest that the true diversity of AMF in the field is much higher than that currently represented in culture collections as sporulating AMF [35,46].

The AMF sequences obtained from the rhizosphere of *V. seyal* were classified into 11 glomeralean groups, all belonging to the Glomus group. According to [8], it is unclear whether each AMF type represents a single morphospecies or whether some morphospecies contain more than one sequence type. Species identification will only be possible once the spores identified in the respective study sites have been sequenced.

However, until a better understanding of the genetic organization of AMF and their taxonomy is achieved, the sequence grouping concept remains the most valid system for delimiting taxa recovered from the field [47]. The diversity of AMF associated with *V. seyal* appears to be relatively low, particularly in saline soils. These findings are in accordance with those of Manga et al. [5] and Ndoye et al. [43], who found only species of the genus Glomus in the rhizosphere of *V. seyal* and *Senegalia senegal*, respectively, in semi-arid soils. This is consistent with previous findings showing that polluted environments can negatively affect the diversity of mycorrhizal fungi [11,48–50]. In comparison, studies in undisturbed environments can reveal dozens of AMF taxa [51]. However, Belay et al. [6] found greater AMF diversity in the rhizosphere of *A. seyal* in Ethiopia, and Dalpé et al. [45] reported five AMF in the rhizosphere of *Faidherbia albida* in Senegal, belonging to the genera Glomus and Gigaspora.

The relationship between functional characteristics and genetic diversity needs to be addressed in order to identify an appropriate level of genetic diversity that is meaningful in an ecological context [52]. The investigation of AMF communities has been hampered in the past by the lack of consensus on a nomenclature system that can be applied to AMF identified by molecular data [10,26,49].

The number of clones analyzed for each soil was probably too small to allow us to detect the maximum diversity within the soils. None of the soil-derived sequences were identical, and many more soil samples need to be analyzed to monitor the full genetic diversity. The sequence groups described in this study, identified by phylogenetic analysis, are partly dependent on the limited number of clone samples. The "unspecified group" may represent sequences for which data are not available in the database. The use of ribosomal genes has a major advantage over other genomic regions in that more and more sequences are becoming available, providing a broad database for the fine tuning of specific molecular probes for monitoring glomalean fungi in the field [53]. Ten new AMF sequence groups from semi-arid soils are described in this study, and one sequence group from the Ngane soil belonged to *R. intraradices*. Identification of the respective fungal spores from

the soil producing these new sequences will require more intensive surveys or alternative spore enrichment culturing.

Discussion of AMF ecology in saline environments has been confused by the need to distinguish between biotic and abiotic effects on fungal distribution and relative abundance [54]. The sequences obtained from the three soils differed in their specificity for different "phylotype groups". The high- and moderate-salinity zones of Ndiafate and Ngane, respectively, consist of unsuitable arable lands due to the effects of salt stress on plants. The AMF diversity observed in the three semi-arid soils was low. The salinity of these soils has negative effects and may be responsible for the low biodiversity of both plants and AMF. The acidic conditions (pH 3.6) observed, particularly in the Ndiafate soil, is a consequence of the high salinity level, which could be responsible for the lower level of diversity in this soil compared to soil of Ngane. All OTUs from the high-salinity soil of Ndiafate were also found in the moderately salty soil of Ngane. However, some specific OTUs, classified as G. sp. 1 and G. sp. 5, were only found in the Ngane soil. These OTUs were not found when the soil became more salty and acidic. The hypothesis could be that a reduction or disappearance of these species is due to the increase in salt and acidity in the Ndiafate soil. On the other hand, G. sp. 3, for which we had only one sequence from the Ngane soil and essential OTUs from the Ndiafate soil, seems to be a "specific" group. This group could be represented by one or several species adapted to the harsh environmental conditions for which no similarity sequences were available in the database. The difference between the AMF communities at the two sites may reflect variations in soil factors, such as pH, nutrient content, moisture content, and temperature, which are known to influence spore distribution [33,38,55–57].

The Bambey region consists of arable land with intensive annual monoculture of peanut (*Arachis hypogaea* L.) and millet (*Pennisetum glaucum* (L.) R. Br.) during the rainy season from July to October. No OTUs recorded in the low-salinity soil of Bambey were found in the moderate- and high-salinity soils of Ngane and Ndiafate, respectively. These findings are consistent with previous reports that the AM fungal community is found to be impoverished in species composition in areas of agricultural intensification [58–61], particularly with a change from crop rotation to monocropping [60].

The lack of statistically significant effects of site, species, and salinity on AMF diversity could be due to several reasons, including potential micro-environmental factors not accounted for in this study or an inherent resilience of AMF species to varying conductivity levels. This study was limited by the scope of environmental parameters measured and the sample size. Future research could expand to include additional soil characteristics and a larger number of samples to increase the power of statistical analyses. Ngane demonstrates both higher diversity (according to the Shannon and Simpson indices) and greater evenness among OTUs, meaning it has a more balanced and diverse community of arbuscular mycorrhizal fungi. Bambey and Ndiafate have comparable diversity and evenness values, suggesting that their fungal communities are moderately diverse but less evenly distributed than Ngane's. These differences could be due to varying environmental conditions or soil characteristics that affect the distribution and abundance of fungal species in each location.

The rarefaction curves obtained showed a much lower diversity than that commonly observed with AMF in the field [62]. The main reason for this could be that the richness of AMF may have been underestimated in this study, and the true ecological diversity is almost certainly higher than observed here.

The molecular polymorphism of Glomales indicates that they exhibit a much higher level of genetic diversity than would be expected from morphological characteristics alone [53]. Typically, AMF show low levels of host specificity [63,64]. However, this study showed particularly high host specificity in *V. seyal* roots, the vast majority of which were

colonized by spores for which no homologous sequences were found in the databases. This specificity could be related to a very high colonization of fungal structures of Entrophospora in the roots of *V. seyal*, making it more difficult to detect other AMF species. In addition, some AMF showed host preference when given the opportunity to colonize different host species, whereas different plant species can vary in the degree to which they form mycorrhizal associations [63,65–67].

5. Conclusions

This study enhances our understanding of AMF ecology by suggesting that many AMF species associated with *V. seyal* plants have not yet been isolated and characterized. The findings of this study showed a low AMF diversity associated with *V. seyal* in non-salty to moderately and high-salinity soils in the semi-arid Senegalese Peanut Basin. These results indicate that the sampling (rhizosphere soils and roots) captured a substantial portion of the OTU diversity present in Ngane, Ndiafate, and Bambey, showing varying levels of richness. The distinct numbers of sequences required for comprehensive diversity coverage suggest differences in microbial community composition among the sites, potentially influenced by local environmental factors or soil characteristics such as salinity level. Additionally, the use of molecular techniques to identify AMF species with greater accuracy may provide more insights into the subtle variations in species distribution. This comparative analysis lays the foundation for further exploration of the factors that determine microbial diversity and host specificity across these sites.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/microbiolres16010019/s1. Table S1: Results of DNA analysis based on the D1 and D2 domains of the LSU rDNA gene identifying AMF OTU associated with *Vachellia seyal* trees in 3 different sites.

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