Evidence of novel plant-species specific ammonia oxidizing bacterial clades in acidic South African fynbos soils

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Ammonia-oxidizing bacteria (AOB) are essential in the biogeochemical cycling of nitrogen as they catalyze the rate-limiting oxidation of ammonia into nitrite. Since their first isolation in the late 19th century, chemolithoautotrophic AOBs have been identified in a wide range of natural (e.g., soils, sediments, estuarine, and freshwaters) and man created or impacted habitats (e.g., wastewater treatment plants and agricultural soils). However, little is known on the plant-species association of AOBs, particularly in the nutrient-starved fynbos terrestrial biome. In this study, we evaluated the diversity of AOBs in the plant canopy of three South African fynbos-specific plant species, namely *Leucadendron xanthoconus*, *Leucospermum truncatulum* and *Leucadendron micro-cephalum*, through the construction of *amoA*-gene clone libraries. Our results clearly demonstrate that plant-species specific and monophyletic AOB clades are present in fynbos canopy soils.

Keywords: *amoA*-gene diversity / Ammonia oxidizers / Fynbos soil / Proteaceae family / Plant–microbe interactions

Introduction

Microorganisms play key roles in the biogeochemical cycling of nitrogen (N) with N-fixation, anammox (anaerobic ammonium oxidation), nitrification, and denitrification processes being exclusively microbially mediated [1]. Nitrification is a two-step biological process transforming ammonia (NH_3) to nitrate (NO_3^-), via nitrite (NO_2^-), by oxidation. It is performed aerobically by ammonia-oxidizing bacteria (AOB) and archaea (AOA) [1, 2]. AOB and AOA are the pivotal N mineralizing guilds in many environments as they perform the first and generally rate-limiting step of nitrification [3].

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Based on 16S rRNA gene phylogeny, AOBs constitute monophyletic groups within the β- and γ-proteobacterial classes [4]. The *amoA* gene, present in the *amoCAB* operon of all AOBs [5], which encodes the active site of the ammonia monooxygenase, has been extensively used to characterize environmental AOB communities (e.g., [2, 4, 6, 7]). Despite exhibiting a lower phylogenetic resolution, this functional marker has been shown to display congruent phylogenies compared to 16S rRNA gene-based analyses [8] and to enable the detection of ammonia-oxidizers even in environments where they are present in low amount [4].

The Cape Floristic Region in South Africa is the smallest of the six Floral Kingdoms in the world [9]. It represents around 6% of South Africa's land area (Fig. 1), but contains over 6000 plant species, 65% of which are endemic to South Africa [9]. The main vegetation type of the Cape Floristic Region is termed fynbos (fine bush) and is dominated by members of the Proteaceae, Ericaceae,

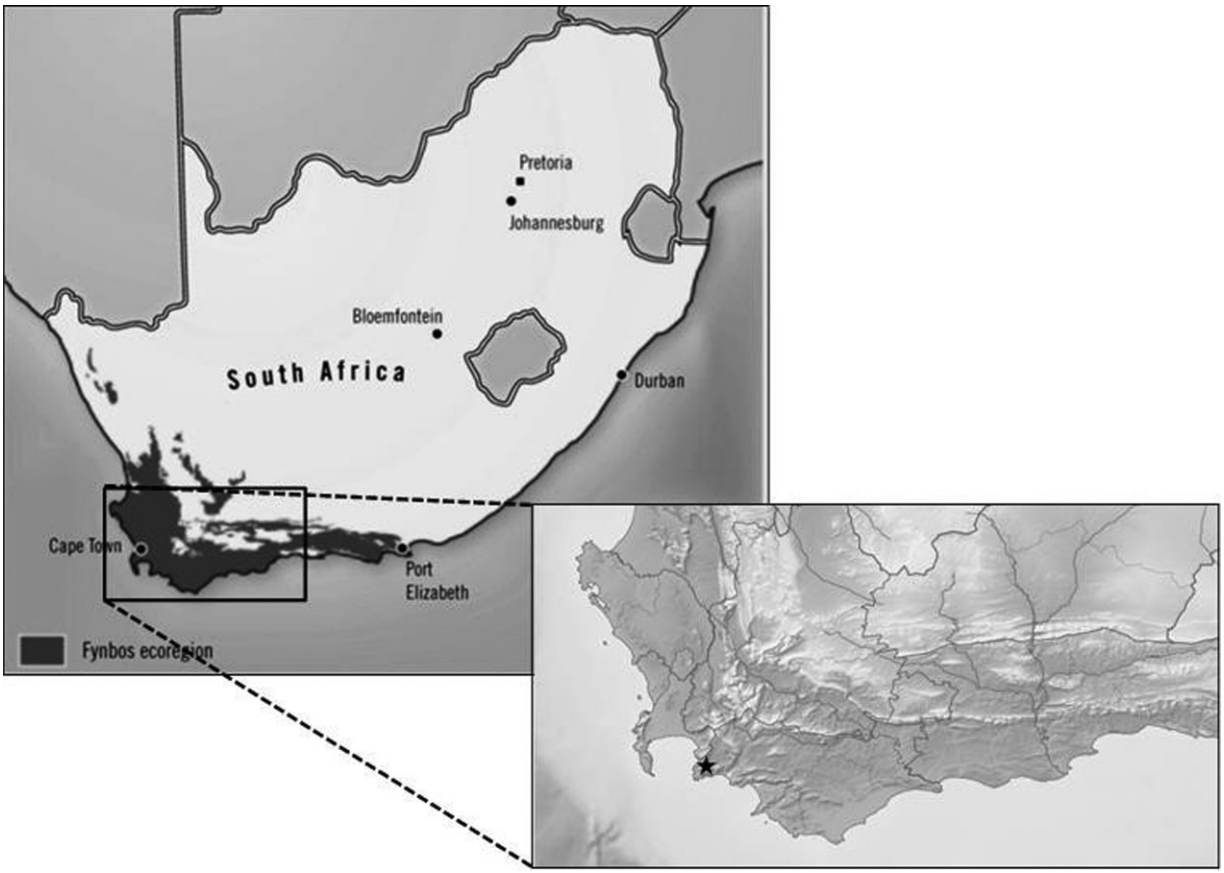

Figure 1. Map displaying the sampling site. The Cape Floral Kingdom (fynbos) is shaded on the map and the Kogelberg Nature Reserve (S34°16.489/E019°02.405), where the samples were collected, is indicated by a black star. The map of South Africa was adapted from a design made by Hugo Ahlenius (UNEP/GRID-Arendal) and available at this site: http://www.grida.no/graphicslib/detail/fynbos-ecoregion-in-south-africa_1320.

and Restionaceae families. Despite the extremely high plant diversity, fynbos soils are typically acidic, of low fertility, and with low nitrogen contents [10]. Due to the acidity of fynbos soils, biogenic ammonia is likely to be largely present in its protonated form (i.e., ammonium [NH_4^+]; [8]). Nevertheless, nitrification by autotrophic bacterial ammonia oxidation has been reported in acid soils [8, 11–13] despite the lack of substrate (i.e., ammonia [NH_3]). It has therefore been suggested that in such environments AOBs probably display urease activity (i.e., produce ammonia from N-rich compounds) to obtain their nitrification substrate [8]. While ammonia-oxidizing archaea (AOA) are expected to be more adapted and abundant in such acidic and oligotrophic fynbos soils [2, 14], we aimed to evaluate the diversity of AOBs in such soils as they have been shown to possess a wide number of survival strategies [15] and to potentially develop mutualistic relationships with other microbial guilds (e.g., heterotrophs) to cope with nutrient limitations [16]. Therefore, we targeted *amoA* genes in the canopy of three indigenous fynbos plants from the Proteaceae family, namely *Leucadendron microcephalum*, *Leucadendron xanthoconus* (same genus), and *Leucospermum truncatulum* (Supporting Information Fig. S1), by constructing *amoA*-gene clone libraries.

Materials and methods

Fynbos plant canopy soil sampling

Soil samples were collected in the Kogelberg Biosphere Reserve (S34°16.489/E019°02.405; Western Cape Province, South Africa, Fig. 1) at 370 m above sea level. Twenty random canopy soil cores (2 cm diameter, to a depth of 6 cm) from each of the three Proteaceae plant species (*L. xanthoconus*, *L. microcephalum*, and *L. truncatulum*; Supporting Information Fig. S1) were collected randomly and aseptically from an area of approximately 800 m^2.

Canopy soil samples from each plant were pooled to enable representative plant-associated AOB community analysis and stored at −80 °C.

Soil pH and ammonium concentration measurements

pH was measured in 10 g soil slurries (1:2.5 soil/deionized water) with a pH meter (Crison Basic 20, Barcelona, Spain). Soil ammonia concentrations were measured according to Ref. [17]. After addition of 1 M KCl (1 g of soil: 10 ml KCl) samples were incubated on a shaker (200 rpm) for 1 h at 4 °C. After incubation, samples were centrifuged for 10 min at 4 °C, filtered using cellulose acetate membrane filters (0.22 μM pore size) and the sample stored at −20 °C prior to colorimetric analysis. Briefly, solution I (8.5 g Na-salicylate and 0.06 g Na-nitroprusside/ 100 ml) was added to the soil sample (1:1 v/v) and vortexed. After addition of solution II (Na-dichloroisocyanurate [1 g L^{-1}] w/v) and further dilution [1:3] with 300 mM NaOH, samples were incubated for at least 30 min in the dark and the absorbance at 690 nm was measured (Spectronline, Biomate3, USA).

Metagenomic DNA extraction, purification, and quantification

Soil metagenomic DNA was extracted as previously described [18]. Due to the presence of PCR inhibitors (e.g., humic acids), the extracted DNA was further purified by electrophoresis in 0.7% (w/v) agarose gels. Metagenomic DNA (>10 kbp) was excised from the gel and re-purified using a Qiaquick PCR purification kit (Qiagen), eluted in 50 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and quantified spectrophotometrically (Nanodrop ND-1000, Delaware, USA).

Bacterial ammonia monooxygenase (amoA) gene PCR amplification

The purified metagenomic DNA was used as a template for PCR using primers targeting the *amoCAB* operon. A nested PCR approach was performed to increase the *amoA* gene detection limit as recommended [19], where the first round primer set 305F (5′-GTGGTTTGGAACRGICARAG-CAAA-3′)/308R (5′-TCCCAGCTKCCGGTRATGTTCATCC-3′) was used to amplify a fragment of about 1.5 kb, which was used as a template for the second round [20]. The second round PCR used the amoA-F1 (5′-GGGGTTTCTAC-TGGTGGT-3′)/amoA-2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′) primer set, generating a 491 bp *amoA* gene product [7].

PCR reactions were prepared in a final volume of 50 μl and contained: 20 mM Tris–HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% (w/v) Triton X-100, 2 mM $MgSO_4$, 200 μM of each dNTP, 0.5 μM of each primer, 10–20 ng of template DNA, and 2 U of Taq polymerase. First round PCR conditions were: 94 °C for 5 min, followed by 25 cycles of 94 °C for 1 min; 55 °C for 1 min, and 72 °C for 2 min, with a final extension at 72 °C for 7 min. The second round PCR conditions were: 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min; 57 °C for 1 min, and 72 °C for 112 s, with a 5 min final extension at 72 °C.

Bacterial *amoA*-gene clone libraries construction and phylogenetic analysis

PCR amplicons were gel-purified using a Qiaquick PCR purification kit (Qiagen) and cloned into the pTZ57R/T vector according to the manufacturer's instructions (Fermentas). Three clone libraries were constructed (i.e., one for each fynbos plant canopy soil sampled) and 90 transformants (i.e., 30 per clone library) were selected by blue–white screening. The presence of correctly sized inserts was confirmed by colony PCR using M13F/R primers targeting the pTZ57R/T vector. Amplified rDNA restriction analysis (ARDRA; using *Hae*III) was used to de-replicate clones. PCR products with unique ARDRA patterns were sequenced using the vector primer M13F. The nonparametric S_{Chao1} estimator was used to estimate the richness and coverage of each *amoA* gene library ([21]; Supporting Information Fig. S2). Phylogenetic affiliations of the partial sequences were initially estimated with the program BLAST. For each unique *amoA* sequence, the 15 closest hits were retained for further analysis.

Deduced amino acid -based phylogenetic trees were constructed with MEGA6 [22] using the distance-based Neighbour-Joining or the character-based Maximum Parsimony (MP) methods [23]. The robustness of the tree topology was evaluated by boot-strap analysis based on 200 replicates [24]. Sequences obtained in this study were deposited in the NCBI GenBank database under accession numbers AM933241–AM933251 and AM933256–AM933268.

Results and discussion

Nitrogen (N) enters terrestrial ecosystems through bacterial N_2-fixation, atmospheric deposition of ammonium and nitrate salts in the form of dissolved dust or particulates in rain water, and fertilization [25]. Bacterial N-fixation leads to the formation of ammonia (NH_3) via nitrogenase activity. The rhizospheric fynbos soils sampled were acidic (pH 5.5, 5.4, and 5.5 for the rhizospheric soils of *L. xanthoconus*, *L. truncatulum*, and *L. microcephalum*, respectively; [10]) and presented low ammonia contents (0.4 [±0.001], 0.5 [±0.003], and 0.6 [±0.004] μg NH_3 g^{-1} soil in the rhizospheric soils of *L. xanthoconus*, *L. truncatulum*, and *L. microcephalum*,

respectively). In these acidic soils (pH <6), ammonia is ionized into ammonium (NH_4^+) [6], and is not available as a substrate for nitrification. Moreover, ammonia oxidation does not commonly occur in acidic environments, and most AOBs grow optimally in a pH range of 7.5–8.0 [4], although some have been observed in acidic soils [4, 13]. Therefore, in such acidic soil environments, AOBs are likely to produce the ammonia necessary for their nitrification process by hydrolysing edaphic N-rich compounds (e.g., urease activity) [8].

Consequently, to increase the sensitivity of amoA gene PCR-detection, we implemented a nested-PCR strategy [19]. The asymptotic shapes of the rarefaction curves for the three *amoA*-clone libraries constructed (Supporting Information Fig. S2) showed that this nested-PCR approach was successful in yielding a high diversity of *amoA* gene sequences in each of the fynbos rhizospheric soils studied, and that these data represent near-saturating coverage. Thus, the number of *amoA* clones selected was adequate to fulfil the aim of this study, i.e., describe the rhizospheric AOB diversity associated with the fynbos plant species selected [21].

All fynbos *amoA* gene sequences belonged to the β-proteobacteria [26] and were closely related (>90% identity) to *amoA* sequences from *Nitrosovibrio*, *Nitrosospira*, and/or *Nitrosomonas* species (Table 1). Based on their deduced amino-acid sequences, all the AmoA sequences were related to β-AOBs of the *Nitrosospira–Nitrosomonas* lineages (Figs. 2 and 3) [26]. This was not unexpected as sequences related to the genus *Nitrosospira* have been shown to particularly dominate in such acidic soil environments [4, 12].

Both distance- (Fig. 2) and character-based (Fig. 3) phylogenetic analyses of AmoA sequences clearly show that the fynbos AmoA rhizospheric sequences formed distinct clades, suggesting that individual plant species (even those from the same genus) select for specific and monophyletic AOB communities. Cultivar-specific AOB communities have previously been observed in near neutral (pH 6.2) rice rhizosphere soils, with microscale differences in O_2 availability identified as a potential driving factor [27]. However, in the latter study, the rice cultivar-specific AOB communities were structurally different, with different assemblages of similar AOB phylotypes [27]. We note that in acidic fynbos rhizospheric soils, plant-specific AOB communities were significantly divergent with unique *amoA*/AmoA clusters (Table 1; Figs. 2 and 3). This strongly suggests that fynbos

Table 1. Fynbos amoA-gene sequence similarities.

Fynbos plant rhizosphere	Accession number	Closest match in genebank/origin [accession number]	% of Identity (453 bases)	Reference
Leucadendron xanthoconus	AM933241.1	*Nitroso vibriotenuis*/Soil (Hawaii, USA) [AY123824.1]	98	[6]
	AM933242.1		98	
	AM933243.1		98	
	AM933256.1		99	
	AM933257.1		99	
	AM933258.1		99	
	AM933259.1		99	
Leucadendron microcephalum	AM933249.1	*Nitrosospira* sp. L115/Peat Bog (Finland) [AY123817.1]	96	[32]
	AM933250.1		96	
	AM933251.1		96	
	AM933264.1		96	
	AM933265.1		96	
	AM933266.1		97	
	AM933267.1		97	
	AM933268.1		97	
Leucospermum truncatulum	AM933263.1	*Nitrosospira* sp. L115/Peat Bog (Finland) [AY123817.1]	91	[32]
		Nitrosomonas sp. Nm51/Sea water [AJ298711.1]	92	[33]
	AM933262.1	*Nitrosovibriotenuis*/Soil (Hawaii, USA) [AY123824.1]	91	[6]
		Nitrosomonas communis Nm2/Soil (Greece) [AJ298705.1]	92	[33]
		Nitrosospira sp. Nsp2/Soil (Germany) [AJ298719.1]	92	[34]
	AM933261.1	*Nitrosovibriotenuis*/Soil (Hawaii, USA) [AY123824.1]	91	[6]
		Nitrosomonas communis Nm2/Soil (Greece) [AJ298705.1]	92	[33]
		Nitrosospira sp. Nsp2/Soil (Germany) [AJ298719.1]	92	[34]
	AM933260.1	*Nitrosovibriotenuis*/soil (Hawaii, USA) [AY123824.1]	91	[6]
	AM933246.1	*Nitrosospira* sp. Nsp2/Soil (Germany) [AJ298719.1]	91	[34]
		Nitrosospira sp. Wyke8/Agricultural soils [EF175099.1]	92	[35]
		Nitrosospira sp. Nsp17/Soil (Iceland) [AY123825.1]	91	[6]

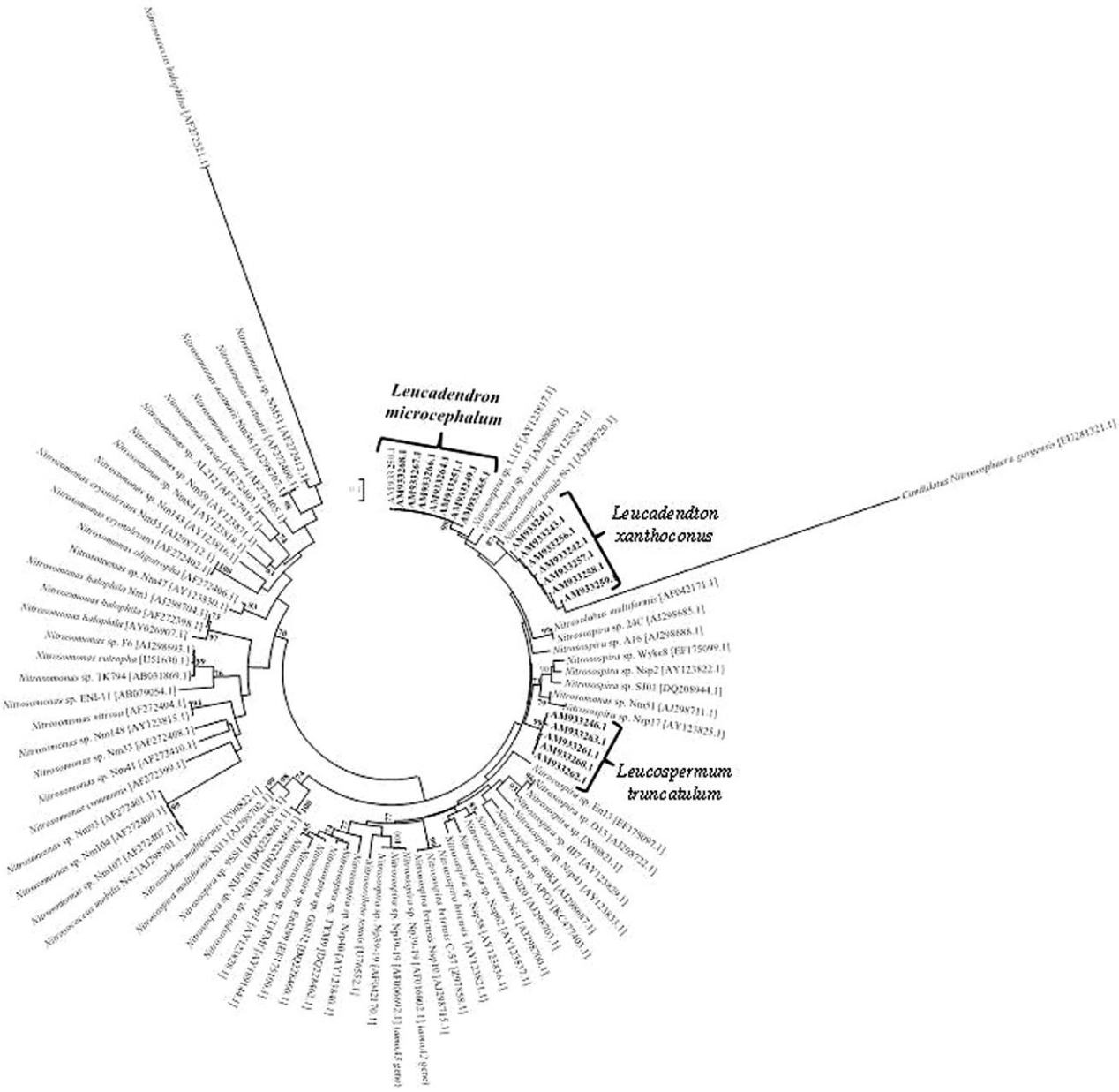

Figure 2. AmoA-based phylogenetic tree of AOB using the Neighbor-Joining (NJ) method. The evolutionary distances were computed using the JTT matrix-based method [36] and are in the units of the number of amino acid substitutions per site. Only boostrap values (200 replicates) $\geq$65% are shown. Sequences obtained in this study are indicated in bold and their associated fynbos plants specified. The analysis involved 92 amino acid sequences. The coding data was translated assuming a standard genetic code table. All positions containing gaps and missing data were eliminated. There were a total of 98 positions in the final dataset.

plants and AOBs have evolved a structured and intimate relationship [28, 29].

Concluding remarks

The direct roles of plant root systems in shaping their associated rhizospheric communities is well established, notably as this niche is created by the release of plant-specific nutrient- and carbon-rich root exudates [28, 30]. AOBs, being autotrophic [8], should, however, not depend on such compounds to thrive and thus be specifically selected by plants; even more in their canopy. Nevertheless, AOBs and heterotrophic bacteria (i.e., the ones selected by plant root exudates) have recently been shown to be associated in mutualistic relationships; with

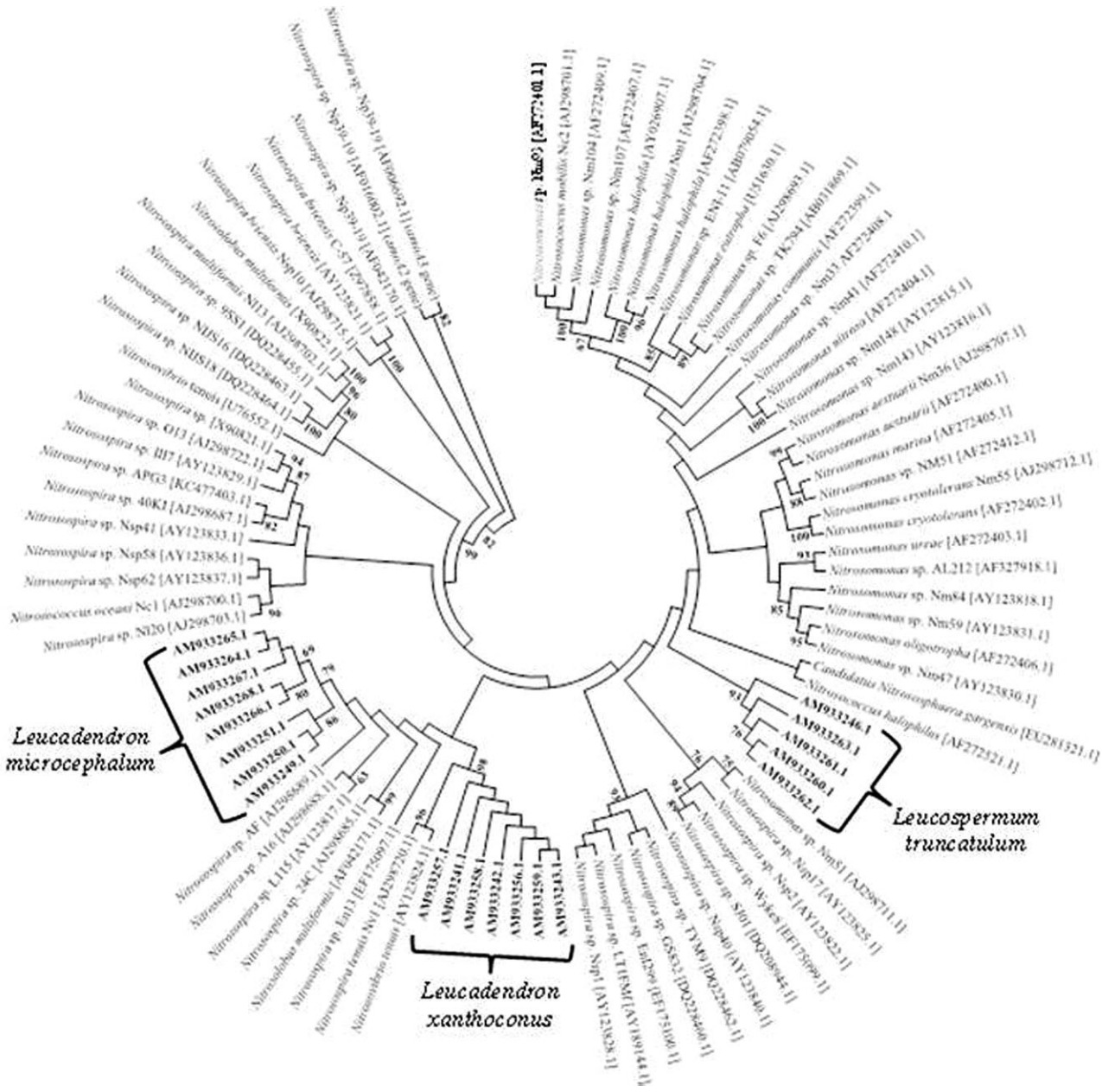

Figure 3. AmoA-based phylogenetic tree of AOB using the MP method. Sequences obtained in this study are indicated in bold and their associated fynbos plants specified. Only boostrap values (200 replicates) $\geq$65% are shown. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm [37] with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 92 amino acid sequences. The coding data was translated assuming a standard genetic code table. There were a total of 1107 positions in the final dataset.

the heterotrophs helping AOBs to acquire an essential nutrient (iron) and AOBs supporting the growths of the heterotrophs by releasing organics [16]. It is thus conceivable that in nutrient-starved canopy fynbos soils [10], such AOB/heterotrophic bacteria association exists.

To conclude, in South African fynbos, above-ground floral communities have been suggested to be implicated in shaping edaphic bacterial and fungal communities [31]. Our study demonstrates that specific functional guilds may show a high level of plant–host specificity. This is consistent with the general concept that plants

stringently manage their associated microbiome through evolutionary selection [29].

Acknowledgments

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Supporting information

Figure S1. Photographs of the fynbos plants sampled. A: *Leucadendron microcephalum.* B: *Leucadendron xanthoconus.* C: *Leucospermum truncatulum* (Courtesy of J.D.W. Lako).

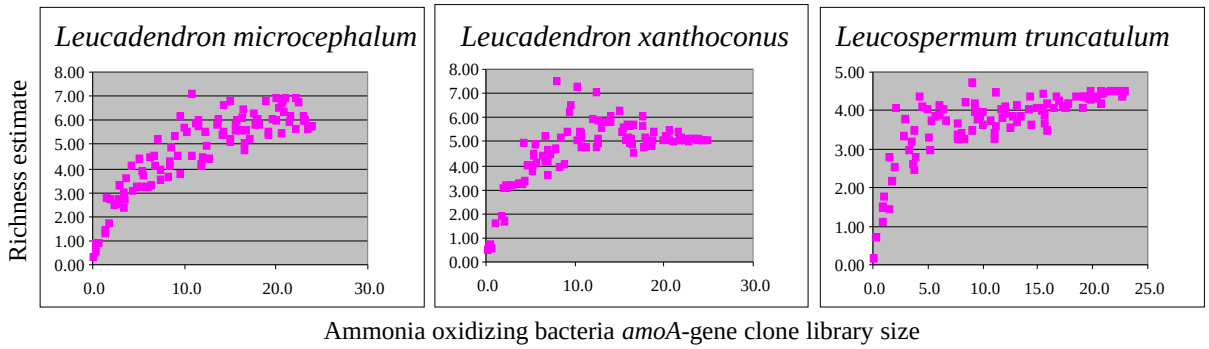

Figure S2. Graphical representation of the non-parametric S_{Chao} estimator for the three amoA-gene clone library constructed. The richness was estimated from OTUs identified in each library by *Hae*III restriction analysis and DNA sequencing.