Analysis of ammonia-oxidizing bacteria associated with the roots of Proteaceae plant species in soils of Fynbos ecosystem

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Abstract

Molecular methods were used to investigate the microbial diversity and community structure of ammonia-oxidizing bacteria (AOB) associated with the roots of the Proteaceae plant family. The identification of ammonia oxidizing bacteria in this ecosystem is of particular interest since Proteaceae are adapted to acidic, low nutrient (e.g. nitrogen) soils. The ammonia monooxygenase operon was used as a molecular marker to identify ammonia-oxidizing bacteria associated with the proteoid roots of the three Proteaceae members and compared to non-plant associated soil. PCR amplification using primer sets targeting the ammonia monooxygenase gene (*amoA* subunits) were used to construct a clone library. Sequence diversity was determined by RFLP analysis of *amoA* to identify major groups of AOB of the β-subclass of *Proteobacteria* in total community DNA, and DNA sequencing and phylogenetic analysis were also applied. DGGE analysis was performed to determine the community structure and distribution of ammonia-oxidizing bacteria in plant associated and non-plant associated soils. The AOB genotypic diversity was similar in the plant-associated samples and non-plant associated soil. All AOB phylotypes belonged to *Nitrosospira* species and clustered with *Nitrosospira* cluster 3. The abundance of the *amoA* was quantified to be approximately 4.2 x 10^7 copies/g of dry soil, using real-time PCR assay. These data suggest that the *Nitrosospira* species are dominant phylotype in that environment. This investigation provides new insights into the relationships between plants and ammonia-oxidizing bacteria in natural Fynbos ecosystems.

Declaration

I declare that **Analysis of ammonia-oxidizing bacteria associated with the roots of Proteaceae plants species in soils of Fynbos ecosystem** is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Mr. Joseph Lako November 2005

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Dedicated in memory of my late brother

Dennis Yogu Wani

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List of Abbreviations

AOB	Ammonia-oxidizing bacteria
AMO	Ammonia monooxygenase
AmoA	Alpha subunit of the ammonia monooxygenase
Anammox	Anaerobic ammonium oxidation
ARDRA	Amplified ribosomal DNA restriction analysis
APS	Ammonium peroxodisulfate
ATCC	American Type Culture Collection
bp	Base pair
BLAST	Basic Local Alignment Search Tool
DGGE	Denaturant Gradient Gel Electrophoresis
dNTPs	Deoxynucleotide tri-phosphate
ē	Electron
EDTA	Ethylendiaminetetraacetatic acid
FISH	Fluorescence *In situ* Hybridization
g	Gram
GC-clamp	Guanosine-cytosine clamp
GeneBank	Nucleotide sequence database
HAO	Hydroxylamine oxidoreductase
IPTG	Isopropyl-β-D-thiogalactoside
kb	Kilo-base
LB	Luria Bertani
mg	Micro-gram
ml	Milliliter
µl	Microliter
NCBI	National center for Biotechnology Information
ng	Nanogram
O.D	Optical Density
PCR	Polymerase Chain Reaction
PVPP	Polyvinylpolypyrrolidone
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute

RT	Room temperature
SDS	Sodium Dodecyl Sulfate
sp.	Species (single)
spp.	Species (plural)
TAE	Tris-acetate-EDTA
TBE	Tris-Boric acid-EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethyldiamine
Tris	Tris (Hydroxymethyl)-aminomethane
U	Units
v/v	volume per volume
v/w	volume per weight
w/w	Weight per weight
X-Gal	5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside

1.1. Introduction

The Cape Floral Kingdom or Floristic Region is the smallest of the six Floral Kingdoms in the world. The Cape Floral Kingdom comprises less than 6% of the South Africa's area, but contains one third of its plant species (Cowling, 1992). The main vegetation type of the Cape Floral Kingdom is called Fynbos, meaning "fine bush" which consists mainly of members of the Proteaceae (*Leucadendron xanthoconus*, *Leucospermum truncatulum* and *Leucadendron microcephalum*), (Photos courtesy of Dr. W.H.L Stafford ARCAM-UWC), Ericaceae and Restioaceae families. The Fynbos biome occupies the majority of the Cape Floristic Region, with over 6000 plant species of which 70% are endemic to South Africa.

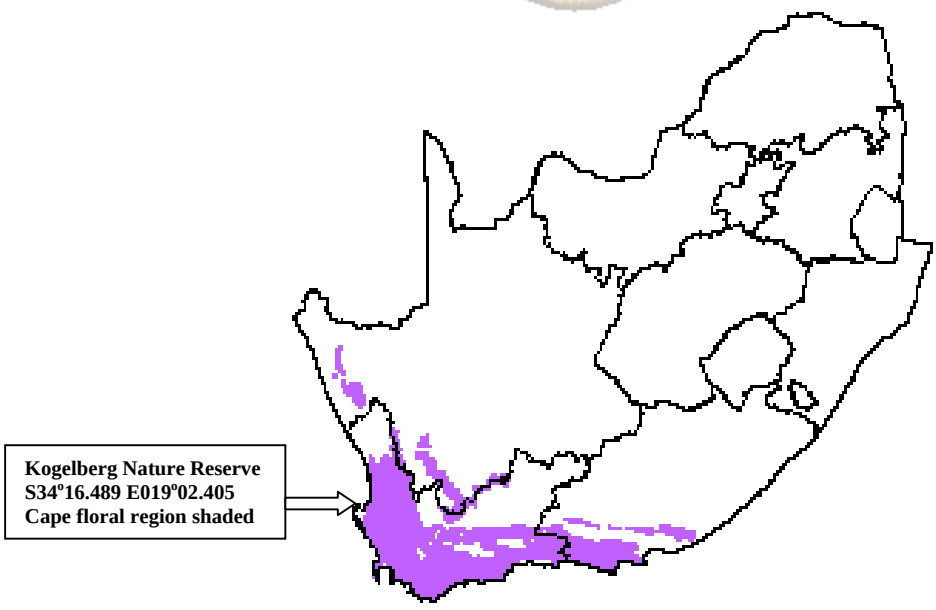


Fig 1.1: Map of South Africa with Cape Floral Kingdom shaded.

Fynbos soil is infertile, with very low nutrient levels (nitrogen, potassium and phosphorous). Fynbos species have adapted to grow in the harsh nutrient-deprived soils, implying a high level of efficiency in locating and absorbing available nutrients. Consequently, many Fynbos plants have developed nutrient-uptake mechanisms such as symbiotic partnerships with bacteria, archaea and fungi (McDonald *et al*., 1995). Bacteria stimulate the production of nodules on the roots of some plant species stimulating the up-take of nitrogen and other ions from the soil. Fynbos soil is characterized mainly by acidic pH (around 6.0), although there are some areas with alkaline soils with pH values as high as 8.0.

The unique plant biodiversity of Fynbos region offers the opportunity of exploring the microbial diversity associated with the roots of protea plant species (Lombard *et al*., 1997), and assessing the role of indigenous plant species in dictating the microbial population with which they are associated.

(**A**) *Leucadendron xanthoconus*

(**B**) *Leucospermum truncatulum*

*(**C**)* Leucadendron microcephalum

*(**D**)* Non- plant associated

Fig 1.2: Representatives of Proteaceae family (Photos courtesy of Dr. W.H.L Stafford ARCAM-UWC).

1.2. Ammonia-oxidizing bacteria

Nitrogen is one of the most important elements in plant nutrition, and the high crop yields obtained by modern agriculture have been significantly facilitated by the application of nitrogen rich fertilizers (Kowalchuk *et al.*, 1998). In the past years, the number of ammonium-rich environments has increased significantly due to human activities (Calvó and Garcia-Gil, 2003). Chemolithoautotrophic ammonia-oxidizing bacteria (AOB) play an essential role in the natural cycling of nitrogen by aerobically transforming ammonia to nitrite with hydroxylamine as an intermediate (Wood, 1986). These chemoautotrophs obtain their energy through oxidation of ammonia. Generally, ammonia-oxidizing bacteria are obligatory chemolithoautotrophs, and

form monophyletic groups within the β and γ-subclasses of the *Proteobacteria* and anaerobes within the Planctomycetales. Researchers have continued to investigate the diversity of ammonia-oxidizing bacteria in natural and engineered environments by applying enrichment and isolation techniques. These efforts have resulted in the description of 16 AOB species (Jones *et al.,* 1988; Koops *et al.,* 1990). The isolates are obligatory aerobic, gram-negative bacteria of the family Nitrobacteraceae. Some heterotrophic bacteria and fungi (Fosht and Verstraete, 1977) can also oxidize ammonia and/or reduced nitrogen from organic components to hydroxylamine, nitrite and nitrate.

Chemolithoautotrophic AOB has been found in many ecosystems, including fresh water, salt water, sewage systems, soil and rocks (Mansch and Bock, 1998; Bothe *et al.,* 2000). These microorganisms occupy diverse habitats and are found in both thermophilic (Egorova and Loginova, 1975) and psychrophilic environments (Wilson *et al.,* 1997; Arrigo *et al.,* 1995). Ammonia-oxidizing bacteria are generally rod-shaped, spherical, spirillar, or lobular are typically Gram-negative, and flagellation of motile cells is polar to sub-polar or peritrichous. Most species are aerobic but can grow at reduced oxygen partial pressure. Since first reports of the successful isolation of chemolithoautotrophic ammonia-oxidizers at the end of the 19th century (Frankland and Frankland, 1890) most pure cultures grow optimally in a pH range of 7.5 to 8.0, although a *Nitrosospira* isolate has been shown to adapt to extreme acidic conditions after exposure to pH fluctuations (De Boer *et al.,* 1995). Tolerance to ammonia concentrations differs between the various species. Temperature and moisture are the

other environmental factors that influence ammonia oxidizer populations (Belser, 1979). Temperature also influences ammonification (mineralization) rates.

The generation time for chemolithoautotrophic ammonia-oxidizing bacteria (AOB) in laboratory culture varies from 8 hours to several days under aerobic conditions. In batch cultures, growth is limited by acidification and accumulation of nitrite (Watson *et al.,* 1989; Koops and Möller, 1992). As a result of poor growth in liquid cultures as well as on solid media, isolation and maintenance of ammonia-oxidizing bacteria is considered to be difficult. Consequently, much of the detailed physiological, genetic and functional data is limited to *Nitrosomonas europaea.*

1.2.1. Taxonomy and distribution of ammonia-oxidizing bacteria

The early taxonomy of ammonia-oxidizing bacteria was entirely based on cell morphology (Watson *et al.,* 1989). Typically, ammonia-oxidizing bacteria are members of the β-subclass of *Proteobacteria* and are classified into five different genera: *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosovibrio* and *Nitrosolobus* (Koops and Möller, 1992). While most of the known strains are closely related members of the β-subclass of the *Proteobacteria* (Teske *et al.,* 1994), a few belong to the γ- *Proteobacteria*. These include two species, *Nitrosomonas oceanus* and *Nitrosomonas halophilus*. To date, only a few γ- *Proteobacteria* ammonia-oxidizing bacteria have been isolated from marine habitats, and none from soil or freshwater habitats. Despite their slow growth ammonia oxidizers demonstrated the ability to

survive in extreme environments such as cold waters. For example, *Nitrosomonas cryotolerans* was isolated from Alaska coastal waters and is capable of growth at -5^{o}C (Jones *et al*., 1988). However, β-*Proteobacterial* ammonia oxidizers have been also detected in permanently ice-covered Antarctic lakes (Voytek and Ward, 1995).

Table 1.2: Soil factors and composition of ammonia-oxidizing bacteria communities (Avrahami, 2003)

Environmental Factor	Cluster	Reference
Acidic pH	*Nitrosospira* cluster 2	Stephen *et al*., 1996; 1998; Kowalchuk *et al*., 2000
Acidic pH	*Nitrosomonas*	Carnol *et al*., 2002
Improved soils high ammonium concentration	*Nitrosospira* cluster 3	Kowalchuk *et al*., 2000
Low ammonium concentration	*Nitrosospira* cluster 4	Kowalchuk *et al*., 2000
Improved soils	*Nitrosospira* cluster 1 and 3 and *Nitrosomonas* cluster 7	Webster *et al*., 2002
Unimproved soils	*Nitrosospira* cluster 1 and 3 *Nitrosomonas* cluster 7	Webster *et al*., 2002
Limitation of moisture	*Nitrosomonas* species exhibit more rapid recovery than *Nitrosospira* species	Hastings *et al*., 2000
Soil irrigated with fertilizer amended water	*amoA Nitrosospira*-like (cluster 3 and 9)	Oved *et al*., 2001
Effluent-irrigated soils improved agriculture soil	*amoA Nitrosospira*-like *amoA Nitrosospira* cluster 1	Oved *et al*., 2001; Avrahami *et al*., 2002

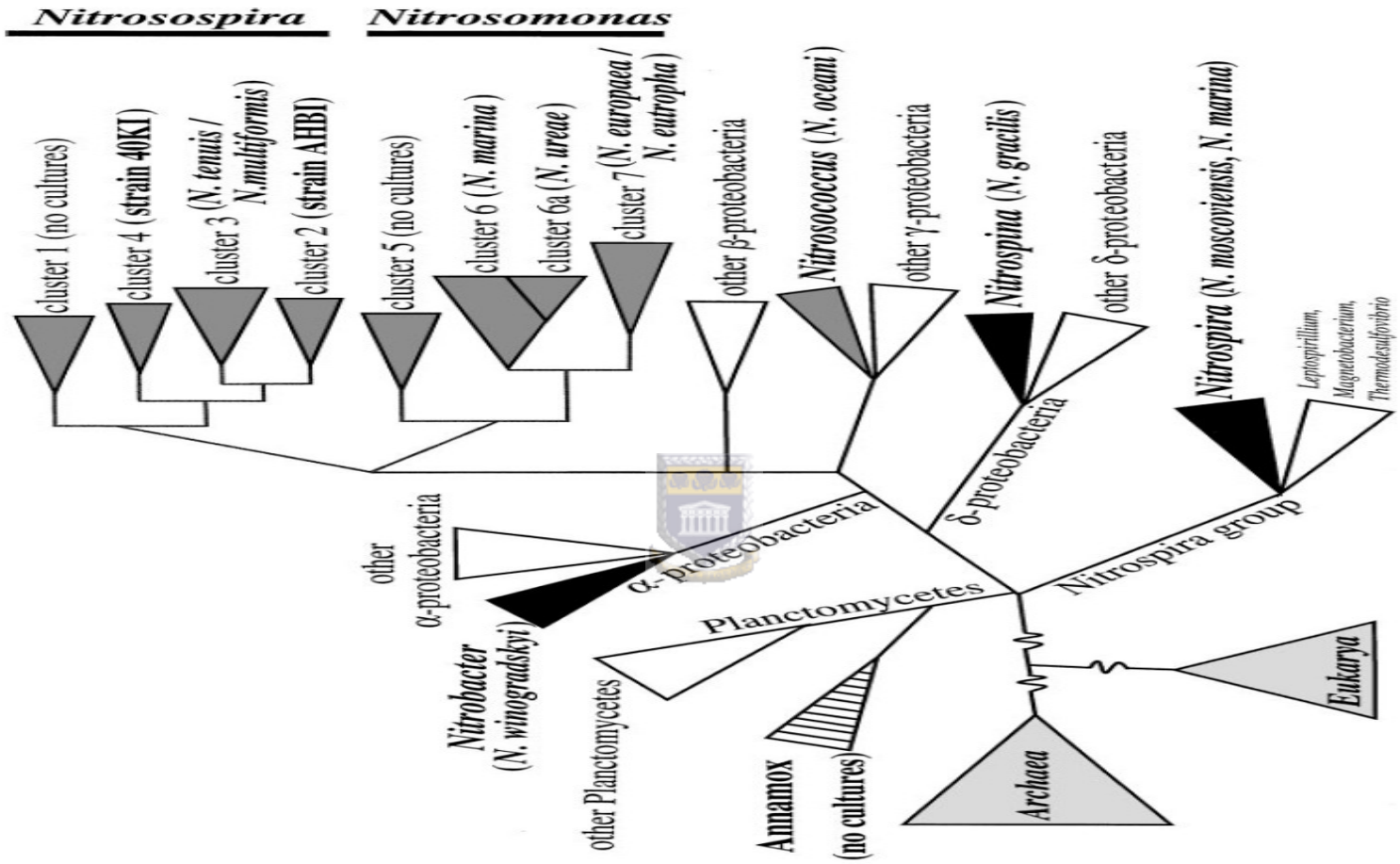


Fig 1.3: Schematic representation of the autotrophic nitrifiers based on 16S rRNA gene sequences (Kowalchuk and Stephen, 2001)

1.2.2. Physiological aspects of ammonia oxidation

Ammonia oxidation was thought to be a strictly aerobic process, requiring molecular oxygen, however, some ammonia oxidizers can readily adapt to low O_2 concentrations. Under anoxic conditions, *Nitrosomonas eutropha* and *Nitrosomonas europaea* can oxidize ammonia in the presence of pyruvate, using nitrite or NO_2 as an electron acceptor (Abeliovich and Vonshak, 1992; Schmidt and Bock, 1997). Growth of ammonia-oxidizing bacteria under anaerobic condition is very slow (doubling time is about 11 days) (Strous *et al.,* 1998), and involves the production of NO, N_2O and even N_2 gases at low levels as by-products of normal nitrification (Zart *et al.,* 2000).

Ammonia-oxidizing bacteria have the ability to grow at low environmental ammonia concentrations and the response after deficiency of ammonium appears to be a major factor in the communities' differentiation of these microorganisms. *Nitrosomonas europaea* were monitored in retentostat and batch experiments by Tappe *et al*. (1999), who showed that *Nitrosomonas europaea* regained activity rapidly after a long period of ammonia starvation, while *Nitrosospira* species responded very slowly.

The autotrophic AOB that facilitate nitrification have been found to be influenced by a variety of factors, which dictate community numbers, diversity and activity *in situ* (Hastings *et al.,* 1998). The pH and moisture are the two other parameters which appear to play an important role in shaping the community structure and in

determining the functional activity of these microorganisms (Bocks *et al.*, 1986). Conversely, lack of moisture in a variety of natural environments has an influence on general microbial growth and activity in soil, leading to decreased mineralization of carbon and nitrogen (Hayes and Swift, 1989). Moisture is known to be rate limiting for nitrification in many ecosystem (Haynes, 1986).

1.2.3. Ammonia-oxidizing bacteria community structure in soil environments

The community structure of ammonia-oxidizing bacteria has been investigated in various soils differing in environmental conditions such as pH, ammonia concentration, and water content. *Nitrosospira* species of clusters 2, 3 and 4 are dominant in neutral soils (Stephen *et al.*, 1996; Kowalchuk *et al.*, 1997; Philips *et al.*, 2000; Burns *et al.*, 1999; Hasting *et al.*, 2000), while *Nitrosomonas* species have been shown dominate in acidic forest soil (Carnol *et al.*, 2002) table 1.2.

Much of our present understanding of soil microbial diversity is based on the extraction and analysis of community (metagenomic) DNA. This approach has facilitated comparative analysis of changes in community structure in relation to environmental factors (Nüsslein and Tiedje, 1998). The application of molecular techniques, in particular analysis of 16S rRNA genes, provides new opportunities for the evaluation of ammonia oxidizing populations. Phylogenetic analysis of 16S rRNA

genes of pure and mixed cultures places ammonia-oxidizing bacteria in three groups (Freitag and Prosser, 2003). An alternative for 16S rRNA as a phylogenetic marker is sequence analysis of *amoA*, which provides comparable phylogenetic taxonomy and valuable additional information related to gene function (Rotthaume *et al.,* 1997). In order to detect the presence of ammonia-oxidizing bacteria in the environment, PCR primers have been designed against conserved regions of the structural gene subunit A (*amoA*) of the ammonia monooxygenase operon (Bothe *et al.,* 2000). Environmental 16S rRNA and *amoA* libraries have extended our knowledge of the natural diversity of ammonia-oxidizing bacteria (Head *et al.,* 1993). The phylogenetic analysis using the *amoA* genes offers a finer-scale level of the resolution (to the strain level) than 16S rRNA sequences for both the γ- and the β-subdivision ammonia-oxidizing bacteria (Purkhold *et al.,* 2000).

1.3. Ammonia monooxygenase (AMO)

AMO is a membrane-bound enzyme in *Nitrosomonas europaea* and other autotrophic ammonia-oxidizing bacteria of the β and γ-subclasses of *Proteobacteria*. The enzyme contains multiple subunits; *amoA, amoB* and *amoC*. All the three AMO genes have been cloned and sequenced from several ammonia-oxidizing bacteria (McTavish *et al.,* 1993). The *amoA* genes are present in multiple copies in most ammonia-oxidizing bacteria. *Nitrosomonas* strains usually carry two gene copies while most *Nitrosospira* strains carry three copies (Klotz and Norton, 1998). Only one copy was found in a

member of the γ-subclass of *Proteobacteria*, *Nitrosococcus oceani* (Alzerreca *et al.*, 1999). However, the variation in copy number of the *amo* operon is less than the variation in number of ribosomal operons in eubacterial genomes (Farrelly *et al.*, 1995).

PCR specific primers used to amplify sequences of the *amo* operon from different environments have been designed to target *amoA*, since this region is highly conserved and encodes the AmoA polypeptide which carries the active site (Rotthauwe *et al.*, 1997). The c-terminus of *amoA* appears to be a suitable target site for primers and probes to discriminate between ammonia-oxidizing bacteria of β and γ-subclasses of the *Proteobacteria* (Alzerreca *et al.*, 1999). The abundance of ammonia-oxidizing bacteria has been assessed by a competitive polymerase chain reaction (cPCR) assay using primers targeting the gene coding the active site polypeptide of ammonia monooxygenase (*amoA*).

AMO is responsible for the conversion of ammonia to hydroxylamine (Hyman and Arp, 1992). Hydroxylamine is then oxidized to nitrite by hydroxylamine oxidoreductase (HAO) in an endergonic reaction. HAO is an unusual enzyme with a highly complex structure, located as a soluble enzyme in the perplasmic space, but anchored in the cytoplasmic membrane. The enzyme catalyzes the oxygenation of a broad range of substrates (Hooper *et al.*, 1997).

AMO and HAO enzymes are necessary for energy conversion during the oxidation of ammonia. The initial oxidation of ammonia, which yields hydroxylamine as a reduced product, is an O_2-dependent reaction catalyzed by AMO:

$NH_3 + O_2 + 2e^- + 2H^+$ --------- $NH_2OH + H_2O$

Hydroxylamine is further oxidized to nitrite by HAO:

$NH_2OH + H_2O$ --------- $NO_2^- + 5H^+ + 4e^-$

Two of the four electrons generated from hydroxylamine are used to support the oxidation of additional ammonia molecules; the other two enter the electron transfer chain and are used for CO_2 reduction and ATP biosynthesis (Wood, 1986).

1.4. Nitrification

Nitrification is a key process in the global nitrogen cycle resulting in nitrogen loss from ecosystems (Fig 1.4), eutrophication of surface and groundwater, and production of atmospherically active trace gases (Norton *et al.*, 2001). Autotrophic ammonium oxidation is involving the two-step conversion of ammonia to nitrite and subsequently to nitrate. The nitrification process is primarily carried out by two groups of autotrophic microorganisms (Fig 1.5), AOB and nitrite-oxidizing bacteria, respectively (Hooper *et al.*, 1997). Ammonia oxidizers (chemolithotrophic) are involved in the first step, and are thought to be the rate-limiting step for nitrification in most systems as nitrite is rarely found to accumulate in the environment (Prosser,

1989; De Boer *et al.*, 1990; 1992). The overall reaction that defines nitrification is the conversion of ammonia to nitrate. Nitrate is most oxidized form of nitrogen that can be utilized by plants and other bacteria

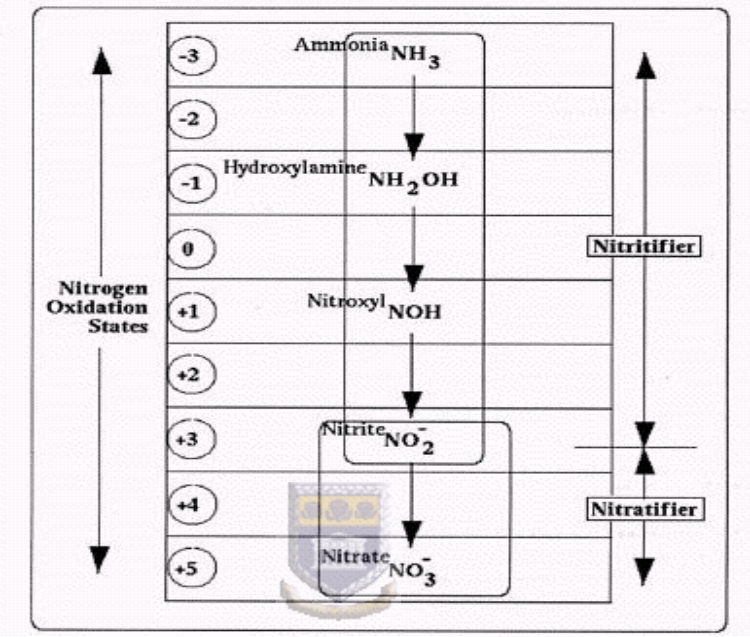


Fig 1.4: Conventional Nitrification Pathway (http://bridge.ecn.purdue.edu/)

Nitrosomonas genera is the most frequently identified genus associated with the first step of nitrification, although many other genera, including *Nitrosococcus* and *Nitrosospira* can also autotrophically oxidize ammonia (Watson *et al*., 1981). Various groups of heterotrophic bacteria and fungi can also carry out nitrification, although at the slower rate than autotrophic microorganisms (Verstraete and Alexander, 1973).

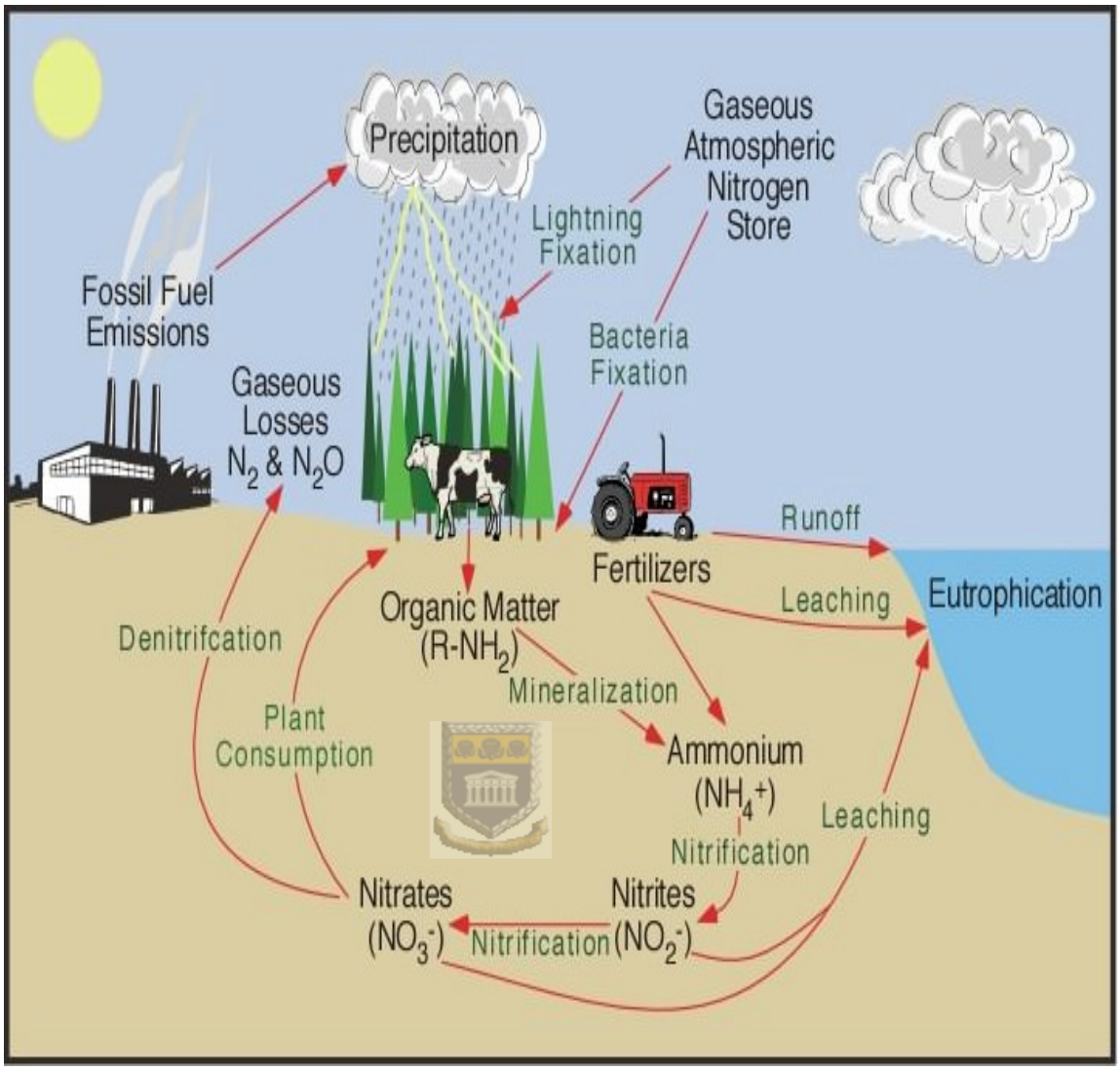


Fig 1.5: Nitrogen cycle (www.physicalgeography.net)

1.5. Denitrification

Denitrification refers to the process in which nitrate is converted to gaseous nitrogen compounds by microorganisms. The process is characterized by consecutive steps

starting from nitrate via nitrite, nitric oxide (NO), nitrous oxide (N_2O) to di-nitrogen (N_2) (Fig 1.5). The ability to denitrify is widespread among bacteria of unrelated systematic affiliations, most likely due to lateral gene transfer in evolution (Stewart, 1988; Zumft, 1992). Denitrification is related to nitrate mineralization and to the newly discovered anaerobic ammonia oxidation (anammox) reaction. Several types of bacteria perform the conversion of nitrate when growing anaerobically on organic matters; they use nitrate rather than oxygen as a terminal electron acceptor.

The two molecular approaches which have been applied to date to study the ecology of denitrification are PCR amplification and DNA hybridization, both targeted against nitrite reductase genes. All hybridization methods are based on known sequences from ammonia oxidizers organisms, the probes have been designed for genus-level and the species-level discrimination (Hovanec and Delong, 1996). Despite the worldwide importance of denitrifiers, in most environments they are poorly identified.

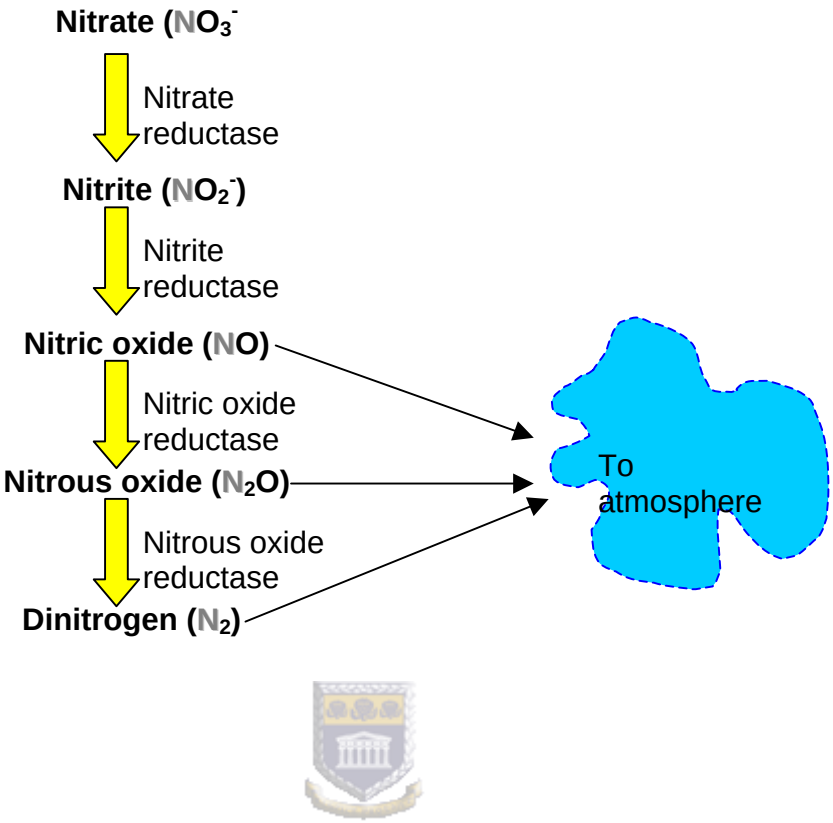


Fig 1.6: Nitrate reduction and denitrification process (Madigan *et al.*, 2000)

1.6. Anaerobic ammonium oxidation (Anammox)

In the agricultural industry, chemical nitrogen fertilizers have been used extensively, resulting in environmental impact including greenhouse gas production, waste water pollution and ozone depletion. Anaerobic ammonia oxidation (Anammox) is the

biological conversion of ammonium and nitrite to di-nitrogen gas under anaerobic conditions, using nitrite as an electron acceptor (Strous *et al*., 1999). While this process is an ideal option for low-cost ammonium removal from concentrated wastewater, its importance in the biological nitrogen cycle has not been established. The loss of nitrate with simultaneous consumption of ammonium and formation of N_2 in a fluidized-bed effluent treatment reactor was reported by Mulder *et al*. (1995). The microorganism that catalyzed this reaction was identified as a *Planctomycete*, but was shown not to be related to known autotrophic nitrifiers (Strous *et al*., 1997).

Planctomycetes are obligately aerobic, Gram-negative, chemoheterotrophic bacteria with mostly spherical, ovoid or pear-shaped cell morphology. They have some unique characteristics such as a lack of peptidoglycan in their cell walls and budding type of reproduction, which distinguish them from other bacteria (Neef *et al*., 1998). *Planctomycetes* are currently represented by only a few cultured and characterized heterotrophic members isolated from aquatic habitats, including fresh water lakes and sea-water. Four genera of these organisms have been described; *Planctomyces*, *Pirellula*, *Gemmata* and *Isosphaera* (Staley *et al*., 1992). A number of essential pathways are not organized as operons, which is unusual for bacteria (Glocker *et al*., 2003).

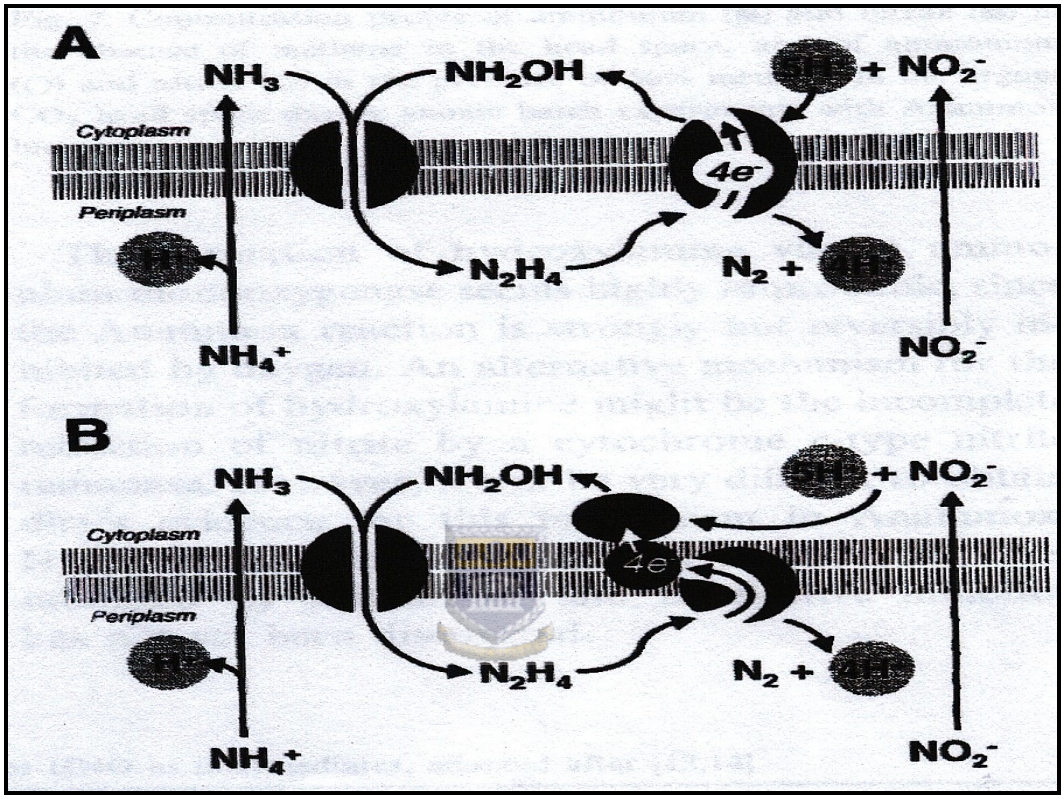


Fig 1.7: Possible reaction mechanisms and cellular localization of the enzyme systems involved in anaerobic ammonium oxidation. **A:** Ammonium and hydroxylamine are converted to hydrazine by a membrane-bound enzyme complex, hydrazine is oxidized in the periplasm to dinitrogen gas, and nitrite is reduced to hydroxylamine at the cytoplasmic site of the same enzyme complex responsible for hydrazine oxidation with an internal electron transport. **B:** Ammonium and hydroxylamine are converted to hydrazine by a membrane-bound enzyme complex, hydrazine is oxidized in the periplasm to dinitrogen gas, the generated electrons are transferred via an electron transport chain to nitrite reducing enzyme in the cytoplasm (Jetten *et al*., 1998).

1.8. Impact of nitrification on the environment

Nitrification has a significant impact on many environments, especially in the pollution of water supplies with nitrite and nitrate. Furthermore, nitrification can produce greenhouse gases (N_2 and N_2O) that may have a significant impact in the earth's climate (Tortoso and Hutchinson, 1990). Ammonia, nitrite and nitrate can typically be found in surface water supplies as a result of natural processes. These natural sources of nitrogen generally have minimal impacts on water supply distribution systems because the concentration of nitrite nitrogen in surface water is normally far lower than the deep ground waters.

The formation of nitrite and nitrate from agricultural sites that contaminate ground and freshwater supplies poses a potential threat to public health (Kowalchuk and Stephen, 2001). During nitrification processes in soil, a significant amount of bicarbonate is consumed in the conversion of ammonia to nitrite. This process involves the release of protons which can lead to significant soil acidification where the ammonia input is high.

The acidification of forest soil can have a damaging effect on plant health, and high levels of nitrification may increase the effects of acid rain (Beiderbeck *et al.*, 1996). Furthermore, some ammonia-oxidizing bacteria can have a damaging effect on the walls of buildings and on the surfaces of monuments, especially in the polluted areas with high levels of nitrogen compounds on the air (Spieck *et al*., 1992).

1.9. Significance of ammonia-oxidizing bacteria

Ammonia-oxidizing bacteria oxidize ammonium containing fertilizers to nitrite, which is subsequently converted to nitrate and nitrite, thereby contributing to fertilizer loss from agricultural soils by producing compounds that are more easily leached from soil (McDonald, 1986).

The reduction of ammonia released into the environment reduces the risk of local oxygen depletion, and helps to prevent eutrophication (Hall, 1986). It has been suggested that AOB populations in the rice rhizophere may facilitate plant nitrogen uptake by co-providing nitrate and ammonium as an inorganic nitrogen source for plant growth (Briones *et al.*, 2003).

Biological ammonia oxidation is a major factor in the removal of nitrogen during waste-water treatment. The process has high environmental significance, as release of untreated waste can result is severe eutrophication of the environment with a coincident increase in water borne human pathogens, especially in highly populated areas (Kowalchuk and Stephen, 2001). Although, waste-water treatment is the most important biotechnological application of ammonia-oxidizing bacteria, other uses are of some significance.

Nitrosomonas europaea has been shown ability to oxidize trichloroethylene (TCE) and other chlorinated aliphatics through the action of ammonia monooxygenase, and

recent studies have been shown that TCE can effectively compete for ammonia monooxygenase, due to the high affinity of this compound for the enzyme (Hyman *et al.,* 1995). However, the chloride ions released during the breakdown of the TCE, inactivate ammonia monooxygenases, thereby reducing the load of TCE that can be degraded (Hyman *et al.,* 1998).

1.10. Molecular Techniques used to investigate the diversity and community structure of AOB

1.10.1. Denaturing gradient gel electrophoresis (DGGE)

DGGE is a powerful technique whereby the sequence diversity of PCR-amplified genes from a large number of samples can be compared in one gel to reveal changes in community structure over time or space. This approach separates DNA sequences based on their melting behavior (Lerman *et al.,* 1984) and allows 95% of single base sequence differences to be detected (Myers *et al.,* 1985). Furthermore, this approach has the ability to separate genomic sequences differing by more than one base (Muyzer *et al*., 1993), although DNA fragments with multiple sequence differences may co-migrate (Lyons, 1994). DGGE has been shown to be a useful method for bacterial community profiling by targeting the 16S rRNA and/or *amoA* genes of AOB (Nicolaisen and Ramsing, 2002). This method is less time consuming for comparing AOB communities than conventional analysis by cloning and sequencing.

Some difficulties have been experienced in studying ammonia-oxidizing bacterial diversity using DGGE of 16S rDNA gene sequence, because of high similarity of the 16S rDNA sequences that makes it difficult to identify closely related ammonia-oxidizing species (Kowalchuk *et al.*, 1998). However, the development of new primers targeting the ammonia monooxygenase (*amoA*) active site region with high specificity has simplified the detection of this group of important microorganisms (Rotthauwe *et al.*, 1997).

1.10.2. Real-Time PCR

Quantification of microbial populations is essential in many aspects of microbial ecology. The development of many quantitative molecular biological techniques such as limiting-dilution PCR (Sykes *et al*., 1992), kinetic PCR (Alard *et al*., 1993) and competitive PCR (Felske *et al*., 1998) has furthered our understanding of quantitative aspects of microbial populations.

Competitive PCR techniques have been commonly used for the quantitative analysis of DNA from different microbial communities (Becker *et al*., 2000). However, this method has the disadvantage of depending on end-point measurements of the DNA produced, and it has been shown to be difficult to accurately assess the initial concentration of the template DNA (Felske *et al*., 1998). Real-time PCR is a highly

sensitive technique that facilitates amplification and quantification of a specific DNA sequence with the detection of the PCR product in real time. Quantification of DNA targets can easily be achieved by determination of the cycle when the PCR product can first be detected. Consequently, this technique appears to be reliable and reproducible for quantifying ammonia-oxidizing bacteria and for evaluating correlations between microbial activities, cell numbers and population changes in time and space (Hermansson and Lindgren, 2001).

1.10.3. Fluorescent *in situ* Hybridization (FISH)

Fluorescent *in situ* Hybridization (FISH) is one of the methods that have been described for directly visualizing AOB (Wagner *et al*., 1995), although this approach has limited application in estimating the abundance of nitrifying bacteria in the natural environment. Hybridization techniques generally use directly extracted DNA as a "probe" for specific detection of various microorganism species (Voordouw, 1998; Wagner *et al.*, 1993; Wagner *et al.*, 1994).

Commonly, the major difficulty involved in molecular probe techniques lies in the design of the probe itself. In addition, FISH analysis relies on the presence of many target sequence within an individual cell, with the result that cells containing small numbers of ribosome are poorly identified. The application of *In Situ* Hybridization approaches to AOB has been most effective in detecting AOB that dominate of the

total bacterial community in environment such as sewage treatment plants (Wagner *et al.*, 1995). However, in the community where AOB do not represent a large proportion of the total microbes.

1.11. The objectives of the study

The major objective of this study was to investigate soil ammonia-oxidizing bacterial diversity and composition associated with plant roots of Proteaceae plants and to compare it with non-plant associated soil. Culture-independent molecular techniques were chosen for the analysis and determination of the community structure and distribution of ammonia-oxidizing bacteria in soil samples. The *amoA* subunit was chosen as the optimum target for PCR-amplification. In addition, quantitative data on soil ammonia-oxidizing bacterial populations were generated using real-time PCR to target the *amoA* gene. These studies aim to enhance our understanding of the relationships between plants and AOB in natural Fynbos ecosystems.

The specific aims of this study are to:

- Identify ammonia-oxidizing bacteria in rhizosphere of Proteaceae soils.
- Determine the diversity and distribution of these bacteria and their specificity for certain Protea plant species.
- Construct genomic libraries to screen for gene variants of *amoA*.
- Determine the ammonia-oxidizing bacteria community structure and composition by using denaturant gradient gel electrophoresis (DGGE) techniques.
- Quantify the numbers of ammonia-oxidizing bacteria in the soil of Fynbos soil samples using real-time PCR.

Table 2.1: Chemical reagents used in study

Chemicals	**Sources**
Agar	Merck, Darmstadt, Germany
40% Polyacrylamide/Bis solution 37:5:1	Fluka, Germany
Ammonium peroxodisulfate (APS)	Bio Rad, München, Germany
Bromophenol blue	Sigma Aldrich, Deisenhofen, Germany
Chloroform	Sigma Aldrich, Deisenhofen, Germany
EDTA	Merck, Darmstadt, Germany
Ethidium bromide	BDH, England
Formamide	Merck, Darmstadt, Germany
Isoamyl alcohol	Merck, Darmstadt, Germany
Isopropanol	Kimix, South Africa
Ethanol	Kimix, South Africa
Polyvinylpolypyrrolidone (PVPP)	Sigma Aldrich, Deisenhofen, Germany
Sodium Dodecyl Sulfate (SDS)	BDH, England
Urea	Bio-Rad, München, Germany
Tris	Fluka, Germany
Methanol	Kimix, South Africa
TEMED	Bio-Rad, München, Germany
Ammonium sulfate	Merck, Darmstadt, Germany
Ammonium acetate	Sigma Aldrich, Deisenhofen, Germany
IPTG	Fermentas, Vilnius, Lithuania
X-Gal	Fermentas, Vilnius, Lithuania
Ampillicin	Fluka, Germany
Magnesium chloride	Saarchem, South Africa
Sodium phosphate	Fluka, Germany
Sephacryl S-300	Pharmacia, Sweden
Bovine Serum Albumin	Roche, Germany
Betaine	Sigma Aldrich, Deisenhofen, Germany
DMSO	RDH, Germany
Triton X-100	BDH, England
Deoxynucleotide triphosphate	Roche, Germany

Orange G loading buffer	Promega, Madison, Wis. USA
Agarose	Bioline, England
Sodium hydroxide	Saarchem, South Africa
Sodium chloride	Kimix, South Africa
Sand quartz	Sigma Aldrich, Deisenhofen, Germany
Potassium chloride	Merck, Darmstadt, Germany
Na-salicylate	Fluka, Germany
Na-nitroprusside	Fluka, Germany
Na-dichloroisocyanurate	Fluka, Germany
Tryptone	Fluka, Germany
Yeast extract	Merck, Darmstadt, Germany
Enzymes	
Taq DNA polymerase	In-house prep. Arcam.
*BsuR*1	Fermentas, Vilnius, Lithuania
*Pst*1	Fermentas, Vilnius, Lithuania
Kits	
QIAquick® gel extraction kit	Qiagen, Hilden, Germany
TA cloning kit	Fermentas, Vilnius, Lithuania
Other materials	
SYBR Green 1	Roche, Germany

2.1. Media used in this study

LB agar Medium (Luria-Bertani Media)

Constituent	**L^{-1}**
Tryptone	10.0 g
Yeast extract	05.0 g
NaCl	10.0 g
Agar	15.0 g

The pH was adjusted to pH 7.0 with 5 N NaOH, made up to 1L.

SOB media

Constituent	**L^{-1}**
Tryptone	20.0 g
Yeast extract	05.0 g
NaCl	0.50 g
250 mM KCl	10.0 ml

The pH was adjusted to 7.0 before autoclaving. The medium was cooled to ~50 ^{o}C and 5.0 ml of filter sterilized 2 M $MgCl_2$ was aseptically added.

SOC Media

Constituent	**L^{-1}**
Tryptone	20.0 g
Yeast extract	05.0 g
NaCl	0.50 g
250 mM KCl	10.0 ml

The pH was adjusted to 7.0 before autoclaving; the medium was cooled to ~50 ^{o}C and the following filter sterilized and added aseptically, 5 ml of 2 M $MgCl_2$ and 20 ml of 1M glucose.

2.2. Ammonia media for growth of ammonia-oxidizing bacteria

Part 1:

Constituent	**L^{-1}**
50 mM $(NH_4)_2SO_4$	6.60 g
50 mM KH_2PO_4	0.41 g
1 M $MgSO_4$	0.75 ml
1M $CaCl_2$	0.20 ml
30 mM $FeSO_4$	0.15 ml
50 mM EDTA	0.15 ml
50 mM $CuSO_4$	0.01 ml

Dissolved in 900 ml of distilled water in a 2 liter flask and autoclaved at 121^{o}C

Part 2:

Constituent	**500 ml^{-1}**
KH_2PO_4	27.22 g
NaH_2PO_4	02.40 g

Dissolved in 500 ml of distilled water, the pH was adjusted to 8.0 with 10 N NaOH, then autoclaved in 100 ml volumes at 121^{o}C.

Part 3:

5% (w/v) $NaCO_3$ (anhydrous) was dissolved in 500 ml of distilled water, and filtered sterilize using a cellulose acetate membrane filter, 0.22 µM pore size.

For the bacteria growth

1x 100 ml aliquot of Part 2 added to 1x 900 ml of Part 1. 8 ml of part 3 added. Finally, 10 ml of 3 day old culture added to new media and incubated on rotary shaker (100- 150 rpm) at 30^{o}C for 4 to 5 days.

Table 2.2: Buffers used in this study

Buffer	**components**	**pH**
6x agarose loading buffer	30% (v/v) Glycerol	
	0.25% (w/v) Bromophenol blue	
	15% (w/v) glycerol	
10x Orange G loading buffer	60% Glycerol	
	0.25% (w/v) Orange G	
1x PCR	50 mM Tris-HCl	8.0
	100 mM NaCl	
	25 mM $MgCl_2$	
	1% Triton X-100	
Inoue	55 mM $MnCl_2.4H_2O$	6.7
	15 mM $CaCl_2.2H_2O$	
	250 mM KCl	
	0.5 M Pipes	
Potassium phosphate buffer	71.7 ml 1 M K_2HPO_4	7.2
	28.3 ml 1 M KH_2PO_4	
Sodium phosphate buffer	93.2 ml 1 M Na_2HPO_4	8.0
	6.8 ml 1 M Na_2HPO_4	
50x TAE	2 M Tris base	8.0
	10 mM Glacial acetic acid	
	0.5 M EDTA	
0.5x TBE	45 mM Tris-borate	8.3
	1 mM EDTA	
TE	10 mM Tris-HCl (pH 8.0)	8.0
	1 mM EDTA (pH 8.0)	

2.3. Bacterial strain

The pure culture of *Nitrosomonas europaea* ATCC 19718 used in this study as a positive control was kindly provided by Hommes Cardley, Department of Botany, Oregon State University, Corvallis, OR 97331-USA. The strain was grown in the dark at 30^{o}C for 3 days in liquid media supplemented with $(NH_4)_2SO_4$ as a nitrogen source. At stationary phase, the culture was harvested as described by Norton *et al.* (1996).

2.4. Environmental sampling

Soil samples were collected from the Kogelberg Biosphere Reserve, Western Cape, South Africa. Soil, pH and temperature profiles were measured at the time of sampling. Three species of the Proteaceae plant family (*Leucadendron xanthoconus, Leucospermum truncatulum* and *Leucadendron microcephalum*) were chosen to compare the diversity of the ammonia-oxidizing bacteria in soil associated with proteoid roots. Non-plant associated soil was used as a control. Soil cores were taken aseptically (2 cm diameter, to a depth of 6 cm) randomly from an area of approximately 800 m^2 (S34^{o}16.489' E19^{o}02.405', S34^{o}16.496' E19^{o}02.398', S34^{o}16.478' E19^{o}02.290', S34^{o}16.460' E19^{o}02.294). The soil samples were stored frozen at –80^{o}C until total DNA was extracted.

2.5. Ammonium concentration measurements

The ammonium concentration was measured by extraction of soil samples with 1 M KCl (1g of soil: 10ml KCl) on a shaker (200 rpm) for 1 hour at 4^{o}C, centrifugation for 10 minutes at 4^{o}C and filtering using cellulose acetate membrane filters, 0.22 µM pore size. Samples were stored at -20^{o}C before colorimetric analysis (Kandeler and Gerber, 1988): solution I (Na-salicylate [8.5g/100 ml]: Na-nitroprusside [0.06g/100 ml]) was added to the soil sample (1:1, v/v) and vortexed briefly for a few seconds. After addition of solution II (Na-dichloroisocyanurate [1g/l] and further dilution [1:3] with 300 mM NaOH), samples were incubated for at least 30 min in the dark. The absorbance at 690 nm was measured spectrophotometrically.

2.6. DNA extraction from soil samples

Extractions of total genomic DNA were performed by a modification of the method described by Miller *et al.*, (1999). Approximately 0.5 g of each soil sample was added to a 2 ml screw-cap plastic vial containing 0.5 g of sterile sand quartz (Sigma S-9887 [50-70 mesh= 0.21-0.30 mm]). 300 µl each of sodium phosphate buffer (pH 8.0) and SDS lysis buffer (10% w/v sodium dodeycl sulphate, 100 mM NaCl, 500 mM Tris-HCl pH 8), was added and mixed gently, followed by 300 µl of chloroform/isoamyl alcohol (v/v). The mixture was vortexed for 120 s at maximum speed (Chiltren MT19), centrifuged (15,000 x g for 5 minutes), and the supernatant transferred to new tubes. Ammonium acetate added to a final concentration of 2.5 M (5/9 volume). The mixture was shaken to ensure thorough mixing, centrifuged at 15,000 x g for 10 min. The supernatant

transferred to the new tube. To precipitate the DNA, 0.6 volumes of isopropanol were added, mixed and incubated at RT for 15 min followed by centrifugation (15,000 x g for 10 min), and the pellet washed with 1 ml of ice-cold 70% EtOH. After further centrifugation (15,000 x g for 10 min), the pellet was allowed to air dry and the DNA was resuspended in 100 µl of TE. DNA extracted by this method was subjected to further purification. The crude genomic DNA samples were loaded onto a 0.7% (wt/vol) agarose gel. After electrophoresis, the genomic DNA bands were excised from the gel and purified using the Qia quick purification Kit (Qiagen). The purified genomic DNA was eluted with TE buffer, and quantitated using the Nanodrop ND-1000 spectrophotometer at 260 nm (Delaware-USA).

2.7. Purification of DNA using Polyvinylpolypyrrolidone (PVPP) minicolumns

Where genomic DNA was purified as described by Berthelet *et al.*, (1996), aliquots (50-150 µl) of the supernatants were further purified by centrifugation at 2,000 xg for 2 minutes through spin column. The columns were filled with about 200 µl of acid-washed PVPP (Sigma P-6755) which was equilibrated in 20 mM potassium phosphate buffer (pH), followed by washing twice with 150 µl of TE (pH 8.0), and centrifuging twice at 2,000 xg for 2 minutes. The columns were dried further by centrifugation at 3,000 xg for 10 minutes, then placed in new 1.5 ml Eppendorf tubes. Subsequently, the 100 µl total genomic DNA was added to the columns and incubated for 1 minute at room temperature, then eluted by

centrifugation first at 3,000 xg for 5 minutes, then at 5,000 xg for 10 minutes in TE buffer (pH 8.0). The DNA concentration of the purified extracts was determined as described in section 2.3.

2.8. Agarose gel electrophoresis

Total genomic DNA fragments and PCR products were separated in 1% and 1.5% (w/v) agarose gels, respectively, prepared in 0.5x TBE buffer (Sambrook *et al*., 1989). Samples were prepared by mixing with 6x concentrated loading buffer (20% (v/v) glycerol and 5 mg/ml bromophenol). Electrophoresis was performed in 0.5x TBE buffer at 100 V. Ethidium bromide solutions (0.5 µg/ml), was added to the agarose gels during the preparation for the staining procedure. The DNA bands were sized according to their migration in the gel as compared to DNA molecular weight markers (e.g., λ DNA cut with *Pst*1 restriction enzyme). Gels were visualized via ultraviolet (UV) light illumination and photographed with a digital imaging system (Alphalmager 2000, Alpha innotech, San Leandro, CA).

2.9. PCR amplification of *amoA* gene fragments

Purified genomic DNA was used as a template for the PCR amplification using primers specific for the ammonia monooxygenase operon. For the first round, primer sets (305F and 308R) (Table 2.4) were used to amplify a fragment of approximately 1.5 kb which was used as a template for the second round, with *amoA* gene specific primer sets were used (amoAF1 and amoARevino) to amplify

a fragment of approximately 491 bp (Table 2.4). Reaction mixtures were prepared in a total volume of 50µl and contained PCR buffer (50 mM Tris-HCl, 100 mM NaCl, and 1% Triton X-100 [pH 8]), 2.5 mM $MgCl_2$, 200 µM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 0.5 µM of each primer, PCR additives (200 mM betaine, 1% BSA, 3% DMSO), 10-20 ng of template DNA and *Taq* DNA polymerase (2.5 U).

PCR thermocycling conditions were: 94^{o}C for 5 min, 25 cycles (94^{o}C for 1 min, 55^{o}C for 1 min, 72^{o}C), and a final extension at 72^{o}C for 7 min for first round amplification, and for *amoA* second round amplification PCR thermocycling conditions were; initial denaturation at 94^{o}C for 3 min, followed with 35 cycles of (94^{o}C for 30 second, 57^{o}C for 30 second, 72^{o}C for 1.20 min), final extension at 72^{o}C for 5 min. PCR products were resolved by electrophoresis on 1.5% (wt/vol) agarose gels in 0.5x TBE buffer with λ DNA cut with *Pst*1 restriction enzyme as a molecular marker. The gels were stained with ethidium bromide (0.5 µg/ml), and examined using a UV transilluminator. PCR amplicons were purified using the Qia quick PCR purification Kit (Qiagen) according to manufacturer's instructions.

2.10. Colony PCR

Libraries of *amoA* gene were screened to confirm that inserts of the correct size were present. DNA template were prepared by resuspending cells in 40µl of the TE buffer (pH 8.0), heating at 99^{o}C for 3 minutes, followed by centrifugation at 16,000 xg for 2 minutes. The recovered supernatant were transferred to a new 0.6

ml Eppendorf tube, and was stored frozen at –20°C until used as a template in a PCR amplification (10 µl in a standard PCR reaction).

Table 2.3: Primers sets used for amplification of *amoA* genes

Primer	Target gene	sequence	Tm	Reference
305F	*amoC*	5'GTGGTTTGGAACRGICARAGCAAA-3'	55°C	Norton *et al.*, 2001
308R	*amoB*	5'TCCCAGCTKCCGGTRATGTTCATCC-3'	55°C	Norton *et al.*, 2001
304R	*amoA*	5'TAYCGCTTCCGGCGGCATTTTCGCCGC-3'	55°C	Norton *et al.*, 2001
amoARevino	*amoA*	5'CCCCTCXGXAAAGCCTTCTTC-3'	57°C	Rotthauwe *et al*., 1997
amoAFinoGC	*amoA*	5'CGCCCGCCGCGCCCCGCGCCCGTCCCGC CGCCCCCGCCCGGGGGXTTXTACTGGT-3'	57°C	Rotthauwe *et al*., 1997
amoAFI	*amoA*	5'GGGGXTTXTACTGGTGGT-3'	57°C	Rotthauwe *et al*., 1997
M13F	598-615*	5'-GTAAAACGACGGCCAGT-3'	50°C	Yanisch-Perron *et al*., 1985
M13R	734-751*	5'-CAGGAAACAGCTATGAC-3'	50°C	Yanisch-Perron *et al*., 1985

*M13 primers position related on pTZ57R/T vector

2.11. Preparation of *Escherichia. coli* competent cells

Competent *E. coli* DH5α cells were prepared and transformed as described by Inoue *et al*. (1990). A single colony of a freshly streaked culture of *E. coli* DH5α

on SOB (Section 2.1) media was inoculated into 20 ml SOC media (Section 2.1) and cultured for 8 hours at 37°C with agitation at 250 rpm. 2 ml of the overnight culture was inoculated into a 250 ml sterile flask at 18^{o}C and incubated overnight with shaking to mid-exponential phase (OD_{600} of 0.4 to 0.55). The cells from 250 ml of the culture were pelleted in polypropylene tubes by centrifugation at 4,000 × *g* for 10 min at 4°C in a J2-21M rotor (Beckman-USA). The supernatant was decanted and the pellet washed twice with transformation buffer. The cells were resuspended gently in 2 ml of ice-cold Inoue transformation buffer, to which 150 µl of DMSO was added. Following incubation on ice for 15 min, the cells were aliquoted into 0.5 ml Eppendorf tubes (50 µl), frozen immediately using liquid nitrogen and stored at -70°C until needed.

2.12. Ligation of *amoA* PCR products

Ligation reactions were performed in a 10µl volume containing the vector (pTZ57R/T) and insert DNA (1 vector:2 insert molar ratio), 1x ligation buffer, 1 U of T4 DNA ligase enzyme, 5% of PEG 4000 and sterile water (Insta/clone kit-Fermentas), according to manufacturer's instructions. The reactions were incubated overnight at 16^{o}C, and subsequently transformed into *E. coli* DH5α competent cells using the heat-shock method (Sambrook *et al.*, 1989).

2.13. Transformation of competent *E. coli* DH5α cells

E. coli DH5α competent cells were transformed by the addition of 1 µl ligated DNA to 50 µl of the competent cells in a sterile 1.5 ml Eppendorf tube. The cells were incubated on ice for 5-10 min prior to the transformation, followed by heat shock at 42^{o}C for 90 seconds and then incubation on ice for a further 2 minutes. After an addition of 950 µl of SOC, the transformation mixtures were incubated for 1 hour at 37°C with shaking at 200-250 rpm to allow recovery. An aliquot (50-100 µl) of the cells was plated on LB- agar plates supplemented with ampicillin (100 µg/ml), IPTG (20 µg/ml), and X-Gal (30 µg/ml) for blue/white selection. Agar plates were incubated overnight at 37°C.

2.14. The *amoA* gene library analysis

Recombinants (white colonies) were randomly picked and putative *amoA* genes re-amplified using *amoA* specific primer sets to confirm that inserts of the correct size were present. PCR products were visualized on 1.5% agarose electrophoresis gels. The clones were screened using Restriction Fragment Length Polymorphism (RFLP); the *amoA* clones were digested with restriction enzyme *BsuR*1 (*Hae*III) (recognition site GG/CC). Digestions were performed by preparing reaction volumes of 15 µl in sterile 0.2 ml Eppendorf tubes. The reactions contained 5 µl of PCR product, the appropriate 10 × buffer (1.5 µl) supplied by the manufacturer (Fermentas), and 1-2 U of the appropriate restriction enzyme. The reactions were typically incubated at 37^{o}C overnight. The digestion products were analyzed by

electrophoresis on 2.5% (w/v) agarose gels. Unique clones were sequenced using the Inqaba Biotechnology sequencing service (Pretoria-South Africa). The complete sequences of the *amoA* library clones were determined by using specific M13 primers targeting pTZ57R/T vector sequences flanking the multiple cloning sites.

2.15. DGGE analysis

The *amoA* fragments were amplified using the amoARevino/amoAFino primer pair (Table 2.3); PCR reaction mixtures were prepared as in Section 2.6. PCR amplification for the first round using primer sets (305F and 308R). Thermocycling conditions were as described in section 2.6 for first round amplification. PCR reaction mixtures were prepared in a total volume of 50µl contained 10-20 ng metagenomic DNA (Section 2.6) with DGGE primers (amoAFino-GC/amoARevino) (Table 2.4). For *amoA* gene second round amplification thermocycling condition; initial denaturation at 94^{o}C for 3 min, followed with 35 cycles of (94^{o}C for 45 second, 57^{o}C for 30 second, and 72oC for 1.20 min), final extension at 72^{o}C for 10 min. 10 µl of PCR products were loaded on 16.5x16.5 cm, 1 mm thick 9% polyacrylamide gels with a 35% to 55% denaturant gradient.

Gels were electrophoresed for 16 h at a constant voltage of 100 V in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA [pH adjusted to 8 with acetic acid]) at a constant temperature of 60^{o}C using a Scie-Plas (V20) system. Gels were stained with 0.5 µg/ml ethidium bromide and were examined under UV

light. Bands of interest were further analysed. For DNA sequence analysis, bands were excised and were briefly washed in 500 µl of H_2O and vortex followed by centrifugation at 16,000 rpm for 2 minutes. The supernatant was discarded and gel slices were resuspended in 50 µl of TE buffer (Table 2.2), and incubated for 24-48 hours at RT. 1 µl of supernatant was used as a template for re-amplification using the same PCR conditions. Re-amplified bands were cloned into pTZ57R/T (InsT/Aclone, Fermentas) according to the manufacturer's instruction and sequenced with M13 primers (Table 2.4).

2.16. Phylogenetic analysis

Chromatograms of DNA sequences were edited using Bio-Edit version 5.0.9 (Freeware) software. Analysis of DNA sequences and homology searches were carried out using software from the National Centre for Biotechnology Information (NCBI). The Basic Local Alignment Search Tools (BLASTn and BLASTp) programmes (Altschul *et al.*, 1997) were used to determine sequence similarity and identity to known species in the GenBank database (www.ncbi.nlm.nih.gov/).

AmoA sequences generated from *amoA* gene libraries were edited and grouped with define sequences for further analysis. Multiple sequence alignments were carried out using ClustalW (Hall, 1999). Alignments were performed for both the nucleotides and predicted amino acids sequences. Translations of *amoA* gene

sequences were performed by using DNAMAN program version. 4.13. Phylogenetic trees based on *amoA* nucleotide and deduced amino acids sequences were constructed using the Phylo-Win program. For phylogenetic analysis of the *amoA* gene sequences, the trees were initially constructed using Neighbour-joining algorithm (Jukes-Cantor algorithm) with more than 40 *amoA* NCBI database sequences to guide phylogenetic placement. Final trees were constructed using the Neighbour-joining algorithm for *amoA* gene sequences and the Kimura two-parameter distance measure. Bootstrapping support values generated from 100 replications was used to establish confidence in the tree topology. Phylogenetic trees were visualized using Treeview software, (version 3.5).

3.1. Introduction

The biological process of ammonia oxidation is central to global nitrogen cycling and is performed by autotrophic ammonia-oxidizing bacteria (AOB) that form a monophyletic group within the β-*Proteobacteria* (Webster *et al*., 2005). Recently, AOB have shown great potential in biotechnological processes such as wastewater treatment (Calvó *et al*., 2005). This group of microorganisms is ecologically essential, being the major group that oxidizes ammonia to nitrite, and is abundant in all environments in which nitrogen is mineralized (Aarkra *et al*., 1999).

In recent years, molecular approaches have been used extensively to characterize microbial populations of AOB in a variety of environments (Amann *et al*., 1995). In analyzing environmental samples many researchers turned to modern molecular tools based on PCR, DGGE, and phylogenetic analysis of the ammonia monooxygenase (*amoA*) gene as a functional marker to study the diversity, distribution and functional activity of this important group of microorganisms in the environment.

The objective of the study reported in this chapter was to use molecular techniques to examine the presence, diversity and distribution of AOB in soils associated with Fynbos plant roots, using the *amoA* gene as a molecular marker (Aakra *et al*., 2001).

3.2. Ammonia concentration measurement

The determination of ammonium concentrations in each soil sample was performed using the method described by Kandeler and Gerber (1988). Comparisons of the ammonium concentration of different soils showed that the highest concentration was found in samples of non-plant associated soil, while the lowest concentrations were recorded in samples of soils associated with the Proteaceae plant species (Fig. 3.1). The ammonium concentrations in plant-associated soil samples were similar (Table 3.1). These data suggest that Proteaceae plant species compete with ammonia oxidizing microorganism for the utilization of free ammonia.

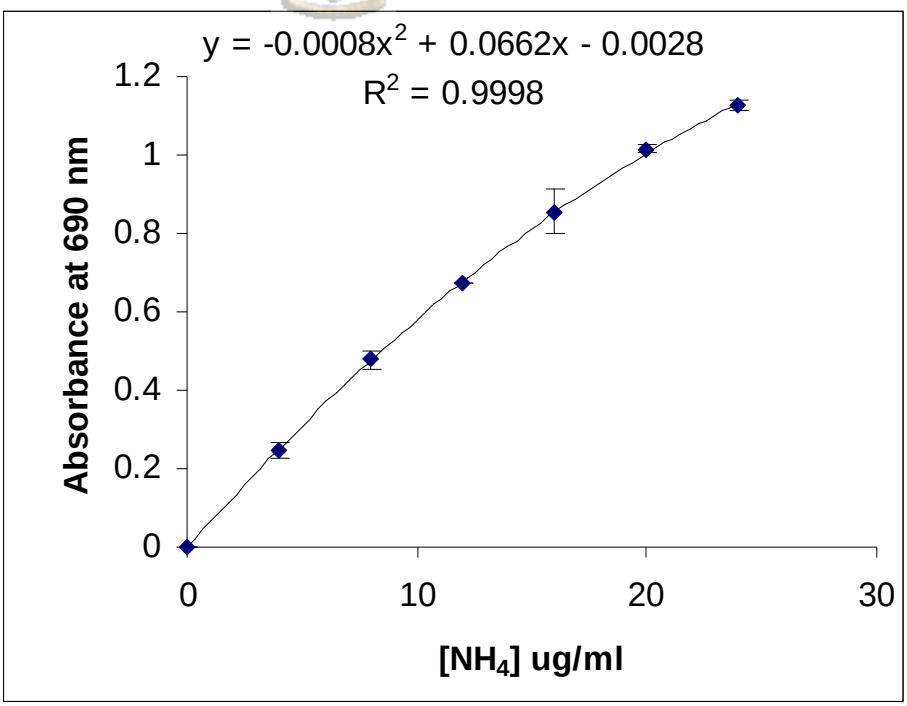


Fig. 3.1: Standard curve of ammonium concentration.

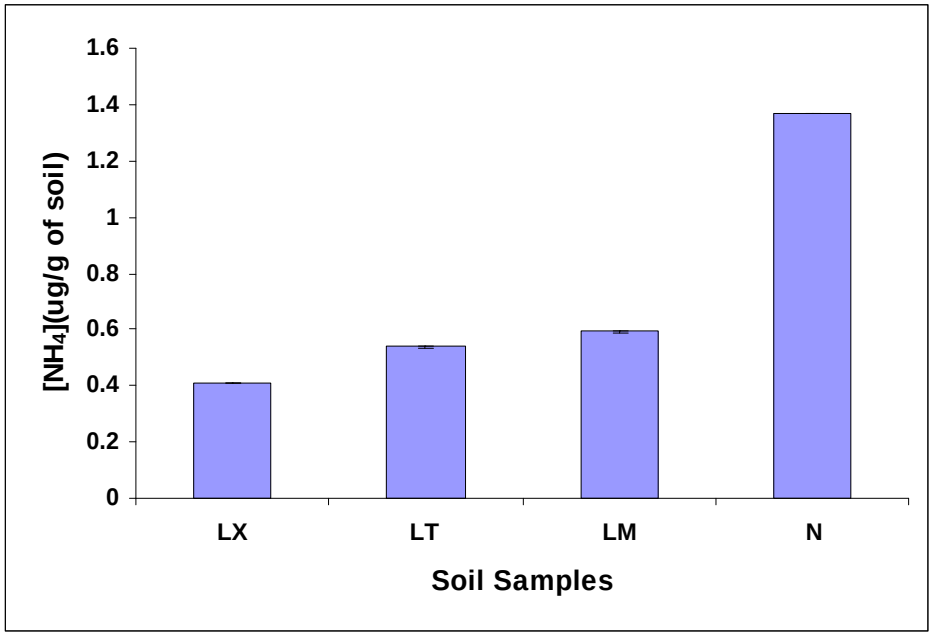


Fig. 3.2: Ammonium concentration values from different samples of Proteaceae rhizosphere soils. LX (*Ld. xanthoconus* rhizosphere), LT (*Ls. truncatulum* rhizosphere), LM (*Ld. microcephalum* rhizosphere) and N (Non-plant associated soil); Error bars show the standard error of triplicate determinations.

Table 3.1: Characteristics of the Proteaceae rhizosphere soils sampled April 2003.

Soil Characteristics	***Leucadendron xanthoconus*-rhizosphere**	***Leucospermum truncatulum*-rhizosphere**	***Leucadendron microcephalum*-rhizosphere**	**Non- plant associated soil**
Ammonium(µg/g soil)	0.4	0.5	0.6	1.3
pH	5.5	5.4	5.5	5.3
Temperature	17^{o}C	15^{o}C	18^{o}C	20^{o}C
Soil texture	Sandy	Sandy	Sandy	Sandy

3.3. DNA extraction

Community DNA was successfully extracted from all soil samples using the method described by Miller *et al*. (1999). The extracted DNA was of high molecular weight, in the range of approximately 10-15 kbp (Fig. 3.3). The crude DNA was further purified by agarose gel electrophoresis and DNA concentrations were determined spectrophotometrically (Section 2.5) (Table 3.2).

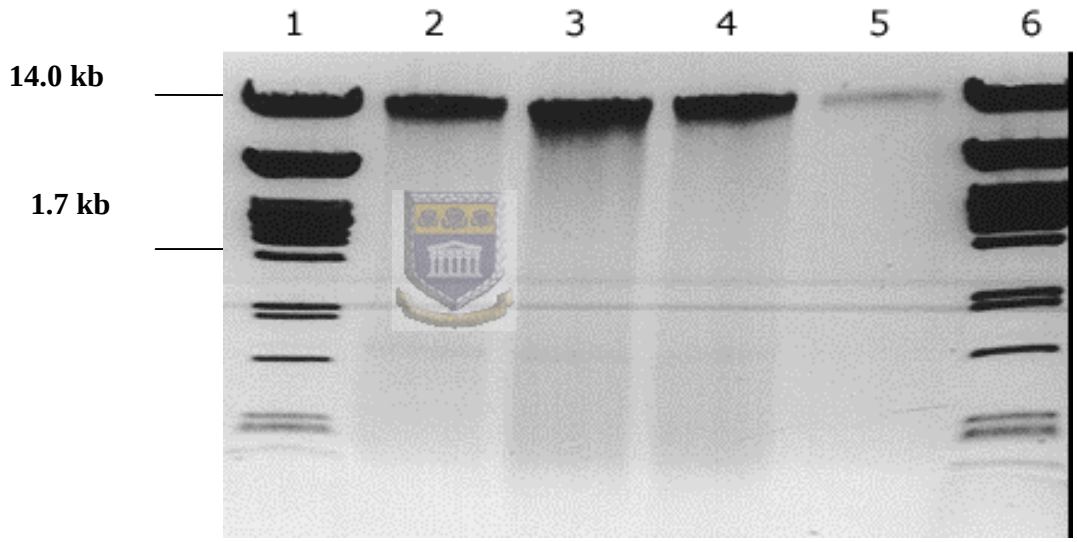


Fig. 3.3: Total genomic DNA from four soil samples collected from Fynbos ecosystems. Lane 1: molecular marker λ DNA cut with *Pst*I restriction enzyme; Lane 2: LX (*Ld. xanthoconus* rhizosphere); Lane 3: LT (*Ls. truncatulum* rhizosphere); Lane 4: LM (*Ld. microcephalum* rhizosphere); Lane 5: N (Non- plant associated soil); Lane 6: molecular marker λ DNA cut with *Pst*I restriction enzyme.

Table 3.2: Concentration and purity of DNA extracts from Fynbos soils

Sample	**Concentration (**ng/µl)	**260/280**
LX	22.2	1.78
LT	15.5	1.75
LM	30.0	1.78
N	12.2	1.87
*NE	10.1	2.81

*NE (*Nitrosomonas europaea*)

3.4. Primers for PCR amplification of the *amoC- amoA* ITS

The PCR primer sets (305F and 304R, Fig. 3.6), targeting the internal transcribed spacer region (ITS) of *amoC-amoA* subunits (Norton *et al.*, 2002), were used to determine the diversity AOB of β-*Proteobacteria* in environmental samples. Comparisons of ITS regions from closely related AOB have been used as a standard for differentiation, since the ITS region shows higher sequence variability than the *amoA* gene sequences from different organisms. Additionally, the size variation of this ITS region has been used to characterize AOB species (Table 3.3)

PCR amplicons were used to construct clone libraries which were screened for variation in the insert size and sequence by restriction fragment length polymorphism (RFLP) (Fig. 3.4 and 3.5). These identified 7 different representative groups suggesting that different AOB genera were present in environmental samples.

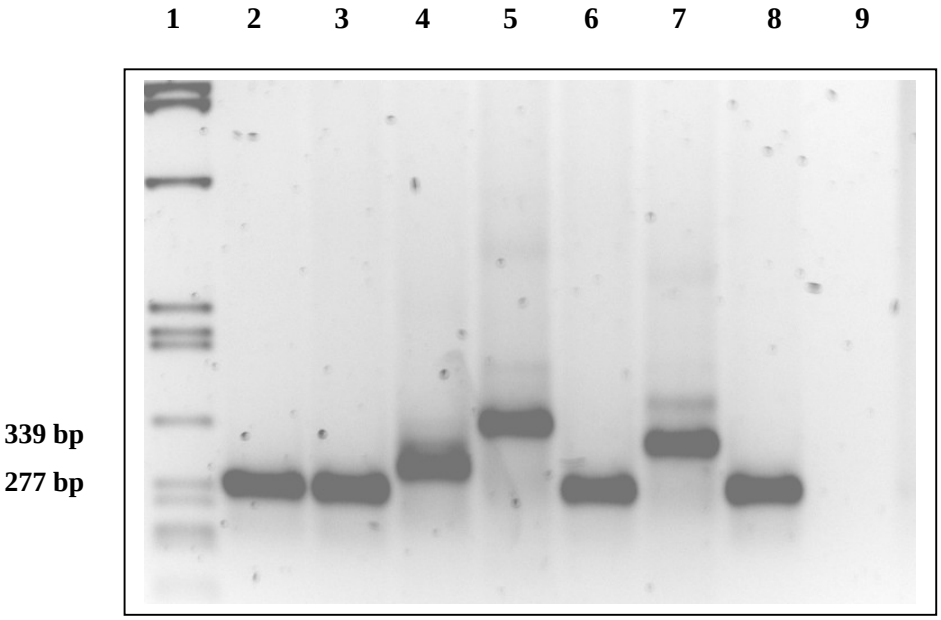


Fig. 3.4: PCR amplicons of the *amoC* - *amoA* intergenic region. Lane 1: molecular marker λ DNA cut with *Pst*I restriction enzyme, Lane 2-4: LX (*Ld. xanthoconus* rhizosphere) clones, Lane 5-7: LT (*Ls. truncatulum* rhizosphere) clones, Lane 8: N (Non-plant associated soil) clone, Lane 9 Negative control.

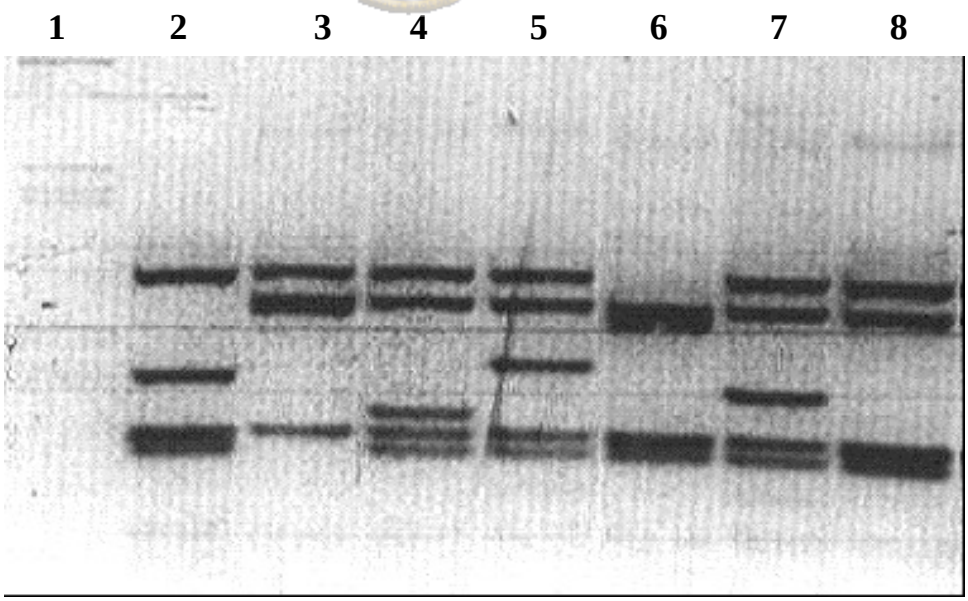


Fig. 3.5: RFLP analysis of *amoA* gene amplicons digested with *BsuRI* (*Hae*III) restriction enzymes. Lane 1: molecular marker λ DNA cut with *Pst*I restriction enzyme, Lane 2-4: LX (*Ld. xanthoconus* rhizosphere) clones, Lane 5-7: LT (*Ls. truncatulum* rhizosphere) clones, Lane 8: N (Non- plant associated soil) clone.

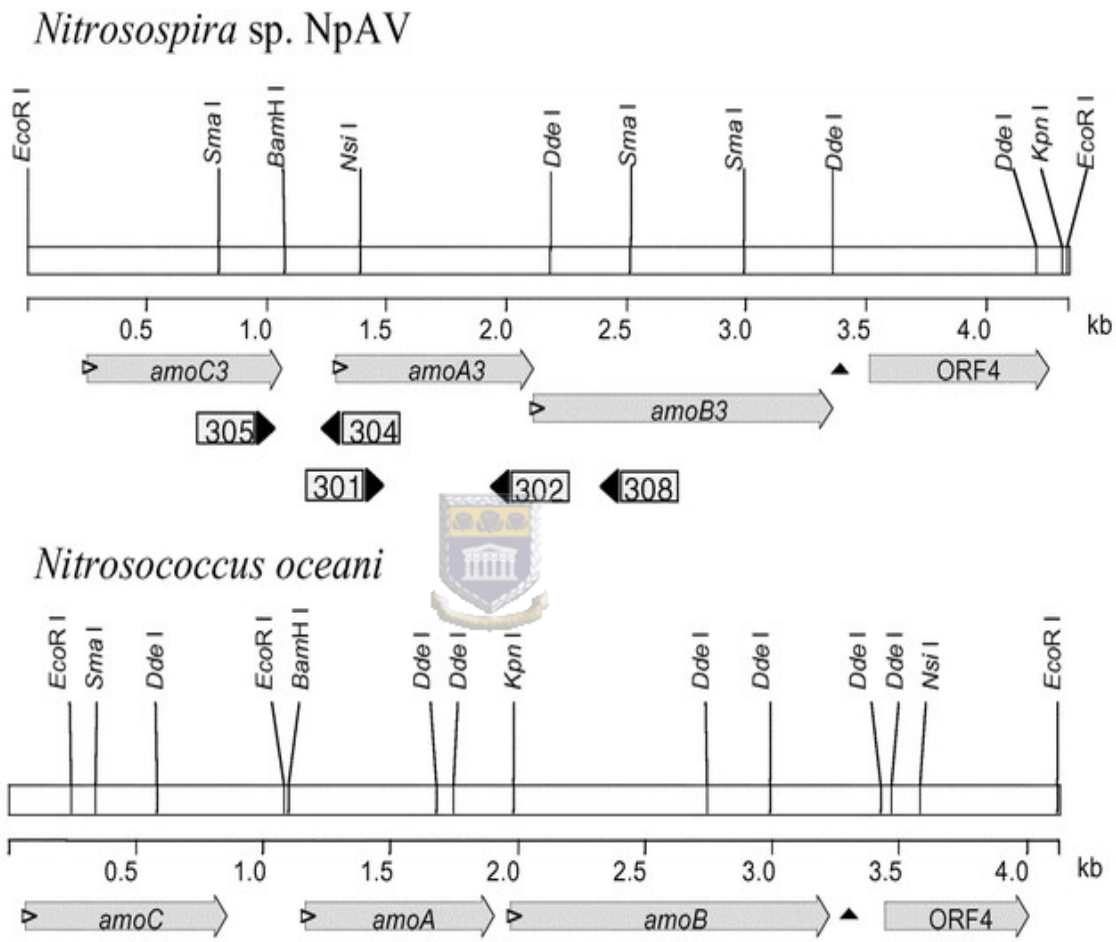


Fig. 3.6: Physical map of the *amo* operon in *Nitrosospira* sp. NpAV and *Nitrosococcus oceani* showing the four ORFs; *amoC*, *amoA* and *amoB*, and ORF4. The target sites for various primers are shown as well as the putative ribosome binding sites (open right-facing triangles) and putative transcriptional terminator (▲) for the *amo* operon reproduced from Norton *et al*., (2002).

Table 3.3: The length of the *amoC* to *amoA* intergenic variable region and the length of amplified region across from the end of *amoC* to *amoA* (From primer 305F to 304R) (Norton *et al*., 2001).

Strain	*amoC-amoA* intergenic region (bp)	305F-304R Amplicons (bp)
Nitrosospira sp. NpAV	223	331
Nitrosospira briensis C-128	263	371
Nitrosospira sp. 39-19	445	553
Nitrosospira tenuis NV-12	427	535
Nitrosospira multiformis 25196	323	431
Nitrosospira multiformis 24C	261	369
Nitrosomonas europaea 19178	163	277
Nitrosomonas eutropha C-19	173	287
Nitrosomonas sp. AL212	174	282
Nitrosomonas cryotolerans	195	305
Nitrosomonas sp. GH22	173	287
Nitrosomonas sp. JL21	173	281

3.5. PCR amplification of the *amoA* gene fragment

An *amoA* gene fragment was amplified using a nested PCR method. The primer sets 305F and 308R were used for the first round to increase the relative abundance of the target *amoA* sequence. Approximately 1500 bp was generated for further amplification (Fig. 3.6). For the second round, the primer sets amoAF1 and amoARevino were used to target a 491 bp fragment of *amoA* gene (Fig. 3.7), which encodes the active site polypeptide of the ammonia monooxygenase enzyme (MeTavish *et al.*, 1993).

The specificity of the primers amoAF1 and amoARevino has been tested by Francis *et al*. (2003) and Purkhold *et al*. (2003). These studies showed that this PCR primer set has a broad coverage and is specific for all β-*Proteobacteria* AOB populations. Furthermore, several studies have shown that *amoA* is an effective molecular marker for identifying ammonia-oxidizing bacteria in environmental samples (Nicolaisen and Ramsing, 2002).

For DGGE analysis, the same primer set was used except that the forward primer had a 40 bp GC-clamp (Table 2.4).

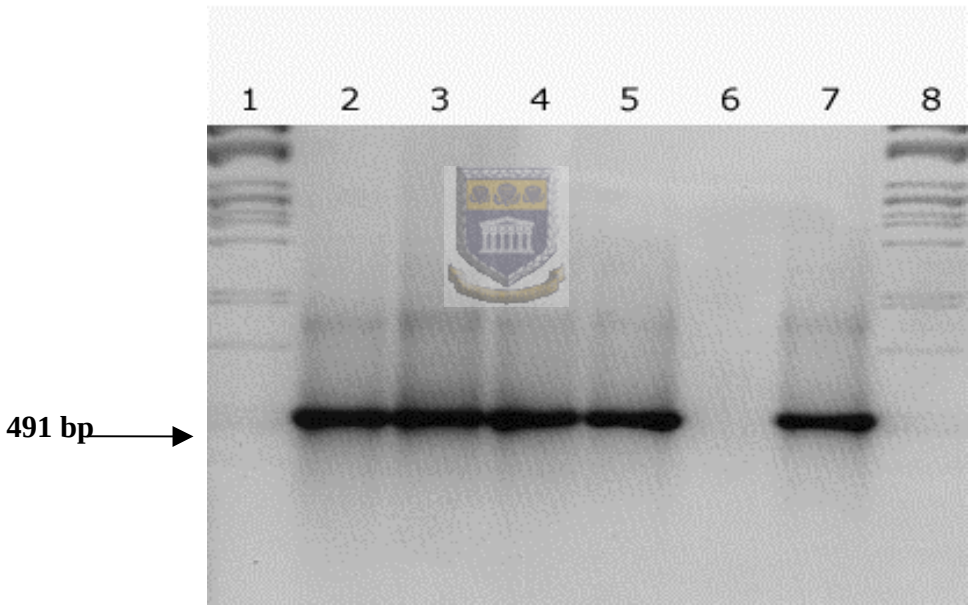


Fig. 3.7: Amplification of the *amoA* gene of ammonia oxidizing bacteria using the amoAFino and amoARevino primer set. The total genomic DNA was used as a template and the PCR products were separated with 1.5% EtBr-agarose gel. Lanes: 1 molecular markers λ DNA cut with *Pst*I restriction enzyme; Lane: 2 LX (*Ld. xanthoconus* rhizosphere); Lane: 3 LT (*Ls. truncatulum* rhizosphere); Lane: 4 LM (*Ld. microcephalum* rhizosphere); Lane: 5 N (Non- plant associated soil); Lane: 5 Negative control; Lane: 6 Positive control (*Nitrosomonas europaea*); Lane: 7 Molecular marker λ DNA cut with *Pst*I restriction enzyme.

3.6. *AmoA* gene library

The amoAFino and amoARevino PCR products were used to construct clone libraries. A total of 120 randomly selected clones from the four different environmental samples, (approximately 30 clones for each library) were subjected to further analyses by RFLP and DNA sequencing.

3.6.1. Restriction Fragment Length Polymorphism (RFLP) analysis

RFLP analysis is the most convenient tool to assess the complexity of AOB communities in environmental samples. RFLP generates profiles based on differences in the *amoA* gene sequences. The tetrameric restriction enzyme *Bsu*RI (recognition site: GG/CC) was used to examine randomly selected *amoA* clones (Horz *et al*., 2000).

The RFLP patterns generated with *Bsu*RI digests have previously been shown to enable different AOB genera to be distinguished (Santomassimo *et al*., 2003). In this study the restriction enzyme *Bsu*RI produced 4-5 distinct patterns (Fig. 3.10). The RFLP analysis allowed the overall diversity of *amoA* gene sequences from the clone libraries to be determined (Fig. 3.8 and 3.9).

AmoA clones yield a range of fragment sizes after restriction digest and electrophoresis (Table 3.4). Two observations can be made from the comparative analysis of RFLP patterns clones of soil samples. Firstly, the 124 bp fragment was

present in all samples except in LT. Secondly, the number of the different RFLP profiles was the lowest in LT soil.

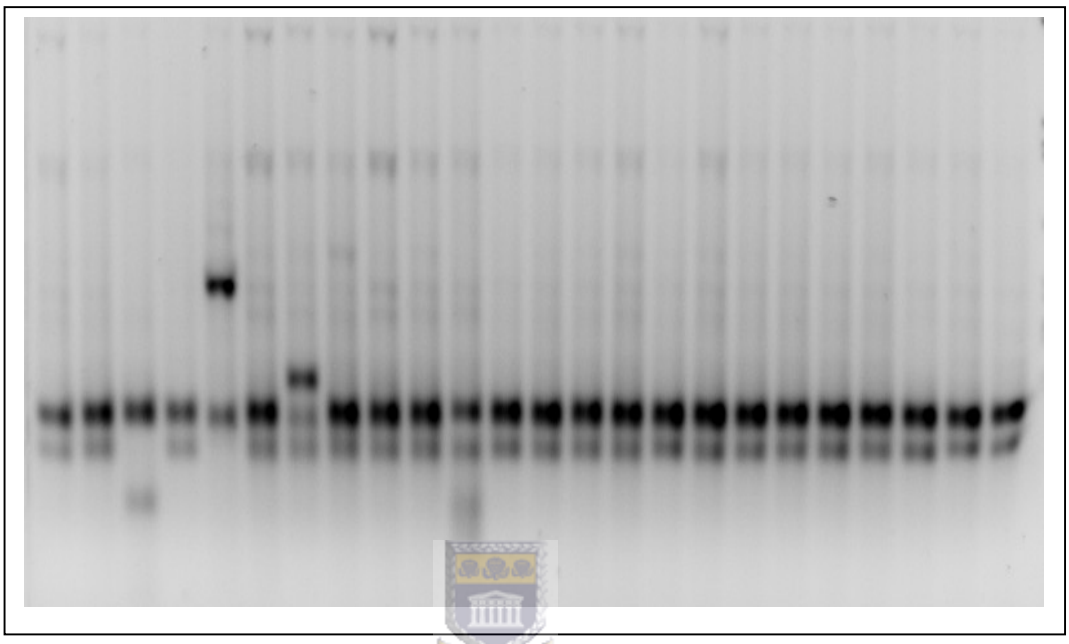


Fig. 3.8: Restriction analysis (RFLP analysis) of *amoA* clones of β-*Proteobacterial* ammonia-oxidizing bacteria with restriction endonuclease *BsuR*1; Lane 1: Molecular marker λ DNA cut with *Pst*I restriction enzyme; Lane 2-25: LX (*Ld. xanthoconus* rhizosphere).

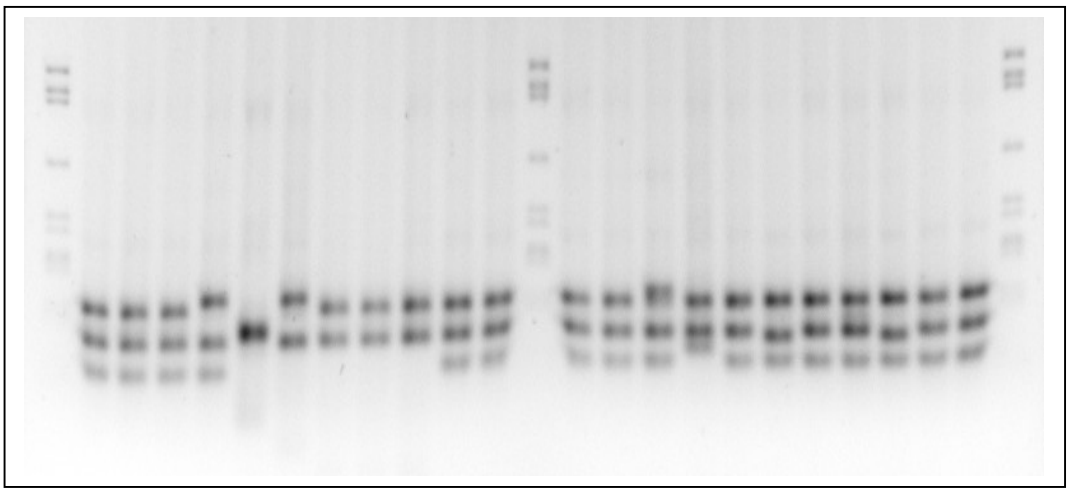


Fig. 3.9: Restriction analysis (RFLP analysis) of *amoA* clones of β-*Proteobacterial* ammonia oxidizing bacteria with restriction endonuclease *BsuR*1; Lane 1: Molecular marker λ DNA cut with *Pst*1 restriction enzyme; Lane 2-12: LT (*Ls. truncatulum* rhizosphere); Lane 13: molecular marker λ DNA cut with *Pst*1 restriction enzyme; Lane 13-24 (*Ls. truncatulum* rhizosphere); Lane 25: molecular marker λ DNA cut with *Pst*1 restriction enzyme

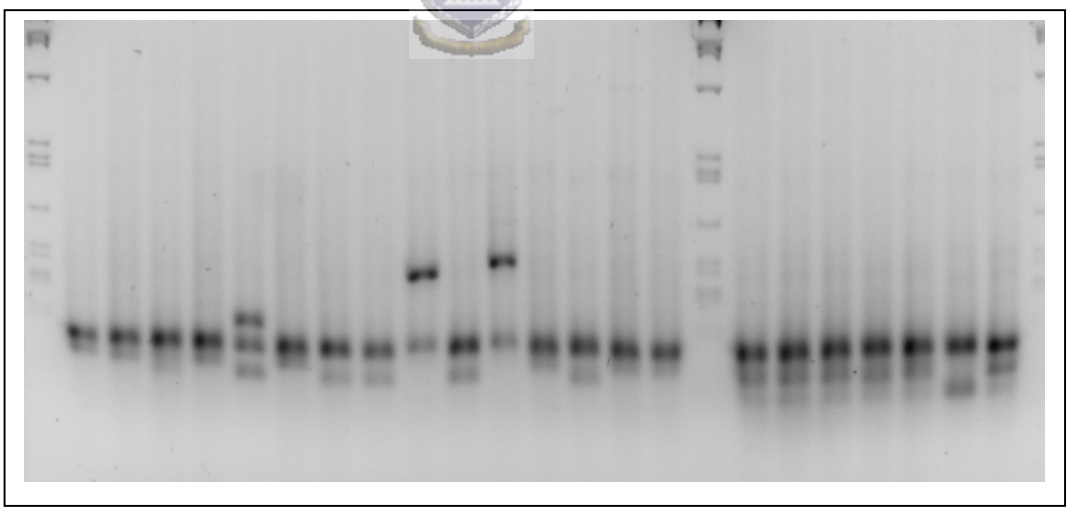


Fig. 3.10: Restriction analysis (RFLP analysis) of *amoA* clones of β-*Proteobacterial* ammonia oxidizing bacteria with restriction endonuclease *BsuR*1; Lane 1: Molecular marker λ DNA cut with *Pst*1 restriction enzyme; Lane 2-12: LT (*Ld. microcephalum* rhizosphere); Lane 13: molecular marker λ DNA cut with *Pst*1 restriction enzyme; Lane 13-24 (*Ld. microcephalum* rhizosphere); Lane 25: molecular marker λ DNA cut with *Pst*1 restriction enzyme.

3.6.2. Clone library collection curves

Collection curves were produced by comparing the number of clones in each *amoA* gene library of each soil samples to the total number of different RFLP patterns that were generated (Fig. 3.12). Analysis of more than 80 clones using the restriction endonuclease *BsuR*I generated four and five different RFLP patterns in the clone libraries. The coverage in each library was calculated using the following equation:

Coverage % = (N-n/N) x 100.

Where N = Number of clones analysed and n = number of the singleton clones (number of clones analysed showing single, unique RFLP)

The clone coverage of each library was determined based on the number of singleton clones from each library (Begon *et al*., 1996). For LX-, LT-, LM-rhizosphere soils, and non-plant associated soil the coverage was 80%, 82%, 77%, and 75%, respectively.

A comparison of the abundance of these RFLP patterns in each library indicated that different *amoA* gene variants dominated each soil sample. DNA sequencing of representative clones of each soil sample, were analysed using DNA club software to identify *amoA* gene variants. These data showed that the *amoA* sequences belong to three distinct groups (Fig. 3.11).

Table 3.4: RFLP analysis of fragment length based on *BsuR*I restriction endonuclease in Proteaceae Rhizosphere soils

Samples	**RFLP patterns**	**Fragment lengths**	**# clones (%)**
Ld. xanthoconus	R1	158/146/124	83%
	R2	158/146/124/25	4%
	R3	158/146/25	4%
Ls. truncatulum	R1	158/149/115	37%
	R2	158/135/135	5%
	R3	158/135	18%
Ld. microcephalum	R1	135/124/111	45%
	R2	135/124/47	5%
	R3	135/124	36%
Non-plant associated soil	R1	135/124/111	50%
	R2	135/124/47	30%
	R3	135/124	15%

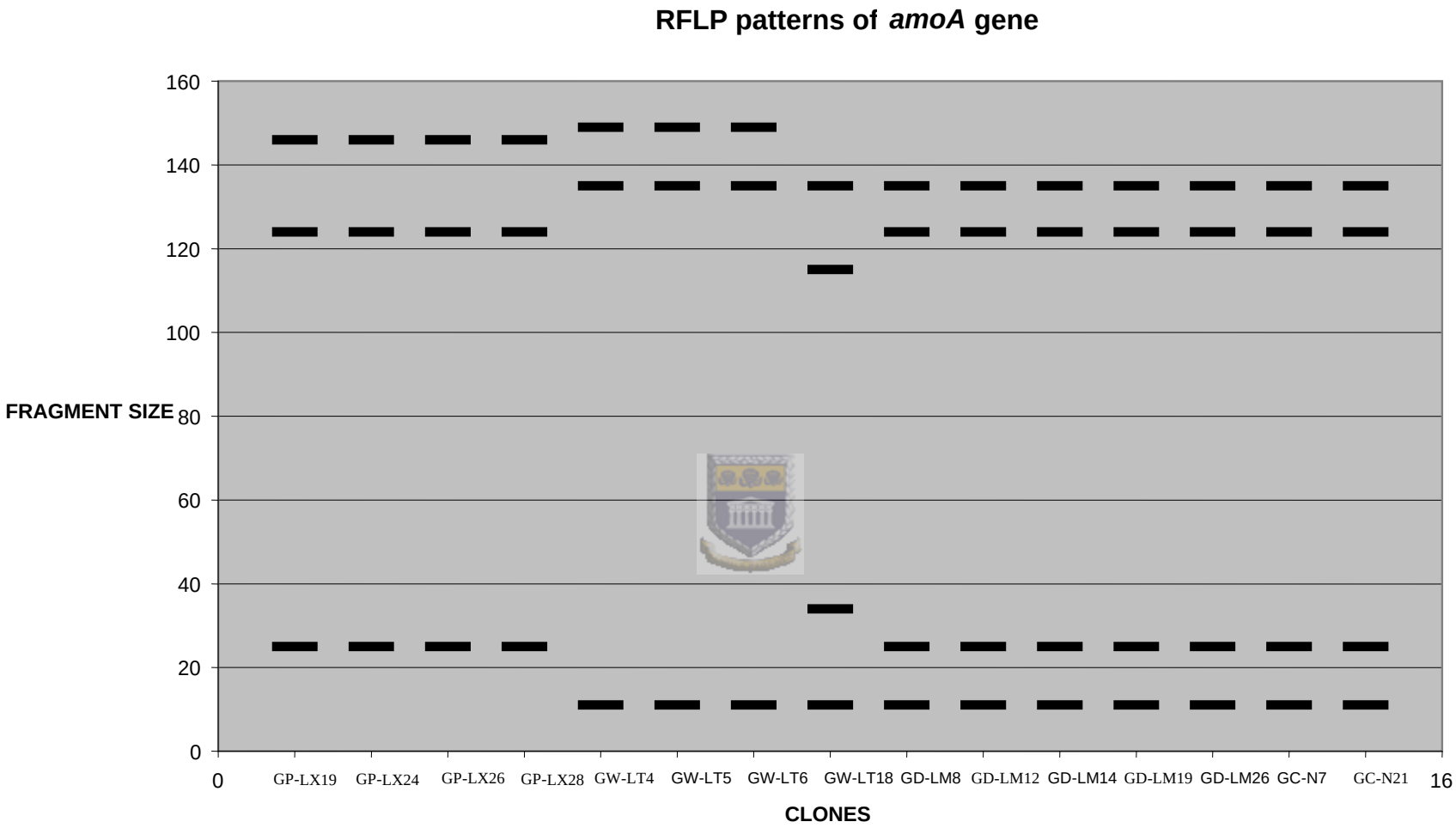


Fig. 3.11: *In silico* RFLP patterns generated from DNA sequence of the *amoA* from of the soil samples. (DNA Club software, Xiongfong Chen, www.imtech.res.in/pub/nsa/dnaclub/dos/)

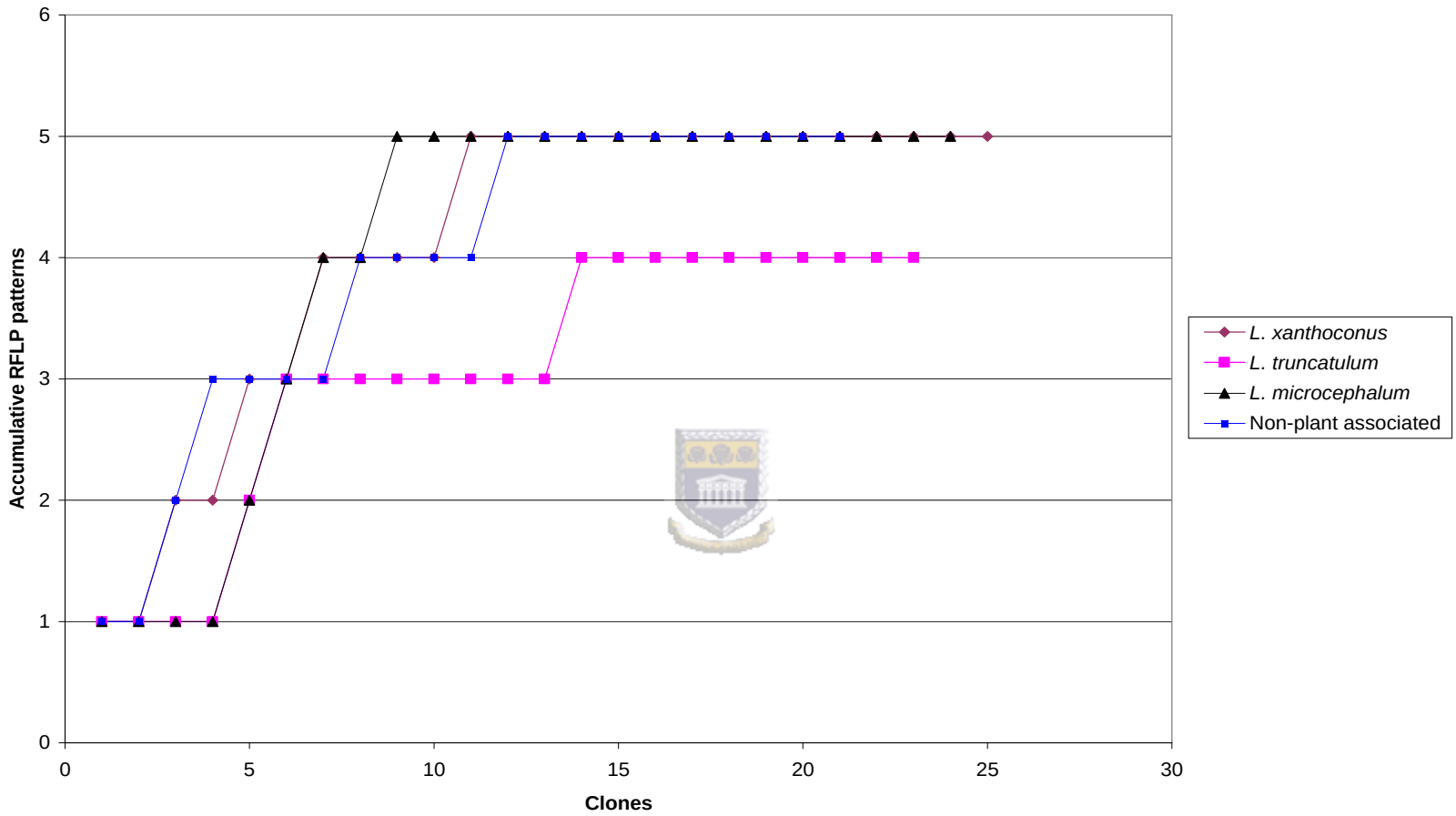


Fig. 3.12: Collection curves of accumulative RFLP patterns of *amoA* clone libraries.

3.7. Phylogenetic Analysis

Molecular approaches based on *amoA* have provided a broad phylogenetic framework to analyse ammonia-oxidizing bacterial communities and there is good agreement analysis using the 16S rRNA or *amoA* as phylogenetic molecular marker.

Almost full length *amoA* gene sequences were obtained from the clone libraries for each soil sample. The NCBI database was searched for most similar sequences using BLAST and sequences with closest similarity downloaded and aligned using ClustalW. This alignment was used to construct phylogenetic trees using the neighbour-joining algorithm. The branching patterns of the trees for nucleotide and deduced amino acid sequences appeared to be the same (Fig 3.20 and 3.21). The bootstrap values were also very similar, but the nucleotide generated tree displayed greater resolution.

Phylogenetic analysis revealed that all clone sequences were grouped with the β-*Proteobacteria* AOB and all sequences clustered with terrestrial *Nitrosospira* species (Avrahami and Conrad, 2003). The phylogenetic trees also showed that three major subgroups of *Nitrosospira* species were identified in the Fynbos environmental samples. The first group is represented by *Nitrosospira multiformis*, the second group by *N. tenuis* and the third group represented by an unidentified bacterium.

Furthermore, clones most similar to *N. multiformis* (AF042171) formed a new subgroup, suggesting that this represents a novel *Nitrosospira* species or sub-species. There was good bootstrap support for this clade in the trees generated from nucleotide alignments (83 %, Fig. 3.20), but the support in the deduced amino acid trees is lower (Fig. 3.21). Approximately 22% of the clone library sequences from the LT samples showed high similarity to an unidentified bacterium (AY667594) from other soil studies (Avrahami and Conard, 2003, Okano *et al*., 2004 and Hawkes *et al*., 2005). The bootstrap support for this unidentified bacterium clade was 68% (Fig. 3.20).

Table 3.5: Nucleotide sequence similarity from BLAST results of *amoA* gene amplicons generated using *amoA* gene specific primers.

#	Clone	Closest match	Accession #	Similarity (%)	E-Value
	Leucadendron xanthoconus				
1	GP-LX19	*Nitrosovibrio tenuis*	AY123824	98% (387/394)	0.0
2	GP-LX24	*Nitrosovibrio tenuis*	AY123824	98% (387/394)	0.0
3	GP-LX26	*Nitrosovibrio tenuis*	AY123824	98% (388/395)	0.0
4	GP-LX28	*Nitrosovibrio tenuis*	AY123824	97% (383/394)	0.0
	Leucadendron truncatulum				
5	GW-LT4	Uncultured AOB	AY369330	98% (382/387)	0.0
6	GW-LT5	*Nitrosovibrio tenuis*	AY123824	98% (348/354)	0.0
7	GW-LT6	Uncultured AOB	AY369330	99% (332/333)	0.0
8	GW-LT18	Uncultured AOB	AY369330	94% (363/384)	6e-148
	Leucadendron microcephalum				
9	GD-LM2	*Nitrosospira sp.* L115	AY123817	94% (367/390)	1e-164
10	GW-LM8	*Nitrosospira sp.* L115	AY123817	95% (358/376)	2e-169
11	GD-LM12	*Nitrosospira sp.* L115	AY123817	95% (379/396)	0.0
12	GD-LM14	*Nitrosospira sp.* L115	AY123817	95% (368/387)	1e-173
13	GD-LM19	*Nitrosospira sp.* L115	AY123817	95% (379/396)	0.0
14	GD-LM26	*Nitrosospira sp.* L115	AY123817	95% (388/391)	0.0
	Non-associated with plants				
15	GC-N7	*Nitrosospira sp.* L115	AY123817	95% (433/453)	0.0
16	GC-N21	*Nitrosospira sp.* L115	AY123817	94% (427/453)	0.0

Table 3.6: Amino acid sequence identity from BLAST results of *amoA* gene amplicons generated using *amoA* gene specific primers

#	Clone	Closest match	Cluster	Accession #	Identity (%)
	Leucadendron xanthoconus				
1	GP-LX19	*Nitrosospira multiformis*	3b	AAC25057	96% (127/132)
2	GP-LX24	*Nitrosospira multiformis*	3b	AAC25057	96% (127/132)
3	GP-LX26	Uncultured AOB	3b	AAR23900	96% (127/123)
4	GP-LX28	*Nitrosospira multiformis*	3b	AAR25057	92% (122/132)
	Leucadendron truncatulum				
6	GW-LT4	Uncultured soil bacterium	3c	CAD62078	95% (123/129)
7	GW-LT5	*Nitrosospira multiformis*	3c	AAC25057	95% (113/118)
8	GW-LT6	Uncultured *Nitrosospira sp*	3c	AAR18237	99% (103/104)
9	GW-LT18	*Nitrosospira multiformis*	3c	AAC25057	75% (117/155)
	Leucadendron microcephalum				
10	GD-LM2	*Nitrosospira multiformis*	3a	AAC25057	93% (122/130)
11	GD-LM8	*Nitrosospira multiformis*	3a	AAC25057	94% (122/129
12	GD-LM12	*Nitrosospira multiformis*	3a	AAC25057	96% (128/132)
13	GD-LM14	*Nitrosospira multiformis*	3a	AAC25057	94% (122/129)
14	GD-LM19	*Nitrosospira multiformis*	3a	AAC25057	96% (128/132)
15	GD-LM26	*Nitrosospira multiformis*	3a	AAC25057	95% (129/135)
	Non-associated with plants				
17	GC-N7	*Nitrosospira multiformis*	3a	AAC25057	96% (155/160)
18	GC-N21	*Nitrosospira multiformis*	3a	AAC25057	93% (150/160)

3.8. DGGE analysis

3.8.1. Modified degenerate PCR primers

The degenerate primers (amoAFino-GC and amoARevino), targeting a stretch of the region that corresponding to nucleotide positions 332 to 349 and positions 802 to 822 of the *N. europaea amoA* gene, were designed and developed by Rotthauwe *et al.* (1997). This primer set specifically targets the *amoA* gene of AOB from the β-*Proteobacteria* group. The primers were slightly modified by replacing the two degenerate bases with inosines in order to amplify all AOB species (Table 2.3). The PCR products generated with these primer sets were compared by DGGE.

3.8.2. Comparative profiling of AOB communities

PCR amplification of the *amoA* gene was used to assess AOB community profiles in soil samples. The banding patterns of amoAFino-GC/amoARevino PCR amplicons, specific for β-Proteobacterial AOB, showed different mobilities, with one major band being present in all samples (Fig. 3.13). However, some doublets were also present, possibly due to the degeneracy in the primers.

The DGGE patterns suggested that only sequences affiliated with the *Nitrosospira* genus were presented since the mobility (55%) of this band corresponded to the *Nitrosospira* genus (Nicolaisen and Ramsing, 2002). These data support the

conclusion from the library clone analyses that *Nitrosospira* species dominate the soil samples.

The DGGE analysis also indicates some sequence heterogeneity, suggesting that different genospecies may be present in the environmental samples. However, this heterogeneity may also have resulted from AOB containing multiple copies (e.g., two or three) of the *amoA* gene with minor sequence variations.

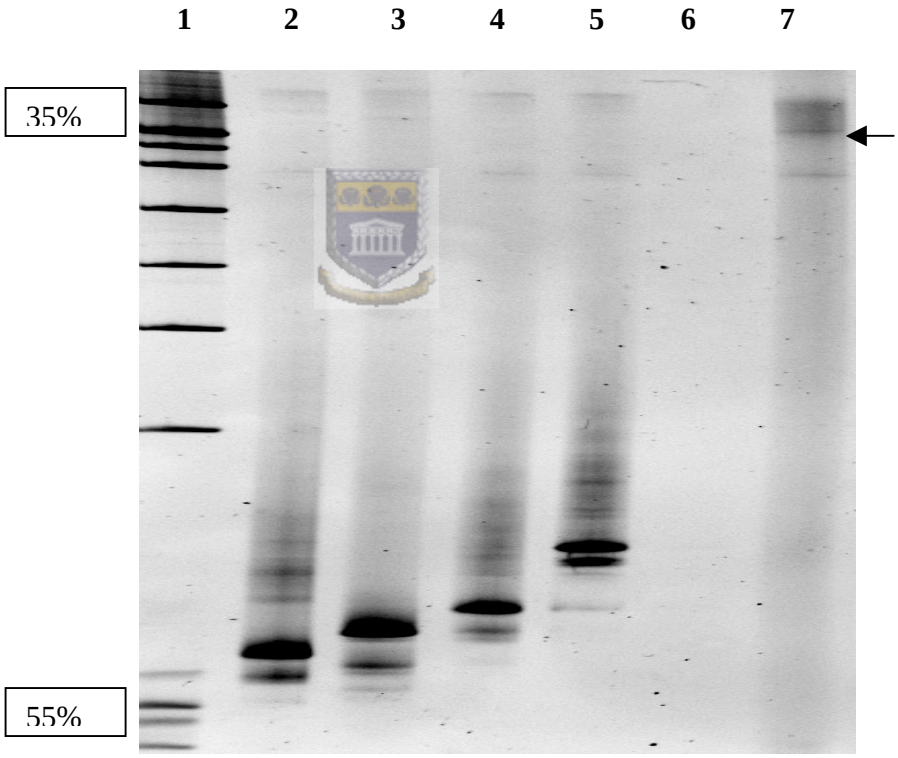


Fig. 3.13: DGGE of DNA samples extracted from Proteaceae rhizosphere soils samples following PCR amplification with the amoAFino-GC and amoARevino primer set. Lane 1: Molecular marker λ DNA cut with *Pst*I restriction enzyme, Lane 2: LX (*Ld. xanthoconus* rhizosphere), Lane 3: LT (*Ls. truncatulum* rhizosphere), Lane 4: LM (*Ld. microcephalum* rhizosphere), Lane 5: N (Non- plant associated soil), Lane 6: Negative control, Lane 7: Positive control (*Nitrosomonas europaea*) indicated by arrow.

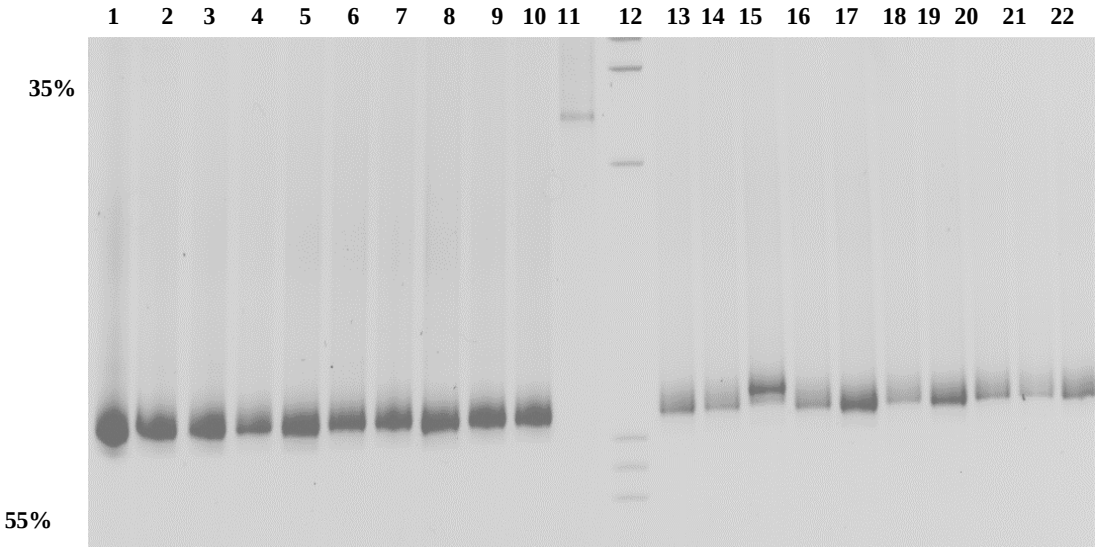


Fig. 3.14: DGGE profile (35% and 55% denaturants) showing partial *amoA* amplicons from the LX (*Ld. xanthoconus* rhizosphere) and LT (*Ls. truncatulum* rhizosphere) soil metagenomic DNA. Lanes 1-10: LX clones; Lane 11: positive control (*Nitrosomonas europaea*) clone; Lane 12: Molecular marker λ DNA cut with restriction enzyme; 13-22: LT (*Ls. truncatulum* rhizosphere) clones

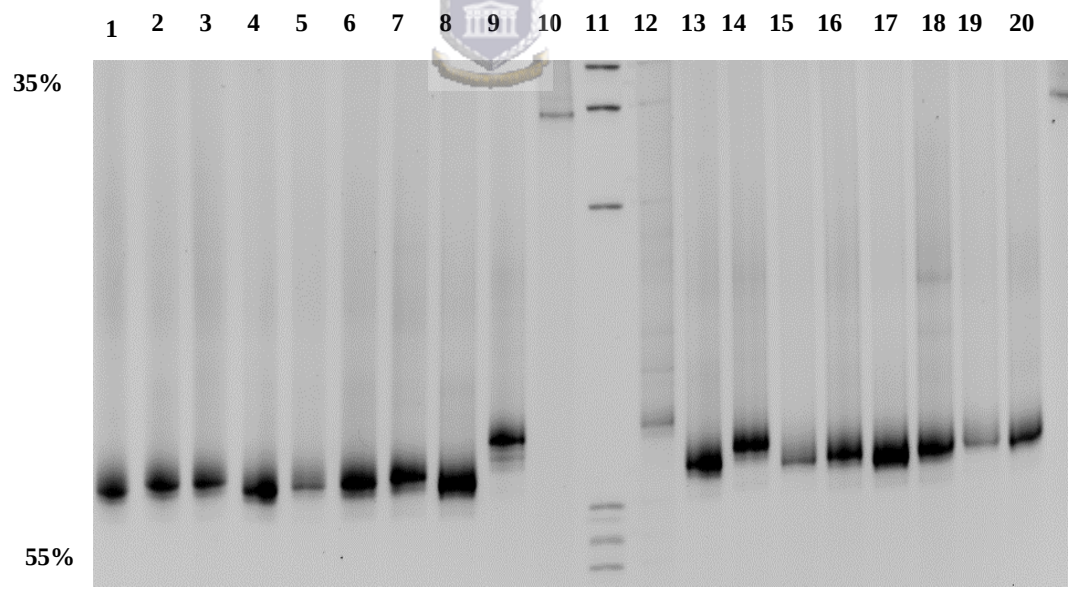


Fig. 3.15: DGGE profile (35% and 55% denaturants) showing partial *amoA* amplicons from the LM (*Ld. microcephalum* rhizosphere) and N (Non- plant associated soil) metagenomic DNA. Lanes 1-9: LM (*Ld. microcephalum* rhizosphere) clones; Lane 10: positive control (*Nitrosomonas europaea*) clone; Lane 11: molecular marker; 12-20: N clones (Non-plant associated soil).

PCR products generated from the environmental soil samples using the DGGE primer set were also used to construct a DGGE clone library. Clones were subjected to further analysis by PCR amplification DGGE and DNA sequencing (Fig. 3.12).

3.8.3. Phylogenetic Analysis of DGGE clones

To determine the identity of banding patterns in the environmental samples, 15 clones were randomly selected from each library and subjected to DGGE analysis and DNA sequencing. The sequence similarity of all clones was relatively high, being 94% to 99% at the nucleotide level and 88% to 98% at the amino acid level. Phylogenetic trees were constructed as previously described (section 3.11).

The sequence of the clones from the *amoA* gene libraries and the dominant DGGE bands clustered together in these trees. These two slightly different approaches therefore provide complementary evidence for the specific phylotypes identified in these soil samples (Section 3.6) (Fig. 3.20 and 3.21). Three clusters or groups can be defined. Cluster 3a representing closely related clones (**pLM5, pLM9, pLM15** and **pLM24**) of sample LM, and clones from sample N (**pN7, pN8, pN11** and **pN15**) with sequence similarities ranging from 88% to 98%. Cluster 3b containing closely related clones (**pLX8, pLX13** and **pLX24**) of sample LX with sequence similarities ranging from 92% to 98% and Cluster 3c is represented by clones **pLT2, pLT10, pLT17** and **pLT22** of sample LT that clustered with an unidentified bacterium.

Table 3.7: Nucleotide sequence BLAST results of DGGE amplicons generated by using *amoA* gene specific primers.

#	Clone	Closest Match	Accession #	Similarity (% nucleotide	E-Value
	Leucadendron xanthoconus				
1	LX8	*Nitrosovibro tenuis*	AY123824	98% (444/453	0.0
2	LX13	*Nitrosovibro tenuis*	AY123824	98% (445/453	0.0
3	LX24	*Nitrosovibro tenuis*	AY123824	96% (439/453	0.0
	Leucospermum truncatulum				
4	LT2	Unidentified bacterium	AY667594	99% (450/452	0.0
5	LT10	Unidentified bacterium	AY667594	99% (430/432	0.0
6	LT17	Unidentified bacterium	AY667590	99% (481/484	0.0
7	LT22	Unidentified bacterium	AY667594	99% (470/473	0.0
	Leucadendron microcephalum				
8	LM5	Uncultured AOB	AY177928	94% (453/476	0.0
9	LM9	Uncultured AOB	AY177928	94% (444/470	0.0
10	LM15	Uncultured AOB	AY177928	94% (449/476	0.0
11	LM24	*Nitrosospira sp.* L115	AY123928	95% (369/386	0.0
	Non- plant associated				
12	N7	Uncultured AOB	AY177928	95% (455/476	0.0
13	N8	Uncultured AOB	AY177928	95% (453/476	0.0
14	N11	*Nitrosospira sp.* L115	AY123817	95% (428/448	0.0
15	N15	*Nitrosospira sp.* L115	AY123817	95% (423/440	0.0

Table 3.8: Amino acid sequence BLAST results of DGGE amplicons

#	Clone	Closest Match	Cluster	Accession nu	Identity (%) of aa
	Leucadendron xanthoconus				
1	LX8	*Nitrosospira multiformis*	3b	AAC25057	98% (161/163)
2	LX13	*Nitrosospira multiformis*	3b	AAC25057	92% (151/163)
3	LX24	*Nitrosospira multiformis*	3b	AAC25057	92% (151/164)
	Leucospermum truncatulum				
4	LT2	Unidentified bacterium	3c	AAT77737	98% (149/152)
5	LT10	Unidentified bacterium	3c	AAT77737	98% (149/152)
6	LT17	Unidentified bacterium	3c	AAT77737	91% (149/163)
7	LT22	Unidentified bacterium	3c	AAT77737	95% (156/163)
	Leucadendron microcephalum				
8	LM5	*Nitrosospira multiformis*	3a	AAC25057	98% (161/163)
9	LM9	*Nitrosospira multiformis*	3a	AAC25057	95% (156/163)
10	LM15	*Nitrosospira multiformis*	3a	AAC25057	95% (155/163)
11	LM24	*Nitrosospira multiformis*	3a	AAC25057	98% (161/163)
	Non- plant associated				
12	N7	*Nitrosospira multiformis*	3a	AAC25057	96% (158/163)
13	N8	*Nitrosospira multiformis*	3a	AAC25057	96% (155/160)
14	N11	*Nitrosospira multiformis*	3a	AAC25057	88% (144/163)
15	N15	*Nitrosospira multiformis*	3a	AAC25057	97% (160/163)

3.9. Bioinformatics analysis of partial *amoA* gene variants

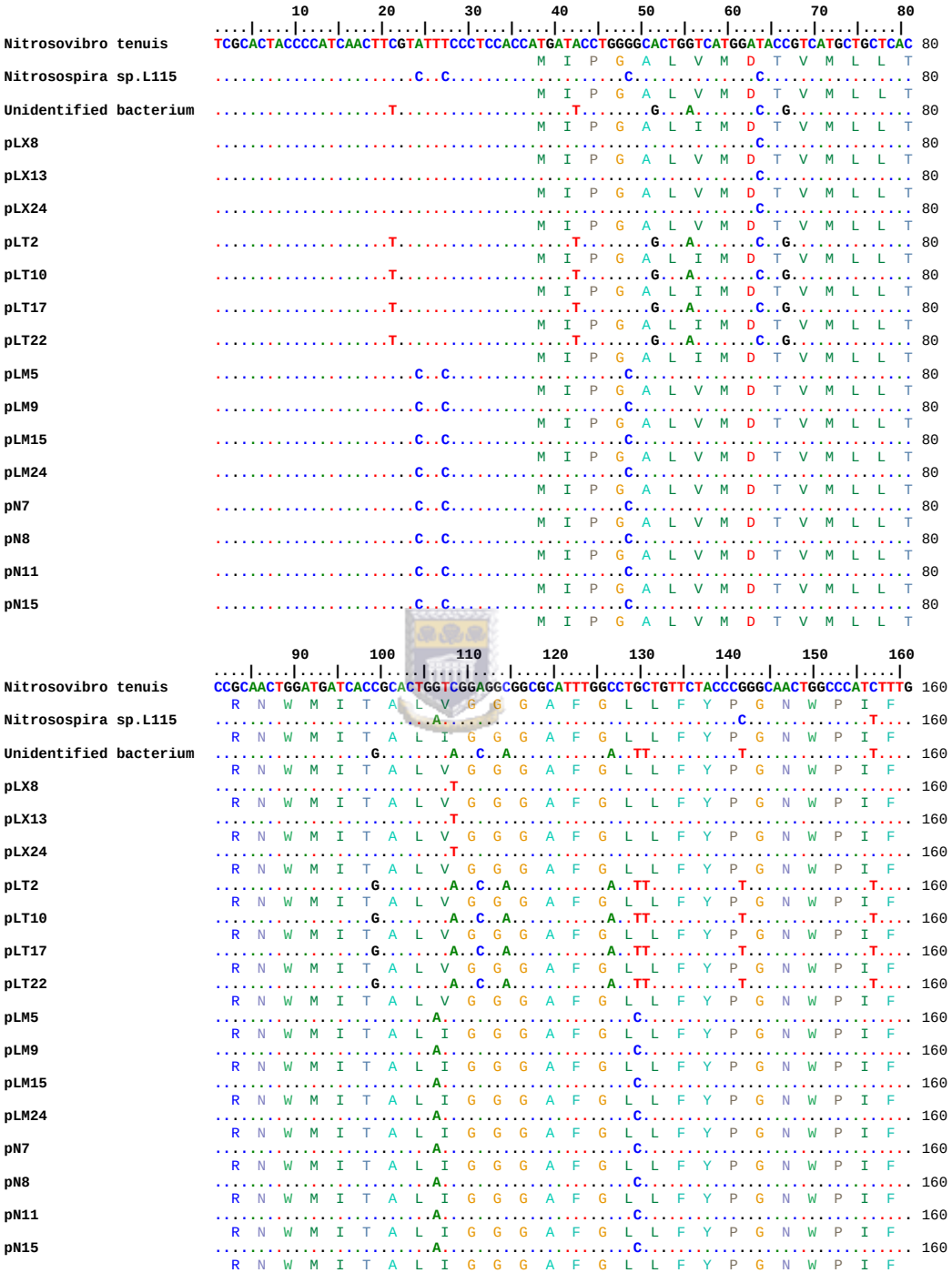
Alignments of *amoA* clone nucleotide sequences retrieved from metagenomic samples showed significant sequence divergence at the third position of triplet codons (Fig. 3.16). The deduced amino acids of the *amoA* gene sequences retrieved from clone libraries were compared with *Nitrosospira amoA* sequences in the GenBank database (Fig. 3.17).

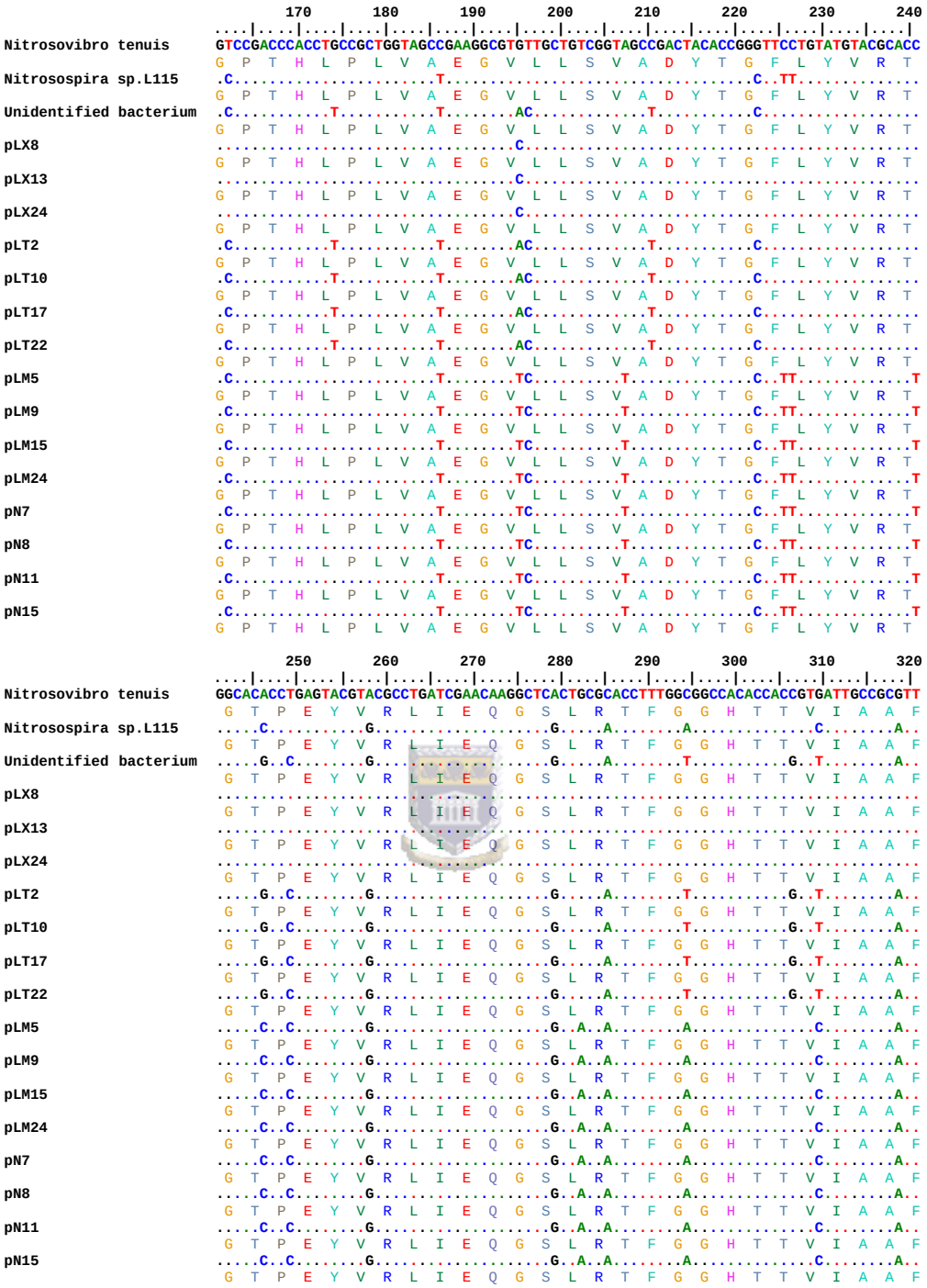
Sequences from soil associated with LT revealed a high level of similarity to an unidentified bacterium (uncultured *Nitrosospira*). These sequences show three amino acid substitutions: Ile at position 134, Cys at position 233 and Phe at position 246 (Fig. 3.16 and 3.17), compared to *Nitrosospira* isolates previously published in the database. However, the LM and N clone sequences exhibited high levels of similarity to *Nitrosospira* sp. L115. LX clone sequences showed almost 99% similarities to *N. tenuis* (U76552), with only a single amino acid difference. Amino acids Val, Thr and Tyr in positions 134, 233 and 246 respectively, in *Nitrosospira* isolates were substituted in all clones retrieved from the LT soil sample (Fig. 3. 17). All these substitutions were identical to those of the unidentified bacterium (Hawkes *et al*., 2005; Carney *et al*., 2004).

It is possible to speculate that these substitutions could play a role in the adaptation of these microorganisms to the acidic low nutrient soils in Fynbos ecosystems. It may be significant that the unidentified bacterium was retrieved from a similar environment in North West of USA, which was characterized with lower nitrogen

content and acidic soils (Hawkes *et al*., 2005). Furthermore, these substituted amino acid residues could be a signature for this new ecotype.

3.9.1. Nucleotide alignment of amoA gene sequences with deduced amino acids shown




170 180 190 200 210 220 230 240
Nitrosovibro tenuis GTCCGACCCACCTGCCGCTGGTAGCCGAAGGCGTGTTGCTGTCGGTAGCCGACTACACCGGGTTCCTGTATGTACGCACC 240
Nitrosospira sp.L115 240
Unidentified bacterium 240
pLX8 240
pLX13 240
pLX24 240
pLT2 240
pLT10 240
pLT17 240
pLT22 240
pLM5 240
pLM9 240
pLM15 240
pLM24 240
pN7 240
pN8 240
pN11 240
pN15 240
250 260 270 280 290 300 310 320
Nitrosovibro tenuis GGCACACCTGAGTACGTACGCCTGATCGAACAAGGCTCACTGCGCACCTTTGGCGGCCACACCACCGTGATTGCCGCGTT 320
Nitrosospira sp.L115 320
Unidentified bacterium 320
pLX8 320
pLX13 320
pLX24 320
pLT2 320
pLT10 320
pLT17 320
pLT22 320
pLM5 320
pLM9 320
pLM15 320
pLM24 320
pN7 320
pN8 320
pN11 320
pN15 320

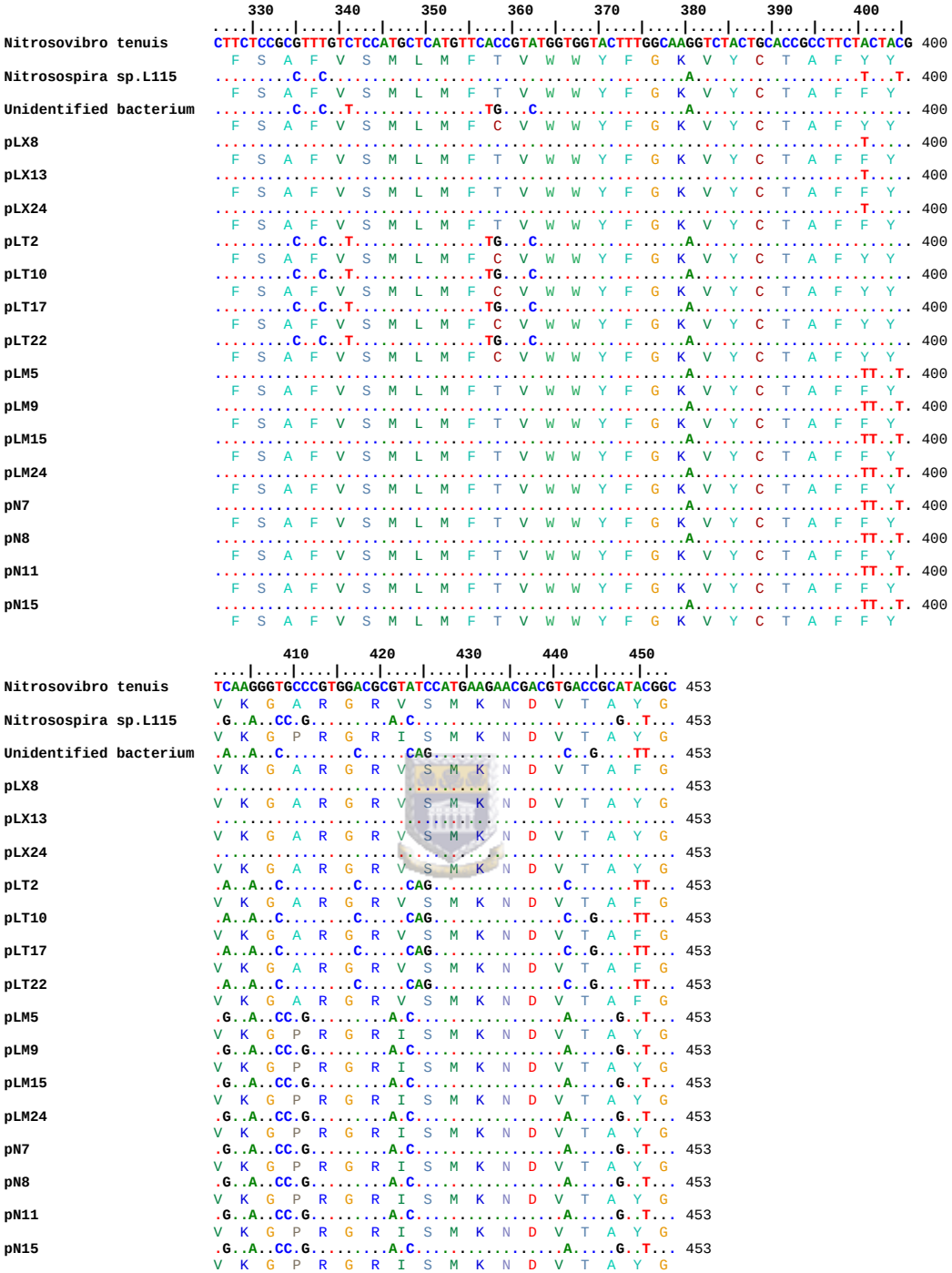


Fig. 3.16: Alignment of nucleotide sequences for 453-bp *amoA* gene fragment with sequences obtained from (GenBank), (*Nitrosovibrio tenuis*, *Nitrosospira* sp. L115 and Unidentified bacterium). Similar nucleotides positions are indicated by dots

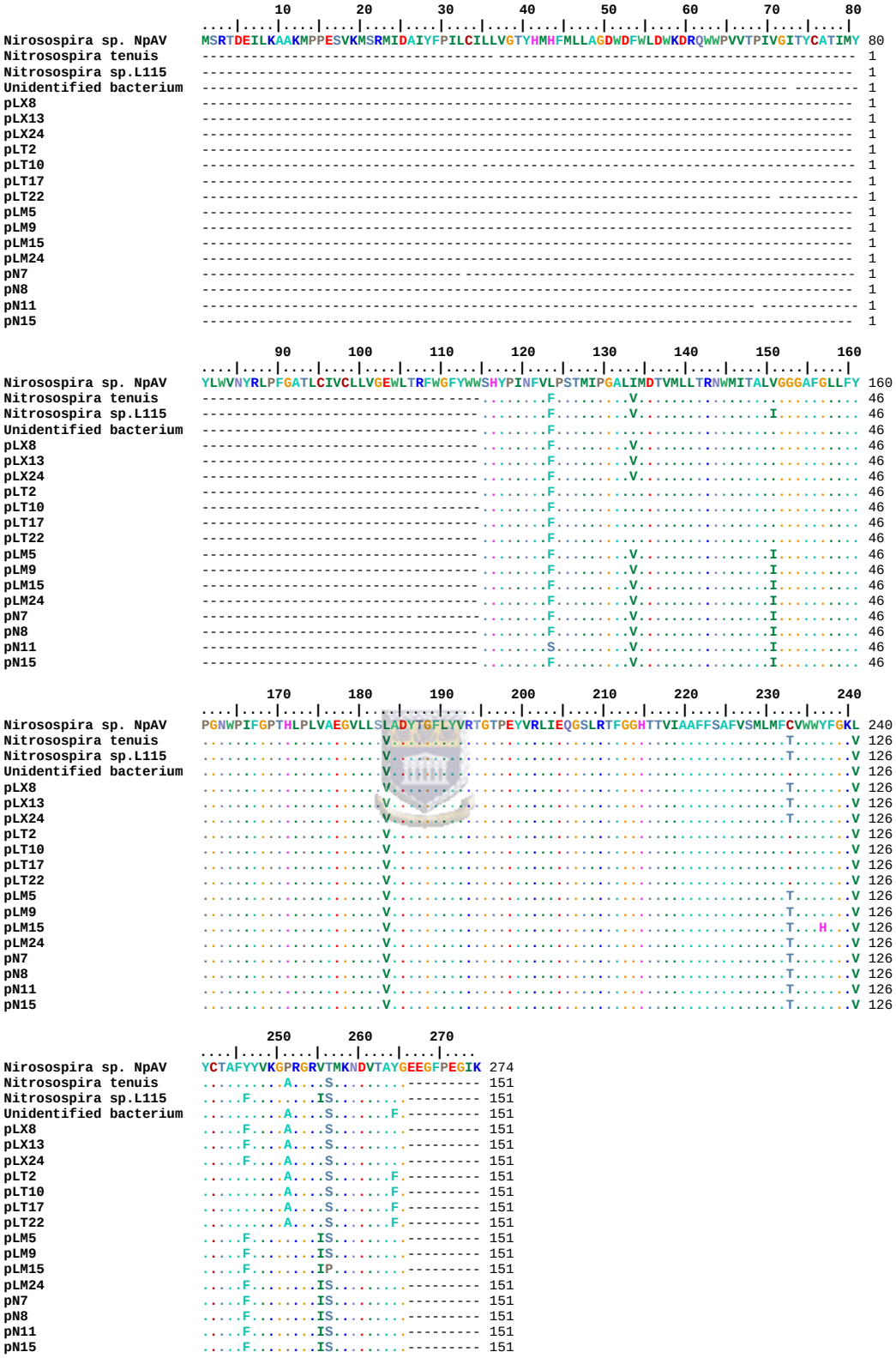


Fig 3.17: Multiple alignment of the deduced amino acid sequences of the partial *amoA* gene fragment, and the full length of *amoA* sequence of *Nitrosospira* sp. NpAV, with sequences of β-subclass Proteobacterial AOB (*N. tenuis*, *Nitrosospira* sp. L115 and unidentified bacterium). Identical amino acid positions are indicated by dots.

3.9.2. Secondary structure predictions of deduced AMO protein

The structural prediction for the full-length AmoA polypeptide from *Nitrosospira* sp. NpAV shows that there are seven highly hydrophobic putative transmembrane-spanning regions and other hydrophilic regions that are predicted to be located in cytosol (Norton *et al*. 2002). A hydrophobicity profile (Kyte and Doolittle, 1982) was generated for the deduced partial *amoA* polypeptide from the LX, LT, LM, and N clone libraries. This enabled a comparison of the putative transmembrane helices (residue 50-55, 75-98 and 130-145), and hydrophilic, surface exposed loops (residue 5-50, 55-75 and 98-133) (Fig 3.18).

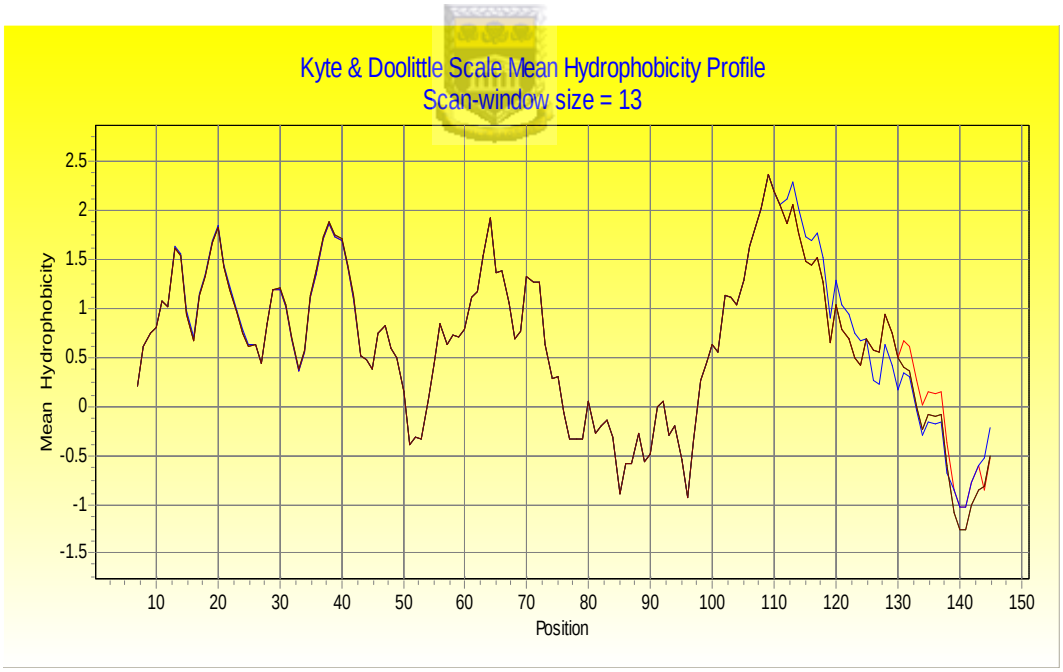


Fig 3.18: Plots showing secondary structure predictions for LX in red, LT in blue, LM in green and N in brown deduced amino acids of AmoA from clone libraries of soil samples. The hydrophobicity plots indicating membrane topology. Horizontal lines above the plots show the position of highly conserved loop residues for AmoA.

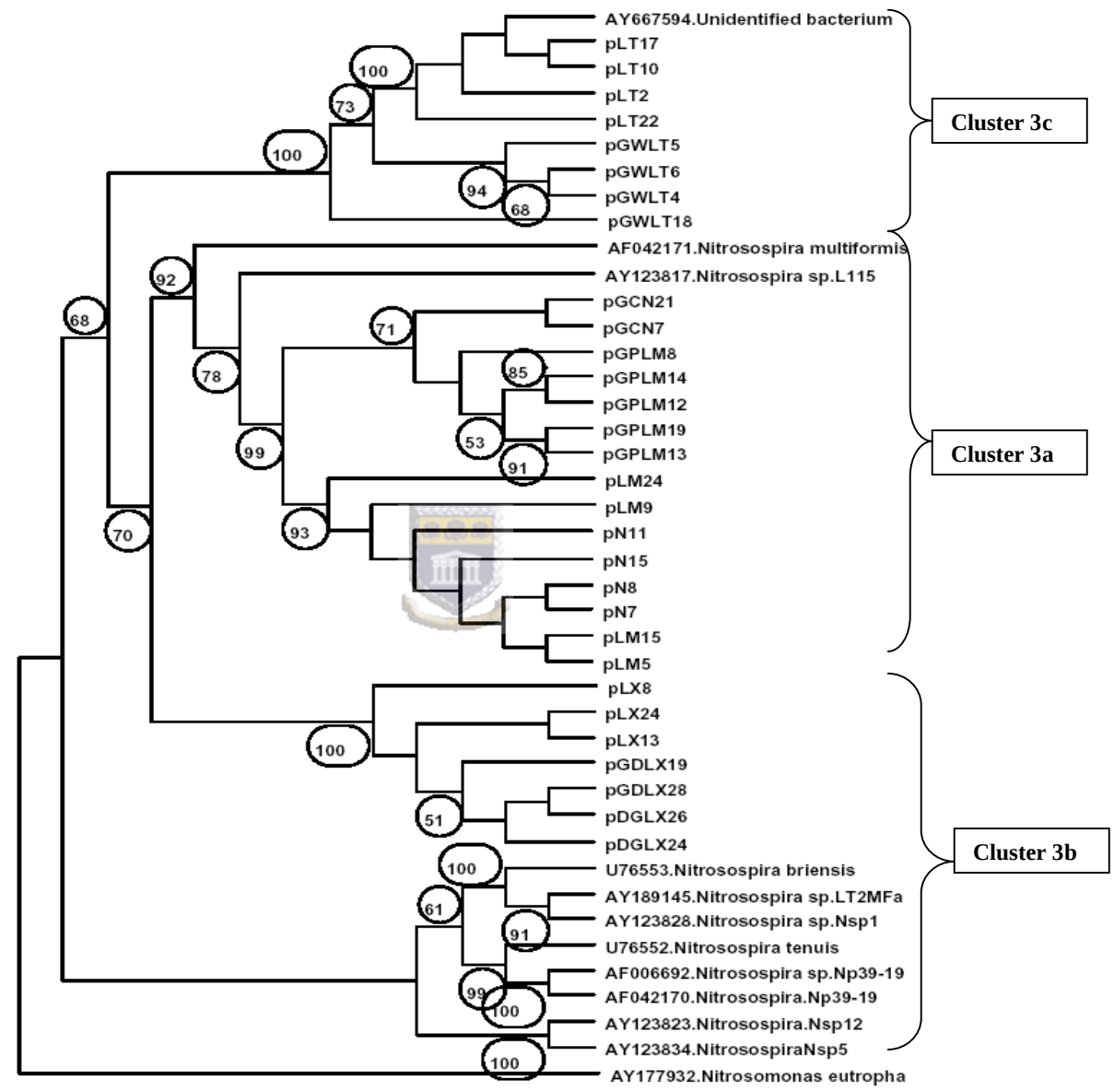


Fig 3.19: Phylogenetic tree constructed for partial *amoA* gene sequences, including both DGGE clones (Table 3.4) and *amoA* gene library clones (Table 3.6). Representative sequences from the NCBI database were included in the alignment and *Nitrosomonas eutropha* (AY177932) was used as an outgroup.

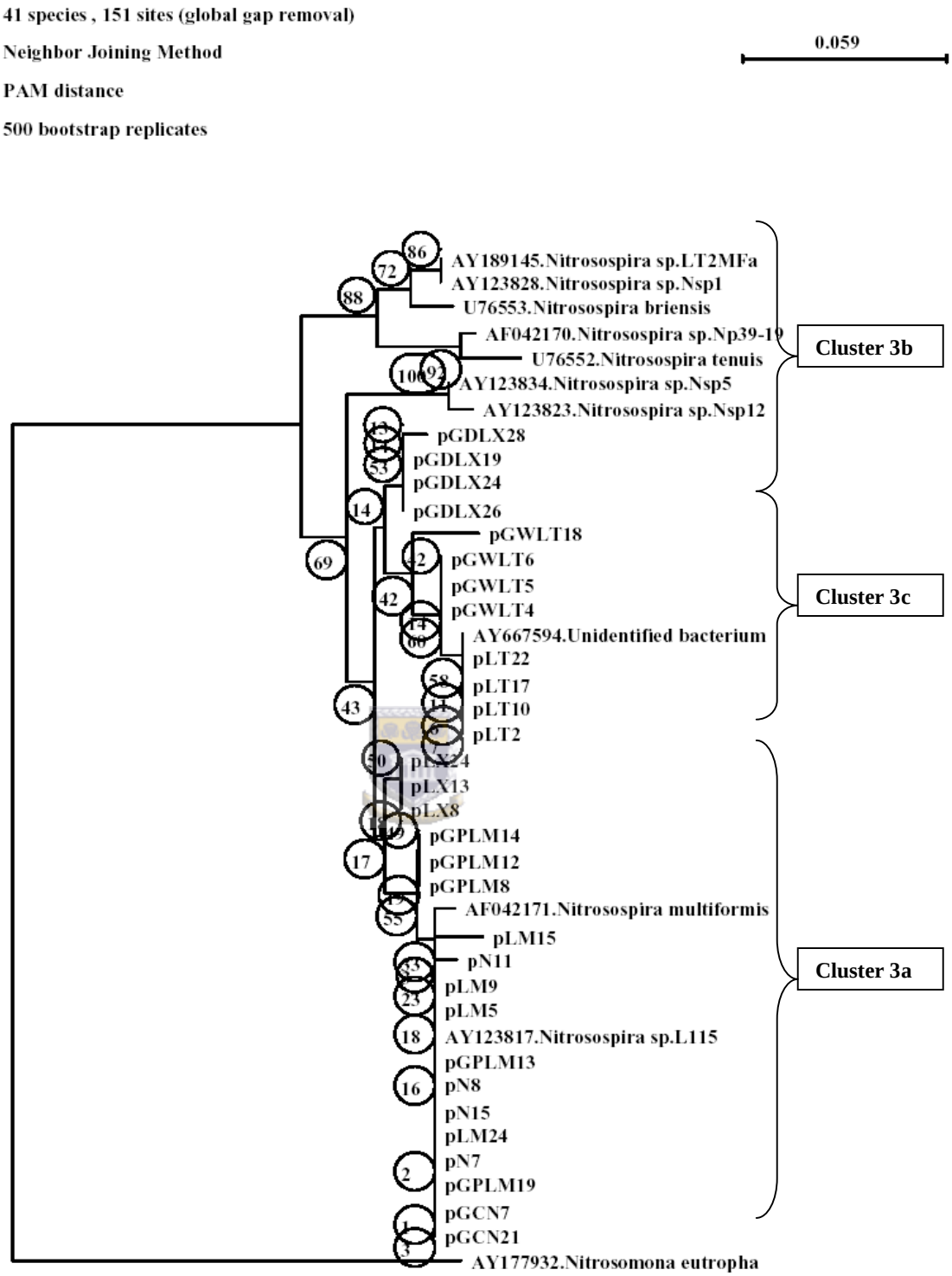


Fig. 3.20: Phylogenetic tree showing the clustering of *amoA* clones sequences from both DGGE and *amoA* gene libraries from soil samples (LX, LT, LM, and N). The tree was generated from *amoA* deduced amino acids, aligned with representative sequences from the NCBI database. The *Nitrosomonas eutropha* (AY177932) was used as an outgroup.

3.10. Discussion

3.10.1. Phylogeny and diversity of AOB

The main aim of this investigation was to explore the potential of *amoA* genes as a functional phylogenetic marker to analyse the community structure of AOB populations in soil associated with the roots (rhizosphere) of Proteaceae plant species. The current study is the first to describe the AOB communities in Fynbos ecosystems. *Nitrosospira* sp. was found to be the dominant species in Fynbos soils and there was no evidence for the presence of *Nitrosomonas* species in this study, reflecting a limited overall AOB community diversity. By comparison, Burns *et al.* (1999) found that AOB sequences from the undisturbed soils were distributed in more than seven distinct phylogenetic clusters. In contrast, our sequences were clustered into only three groups of *Nitrosospira* sp. This is unlikely to be due to primer specificity since these primers were designed to encompass all known β-*Proteobacterial* AOB (Rotthauwe *et al*., 1997), and previous studies have yielded sequences of both groups (Nicolaisen and Ramsing, 2002; Purkhold*et al*., 2003).

All the clone sequences retrieved from this environment are most similar to *Nitrosospira* species, and their similarity values ranged from 94% to 98% (Table 3.5 and 3.7). Homology at the amino acid level was relatively low at 75% to 99% (Table 3.6 and 3.8). Phylogenetic analysis identified three distinct clusters, all showing good bootstrap support (>68% Fig. 3.20) based on *amoA* nucleotide sequences. These are therefore likely to represent different *Nitrosospira* species, represented by

Nitrosospira multiformis (AF042171, cluster 3a) *Nitrosospira tenuis* (U76552, cluster 3b) and an unidentified bacterium (AY667594, cluster 3c).

Interestingly, the Finnish forest soil in which the unidentified bacterium (cluster 3c) was identified represents a similar soil ecotype to Fynbos, since both contain a low ammonium concentration and a low soil pH (pH 4.5). The low level of nitrification in this system was thought to be due to the both the unfavourable soil pH and the limitation of ammonia (Laanbroek and Woldendorp, 1994). Since *Nitrosospira* sp. have been detected in both low and high pH environments (Aakra *et al*., 2000) this cluster may represent a new group of acid-tolerant clade. The isolation of this species would enable definitive assignment of this novel *Nitrosospira.*

3.10.2. Distribution of AOB

The analysis of the *amoA* metagenomic clone libraries of the four different soil samples revealed some interesting differences in distribution. RFLP analysis of sequenced clones agreed with *in silico* restriction digestion patterns. The unidentified bacterium (cluster 3c, Fig. 3.19) (Hawkes *et al*., 2005), was only dominant in LT soil and could be defined by the presence of a 149 bp fragment and the absence of a 124 bp fragment, which corresponded to loss of the *BsuR*1 restriction site (position 124-127, GG/CC Fig. 3.16). This 124 bp fragment was the dominant RFLP of clones from the LX, LM and N libraries. Furthermore, LX sequences clustered only with *N. tenuis* (cluster 3a) while LM and N sequences clustered with *N. multiformis* (cluster 3b).

This indicates that the different members of the Proteaceae plant family (*Leucadendron* and *Leucospermum* sp.) have different specific AOB associations. It therefore follows that the plants may be significant determinants of AOB community composition. This would not be unexpected, since plant roots are known to effect soil chemistry and microflora (Paul and Clark, 1989). It has been recently suggested that the community structure of AOB in the rhizosphere may have an influence on the plant nitrogen uptake by co-providing nitrate at high rate (Briones *et al*., 2003).

The differences in plant specific associations were based on comparative analysis of *amoA* clone libraries and DGGE profiles. Although the estimated coverage for the libraries was >75%, uncertainties in log-normal distribution of species and bias in PCR amplification mean that these differences are not fully conclusive. A quantitative method such as real-time PCR or FISH with DNA primers/probes specific for cluster 3a, 3b, and 3c would provide more conclusive evidence of these plant-specific associations.

The highest matches for the AOB species found in this study are from soils with very different plant communities and wide geographical regions [e.g., an agriculture field in California (AY445618, Okano *et al*., 2004), a coniferous forest in New Mexico (AY819625), and a meadow in Germany (AY249706, Avrahami and Conard, 2003)]. As additional sites are studied and new sequences added to the database, more information of AOB distribution in different environment will be

provided, and this may facilitate the identification of geographical ecotype and plant specific AOB populations.

4.1. Introduction

Autotrophic ammonia-oxidizing bacteria (AOB) are of great importance in the nitrogen cycle, particular in the nitrification process. Since oxidation of ammonium to nitrate is essential for making nitrogen available to most plants, it is important to quantify the abundance of AOB microbial populations. This has previously been attempted using several different techniques including competitive PCR (cPCR) (Mendum *et al*., 1999) and fluorescence *in situ* hybridization (FISH) (Biesterfeld *et al*., 2001). However, these methods are unreliable and irreproducible for many reasons. For example, FISH is time consuming and difficult to use in soil and requires active cells, while competitive PCR requires time-consuming optimization and extensive post-PCR analyses (Diviacco *et al*., 1992).

The real-time PCR assay has been widely applied to the quantification of microbial populations, particularly in medical research for diagnosis of infectious diseases and in food microbiology for the detection of pathogens (Mackay, 2004).

The objective of this chapter was to quantify soil AOB populations of LX, LT, LM and N, using a real-time PCR targeting the *amoA* gene. This method will be useful for establishing correlations between microbial activities and cell numbers in the soil environment.

4.2. Real-Time PCR assay

PCR was monitored in real time using the LightCycler I instrument (Roche, Germany) and SYBR Green as the fluorophore for the detection of double stranded DNA (PCR product). PCR was performed in a 20 µl reaction that contained 1 ng of template DNA, 10 µl of ABsolute^{TM} QPCR$^{®}$ Green Mix, 0.1mg/ml final concentration of BSA, and 0.5 µM of each primer.

The PCR thermocycling conditions for *amoA* gene quantification were: enzyme activation at 95^{o}C for 15 minutes, and then 40 cycles consisting of 10 seconds at 95^{o}C and 9 seconds at 58^{o}C, 10 seconds at 72^{o}C. Primers targeting the *amoA* gene (amoAFI/amoARevino) were used (Table 2.4).

4.2.1. Quantification

All metagenomic DNA samples from rhizosphere Proteaceae soils and non-plant associated soil were analysed in duplicate. The increase in fluorescence abovethe baseline indicates the detection of accumulated PCR product. The parameter C_T (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. A plot of the log of initial target copy number for a set of standards versus C_T is a straight line (Higuchi *et al*., 1993). Quantitation of the amount of target (*amoA* gene copy number) in unknown samples is accomplished by measuring C_T using the equation: (y=2.610x +53.66) from the linear calibration curve.

Unfortunately, SYBR green I detects all double-stranded DNA, including primer dimers and other undesired products so that the detection is limited by the specificity of amplification. However, because the melting curve of a product is dependent on GC content length and sequence, PCR products can be distinguished by their melting curves. Therefore, melting curve analysis was used to differentiate specific from nonspecific PCR products (Ririe *et al.* 1997) prior to analysis. The real-time PCR analysis showed that the melting points of all *amoA* gene amplicons from soil samples were different from that of the positive control amplicon (Fig. 4.1). This could be attributed to high GC contents in the soil DNA sequences. The C_T values for *amoA* gene was then determined for metagenomic DNA and for the control *Nitrosomonas europaea* control genomic DNA template.

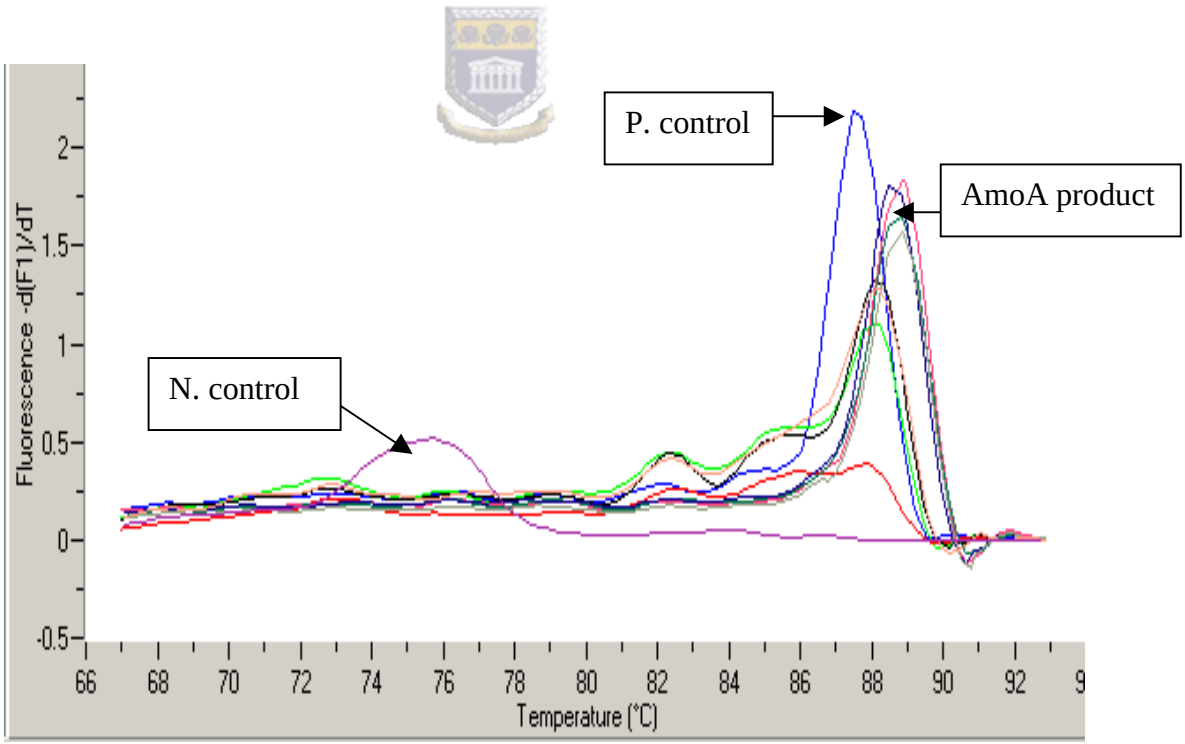


Fig. 4.1: Melting-curve analysis of *amoA* gene real-time PCR amplification products. LX in pink, LT in green, LM in dark blue, N in light green, Negative control in purple and positive control in blue.

Total genomic DNA was extracted from a pure culture of *Nitrosomonas europaea* (ATCC 19718) that has two copies of *amoA* gene and one copy of rRNA gene (Aakra *et al*., 2001). A standard curve to show the relationships between *amoA* copy numbers and C_T values was constructed with serial dilutions of a known copy number of the *amoA* gene, and was calculated using LightCycler software. The *amoA* gene copies in the metagenomic DNA samples were quantified using this standard curve and the C_T values obtained (Table 4.1). AOB population size was quantified by dividing the value of the *amoA* copy numbers by 2.5. This value was chosen because *Nitrosospira* phylotypes dominated our clone libraries (Chapter 3) and *Nitrosospira* sp. have previously been shown to contain 2 or 3 copies of the *amoA* gene (Norton *et al*., 2001).

Table 4.1: Quantification of *amoA* gene copies/g dry soil

sample	Metagenomic DNA concentration (ng/µl)	Calculated concentration of *amoA* gene	Copy number of *amoA* gene/g dry soil
NE	10	1.587E+10	3.2×10^{10}
LX	12	3.943E+09	9.1×10^{7}
LT	12	1.948E+09	1.7×10^{7}
LM	12	0.875E+09	1.9×10^{7}
N	12	3.516E+09	8.2×10^{7}

*NE (*Nitrosomonas europaea*) as a positive control from pure culture.

The average ammonia-oxidizing bacterial population sizes in soil samples were approximately 4.2 x 10^7 and 8.2 x 10^7 copies/g dry soil for Protea plant species and non-plant associated samples, respectively. These values are relatively similar compared to those reported by Hawkes *et al*. (2005) 1.0 x 10^8copies/g of dry soil.

Table 4.2: Comparison of the *amoA* copy numbers in different environments

samples	*amoA* copies/g of dry soil	References
Fynbos ecosystem	4.2 x 10^7	This study
Soil DNA	1.0 x 10^8	Hawkes *et al*., 2005
Arable soil	1.6 x 10^8	Hermansson and Lindgren, 2001
Agricultural soil	0.7 x 10^7	Okano *et al*., 2004

The quantification of the *amoA* gene is informative in studying the relationship between microbial community abundance, activity and ecological function in terms of nitrogen cycling processes. In this study the microbial population size is correlated to the ammonium concentrations in the Fynbos environment, which typically has lower nitrogen content than other environments (Hawkes *et al*., 2005).

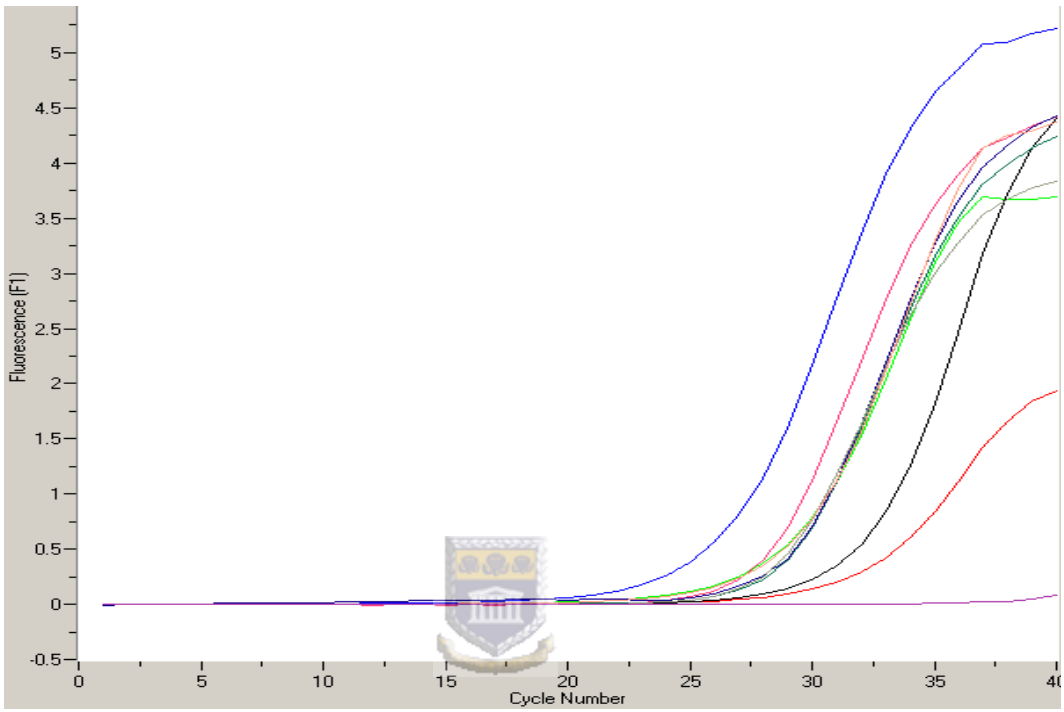


Fig. 4.2: *amoA* gene amplification product of from soil samples (LX, LT, LM and N) and the positive control (*Nitrosomonas europaea*).

4.3. Discussion

A number of studies have shown the value of the real-time PCR assay for estimating the abundance of bacteria in soil, waste-water and marine communities (Harms *et al*., 2003). However, this process can be complicated due to a number of factors such as very low concentration of target DNA and the presence of PCR inhibitors. AOB have been shown to possess only one rRNA gene copy per genome, but more than one copy of the *amoA* gene (Aakra *et al*., 1999). In addition, the *amoA* gene copy number differs between AOB species. For all known *Nitrosospira* isolates, the genome contains 2-3 copies of *amoA* gene (Norton *et al*., 2001). Therefore, the use of real-time PCR targeting the AOB 16S rRNA gene may be a more reliable method for the quantification of AOB compared to the *amoA* gene. Quantification of the AOB in the Fynbos soil environment yielded values in the order of 10^7-10^8 (Table 4.1). The number of AOB found in the LX, LT, LM rhizosphere soil and N non-plant associated soil are lower, than the numbers found in the previous studies (Okano *et al*., 2004; Hermansson and Lindgren, 2001; Hawkes *et al*., 2005) (Table 4.2).

The data generated in this chapter, was preliminary analyses for the quantification of AOB populations from the Fynbos soil environment. Time constraints precluded detailed analyses, and as a result part of the future work would focus on generating accurate data for reliable quantification of abundance of AOB populations. This would then allow the comparison between the AOB populations from the Fynbos ecosystem, and total bacterial population.

General Discussion

Molecular approaches targeting the *amoA* gene as functional marker were used to investigate the diversity, distribution and community structure of the β-subclass of *Proteobacteria* ammonia-oxidizing bacteria (AOB). The gene encoding the active site A subunit of the ammonia monooxygenase (AMO) has increasingly been exploited as a molecular marker for cultivation-independent analyses of AOB diversity. Different primer sets for the amplification of *amoA* gene fragments have been used, but the primers used by Rotthauwe *et al*. (1997) have been shown to be specific for all known members of the β-subclass of Proteobacterial AOB. These degenerate primers were successfully used in this study to amplify the *amoA* fragment from Fynbos soil samples. A nested PCR approach was required to provide the sensitivity to detect low numbers of the *amoA* operon in metagenomic DNA.

To evaluate if the *amoA* gene allows fine-scale resolution of closely related AOB populations, as previously suggested by Rotthauwe *et al*. (1997), degenerate primers were used with slight modifications. Two degenerate bases were replaced with the inosine to reduce primer degeneracy while still allowing base-pairing to both purine and pyrimidine bases. These primers have previously been shown to amplify both *Nitrosomonas* and *Nitrosospira* genera. The detection of *amoA* gene sequences amplified from metagenomic DNA provided evidence for the presence of β-subclass Proteobacterial AOB in Fynbos soils, although the detection of *amoA* mRNA by using RT-PCR or a proteomics approach would give additional data on the activity of AOB in this environment. It is noteworthy that no

Nitrosomonas species sequences were retrieved from Fynbos metagenomic DNA, and clone libraries identified *Nitrosospira* as the dominant phylotype in Fynbos soil metagenomic DNA Furthermore, phylogenetic analyses showed that only sequences closely related to *Nitrosospira* cluster 3 were present.

The overall diversity of AOB was low compared to other soil and terrestrial environments (Hawkes *et al*., 2005), where other AOB phylotypes were commonly detected in soils. Further, our data suggested that soil AOB diversity and composition was sensitive to changes in plants diversity. There was, however, no clear relationship between the ammonia concentration measured in soils and the *amoA* phylotypes identified. It is of interest that both LM and N contained *Nitrosospira tenuis* phylotypes and this may be correlated with the relatively higher ammonia concentrations in these soil samples.

DGGE analysis of *amoA* amplicons is a reliable screening tool to investigate AOB diversity in a wide range of environments (Nicolaisen and Ramsing, 2002). Data obtained from DGGE analysis was consistent with that obtained from the *amoA* clone libraries, confirming that only *Nitrosospira* cluster 3 species were present in LX, LT, LM and N soils and that plant-specific AOB associations existed.

Clone libraries often lead to the detection of sequences not recognized by DGGE, since the latter is based on cloning of the major bands only, which results in a lower sensitivity (Nicolaisen *et al.,* 2004). Therefore, it is essential to combine the

analyses of DGGE and clone libraries to investigate the community structure of AOB.

The quantification of *amoA* genes in soil samples of Proteaceae plant rhizospheres and non-plant associated soil was a further objective of this study. Using a real-time PCR assay, the potential of this technique for quantifying AOB populations in Fynbos soils was successfully demonstrated. The abundance of the *amoA* gene was calculated to be approximately 4.2 x 10^7 copies/g of dry soil. These data suggested that AOB abundance is relatively low compared to previous reports. Future work should focus on the estimation of the total bacterial abundance by targeting the 16S rRNA gene so that the AOB abundance can be compared to the total bacterial abundance.

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