Characterization and Identification of Microbial Communities in Pigeon Droppings using Culture-Independent Techniques

BY

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(Baccalaureus Technologiae Biotechnology)

Dissertation submitted in fulfilment of the requirements for the degree

Magister Technologiae (M.Tech.) Biotechnology

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2015

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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ACKNOWLEDGEMENTS

I would like to express my gratitude to the following people;

My supervisor, Professor M. Pillay for his mentorship, advice and support for the duration of this study. Thanks Prof!!

My co-supervisor, Dr. Naser Aliye Feto for his advice and intellectual input.

Cano Ssemakalo for suggesting this topic, the initial discussions, advice and bringing this study to fruition.

My colleagues, lab technicians (*) and friends: Gabriel, Losika, Manzi, Joseph, Muamba, Wambo, Pule, Amara, Herman, Sibusiso, Doreen*, Samkeliso*, Nnana*, Patience and Basadi for making life inside and outside the lab bearable. *May the power of the universe stay with you*. Hehe!

A special thanks to my aunt, Usinah Tshekiso, for all she has done for me. My family, for their support and encouragement.

DEDICATION

This work is dedicated to ALL of my family.

"Í never aged while giving time a test"

ABSTRACT

Pigeon droppings, found in abundance in most cities and towns where pigeons are found, are a source of potential yeast and molds into the environment. Invasive fungal infections are a cause of morbidity and often mortality in immunocompromised individuals. The objective of this study was to the identification of bacterial and mold agents from pigeon droppings. Pigeon droppings samples were collected from three locations during the winter and summer months and studied for the occurrence of bacteria, yeast and molds by utilising culture-independent techniques. Amplification of the 16S rDNA gene and the internal transcribed spacer (ITS) region, cloning and ARDRA and DGGE were used for the characterisation of the microbial populations followed by sequencing. Several mold and yeasts, as well as bacteria were found to be present in pigeon droppings, which can spread into the environment and be transmitted to immunocompromised individuals and children.

DGGE analysis of the bacterial communities revealed banding patterns that clustered all but one winter samples and all summer samples, showing a high similarity among the microbial members in both seasons and sample locations. Fungal DGGE analysis revealed clusters that grouped summer and winter samples from Johannesburg and Pretoria while VUT samples were clustered on their own. From the identification of fungal and bacterial DNA, *Cryptococcus* species was the majority of fungi isolated from the dropping samples. *Geotrichum*, *Kazachstania* and *Fusarium* species were isolated from phylotypes obtained from ITS amplicons analysed by ARDRA. *Lactobacillus* and *Enteroccoccus* species, organisms usually found in the gastrointestinal tract were the common bacterial members identified. The results showed no difference in microbial communities across all sample locations, while seasonal changes also had no impact in microbial community patterns.

Keywords: Pigeon droppings, diversity, DGGE, ARDRA

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LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ARDRA	Amplified ribosomal DNA restriction analysis
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
CTAB	Hexadecyltrimethylammonium bromide
DGGE	Denaturing gel gradient electrophoresis
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate
HIV	Human immunodeficiency virus
ITS	Internal Transcribed spacer
O.D	Optical density
OUT	Operational taxonomic units
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
µ	Micro
°C	Degrees Celsius

CHAPTER 1

INTRODUCTION

1.1 Background

Humans are in contact with feral pigeons that are found in most cities and towns worldwide (Haag-Wackernagel & Moch 2004; Heddema *et al.* 2006). The absence of predators and an unlimited supply of food have contributed to large populations of pigeons and the accumulation of pigeon droppings in places where they live (Haag-Wackernagel S.a). Their faeces are continuously deposited near their roosting, breeding and feeding sites that includes man-made structures such as city towers, monuments and buildings (Magnino *et al.* 2009). There are concerns of the damaging nature of pigeon droppings on buildings and monuments and environmental hygiene (Magnino *et al.* 2009). Pigeon droppings consist of nitrogen, phosphorus, potassium as well as other minerals. These constituents provide an excellent sanctuary that supports the growth of various microorganisms (Nyakundi & Mwangi 2011). Feral pigeons may directly and indirectly act as vectors for the transmission of various human and animal pathogenic microorganisms, although the pigeon is not usually the source of the pathogens (Haag-Wackernagel & Moch 2004; Zarrin *et al.* 2010).

Humans and animals may contract infections as a result of being exposed to airborne particles containing spores from bird droppings (Nyakundi & Mwangi 2011) while the dust is usually a cause of asthma (Haag-Wackernagel & Moch 2004). Exposure of infection to humans occurs in squares, public gardens, parks and markets. The tendency of pigeons to roost on roofs, balconies and window sills also brings them closer to humans (Magnino *et al.* 2009). Some microorganisms isolated from pigeon droppings are known to be pathogenic agents in immunocompromised and immuno-deficient individuals although immunocompetent individuas have also been reported to be infected from these organisms (Hamasha *et al.* 2004; Kwang & Soo 2005; Millar *et al.* 2007). Some organisms isolated from pigeon droppings that were previously not known to be pathogenic have continued to show increases in rates of infection and mortality in immunocompromised individuals (Warnock 2007). These changes have been attributed to increasing immunocompromised individuals and climate change that has shifted the prevalence of these microorganisms beyond their geographic localities (Panackal 2011). Therefore, it appears that the risk of illness associated with pigeon droppings from opportunistic microbes are largely a function of the immnune status of the host and the

incidence of opportunistic microorganisms (Haag-Wackernagel & Moch 2004). The majority of people contacting invasive mycoses have been those with HIV/AIDS (Human Immunodeficieny Virus/ Acquired Immune Deficiency Syndrome) and cancer (Warnock 2007).

South Africa has approximately 6.4 million people or 12.2% of the population living with HIV (Shisana *et al*. 2014). *Cryptococcus neoformans* is the most common fungal infection in South Africa (Govender *et al.* 2011). Frequently isolated from pigeon droppings, the organism causes meningitis in immunocompromised individuals (Litvintseva *et al.* 2011) and is known to infect a million people annually in Sub-Saharan Africa with two thirds of that number dying as a consequence. It is reported that up to 90% of HIV/AIDS patients contract opportunistic fungal infections due to a weakened immune system and that 10 to 20% die as a direct consequence of these infections (Otang *et al.* 2012). This represents a very high number of immunocompromised people who are sucseptible to infections or complications due to opportunistic pathogens.

In South Africa, no information exists on the variety and ecological occurrence of microbial populations from pigeons and their droppings. In most studies, the isolation, identification and characterization of both fungi and bacteria from fungi and bacteria were usually conducted on culture based techniques (Hamasha *et al.* 2004; Kwang & Soo 2005; Pedroso *et al.* 2007; Silva *et al.* 2009; Liaw *et al.* 2011; Huang & Lavernburg 2011; Wu *et al.* 2012; Silva *et al.* 2012). However, there are microbes which are viable but non-culturable. Therefore the conventional culture based techniques are not able to detect unculturable microbes, even if they are viable. Culture-independent techniques, on the other hand, are better able to target microbes regardless of their culturability or viablity as the techniques only target the nucleic acids. These methods also have a higher sensitivity and specidficity than conventional culturing techniques (Anderson & Cairney 2004; Smalla *et al.* 2007).

Culturable microorganisms are just a small portion of all the possible microorganisms existing in an environmental sample due to the fact that a large portion of all organims are not culturable (Giannantonio *et al.* 2009). Selectivity of media and a lack of knowledge on the conditions under which most organims grow, viability and sampling are critical limitations when trying to gather an understanding of microbial diversity and community structure (Muyzer 1999; Kozdrój & Elsas 2000; Tsiodras *et al.* 2008).

Culture-independent techniques have continued to overcome problems associated with selective cultivation of organisms from natural systems (Marzorati *et al.* 2008). Molecular methods utilising the 16S rDNA gene and the internal transcribed spacers regions have been used to identify microbial communities from diverse environments (Giannantonio *et al.* 2009). Polymerase chain reaction (PCR) is at the core of most molecular biology methods due to its simplicity, specificity, and sensitivity (Luo & Mitchell 2002). The general strategy for genetic fingerprinting basically consists of extraction of nucleic acids, amplification of genes such as the ribosomal DNA units and analysis of the PCR products by techniques such as Denaturing Gradient Gel Eletrophoresis (DGGE) (Muyzer 1999).

South African cities, just like other cities and towns around the world are host to populations of feral pigeons. The country has been facing an unprecedented rise in the number of HIV/AIDS positive and cancer patients (Bradshaw *et al.* 2010). Pigeon droppings as a source of infections to the immune-compromised have not been investigated for the prevalence and occurrence of opportunistic mycotic and bacterial agents. Therefore, the study was proposed to investigate the occurrence of microbial communities using culture-independent techniques.

1.2 Research aim

The aim of the study was to investigate the diversity of bacterial and fungal communities in pigeon droppings using culture independent techniques.

1.3 Research objectives

1. To collect pigeon droppings from three different areas during summer and winter.
2. To extract DNA from the droppings using the CTAB method.
3. To amplify the 16S rDNA gene and assess the bacterial diversity in the pigeon droppings using DGGE and PCR-RFLP.
4. To amplify the Intergenic Spacer region and access the fungal diversity of the mixed template DNA using DGGE and PCR-RFLP.
5. To identify pathogenic microorganisms that may exist in the pigeon droppings by cloning and sequencing of the dominant organisms.

CHAPTER 2

LITERATURE REVIEW

2.1 The feral pigeon

The feral pigeon is a descendant of the wild rock dove and is one of the first animals to be domesticated by man (Haag-Wackernagel 1995). Pigeons were introduced to cities through domestication. Breeders selected pigeon breeds that were tame and had high breeding rates. These characteristics have contributed to the increase in the number of feral pigeons and their adaptation to urban areas (Magnino *et al.* 2009). Feral pigeons have adapted to urban settlements in their natural range and areas where they are transported as captive birds and introduced to the region (Giunchi *et al.* 2012). Currently, they are found in almost all urban settlements in the world. The ease with which the feral pigeon adapted and increased in numbers within urban areas could be attributed to the availability of food. Firstly, pigeons are extremely adaptable birds; this character has allowed them to adjust to atypical breeding places within human structures. Feeding them is also one of the most pleasurable experiences for people in cities (Haag-Wackernagel S.a).

The presence of feral pigeons in urban areas and their interaction with human life and activities can be pronounced in many ways, ranging from harmless birds to harmful pets (Giunchi *et al.* 2012). They are reared by pigeon fanciers for recreational and ornamental value as pets and are fed by people and tourists in urban areas. In addition these birds also offer a cleaning up function by picking and eating discarded food (Magnino *et al.* 2009). On the contrary, they have been shown to be a hazard to human health and well-being and they contribute to damage to buildings and monuments (Marques *et al.* 2007; Tarsitano *et al.* 2010; Albureesh 2011). They can also be a source of accidents of varying nature, such as the occasional slipping on surfaces covered with pigeon droppings to more serious problems such as bird-strike on aircrafts (Giunchi *et al.* 2012).

2.2 Feral Pigeon droppings

A pigeon produces around 12 kg of faeces per year. This is shed on roofs, monuments, statues, streets and sidewalks (Magnino *et al.* 2009). In birds the excreta is moved to the terminal portion of the alimentary canal called the cloaca where it is expelled. Bird faeces are composed of three separate components mixed in the cloaca. The components consist of coloured, solid and coiled faeces. The first part is a creamy and whitish coloured solid urate that is a result of the digestion and metabolism of proteins in the bird digestive system. The second part is the

watery urine made up of uric acids from the kidney (De-Ruiter 2009). Pigeon droppings are corrosive and have been shown to be the most acidic of all bird droppings. This has been attributed to the high uric acid content The uric acid is responsible for the accelerated rate of corrosion and deterioration of monuments made of metals and marble buildings (Vasiliu & Bruiana 2010).

2.3 Pigeon droppings and microorganisms

Pigeon droppings are a suitable environment for the growth of fungi and bacteria (Haag-Wackernagel & Moch 2004; Chee & Lee 2005; Magnino *et al.* 2009; Huang & Lavernburg 2011; Nyakundi & Mwangi 2011; Giunchi *et al.* 2012). These microorganisms are a source for the contraction of diseases for humans (Haag-Wackernagel & Moch 2004). The transmission of pathogenic and opportunistic microorganisms through pigeon droppings to humans depends on: the incidence of pathogenic organisms in the droppings, the survival of these organisms in the droppings and the distribution of these droppings in locations where humans are likely to come in contact with them (Feare *et al.* 1999). The susceptibility of humans to infection from potential pathogenic agents from pigeon droppings is dependent on the state of their immune system (Feare *et al.* 1999).

2.3.1 Mode of Infection

The inhalation of aerosolized organisms or spores from dry weathered droppings, ocular secretions or dust from the wings of the birds are the common methods through which pathogens are transmitted to humans. Other methods of pathogen transmission may be through biting of humans (Haag-Wackernagel & Moch 2004). The transmitted organisms are capable of causing diseases in both immunocompromised and immunocompenent individuals (Wu *et al.* 2012). Immunocompromised individuals have a 100-fold greater risk of contracting diseases from pigeons and their excreta (Haag-Wackernagel & Moch 2004).

2.3.2 Immunity and response

Immunity is commonly used as a measure of resistance to infection. A compromise in immunity can therefore be defined or related to individuals or patients without the ability of defence to infections while immunodeficiency applies to those affected by diseases or conditions in which their defence or immune system is weakened. This can either result from a condition or disease that occurs before and during birth (congenital) or in the first month of life or resulting from the abnormality of a normal system (Mahon & Stiller 1987).

Individuals become immunocompromised due to an acquired or inherited immune deficiency disorder from a number of conditions; acquired immune deficiency often occurs in patients

undergoing blood and marrow transplantation, solid-organ transplantation and major surgery, those with AIDS, neoplastic disease, advanced age, patients receiving immunosuppressive therapy and premature infants (Pfaller *et al.* 2006). The number of immunocompromised patients is on the rise. This in part could be ascribed to a repression of immunity due to cancer treatment, organ transplants, and HIV infection (Soltani *et al.* 2013). Immunocompromised individuals are predisposed to infections from bacteria, fungi and viruses (Soltani *et al.* 2013).

It has been reported that AIDS patients are at a higher risk or constitute the majority of people contracting invasive fungal infections (Warnock 2007). The past twenty years has seen a dramatic increase in the number of emerging fungal pathogens due to the global HIV epidemic. The rise in infections of unknown fungal pathogens and those that have always been known to be opportunistic pathogens has increased along with the number of immunocompromised individuals (Wu *et al.* 2012). However, there has been a decline in the number of immunocompromised individuals succumbing to infections resulting from opportunistic pathogens in developed countries. This has not been the case for other parts of the world, including some parts of Asia and particularly Africa (Park *et al.* 2011).

Sub- Saharan Africa carries the highest burden of the AIDS epidemic, with more than 60% of the world's HIV-infected population. South Africa has the largest number of individuals living with HIV in a single country (Patel *et al.* 2006; Govender *et al.* 2011; Otang *et al.* 2012). From this perspective, the large prevalence of immunocompromised individuals in South Africa due to the AIDS epidemic and the growing problem of cancer (Mayosi *et al.* 2009) is a burden to the health sector and may result in an increase in morbidity (McCarthy *et al.* 2006).

2.4 Pigeons, droppings and disease-causing agents

There is a long recognized link between pigeons and diseases (Haag-Wackernagel & Moch 2004; Magnino *et al.* 2009). Epidemiological studies carried out on pigeon populations revealed at least 100 organisms that are pathogenic to humans, eight of them being viruses, 41 bacteria, 55 fungi and 6 protozoa (Haag-Wackernagel & Moch 2004). *Chlamydophila psittaci*, *Aspergillus* spp., *Candida parapsilosis*, *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Toxoplasma gondii* cause a significant risk of infection in humans. These studies showed a notable lack of transmission of bacteria and viruses to humans (Haag-Wackernagel & Moch 2004; Magnino *et al.* 2009).

Other fungi such as *Aspergillus* spp., *Mucor* spp., *Candida* spp., *Rhodotorula* spp., *Geotrichum* spp. and *Trichosporon* spp. have continually been isolated from pigeon droppings (Khosravi

1997; Costa *et al.* 2010), while isolations of *Penicillium, Mucor, Rhizopus, Paecilomyces, Fusarium, Alternaria,* and *Cladosporium* are becoming common (Soltani *et al.* 2013). These fungi have been classified as emerging opportunistic pathogens (Miceli *et al.* 2011) due to their increased infection and mortality rates in immunocompromised individuals, particularly in patients with AIDS and cancer (Miceli *et al.* 2011).

2.4.1 Pigeons as a vector and source

Some of the microorganisms usually found in pigeon droppings and other birds cause common infections, while some cannot be associated with pigeons (Haag-Wackernagel & Moch 2004; Magnino *et al.* 2009; Zarrin *et al.* 2010). Others continue to gather interest due to their presence, survival and growth in pigeon droppings, raising questions about the role pigeons play in their distribution. For instance, the multiplication of *C. neoformans* is repressed at 42°C, which is reported to be the internal temperature of the pigeon. Furthermore, the viability of this microorganism is also affected by a high alkaline environment such as that found in fresh pigeon droppings. On the contrary, a study by Wu *et al.* (2012) found *Cryptococcus* in fresh and wet droppings, highlighting that fungal cells could have survived and passed though the digestive tract of pigeons. This microorganism and various yeasts can be isolated from weathered pigeon droppings, an environment considered unfavourable for their growth (Zarrin *et al.* 2010).

Other microorganisms have been reported to cause diseases in birds and are also transmissible to humans. *Cryptococcus psittaci*, for example, is known to cause avian chlamydosis (Heddema *et al.* 2006). This microorganism is able to persist in the environment for months and is responsible for chlamydia in humans (Doosti & Arshi 2011). Human infections can result following brief exposure to the contaminated excretions or secretions of infected birds (Doosti & Arshi 2011). Another organism, *Campylobacter*, has been isolated from pigeon droppings and is known to enter the environment through excretions (Vázquez *et al.* 2010). These findings highlight the role played by pigeons as vectors of disease causing organisms.

2.4.2 Survival of organisms in pigeon droppings and transmission

Although the uric acid in pigeon droppings is considered inhibitory to microbial growth and multiplication (Zarrin *et al.* 2010), microorganisms can survive and be transmitted from the droppings (Soltani *et al.* 2013). Some bacteria and fungi are known to have the ability to catabolize uric acid and its derivatives to take advantage of its stored nitrogen, carbon and energy source (Lee *et al.* 2013). This ability to fully degrade uric acid to ammonia together with nitrogen catabolic enzymes are generally found in some bacteria, fungi and most plants.

This makes ammonium the most readily assimilated and preferred nitrogen source for most of these bacteria and fungi (Lee *et al.* 2013). This factor is highlighted in two of the most studied organisms from pigeon droppings, *Hiptoplasma capsulatum* and *C. neoformans* that are able to survive and remain virulent in these droppings (Fischer *et al.* 2009; Ferreira & Raso 2012; Leite-Jr *et al.* 2012).

Species of *Cryptococcus* are known to infect and cause fatal meningitis in immunocompromised individuals, with close to a million infected annually and up to a third of the number dying as a result (Lee *et al.* 2013). The organism's ecological niche is the purine-rich pigeon droppings. The uric acid present in pigeon droppings also assists in the production of a polysaccharide capsule that prevents phagocytosis and aids in the virulence of the organism. This virulence factor can have a detrimental effect in the immune response of a host during infection (Lee *et al.* 2011; Lee *et al.* 2013).

2.4.3 Factors contributing to infections and disease mechanisms

Transmission of pathogens from birds to humans is a complex issue. Transmission depends on several factors including the stability of the pathogen in the environment and environmental factors such as temperature and humidity (Tsiodras *et al.* 2008). For instance, acidic pH, cationic salts, temperatures ranging from 18°C to 37°C and 12% moisture allow the fungus *Hiptoplasma capsulatum* to survive in pigeon droppings. *Histoplasma capsulatum* is a dimorphic fungus which is endemic in certain areas of North, Central, and South America, Africa, and Asia (Fischer *et al.* 2009). Although first isolated in chicken excreta, the fungus predominantly lives in pigeon droppings and soil contaminated with pigeon droppings and is stable for many years in this acidic environment. It remains in this environment in its mycelial form, transitioning to a yeast form in the respiratory tract upon inhalation. Its survival thus effects transmission and infection based on its survival and amount of inoculum in droppings. Exposure to a heavy inoculum load of *H. capsulatum* could lead to infection in an immune-competent host, while even a brief exposure to even a small amount of inoculum causes infection in an individual with declining immunity (Fischer *et al.* 2009)

2.5 Seasonality and host specificity

The occurrence of fungal and bacterial infections is often influenced by seasonal climatic conditions and warming pattern differences (Panackal 2011). The same can also be said about the host behaviour and vulnerability, and the incidence of a particular pathogen. For instance, light-dark cycle changes for fungi can affect their incidence based on weather-related conditions, which are also different in regions where the particular organism occurs (Panackal

et al. 2010). This can be seen in dimorphic fungi, where a temperature induced transition between growth phases occurs, metamorphosing to their parasitic form that makes them virulent (Brooks 2011). Some researchers include global climate change as the reason for the occurrence of invasive human pathogens, particulary fungi, elsewhere of their regional or geographic locations. Panackal (2011) proposed that the incidence of *Cryptoccoccus gattii*, a species found in the semi-tropical climates of South Africa and Australia, in some parts of the US, with a temperate climate is due to the influence of climate change. The organism has found a new niche environment since the temperatures have become increasingly warm.

2.6 Studies on the occurrence of pathogenic microorganisms on pigeon droppings

Although there are studies on the environmental occurrence and identification of pathogens in pigeon droppings in other countries (Chee & Lee 2005; Costa *et al.* 2010), there are no such reports in South Africa. The isolation and composition of microorganisms in pigeon droppings can be used to provide information about the nature and variety of organisms present within the faecal sample (Wu *et al.* 2012). Many studies have focused on known pathogens associated with pigeon droppings, while few have tried to explore the diversity of these organisms. Most studies on the environmental occurrence of pathogens and opportunistic pathogens from pigeon droppings have largely focused on *Cryptococcus neoformans*, and to an extent *Chlamydia pscittaci* and *H. capsulatum*. A few mainly assessed the diversity of fungi while a few looked at bacteria.

Costa *et al.* (2010) isolated *C. neoformans, C. laurentii, Candida* spp, *Rhodotorula mucilaginosa*, and *Trichosporon* spp. from pigeon droppings, confirming that urban pigeons are a potential source of pathogenic yeasts. Khosravi (1997) collected 983 specimens of pigeon droppings in different regions of northern Iran and isolated the saprophytic fungi *Aspergillus, Candida, Mucor* and *Penicillium* spp. from the samples. *Penicillium* spp. was the most frequently isolated saprophyte followed by *Aspergillus* spp., *Mucor* spp., *Rhizopus* spp., *Altenaria* spp., *Fusarium* spp., *Cladosporium* spp. and *Paecilomyces* spp. in pigeon droppings from a study in Iran (Soltani *et al.* 2013). These fungi can be dangerous in immunocompromised persons (Soltani *et al.* 2013). *Mycobacterium* spp. have also been isolated from pigeon droppings in Japan (Tanaka *et al.* 2005).

Microbial communities in environmental samples can be accessed in two ways. Conventional plating of cultivatable microorganisms and molecular methods (Grantina *et al.* 2011). Culturing in media were used to isolate microorganisms in most studies involving pigeon droppings. Molecular techniques have also been employed when targeting individual

organisms to determine their prevalence in pigeon droppings in specific locations. Molecular techniques are usually preceded by culturing in specific media. For example, the *OmpA* gene was used for genotyping *C. psittaci* (Heddema *et al.* 2006). The ribosomal genes were used to determine the composition of yeasts in pigeon droppings (Wu *et al.* 2012).

Characterizing and understanding the microbial community and diversity in pigeon excreta may provide information needed on the risk of contracting diseases (Magnino *et al.* 2009). Although the isolation of microorganisms by culturing allows for more elaborate physiological characterization of the isolates, it does not describe the general microbial diversity of the samples (Gomes *et al.* 2003).

2.7 Methods used to study microbial communities

2.7.1 Diversity explained

In microbiological terms, diversity refers to the variability and complexity of microorganisms at diverse levels of biological organisations (Fakruddin & Mannan 2013). This includes the genetic variability among species, the richness as well as evenness of taxa in communities (Fakruddin & Mannan 2013). Species richness is a score or total number of species present, while evenness is a measure of distribution among the species. In theory, this can be considered as the amount and distribution of genetic information in a community (Torsvik *et al.* 1998). Diversity studies can therefore be used to provide ecological information that may be used to understand structures of the microbial community. This information can also be used as a parameter for the stability of a given community, and be used to measure successions or changes due to stresses in the community (Fakruddin & Mannan 2013).

A community can be made up of different organisms that have similar processes or occupy the same niche. The diversity within a community can be influenced by abiotic and or biotic factors. Factors such as pH, temperature, heavy metals, aerobic or anaerobic conditions to mention a few, can create environmental variations in different ways resulting in shifts of the diversity profile (Fakruddin & Mannan 2013).

2.7.2 Methods used to study diversity

Different methods can be used in the evaluation of diversity. The main methods are culture-dependent and culture-independent (Grantina *et al.* 2011). Each method has its advantages and disadvantages.

2.7.3 Culture-dependent techniques

The process of working with a representative of a sample, isolating pure cultures and identification of the isolates can be a lengthy process (Grantina *et al.* 2011). Culturing of microorganisms involves the use of media which are formulated to provide the sugar, protein and mineral content to grow the required microorganisms. In this case, dilution of the sample is required to reduce the number of the growing colonies to a controllable level (Hill *et al.* 2000).

The greatest limitation of this method is that less than 1% of microorganisms are cultivable when using typical culturing techniques (Millar *et al.* 2007; Rastogi & Sani 2011). In this case, only the culturable organisms are accounted for while fastidious and those in the unculturable state will not be detected (Hill *et al.* 2000). Lack of knowledge on the conditions under which most organisms readily grow, selectivity of the media and formulations used and viability of organisms in the media are some of the shortfalls of this method (Muyzer 1999; Kozdrój & Elsas 2000; Tsiodras *et al.* 2008). Morphological characteristics are not sufficient for establishing a detailed classification of microorganisms (Fakruddin & Mannan 2013). Growth of some organisms like fungi, require specific knowledge of their growth, behaviour and morphology. Secondly, the methods involved tend to be time consuming and expensive, with the entire analysis depending wholly on the growth of the organism. In the case of dimorphic organisms, the variability can make it difficult to correctly identify with culturing techniques (Brooks 2011).

In contrast to culturing techniques, molecular techniques allow and offer more genetic heterogeneity of microbial communities while providing the capacity to identify microorganisms without the need for cultivation (Cho *et al.* 2003; Grantina *et al.* 2011). These techniques afford the chance to characterize and identify the diversity of environmental organisms in a wide variety of niches, and have continued to revolutionize diversity studies (Piterina *et al.* 2010; Su *et al.* 2012).

By directly extracting and amplifying microbial genes, one can obtain a more reliable portrayal of diversity (Burr *et al.* 2006), while eliminating the bias and restriction of the selective nature of media in providing for culturable and non-culturable organisms (Marzorati *et al.* 2008).

2.8 Molecular methods employed for diversity and characterization studies

The potential of molecular methods lies in their allowance for comparative studies across different environments as well as allowing for accurate identification of species that often have common morphological characteristics (Marano *et al.* 2012). Molecular based methods can

therefore be considered more accurate when compared with culture based techniques (Marano *et al.* 2012).

The simplicity, specificity and sensitivity of molecular methods, is based on the polymerase chain reaction (Luo & Mitchell 2002). Molecular methods allow the rapid and comprehensive processing of samples from all kinds of environments. In the case of community members, molecular methods are also able to detect all the organisms due to the fact that nucleic acids are available throughout all stages of the microorganisms life cycle (Marano *et al.* 2012). Diversity is thus easily estimated without having to isolate the microorganisms. The use of genes, where differences in genes can be detected has now become a framework for measuring diversity (Fakruddin & Mannan 2013).

A number of approaches are available for measuring molecular microbial diversity. The common strategy for genetic fingerprinting of microbial communities involves: extraction of nucleic acids, ampification of target genes by PCR, and the analysis of PCR products by genetic fingerprinting techniques (Muyzer 1999).

2.8.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction is a highly sensitive method for analysis of microbial DNA extracted from environmental samples. PCR allows for the detection and amplification of gene sequences, specific to particular microorganisms, and its value lies in the identification of species from amplified products without the need for culturing of microorganisms (Boccuzzi *et al.* 1998). The characterization of microbial communities is achieved without prior knowledge of morphology of individuals from the community (Marchesi *et al.* 1998). A PCR product generated from environmental samples is thus considered to replicate the microbial gene signatures from all organisms present within the sample (Rastogi & Sani 2011).

The molecular approach, usually termed partial community DNA analysis (Ranjard *et al.* 2000), is a culture-independent method consisting of the analyses of whole genomes or selected genes such as 16S, 18S rDNA and the internal transcribed spacers (ITS) for prokaryotes and eukaryotes (Rastogi & Sani 2011).

The detection of sequence specific characteristics such as length variability or nucleotide polymorphisms are some of the specific characteristics that can be used to function as genetic markers and can be employed to discriminate individuals in culture-independent analyses of samples (Xu 2006; Enkerli & Widmer 2010a).

PCR amplifications are carried out either with random, specific or universal primers. Conserved regions of the locus of interest can be used to design universal primers that allow the detection of a wide range of organisms across phylogenetically related groups (Enkerli & Widmer 2010b). Molecular methods using the 16s rDNA gene have been used to identify microbial commuties from diverse environments (Giannantonio *et al.* 2009; Piterina *et al.* 2010) .

2.9 Universal genes employed for phylogenetic analyses

2.9.1 16S rDNA

The use of the DNA that codes for the ribosomal RNA and the subsequent sequencing of the amplified product have proven valuable for identifying bacterial organisms where other methods fell short. The 16s rDNA gene, commonly used in identifying bacterial species, has found widespread use due to its highly conserved nature as well as moderate copy number depending on the genus. It is found in all bacteria and accumulates mutations at a slow constant rate (Millar *et al.* 2007). The 16s rDNA molecules possess critical structural differences based on certain conserved regions of sequence in all bacteria. These rDNA sequences offer unique signatures to any bacterium and useful information about relationships between bacteria can be deduced from the highly variable portions of the gene (Chakravorty *et al.* 2007). There are over 90 000 deposited sequences of the 16S DRNA gene and an unknown strain can easily be compared against the previously deposited sequences (Clarridge 2004). To amplify and further analyse the hyper-variable regions within the 16S rDNA gene sequences, broad-range PCR primers can be designed to recognize the conserved gene sequences flanking the hyper-variable regions. The primers used in this case are often referred to as universal primers (Chakravorty *et al.* 2007).

2.9.2 Internal transcribed spacer region (ITS)

In contrast to bacteria, taxonomic identification of fungi has often been centred on the amplification of the eukaryotic ribosomal small unit, the 18S rRNA. The shortfall of using this gene is the fact that identification of organisms is only limited to the family or genus level. This is because of the lack of variation within the 18 rRNA genes of closely related fungal species. The evolution of the kingdom fungi occurred over a much shorter compared to bacteria, hence the lack of variation (Anderson & Cairney 2004).

In fungi, rRNA genes occur as tandem repeats, with one to several hundred repeat copies per genome (Viaud *et al.* 2000). The internal transcribed spacer (ITS) region is located between the 18S rRNA and 28S rRNA genes, and includes the ITS1 and ITS 2, the non-coding but

variable sequences (Diguta *et al.* 2011), and integrates the 5.8S rRNA gene, the conserved coding region (Bellemain *et al.* 2010).

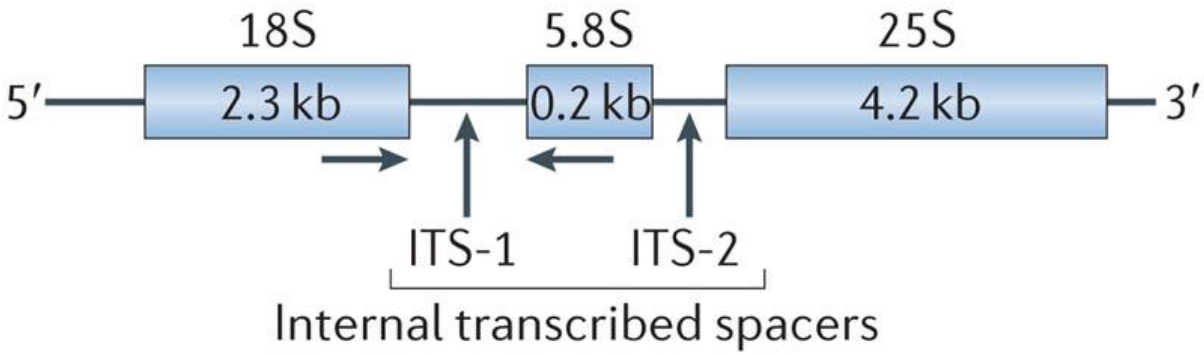

Figure 2.1: Fungal Ribosomal DNA internal transcribed spacers region (Underhill & Iliev 2014).

Non-coding rDNA spacer regions, such as the ITS, benefit from a fast rate of evolution, resulting in greater sequence variation amongst closely related species compared with the more reserved coding regions of the DNA gene cluster. For this reason, a much greater taxonomic resolution is achieved using fungal ITS sequences rather than sequences generated from coding regions (Anderson & Cairney 2004). For fungal identification, there are ITS sequences deposited in public sequence databases that are available as reference material (Bellemain *et al.* 2010).

2.9.3 Cloning and library construction

Cloning of large fragments of DNA directly from microbes in natural environments provides an opportunity to access metagenomic DNA. In this procedure, the sequences amplified from the environmental samples are separated by cloning so they can be individually characterized using PCR-RFLP or sequencing methods (Ranjard *et al.* 2000). By amplification of all DNA and the screening of the created libraries, this approach offers a basis for genomic analysis of even the uncultured microorganisms (Rondon *et al.* 2000).

The method involves the ligation of the amplified gene fragments into a suitable plasmid vector, which is then transformed into competent cells, usually *Escherichia coli* (Sambrook *et al.* 1989; Gonzalez *et al.* 2003). The more common ligation of environmental amplicons into a vector is done with the universal TA cloning method. The approach is dependent on the sticky ends made by *Taq* DNA polymerase. The polymerase adds a single 3'adenosine overhang to

the amplified PCR products. The vectors chosen should also possess a vector-specific 3' thymidine overhang. The ligation of the vector and amplicon is then catalysed by DNA ligase or Topoisomerase I (Leigh *et al.* 2010).

The ligated vectors can then be introduced into competent cells followed by plating onto selective media, commonly basing the selection on the usage of antibiotic-resistant genes within the vector that are expressed when the transgenic vector is up taken by the cell. The colonies obtained from the individual transformed cells produce copies of each single sequence which can then be extracted and followed by analysis of the individual sequences (Lee *et al.* 2007; Leigh *et al.* 2010; Kluber *et al.* 2011). Although the cloning/sequencing approach is effective and desired, it takes up a lot of time, is labour intensive and very expensive (Burr *et al.* 2006; Steven *et al.* 2007). The screening of clones can consequently be followed by other techniques that aim to characterise the sequences (Ranjard *et al.* 2000). These techniques, also based on PCR-amplification are called genetic fingerprinting and can also be used to detect microbial diversity within the environmental samples (Gonzalez *et al.* 2003).

2.10 Genetic fingerprint techniques

The principle of these techniques is centred on the determination of diversity of the amplified sequences based on the electrophoretic migration differences on agarose or polyacrylamide gels, depending on their size or sequence (Ranjard *et al.* 2000). Complex band profiles are produced, yielding a descriptive analysis of the community of the organisms targeted by the primers used (Ranjard *et al.* 2000).

The characterization or screening of clones with molecular fingerprinting techniques has increased in popularity, with amplified ribosomal DNA restriction analysis (ARDRA) (Riggio *et al.* 2007; Leigh *et al.* 2010; Santos *et al.* 2010) and DGGE (Handschur *et al.* 2005; Burr *et al.* 2006; Thornhill *et al.* 2010) being the more common approaches used to select one or several clones from large clone libraries and explore the diversity within environmental samples.

2.10.1 Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP)

Amplified ribosomal DNA restriction analysis (ARDRA) is a method used to study microbial diversity that is based on DNA polymorphisms (Kowalchuk *et al.* 2002) and makes use of the activity of restriction endonucleases (REs) or restriction enzymes and the differences in recognition sites of the enzymes on DNA sequences. The targeted sequences are selected and amplified, followed by digestion using REs, usually tetra-cutters, and the resulting fragments

separated by electrophoresis on high-density agarose or polyacrylamide gels. Genotypic groups are then created for the community by using the resulting profiles (Sklarz *et al.* 2009).

Compared to other techniques, the method does not require a lot of equipment, making it simple and affordable. The method provides a genetic fingerprint of communities, populations or phylogenetic groups. Usually at least two or more restriction enzymes are used, while fragments obtained can then be analysed as separate or combined data sets (Grantina *et al.* 2011). Two of the key factors or parameters that describe community structure and diversity, species richness and evenness, are qualitatively determined from this technique, based on the sequence differences from the population analysed (Liu *et al.* 1997).

Specific RLFP patterns are produced from the analysis of the specific regions of rDNA, which can then be used to create and define operational taxonomic units (OTU). The number of OTUs present in a community is then used to describe the community structure and the abundance of individual clones within each OTU (Moyer *et al.* 1994; Ranjard *et al.* 2000). However, ARDRA does not provide information about the identity of the microorganisms present (Ranjard *et al.* 2000), and can be a restricted tool for the sole use of demonstrating the presence of specific phylogenetic groups or for estimating species richness and evenness (Liu *et al.* 1997).

2.10.2 Denaturing gradient gel electrophoresis

PCR-DGGE is an electrophoretic method that allows one to describe the genetic diversity of total microbial communities by separating mixtures of PCR products that are of the same length but differ only in sequence. The use of DGGE in microbial ecology was established by Muyzer and co-workers (Muyzer *et al.* 1993). The power of this technique lies in the separation of double stranded DNA molecules by exploiting their melting behaviour. Universal primers that target a conserved region of the community DNA are used (Muyzer 1999; Gafan *et al.* 2005).

To one of the primers is attached a GC rich sequence, commonly referred to as GC clamp. The clamp, about 40 bases long, is attached to all amplicons generated during PCR. It is attached to ensure that a part of the amplified DNA remains double-stranded during electrophoresis. The electrophoresis takes place through a polyacrylamide matrix that is increasingly denaturing. As the migration of the double-stranded DNA proceeds, they encounter a concentration of denaturant, which will halt their mobility (Green *et al.* 2010; Rettedal *et al.* 2010). In theory, any small subunit rDNA gene found in the mixed template DNA extracted

from the sample could be specifically amplified and resolved on a DGGE gel (Jackson *et al.* 2000). After the separation of the amplicons from the rDNA by DGGE, they can then be excised, cloned and sequenced (Kozdrój & Elsas 2000). This technique can be used to analyse several or multiple samples at the same time. This allows for the monitoring of complex changes that microbial communities could undergo, due to variations brought about by changing seasons or time (Muyzer 1999; Lopez *et al.* 2003; Poulsen *et al.* 2005).

A combination of cloning libraries, RFLP/ARDRA and DGGE techniques have been used in studying fungal and bacterial communities in environmental samples. Although clone library creation and RFLP analysis as a fingerprinting method serves as a better tool for diversity in place of cultivation of microorganisms, the need to construct the libraries and screen them is demanding in terms of labour. DGGE is a means of bypassing the labour used for detecting and exploring species diversity and community structure (Liu *et al.* 1997). The resulting DGGE profiles can then be analysed by use of multidimensional scaling and principal component analysis, which group them according to set criteria. Mostly, hierarchical cluster analysis are often employed to determine similarities in the obtained data as well as measuring community diversity using diversity indices (Gafan *et al.* 2005). Diversity indices, particularly the Shannon index has been employed for the measurement of diversity (Hill *et al.* 2003).

2.10.3 Sequencing

DNA sequence data offers a more accurate and definitive way to identify microbes. Phylogenetic classification is therefore based on the variable regions of the DNA sequences among different organisms. Sequencing of a comprehensive range of PCR products has the possibility to detect almost any bacterial species if 16S rDNA is used. The extensive known sequences in the GenBank and other databases can be used to compare the resulting sequences, helping in identifying the unknown organism (Millar *et al.* 2007). Usually, sequence identification by sequencing and database comparisons are assigned to phylum, class, order, family, subfamily or genus, or species at sequence similarity cut-off values of 80, 85, 90, 92, 94, or 97%, respectively (Rastogi & Sani 2011).

2.11 Limitations to molecular methods

PCR amplification is dependent on the extraction of good quality nucleic acid. The type of extraction method chosen or used should be appropriate to avoid shearing of DNA, and provide good quality and quantity DNA. For environmental samples, particularly soil and plants, phenolics and humic acids or substances are co-extracted along with the DNA. This has a detrimental effect on the PCR amplification of DNA as they can inhibit the function of the *Taq*

polymerase (Kreader 1996; Zhou *et al.* 1996). PCR bias, where certain templates in the pool are preferentially amplified is a limitation in molecular approaches. This occurs due to the low GC content in some templates, differential accessibility of rDNA genes within some genomes or the probability of amplification due to the copy numbers within the genome (Polz & Cavanaugh 1998; Gonzalez *et al.* 2012).

Strategies to counter these limitations include serial dilution of the DNA and titration with $MgCl_2$. This can, however, lead to low reproducibility in amplification, lack of amplification of low count genetic targets lost through dilution and reduced resolution power of DNA based techniques (Piterina *et al.* 2010). Addition of adjuvants or additives such as bovine serum albumin (BSA), betaine, DMSO (dimethyl sulfoxide), formamide and T4 gene 32 protein to PCR to protect DNA polymerases from inhibitors have been successfully used (Kreader 1996; Anderson & Cairney 2004; Enkerli & Widmer 2010b; Farell & Alexandre 2012). The adjuvant or additive to be used is selected by empirical screening of numerous compounds in the PCR reaction until successful amplification is achieved. In other cases, the experience of other researchers working with a similar sample is used based on the type of impurities or inhibitors expected or known to be found within the sample niche (Piterina *et al.* 2010). To reduce PCR bias, a number of approaches can be used. High template concentrations can be used in the PCR amplification of the gene pool. Secondly, a number of PCR amplification replicates can be combined to minimize PCR drift and thus bias (Polz & Cavanaugh 1998).

Nested/semi-nested PCR approach can also be used. This approach works by using two sets of primers, with the first set of primers aimed at producing a PCR product larger than the second set. The first PCR product is then used as a template to amplify an internal region of the DNA in the following amplification stage. The primers in the second PCR set can be different to the first set (nested) or one of the primers can be the same as the first set (semi-nested). Increase in sensitivity of detection can be achieved if the primers in the second reaction are species-specific (Millar *et al.* 2007).

2.12 Summary

Pigeons and humans have continued to live in close contact since their domestication by man. These birds have become a risk to human health as they are vectors of opportunistic as well as pathogenic organisms. The continually increasing numbers of diseases affecting immunocompromised individuals have made these organisms even more important, and the need for studying and reviewing these communities of organisms has become apparent.

Culture-dependent techniques have continued to be employed for the analysis of organisms from pigeon droppings, although these techniques target a mere 1% of the organisms of existing microorganisms. Various studies have shown that organisms that are hard to culture and are represented by few genera always constitute a part of the community and would otherwise be hard to depict with culture-dependent techniques.

These findings therefore propose or suggest that molecular techniques that bypass the necessity for isolation and cultivation are highly appropriate for in-depth characterization of environmental microbial communities (Rastogi & Sani 2011). The use of evolving, yet highly conserved genes makes them accessible for many methods like ARDRA, DGGE, cloning and sequencing (Weidner *et al.* 1996).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection

Old, weathered and fresh pigeon droppings were collected from 3 locations including Burghers Park in Pretoria, the South African High Court and Ghandi square in Johannesburg and Vaal University of Technology (VUT) campus in Vanderbijlpark. Parks were selected because they represent common places where pigeons and humans are in regular contact. The droppings were collected in August 2012 during winter and April 2013 in summer for the study. Samples were collected by the random sampling method. The samples were ground to a powder and mixed to a homologous mixture. Table 3.1 contains the labels used for the sample and the descriptions.

Table 3.1: Identification and description of samples used in the study

Sample label	Description	Location	GPS coordinates	Season collected
1	Dry droppings	Johannesburg	26.1208°S, 28.0243°E	Winter
2	Dry droppings	Pretoria	25.7539°S, 28.1925°E	Winter
3	Dry droppings	VUT	26.7118°S, 27.8629°E	Winter
4	Wet/fresh droppings	Johannesburg	26.1208°S, 28.0243°E	Winter
5	Wet/fresh droppings	Pretoria	25.7539°S, 28.1925°E	Winter
6	Wet/fresh droppings	VUT	26.7118°S, 27.8629°E	Winter
7	Dry droppings	Johannesburg	26.1208°S, 28.0243°E	Summer
8	Dry droppings	Pretoria	25.7539°S, 28.1925°E	Summer
9	Dry droppings	VUT	26.7118°S, 27.8629°E	Summer
10	Wet/fresh droppings	Johannesburg	26.1208°S, 28.0243°E	Summer
11	Wet/fresh droppings	Pretoria	25.7539°S, 28.1925°E	Summer
12	Wet/fresh droppings	VUT	26.7118°S, 27.8629°E	Summer

3.2 DNA isolation

DNA was extracted according to Zhou et al. (1996) with modifications. All samples were ground with a pestle and mortar in the presence of liquid nitrogen. About 5 g of the sample was mixed with 13.5 ml of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB) and 100 µl of proteinase K (20 mg/ml) in Oakridge tubes by horizontal shaking at 225 rpm for 30 min at 37°C. After the shaking treatment, 1.5 ml of 20% (w/v) SDS was added, and the samples were incubated in a 65°C water bath for 2 h with gentle end-over-end inversions every 15 to 20 min. The supernatants were collected after centrifugation at 6,000 x *g* for 10 min at room temperature and transferred into 50-ml centrifuge tubes. The pellets were extracted two more times by adding 4.5 ml of the extraction buffer and 0.5 ml of 20% SDS, vortexed for 10 s, followed by incubation at 65°C for 10 min, and centrifuged as before. Supernatants from the three cycles of extractions were combined and mixed with an equal volume of chloroform-isoamyl alcohol (24:1, v/v). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1h. The pellet of crude nucleic acids was obtained by centrifugation at 16,000 x *g* for 20 min at room temperature, washed with cold 70% ethanol, and re-suspended in sterile deionized water, to give a final volume of 500 µl. The quality of the DNA was then confirmed by gel electrophoresis on a 1% Tris-Borate-EDTA agarose gel with ethidium bromide (EtBr) under ultraviolet light. DNA concentrations were determined for all extracts using the NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA). The extracted DNA was diluted 1:20 using sterile DNase free water and stored at -20°C until required.

3.3 DNA amplification

DNA was amplified using a C1000 thermal cycler (Bio-Rad, Hercules, CA). The bacterial 16s rRNA gene and fungal ITS rDNA were targeted using specified universal primers (Table 3.2). A PCR ready-to-use cocktail containing dNTPs (0.2 mM of each dNTP at 1X) $MgCl_2$ (2mM at 1X) and KAPA 2G Robust Hotstart DNA Polymerase (Kapa Biosystems, Wilmington, NC) was used together with 1.25 µl of 100 ng/ml BSA, sterile distilled water and 3 µl of template DNA to give a final reaction volume of 25 µl. Three PCR products were amplified and pooled together for each sample to avoid PCR bias and recovering better concentrations during purification.

Table 3.2: Primers used for the amplification of targeted genes in the study

Target	Primer Sequence (5'to 3')	Reference
Bacterial 16s rRNA		
27f	AGAGTTTGATCCTGGCTCAG	Burr *et al.* (2006)
1492r	GGTTACCTTGTTACGACTT	Burr *et al.* (2006)
341f	CCTACGGGAGGCAGCAG	Muyzer *et al.* (1993)
518r	ATTACCGCGGCTGCTGG	Muyzer *et al.* (1993)
Fungal ITS		
ITS1f (forward)	CTTGGTCATTTAGAGGAAGTAA	Anderson *et al.* (2003)
ITS2	TCCTCCGCTTATTGATATGC	Valášková & Baldrian (2009)
1TS4 (reverse)	TCCTCCGCTTATTGATATGC	Anderson *et al.* (2003)
Vector Primers		
M13f	CAGGAAACAGCTATGAC	Alexander & Imhoff (2006)
M13r	GTAAAACGACGGCCAG	Alexander & Imhoff (2006)
GC-clamp	CGC CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G	Muyzer *et al.* (1993)

3.3.1 PCR amplification of the 16S rRNA gene

The amplification of the 16s rDNA was done under the following conditions: initial denaturation step at 95°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min and a final extension step at 72°C for 10 min.

3.3.2 PCR amplification of the Internal Transcribed spacer region (ITS) gene

Amplification of the ITS region was carried out in the thermal cycler as follows: initial denaturation step at 95°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min and a final extension step at 72°C for 10 min.

Amplified products were confirmed by gel electrophoresis on a 1% (w/v) TBE agarose gel. Five microliters of the PCR product was mixed with 1 µl loading dye (KAPA 6X Loading dye) and loaded into a gel well. Three microliters of DNA ladder (KAPA) was also loaded onto the gel to estimate the size of the fragments. Electrophoresis was done for 60 minutes at 90 V using 1X TBE buffer. Ethidium bromide (10 microliters/milliliter) was added to the gel for visualization under UV light. Gel images were captured using an imaging system (Bio-rad Gel Doc Imaging system, Hercules, CA).

3.4 Cloning

3.4.1 Preparation of chemically competent cells using the rubidium chloride method

One milliliter of overnight *Escherichia coli* DH10B strain was inoculated into 100 ml of Psi broth and incubated at 37°C in a shaking incubator until the absorbance measured 0.48. The culture was then incubated on ice for 15 min, followed by centrifugation at 4000 xg for 5 min. The supernatant was discarded and 40 ml of TfbI buffer (30 mM KOAc, 100 mM RbCl, 10mM $CaCl_2$, 50 mM $MnCl_2$, 15 % (w/v) glycerol; pH 8) was added followed by 15 min incubation on ice. The tube was then centrifuged (Eppendorf, San Diego, CA) at 4000 xg for 5 min, the supernatant discarded and 4 ml TbfII (10 mM MOPS, 10 mM RbCl, 75mM $CaCl_2$, 15 % (w/v) glycerol; pH 6.5) was added. The tube was incubated on ice for 15 min. one hundred microliters of the suspension was then transferred into microcentrifuge tubes, quick- frozen using liquid nitrogen and stored at -80°C.

3.4.2 Ligation and transformation

PCR products were first purified using the Wizard SV Gel and PCR Clean-up kit (Promega, Madison, WI) following the manufacturer's instructions. The purified amplicons were then ligated into the plasmid vector PCR 4.0 using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

This procedure consisted of ligating the DNA into the PCR4-TOPO vector followed by transformation of competent *E. coli* DH10B cells that had been prepared in-house. The maximum recommended incubation times (30 minutes) from the manufacturer's instructions were used for each step with 3 µl of PCR product. pUC19 plasmid and "vector only" reactions were set as controls for all cloning work. The transformed cells were plated on Luria-Bertani (LB) plates containing 50 µg/ml of Kanamycin.

3.4.3 Analysis of positive clones by PCR

Colonies with inserts were checked using PCR amplification with M13 (vector) primers (Table 3.2) in a colony PCR procedure. Colonies were screened for inserts by direct amplification using 0.5µM M13 F and R primers (Invitrogen) in colony PCR. Pipette tips were used to pick out transformed *E.coli* colonies which were then introduced into a 25 µl PCR reaction mix (without BSA). The following thermal conditions were used; initial denaturation step at 95°C for 10 min followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension step at 72°C for 5 min. Positive clones containing the correct size of amplicons for both I6S rDNA and ITS genes were further evaluated by restriction digestion.

3.5 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

To examine the ARDRA patterns, 1µl of a 1:10 dilution of the PCR product amplified by the M13 primers (10µM) was re-amplified by using the 27f and 1492r primers for 16S rDNA gene while ITS1f and ITS4 were used to amplify the ITS clones with the same PCR conditions as described above.

The 16S rDNA fragment was digested with two selected tetra-cutter restriction endonucleases (REs), *Hae*III and *Hin*fI while ITS amplified product was digested with *Hin*fI and *Mbo*I. For a 20 µl reaction mixture, a master mix consisting of the restriction enzyme (RE) 1µl (1.0 U) (Promega, Madison, WI)); 2µl Restriction buffer (10 X) (Promega); 0.5µl of acetylated BSA (10µg/µl) (Promega); 4µl PCR amplicon and 12.5µl of Milli-Q water (Merck, Germany).

The reaction mixture was incubated at 37 °C for 3 h. the restriction digestion products were resolved on 2.5% agarose gel in 1X TBE at 75 V for 3 h and visualized on a UV transilluminator (Bio-Rad). Clones that had the same restriction pattern for any one enzyme were assigned to the same ARDRA group (represented by alphabets) while all clones with both the same *Hae*III and *Hin*fI or *Hae*III and *Mbo*I restriction patterns were assigned to one ARDRA OTU.

3.6 Nested PCR-Denaturing gel gradient electrophoresis (DGGE)

A nested PCR-DGGE approach was used for amplification of the products for DGGE analysis. One microliter of PCR products resulting from the amplifications stated in section 3.3 were used for the analysis. Primer sets 341F and 518R and ITS1F and ITS2 were used for amplification of 16S rDNA and ITS regions, respectively. The primers 341F and ITS1F contained a GC clamp (Table 3.1) at the 5'end for DGGE analysis.

PCR of bacterial samples for DGGE was carried out with the temperature profile as follows: an initial denaturation step of 3 min at 95°C, followed by 10 cycles of 94°C for 30 s, 65-55°C for 30 s with a decrease of 1°C after every cycle and 72°C for 30 s. An additional 15 cycles were allowed at the constant annealing temperature of 55°C. The final extension step was for 10 min at 72°C. In all, only 25 cycles of PCR were allowed to avoid excessive amplification of dominant fragments. Cycling conditions for fungal PCR-DGGE were as follows: 94°C for 5 min, 35 cycles of; denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and a final elongation step at 72°C for 10 min.

A DCode Universal Mutation Detection System (Bio-Rad) was used for DGGE analysis. Twenty microliters of PCR products was mixed with an equal amount of 2 X loading dye and resolved in an 8 and 9% (w/v) polyacrylamide denaturing gradient gels in 1X TAE buffer for bacteria and fungi, respectively. The denaturing gradients were prepared with 35-55 % and 40-60% gradients of denaturants (urea and formamide) for ITS and 16S rDNA respectively.

Electrophoresis was run at 60°C, first for 10 min at 20V and then for 17h at 70V. The gels were stained with ethidium bromide in 1X TAE buffer for 15 minutes and destained for 25 minutes. The gel was then viewed under a UV transilluminator.

3.7 Excision of DGGE bands and sequencing

Bands of interest were then excised from the polyacrylamide gel using sterile surgical blades and placed in 0.2 ml Eppendorf tubes with 30 µl sterile distilled water and kept in the 4°C fridge overnight to allow the DNA to be released into the water. The DNA was then amplified using the DGGE primers without the GC clamps. PCR conditions for excised bands from DGGE were the same as that described above. The annealing temperature for both 16S rDNA and the ITS region was 58°C. The resulting individual samples were then sent to Inqaba Biotech (Pretoria, South Africa) for sequencing using the Sanger sequencing method.

3.8 Data analyses

3.8.1 DGGE Analysis

The Gel2k software program (Norland 2004) was used to analyze DGGE patterns using Jaccard co-efficient (Jaccard 1908) to calculate similarities among band patterns based on the absence/presence of bands and their positions

3.8.2 ARDRA analysis

Representatives of ARDRA phylotypes or OTUs from the fungal sequences were selected for sequencing. The ARDRA data obtained in the study was cumulatively used for rarefaction

analysis to determine whether the number of colonies picked for every microbial population was sufficient to give a reliable reflection of the diversity in the community.

3.8.3 Sequence analysis

All the DNA sequences were checked for chimera detection using DECIPHER (Wright *et al.* 2012), and compared to database sequences by using BLAST (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov). The sequences were aligned together with similar sequences from the GenBank using T-Coffee and MAFFT online alignment programmes. All chimeric sequences were excluded.

The aligned sequences were then exported and used to determine phylogenetic relationships for the 16S rDNA and ITS genes. A neighbor-joining method (Saitou & Nei 1987) was used to infer relationships for both genes using Mega 6 programme (Tamura *et al.* 2013). The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor 1969) for ITS and Kimura-2-parameter for 16S rDNA in a bootstrap analysis of 1000 replicates.

CHAPTER 4

RESULTS

4.1 Introduction

In this study, DNA was extracted from pigeon dropping samples collected from three locations during the winter and summer seasons. Bacterial and fungal DNA was amplified by targeting the 16S rDNA and ITS regions, respectively. Diversity within the resulting amplicons was assessed by use of cloning libraries, ARDRA and DGGE fingerprinting. Some of the sequences derived from the fingerprinting methods were used for sequencing and identification of the microorganisms.

4.2 DNA isolation

Total genomic DNA was successfully extracted (Fig. 4.1) using the hot-lysis CTAB method as described (Section 3.2). The DNA extracted had a dark brown colour signalling co-extraction of humic acids. Purification methods were employed to remove the colour and other co-extracts. However, the purification step resulted in loss of DNA and was omitted. The quality and quantity of the DNA extracted was not determined spectrophotometrically due to known interference of humic acids with spectrophotometric measurements of DNA. The extracted DNA was diluted in a 1:20 dilution and used for PCR amplification and other analysis.

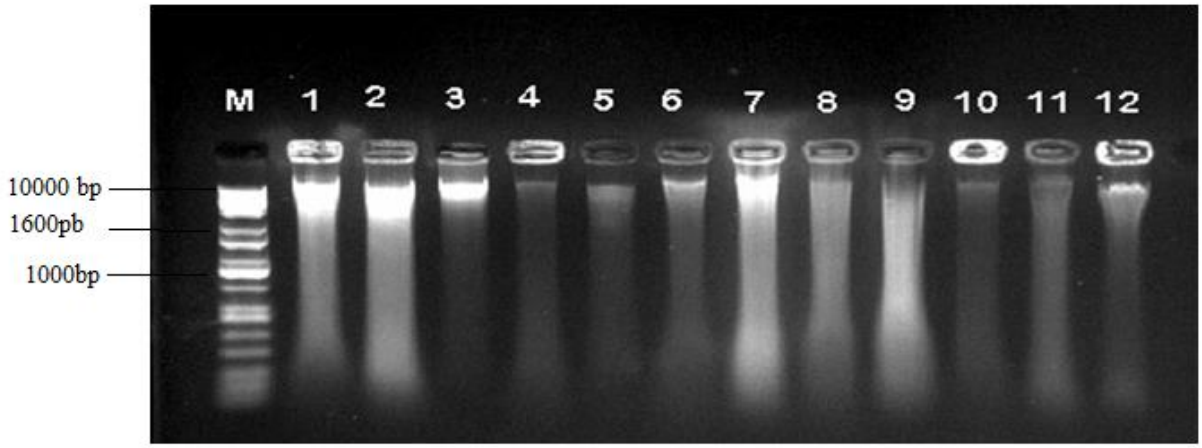

Figure 4.1: Gel electrophoresis showing DNA extracted from pigeon dropping samples collected for this study. (M=100bp molecular weight marker. The numbers represent the sample names in Table 3.1).

4.3 DNA amplification

The amplified products of the 16S rDNA gene is shown in Fig. 4.2. The 16S rDNA amplified product was approximately 1500bp for all samples.

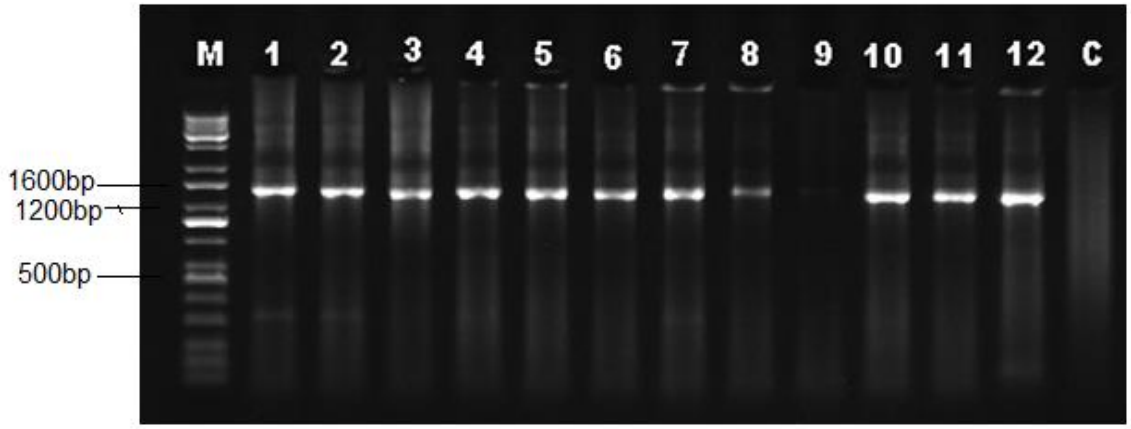

Figure 4.2: Agarose gel 1% (w/v) showing electrophoresis of amplified 16S rDNA products from pigeon droppings. (The numbers represent the sample names in Table 3.1).

These amplicons were then used in a nested PCR-DGGE amplification reactions targeting the V3 region of the 16S rRNA gene with primers 341f-GC and 518r as detailed in section 3.6. The PCR products were approximately 233bp for all samples (Fig. 4.3)

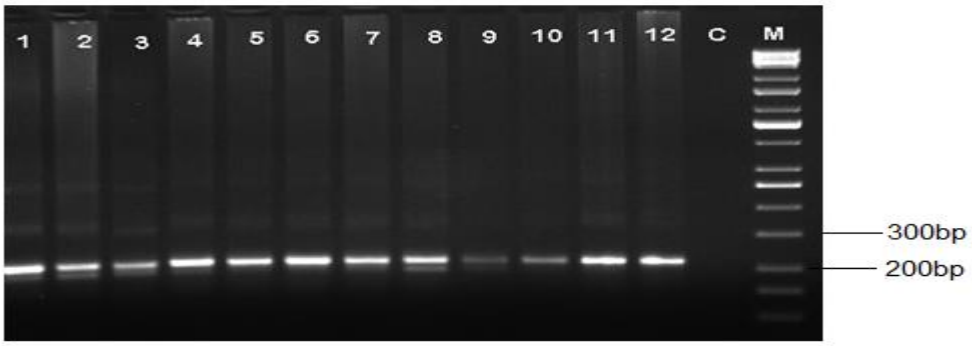

Figure 4.3: Agarose gel showing 16S rDNA fragments amplified for DGGE analysis in a nested PCR reaction. (M=100bp molecular weight marker. The numbers represent the sample names in Table 3.1).

The amplified products of the ITS regions are shown in Fig. 4.4 and ranged from 500 to 900 bp. (The dilution of the sample to reduce the amount of co-extracted humics and the addition of BSA to enhance amplification proved to be successful).

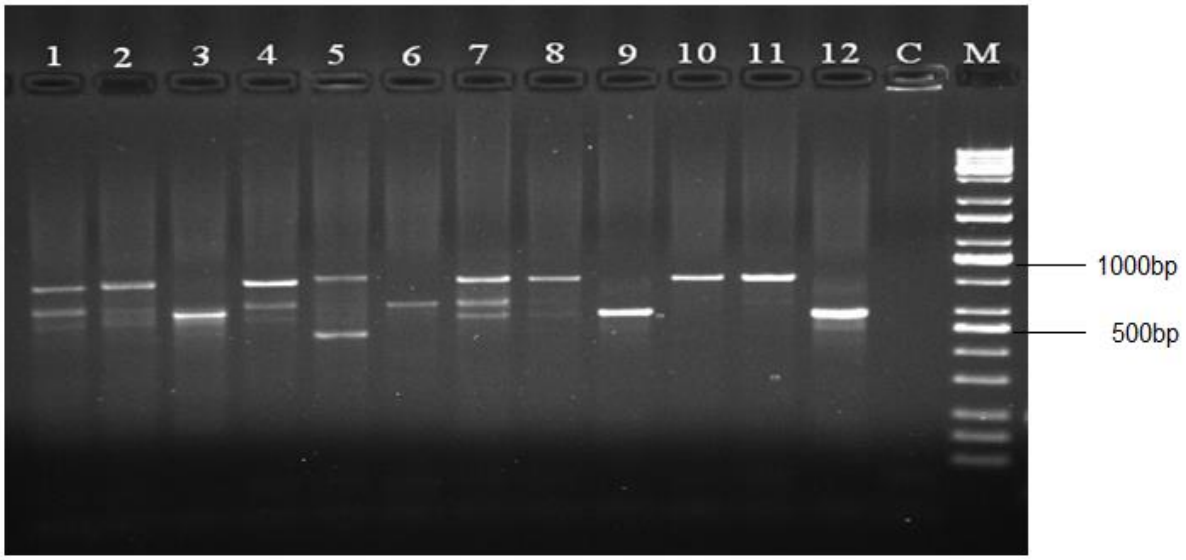

Figure 4.4: Agarose gel 1% (w/v) showing ITS amplified fragments on samples from pigeon droppings. (M=100bp molecular weight marker. The numbers represent the sample names in Table 3.1).

4.4 Cloning and verification of inserts

Transformed cells were grown on LB agar (Fig. 4.5). Ten randomly selected colonies were picked from the 16SrDNA and ITS clone libraries to check for correct sized inserts (Fig. 4.6 and 4.7). For colonies from the 16S rDNA clone libraries, amplicons were considered to be correct if their size was approximately 1650 bp, while the ITS amplicons were considered positive if they ranged between 500-900 bp. Only positive clones were used for further analysis using restriction digestions. Forty eight and 51 clones were selected for further analysis from the winter and summer samples, respectively, for the 16S rDNA clone library. Fifty three and 51 clones were selected from winter and summer samples, respectively, from the ITS clone library.

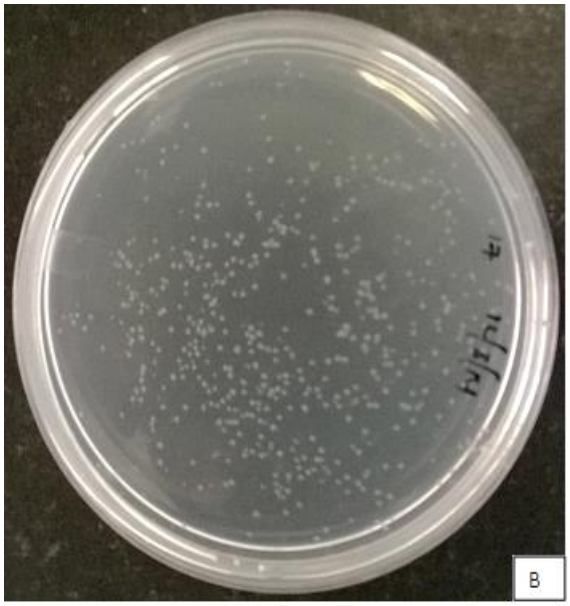

Figure 4.5: Representative plate showing transformed *E.coli* cells grown on LB agar. The pCR 4-TOPO vector used allows for direct selection of recombinants by disruption of the lethal E.coli gene, *ccd*B. Ligating a PCR product disrupts the expression of the gene, permitting growth of only positive recombinants upon transformation, making blue/white screening unnecessary.

Colony PCR was then performed on randomly selected colonies to confirm the correct size of the inserts using M13 primers for 16S rDNA clones (Figure 4.6) and ITS primers for ITS clones (Figure 4.7). Only positive clones were selected for further analysis using ARDRA.

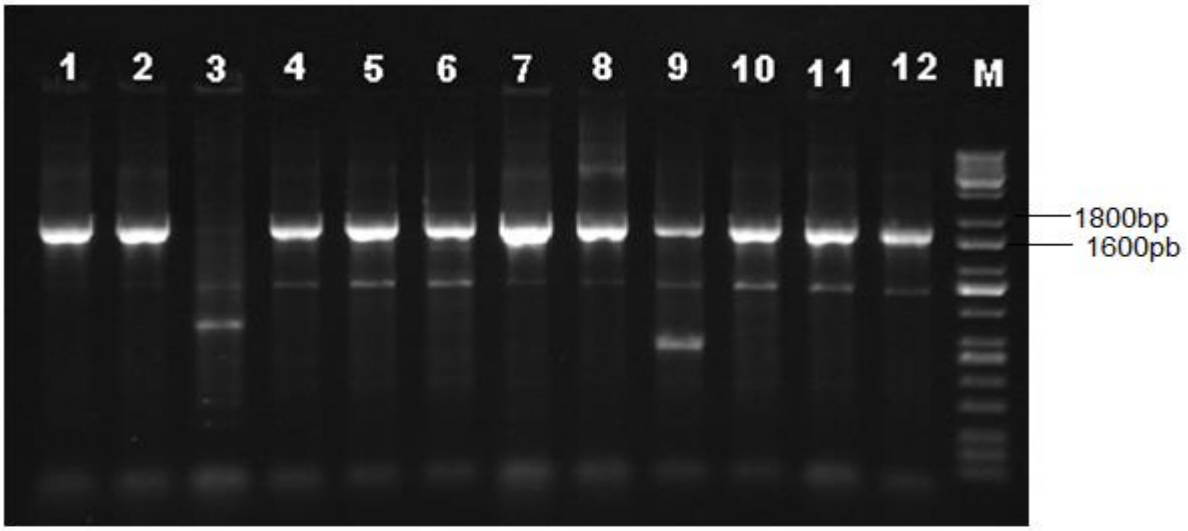

Figure 4.6: Representative 1% (w/v) agarose gel showing electrophoresis of PCR products showing fragment inserts using M13 primers from clones created from the 16S rDNA samples.

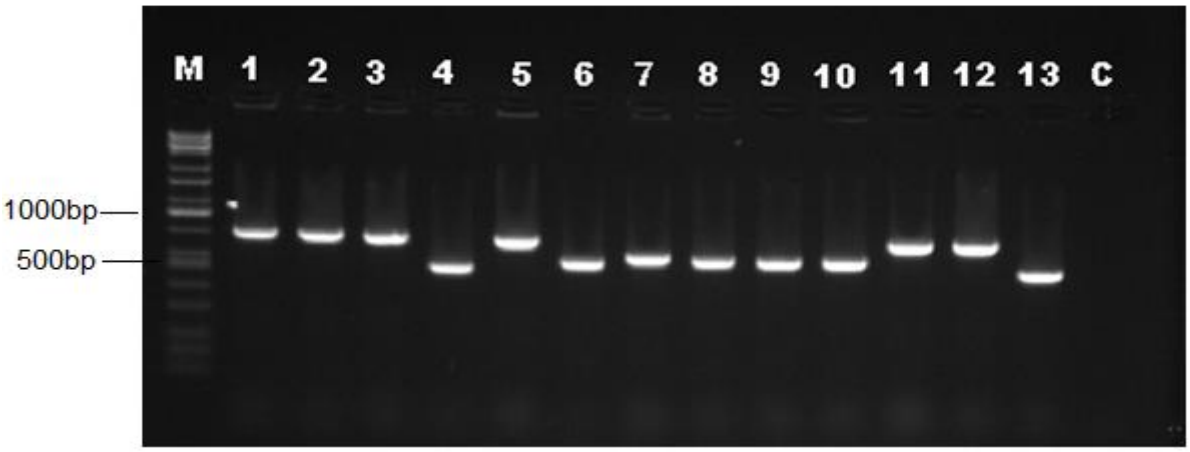

Figure 4.7: Representative electrophoresis gel confirming the presence of transformation inserts from ITS samples.

4.5 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Positive clones were selected for restriction analysis of PCR products using the two tetrameric restriction endonucleases. For all ARDRA analysis, bands that were smaller than 100pb were disregarded in band pattern analysis.

The banding patterns for the *Hae*III and *Hin*fI digestions of the 16S rDNA gene are shown in Fig. 4.8 and 4.9, respectively.

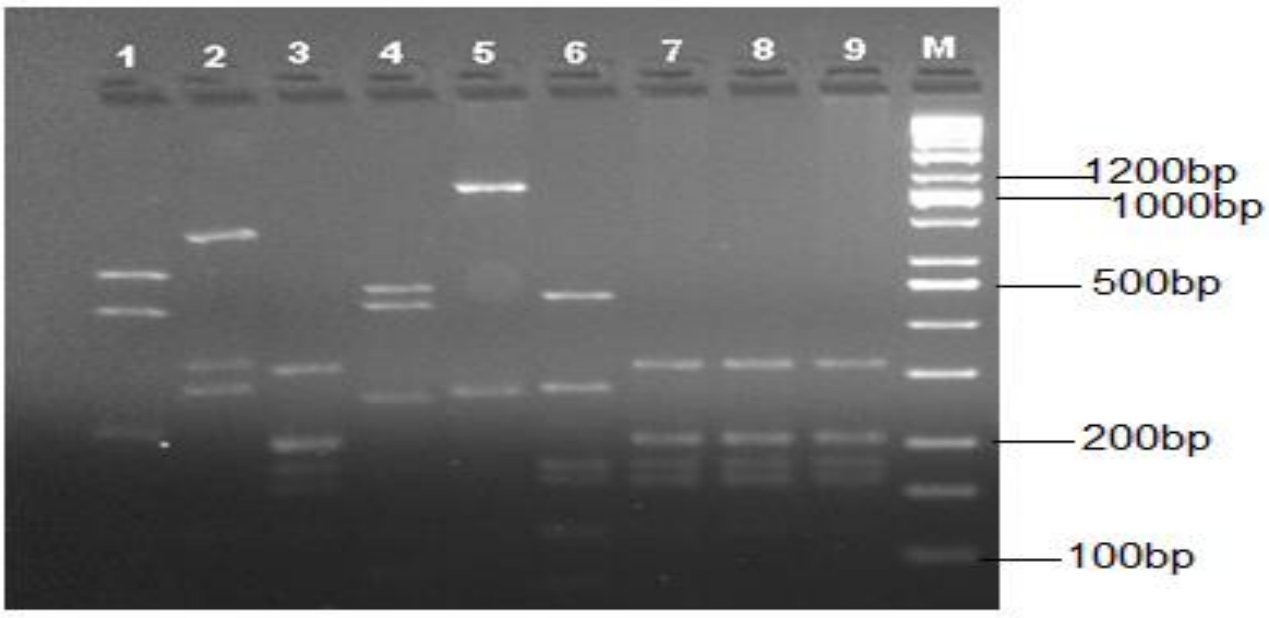

Figure 4.8: Representative gel electrophoresis of *Hae*III restriction digestion for 16S rDNA clone inserts.

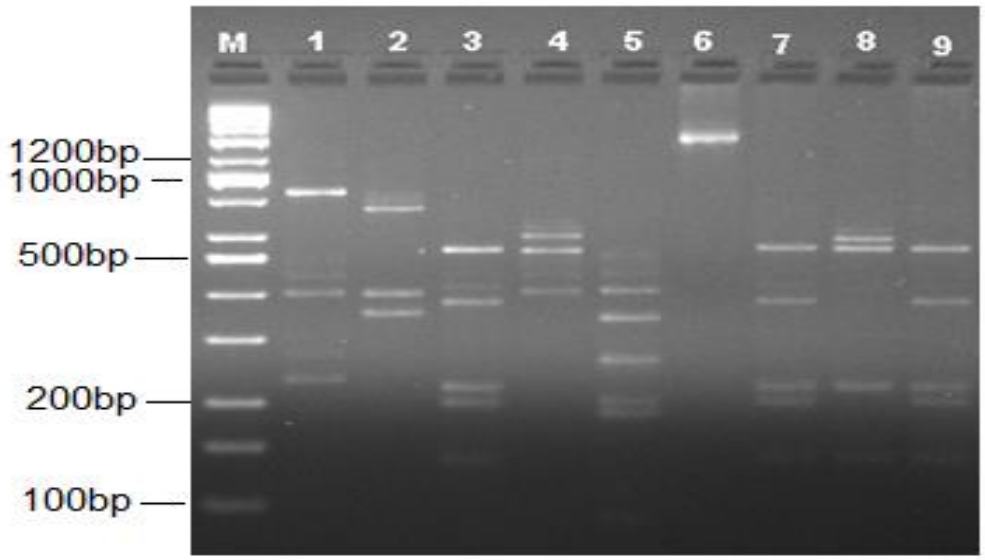

Figure 4.9: Representative gel electrophoresis of *Hin*fI restriction digestions for 16S rDNA clone inserts.

Banding patterns that were similar for all the samples after restriction digestion were grouped together and considered an Operational taxonomic unit (OTU), a term used to refer to an individual strain or phylotype.

Thirteen ARDRA groups were identified for the *Hae*III (Fig. 4.10) and *Hin*fI (Fig. 4.11) digestions.

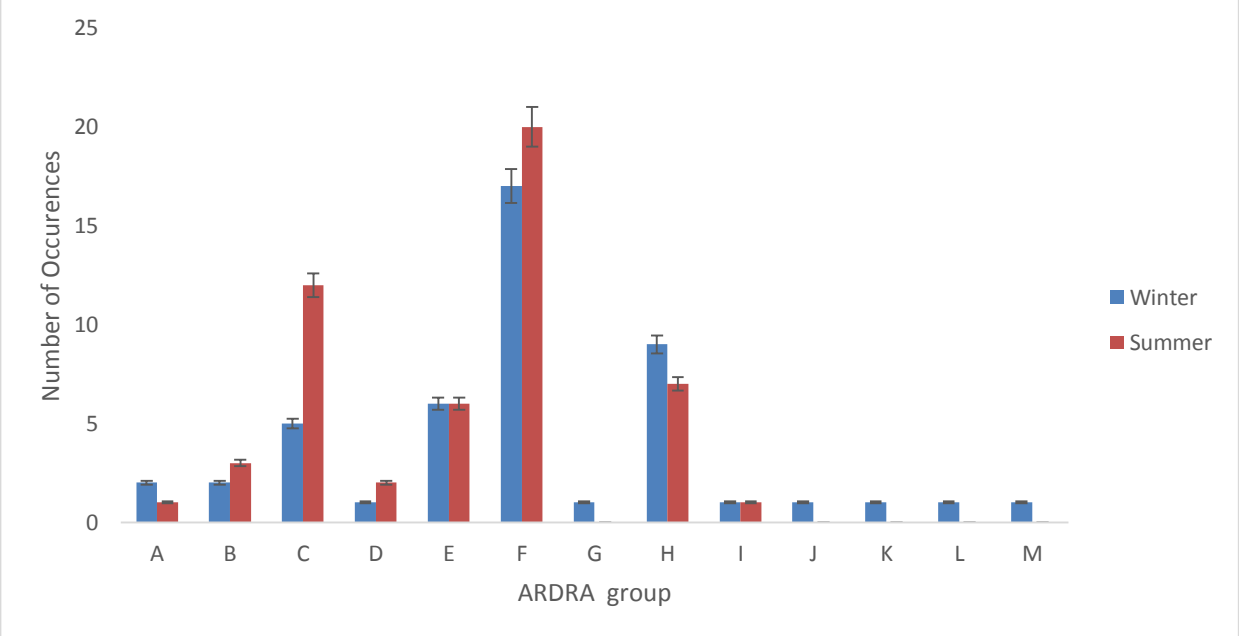

Figure 4.10: ARDRA groups derived from 16S rDNA for winter and summer samples using the enzyme *Hae*III.

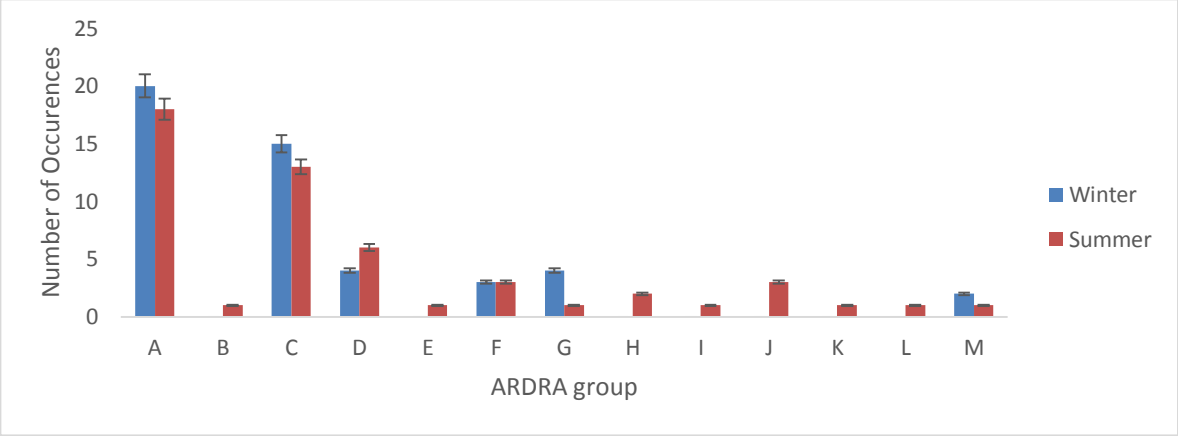

Figure 4.11: ARDRA groups obtained from 16S rDNA for winter and summer samples using the enzyme *Hin*fI.

The digestion of 16S rDNA gene for all the samples yielded 26 phylotypes or OTUs (Fig 4.12). Each season had 18 phylotypes, with 16 being unique and 10 being common to the both seasons. Only two phylotypes appeared more than two times in both seasons. OTU 14 contained the vast majority or 35% of clones (35 of the 99 clones), followed by OTU 19 (15%), making up 50% of the clone libraries. These were the common phylotypes derived from all three locations during both the summer and winter season. The rest of phylotypes occurred either once or twice in both seasons while a few only occurred once in only one season for all three locations.

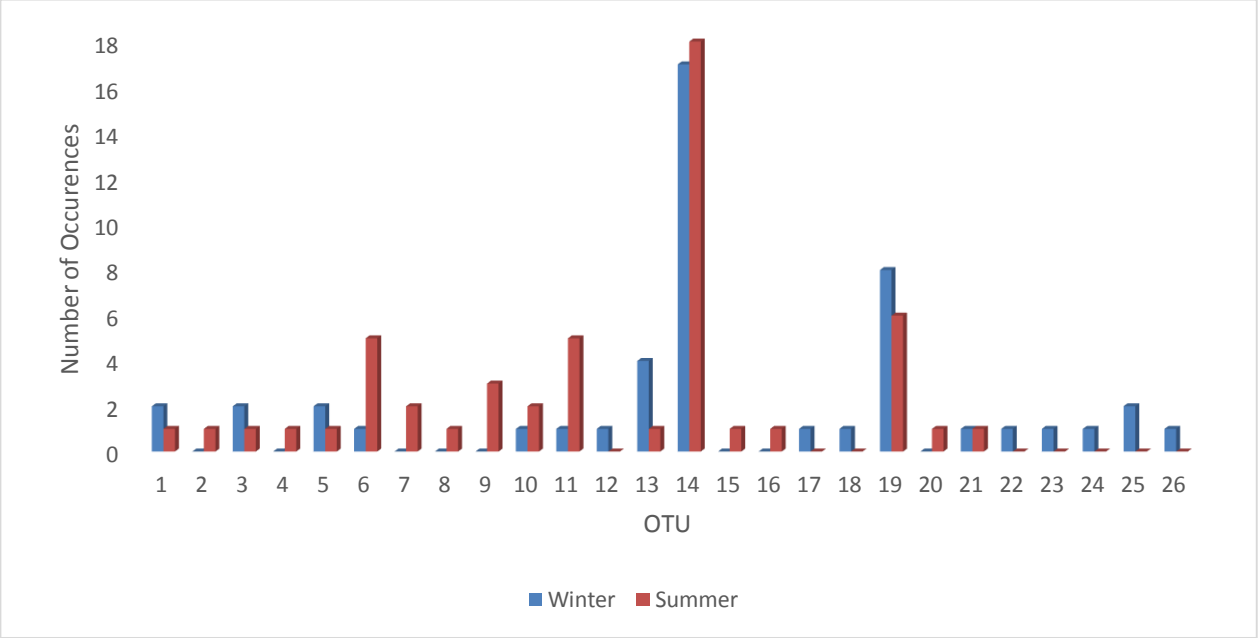

Figure 4.12: Unique OTUs derived from 16S rDNA. A total of 26 OTUs were identified for the summer and winter clones.

For ARDRA analysis of ITS clone libraries, *Hae*III and *Hin*fI were also used. However, the banding patterns with *Hae*III showed no distinct profiles for the different amplicons a since many fragments remained undigested (Fig. 4.13).

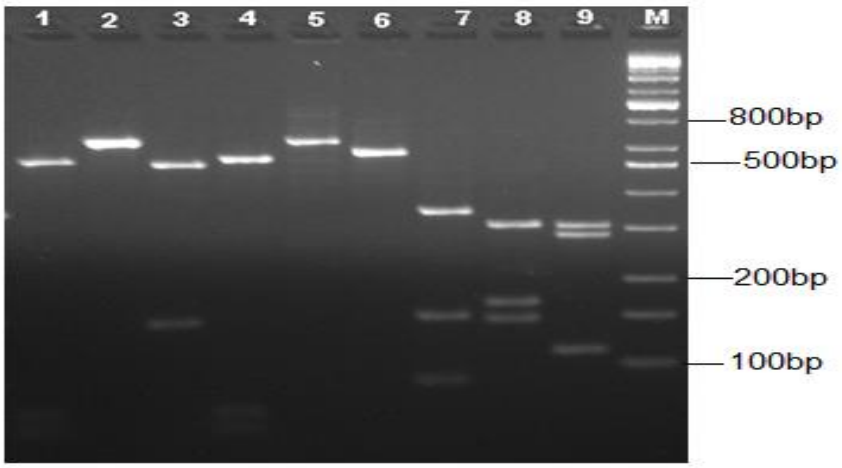

Figure 4.13: Agarose gel showing *Hae*III RFLP digestion patterns for ITS clone inserts.

*Mbo*I was therefore used in combination with *Hin*fI. The two endonucleases produced patterns that were used for comparison (Figure 4.14 and 4.15).

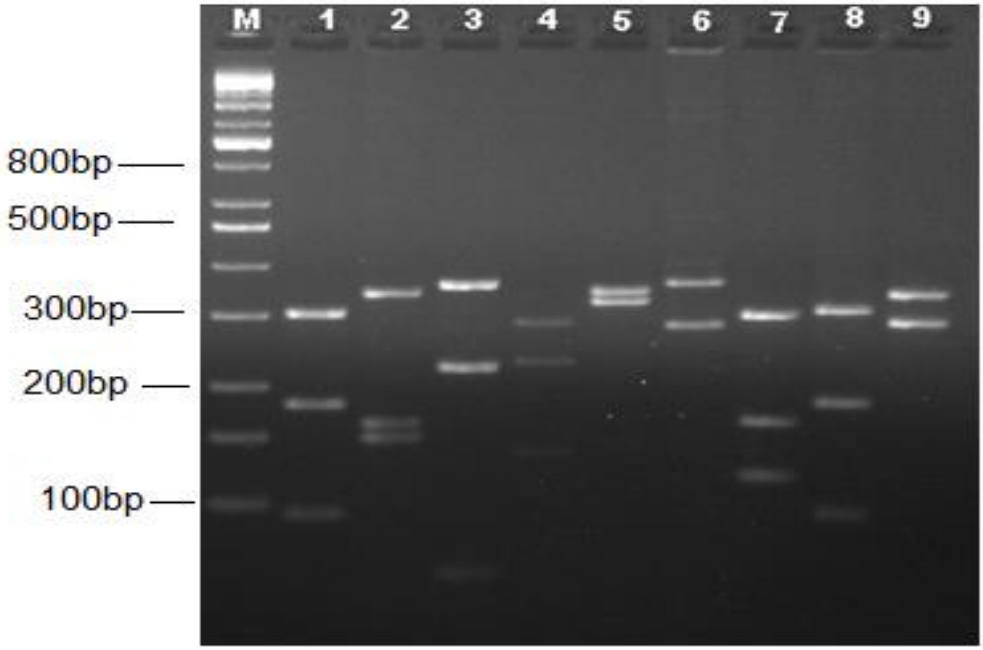

Figure 4.14: Agarose gel showing of *Hin*fI PCR-RFLP patterns for ITS clone inserts

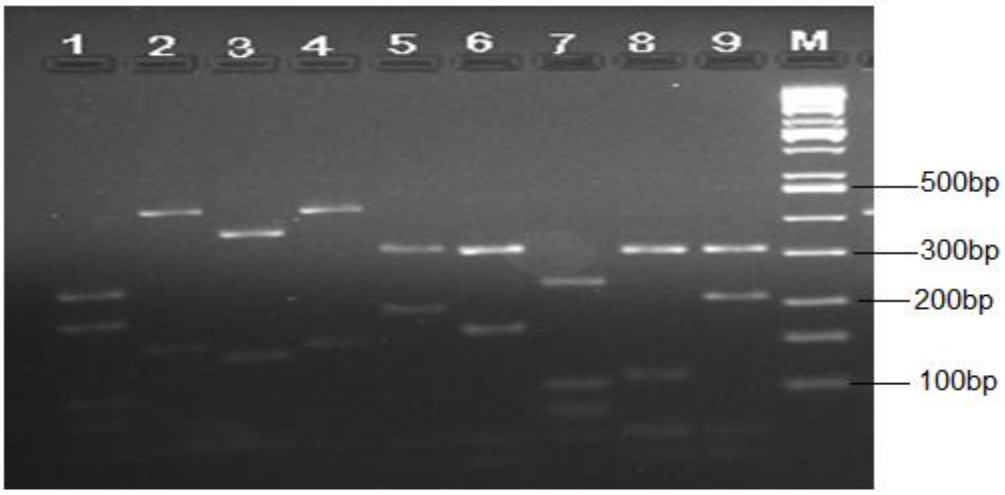

Figure 4.15: Agarose gel showing restriction patterns obtained from ITS amplicons digested with *Mbo*I

Sixteen ARDRA groups were obtained with *Hin*fI (Fig. 4.16) while 12 resulted from *Mbo*I digestion (Fig. 4.17). A total of 41 phylotypes were derived from all clones (Fig. 4.18). Of the 41 phylotypes, 16 were unique to winter samples while 13 were unique to summer samples. Only 11 phylotypes were common among the two seasons. Four of these common phylotypes occurred more than once.

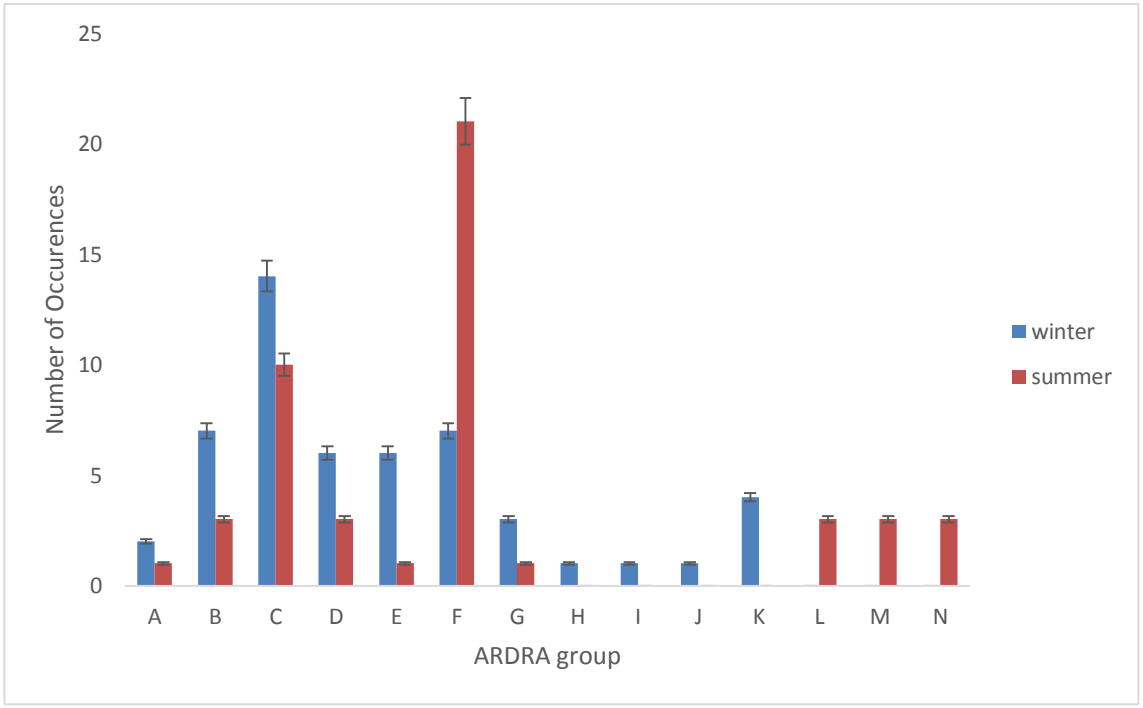

Figure 4.16: ARDRA groups derived from 16S rDNA samples using the enzyme *Hin*fI.

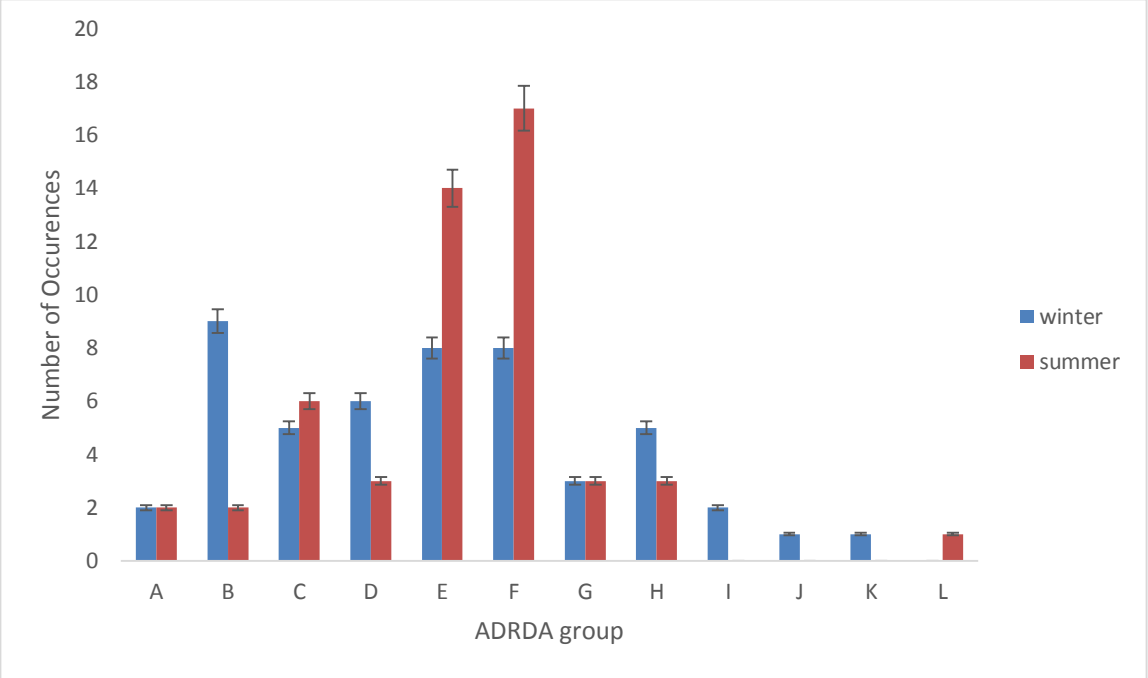

Figure 4.17: ARDRA groups derived from ITS clones using the enzyme *Mbo*I.

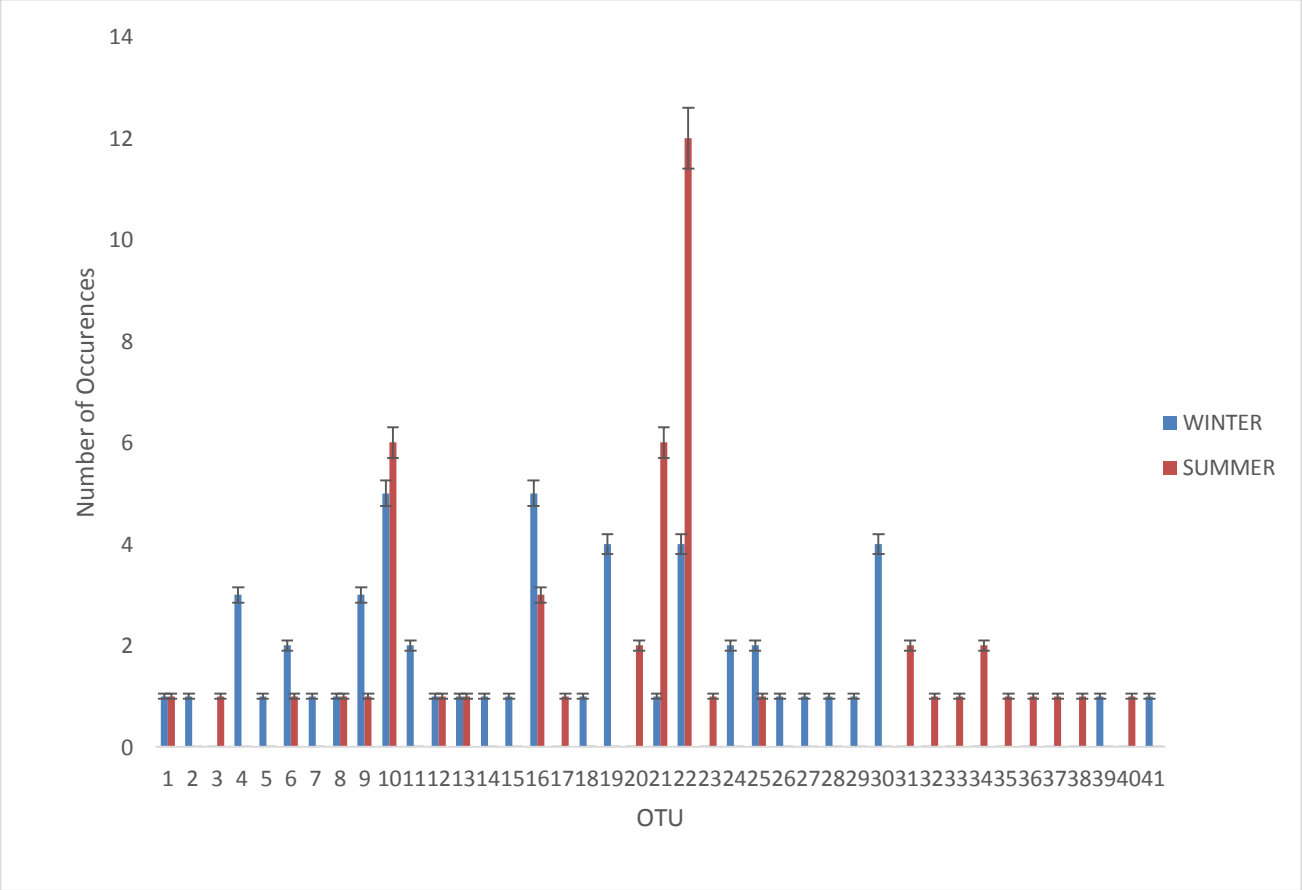

Figure 4.18: Unique OTUs derived from ITS clones

ITS clones belonging to some of the common and selected unique phylotypes were sequenced. A total of 40 clones were sequenced. The resulting sequences were compared against sequences in the GenBank. Table 4.1 shows the sequenced clones and their nearest match from the GenBank.

Table 4.1: Clones from the ITS libraries and their nearest match from GenBank

Sequence Identification	GenBank ID	Accession Number	Percentage (%) similarity (no. of bp)	E-value
1_its1f	*Galactomyces geotrichum*	AJ27945.1	98% (367/373)	6e-180
2_its1f	*Galactomyces geotrichum*	AJ279451.1	99 % (364/369)	6e-180
3-its1f	*Metschnikowia pulcherrima*	AY301026.1	97% (370/382)	1e-175
4_its1f	*Geotrichum candidum* strain WLL1	KJ817904.1	99% (360/365)	9e-178
5_its1f	*Leptosphaerulina australis*	JN712494.1	99% (527/532)	0.0
6_its1f	*Cystofilobasidium lari-marini*	AY052494.1	98% (575/585)	0.0
8_its1f	Uncultured *saccharomycete* clone	EF087980.1	99% (447/450)	0.0
9_its1f	*Epicoccum* sp.	KF128843.1	100% (538/538)	0.0
10_its1f	*Epicoccum* sp.	JQ388284.1	99% (530/535)	0.0
11_its1f	*Arxiozyma telluris* 18S rRNA gene (partial)	AJ853763.1	99% (296/298)	3e-149
13_its1f	*Cryptococcus magnus* strain	JQ425371.1	97% (634/654)	0.0
14_its1f	*Epicoccum* sp. strain HS-1	JQ388284.1	99% (543/549)	0.0
A_its1f	*Cryptococcus magnus* strain	JQ425371.1	99% (633/641)	0.0
B_its1f	Uncultured *endophytic fungus* clone	EF504508.1	99% (610/612)	0.0
C_its1f	*Cryptococcus kuetzingii* strain	AF145327.2	99% (606/608)	0.0
D_its1f	Uncultured *endophytic fungus* clone	EF504508.1	99% (611/612)	0.0
E_its1f	*Kazachstania telluris* CBS 2685	NR_111115.1	85% (482/566)	4e-179
F_is1f	*Cryptococcus albidus*	KC254020.1	99% (610/614)	0.0

G_its1f	*Fusarium equiseti* isolate	KJ562376.1	99% (544/545)	0.0
H_its1f	*Cryptococcus albidus* isolate	KC295595.1	99% (604/606)	0.0
I_its1f	*Cryptococcus albidus* isolate	KC295595.1	99% (603/605)	0.0
J_its1f	*Cryptococcus diffluens* isolate	KC152904.1	99% (604/607)	0.0
K_its1f	*Filobasidium uniguttulatum* isolate	KF958247.1	99% (599/601)	0.0
L_its1f	*Cryptococcus* sp.	JN255513.1	98% (598/610)	0.0
M_its1f	*Filobasidium uniguttulatum* isolate	KC152903.1	99% (608/610)	0.0
N_its1f	Uncultured *ascomycete* clone	EU489889.1	99% (548/555)	0.0
O_its1f	*Filobasidium uniguttulatum* isolate	KC152903.1	99% (608/611)	0.0
P_its1f	*Holtermanniella watticus* isolate	JQ857031.1	99% (547/550)	0.0
Q_its1f	*Pseudeurotium bakeri* strain	GU934582.1	99% (553/557)	0.0
R_its1f	*Cryptococcus magnus* strain	JQ425371.1	99% (629/636)	0.0
S_its1f	*Kazachstania telluris*	NR_111115.1	85% (462/545)	2e-169
T_its1f	Uncultured *endophytic fungus* clone	EF504508.1	99% (609/612)	0.0
U_its1f	Uncultured *fungus* clone	KF800449.1	100% (602/602)	0.0
V_its1f	*Clavispora lusitaniae*	FJ183442.1	99% (371/373)	0.0
W_its1f	*Ascomycota* sp.	HM535402.1	99% (514/520)	0.0
X_its1f	*Geotrichum* sp.	AY513953.1	99% (361/362)	0.0
Y_its1f	*Clavispora lusitaniae* strain	EF221824.1	98% (368/376)	9e-178
Z_its1f	*Geotrichum candidum* strain	KJ817904.1	99% (362/363)	0.0

4.6 Phylogenetic analysis of ARDRA derived sequences

Figure 4.19 shows the evolutionary relationships of the sequenced clones from the ITS amplicons and their nearest matches from the GenBank.

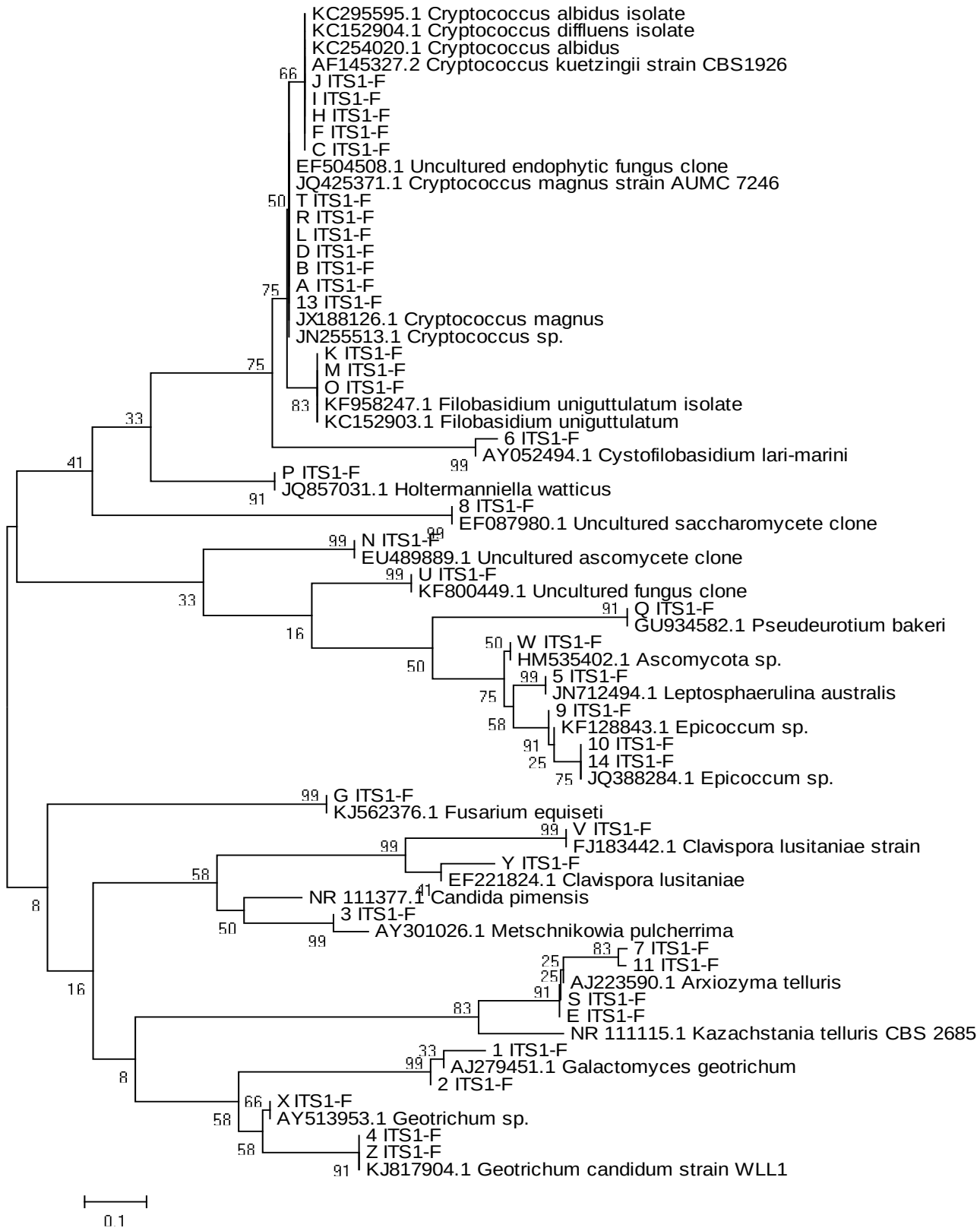

Figure 4.19: Phylogenetic relationships built on sequences of ITS amplicons isolated from pigeon droppings

4.7 Rarefaction analysis

Rarefaction analysis was used to determine if the number of clones selected for analysis were sufficient to provide a reliable representation of the diversity of microbial populations from the sample groups**.** Table 4.2 shows a summary of all the phylotypes from ARDRA analysis of the constructed libraries. This information was used to create rarefaction curves (Fig. 4.20).

Table 4.2: Summary of cumulative number of clones for all clone libraries for the two seasons and the phylotypes derived.

Clone Library	16S winter clones	16S summer clones	ITS winter clones	ITS summer clones
Number of clones	48	52	53	51
Number of phylotypes/OTU	18	18	28	24

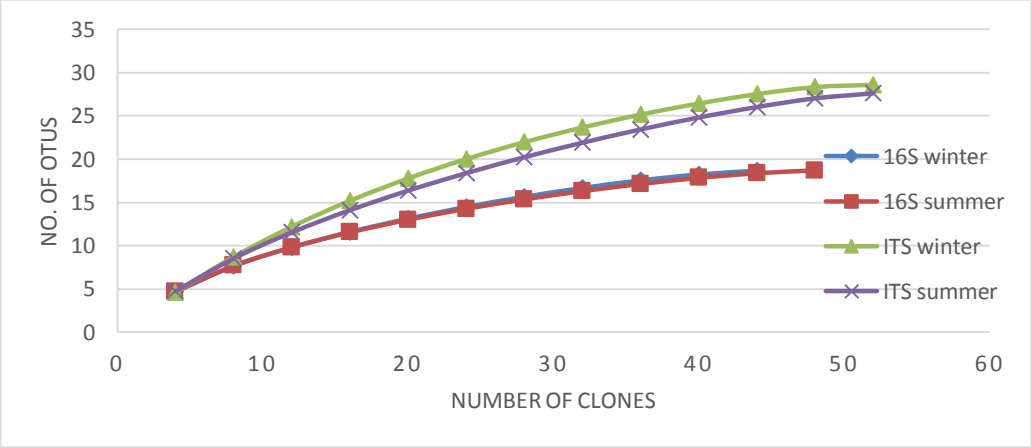

Figure 4.20: Rarefaction curves constructed for cumulative winter and summer 16S and ITS clone libraries.

Rarefaction analysis for all the clones derived from the four clone libraries did not show a plateau, which suggested that an insufficient number of clones were sampled for ARDRA analysis. The 16S clone library showed a less reliable representative of the microbial population. Therefore, diversity indices were not applied for all samples.

4.6 Denaturing gradient gel electrophoresis

Community bacterial and fungal diversity of the faecal samples collected from the three locations between the winter and summer season were analysed using DGGE.

Separation of PCR products obtained from the nested PCR-DGGE amplification produced reproducible banding patterns for all samples (results not shown). Each band was considered to represent a unique or specific fungal and bacterial species for ITS and 16S rDNA analysis, respectively. Several bands were observed in many samples while some were unique to particular samples. High intensity bands were observed in some samples, whereas in some samples the bands appeared to be faint or absent altogether.

The banding patterns obtained from the 16S rDNA DGGE gel (Fig. 4.21) were graphically reproduced (Fig. 4.22) using the Gel2K program. The banding patterns for each sample were compared against all samples and used to construct a dendrogram. The number of bands in individual samples ranged between 8 and 11 bands. Common and unique bands were selected for excision and sequencing (Fig. 4.23).

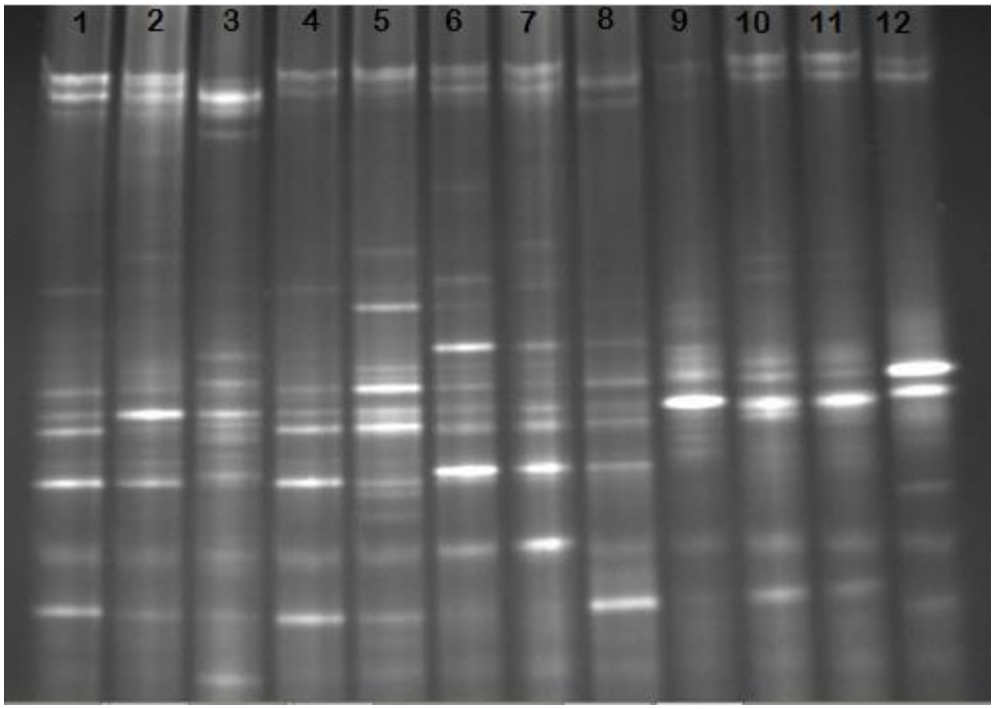

Figure 4.21: DGGE fingerprinting of bacterial communities from genomic DNA extracted from pigeon droppings. (The numbers represent the sample names in Table 3.1).

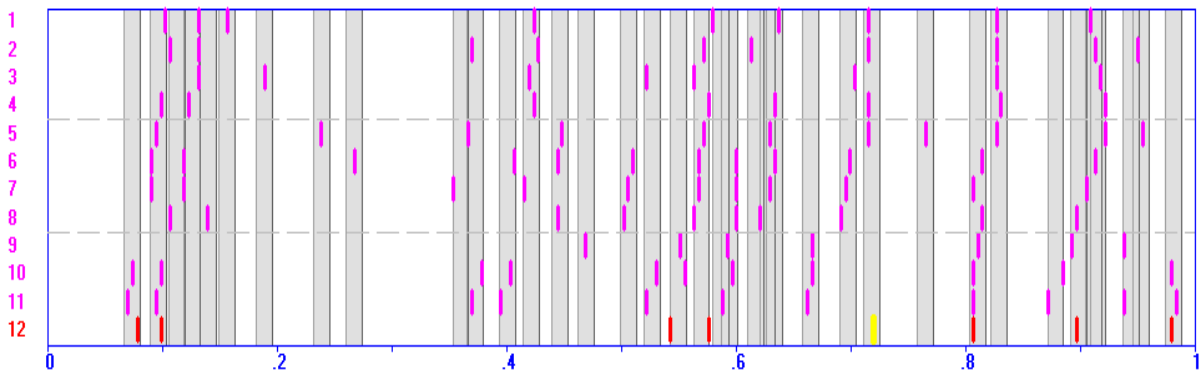

Figure 4.22: Graphical band patterns of 16s rDNA DGGE profiles. (The numbers represent the sample names in Table 3.1).

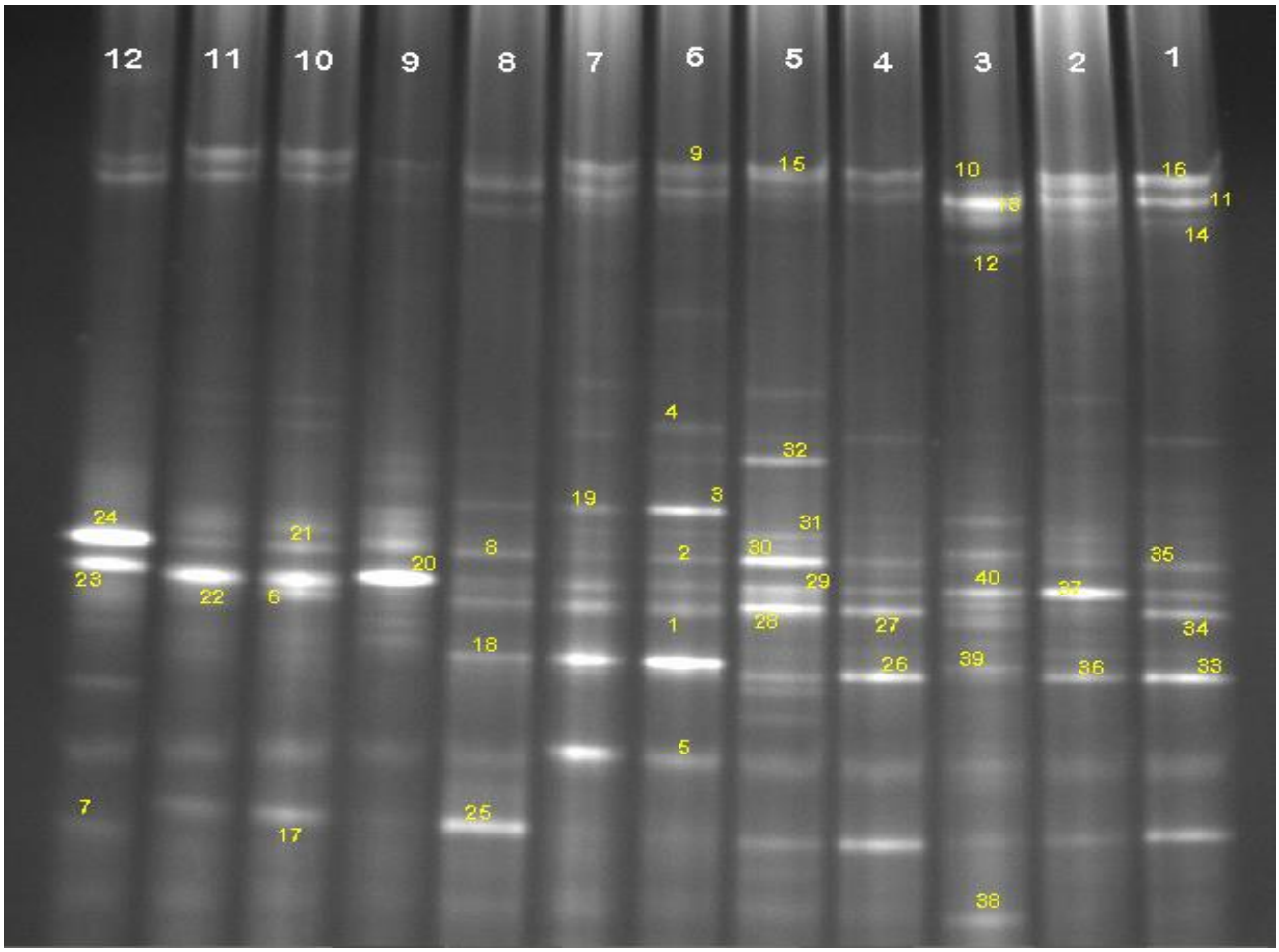

Figure 4.23: Illustration of the position and labelling of DGGE bands that were excised and r sequenced. (The lane numbers represent the sample names in Table 3.1).

The Gel2k programme used the Jaccard coefficient calculated over a complete-link setting to compare the DGGE fingerprints and generate the dendrograms. For the 16S rDNA gene, the dendrogram (Fig. 4.24) showed two different clades. The first clade grouped all winter samples while the second clade grouped all summer samples and contained one winter sample (Figure 4.24). The first clade contained the Johannesburg and Pretoria old pigeon dropping samples and fresh droppings clustered closely together showing a high similarity in banding patterns between the samples. Some bands were, however, unique between the weathered and fresh droppings.

In general, weathered and fresh faecal samples collected in winter showed no distinct differences in banding patterns between the Johannesburg samples while the Pretoria and VUT sample showed marked differences in band numbers and size (Fig. 4.24).

Clade two showed two sub-clades. Sub-clade I grouped two old faecal samples collected in Johannesburg and Pretoria. The third sample was a fresh faecal sample from VUT. Sub-clade II grouped all fresh faecal samples from the three locations and one old faecal sample from VUT.

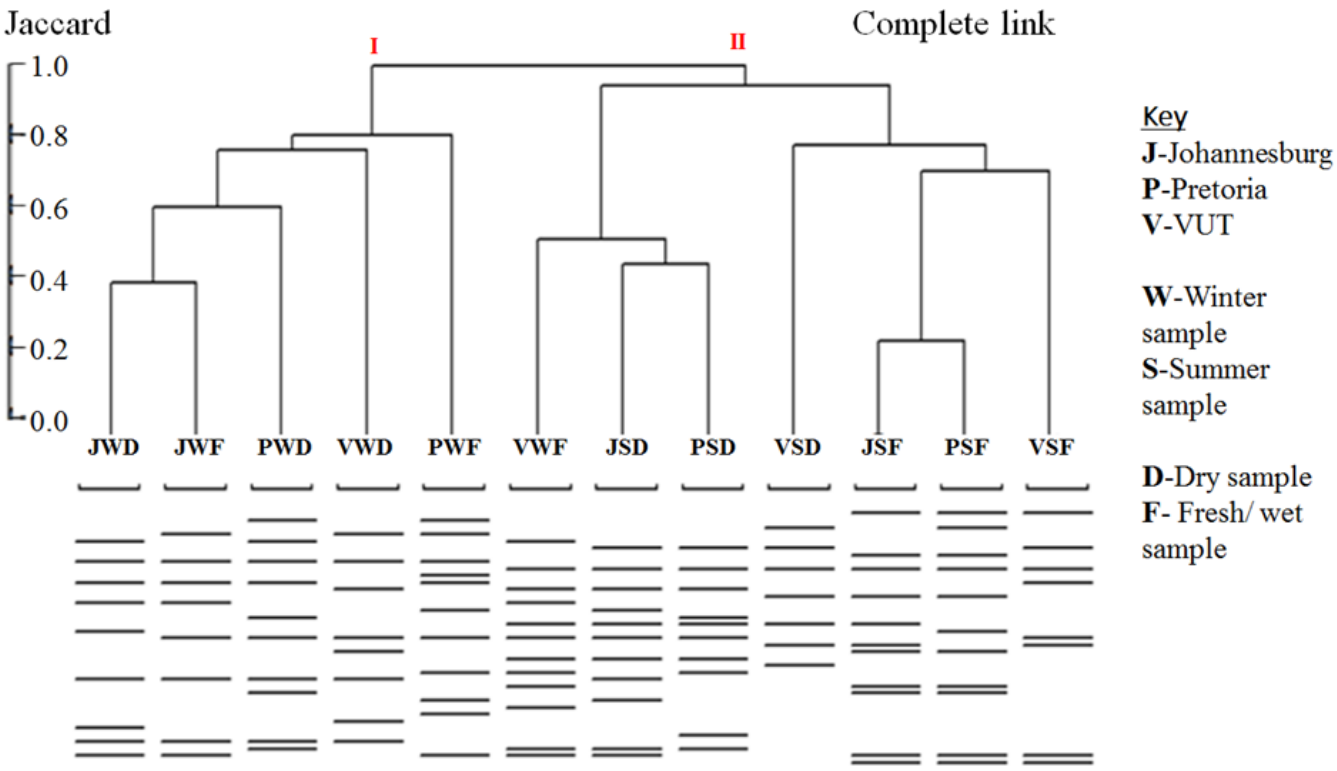

Figure 4.24: Cluster analysis of 16s DGGE profiles

Forty bands that were either unique or common to the different samples (Fig. 4.23) were excised from the gel, re-amplified, purified and sequenced. The sequences were used to match known sequences in the GenBank (Table 4.3). Only one sample (40) was considered a chimera while 8 were indecipherable. These 9 samples were considered to be chimeras and excluded from construction of phylogenetic trees. Only those that showed ≥85% similarity were used to create the phylogenetic tree (Fig 4.26).

Table 4.3: List of excised bands from DGGE analysis of 16S rDNA and their closest match from the GenBank.

Sequence Identification (band no)	GenBank ID	Accession Number	Percentage (%) similarity (no. of bp)	E-value
1	Uncultured bacterium clone	JQ337341.1	94% (150/160)	1e-58
2	*Carnobacterium* sp.	emb|AM111051.1	86% (131/153)	5e-36
3	Uncultured bacterium clone	gb|JF692705.1|	81% (112/138)	2e-23
4	*Psychrobacter* sp.	gb|HM216576.1|	96% (152/158)	1e-63
5	*Psychrobacter* sp.	JQ800071.1	100% (150/150)	1e-69
6	Uncultured *Lactobacillus* sp. isolate DGGE gel band SBL18	gb|JF427679.1	92% (136/148)	4e-50
7	Uncultured bacterium isolate DGGE gel band A18	gb|KC991211.1	91% (137/150)	8e-53
8	Uncultured bacterium clone	gb|FJ365167.1	82% (131/160)	4e-32
9	*Psychrobacter* sp.	gb|JQ800071.1	97% (153/158)	7e-67
10	Uncultured bacterium isolate DGGE gel band L6B8	gb|GQ289450.1	87% (137/158)	5e-43
11	Uncultured *Janthinobacterium* sp. isolate DGGE gel band A10	gb|HQ877795.1	87% (132/152)	3e-39
12	Uncultured bacterium isolate DGGE gel band DNSV1	gb|HM640012.1	77% (114/149)	2e-16
13	Uncultured bacterium clone PeHg37	gb|FJ374254.1	74% (110/148)	1e-11

14	Uncultured *Vibrio* sp. clone YDB21	gb|DQ452589.1	83% (125/151)	1e-30
15	Uncultured bacterium clone HB3-10	gb|FJ719280.1	90% (137/152)	2e-48
16	Uncultured bacterium clone 1-9E	gb|EU289466.1	84% (125/148)	3e-33
17	*Streptococcus lutetiensis* strain CG49	dbj|AB849356.1	95% (140/147)	3e-58
18	Uncultured bacterium clone feline jejunum 2FJ-55	gb|EU877821.1	85% (137/161)	5e-37
19	Uncultured bacterium isolate DGGE gel band IMCUGYMSD3	gb|KC470710.1	88% (127/145)	1e-38
20	Uncultured *Enterococcus* sp.	emb|AM711881.1	99% (155/157)	1e-70
21	*Lactobacillus sakei* strain	gb|JN851763.1	98% (148/151)	8e-66
22	*Lactobacillus sakei* strain	gb|JN851763.1	98% (148/151)	8e-66
23	Uncultured *Escherichia* sp. isolate DGGE gel band zzq-9	gb|JQ828856.1	91% (136/150)	1e-49
24	*Pseudomonas* sp.	gb|JQ766116.1	98% (156/159)	4e-70
25	Uncultured *Streptococcus* sp. isolate DGGE gel band CP6	gb|KC770782.1	99% (156/158)	4e-70
26	*Oceanobacter kriegii* strain NBRC 15467	NR_113758.1	91% (136/150)	2e-48
27	Uncultured bacterium clone 5sto25	gb|HQ701656.1	94% (130/138)	3e-51
28	Uncultured *Lactobacillus* sp. clone	gb|JQ961318.2	95% (142/149)	2e-59
29	*Oceanobacillus* sp.	gb|KJ187451.1	86% (126/146)	4e-38
30	*Enterococcus faecium* strain	gb|KJ919969.1	99% (154/155)	1e-69
31	*Lactobacillus agilis* strain JCM 1187	NR_113259.1	99% (150/152)	6e-67
32	Uncultured Weissella sp. gene for 16S ribosomal RNA, partial sequence, clone: 3X63	dbj|LC002947.1	97% (142/147)	6e-61
33	*Oceanobacter kriegii* strain NBRC 15467	NR_113758.1	88% (133/151)	4e-44

34	Uncultured bacterium clone ncd1860g12c1	gb|JF161361.1	82% (131/160)	9e-34
35	Uncultured bacterium clone rRNA368 16S ribosomal RNA gene, partial sequence	gb|AY959141.1	89% (133/149)	3e-45
36	Gamma proteobacterium r61 gene for 16S rRNA, partial sequence	dbj|AB470941.1	81% (124/153)	2e-30
37	*Enterococcus columbae* strain NBRC 100677 16S ribosomal RNA gene, partial sequence	NR_113926.1	99% (153/154)	4e-70
38	Uncultured *Bacillus* sp. isolate DGGE gel band	gb|JX163872.1	91% (71/78)	2e-17
39	Uncultured bacterium isolate DGGE gel band	gb|GQ200091.1	92% (138/150)	3e-51
40	Uncultured *Enterococcus* sp.	emb|AM711881.1	93% (140/150)	7e-54

To assess the distribution of the identified bands excised from the DGGE analysis, classification information obtained from chimera checks using Decipher was used to classify the organisms identified according to their bacterial domains (Fig. 4.25). Twelve bacterial domains, *Gammaproteobacteria*, *Bacilli*, *Psychrobacter*, *Lactobacillales*, *Proteobacteria*, *Carnobacteria*, *Streptococcus*, *Enteroccocus*, *Lactobacillus*, *Pseudomonas*, *Firmucutes* and *Weisella Leconostoceace* were classified. The 9 chimeric and undecipherable sequences were classified as unclassified bacteria.

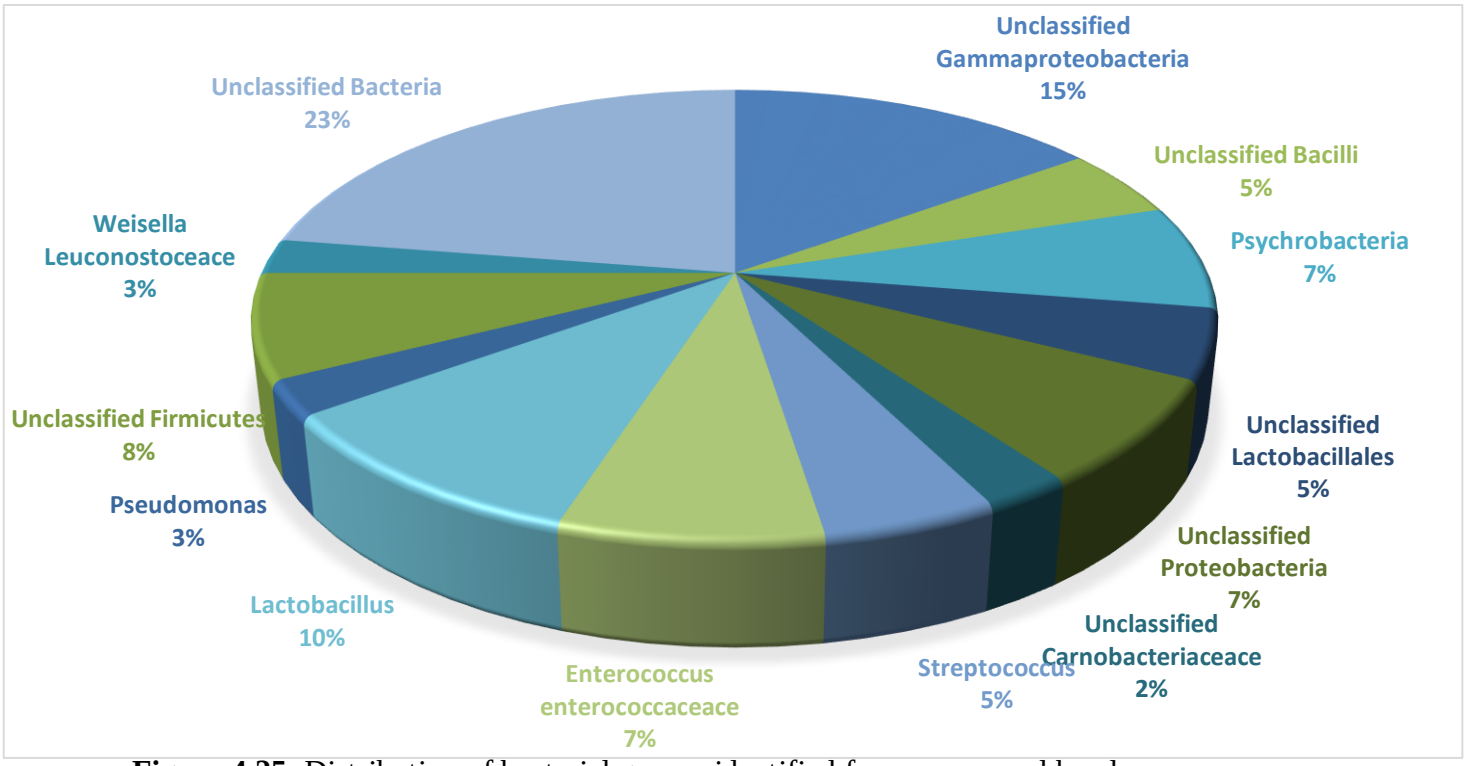

Figure 4.25: Distribution of bacterial groups identified from sequenced bands.

Figure 4.26 illustrates the neighbour-joining phylogenetic tree of the obtained 16S rDNA sequences along with their closest match from the GenBank.

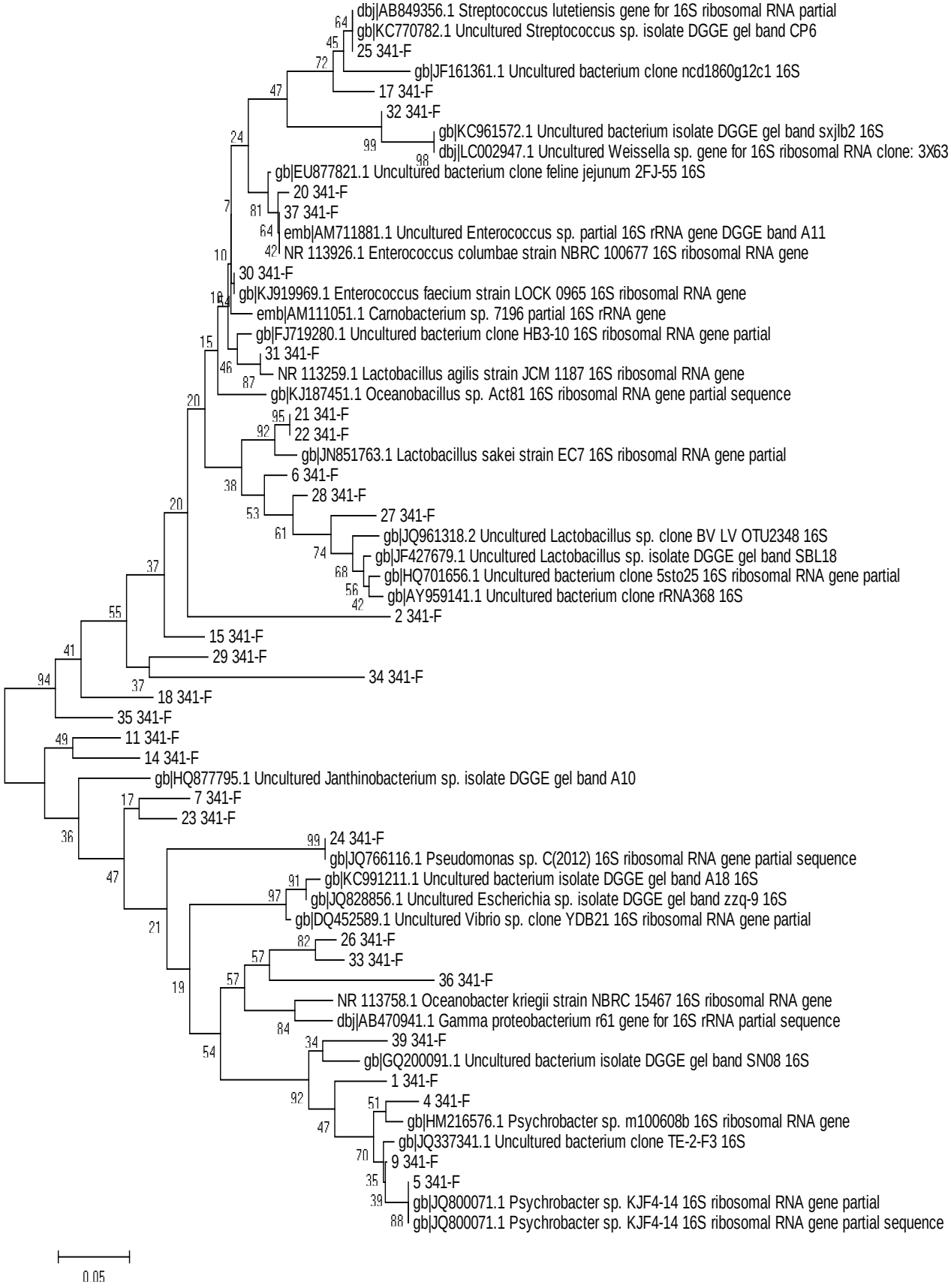

Figure 4.26: Evolutionary relationships built on sequences of 16S RDNA amplicons of derived from pigeon droppings.

Figure 4.27 shows the DGGE fingerprint derived from the amplification of the ITS region. The gel obtained was reproduced graphically (Fig 4.28) for analysis.

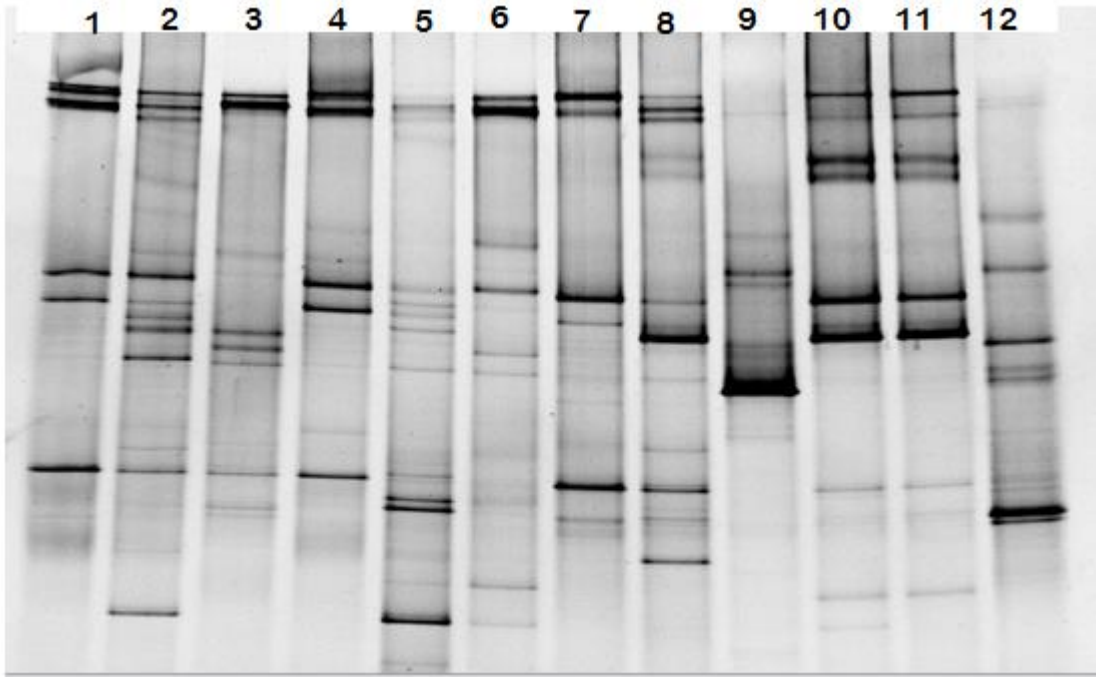

Figure 4.27: DGGE fingerprint patterns of the amplified ITS region. (The lane numbers represent the sample names in Table 3.1).

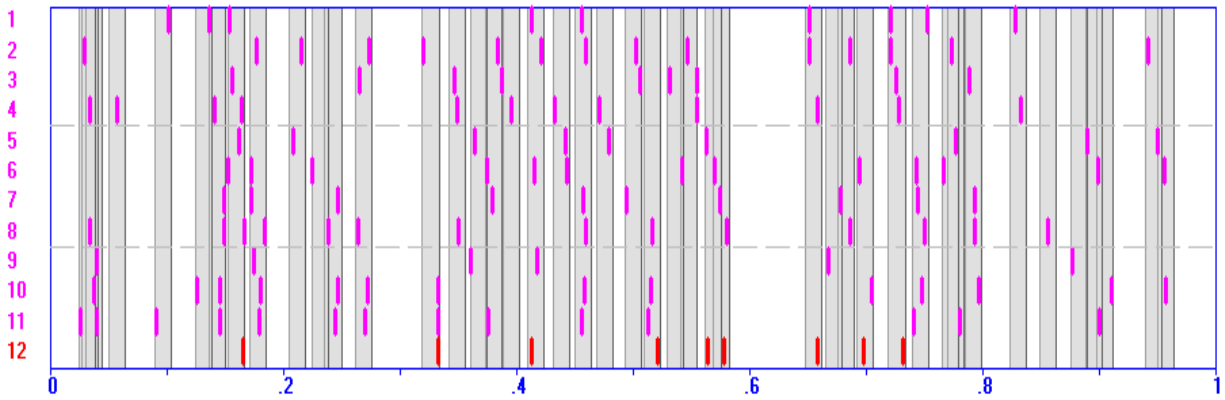

Figure 4.28: Graphical band patterns of DGGE profiles from the ITS amplicons

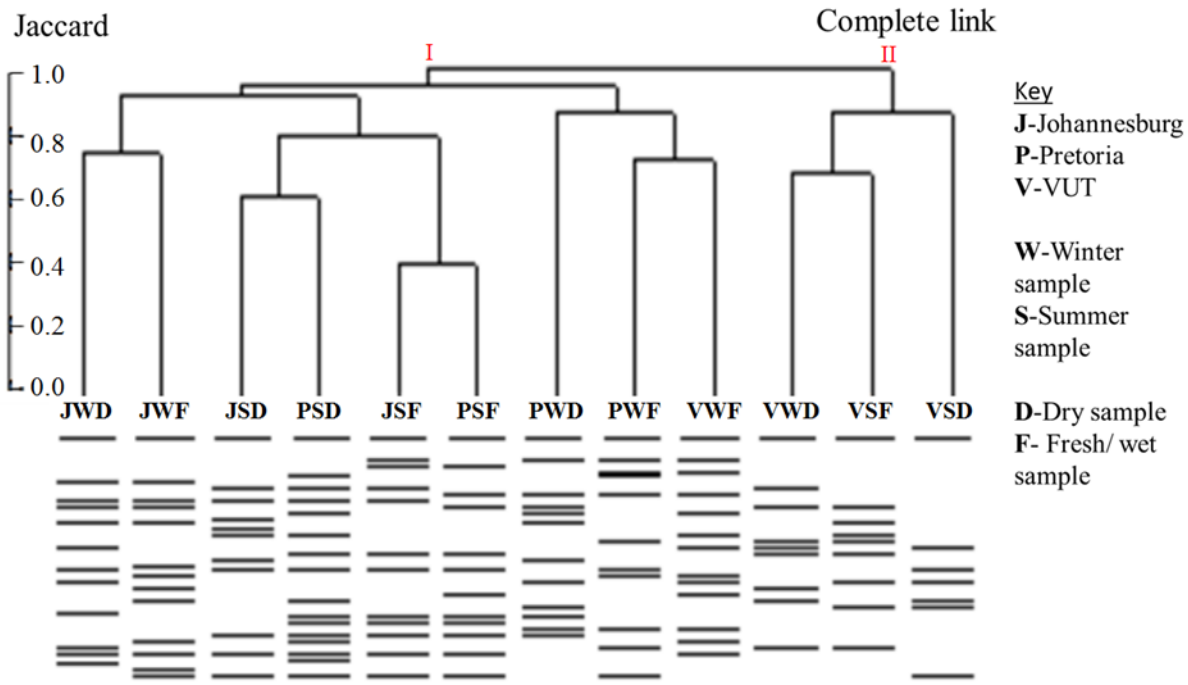

Figure 4.29: Cluster analysis of ITS profiles from DGGE analysis

The dendrogram derived from the ITS-DDGE patterns displayed two major clades (Fig. 4.29). The first clade (labelled I) showed two sub-clades with the first containing Johannesburg samples, fresh and dry, collected in winter. The second sub-clade grouped summer samples, both fresh and dry, collected from Pretoria and Johannesburg.

The second clade (labelled II) included all the three samples from VUT. These samples had the least number of bands compared to clade 1 (Fig. 4.29). There were minor differences in the number of bands between the winter and summer samples.

Bands from the ITS DGGE analysis gel were excised for sequencing. However, multiple amplicons appeared on the gel during re-amplification. This suggested there was a lot of co-migration of bands during DGGE. Therefore, the excised DNA could not be used for sequencing and identification.

The number of dominant bands per sample for both 16S rDNA and ITS samples in the two seasons (summer and winter), and the two types of samples (old weathered and fresh wet faecal droppings) is shown in Table 4.4. Overall, there was very little or no change in the number of bands or species across all sample types and seasons, suggesting that the type of organisms present in all the samples is not influenced by the season and the time and exposure of the droppings to the environment.

CHAPTER 5

DISCUSSION

5.1 DNA Extraction

The choice of method of DNA extraction is an important step when working with environmental samples. This affects the quality and quality of the extracted DNA depending on the targeted organisms (Wu *et al.* 2009). When working with environmental samples, complete lysis of all organisms, from Gram-negative to Gram-positive bacteria as well as spores and fungi is important. Organic samples, particularly faecal samples, are usually a challenge in extracting pure nucleic acids due to co-extracted substances with inhibitory biochemical reactions and low DNA yields (Burgmann *et al.* 2001; Yu & Morrison 2004; Tang *et al.* 2008). These co-extracted substances are a mixture of complex polyphenolics produced during the decomposition of organic matter, and are present in soil and water and usually contaminate any material exposed to the environment (Kreader 1996). In this study, the hot lysis method (Zhou et al. 1996) using 2% CTAB with an extended incubation period of two hours produced suitable high molecular weight DNA (Fig 4.1). The isolated DNA had an intense brown colour that indicated the presence of humic acids as reported by Matheson *et al.* (2010). The quality and quality of the extracted DNA were not measured. Humic acids have a high absorption coefficient in the Ultraviolet wave range, which affects the quantification of nucleic acids by UV spectrophotometry, sometimes even leading to overestimation of concentrations of extracted DNA (Zipper *et al.* 2003).

5.2 DNA amplification

16S rDNA and ITS gene amplification products were obtained from specific primers by use of PCR. Initial attempts to amplify the DNA failed until dilutions (1:20) of the extracted DNA and addition of bovine serum albumin (BSA), which led to successful amplification in the PCR reactions. BSA is acts an enhancer of PCR amplification since its prevents inhibitors co-extracted with DNA from interacting with DNA polymerase during amplification (Farell & Alexandre 2012) while dilutions are done to reduce the contaminants.

The primers selected to amplify the 16S rDNA gene of the bacterial samples produced the expected fragment size of approximately 1500 bp (Fig. 4.2) as observed in other studies (Dees & Ghiorse 2001; Yang *et al.* 2007), while nested amplification of the variable V3 region of the 16S rDNA produced a single fragment of 233 bp (Fig.4.3) as reported by (Muyzer *et al.* 1993).

The specific primers that targeted the ITS regions of fungal isolates in the faecal samples produced amplicons of between 500 and 900 bp (Fig. 4.4). This is consistent with the ITS profiles of fungal isolates and environmental samples in other studies (Chen & Cairney 2002; Anderson & Parkin 2007).

A PCR-DGGE nested approach was chosen to improve the specificity of amplification and reduce the formation of spurious by-products as reported by Muyzer *et al.* (1993); Lu *et al.* (2013).

5.3 Cloning and verification of inserts

Following cloning (Fig. 4.5), verification of the 16S rDNA and the ITS inserts corresponded to the expected sizes (Fig 4.6 and Fig. 4.7), respectively. However, a few colonies without the inserts were also observed on the media. This is perhaps due to frame shifts events which resulted in the disruption of the lethal gene reading frame (Invitrogen 2012).

5.4 Amplified ribosomal DNA restriction analysis (ARDRA)

Analysis by ARDRA technique allowed for phylotype/OTU affiliations from the rapid assessment of the large number of clones. Tetracutter endonucleases, theoretically known to cut after every 256bp, were selected due to their suitability to reveal enough restriction fragments, therefore revealing sequence polymorphisms (Durieux and Simon 2002). Clones with the same restriction pattern for any one enzyme were assigned to the same ARDRA group while all clones with similar *Hae*III and *Hin*fI restriction patterns were assigned to the same OTU.

Digestion of the 99 cloned 16S rDNA fragments with *Hae*III and *Hin*fI produced both similar and variable restriction patterns among the samples. Thirteen ARDRA groups were identified for both *Hae*III and *Hin*fI (Fig 4.10 and 4.11) restriction endonucleases, producing a total of 26 OTUs (Fig. 4.12). A seasonal effect on the number of OTUs was observed in this study. Of the 26 phylotypes, each season had 16 were unique and 10 were common to the both seasons. Only two phylotypes appeared more than two times in both seasons. Similar studies involving avian faeces are not reported in the literature. Therefore it is not possible to make any comparisons with other studies with regards to the number of OTUs identified in this study. This study used 99 clones and only two restriction enzymes. Perhaps using a greater number of clones and more enzymes could have produced more OTUs. Fifty percent of the clones were accounted for by two OTUs. For example, OTU 14 contained the majority (35%) of clones, followed by OTU 19 (15%). The latter two OTUs were the most common phylotypes found

in all three locations during both the summer and winter season. The rest of the phylotypes occurred rarely in both seasons and locations. This suggested that there are certain dominant organisms found in pigeon droppings irrespective of seasons.

Digestion of the ITS region using identified 16 ARDRA and 12 ARDRA groups using *Hin*fI and *Mbo*I, respectively. The two enzymes generated 41 phylotypes from the analysis of 104 clones, showing a relatively large number of phylotypes. Eleven of the phylotypes were common in the winter and summer season. Sixteen phylotypes were restricted to the winter season while 13 were only found in summer.

The DNA representing some of the forty phylotypes were sequenced and compared to sequences in the GenBank using BLAST analysis. The results (Table 4.1) showed that the majority of the organisms were yeasts and one was a mould. The results showed 17 fungal species, 4 genera as well as 6 unidentified species. This discussion focusses only on medically relevant organisms.

Non-neoformans *Cryptococcus* spp. were the dominant organisms identified among the fungal isolates in the pigeon droppings. Non-neoformans Cryptococci have been identified in various environmental sources such as air, soil, pigeon droppings and food items and can become opportunistic pathogens. Their pathogenesis is similar to that of *Cryptococcus neoformans* (Miceli *et al.* 2011). It is known that these are pathogenic organisms that are transmitted to humans through pigeons (Haag-Wackernagel & Moch 2004). For example *C. albidus*, which was previously considered non-pathogenic, have continuously and increasingly been described in infections involving immunocompromised hosts (Miceli 2011).

In this study *Cryptococcus* species were identified in samples collected during the winter and summer. Winters are dry with low temperatures while it is rainy in the summer with slightly high temperatures. Interestingly, related studies done in India by Randhawa *et al.* (2005) and (Granados & Castaneda 2005) in Brazil showed a decline of *Cryptococcus* spp. in the extreme hot summer compared to the mild spring. The two studies suggested that wet months, with few hours of sunlight and less extreme temperatures to slightly higher temperatures favoured the occurrence of the *Cryptococcus* species than dry months. Both seasons proved to be suitable for *Cryptococcus* species in our study. This is perhaps due to the relatively mild climate conditions in South Africa.

The phylogenetic tree (Fig. 4.19) showed that the *Cryptococcus* species formed one distinct clade and two very closely related groups showing the close genetic relationships among these

species. The Cryptococcus species were also closely related to *Filobasidium uniguttulatum.* The latter species is also referred to as *Cryptococcus uniguttulatum* (Guffogg *et al.* 2004) the first non-*Cryptococcus* species reported for human infections (Miceli *et al.* 2011).

The phylogenetic tree (Fig 4.19) showed that *Cryptococcus* was distantly related to *Cystofilobasidium lari-marini* and *Holtermaniella watticus.* Guffogg *et al.* (2004) reported that species of *Cryptococcus* are polyphyletic in nature and was represented in all four clades of yeast (*Tremellales, Trichosporonales, Filobisidiales* and *Cystofilobasidiales*) in their study of fungal taxonomy. This study provides partial support for this hypothesis.

Yeasts of the *Candida* genus were also identified in the pigeon droppings. The genus *Candida* has been found in pigeon droppings in a number of studies (Khosravi 1997; Costa *et al.* 2010; Soltani *et al.* 2013). The genus has been found to cause infections in immunocompromised individuals, although the yeast can be found as normal flora on the skin and digestive tract of humans and animals (Soltani *et al.* 2013). This study also identified *Clavispora lusitaniae,* a teleomorph of *Candida lusitaniae* (Baker *et al.* 1984). This yeast has been documented and known to infect humans with registered cases in mostly immunocompromised individuals (Miceli, 2011). Of greater concern is that it is showing resistance to antibiotics (Merz *et al.* 1992).

The most common organism was identified as *Kazachstania (Arxiozyma) telluris.* It was found in all the locations and during all seasons. This yeast is commonly associated with soil in South Africa but it is also found in the nasal passages of pigeons (Kurtzman *et al.* 2005). The yeast can grow at temperatures between 20 and 30°C, and is usually found in the alimentary canal of warm-blooded animals (James *et al.* 2001). Although labelled a psychrophobic yeast (James *et al.* 2001), this organism was also found in fresh and dry pigeon droppings collected in summer. Opportunistic organisms usually show distinct regional incidence patterns throughout the world and exhibit different epidemiologic features. This usually depends on the geographic region. This could be true for *K.* (*Arxiozyma*) *telluris* since it has been found in different environments and at different temperatures.

Fusarium equiseti was identified from pigeon droppings in this study (Table 4.1). *Fusarium* species, commonly known to be plant pathogens, are also known to cause infections in humans. Allergies in immune-competent individuals, and mycotoxicosis as a result of the pathogenicity of the *Fusarium* species are common (Nucci & Anaissie 2007). *Fusarium* is, however,

classified with other emerging opportunistic fungi that cause a variety of infections in immunocompromised individuals (Soltani *et al.* 2013).

Geotrichum species was also identified in this study (Fig 4.19). *Geotrichum* species are rare opportunistic pathogens that are found in the environment as well as in humans as colonisers (Miceli 2011). Phylogenetic analysis (Fig 4.19) showed that *Epicoccum* sp. clustered along with *Pseudeutorium bakeri, Leptosphaerulina australis, Metschnikowia pulcherrima* as well as other unidentified ascomycetes. These organisms were found in pigeon droppings in this study. Some are mainly associated with plant debris and soil and have no relevance as medically important fungi.

The highlight of this study is that pigeon droppings do act as a reservoir for bacterial and fungal isolates. The ADRA techniques was useful in screening the cloned libraries and identifying phylogenetic clusters from pigeon droppings. Although the technique offers no advantage in revealing information about the identity or type of microorganisms present in a particular sample (Gich *et al.* 2000), it is reliable in assessing the genotypic changes or community changes in microbial communities brought about by environmental conditions.

The number of clones analysed per location was too low to infer diversity. This was proven by the use of cumulative (including all samples under one location and sample types to their particular season) rarefaction analysis (Fig. 4.20). This is because the number of phylotypes observed is sensitive to the number of samples (clones in this case) analysed and sufficient sampling becomes important in determining phylogenetic clusters with ARDRA. For instance, a particular organism identified in only one location in a particular season could not conclusively be interpreted as unique to that location. Therefore a larger number of clones will have to be sampled from each location to reflect the diversity among all the locations and seasons. The number of restriction enzymes used in a study using ARDRA for phylotypic information of environmental isolates is also critical since the use of more REs reflects better phylotypic information (Sklarz 2009).

5.5 DGGE Analysis

DGGE analysis is an inexpensive, rapid and reliable method for analysis of microbial diversity in environmental samples. The method was chosen because it eliminates bias by showing representation of whole microbial communities and provides a reliable characterization of microbial population differences. The use of a hierarchical cluster analysis for examination of DGGE profiles is often used to demonstrate similarities in the data obtained (Gafan *et al.* 2005).

The Gel2k software and CLUST programme (Norland 2004) were used to analyse DGGE gels and uses clustering algorithms to group banding profiles of the individual species in each of the samples according to the similarity in community composition. Samples from similar samples would therefore be expected to group together to show comparable communities in the dendrograms.

For 16S rDNA, the DGGE patterns did not vary greatly between samples collected in the summer and winter for all locations (Fig 4.21 and Fig 4.22). However, there were differences in banding patterns within locations. For example, in Figure 4.23 the Johannesburg (1, 4) and Pretoria (2, 5) samples showed differences in cluster analysis of banding patterns although they were collected in the winter (Fig 4.25). This probably means that different organisms were present in the pigeon droppings at different times in the same season. These results also suggest that there is little species diversity with the different seasons. These results are consistent with the results obtained from ARDRA analysis. Although 10 phylotypes were common to all seasons and locations eight phylotypes each appeared in each season in some locations. This suggested that there is little species diversity in the DGGE analysis. The intense bands observed in Fig 4.20 may suggest that some species are in abundance within a community of organisms. However, this assumption may not be clear due to bias in the extraction-amplification procedures preceding DNA fingerprinting (Gafan *et al.* 2005).

The 40 excised bands from the DGGE profiles of the 16S rDNA identified a number of bacteria in the pigeon droppings (Table 4.3). These belonged to 13 bacterial groups (Fig.4.23). The most common species belonged to the genus *Lactobacillus.* Organisms of this genus are known to occur in the gastrointestinal systems of most animals. Species of *Lactobacillus, Enterococcus, Clostridium, Fibidobacterium* and *Bacillus* have been isolated from fresh or new pigeon droppings (Veld & Berrens 1976). Seven percent of the samples were comprised of *Enteroccoccus* (Fig 4.23). *Enterococcus* species such as *E. fecalis, E. feaecium* and *E columbiae* as well as *Escherichia* spp. have been reported in a study by Radimersky *et al.* (2010) in pigeon droppings in Czech Republic. Found in the gastrointestinal tract, these organisms have been shown to be opportune pathogens and documented to show antibiotic resistance (Radimersky *et al.* (2010).

Some of the bacterial species identified in this study are not common in pigeon droppings. Bacteria such as *E. coli, and Streptococcus* been found in a number bird species and are carriers of human pathogens. However, organisms such as *E. coli, Streptococcus* spp. (found in this

study) have not been found in pigeon droppings. This occurrence can been attributed to the interaction between worldwide migration patterns of birds and in different habitats (Ryu *et al.* 2014).

A number of unclassified bacteria were identified from the excised bands (Table 4.3). The phylogenetic tree (Fig 4.24) showed a number of these clones appeared in separate groups from the known taxa. The fact that only small fragments can be separated in DGGE may limit sequence information. Moreover, possible intra-specific or intra-isolate heterogeneity of rDNA genes can give rise to multiple banding patterns for one species (Michaelsen et al., 2006; Nakatsu et al., 2000), as shown by the multiple bands for *Psychrobacter* sp. in this study (Fig. 4.20: lane 6, bands 4, 5 and 9).

For the ITS-DGGE analysis, there were slight differences in banding patterns between samples collected in the winter and summer (Fig 4.27 and Fig. 4.28). The dendrogram derived from the patterns showed two main clades that showed differences between VUT and the other sample locations, Johannesburg and Pretoria (Fig. 4.29). The first clade contained samples of both seasons from Johannesburg and Pretoria and one VUT sample that were separated into two sub-clades. The first clade clustered Johannesburg and Pretoria, fresh and dry samples collected from both seasons. The second sub-clade contained Pretoria and one VUT sample, wet and dry, all collected in winter. The second clade contained only 3 samples, being VUT samples (Fig 429). This suggested there was a high similarity among samples from Johannesburg and Pretoria samples as compared to VUT samples.

This study could not establish the identity of some of the common and unique bands in the banding profiles of DGGE analysis of the ITS gene due to co-migration during DGGE. Different DNA sequences may have similar motilities due to identical GC contents (Muyzer *et al*. 1999), and, therefore, one band may not necessarily represent a single species (Gelsomino *et al.* 1999). The close relationship among the different identified fungal isolates, particularly the *Cryptococcus* species and its polyphyletic nature shown in the phylogenetic tree (Fig. 4.19) could be used as proof of co-migration showing lack of clear separation of closely related organisms.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion remarks

This study looked at the occurrence of bacterial and fungal communities present in pigeon droppings collected in two seasons, specifically winter and summer. On the basis of a literature search, this is the first study in South Africa that examined pigeon droppings at the community level.

The use of DGGE and ARDRA as fingerprinting methods for microorganisms present in pigeon droppings proved to be useful tools for characterising these microbial communities. ARDRA provided an opportunity to assess the phylotypes present within the genomic DNA extracted from bacterial and fungal species within pigeon droppings. For bacteria, 26 phylotypes (OTUs) were identified from 99 clones while 41 phylotypes were identified from 104 clones derived from fungi. Little to no effect was detected on the microbial populations in the two seasons. DGGE was used to assess the total microbial change and allow for direct comparison of the location, season as well as species present in pigeon droppings. The results showed that there was very little difference in the community structure in pigeon droppings during the two seasons as well as locations.

The main bacterial species identified from the pigeon droppings included, *Enteroccocus* sp., *Streptococcus* sp., *Escherichia* sp., and *Lactobacillus* spp. Fifteen of the sequences obtained were unidentified as result of the short sequence information obtained from the targeted region for DGGE analysis while.

Fungal species identified included mainly *Cryptococccus* spp., Geotrichum spp., *Candida* spp., *Fusarium equiseti* and 10 unidentified species as well as medically irrelevant species were also identified.

This research provided information on the major bacterial and fungal species present in pigeon droppings. It sets a blueprint for further studies involving pigeon droppings since it is the first study to holistically involve molecular techniques, eliminating the culturing approach.

The tendency of pathogenic organisms to be opportunistic in immunocompromised individuals, creates a health risk to individuals living in the sampling sites and perhaps the entire population of South Africa since pigeons are present in the country.

In summary, the findings of this study reinforces the hypothesis that pigeon droppings are an important factor to be considered for yeast and bacterial infections in the urban environment. This study is the first to look at pigeon droppings by utilising culture-independent techniques and eliminating culturing altogether. It is also the first study to assess the influence of seasons on the microbial groups found in pigeon droppings, as well as identify bacteria in the samples since most studies have focussed on yeasts and fungi. The results showed that seasonal changes and the type of sample (wet or dry) had little or no effect on the occurrence and variety of microbial groups. The occurrence of opportunistic pathogens in the public places from which samples were collected creates a disease risk for immunocompromised people.

6.2 Recommendations

The results and discussion from this study has led to the following recommendations:

- Increasing the number clones and restriction enzymes for ARDRA analysis may perhaps identify other species in pigeon droppings. A larger sample size would make it possible to look at species richness and other indices like coverage and abundance.
- Another approach could be combination of DGGE analysis to analyse clones as well as the environmental sample as a tool for screening the clone libraries. In this approach, migration of the individual and randomly pooled clones are analyzed by DGGE, and the migration patterns compared to the conventional DGGE profile produced directly from environmental DNA enables the particular clones to be sequenced from the library without worrying about separation of co-migrating organisms as well as the limited sequence information of the excised bands from the gel.
- For DGGE application, primers that target other regions of the 16S rDNA gene should also be employed since a comparison of the different fingerprints would give a better understanding of the communities involved. Amplification of a longer fragment, for instance, V1 to V3 region, would also provide more information necessary for identification of the organism.
- A comparison could be made between culture-dependent and culture-independent approaches to determine the advantages and disadvantages of these methods. It would also offer a chance to study virulence factors of the different *Cryptoccocus* species from their growth on media by colour characteristics on different media as well as microscopy.

CHAPTER 7
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