

Molecular characterisation of the causal agent of bacterial leaf streak of maize

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Declaration

I declare that this dissertation submitted for the degree of *Master of Science in Environmental Sciences* at the North-West University, Potchefstroom Campus, has not been previously submitted by me for a degree at this or any other university, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

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Date

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Abstract

All members of the genus *Xanthomonas* are considered to be plant pathogenic, with specific pathovars infecting several high value agricultural crops. One of these pathovars, *X. campestris* pv. *zeae* (as this is only a proposed name it will further on be referred to as *Xanthomonas* BLSD) the causal agent of bacterial leaf streak of maize, has established itself as a widespread significant maize pathogen within South Africa. Insufficient information about the present distribution of the pathogen is available. The main aim of the study was thus to isolate and characterise the pathogen using molecular methods. Results demonstrated that the causal agent of bacterial leaf streak disease (*Xanthomonas* BLSD: potentially *X. campestris* pv. *zeae*) was widely distributed within the major maize cultivation regions of South Africa. Most of the isolates collected originated from the Highveld maize production provinces (North West, Free State, Gauteng and Mpumalanga provinces) as well as from irrigated maize fields in the Northern Cape province. The *XgumD* gene marker was used to determine if the isolates belonged to the genus *Xanthomonas*. The *gumD* gene fragment is located within the *gumB-gumM* region of the operon and is conserved among *Xanthomonas* species. This gene fragment is partially responsible for xanthan production. This marker was amplified from all isolates and a selected number were sequenced. The marker was only able to confirm that the causal agent was a member of the genus *Xanthomonas*. PCR methods were used for the characterisation of the isolates. This included PCR and sequencing of ribosomal RNA- *gyraseB* and *gumD* genes. A fingerprinting method BOX-PCR was also employed. Good quality DNA of sufficient quantities was obtained from the various isolates. Amplification produced no non-specific amplification products. This resulted in good quality sequences that could be analysed using bioinformatics tools. Phylogenetic analyses of the ribosomal RNA and *gyraseB* genes could not detect differences amongst the 47 *Xanthomonas* BLSD isolates. However, these genes were able to distinguish between the type strain of these isolates and various *Xanthomonas* species and pathovars. From all three neighbour joining trees the *Xanthomonas* BLSD isolates had close association with *X. axonopodis* pv. *vasculorum* strain ATCC 35938. For the 16S rRNA gene there exists no sequence differences between *Xanthomonas* BLSD and *X. axonopodis* pv. *vasculorum* strain ATCC 35938. A single nucleotide difference was observed between *Xanthomonas* BLSD and *X. axonopodis* pv. *vasculorum* strain ATCC 35938 for the 23S rRNA gene. The *gyraseB* gene detected a total of six nucleotide variations between these two *Xanthomonas* species. For all of the phylogenetic trees there was no clustering of *Xanthomonas* BLSD with *X. campestris* pathovars.

Genetic profiling (via BOX-PCR) based on present/absent analysis revealed no variations amongst the *Xanthomonas* BLSD isolates. All isolates shared an identical pattern produced by 12 distinct PCR products. This profiling technique did differentiate between the isolates of *Xanthomonas* BLSD and *X. axonopodis* pv. *vasculorum* strain ATCC 35938. Their profiles shared common bands, but differed in the number and overall pattern of the bands. These results suggest two main conclusions: (i) *Xanthomonas* BLSD has a clonal origin with geographical separation not impacting genetic variation. The fact that all the isolates appear to be clonal may imply that when resistant maize cultivars are developed these should be resistant to all isolates of the pathovar irrespective of their geographical origin. This is a suggestion that will have to be corroborated using more isolates and additional genetic fingerprinting techniques (ii) the *Xanthomonas* BLSD isolates from this study may not belong to *X. campestris*. Further studies using other markers should be conducted to determine the real identity of *Xanthomonas* BLSD.

Keywords – *Xanthomonas*, bacterial leaf streak disease, *X. campestris* pv. *zeae*; maize, ribosomal RNA; *gyraseB*; *X. axonopodis* pv. *vasculorum* strain ATCC 35938; BOX-PCR profiling; clonal origin.

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Chapter 1 - Literature Review and Research Rationale

1.1 The origin of *Zea mays* subsp. *mays*

Maize is indigenous to Mesoamerica and was probably first cultivated by American Indians thousands of years ago (Tenaillon and Charcosset, 2011). Even to this day it is still grown due to its nutritional value and capacities, ease of cultivation, adaptability and the shortness of its vegetative cycle (Glover and Mertz, 1987; Du Plessis, 2003; Bänziger *et al.*, 2006; Verheye, 2010). The name maize possibly originated from the Mayan word 'mahiz' or was derived from the Spanish connotation 'maiz'. The Mayans were also responsible for its dispersal in Central and South America. In 1492 Columbus (re)discovered this new grain in Cuba from where it was exported to Europe and from there to Africa, Asia and the rest of the world (Tenaillon and Charcosset, 2011).

The numerous modern races and types of maize possibly resulted from the selective breeding, backcross breeding and/or interbreeding of teosinte (*Zea mays* subsp. *parviglumis* and ssp. *mexicana*), this is known as the "Teosinte Hypothesis" which claims teosinte is the sole progenitor of maize (Beadle, 1978 cited by Doebley, 2001; Matsuoka *et al.*, 2002). Others hypothesize that the domestication of maize was aided by the hybridization of teosinte with a variety of wild grasses, including pod corn (*Zea tunicata*), gamagrass (*Tripsacum* spp.) and possibly other grasses from the *Andropogoneae* tribe (family *Poaceae*). The latter is known as the "Tripartite Hypothesis" (Mangelsdorf, 1974 cited by Doebley, 2001). Several thousand years of domestication resulted in the absolute dependency of maize on man for its propagation and cultivation (Vollbrecht and Sigmon, 2005).

The differences between maize and teosinte with regards to chromosomes, gene structures and nucleotide sequences are no greater than that between two maize varieties, moreover maize and teosinte are completely interfertile (Kato, 1976; Doebley, 1990; Collins, 1920 cited by Doebley, 2004). Phylogeny based on microsatellite DNA of maize confirms that all maize types clustered in a single monophyletic lineage which is derived from a wild grass indigenous to Mesoamerica, namely teosinte (*Zea mays* subsp. *parviglumis*) (Matsuoka *et al.*, 2002).

Teosintes are perennial and tropical and they are expected to come in contact with pathogens and therefore must be able to tolerate disease and insect attack. As a result teosinte must possess a large number of resistance and defence related alleles which could be incorporated into modern, annual temperate maize varieties (Wilkes, 1977; Ellstrand *et al.*, 2007; Ross-Ibarra *et al.* 2009). As maize originated from teosinte as well as being able to integrate with it, the possibility exists that genes providing resistance to certain pathogens may also be incorporated into maize varieties. Teosinte may be very important in terms of improving the protein content of modern maize varieties (Wang *et al.*, 2005; Wang *et al.*, 2008; Flint-Garcia *et al.*, 2009).

Kernels of teosinte are small, few and are not fused to form ears and are entirely surrounded by an exocarp (Holst *et al.*, 2007). The breeding was based on the selection of teosinte mutants with cobs which possessed multiple rows of kernels and kernels with reduced or absent exocarps (Dorweiler *et al.*, 1993; Dorweiler and Doebley, 1997; White and Doebley, 1998; Doebley, 2004). The diversification of teosinte into a primitive maize form was due to variations and mutations in as few as five genes; these mutations controlled different traits (Beadle, 1980 cited by Doebley, 2004).

Seeds/kernels of the genus *Zea* have changed drastically over time and this might have also radically altered associated microbial communities. However, it was concluded that there was no significant difference in the amount of endophytic diversity observed in wild versus domesticated *Zea* species. As a matter of fact endophytes occurring in wild ancestors persist even in domesticated maize. Endophytes may reflect phylogenetic relationships amongst the genus *Zea*; due to the existence of host genotype-specific endophytes. There is conservation of endophytes in *Zea* across boundaries of domestication, evolution, ethnography (migration) and ecology (Johnston-Monje and Raizada, 2011).

1.2 Maize requirements

Agricultural crops develop and grow optimally within certain temperature and moisture ranges; these are usually crop specific (Schulze, 1997). The most significant environmental factors influencing maize agriculture are: (i) daily maximum and minimum temperatures, (ii) soil type and fertility, (iii) soil moisture levels, (iv) the ambient humidity surrounding the plant and wind movement, (v) the day length along with light intensity, (vi) air quality and pollution, (vii) competing plants and (viii) the pathogen-insect complexes (Verheye, 2010). When environmental conditions are unfavourable and fluctuate outside the tolerance ranges of

plants they produce stress on plants resulting in weakened plants which are more susceptible to disease (Boyer, 1995; McElrone *et al.*, 2001; Garrett *et al.*, 2006; Verheye, 2010).

The optimum temperature for growing maize is average daily summer temperatures of 23°C and higher. Temperatures higher than 32°C, along with water stress due to limited precipitation and/or high evaporation rates severely effects maize production (Du Toit, 1997). High yields may only be obtained if soil, nutrient and climatic conditions are favourable during all subsequent development stages of the maize plant (Sun *et al.*, 2007). Germination is optimal when temperatures fluctuate between 18°C and 21°C; however, it is severely hampered when temperatures rise above 21°C and plunge below 13°C. Maize cannot be cultivated where the mean minimum temperature drops below 10°C and/or where daily temperatures exceeds 45°C for prolonged periods. The rate of leaf elongation, leaf area, shoot biomass and the photosynthetic CO₂ assimilation rate and ultimately yield is seriously decreased if and when daily temperatures exceed 35°C. Above 40°C pollen is damaged and grain development and setting is reduced (Du Toit, 1997; Verheye, 2010).

Maize requires about 450 mm to 600 mm of water per season, which is obtained from soil moisture reserves and/or precipitation. During the growing season precipitation should be well distributed and followed by full days of sunlit warm weather between these rainstorms. In order to avoid yield loss due to moisture stress water reserves need to be supplemented when the annual rainfall decreases below $\pm$ 350 mm (Verheye, 2010). The harvest obtained at any stage is directly equivalent to the soil and climatic conditions which persisted during the growing season (Du Toit, 1997). Yield is also impacted by management strategies, crop genetics and other biotic stresses (Sun *et al.*, 2007). About 15 kg of grain is produced for each millimetre of water used. A mature plant would have consumed up to 250 l of water during its growth cycle (Du Plessis, 2003). Maize has an average frost-free maturing period of 140 days or more, depending on the type or variety of maize. No other crop utilises sunlight more efficiently than maize, and its yield per hectare is the highest of all grain crops (Du Plessis, 2003).

1.3 Maize production

Southern Africa is the major producer of maize in Africa (BFAP, 2012; USDA, 2012). Maize is the principal food source for the South African population at large (Durand, 2006). The bulk of maize produced in South Africa is allocated towards human consumption, whilst the

rest is either used as animal feed and/or for industry, such as chemical and ethanol production (BFAP, 2010; USDA FAS, 2012; DAFF, 2012a). The quality and quantity of maize yields are determined by a range of factors, both abiotic and biotic, when these factors develop into unfavourable conditions maize production may severely be affected (Stuckey *et al.*, 1993; Du Toit *et al.*, 1999). The effects of biotic stressors, in particular plant pathogens, vary from a few symptomatic leaves to severe epidemics in which large areas of field crops may be destroyed. The current deficiencies in world food supply are intensified by disastrous plant diseases (Strange and Scott, 2005). Currently South Africa experiences extremely low levels of malnutrition (less than 5% of the population is underfed). It is not expected that South Africa will endure any kind of food deficiencies due to climate change. If and when shortages do arise due to reduced crop yield and/or population changes, it will be counteracted by minimizing or completely halting crop exports and decreasing the amount of crops which is assigned towards industry and animal feed (FAO, 2008; Arnell *et al.*, 2010).

During the marketing year (MY) of 2009/10 the total hectares of maize planted in South Africa was estimated at 2.8 million hectares while the total maize production was 13.3 million tons. Production during this period was the second largest in the history of maize production in South Africa (USDA, 2012). The country's total maize production for the 2010/11 marketing year was 10.9 million tons, a reduction of nearly 19% from that of the previous season. In this production season the area has increased to approximately 2.9 million hectares. For the 2011/12 season a total of 3.1 million hectares were planted with a production of 12.1 million tons. A projected 11.4 million tons of maize was expected to be produced on approximately 3 million hectares for the 2012/13 period (USDA FAS, 2012; DAFF, 2012a).

Due to satisfactory conditions in most of the maize production regions of South Africa, continuous improvement of agricultural technologies and equipment, along with exceptional management practices as well as excellent yielding varieties the predicted long term trend for South African maize production is the production of more maize on smaller areas (USDA FAS, 2012). Over time fluctuations are experienced within South African maize production; these are determined by some of the following factors such as cultivars planted, weather conditions, management strategies, input costs and monetary maize value (Du Toit, 1997).

The maize industry is also responsible for generating large amounts of foreign exchange through the export of maize. South Africa mainly exports to Botswana, Lesotho, Namibia and Swaziland (the BLNS countries) as well as Zimbabwe, Kenya, Mozambique, Zambia, and Mauritius and in some seasons to Japan and Mexico. For the 2010/11 season South Africa

has exported 2.2 million tons of maize, it is estimated that 2 million tons were exported for the 2011/12 marketing year and finally it was predicted that 1.5 million tons of maize would be exported for the 2012/13 season (USDA FAS, 2012; BFAP, 2012; DAFF, 2012a).

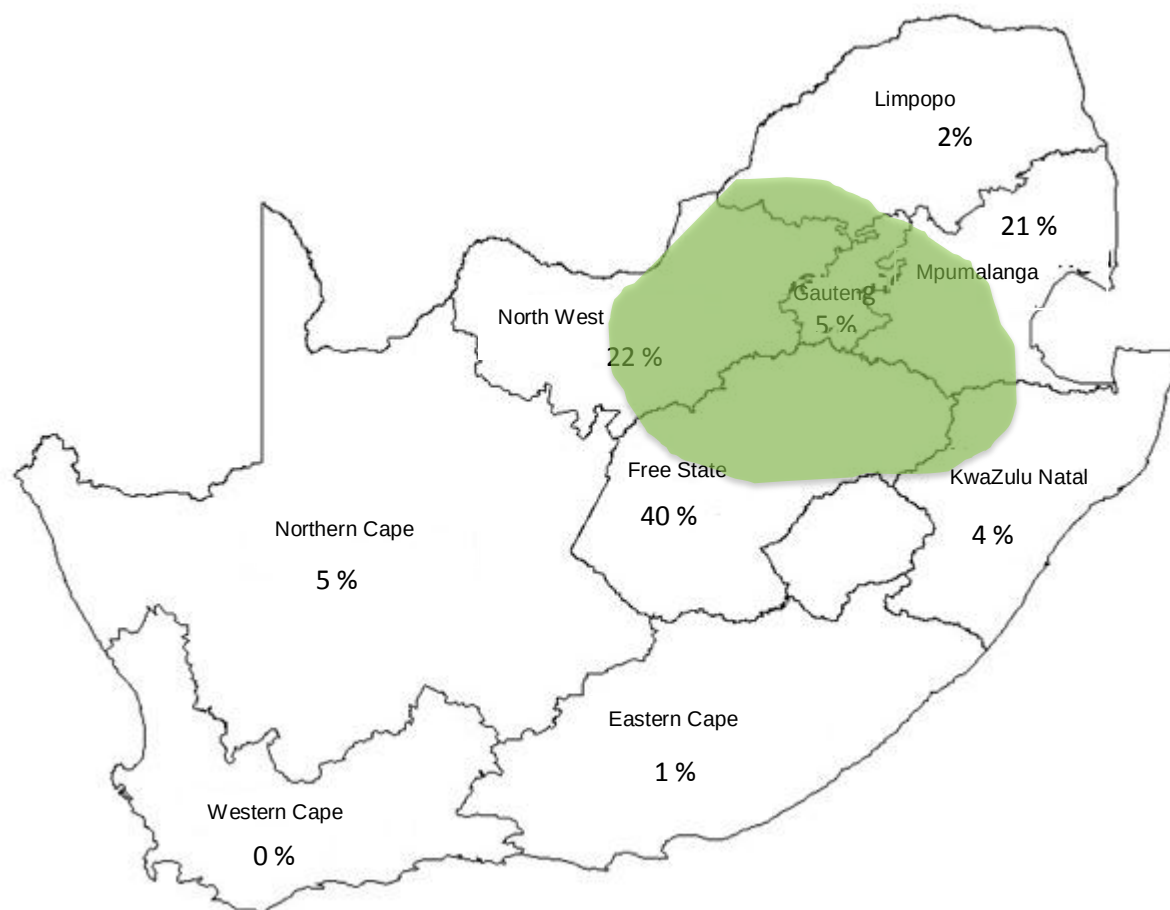


Figure 1 - The major maize cultivation regions of South Africa (indicated by the green zone) and also the percentage contribution of each province towards SA's maize production (JAWF, 1999; DAFF, 2012a).

Although maize is mainly produced on non-irrigated dry land there is a portion of less than 10% that is produced under irrigation. South Africa is divided into 36 grain production areas. The winter rainfall areas, namely the Western Cape and the Eastern Cape form part of regions 1 to 9. Griqualand West is region 10. Region 11 (Vaalharts) as well as areas 12 to 20 are all situated in the North West province. Regions 21 to 28 are situated within both the Free State and North West provinces. Mpumalanga constitutes regions 29 to 33. Region 34 is placed within the Gauteng province, region 35 within Limpopo and region 36 within Kwazulu-Natal (Du Toit, 1997; DAFF, 2012a). Although maize is cultivated throughout South Africa more than 60% are obtained from only the North West and Free State provinces.

This also constitutes an area of 65% of the total agricultural land of the country. Other provinces contribute to a lesser extent towards South Africa's maize production (as seen in Figure 1).

1.4 The South African climate

South Africa is situated in the subtropical high pressure zone, this is an atmospheric region dominated by dry descending wind, and for this reason the country generally has a hot and dry climate. South Africa's average annual rainfall is less than 500 mm, with an uneven distribution (Preston-Whyte and Tyson, 1993; Benhin, 2006; DoA, 2007). The western regions experience dry, desert like circumstances, whilst the eastern areas are subjected to humid, subtropical conditions (DoA, 2007). South Africa is further subdivided into three distinct regions according to rainfall patterns, the summer rainfall area, the winter rainfall area and the regions which receive rainfall throughout the year (Benhin, 2006; Schulze and Maharaj, 2007). The precipitation of South Africa is extremely variable and to some extent insufficient. This variability leads to serious losses in yield during years of inadequate rainfall and largely determines which water resources get allocated towards agriculture (Cook *et al.*, 2004).

The largest sections of the North West and Free State along with Gauteng and the eastern parts of the Mpumalanga province constitute the Highland region. It is an inland plateau and plain with low to moderate relief which ranges in altitude from 900 m to 1800 m above sea level (Schulze, 1997). The soil of these areas is of a sandy, clay and loam texture, with soil depths ranging from 400-1200mm; ideal for the cultivation of maize (Schulze, 1997). This region is responsible for 90% of the country's maize production (Du Toit *et al.*, 1999) and can be said that it is the country's bread basket in terms of food security for the South African population.

The Highveld is an early to mid-summer rainfall area which receives almost all of its precipitation during the months of October through to March (Schulze, 1997). Within the Highveld three climatic regions are existent. These are based on spatial rainfall and temperature variances and include (i) the dry/warm western region, (ii) the temperate eastern region and (iii) the wet/cool eastern region (ARC-GCI, 2008; Benhin, 2008). The western areas receive an average precipitation of up to 600 mm (with the Northern Cape being the driest receiving only between 200 mm and 500 mm), whilst the eastern regions receive between 600 mm and 1400 mm. Therefore, during the summer months the rainfall

increases in a west to east direction (Preston-Whyte and Tyson, 1993; Du Toit *et al.*, 2002; Benhin, 2006; Deichmann and Eklundh, 1991 cited by Benhin, 2006). In general the dryer western regions receive higher solar radiation during the summer months, which ranges from 32-34 MJ.m⁻².day⁻¹, than the eastern parts which receive 28-30 MJ.m⁻².day⁻¹ (Schulze, 1997). During the summer months (December – March) the average daily maximum temperatures for the west ranges from 28°C to 30°C, whilst those of the east ranges from 26°C to 30°C; both share an average daily minimum temperature of between 12°C and 16°C for these months (Schulze, 1997).

The production of cereal crops in the southern African region, in particular South Africa, may be severely affected by climate change (Perks, 2001). Over the past years South Africa had an approximate 2% temperature increase and at least a 6% decrease in precipitation. All provinces experienced a certain percentage decrease in rainfall during the past years, the Northern Cape -21.4%, North West -11.3%, Free State -3.5%, Mpumalanga -5.7% and Gauteng -7.1%. They were also subjected to temperature upsurges, Northern Cape 1.7%, North West 2.3%, Free State 1.7% and Gauteng 4.0%, whilst Mpumalanga experienced a decrease in temperature -2.1% (Blignaut *et al.*, 2009). It was estimated that each 1% decline in precipitation is likely to lead to a 1.1% reduction in maize production (Blignaut *et al.*, 2009).

It is expected that South Africa will experience a further 5% – 10% decrease in mean annual rainfall over the next 50 years and an average of 0.19°C increase in summer temperatures per decade (even as much as 1°C to 3°C) (Hewitson, 2001; Durand, 2006). A mean temperature increase of 21.4°C to 21.6°C would result in an average maize yield increase of 0.4%. Yield would keep on increasing until a certain temperature, thereafter it will decrease drastically with further increases in temperature. Maize yield would be reduced by approximately 4% when mean precipitation experience a 10% reduction. The combined effect of changes in temperature and rainfall on maize yields is determined by both the degree and direction of each of the changes. The total impact of a marginal decrease in precipitation along with a marginal increase in temperature on maize yield will be detrimental, as the effect of reduced precipitation on maize yields is far greater than the effect of increased temperature (Akpala *et al.*, 2008).

Potentially climate change will also alter the plant environment in the following ways (Downing *et al.*, 1996), increased levels of atmospheric CO₂ along with local warming will lead to increased growth and development along with higher yields per unit of water required (Kimball *et al.*, 2002). However, higher temperatures in combination with decreased

precipitation and uneven water supply (Schulze *et al.*, 2005) will affect the distribution of agro-ecological zones. The total maize production area will probably contract along the western borders (especially those of the North West and Free State provinces) of the current maize production region and/or the agricultural production belts for different crops may shift (Perks and Schulze, 2000). It is expected that South Africa will experience a delay in the onset of the rainy season along with an early end to the rainy season. Future drought is proposed to increase over the current arid regions as well as projected desertification extending in an eastward direction (Shongwe *et al.*, 2009). Current highly productive areas may experience a radical reduction in productivity, whilst previous minor production areas may experience a considerable increase in productivity (Reilly, 1996; Reilly and Schimmelpfennig, 1999). It is anticipated that the maize yields obtained from the western parts of the Highveld region are expected to decrease and become less predictable (Du Toit *et al.*, 1999). As agricultural regions become drier the need for irrigation will increase drastically; already 60% of total water reserves are allocated towards irrigation of agricultural land and it is the largest sole consumer of water (Blignaut *et al.*, 2009). Increases in temperature along with variations and fluctuations in regular distribution patterns of precipitation and other climate elements may ultimately result in aggravated water stress (Schulze *et al.*, 2005). Early to extra-early maturing maize cultivars may be planted due to their resilience towards climate variability (Amouzou *et al.*, 2013).

The South African agricultural sector will be severely affected by climate change in the following ways, decrease in water availability, shifts in seasonal temperatures and climatic patterns, an increase in the incidence, activity and distribution range of pests and diseases (maize pathogens in particular). It is anticipated that elevated CO₂ levels may increase the incidence and severity of some diseases, particularly those caused by necrotrophic pathogens (Chakraborty *et al.*, 2000; Chakraborty and Newton, 2011; Eastburn *et al.*, 2011). Climate change may also alter host physiology (in order to alter the microclimate in such a way that it favours pathogen development and colonization), resistance and pathogen development stages (disease infection cycles) and rates. It is expected that higher temperatures will accelerate the collapse of plant disease resistance via higher disease pressure and/or altered resistance gene efficiency in several host-pathogen systems. Although phytopathogens rely on leaf wetness for infection, an increase in temperature will more than compensate for the decline in precipitation, the reason for this being that infections would initiate much earlier in the growing season, thereby providing more time for epidemics to develop (Garrett *et al.*, 2006; Webb *et al.*, 2010; Newton *et al.*, 2011).

Higher temperatures decrease generation time resulting in a higher number of generations per season. Generation time regulates the severity of plant diseases in two ways, (i) accelerating and increasing inoculum levels and/or (ii) affecting the rate and frequency of pathogen evolution (gene flow) and a pathogen's ability to adapt to the environment (Legreve and Duveiller, 2010). Diversity within pathogen populations (due to exchange and flow of genes) leads to variation in host resistance, pathogen virulence and interactions (pathogen complexes). These variations may result in the increased importance of previously unimportant irrelevant diseases, increase the potential introduction of new diseases or pathogen emergence and the introduction of pathogens into new environmental niches; relying on the distribution of populations and environmental conditions which are influenced by climate change (Legreve and Duveiller, 2010).

Higher average daily temperatures along with extended periods of warm weather may have a rapid and prolific effect on the short life cycles of insects, their mobility and high reproductive potential (Ladányi and Horváth, 2010). This scenario may affect the severity of phytopathogen outbreaks as many plant diseases are transmitted by sucking insects. Climate change may also result in an increase in some pathogen epidemics and a decrease in others and the emergence of new diseases. Additionally, the efficiency of control strategies is expected to be affected by climate change (Ladányi and Horváth, 2010; Juroszek and Von Tiedemann, 2011).

1.5 Pathogenicity of Phytoba**cteria**

Infections by foliar phytopathogens result in a loss of available photosynthetic leaf tissue through necrosis - the end result is a decline in photosynthetic assimilate production, translocation and accumulation and thus reduce yields. Further effects include alterations in metabolism (photochemistry, electron transport and the photosynthetic carbon reduction cycle, reallocation of photoassimilates to supply the pathogen with nutrients) and gas diffusion (stomatal closure) (Berger *et al.*, 2007; Kocal *et al.*, 2008; Bilgin *et al.*, 2010; Garavaglia *et al.*, 2010).

Phyto**ba**cteria (in particular *Xanthomonas*) initially grow epiphytically and then gain access to the host through natural openings such as stomata and hydathodes or through wounds. Once inside the leaf they spread to the intercellular spaces of the plant tissue (mesophyll) and/or the xylem where they multiply and systemically colonize the plant (Hugouvieux *et al.*, 1998; Dangl and Jones, 2001; Buttner and Bonas, 2002a; Buttner and Bonas, 2002b).

Fluctuations in humidity cycles aid the entry through the hydathodes. During periods of high humidity bacteria colonize guttation droplets which form at the hydathodes by exudation. After a decrease in humidity guttation fluid together with the bacteria are then withdrawn into the plant (Ryan *et al.*, 2011). These bacteria are hemibiotrophic pathogens which initially feed on living host tissue, but cause the death of plant cells at later infection stages (Buttner and Bonas, 2010).

Substances such as bacterial toxins and extracellular degradative enzymes which are essential for the establishment of disease are termed pathogenicity factors. Those substances which only enhance the development of disease, but are not required for disease induction, are called virulence factors (Buttner and Bonas, 2010). Virulence factors, such as lipopolysaccharide (LPS) (activates pathogenesis related proteins, glucanases) and extracellular polysaccharides (such as xanthan) may enhance disease development if expressed in the determined location and at the appropriate stage and level of infection (Dow and Daniels, 2000). LPS also protect the bacteria against environmental stressors (Kingsley *et al.*, 1993), but also induce plant defences which restrict bacterial growth and/or decrease the delivery of type III effector proteins due to plant cell wall alterations (Dow *et al.*, 2000).

After entering the plant, the phyto bacterial cells adhere to plant cell receptors in order to translocate their proteins across their plasma membranes, through the host's cell wall into the cell's cytosol. Adhesins from *Xanthomonas* spp. include *XadA* and *XadB*, autotransporter homologs, filamentous hemagglutinin-like proteins and proteins predicted to be involved in type IV pilus synthesis (Da Silva *et al.*, 2002) For this they require a specialized secretion system (Buttner and Bonas, 2010). Currently six protein secretory systems are recognized for Gram-negative bacteria and in particular for *Xanthomonas*. These are classified on the basis of their structure, their function and the recognition of secretion substrates (Preston *et al.*, 2005; Gerlach and Hensel, 2007).

These include: type I or ATP-binding cassette (ABC) systems. This system secretes toxins, proteases, lipases and other degradative enzymes. The secretion is through the periplasmic membrane fusion protein which consists of a transporter in the inner membrane and a channel in the outer membrane (Gerlach and Hensel, 2007). Type II or general secretory pathway systems secrete toxins, extracellular enzymes and cell wall degrading enzymes. The enzymes include the following, cellulases, lipases, cellobiosidases, endoglucanases, polygalacturonases, xylanases and proteases. The enzymes pass through the periplasmic pseudopilus (Jha *et al.*, 2005; Johnson *et al.*, 2006). The substrates of the type II secretion

systems (TISS) may also prompt certain plant defence responses such as callose deposition in the cell wall (Jha *et al.*, 2005). Type III secretion systems (TIISS) secrete extracellular components of the TIISS system and effector proteins via the extracellular pilus, a multimeric transmembrane channel (Ghosh, 2004). Type IV secretion systems (which include bacterial conjugation systems) secrete extracellular components of the TIVSS and include DNA and/or proteins such as adhesins (Cao and Saier, 2001; Juhas *et al.*, 2008). The type V secretion system secretes via a protein channel in the outer membrane and autotransporters and two-partner secretion systems (Henderson *et al.*, 2004; Gerlach and Hensel, 2007; Cascales, 2008). Another secretion system which transports proteins and/or DNA in eukaryotic cells is the multicomponent secretion type VI system (Filloux *et al.*, 2008; Wu *et al.*, 2008).

Proteins exported by the type-II and V pathways are translocated through the inner membrane via the *sec* dependent general export mechanism and then transported across the outer membrane by means of specialized secretory apparatus (Pugsley, 1993; Hueck, 1998). Type I, III and IV pathways do not associate with proteins which are secreted by the *sec* pathway as they secrete proteins directly across both membranes, thereby bypassing the *sec* dependent pathway (Hueck, 1998).

Type ISS is common in most phyto bacteria, while type IISS is mutual in Gram-negative bacteria (Pugsley, 1993). These export proteins, toxins (supress defence genes, block development of plastids), extracellular enzymes (degrade cell walls) and other virulence factors into the host's tissue.

The most important secretory pathway which phyto bacteria rely upon is the type III secretion system (Galan and Collmer, 1999; Arnold *et al.*, 2003; Ghosh, 2004). This secretion system occurs in all or most Gram-negative pathogenic bacteria including the genera, *Erwinia*, *Pantoea*, *Ralstonia*, *Pseudomonas* and *Xanthomonas* (Alfano and Collmer, 1997). This system transports proteins which it is composed of (harpins/pilins), proteins which regulate the secretion process and the effector proteins (Alfano and Collmer, 1997). The effector proteins (virulence factors) interact with *R*-gene proteins in order to restrain and alter host defence responses and physiology (Pugsley, 1993; Van Gijsegem *et al.*, 1993; Cornelis and Van Gijsegem, 2000).

Originally the different types of effector proteins were not discovered through mutant phenotypes which lacked a particular virulence function. The deactivation of individual effector genes usually does not considerably affect bacterial virulence. The latter may be

due to possible functional redundancies among the effector proteins (Noel *et al.*, 2002; Vivian and Arnold, 2000). Their existence was proven through their ability to induce specific defence responses in resistant plants which possess the corresponding *R* genes (White *et al.*, 2000). Thus, avirulence factors place a restriction upon the pathogen's host range. These normally determine specificity at the pathogen's race level (pathovar races are different strains of a particular pathovar which are distinguished by their ability to infect different varieties of the same host). Such proteins also initiate defence responses in particular host varieties that possess a specific resistance gene. Effectors may also determine specificity at the level of plant species (Ryan *et al.*, 2011).

Type III effector proteins of *Xanthomonas* spp. includes the following - *AvrRxv*, *AvrBsT*, *AvrXv4* and *XopJ* with proposed cysteine proteases or acetyltransferases activities. There are also *AvrBs3*, *Avrb6* and *AvrXa7* which may manipulate the host cell transcriptome more directly. *AvrBs2* has proposed glycerophosphoryldiester phosphodiesterase activity and *XopE1*, *XopE2* has suggested transglutaminases functions (Van den Ackerveken *et al.*, 1996; Mudgett, 2005; Gurlebeck *et al.*, 2006; Kay and Bonas, 2009). The definite function of the following effectors are still unknown, however they strongly contribute to the multiplication of the bacteria in the plant, symptom development, and epiphytic survival - *AvrBs1*, *AvrRxo1*, *AvrXccC*, *AvrXv3*, *XopX*, *XopB*, *XopC*, *XopD*, *XopF1*, *XopF2*, *XopK*, *XopL*, *XopN*, *XopO*, *XopP*, *XopQ*, *XopR*, *XopX*, *XopZ* (Kay and Bonas, 2009). The majority of sequenced *Xanthomonas* spp. genomes comprise a core set of nine genes that encode type III effectors *XopR*, *avrBs2*, *XopK*, *XopL*, *XopN*, *XopP*, *XopQ*, *XopX* and *XopZ*. However for *X. albilineans* no effectors have been identified (Ryan *et al.*, 2011).

The type IIISS is encoded by *hrp* (*hypersensitive response* and *pathogenicity*) genes; they are organized in pathogenicity islands of more or less 20 genes with several operons (Hueck, 1998; Cornelis and Van Gijsegem, 2000; Arnold *et al.*, 2003). The *hrp* gene cluster is often flanked by several type III effectors and other types of virulence related genes. Additional type III effector genes are dispersed throughout the rest of the bacterial genome either in clusters or singly (Arnold *et al.*, 2003). These genes are required for disease development, the induction of a hypersensitive response in resistant plants and non-hosts, and they enable the bacterial pathogens to reproduce exponentially within their host (Hueck, 1998); but are not found in non-pathogenic species of *Xanthomonas* (Willis *et al.*, 1990; Leite *et al.*, 1994). These genes also encode the *hrp* pilus which is connected to the TIISS translocon. The latter is a proteinaceous transmembrane channel which inserts into the eukaryotic plasma membrane and facilitates the translocation of effector proteins (Buttner & Bonas, 2002a; Roden *et al.*, 2004; Weber and Koebnik, 2006; White *et al.*, 2009).

Based on similarities in *hrp* operon structures and the regulation of their gene expression, the *hrp* genes of the phyto bacteria are divided into two main groups. The *hrp* genes of *Erwinia*, *Pantoea* and *Pseudomonas* assemble in group I whilst those of *Xanthomonas* and *Ralstonia* species form group II (Alfano and Collmer, 1997).

HrpL – from the ECF (extracytoplasmic function) family of alternative sigma factors regulate the expression of the *hrp* genes of group I (Xiao *et al.*, 1994; Wei and Beer 1995; Kim *et al.*, 1997; Frederick *et al.*, 2001). The induction and expression of *hrpL* requires *hrpS* and *hrpY* in *Erwinia* spp. and *Pantoea* spp. and *hrpS* as well as *hrpR* in *Pseudomonas* spp. (Wei *et al.*, 2000; Hutcheson *et al.* 2001; Chatterjee *et al.*, 2002; Merighi *et al.* 2003).

The majority of group II *hrp* operons are activated by an AraC-like activator, *hrpB* in *Ralstonia* and *hrpX* in *Xanthomonas* spp. (Kamdar *et al.*, 1993; Wengelnik and Bonas, 1996; Wengelnik *et al.*, 1999; Cunnac *et al.*, 2004). The activation of *hrpX* and *hrpB* requires *hrpG* proteins in *Xanthomonas* spp. and *Ralstonia* species (Wengelnik and Bonas, 1996; Brito *et al.* 1999; Buttner *et al.*, 2002C; Buttner *et al.*, 2007).

PhcA is a negative regulator of the *hrpG* protein (Genin *et al.*, 2005). This protein (PhcA) is a transcriptional regulator that directs the expression of multiple virulence factors including extracellular polysaccharides, several cell wall degrading enzymes and bacterial motility (Schell, 2000).

At least nine of the *hrp* genes are conserved (known as *hrc* for *hrp* conserved) in both groups and these encode components of the type III secretion system (Bogdanove *et al.*, 1996; Hueck, 1998). *Hpa* (*hrp* associated) genes also form part of the *hrp* pathogenicity island. These contribute to the pathogenic interaction with the plant (Huguet *et al.*, 1998; Buttner *et al.*, 2004; Lorenz *et al.*, 2008; Buttner and He, 2009).

Hrp genes along with avirulence (*avr*) genes are associated with the expression of pathogenicity and host specificity and range at species, race and pathovar level. The *avr* proteins which are transported by the secretion system along with *hrp* proteins induce rapid cell death and ultimately HR (Hypersensitive Response) in the host. *Avr* proteins partly determine compatible host/bacteria interactions (Klement, 1982; Dangl *et al.*, 1996). Resistance in the form of HR is the result of recognition by the plant's specific receptor molecules (encoded by resistance, *R*, gene) of the elicitors (specific signal molecules) produced by the *avr* genes of the bacteria. *Avr* genes possibly promote pathogen growth, virulence and the development within a susceptible host (Bogdanove *et al.*, 1998a; Bogdanove *et al.*, 1998b; Huguet *et al.*, 1998).

The level of *hrp* gene expression during bacterial infection is influenced and regulated by environmental factors (including temperature and pH) as well as various host factors. The environmental conditions presumably simulate the physiological environment encountered by bacteria during infection (Schulte and Bonas 1992; Wei *et al.*, 1992; Xiao *et al.* 1992; Tang *et al.*, 2006). *Hrp* gene expression is inhibited by high pH and osmolarity as well as complex carbon and nitrogen nutrient sources, but is induced by acidity, low osmotic pressure and simple sugars. Optimal expression of the type IISS genes are obtained when the apoplastic phyto bacteria grow at a temperature of 20°C to 30°C (van Dijk *et al.*, 1999).

1.6 Phytopathogens of maize

Phytopathogens, in particular those affecting maize, are distributed all over the world (Krawczyk *et al.*, 2010). These plant pathogens cause diverse and devastating diseases in various different plants, of which one of the most important from a food security point of view is maize.

The following maize pathogens occur worldwide or at least have a large distribution range, *Pseudomonas avenae* subsp. *avenae* which causes bacterial leaf blight of maize, bacterial stripe and leaf spot caused by *Pseudomonas andropogonis*, holcus spot caused by *Pseudomonas syringae* pv. *syringae* and *Erwinia carotovora* subsp. *carotovora*, and *Erwinia chrysanthemi* pv. *zeae* the causal agents of bacterial stalk and top rot, respectively (Claflin, 2000; Giester and Rees, 2004; Schaad *et al.*, 2008).

Stewart's wilt the disease caused by the pathogen *Pantoea stewartii* subsp. *stewartii*, was recorded in various countries including the USA, Brazil, Italy, Peru, Poland, the former Soviet Union, Romania, Thailand and Vietnam, but not from South Africa (Mergaert *et al.*, 1993; Claflin, 2000; Roper, 2011; Mojtaba *et al.*, 2012).

Other *Pantoea* species are usually epiphytic, endophytic or opportunistic plant pathogens. *Pantoea ananatis* associated with leaf spot disease was reported to occur in South Africa (Goszczyńska *et al.*, 2007), Poland (Krawczyk *et al.*, 2010), Brazil (Paccola-Meirelles *et al.*, 2001) and Mexico (Pérez-y-Terrón *et al.*, 2009). *Pantoea agglomerans* causing leaf blight and vascular wilt of maize and sorghum was reported in Mexico (Morales-Valenzuela *et al.*, 2007) and *Enterobacter cloacae* subsp. *dissolvens* (Hoffman *et al.*, 2005; Grimont and Grimont, 2006) the causal agent of bacterial stalk rot of maize.

Other phyto bacteria of maize still have a restricted distribution range. *Clavibacter michiganensis* subsp. *nebraskensis* the causal organism of Goss's bacterial wilt or blight (Smidt and Vidaver, 1986; Ruhl *et al.*, 2009) and chocolate spot caused by *Pseudomonas syringae* pv. *coronafaciens* which are both currently restricted to the USA (Ribeiro *et al.*, 1977; Barta and Willis, 2005; Janse, 2005).

Bacterial leaf streak caused by *Xanthomonas* BLSD has only been recorded in South Africa (Coutinho and Wallis, 1991). Several *Xanthomonas* species form part of the top ten list of causal organisms of bacterial diseases of plants. However, their rank is dependent on the specific species and pathovars which infect a particular host. *Xanthomonas campestris* pathovars are ranked fifth and are responsible for various diseases in a range of crops worldwide. The fourth and sixth positions are claimed by specific *Xanthomonas* species, namely those which are pathogens of rice, *Xanthomonas oryzae* pv. *oryzae* and those which infect cassava plants, *Xanthomonas axonopodis* pv. *manihotis*, respectively (Mansfield *et al.*, 2012).

1.7 The Xanthomonads

The term Xanthomonads refers to the genera *Xanthomonas*, *Stenotrophomonas* and *Xylella*. The latter two genera were excluded from the *Xanthomonas* group based on phylogenetic analyses of rDNA as well as ITS sequences (Pieretti *et al.*, 2009; Yakoubou and Cote, 2010a).

Xanthomonas are members of the class γ -*proteobacteria*, which also include the genera *Pseudomonas* and *Pantoea* (Saddler and Bradbury, 2005). This class of bacteria has three characteristics in common, (i) they colonize the intercellular spaces of plants, (ii) are capable of killing plant cells and (iii) possess *hrp* genes for translocation of pathogenicity elements. Many of the pathogens in the class are host specific and are recognized by a variety of symptoms (Alfano and Collmer, 1997).

The genus *Xanthomonas* is diverse in its phytopathogenicity and is therefore an economically important group of bacterial plant pathogens (Starr, 1981; Hayward, 1993; Vauterin *et al.*, 1995; Jackson, 2009). In general these organisms are Gram-negative, straight single rods, mobile by means of single polar flagella, non-capsulated and strictly aerobic. Additionally they are chemoorganotrophic, oxidase negative and catalase positive. They are also able to use a variety of carbohydrates and salts and organic acids as sole

carbon sources (Bradbury, 1984). The temperature range of this organism is between 10°C and 37°C, with an optimum growth temperature of 28°C (Coutinho and Wallis, 1991).

Bacterial growth is in the form of convex, yellow coloured mucoid colonies. They are yellow due to the presence of the pigment xanthomonadin, which darkens with age. This pigment may protect the pathogen from excessive light exposure (Poplawsky *et al.*, 2000). The name of the genus is derived from this yellow coloured, membrane bound, brominated, aryl-polyene pigments. It originated from the Greek word, *xanthos*, meaning yellow and *monas*, meaning entity (Starr and Stephens, 1964; Starr *et al.*, 1977; Coutinho and Wallis, 1991). As the production of these pigments is specific to the genus *Xanthomonas* they may be used as chemotaxonomic as well as diagnostic markers (DNA probes/primers based on the xanthomonadin genes) to distinguish between pigmented and non-pigmented mutants of Xanthomonads and other yellow pigmented bacteria (Starr and Stephens, 1964; Moffet and Croft, 1983; Schaad and Stall, 1988; Poplawsky *et al.*, 1993). Variation in *Xanthomonas* pathovars may also be illustrated by a unique RFLP pattern in the *pig* region (cluster of genes encoding xanthomonadin) (Poplawsky *et al.*, 1993).

This genus is composed of numerous species and even more pathovars within the species. The species and pathovars are usually host and tissue specific (Hayward, 1993; Van den Ackerveken *et al.*, 1996; Parkinson *et al.*, 2009; Ryan *et al.*, 2011). The term pathovar (pv.) refers to strains with similar traits which are only distinguishable at the intraspecific level on the basis of their pathogenicity to one or more host plants (Dye *et al.*, 1980; Hayward, 1993; Vauterin *et al.*, 1995). At present the taxonomic position of this genus is based upon DNA-DNA hybridization (revealed 20 DNA homology groups), the analysis of 16S–23S rDNA (ITS - Intergenic Spacer Sequences) and a combination of other molecular profiling techniques, including rep-PCR, AFLP and others (Rademaker *et al.*, 2000; Goncalves and Rosato, 2002; Rademaker *et al.*, 2005; Ryan *et al.*, 2011).

The pathovar classification system was not always in existence. In the past classification relied upon the “new host, new species” concept, relative to each new variant of the genus *Xanthomonas* discovered. These were classified as a separate species due to differences in host range and/or disease symptoms produced (Starr, 1981). The pathovar classification system has certain limitations. These include, (i) the incomplete database on the host range of strains of a specific pathovar (due to insufficient host range studies and the lack of numerous cross-inoculation studies), (ii) the presence of substantial heterogeneity within a number of pathovars (Murata and Starr, 1973; Vauterin *et al.*, 1990; Palleroni *et al.*, 1993; Rademaker *et al.*, 2000) and (iii) the fact that possible nonpathogenic *Xanthomonas*, may be

isolated from healthy or diseased plants, cannot be classified in the pathovar system (Vauterin *et al.*, 2000).

The following *Xanthomonas* species are recognized - *Xanthomonas fragariae*, *X. populi*, *X. oryzae*, *X. albilineans*, *X. sacchari*, *X. vesicatoria*, *X. axonopodis*, *X. vasicola*, *X. codiae*, *X. hortorum*, *X. translucens*, *X. bromi*, *X. campestris*, *X. cassavae*, *X. cucurbitae*, *X. pisi*, *X. melonis*, *X. theicola* and *X. hyacinthi* (Vauterin *et al.*, 2000). Recently an additional 7 species were recognised, *X. perforans*, *X. euvesicatoria*, *X. alfalfae*, *X. fuscans*, *X. citri*, *X. arboricola* and *X. gardneri* (Ryan *et al.*, 2011). Within this species structure the following anomalies arise regarding pathogenic traits; isolates in different genomic groups which infect the same host(s) (convergent evolution) and isolates in the same genomic group which infect different hosts or the same host differently (divergent evolution) (Rademaker *et al.*, 2005). The genus *Xanthomonas* is considered to be monophyletic (that is a species which has diversified from a single lineage) and first arose as a monocot pathogen. The majority of *Xanthomonas* possess limited sequence variation, which is indicative of rapid and extensive pathovar diversification that has occurred in relatively recent times. *Xanthomonas* lineages possess the ability and potential to diversify and exploit new plant hosts (Parkinson *et al.*, 2009).

The *Xanthomonas* genome is a single circular chromosome which ranges in size from 4.8 Mb to 5.3 Mb (depending on the species, *X. albilineans* has a genome size of 3.7 Mb considerably smaller due to the absence of various genes), with a GC content of more than 60%. The gene content is similar amongst all species. It is estimated that the genome encodes more than 4 000 proteins, these include those responsible for energy production and for most other cellular functions. Genes which are encoded include pathogenesis associated gene clusters which encodes the type II secretion system (*xps*) and (*rpf*) which regulates the synthesis of pathogenicity factors. The hypersensitive response and pathogenicity (*hrp*) genes which encode the type III secretion system and the *gum* genes which encode the synthesis of the extracellular polysaccharide xanthan are however lacking within the species *Xanthomonas albilineans* (Pieretti *et al.*, 2012). Other encoded genes include those which are responsible for host recognition by the pathogen, pathogen adhesion to the plant surface, invasion and colonization of the host tissue, acquiring of nutrients and counteracting plant defence responses (avirulence genes). *Xanthomonas* spp. may possess additional plasmids which encode factors and functions associated with virulence. These include type III effector proteins, secreted extracellular enzymes and type IV secretion systems (Comas *et al.*, 2006; Lima *et al.*, 2008; Buttner and Bonas, 2010).

Xanthomonas species and pathovars are the cause of several plant diseases of both crops and ornamentals (an estimated 392 plant species are affected - 124 monocot species and 268 dicot species). These include leaf and fruit spots, leaf streak, rot, blight, vascular wilt and canker (Leyns *et al.*, 1984; Hayward, 1993; Kay and Bonas, 2009; Bogdanove *et al.*, 2011). Although the genus at large is capable of infecting a broad host range, the individual species and pathovars are, however, very host and tissue specific (Vauterin *et al.*, 1995).

Xanthomonads affect a whole range of members from the *Poaceae* family, including forage grasses (Sudan grass, brome grasses, barley) and cereal grains (sorghum, millet, oats, wheat, rye, rice and maize) (Malvick, 1991). Xanthomonads – the causal bacteria of leaf streak or stripe blight - are widely distributed and destructive on several types of sorghum, Sudan grass, pearl millet and foxtail millet (causal organism *X. vasicola* pv. *holcicola*), barley, wheat, rye, and oats (causal organisms *X. translucens* pv. *translucens*, pv. *undulosa*, pv. *cerealis*, pv. *secalis* and pv. *poae*) (Boosalis, 1952; Egli and Schmidt, 1982; Bragard *et al.*, 1997; Parkinson *et al.*, 2009; Raja *et al.*, 2010), rice (causal organisms *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas oryzae* pv. *oryzicola* (Niño-liu *et al.*, 2006). Sugarcane may be affected by gumming disease and leaf scald caused by *X. axonopodis* pv. *vasculorum* (Dookun *et al.*, 2000; Destefano *et al.*, 2003; Parkinson *et al.*, 2009) and *Xanthomonas albilineans* (Pan *et al.*, 1997; Champoiseau *et al.*, 2006; Pieretti *et al.*, 2012; DAFF, 2012b), respectively.

As the maize leaf streak pathogen differs from other *Xanthomonas* species and pathovars (which infect members of the *Poaceae* family) regarding many characteristics and host specificity tests, it was proposed to be regarded as a distinct pathovar and be named *Xanthomonas campestris* pv. *zeae* (Coutinho and Wallis, 1991).

Concluding, *Xanthomonas* BLSD is able to infect maize, *X. campestris* pv. *holcicola*/*X. vasicola* pv. *holcicola* infects both maize and sorghum, *X. vasicola* pv. *vasculorum* infects both maize and sugarcane, whilst *X. campestris* pv. *vasculorum*/*X. axonopodis* pv. *vasculorum* is capable of infecting maize, sorghum and sugarcane (Qhobela *et al.*, 1990; Aritua *et al.*, 2009; Wasukira *et al.*, 2014). DNA homology studies along with others recognized the close similarity of *X. axonopodis* pv. *vasculorum* strains to *X. vasicola* (Vauterin *et al.*, 1995; Dookun *et al.*, 2000).

1.8 *Xanthomonas* BLSD the causal agent of bacterial leaf streak (BLS) of maize

This disease was first reported in South Africa in 1949, and to date it is still restricted to South Africa where it is widespread within the drier regions of the country (Dyer, 1949). The importance of this plant pathogen has increased in the last few seasons due to its continued incidence, severity and spread on maize crops in South Africa. Previously a commercial variety of maize was withdrawn due to its susceptibility to the disease (Coutinho and Wallis, 1991).

X. campestris may survive in post-harvest crop residues for several months. Bacteria in exudates on infected leaves and crop residues (even within the soil microhabitat) remain dormant during dry periods and will infect the host when host is available and climatic conditions become favourable for infection and disease development. Primary infections typically occur during the seedling stages, while secondary infections occur on younger leaves during the growing period. This pathogen (as is the case with many *Xanthomonas* species) may be disseminated from field to field by rain splash, wind, overhead irrigation, by contaminated soil, possibly by sucking insects, alternate weed hosts and by direct contact between plants. It is still unknown whether or not it is seedborne (Schaad and Dianese, 1981; Jones *et al.*, 1986; Dzhililov and Tiwari, 1995; López *et al.*, 1999; Malavolta *et al.*, 2000; Gent *et al.*, 2005; Mwebaze *et al.*, 2006; Gitaitis and Walcott, 2007; Darsonval *et al.*, 2008; Ryan *et al.*, 2011).

The characteristic symptoms of *Xanthomonas* BLSD occur on maize leaves with wavy, irregular margins and 2 mm to 3 mm broad yellow-brown lesions along the veins (figure 2). In severe cases these lesions may extend the entire length of the leaf often coalescing to form large necrotic regions, the end result is a loss in available photosynthetic area.

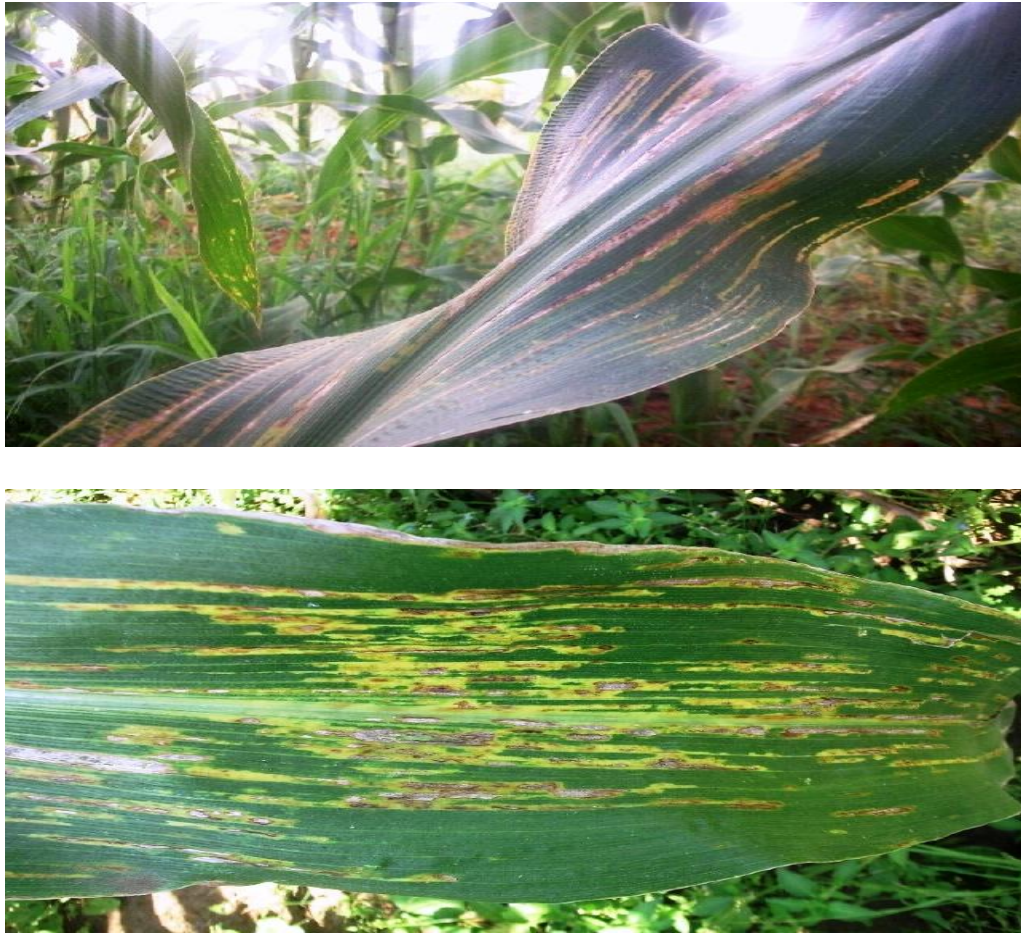


Figure 2 - The characteristic symptoms of a *Xanthomonas* BLSD infection.

Yellow droplets of bacterial exudates (xanthan) ooze from these lesions (Coutinho and Wallis, 1991). When dry, the droplets form yellowish, gummy drips or dry flakes. Xanthan is a polymer of repeating pentasaccharide units with a cellulose backbone and trisaccharide side chains (Becker *et al.*, 1998). Daily temperatures exceeding 32°C promotes and enhances symptom development, whilst a humid climate as a result of rain or irrigation enhances the incidence and spread of the disease. Up to 40% of leaf tissue may be destroyed by this bacterium (Nowell, unpublished, cited by Qhobela *et al.*, 1990); this is detrimental to maize production as a loss in photosynthetic area results in a loss in photosynthate production, translocation and accumulation.

1.9 The function of xanthan

The chromosomal regions *xpsIII*, *xpsIV*, *xpsVI* and a 35.3 kb gene cluster (*xanA* and *xanB*) are responsible for the first phase of xanthan biosynthesis (Hotte *et al.*, 1990; Koplin *et al.*, 1992; Harding *et al.*, 1993). These regions comprise gene functions involved in the synthesis of the glucose and mannose nucleotide precursors. Proteins associated with the successive

stages of xanthan synthesis, assembly of the pentasaccharide repeating unit, polymerization and the export of xanthan are directed and encoded by the *xpsI* or *gum* region (Sutherland, 1993; Denny, 1995; Harding *et al.*, 1995). The *gum* operon consists of a region of twelve products of approximately 16 kb, *gumB-gumM*. Additional co-transcribed open reading frames (ORFs) *gumA* and *gumN-gumP*, are located downstream of *gumB-gumM*, respectively (Katzen *et al.*, 1998; Vojnov *et al.*, 2001; Yoon and Cho, 2007). The gum genes of numerous *Xanthomonas* spp. contribute to epiphytic survival and/or bacterial growth *in planta* and development of disease symptoms (Katzen *et al.*, 1998; Dunger *et al.*, 2007).

Xanthan is an extracellular polysaccharide (EPS) which protects (by forming a physical barrier) the bacterium from freezing, desiccation, the effects of UV light and from bacteriophages (Sutherland, 1993; Jackson, 2009). Xanthan is required during early stages of infection in leaf mesophyll tissue, but is profusely produced at later stages of pathogenesis in tissue undergoing necrosis (Newman *et al.*, 1994; Vojnov *et al.*, 2001). Xanthan usually causes the wilting of leaves by either blocking xylem vessels resulting in their rupture due to high osmotic pressure or by restricting water flow and/or by increasing cell membrane leakage. Thus, this extracellular polysaccharide encourages water saturating of the intercellular spaces which promotes bacterial colonization (Denny, 1995; Kiraly *et al.*, 1997; Vidhyasekaran *et al.*, 1989 cited by Vidhyasekaran, 2004).

Xanthan production also increases the pathogenicity of *Xanthomonas* as it shields the bacteria and promotes biofilm formation which protects it from bacteriostatic substances. It prevents direct morphological contact between bacterial cells and the plant's cell wall, thus preventing the activation of various plant defence reactions and mechanisms (Stoodley *et al.*, 2002; Dow *et al.*, 2003; Ramirez *et al.*, 1988, cited by Born, 2005). Additionally xanthan suppresses local plant defence by inhibiting callose deposition; it promotes and facilitates the dissemination of the pathogen (Braun, 1990; Saile *et al.*, 1997; Yun *et al.*, 2006). Thus disease development may be enhanced when expressed in the determined location and at the appropriate stage and level of infection (Dow and Daniels, 2000).

In general foliar plant pathogenic bacteria, and in particular those affecting cereal crops, may be controlled by various control interventions. These include the planting of resistant varieties (either specific and/or polygenic resistance) and the inhibition of the pathogen's virulence mechanisms. Crop rotation reduces the pathogen's inoculum levels and tillage buries crop residues as these pathogens usually overwinter in debris (Dyer, 1949; Malvick, 1991). The exclusion of the pathogen or infected plant material through quarantine methods will prevent spread of the causal organism. Improved irrigation management will reduce the

spread of the disease through splashing water drops (Schaad and Alvarez, 1993). Elimination of potential alternate weed hosts (especially members of the *Poaceae* family) will reduce potential inoculum sources (Parkinson *et al.*, 2009). Decreasing stand density is another control method, as fewer plants lead to a reduction in relative humidity which might otherwise favour disease development (Burdon and Chilvers, 1982; Stuckey *et al.*, 1993; Strange and Scott, 2005). In order to sufficiently control a phyto-bacterial pathogen population an integrated disease management strategy needs to be employed which includes all possible interventions to prevent disease spread and epidemics (Pedigo, 2002).

Control of plant pathogens may develop new challenges, if the pathogens populations are variable both spatially and temporally and in genotype; inevitably their gene composition would change, often overcoming conventional inbred resistance and/or transgenic resistance (Strange and Scott, 2005). Host plant resistance may be maintained through the promotion of genetic diversity in the crop, this may be achieved through several mechanisms; developing monogenic resistance that following its collapse is replaced by a second cultivar comprising another gene for resistance (Russell, 1978), merging multiple genes either through stacking or pyramiding them into a single cultivar (Pedersen and Leath, 1988), incorporating genes for monogenic resistance into several isolines and integrating these into multiline cultivars (Browning & Frey, 1981; Johnson, 1984; Mundt, 2002; Lannou, 2001) and promoting cultivar mixtures (Wolfe, 1985); developing polygenic resistance which is then combined with monogenic resistance (Browning, 1980); geographic and/or temporal arrangement of different resistant cultivars so that biotypes originating in one region or period are avirulent in nearby areas or future periods (Browning *et al.*, 1977) will help sustain the use of resistance in an integrated control program.

1.10 Genotypic methods

Several steps are involved in the identification and analysis of plant diseases caused by phytopathogens (Schaad and Stall, 1988). These include the isolation of the suspected pathogen from diseased tissue, the purification of the culture, microscopic examination, phenotypic and molecular analyses (Goszczyńska and Serfontein, 2000; Houpiikian and Raoult, 2002; Alvarez, 2004). Phenotypic methods depend upon the availability of pure culture and rely upon colony morphology and biochemical profiling. These methods used for bacterial identification have major limitations, such as various strains of an organism may have different biochemical profiles (strain variation within a species) thereby promoting confusion in identification. Other disadvantages include organisms with biochemical

characteristics which do not comply with patterns of any known genus and/or species (isolates which exhibit unusual phenotypic profiles) are occasionally encountered. These methods are also not suitable for fastidious and uncultivable organisms (Olsen and Woese, 1993; Hauben *et al.*, 1997; Drancourt *et al.*, 2000; Nowbuth *et al.*, 2005).

Phenotypic methods used to characterise *Xanthomonas* species and/or pathovars include amongst others, fatty acid methyl ester analysis, LPS analysis, carbon source/substrate usage (Biologs) and protein profiling (Vauterin *et al.*, 1991; Chase *et al.*, 1992; Vauterin *et al.*, 1992; Jones *et al.*, 1993; Ojanen *et al.*, 1993; Yang *et al.*, 1993). It was previously noted that physiological characteristics and biochemical properties are inadequate to distinguish between most *Xanthomonas* species and in particular *Xanthomonas campestris* pathovars as they are phenotypically uniform (Dye, 1962; Coutinho and Wallis, 1991). Due to the limitations of phenotypic methods a number of different genotypic methods may be used for the identification and classification of microbes, particularly the *Xanthomonas* spp. Each of these methods allows for a certain level of phylogenetic classification, from the family, genus, species, subspecies to the strain level (Louws *et al.*, 1996). Each different method is considered on the basis of its relevance, advantages, disadvantages, ease of application, reproducibility, equipment requirements and level of resolution (as seen in figure 3) (Akkermans *et al.*, 1995).

Family	Genus	Species	Subspecies	Strain
		DNA sequencing		
		16S rDNA sequencing		
		ARDRA		
		DNA-DNA reassociation		
		tRNA-PCR		
		ITS-PCR		
		RFLP LRFPA PFGE		
		Multilocus Isozyme		
		Whole cell protein profiling		
		AFLP		
		RAPD APPCR		
		Rep-PCR		

Figure 3 - Resolution power of various DNA techniques involved in diagnostics, phylogenetics and genomic profiling (derived from Louws *et al.*, 1996).

A polyphasic approach is suggested and occasionally compulsory when definite species identification, description of new species, population genetics analysis and establishing phylogenetic relationships among closely related species are considered (Roumagnac *et al.*, 2004; Ah-You *et al.*, 2009). Genotypic analyses require that the following techniques are used; (i) analyses of ribosomal genes (such as 16S, 23S and/or ITS rDNA) (Hauben *et al.*, 1997; Moore *et al.*, 1997; Goncalves and Rosato, 2002; Adhikari *et al.*, 2012), (ii) protein encoding genes (such as *gyraseB*) (Parkinson *et al.*, 2007; Parkinson *et al.*, 2009), (iii) DNA-DNA hybridization (Vauterin *et al.*, 1995; Rademaker *et al.*, 2000; Rademaker *et al.*, 2005), (iv) genomic profiling (ARDRA, RFLP, AFLP, RAPD, REP-PCR) (Qhobela *et al.*, 1990; Smith *et al.*, 1994; Restrepo *et al.*, 1999. Massomo *et al.*, 2003.), (v) MLSA (Young *et al.*, 2008; Fargier *et al.*, 2011), (vi) MLST and even (vii) genome wide analysis (Snel *et al.*, 1999; Da silva *et al.*, 2002; Rodriguez *et al.*, 2012.)

1.10.1 Specific markers

In previous studies specific markers were designed and applied for the detection and identification of the *Xanthomonas* spp. These may be genus or species specific, based on the genes which encode xanthan (Adriko *et al.*, 2012; Mbega *et al.*, 2012), xanthomonadin (Poplawsky *et al.*, 1993) the *hrp* system (Leite *et al.*, 1994; Park *et al.*, 2004; Berg *et al.*, 2005; Lewis *et al.*, 2010), the ITS region (Adachi and Takashi, 2000; Yakoubou and Côté, 2010b; Mbega *et al.*, 2011) and/or the *gyraseB* protein (Araújo *et al.*, 2013). These molecular markers may be applied for the detection and identification of a specific pathogen and/or groups of pathogens, even before disease symptoms become visible and these may play a crucial role in disease monitoring and management.

1.10.2 Ribosomal DNA analyses

The rRNA operon usually exists as multiple copies within a cell and is composed of three conserved gene domains, the 16S rRNA gene ($\pm 1\ 500$ nucleotides), the larger 23S rRNA gene (± 2900 nucleotides) and the 5S rRNA gene (± 120 nucleotides). The rRNA operon is transcribed into one pre-rRNA transcript that includes the following components in the 5' to 3' order: 16S, ITS, tRNA, ITS, 23S, ITS and 5S rRNA sequences (Gurtler and Stanisich, 1996; Woodson and Leontis, 1998; Klappenbach *et al.*, 2001; Clarridge, 2004).

There are two theories which attempt to elucidate the existence of multiple copies of rRNA operons within a genome. They have a multiplier effect on translation, resulting in the rapid growth of the bacterium in response to environmental changes; bacterial species that divide quickly tend to have more copies of rRNA genes for the rapid production of ribosomes. Secondly, functional differentiation between rRNA operons permit differential expression of rRNA operons in reaction to environmental change; species enclosing multiple rRNA operons may be more adaptable to changing environmental conditions (Gaal *et al.*, 1997; Klappenbach *et al.*, 2000; Klappenbach *et al.*, 2001; Acinas *et al.* 2004). However, it still has to be explored whether or not these paralogous copies of rRNA operons are co- or differentially expressed in bacteria under various environmental conditions (Case *et al.*, 2007).

The first hypothesis' feasibility is reliant upon the occurrence of genetic drift which would have to be countered by gene adaptation to produce rRNA operons with minute sequence heterogeneity scattered randomly across their length. The second hypothesis expects that the selection of functionally differentiated rRNA operons would result in the accumulation of heterogeneous positions at particular sites and differentiated secondary stem-loop structures between rRNA molecules; these heterogeneities may have resulted from the accumulation of neutral nucleotide substitutions or recombination (Dahllof *et al.*, 2000; Acinas *et al.*, 2004; Santos and Ochman, 2004; Case *et al.*, 2007). The impact which such ribotypes would have on phylogeny were assessed through the comparison of the 16S rDNA phylogenetic and evolutionary characteristics to those of a single copy protein encoding gene. It was determined that intragenomic heterogeneity influenced 16S rRNA gene tree topology, phylogenetic resolution, and operational taxonomic unit estimates at the species level and/or beyond (Case *et al.*, 2007). The protein encoding gene provided similar phylogenetic resolution to that of the 16S rRNA gene at all taxonomic levels, except for between closely related species and subspecies levels; at these levels it provided better resolution (Case *et al.*, 2007; Nochi *et al.*, 2009). Thus, information provided by the 16S rRNA gene serves to complement those provided by single copy protein encoding genes. For some closely related bacteria intragenomic heterogeneity may be as substantial as intergenomic heterogeneity; therefore caution is needed when interpreting phylogenies derived from rDNA gene sequences at these taxonomic levels (Cilia *et al.*, 1996; Case *et al.*, 2007).

Prokaryotic identification and comparative phylogenetic analyses were originally based upon the employment of 5S rDNA sequence data. However as research progressed 5S rDNA sequencing was replaced by 16S rDNA sequencing as 5S rDNA sequencing contains limited information due to its short length (Fox *et al.*, 1980; Cilia *et al.*, 1996; Rudi *et al.*, 2007).

Additional reasons which promotes the use of this ribosomal gene include: the 16S rRNA gene is universally present in almost all bacteria with a conserved function (relatively slow rates of evolution – considered to be the most conservative of all genes), due to the availability of deposited sequences for comparison and the presence of universal primers, the relative ease and speed of sequencing and conserved regions which permit prompt alignment whilst variable sections allows for discrimination between related taxa. The function of the 16S rRNA gene has not changed over time. Little evidence exists for its lateral transfer (however, its potential transfer between species have been artificially demonstrated), indicating that sequence alterations are a more accurate measure of time. The 16S rRNA gene is large enough for informational purposes and is therefore more precise and easier for bacterial identification (even direct amplification from infected/diseased tissue) than using phenotypical and biochemical methods (Weisburg *et al.*, 1991; Patel, 2001; Petti *et al.*, 2005; Mignard and Flandrois, 2006).

Species delineation and differentiation is not only supported by 16S rDNA sequencing, but it's also considered to form part of bacterial taxa description (taxonomy) and (re)classification process and is necessary for establishing phylogenetic relationships (Busse *et al.*, 1996; Fredericks and Relman, 1996; Hauben *et al.*, 1997; Woese, 2000; Christensen *et al.*, 2005; Rajendhran and Gunasekaran, 2011).

Bacterial 16S rRNA genes usually consists of eight highly conserved regions, U1-U8, and nine hypervariable regions, V1-V9, which display significant sequence diversity among different bacterial species and/or genera. These may be exploited for species identification (Van de Peer *et al.*, 1996; Baker *et al.*, 2003) and to rapidly detect specific bacterial species. It may also be used for differentiation between closely related organisms. Conserved sections permit PCR amplification of sequences using universal primers and are suitable for resolving distant relationships (Lane *et al.*, 1985; Hauben *et al.*, 1997; Marchesi *et al.*, 1998; Yang *et al.*, 2002; Clarridge, 2004).

These 16S rRNA genes have been used to identify, differentiate and phylogenetically group phytobacterial pathogens of the genera *Erwinia*, *Pantoea* and other *Enterobacteriaceae*. The resolution power, however, diminishes at species level (Hauben *et al.*, 1997; Hauben *et al.*, 1998; Christensen *et al.*, 2005). The 16S rDNA sequences among members of the *Enterobacteriaceae* family are mostly conserved; however minor variations exist within a species and amongst operons of closely related species (Stackebrandt and Goebel, 1994). The ITS region possesses more sequence variability (due to the insertion or deletion of sequences (homologous recombination) (Klappenbach *et al.*, 2000; Sadeghifard *et al.*, 2006;

Yakoubou and Côté, 2010a) and is increasingly being used to supplement 16S rDNA analysis (Nagpal *et al.*, 1998). The ITS region has a functional role in ribosomal RNA maturation and transcriptional anti-termination (Apirion and Miczak, 1993). In general the ITS regions include (multiple rRNA operons) *rrnA*, *rrnB*, *rrnC*, *rrnD*, *rrnE*, *rrnH*, or *rrnG* which encode different tRNA's. Primers which are useful for phylogenetic studies are also designed from these specific operons (Gurtler and Stanisich, 1996; Acinas *et al.*, 2004; Mbega *et al.*, 2011).

Differentiation of closely related species is based either upon direct sequencing and/or the analysis of restriction patterns or fragments and/or different lengths (length polymorphisms) (Grimont and Grimont, 1986; Kostman *et al.*, 1992; Gurtler and Stanisich, 1996; Goncalves and Rosato, 2002; Rajendhran and Gunasekaran, 2011). However, the ITS region is also subjected to sequence heterogeneity in terms of the type and number of tRNA genes present (Boyer *et al.*, 2001). Phylogenies derived from both 16S rDNA sequences and ITS are generally consistent (Mehta and Rosato, 2001; Ferrera *et al.*, 2007), whilst providing even improved resolution or clustering in some cases (Goncalves and Rosato, 2002).

The representativeness and reliability of the 16S rRNA gene in bacterial phylogeny has been established and confirmed through the sequencing of various bacterial genomes and comparison between genome and 16S rDNA gene phylogeny (Snel *et al.*, 1999). The 16S rDNA sequences allows for genus identification in up to >90% of cases and identification of 65–91% of these at the species level, however from 1 to 14% of isolates remain unidentified after analysis (conventional method) (Tang *et al.*, 1998; Drancourt *et al.*, 2000; Mignard and Flandrois, 2006; Janda and Abbott, 2007). The MicroSeq 500 16S rDNA-based identification system allows for species identification of up to 89.2% (Woo *et al.*, 2003). Differentiation at the species, subspecies or strain level may not always be achieved and therefore alternative genes may be needed (Sontakke *et al.*, 2009).

The upsurge in the number of recognized taxa is accredited to the simplicity of performing 16S rDNA gene sequencing as opposed to the more demanding analysis of DNA-DNA hybridization studies (Neefs *et al.*, 1991; Olsen and Woese, 1993; Janda and Abbott, 2002; Janda and Abbott, 2007). With the identification of genera and species some difficulties may arise and these include the recognition of new taxa, limited sequences deposited in nucleotide databases, species sharing similar and/or identical 16S rDNA sequences or nomenclature problems arising from multiple genomovars (DNA groups) allocated to single species or complexes (Janda and Abbott, 2007). Sequencing of the 16S rDNA gene is extremely useful for classification purposes; however it has limited phylogenetic capability at

the species level and poor discriminatory power for some genera where 16S rDNA analysis is inadequate, DNA relatedness studies are required to provide absolute resolution to taxonomic issues (Mignard and Flandrois, 2006; Woo *et al.*, 2008). Bacterial identification based upon 16S rDNA sequences depends on substantial interspecies differences and minor intra-species differences. Therefore, this technique would be insufficient for differentiating bacterial species which share almost the same 16S rDNA sequence (Clayton *et al.*, 1995; Woo *et al.*, 2008).

The accurate interpretation of sequence data depends upon the length and quality of sequences, the programs used for analysis and species assignment based on similarity percentages. A minimum of 500-525 bp (which includes the variable 5' region) are required for the identification of some groups of bacteria, while the ideal length is between 1 300 to 1 500 bp and all of the same length (Drancourt *et al.*, 2000; Christensen *et al.*, 2005; Janda and Abbott, 2007; Woo *et al.*, 2008). However, phylogenetic dendograms generated from either the 1 500 bp 16S rRNA gene sequence or from the partial 500 bp 16S rRNA gene sequence were compared and revealed highly comparable phylogenies (Clarridge, 2004).

The criteria for species identification require a minimum of 99% sequence similarity and an ideal value of 99.5%. The sequence match the type/reference strain of a species that has undergone DNA-DNA hybridization studies (Janda and Abbott, 2007). Similarity scores rely on the lengths of the sequences and on the number of gaps introduced in the query sequence in order to enhance the score (Drancourt *et al.*, 2000). In some cases 16S rDNA sequence similarity (even at very high levels) does not imply identity or precision in microbial identifications. This may be due to some problems which may arise as a result of bacterial nomenclature and taxonomy (16S rDNA cannot distinguish between recently diverged species) (Tang *et al.*, 1998; Petti, 2007).

Interpretation of sequencing data is severely affected and compromised if sequence quality is low, even if the sequence possesses 1% (15 positions) undetermined positions. Therefore culture purity is of utmost importance to limit or eliminate the formation of chimeric molecules (Wang and Wang, 1997; Wang *et al.*, 1999; Drancourt *et al.*, 2000; Janda and Abbott, 2002; Clarridge, 2004). It has been suggested to introduce cut-off similarity levels ranging from 97% for the genus level to 99% for the species level with the use of an ungapped program (Drancourt *et al.*, 2000; Janda and Abbott, 2002; Stackebrandt *et al.*, 2002; Clarridge, 2004). However, these cut-off points are dependent upon genes, fragments and genera (as different bacterial species are likely to evolve at different rates). Furthermore, similarity percentages are also software-dependent and are altered both by the software parameters

and by the length of the compared sequences (Clarridge, 2004). Through using these criteria, it is difficult to determine the species identity with sequences which have very similar distances to the closest and second closest matches, particularly those within 0.5–1% (Woo *et al.*, 2008). Species assignment may be difficult therefore determining relatedness is simplified by alignment and constructing a phylogenetic tree (Clarridge, 2004).

The 16S rRNA gene sequencing as an instrument for microbial identification is reliant upon two essentials, the deposition of complete unambiguous nucleotide sequences into databases and assigning the exact taxon/species/gene to each sequence (Kolbert and Persing, 1999; Janda and Abbott, 2007). Not only do 16S rDNA analyses aid in identification, but also contribute to the improved understanding of the epidemiology and pathogenic role of bacteria and spatial variation of bacterial species (Drancourt *et al.*, 2000; Mignard and Flandrois, 2006; Tringe and Hugenholtz, 2008).

Organisms may be genotypically and/or ecologically diverse whilst having identical 16S rDNA gene sequences (Adhikari *et al.*, 2012), alternatively, a single monophyletic species may even possess multiple intragenomic copies which can differ in sequence (ribotypes) (Clayton *et al.*, 1995). Even clusters of sequences with 99% sequence identity may reveal extensive ribotype microdiversity. These may potentially bring about important ecological differentiation (Acinas *et al.*, 2004).

Other shortcomings of using this gene (16S rRNA gene) for identification and phylogeny include: (i) it spans a very small portion compared to the entirety of genome size, (ii) it lacks informative characters and the differentiation of closely related strains of bacteria, (iii) the resolution of evolutionary trees may be complicated due to its slow rate of evolution (Rogall *et al.*, 1990; Bennasar *et al.*, 1996) (iv) the existence of insertions and deletions (indels) within this gene may present problems for sequence alignments and (v) selection on secondary structures may cause sequence convergence and saturation, thereby misrepresenting the actual relationships among organisms (Hillis and Dixon, 1991; Dixon and Hillis, 1993). Lastly, (vi) the degree to which rDNA sequences are resistant to horizontal gene transfer along with whether any single gene can accurately represent taxonomic phylogeny and the highly variable evolution rates both among and within genes are questionable (Santos and Ochman, 2004).

The 23S rDNA gene offers the same advantages as the 16S rRNA gene (namely, universal distribution, conserved function and variable regions). In contrast to 16S rDNA sequences, 23S rDNA sequences are longer, may contain unique insertions and/or deletions and are

therefore better equipped for phylogenetic resolution (discriminating even between closely related species/subspecies). This is due to higher sequence variation (Ludwig and Schleifer, 1994; Ludwig *et al.*, 1995; Kolbert and Persing, 1999; Hunt *et al.*, 2006). Phylogenies derived from both 16S rDNA and 23S rDNA sequences are in accordance and a polyphasic approach in which both are used could be more informative than individual genes (Arahal *et al.*, 2002; Bavykin *et al.*, 2004; Nochi *et al.*, 2009).

Genes are suitable for identification and phylogenetic analyses, (i) if they are dispersed throughout the genome, (ii) present in a single copy, (iii) not prone to recombination or horizontal gene transfer and (iv) if they are scattered amongst bacterial taxa in order to predict overall genome relations with accuracy (Yamamoto and Harayama, 1996; Yamamoto *et al.*, 2000; Stackebrandt *et al.*, 2002; Zeigler, 2003). However, only a very small portion of genes evade recombination or horizontal gene transfer. As gene function correlates with genetic recombination, genes with a critical function such as DNA replication, transcription, translation and information processing, constitute large multiplex systems, interact with other proteins and are therefore less prone to horizontal gene transfer (Rivera *et al.*, 1998; Jain *et al.*, 1999). However, operational genes, for instance those implicated in metabolic processes, are more likely to be subjected to genetic recombination (Rivera *et al.*, 1998; Jain *et al.*, 2003).

1.10.3 Protein encoding genes – *GyraseB*

Protein encoding genes may be successfully applied in the classification of the ecological/geographical diversity of bacteria even more effectively than DNA-DNA hybridization (Vandamme *et al.*, 1996). Protein encoding genes are the only genes able to adequately change rapidly over time (Palys *et al.*, 1997; Nei and Kumar, 2000).

The *gyraseB* gene is about $\pm$ 860 kb long (Young *et al.*, 2008), is a single copy gene present in all bacteria which encodes the ATPase domain of DNA gyrase, an enzyme essential for DNA replication (Huang, 1996). Specifically, DNA gyrase, is the enzyme responsible for introducing negative supercoils into bacterial chromosomes and which also plays a crucial role in chromosomal replication (Watt and Hichson, 1994; Champoux, 2001).

The use of additional or alternative markers, such as protein encoding genes (in this case *gyrB* genes), are strongly suggested as these are able to detect phylogenetic relationships without bias and a loss in phylogenetic resolution due to the presence of intragenomic heterogeneity (and the lack of sequence heterogeneity, as they are single copy genes)

which would otherwise go undetected by using the 16S rRNA gene (Case *et al.*, 2007; Mondal *et al.*, 2013). Comparative analysis of rRNA and *gyrB* sequences demonstrates excellent correlation for the grouping of bacterial species or species groups. The single-copy *gyrB* gene evolve faster, are relatively conserved but display greater nucleotide variations than the rRNA genes and thus provides higher resolution even at pathovar level (Bavykin *et al.*, 2004; Yin *et al.*, 2008; Nochi *et al.*, 2009; Parkinson *et al.*, 2009). Phylogeny of *Xanthomonas* derived from the *gyraseB* gene confirms a monophyletic group derived from a common ancestor, which is in accordance with data obtained from 16S rRNA genes and 16S–23S intergenic sequences. Sequence alignments revealed the absence of any insertions or deletions, which is consistent with a single origin for the genus (Parkinson *et al.*, 2007).

For the phylogenetic analysis of protein encoding genes both the amino acid and nucleotide sequences may be used. Alignment of proteins allow for the resolution of relationships at higher taxonomic levels (domain or phylum) when one or more codon positions are saturated, whilst the alignment of nucleotide sequences allow for the differentiation between extremely closely related organisms (species, subspecies level or lower) (Thompson *et al.*, 2005; Case *et al.*, 2007).

Protein encoding genes possess additional advantages, (i) it's universally present in all prokaryotes, (ii) the ability to design primers and probes of differing specificities due to the presence of variable and conservative regions, (iii) relatively resistant towards horizontal gene transfer as it's a housekeeping gene and (iv) it's a large fragment containing plentiful phylogenetic information (even after removal of regions that are difficult to align) (Yamamoto and Harayama, 1996; Case *et al.*, 2007).

1.10.4 Genomic profiling - the repetitive extragenic palindromic PCR technique

Genomic fingerprinting of microbes are a series of techniques used to illustrate similarities, or variations whatever the case may be, between different microbial species and/or strains. It is useful in (i) identification of bacterial strains, (ii) analysing taxonomic/genetic diversity of bacterial groups and for (iii) the determination of the phylogenetic structure (classification) of bacterial populations. Other uses include the determination of endemism, epidemiology and biogeography (Louws *et al.*, 1994; Versalovic *et al.*, 1994; Rademaker *et al.*, 2000). Rep-PCR based genomic profiling has the ability to group and distinguish between intrapathovar

diversity within *Xanthomonas* species. These can be grouped as follow, (i) pathovars with identical strains and/or pathovars with strains which almost share identical profiles, (ii) pathovar strains with unique profiles, but sharing multiple bands or (iii) pathovar strains may be divided into groups where none share any common banding patterns (Louws *et al.*, 1994).

Three types of repetitive sequences are recognized namely the 35-40 bp repetitive extragenic palindromic (REP) sequences, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequences and the 154 bp A1R-based repetitive extragenic palindromic (BOX) elements (Stern *et al.*, 1984; Sharples and Lloyd, 1990; Lupski and Weinstock, 1992; Versalovic *et al.*, 1994).

The primers are designed to bind to the consensus sequence at one end of a rep-sequence. Primers then begin to consecutively synthesize a new strand from one rep sequence to another rep sequence; any further elongation is however blocked by the bound primers. All newly synthesized strands remain as separate entities (selective amplification). Through agarose gel electrophoresis the amplified PCR products may be analysed – by assessing whether certain bands (DNA fragments) are present while others are absent. Thus, profile analysis is based on the number and position of bands and the overall banding pattern (Versalovic *et al.*, 1991).

This rep-PCR genomic fingerprinting technique is extremely reliable, reproducible, rapid and highly discriminatory (Louws *et al.*, 1994; Versalovic *et al.*, 1994). Rep-PCR fingerprinting exploits DNA primers that are complementary to naturally occurring, highly conserved, repetitive DNA sequences which are present in numerous copies in the genomes of most Gram-negative and several Gram-positive bacteria (Lupski and Weinstock, 1992). In the genus *Xanthomonas*, rep-PCR has revealed both inter and intrapathovar diversity, and have been used extensively to distinguish between species and subspecies (Louws *et al.*, 1996; Vauterin *et al.*, 1995; Valverde *et al.*, 2007; Zamani *et al.*, 2011).

1.11 Research Rationale

As there is a paucity of information regarding the pathogen's distribution range and whether or not different strains of the pathogen (intrapathovar variation) are responsible for increased maize infections, the following objectives were set forth:

(I) to determine the distribution range of the pathogen through seasonal surveys throughout the maize producing regions, (II) to identify and rapidly detect the causal organism, (III) to assess the ability of ribosomal genes and *gyraseB* to differentiate amongst the *Xanthomonas* BLSD isolates, (IV) to determine possible variation (intrapathovar diversity) within the isolate population and (VI) to determine whether or not these molecular methods are capable of differentiating between *Xanthomonas* BLSD and the closely related *X. axonopodis* pv. *vasculorum*, which is also capable of infecting maize.

Chapter 2 - Materials and Methods

2.1 Sample site and collection

Maize leaves displaying characteristic longitudinal streak symptoms were collected primarily from the warm, dry maize production regions of South Africa, as these climatic conditions favour disease development and incidence (Dyer, 1949; Coutinho and Wallis, 1991). Isolates (47) were collected from the interior parts of South Africa, mainly the North West, Free State, Northern Cape and Gauteng provinces (figure 4) (JAWF, 1999; DAFF, 2012a). Almost all of the isolates were collected from non-irrigated maize fields, except for those obtained from the Northern Cape as well as the southern parts of the Free State. Sample site coordinates were determined using a GPS (Garmin nuvi CAN 310, USA).

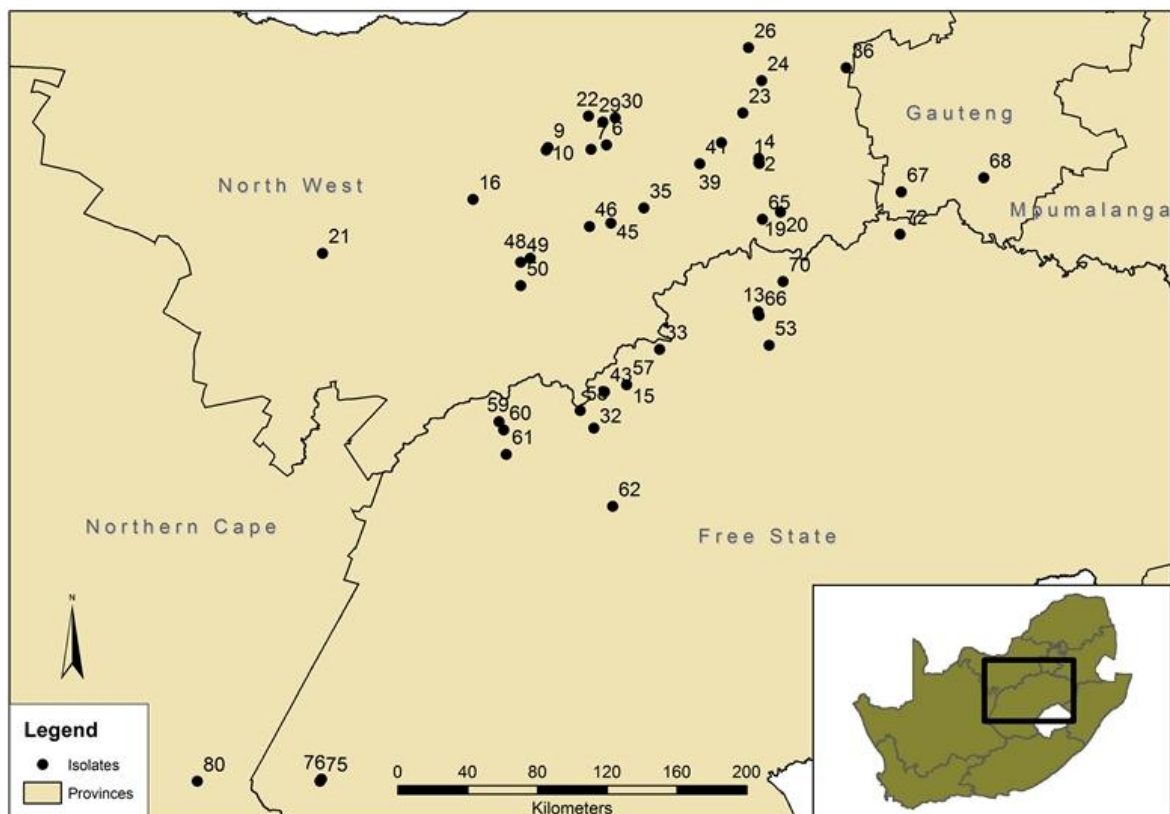


Figure 4 - Mapping the geographical origin of the 47 selected isolates of *Xanthomonas* BLSD.

2.2 Bacterial isolation, purification and pathogenicity testing

Before any molecular analyses could commence the causal agent had to be isolated. Two methods were used for bacterial isolation.

Firstly, diseased tissue was aseptically dissected into smaller pieces followed by surface sterilization - to eliminate all epiphytic bacteria. Sterilization consisted of inserting the pieces of each geographically distinct leaf sample in separate McCartney bottles. Firstly, 5 min in a sodium hypochlorite solution, followed by 3 min in absolute ethanol and a final rinse step of 3 min in sterilized water. The pieces were then plated on glucose yeast calcium carbonate agar (GYCA) (components from Biolab, South Africa) (Schaad *et al.*, 2001).

The previous technique failed to isolate the causal organism from most of the leaf samples, therefore another technique was used, namely the serial dilution method which included the following processes. Firstly, only bacterial leaf streak symptoms were excised and sterilised for 1 min in a sodium hypochlorite solution. A 1 min wash in absolute ethanol followed with a final rinse step of 1 min in sterilised water. The diseased tissue was then ground and crushed using a sterilised mortar and pestle, within 10 ml sterilised water. Dilution series of up to 10^{-3} were prepared and plated on GYC agar.

For both techniques growth occurred after an incubation period of 72 hours at 28°C. Only fluorescent to brightly yellow coloured, convex, mucoid colonies were selected and purified. Due to the first technique's inability to isolate all of the targeted bacteria a total of 47 isolates were selected for further analyses.

Koch's postulate was performed with these purified isolates to determine pathogenicity (Nienaber, 2014). Two weeks after artificial inoculation characteristic leaf streak symptoms developed. The pathogen was again detected and identified and as such the pathogenicity of *Xanthomonas* BLSD was confirmed. Other bacteria which were also isolated from maize leaf samples, namely *Pantoea ananatis* and *Pseudomonas spp.* were selected as controls and did not produce any symptoms on maize leaves. These were therefore considered to be endophytic.

Xanthomonas axonopodis pv. *vasculorum* (ATCC 35938) (extracted from sugarcane) was selected as reference, as no *Xanthomonas* BLSD strains are currently available from any bacterial culture bank. Additionally, it is also capable of infecting maize (Lewis *et al.*, 2010; Adriko *et al.*, 2012). Within this study it was noted that the reference strain *X. axonopodis* pv. *vasculorum* ATCC 35938 also produced minor symptoms on maize leaves when compared

to those produced by *Xanthomonas* BLSD isolates. The overlap in host range prompt the selection of *X. axonopodis* pv. *vasculorum* strain ATCC 35938 as the ingroup taxon to be compared with the isolates of *Xanthomonas* BLSD in order to note possible variation between these two pathovars. *Pantoea stewartii* subsp. *stewartii* (DSM 30176) was selected as outgroup as it's also from the same family, *Enterobacteriaceae*, but from a different genus (*Pantoea*) producing similar disease symptoms on maize leaves (Roper, 2011).

2.3 Genomic DNA isolation

DNA was extracted directly from pure bacterial colonies by means of a genomic DNA extraction kit (NucleoSpin®Tissue) (Macherey-Nagel, Germany). The quality and quantity of the isolated DNA was determined by the NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, USA).

2.4 DNA amplification

PCR-based molecular analyses followed DNA extractions. For initial bacterial identification and detection of the genus *Xanthomonas* a specific marker, *XgumD*, was assessed. Ribosomal genes (16S rRNA; 23S rRNA), along with the *gyraseB* gene were used to assess population structure of the isolates and for phylogenetic analyses. PCR of repetitive elements were used to identify possible variation within the genomic composition of the 47 isolates and to discern them from *Xanthomonas axonopodis* pv. *vasculorum* and *Pantoea stewartii* subsp. *stewartii*. The reagents as well as their quantities needed for these analyses are summarised within table 1.

Table 1 - The different types of PCR and their constituents for each of the samples.

Reagents	<i>XgumD</i> ;16S rRNA; 23S rRNA; <i>gyraseB</i>	BOX
Ultra-pure/PCR grade water (Thermo Scientific, USA)	10 µl	9.375 µl
Additional <i>Taq</i> polymerase (5 U/µl) (Fermentas Life Science, USA)	-	0.375 µl
MgCl ₂ (25 mM) (Fermentas Life Science, USA)	-	0.5 µl
BSA - Bovine serum albumin (0.5 mg/ml) (Fermentas Life Science, USA)	-	1 µl
Primers (Forward; Reverse) (10 µM) (LTC Tech, South Africa)	0.5 µl	0.25 µl
Double strength PCR Master Mix (consists of 0.05 U/µl <i>Taq</i> polymerase, 0.4 mM dNTPs, 4 mM MgCl ₂) (Fermentas Life Science, USA)	12.5 µl	12.5 µl
DNA sample (25 ng/µl)	2 µl	1 µl
Total Volume (µl)	25 µl	25 µl

The following primers and protocols were used for each of the various PCR reactions: A C1000 Thermal Cycler (Bio-Rad, UK) was used for PCR.

2.4.1 *XgumD* PCR

Primers: *XgumD*-F - 5'GGCCGCGAGTTCTACATGTTCAA'3 and *XgumD*-R - 5'CACGATGATGCGGATATCCAGCCACAA'3. Cycling conditions: 3 min at 95°C; followed by 40 cycles, each consisting of 30 sec at 95°C, 15 sec at 65°C, and 15 sec at 72°C; and a final 3 min extension at 72°C (Mbega *et al.*, 2011).

2.4.2 16S rDNA PCR

Primers: 27F - 5'AGAGTTTGATCMTGGCTCAG'3 and 1492R - 5'TACGGYTACCTTGTTACGACTT'3. Cycling conditions: initial denaturation of 95°C for 5 min; 95°C for 30 sec, 52°C for 30 sec, 72°C for 1 min (repeat these 3 steps 30 times) and 72°C for 5 min (derived from Hauben *et al.*, 1997; Lane, 1991).

2.4.3 23S rDNA PCR

Primers: 129F - 5'CYGAATGGGGVAACC'3 and 2441R - 5'ACCGCCCCAGTHAAACT'3. Cycling conditions: 3 min at 94°C; followed by 30 cycles, each consisting of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C; and a final 5 min extension at 72°C (Hunt *et al.*, 2006).

2.4.4 GyraseB PCR

Primers: *XgyrB*1F - 5'ACGAGTACAACCCGGACAA'3 and *XgyrB*1R - 5'CCCATCARGGTGCTGAAGAT'3. Cycling conditions: denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 10 min (Young *et al.*, 2008).

2.4.5 BOX PCR

Primer: BOX A1R -5'CTACGGCAAGGCGACGCTGACG'3. Cycling conditions: 95°C for 5 min, followed by 35 cycles of, 1 min at 94°C, 1 min at 50°C, 8 min at 65°C and a final extension step of 8 min at 65°C (Louws *et al.*, 1994; Versalovic *et al.*, 1994; Rademaker *et al.*, 2000).

2.5 Agarose gel electrophoreses of PCR products

The visualization of the PCR products was carried out using agarose gel electrophoresis; A PowerPacHC system was used (BIO-RAD, UK). Gene fragment sizes were determined through comparison to a loaded 1 Kb molecular weight marker (O'GeneRuler) (Fermentas Life Science, USA). PCR product (10 µl) was mixed with 3 µl 6X Orange loading dye (Fermentas Life Science, USA) containing 3 µl GelRed 10000x (Biotium, USA). A 1.5 % (w/v) agarose (Lonza, USA) gel submerged in 1 x TAE buffer (40 mM Tris-HCl, 1 mM EDTA [pH 8], 20 mM Acetic Acid) was used for all the various electrophoresis analyses. Electrophoresis was carried out at 80 V for 1 hour for the 16S rRNA, 23S rRNA and *gyraseB* gene products and at 60 V for 2 hours for the *XgumD* and BOX PCR products. After electrophoresis fragment size and banding patterns were examined visually by means of the ChemiDoc MP Imaging System (BIO-RAD, USA) and Image Lab 4.0.1 2011 software (BIO-

RAD, USA). This was done to establish whether or not fragments were of the correct size and banding patterns were used to identify possible variance amongst the 47 isolates of *Xanthomonas* BLSD.

2.6 Clean-up of PCR products and sequencing

PCR products were cleaned using a NucleoSpin® Gel and PCR Cleanup Kit (Macherey-Nagel, Germany). Manufacturer's instructions were followed for the cleanup, with a few alterations. Two sequential wash steps were performed using the provided wash buffer (NT3), in order to minimize chaotropic salt concentrations. Residual ethanol was totally removed by centrifuging the columns for an additional 4 min and then incubating them for 5 min at 70°C in a dry heat bath. A high DNA yield was obtained by multiple elution steps, by heating to 70°C and incubating for 5 min. Cleanup efficiency was assessed by NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, USA).

Sequencing of PCR products was performed by Inqaba Biotech, South Africa, with the use of an ABI 3130 Genetic Analyser (Applied Biosystems, USA). A sequencing reaction setup consisted of a BigDye Terminator v3.1 Cycle Sequencing Reaction Mix (Applied Biosystems, USA), BigDye Terminator v1.1, v3.1 5X Sequencing buffer (Applied Biosystems, USA), appropriate primer and concentration (only reverse sequencing was performed), isolate DNA and nuclease free water (Thermo Scientific, USA). Cycling conditions of a sequencing reaction consisted of an initial denaturation of 96°C for 1 min; followed by 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min (repeat these 3 steps 25 times). Following the initial sequencing reaction was a second cleanup step with a ZR DNA Sequencing Cleanup Kit (Zymo Research, USA) and was conducted according to the manufacturer's instructions.

2.7 Statistical analysis

Chromatograms were viewed and edited using Geospiza FinchTV (version 1.4) and BioEdit Sequence Alignment Editor Software (version 7.1.7) (Hall, 1999).

Sequence identities were obtained by comparisons to those in the BLASTN database (<http://www.ncbi.nlm.nih.gov/BLAST>). Ribosomal RNA and *gyraseB* sequences were aligned, respectively, via ClustalW (Thompson *et al.*, 1994 Thompson *et al.*, 2002; Larkin *et al.*, 2007). The software MEGA 5 (Kumar *et al.*, 2004 Tamura *et al.*, 2007) was used to construct phylogenies with the neighbour-joining statistical method (Saitou and Nei, 1987).

The reliability of these dendrograms was assessed by bootstrap analysis (1000 replications) (Hillis and Bull, 1993).

For BOX generated profiles the presence or absence of each band was converted into binary data, scored as 1 for presence, and 0 for absence via TotalLab Phoretix 1D Pro software (version 11.7), the trial version (Nonlinear Dynamics, USA). Genetic relationships amongst isolates were determined by Jaccard's similarity coefficients followed by UPGMA (Unweighted Pair Group Method using Arithmetic averages) clustering analysis (Michener and Sokal, 1957; Van Dongen and Winnepenninckx, 1996; Gronau and Moran, 2007).

Chapter 3 - Results and Discussion

3.1 Distribution and severity of *Xanthomonas* BLSD

Species from the genus *Xanthomonas* rank amongst the top ten phyto-bacterial pathogens which affect a wide range of agriculturally important crops (Leyns *et al.*, 1984; Hayward, 1993; Vauterin *et al.*, 1995; Mansfield *et al.*, 2012). The importance of *Xanthomonas* BLSD has increased in the last few years due to its continuous incidence and severity on maize crops. This disease needs to be adequately researched and understood as further spread and increased severity may be detrimental towards maize production in South Africa. Previously a commercial maize variety was withdrawn due to its susceptibility to bacterial leaf streak (Coutinho and Wallis, 1991). Necrosis of photosynthetic tissue may be as high as 40% (Nowell, unpublished cited by Qhobela *et al.*, 1990). Necrosis results in a decline of photosynthate production, translocation and accumulation and ultimately a decreased yield (Kocal *et al.*, 2008; Bilgin *et al.*, 2010; Garavaglia *et al.*, 2010).

The incidence and severity of the disease may vary from field to field. The onset of this disease as with many others depends on the susceptibility of the maize hybrid, variety or cultivar and the age of the plant, the presence of a virulent race/strain of the pathogen and favourable environmental and climatic conditions (Browning *et al.*, 1977; Schaad and Alvarez, 1993; Stuckey *et al.*, 1993; Franken, 1992, cited by Bila *et al.*, 2013). Symptoms varied from a few streaks, to entire leaves being covered in longitudinal streaks and even in most severe cases the leaves were hanging in tatters with wilting occurring. The localities of the farms where diseased plants were collected are provided in figure 5. Locality of the isolate numbers provided in figure 4. All the plants that were collected had the typical symptoms as indicated in figure 2. The isolates produced yellow colonies on the selective GYC agar (Section 2.2)

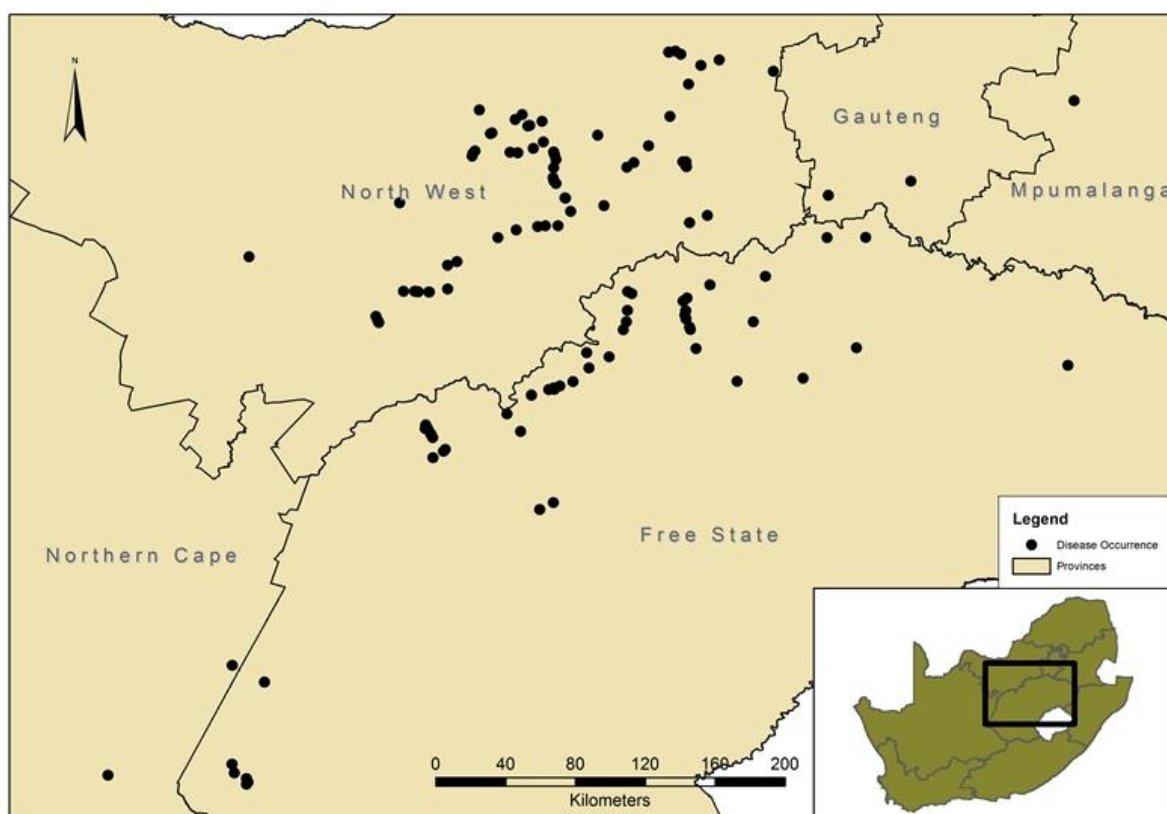


Figure 5 - Mapping the distribution range of *Xanthomonas* BLSD incidence within the maize production regions of South Africa.

The incidence and severity of the disease is mainly reported from the dry, non-irrigated lands in the North West and Free State provinces (Figure 5). These provinces contribute 60% towards South Africa's maize production annually. A few isolated cases were also reported from the Gauteng (contributing 5% to SA's maize yield) and Mpumalanga (contributing 21% to SA's total maize yield) provinces as well as from irrigated fields in the Northern Cape (contributing 5% towards SA's maize yield) (Du Toit, 1997; JAWF, 1999; DAFF, 2012a). Altogether these regions contribute over 90% towards South Africa's maize production. In a previous study, maize leaves displaying similar symptoms were even obtained from as far as the KwaZulu Natal province (Coutinho and Wallis, 1991). The incidence of the disease is thus wide spread.

All of these maize production regions in South Africa form part of the summer rainfall area (Schulze, 1997). The western regions experience dry, desert like circumstances, while parts of the eastern areas are subjected to humid, subtropical conditions (ARC-GCI, 2008; Benhin, 2008). The western areas receive an average annual precipitation of up to 600 mm, whilst the eastern regions receive between 600 mm and 1400 mm. Therefore, during the summer months the rainfall increases in a west to east direction (Preston-Whyte and Tyson, 1993;

Schulze, 1997; Du Toit *et al.*, 2002; Durand, 2005 cited by Benhin, 2006; Deichmann and Eklundh, 1991 cited by Benhin, 2006).

During the summer months (December to March) the average daily maximum temperatures for the west ranges from 28°C to 30°C, whilst those of the east ranges from 26°C to 30°C; both share an average daily minimum temperature of between 12°C and 16°C for the summer months (Schulze, 1997). The growth and dissemination of this pathogen may be explained by the climatic (in particular temperature) conditions and patterns of these areas as well as the physiology of its host. Precipitation aid infection and dissemination (Ryan *et al.*, 2011), whilst temperatures ranging from 28°C to 37°C favours disease development (Nowell, unpublished, cited by Qhobela *et al.*, 1990; Coutinho and Wallis, 1991). These climatic conditions are also required for the optimal growth and development of its host, maize (Du Toit, 1997; Du Toit *et al.*, 1999).

Xanthomonas BLSD may survive in post-harvest crop residues for several months due to the encapsulation by the exopolysaccharide xanthan. Xanthan acts as a physical barrier protecting the bacterium from the elements such as freezing, desiccation and bacteriostatic substances (Jackson, 2009). All the BLSD isolates in the present study produced xanthan and were potentially also protected in a similar manner. The pathogen remains dormant during dry periods and recommences with active development when conditions become favourable again. Primary infections typically occur during seedling stages, whilst secondary infections occur on younger leaves during subsequent growing periods. This pathogen may be disseminated from field to field by rain splash, overhead irrigation, the formation of aerosols and by direct contact between plants. It is still not known whether or not it is seedborne. However, many *Xanthomonas* species/pathovars are indeed seedborne (Schaad *et al.*, 1980; Weller and Saettler, 1980; Bhat *et al.*, 2010; Ryan *et al.*, 2011). If it is established that this pathogen is indeed seedborne it might contribute to broadening its distribution range especially when phytosanitary protocols are not adhered to. The current distribution range of the pathogen may be attributed to the accidental spread of infected plant material through anthropogenic activity, windblown infected debris and possible insect transmission.

As climate change is inevitable and inexorable, higher temperatures along with decreased precipitation may result in the possible shift of the current maize production belt (Perks and Schulze, 2000) and therefore also the distribution ranges of the pathogen. It is anticipated that elevated CO₂ levels may increase the incidence and severity of some diseases, particularly those inducing necrosis; (Chakraborty *et al.*, 2000; Erb *et al.*, 2008; Eastburn *et*

al., 2011). *Xanthomonas* spp. are such pathogens. Climate change may also alter host physiology (in order to alter the microclimate in such a way that it favours pathogen development and colonization), resistance and pathogen development stages (disease infection cycles) and rates. Higher temperatures may accelerate the collapse of plant disease resistance via higher disease pressure and/or altered resistance gene efficiency in several host-pathogen systems. Although phytopathogens rely on leaf wetness for infection, an increase in temperature will more than compensate for the decline in precipitation. The reason for this being that infections would initiate much earlier in the growing season, thereby providing more time for epidemics to develop (Garrett *et al.*, 2006; Webb *et al.*, 2010; Newton *et al.*, 2011). Thus, climate change may affect the distribution and severity of bacterial leaf streak disease.

In general higher temperatures decrease generation time resulting in a higher number of generations per season. Generation time regulates the severity of plant diseases in two ways, (i) accelerating and increasing inoculum levels and/or (ii) affecting the rate and frequency of pathogen evolution (gene flow) and a pathogen's ability to adapt to the environment (Legrève and Duveiller, 2010). Diversity within pathogen populations (due to exchange and flow of genes) leads to variation in host resistance, pathogen virulence and interactions (pathogen complexes). These variations may result in the instigation of new diseases or epidemics and the introduction of pathogens into new environmental niches; relying on the distribution of populations and environmental conditions which are influenced by climate change (Legrève and Duveiller, 2010).

Higher average daily temperatures along with extended periods of warm weather may have a rapid and prolific effect on the short life cycles of insects, their mobility and high reproductive potential (Ladányi and Horváth, 2010). This scenario may affect the severity of phytopathogen epidemics as many plant disease are transmitted by sucking insects. *Xanthomonas* BLSD may also be insect transmitted as is the case with other *Xanthomonas* spp. (Kaiser and Vakili, 1978). Additionally, the efficiency of control strategies is expected to be affected by climate change (Ladányi and Horváth, 2010; Juroszek and Von Tiedemann, 2011).

3.2 DNA quality and quantity

DNA quality was extremely high ranging from the desired nucleic acid absorption spectra of 1.8 to 2.1. DNA quantity varied from sample to sample with the lowest being 29 ng/μl and the highest being 375 ng/μl. Before any PCR analyses could commence the DNA concentration of all the samples had to be standardised to 25 ng/μl.

3.3 Isolated bacteria

Prior to molecular analyses the causal microorganism had to be isolated and purified. Initially a direct plating of diseased leaf tissue on selective agar was used as an approach to isolate the bacterium. A number of bacterial species were isolated (Table 2) and persistently dominated and overgrew the slow growing pathogen (*Xanthomonas* BLSD). Complications with phytobacterial enumeration often arise when trying to isolate pathogens in pure culture, even if semi-selective media are used, due to the presence of these non-targeted species (Narayanasamy, 2011). None of these bacterial species produced any symptoms when subjected to pathogenicity tests to complete Koch's postulates. These bacterial species were therefore considered to be endophytic – microorganisms detected in surface sterilised plant tissue (Hallmann *et al.*, 1997) and are non-pathogenic avirulent species (Johnston-Monje and Raizada, 2011). The subsequent technique (the plate dilution method) yielded almost all of the *Xanthomonas* BLSD isolates which were selected on the basis of colony morphology (convex, yellow mucoid colonies) from heterogeneous bacterial plates.

Table 2 - Endophytic bacterial genera isolated from maize leaves and identified via 16S rRNA gene sequencing.

Isolate	Sequence length (bases)	Query Coverage	Maximum Identity (%)	E Score	Identified Species
E1	635	100	100	0.0	<i>Pantoea agglomerans</i>
E2	640	100	99	0.0	<i>P. ananatis</i>
E3	615	100	100	0.0	<i>P. vagans</i>
E4	625	100	100	0.0	<i>P. stewartii ssp. indologenes</i>
E5	650	100	99	0.0	<i>Enterobacter cloaceae</i>
E6	630	100	99	0.0	<i>E. cowanii</i>
E7	615	100	100	0.0	<i>Pseudomonas putida</i>
E8	615	100	100	0.0	<i>P. poae</i>
E9	635	100	100	0.0	<i>Microbacterium testaceum</i>
E10	625	100	99	0.0	<i>M. paraoxdans</i>
E11	630	100	99	0.0	<i>Curtobacterium flaccumfaciens</i>
E12	620	100	99	0.0	<i>Stenotrophomonas maltophilia</i>
E13	625	100	99	0.0	<i>Sphingomonas yubuuchiae</i>
E14	625	100	99	0.0	<i>Paenibacillus hunanensis</i>
E15	630	100	99	0.0	<i>P. xylanexedens</i>

These species were arranged from being the most abundant (at the top) to being the scarcest.

These bacterial endophytes either live commensally within the host and/or perform beneficial functions for the host (mutualistic symbiosis) (Hallmann *et al.*, 1997; Johnston-Monje and Raizada, 2011). The presence of these endophytes within domesticated maize is attributed to the fact that they originated within their wild teosinte ancestors (Johnston-Monje and Raizada, 2011). These endophytes are frequently detected within the phyllosphere of members from the *Poaceae* family (Schroth *et al.*, 1992; Behrendt, 2003) and particularly within maize (Fisher *et al.*, 1992; Hinton and Bacon, 1995; Palus *et al.*, 1996; Rai *et al.*, 2007; Rijavec *et al.*, 2007; Roesch *et al.*, 2008).

A set of environmental and genetic factors are presumed to dictate and enable a specific bacterium to become endophytic (Reinhold-Hurek and Hurek, 1998). In general plant microbes may be endophytic and non phytopathogenic, whilst others are opportunistic plant pathogens (Torres *et al.*, 2008; Krawczyk *et al.*, 2010). However, some endophytes may even become pathogenic, for example when the plant is stressed the plant/endophyte relationship balance shifts or if virulent strains of the species are present (Tadych and White, 2009). In some cases *P. ananatis* is associated with leaf spot disease (Paccola-Meirelles *et al.*, 2001; Goszczynska *et al.*, 2007; Pérez-y-Terrón *et al.*, 2009; Krawczyk *et al.*, 2010), whilst *P. agglomerans* causes leaf blight and vascular wilt of maize (Morales-Valenzuela *et al.*, 2007).

Enterobacter cloacae is the causal agent of bacterial stalk rot of maize (Hoffman *et al.*, 2005). In this study the latter two were only endophytic.

Endophytes may be seedborne, vector transmitted, whilst others have mechanisms to colonise plants (Rosenblueth and Martínez-Romero, 2006; Hardoim *et al.*, 2008) and may enter through cracks in the roots and/or through leaf stomata (McCully, 2001). Endophytic bacterial populations are conditioned by biotic and abiotic factors and are protected from external factors as they live within their host (Seghers *et al.*, 2004). Once inside a plant the endophytic bacteria may spread from the point of entry throughout the entire plant (Rosenblueth and Martinez-Romero, 2006). Endophytic bacteria primarily colonise intercellular spaces of plant tissue, but they may also live intracellular and even within the vascular system (Tadych and White, 2009).

Species composition of endophyte assemblies and infection incidences differ according to (i) host species, (ii) growth phase of the host, (iii) tissue type, (iv) age of tissues, (v) associated vegetation (host habitat), (vi) environmental conditions and (vii) site characteristics. Typically, only one to a few species dominates the endophyte community (in the case of this study; *Pantoea*, *Enterobacter* and *Pseudomonas* species), whilst the large majority of species are rare (examples from this study, as seen in table 2; *Curtobacterium*, *Stenotrophomonas*, *Sphingomonas* and *Paenibacillus* species (Johnston-Monje and Raizada, 2011). The distribution of the rare and incidental species are influenced more by site than by host and the number of these species isolated is relative to the intensity and time of sampling (Sturz *et al.*, 2000; Sturz and Nowak, 2000; Lodewyckx *et al.*, 2002; Tadych and White, 2009).

Numerous endophytic bacterial groups are conserved among the genus *Zea*. These are referred to as the core micro biota of an organism which is associated with healthy host functioning (Delmotte *et al.*, 2009). The possible function of these endophytes includes the following, (i) stimulating plant and root growth through auxin production, (ii) nitrogen fixation and assimilation, (iii) phosphate solubilisation, (iv) iron absorption and chelation, (v) secretion of RNase, pectinase and/or cellulase, (vi) suppressing pathogens (antibiotic production, competition, parasitism), (vii) catabolising the precursor of ethylene (deaminase activity), (viii) acetoin/butanediol production, (ix) promoting and affecting yield possibly through photosynthate allocation, (x) removing of contaminants (phytoremediation) and (xi) increase host plants resistance to plant pathogens and parasites (Sturz and Matheson, 1996; Preston *et al.*, 2004; Feng *et al.*, 2006; Ryan *et al.*, 2008; Berg, 2009; Weyens *et al.*, 2009; Compant *et al.*, 2010; Ma *et al.*, 2011).

3.4 *Xanthomonas* detection

Only purified yellow, convex, mucoid colonies were selected as their colony morphology is characteristic of that of the genus *Xanthomonas* (Starr, 1981; Coutinho and Wallis, 1991). Through carrying out greenhouse pathogenicity tests to prove Kochs postulates it was determined that these bacterial isolates were the only isolates to produce the distinctive streak patterns on the leaves. Prior to further molecular analyses on these isolates, a more rapid and economical technique was needed for the definitive identification of the pathogen. The technique that was evaluated and ultimately applied for this purpose was the molecular marker *XgumD*.

This marker detects the presence of the gene fragment *gumD*, which forms part of the *gum* operon, and is partially responsible for xanthan production (Mbega *et al.*, 2011). The *gumD* gene fragment is located within the *gumB-gumM* part of the operon and this region is conserved among *Xanthomonas* species (Katzen *et al.*, 1998; Vojnov *et al.*, 2001; Yoon and Cho, 2007).

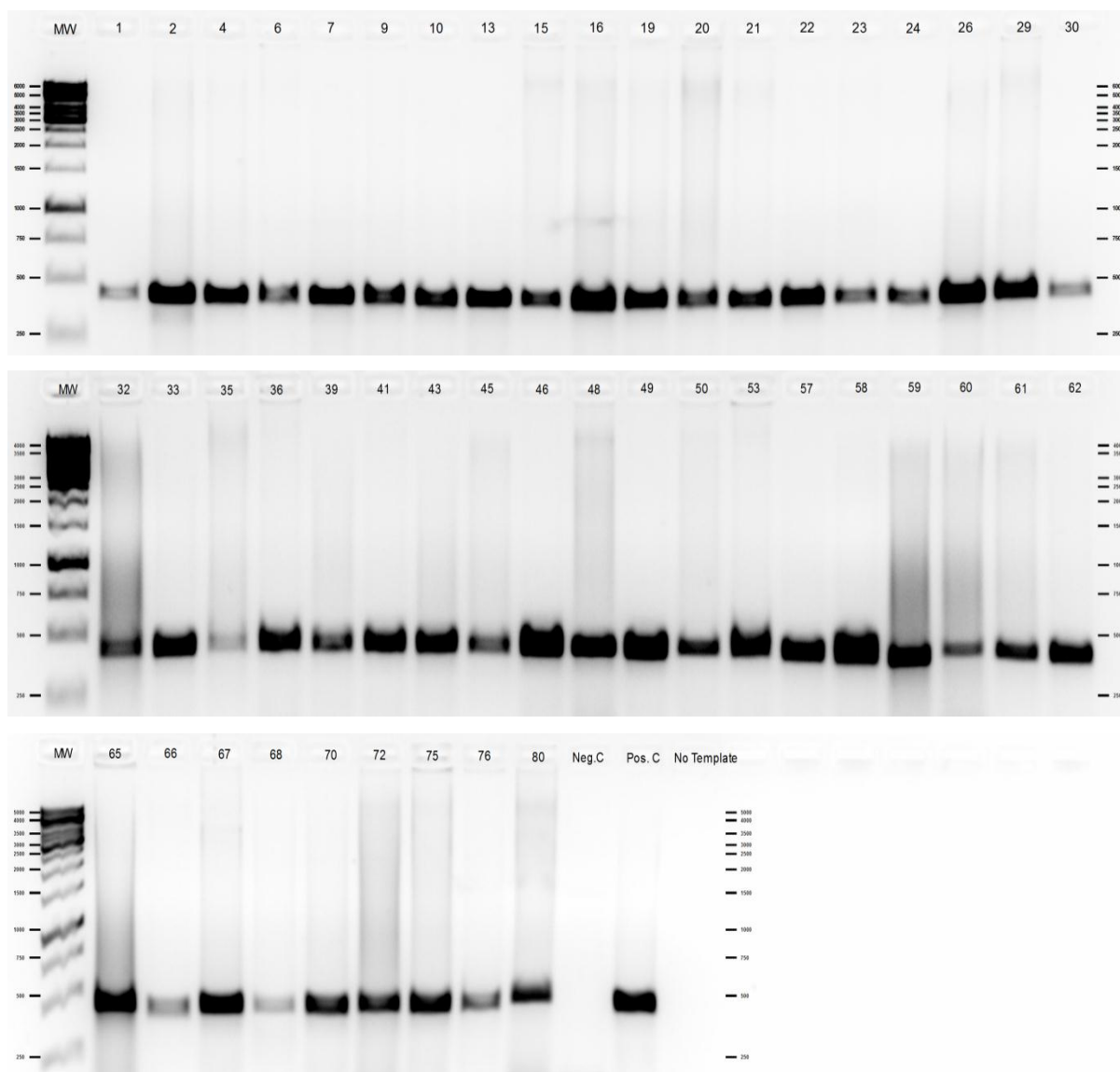


Figure 6 - Detecting *Xanthomonas* BLSD through the use of the molecular marker *XgumD*. *Pantoea stewartii* subsp. *stewartii* was used as the negative control (Neg.C) and *Xanthomonas axonopodis* pv. *vasculorum* strain ATCC 35935 as positive control (Pos.C).

Amplicons of the *XgumD* marker ranged in size from 402 bp to 490 bp (figure 6). These fragment sizes were in accordance with those obtained in a previous study from the detection of bacterial leaf spot of tomatoes caused by a *Xanthomonas* sp. (Mbega *et al.*, 2011). For a definite identification these PCR products were also sequenced, compared with those in the BLAST database and were determined to be part of the *Xanthomonas gumD* gene. No sequence polymorphisms could be detected amongst the *Xanthomonas* BLSD isolates. However, minor polymorphisms (two base substitutions) existed between the sequenced *gumD* gene fragment of *Xanthomonas* BLSD and *X. axonopodis* pv. *vasculorum* strain ATCC 35935.

Therefore, this marker was only able to detect and identify that the causal agent was a member of the genus *Xanthomonas*; differences amongst sequences might be used to distinguish between pathovars. However, this marker might still be valuable in terms of managing the disease by detecting the presence of *Xanthomonas* in maize fields even before visualisation of any symptoms and detecting its possible presence on or within seed whatever the case may be. A large number of markers for the detection of the genus as a whole and specific markers for the detection of a number of individual *Xanthomonas* species have already been designed, but none for this particular pathovar. A pathovar specific marker may be designed from the more variable regions of the operon – the *gumA* and *gumN-gumP* or even from the *hrp* gene cluster. In order to do so it would be essential to include other pathovars which are also known to be capable of infecting maize, namely, *X. axonopodis* pv. *vasculorum* and *X. vasicola* pv. *holcicola* in the analysis (Coutinho and Wallis, 1991; Lewis *et al.*, 2010; Adriko *et al.*, 2012).

3.5 Success of PCR analysis of *Xanthomonas* BLSD

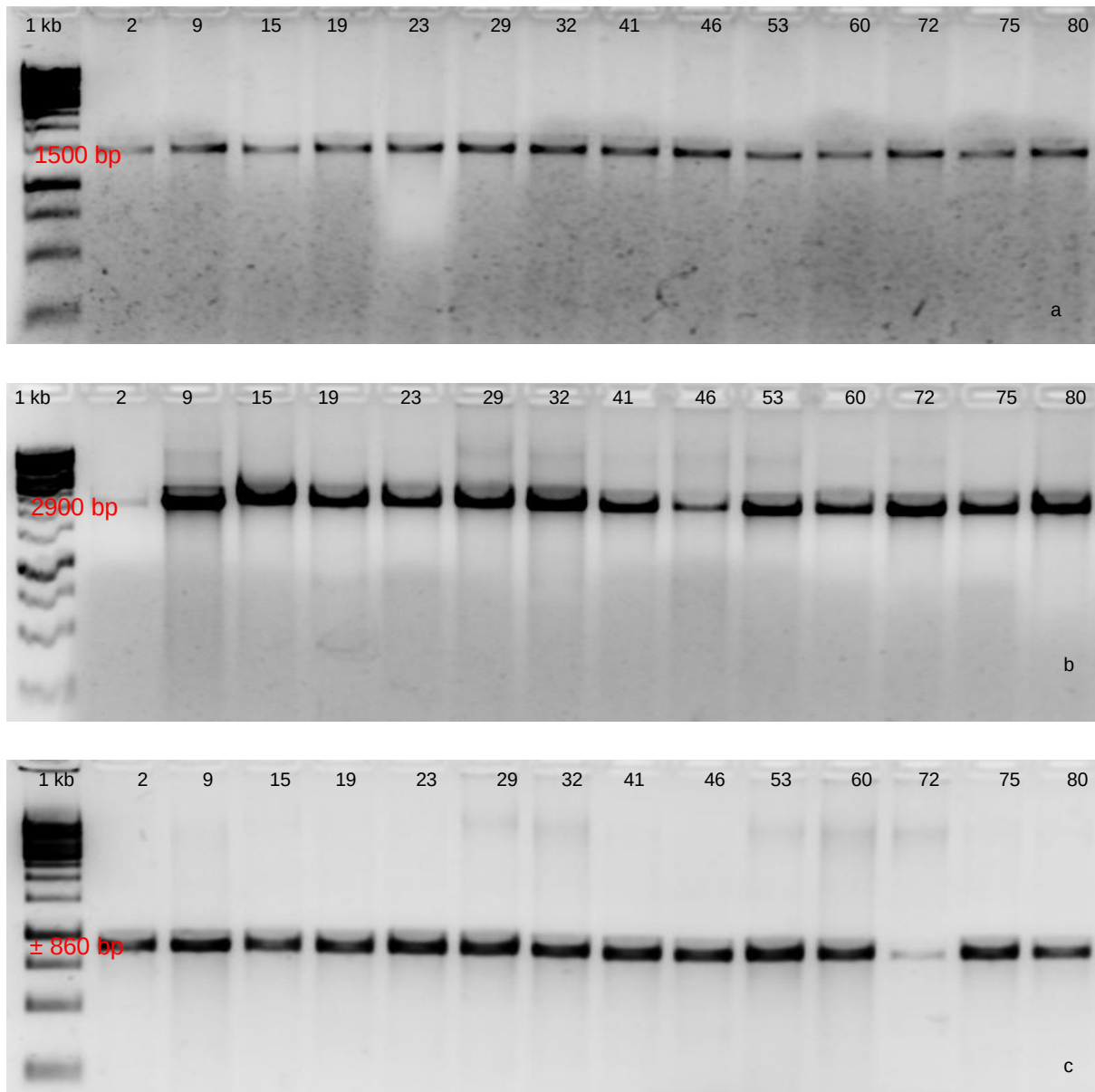


Figure 7 - Agarose gels depicting size differences in PCR products, all dependent on the type of PCR amplicon, of a selected number of isolates. a – 16S rRNA gene; b - 23S rRNA gene; c – *gyraseB* gene.

In all three gels (figure 7) it is evident that the amplicons were free from non-specific amplification products. The quality and quantity was thus sufficient for sequencing of the amplicons. These amplicons were sent to the service provider and they sequenced it using the protocol described in Section 2.6.

Sequences were obtained and edited accordingly where necessary. Little to no base editing was needed as the majority of sequences yielded excellent peaks with little to no background noise (figure 8).

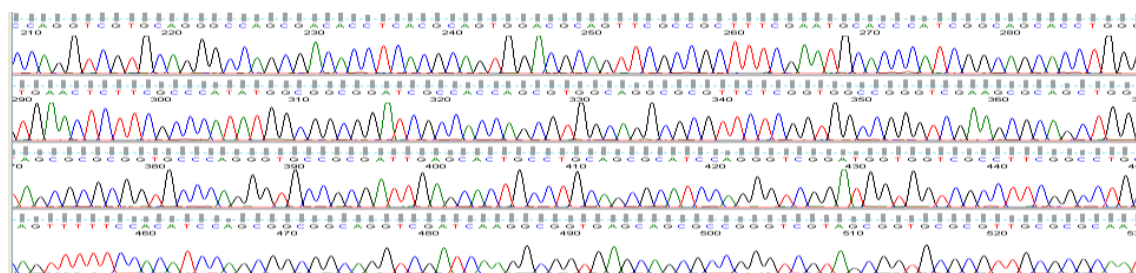


Figure 8 - A section of a sequence chromatogram of one of the genes analysed in this study.

However, the first few bases as well as the bases at the end of the sequences had to be excised as these produced ambiguous peaks. The end result was 16S rDNA fragments of $\pm$ 800 base positions, 23S rDNA fragments of $\pm$ 700 base positions and the *gyraseB* gene fragment with a length of 700 base positions. These were then BLASTn searched using the NCBI web protocol (Section 2.7).

3.6 Ribosomal DNA analyses

3.6.1 16S rRNA gene analysis

The 16S rRNA genes usually consists of eight highly conserved regions, U1-U8, and nine hypervariable regions, V1-V9, which display significant sequence diversity among different bacterial species and/or genera. These may be exploited for species identification (Van de Peer *et al.*, 1996; Baker *et al.*, 2003) and to rapidly detect specific bacterial species. It may also be used for differentiation between closely related organisms. Conserved sections permit PCR amplification of sequences using universal primers and are suitable for resolving distant relationships (Hauben *et al.*, 1997; Maidak *et al.*, 1999; McCabe *et al.*, 1999; Drancourt *et al.*, 2000; Clarridge, 2004).

The delineation and differentiation of species are not only supported by 16S rDNA sequencing. It also forms an integral part of bacterial taxa description (taxonomy), the (re)classification process and is necessary for establishing phylogenetic relationships (Amann *et al.*, 1995; Hauben *et al.*, 1997; Kolbert and Persing, 1999; Ludwig and Klenk, 2001; Mignard and Flandrois, 2006). Phylogenetic clustering obtained from 16S rDNA

sequences closely correlates even with those derived from whole genome phylogenies (Snel *et al.*, 1999).

Sequences (from the 16S rRNA gene) were of a suitable length and of high quality which are essential for accurate interpretation (Drancourt *et al.*, 2000; Christensen *et al.*, 2005; Janda and Abbott, 2007; Woo *et al.*, 2008). Sequencing was only done in the reverse direction. All partial sequences of the BLSD isolates shared a 100% similarity when aligned; therefore isolate number 36 was selected as the type strain and named *Xanthomonas* BLSD Type Strain 36. As of yet no sequence data is available on this specific species/pathovar, this is possibly due to the fact that it has not yet been recognised as a novel pathovar. Sequence similarity could be established for *Xanthomonas* BLSD Type Strain 36 and *Xanthomonas campestris* sequences from the NCBI database. However, a query coverage of a 100%, along with an E value score of 0.0 and a 100% maximum identity was obtained with *Xanthomonas arboricola* pv. *juglandis* strain LMG 747 and *X. hortorum* strain LMG 733, *X. axonopodis* pv. *vasculorum* strain KNU 28186 and strain KNU 28187 and *X. vasicola* pv. *vasculorum* strain CPO 12.013 and strain CPO 12.0012. These similarities were, however, dependent upon length of sequence and segment of sequence selected for alignment. Aligned partial sequences of *Xanthomonas* BLSD Type Strain 36 and *X. axonopodis* pv. *vasculorum* strain ATCC 35938 were of an identical nature, with no nucleotide variance.

To determine the phylogenetic relationship between *Xanthomonas* BLSD isolates from this study and other xanthomonads, several *Xanthomonas* species were obtained from GenBank and imported into the described bioinformatics software (Section 2.7). Analysis of 800 nucleotides of each representative was conducted, using neighbor-joining and 1000 bootstrap methodology. *Stenotrophomonas* sp. was selected as the out-group. The resultant tree is provided in figure 9.

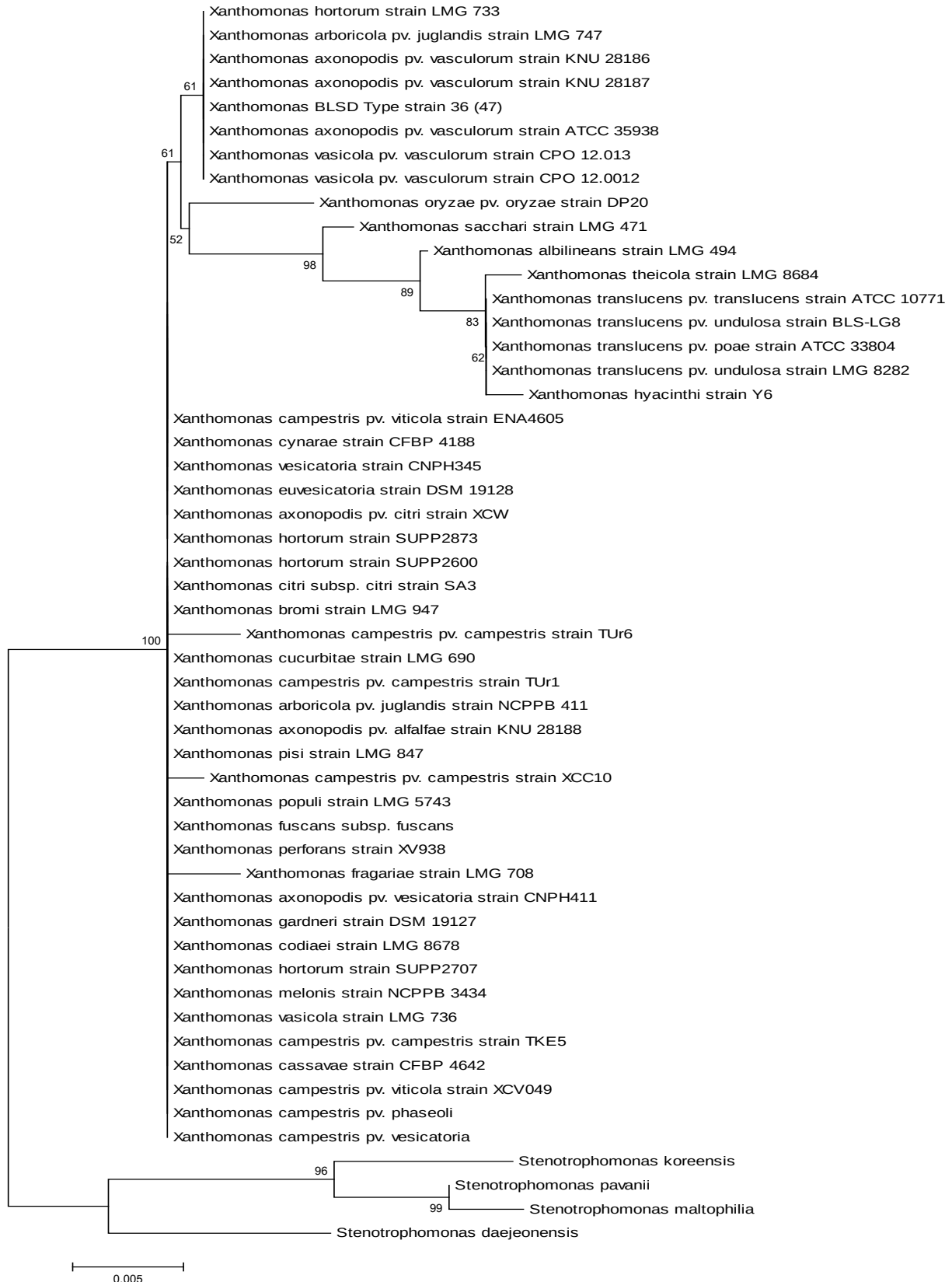


Figure 9 - Phylogeny constructed from partial 16S rDNA sequences for several *Xanthomonas* species and pathovars and the placement of *Xanthomonas* BLSD within the tree diagram.

No sub-clustering or groupings were observed amongst the *Xanthomonas* BLSD isolates based on their aligned 16S rDNA fragments. The 16S rRNA gene was able to differentiate between the different *Xanthomonas* species (eg. between *X. campestris*, *X. axonopodis*, *X. albilineans*, *X. translucens* and the other species), between the different pathovars of a *Xanthomonas* species (eg. *X. translucens* pathovars *undulosa*, *translucens* and *poae*) and to an extent between different strains of the same pathovar (eg. *Xanthomonas axonopodis* pv. *vasculorum* strain KNU 28186, KNU 28187 and strain ATCC 35938) as seen in figure 9. In the phylogenetic tree *Xanthomonas* BLSD clustered with *X. axonopodis* pv. *vasculorum*, *X. vasicola* pv. *vasculorum* strain CPO 12.013 and strain CPO 12.0012, *Xanthomonas arboricola* pv. *juglandis* strain LMG 747 and *Xanthomonas hortorum* strain LMG 733. The latter two species are not known to infect maize, however, *X. axonopodis* pv. *vasculorum* and *X. vasicola* pv. *vasculorum* do infect maize and other grass species (Dookun *et al.*, 2000; Destefano *et al.*, 2003; Parkinson *et al.*, 2009; Wasukira *et al.*, 2014). The classification of *X. campestris* pv. *zeae* may therefore be incorrect and warrants further analysis.

Several possible reasons may explain the lack of sequence variance amongst the *Xanthomonas* BLSD isolates. It might be an indication of the highly similar/identical nature of their 16S rDNA fragments. This group of isolates do not only constitute part of the same genus, which are already highly homogeneous (Hauben *et al.*, 1997), but they may also be of the same pathovar. It is also known that organisms may be genotypically and/or ecologically diverse whilst possessing identical 16S rRNA gene sequences (Clayton *et al.*, 1995; Thompson *et al.*, 2005).

Partial gene sequences were used for phylogenetic construction. The ideal sequence length ranges from 1 300 to 1 500 bp and preferably all of the same length (Drancourt *et al.*, 2000; Christensen *et al.*, 2005; Janda and Abbott, 2007; Woo *et al.*, 2008). Therefore, it might have lacked the necessary length which may have comprised additional informative characters such as, deletions, insertions and/or substitutions. The possible presence of these might have further differentiated between the isolates. However, phylogenies derived from either full length (1500 bp) 16S rRNA gene sequences or from partial (500 bp) 16S rRNA gene sequences were compared in a previous study and revealed highly similar phylogenies (Clarridge, 2004). Therefore, in this case additional sequence length may not have enhanced the phylogenetic resolution of the *Xanthomonas* BLSD isolates. Examples are presented below that support the claims made in the preceding section of this paragraph.

In a past study piloted by Hauben *et al.* (1997) the 16S rRNA gene was used to compile phylogenetic relationships amongst all validly described species of the genus *Xanthomonas*. It was determined that the species of the genus revealed relatively high levels of overall sequence similarity. The levels of 16S rDNA sequence similarity between the species ranged from 98.9% to a 100%. The number of nucleotide differences ranged from 0 to 16. Therefore, sequence differences amongst these species may not always be sufficient to infer internal phylogenetic relationships. Although these species formed a highly homogeneous cluster within the genus *Xanthomonas*, all of the 15 species were described as separate genospecies on the basis of DNA-DNA hybridization data (Vauterin *et al.*, 1995).

This study of Hauben *et al.* demonstrated that *X. sacchari* formed its own cluster with a 98.9% 16S rDNA sequence similarity and displayed 19 nucleotide differences with the previous cluster. The 16S rRNA gene segregated *X. albilineans*, *X. translucens*, *X. hyacinthi* and *X. theicola* into a third cluster. These species exhibited three to seven nucleotide differences. *X. sacchari* shared a sequence similarity of 98.2%, and 26 to 28 nucleotide differences with the third cluster. Discrimination between the species of the third cluster is also complicated due to limited variation in the 16S rDNA sequence. However, as a group these species (from the third cluster) are distinctly separated from the other *Xanthomonas*. They share a sequence similarity of 97.8% with the first cluster, corresponding to 32 to 42 nucleotide differences. The average similarity value was 98.2%, which corresponds to an average of 14 common nucleotide differences. This result illustrates the homogeneity of the genus (Hauben *et al.*, 1997). The latter study investigated the relatedness among *Xanthomonas* species and not variation among pathovars.

In a study conducted by Moore *et al.* (1997), pairwise alignments and comparisons of the 16S rDNA sequences of several *Xanthomonas* species, along with phylogenetic analysis confirmed that Xanthomonads consisted of a monophyletic lineage within the γ -*Proteobacteria*. It was also confirmed that amongst the *Xanthomonas* species, species-specific primary sequence differences were existent and persistent. Sequence variation amongst these different species was low, ranging from one to 35 nucleotide differences. However, little to no 16S rDNA sequence variation could be detected amongst the pathovar type strains of the various species (Moore *et al.*, 1997).

Popovic *et al.* (2013), determined that various strains of *X. campestris* pv. *campestris* isolated from different *Brassica oleracea* members shared 99% 16S rDNA sequence similarity with a type strain of *Xanthomonas campestris* pv. *campestris*. However, when a

profiling technique was applied more variation could be detected, thereby illustrating that the phylogenetic resolution of the gene (16S rDNA) below the levels of species and subspecies is limited.

In a study by Lee *et al.* (2008), 16S rDNA sequences were used to demonstrate differentiation amongst various *X. axonopodis* pathovars and strains. The analysis of their partial 16S rDNA sequences revealed high levels of similarity amongst the *Xanthomonas* pathovars/strains. However, the constructed dendrogram illustrated defined clustering amongst all of the strains. *X. axonopodis* pv. *aurantifolii* (South American origin) and *X. axonopodis* pv. *citrumelo* (North America origin) grouped together with a 99% similarity. The 16S rRNA gene was also capable of differentiating between the *X. axonopodis* pv. *citri* pathovars (Asiatic origin) and the pathovars *X. axonopodis* pv. *aurantifolii* and *X. axonopodis* pv. *citrumelo*. However, this gene was not able to differentiate between the different pathovar types/strains of *X. axonopodis* pv. *citri*.

Adhikari *et al.* (2012), determined that the 16S rRNA gene sequences were successful in its ability to differentiate between the pathovars of *X. translucens* and other *Xanthomonas* pathovars as well as type strains of *Xanthomonas* species. However, it lacked the necessary resolution to distinguish between *X. translucens* pathovars which infect a variety of small grains.

The ability of the 16S rRNA gene to genetically discriminate between geographically distinct *X. axonopodis* pv. *punicae* isolates/strains were also assessed. All fifteen strains collected clustered together sharing 100% identity, suggesting that geographical distance does not inevitably bring about variation in the strains of the pathovar. The homogeneous population was also indicative of similar disease severity across boundaries (Mondal *et al.*, 2013).

Thus the findings of the present study on the effectiveness of 16S rDNA sequences to identify and inability to differentiate between isolates of *Xanthomonas* BLSD pathogens from different geographical areas remain unclear. As the 16S rRNA gene did identify the isolates as *Xanthomonas*, but could not establish a similarity to *X. campestris* pv. *zeae* as strains of this pathovar does not yet exist within the NCBI Blastn database. The 16S rRNA gene did differentiate amongst several *Xanthomonas* species and pathovars. The ability of this gene to differentiate amongst strains of a particular pathovar is challenging as also seen from literature. In the following sections 23S rRNA and *gyraseB* gene sequences were also evaluated for this purpose.

3.6.2 23S rRNA gene analysis

The 23S rRNA gene offers the same advantages as the 16S rRNA gene (namely, universal distribution, conserved function and variable regions). Additionally, 23S rDNA sequences are longer, may contain unique insertions and/or deletions (more sequence variation) and may therefore be better equipped for phylogenetic resolution (discriminating even between closely related species or subspecies) (Van Camp *et al.*, 1993; Kolbert and Persing, 1999; Ludwig and Schleifer, 1994; Hunt *et al.*, 2006).

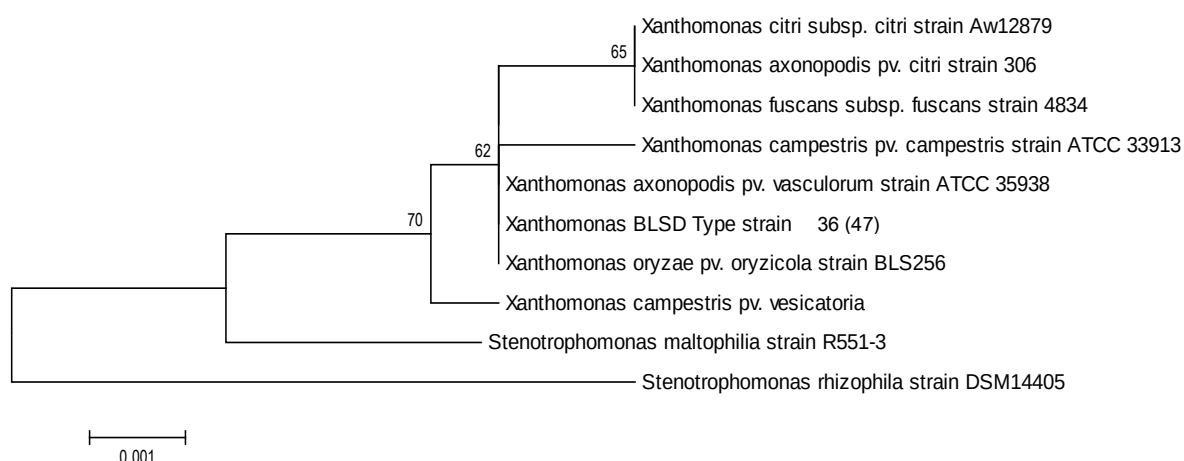


Figure 10 - Phylogeny constructed from partial 23S rDNA sequences for several *Xanthomonas* species and pathovars and the placement of *Xanthomonas* BLSD within the tree diagram.

The neighbour-joining method with a 1000 replicates was used for analysis. Fragment length consisted of 700 bp. Several *Xanthomonas* spp. and pathovars were selected as ingroup, whilst *Stenotrophomonas rhizophila* and *S. maltophilia* was selected as the out-group.

Hundred percent sequence similarity to sequences in NCBI Database could not be obtained with this particular isolates of *Xanthomonas* BLSD. The main reason is that until the present study no 23S rRNA sequence data existed for this pathogen. However, a query coverage of a 100%, along with an E value score of 0.0 and a 99% sequence similarity was obtained with *X. oryzae* pv. *oryzicola* sequences corresponding to a single nucleotide variation (similarity is dependent upon sequence length and the sequence fragment selected). There was also a one base variation between the aligned partial sequences of *Xanthomonas* BLSD Type Strain 36 and *X. axonopodis* pv. *vasculorum* ATCC 35938.

Again no sub-clustering was observed amongst the *Xanthomonas* BLSD isolates based on their partial 23S rDNA fragments. As with the 16S rRNA gene there was no sequence variance amongst the isolates. This may support the notion of the highly similar/identical nature of their 23S rDNA fragments. As only partial gene sequences (700 bp) were used for phylogenetic construction; sequence information might have been lost. Similarities might also be attributed to the possible transfer of rRNA genes between the pathovars. This gene was also capable of distinguishing between different *Xanthomonas* species, even though a fewer *Xanthomonas* species were available for comparison. *Xanthomonas* BLSD Type Strain 33 grouped with *X. oryzae* pv. *oryzicola* and with *X. axonopodis* pv. *vasculorum* ATCC 35938. With both sets of rRNA gene analysis (16S rRNA and 23S rRNA) *Xanthomonas* BLSD grouped with *X. axonopodis* pv. *vasculorum* ATCC 35938, suggesting a closer relationship to the *X. axonopodis* species, as opposed to *X. campestris*.

No comparisons could be made between phylogenies derived from this study and previous work, as little to no phylogenetic data is available on *Xanthomonas* species based on 23S rRNA genes. However, phylogenies derived from both 16S rDNA and 23S rDNA sequences are in accordance and a polyphasic approach in which both are used could be more informative than the individual genes (Arahal *et al.*, 2002; Bavykin *et al.* 2004; Nochi *et al.*, 2009). As no definite answer could have been obtained with both of these ribosomal sequences regarding possible variation amongst the isolates, the resolution power of the *gyraseB* gene was assessed.

3.7 GyraseB analysis

This protein encoding gene was selected as it exists as single copy within the genome of bacteria. It is therefore, not subjected to the formation of ribotypes; that is intragenomic heterogeneity within the same gene such as is the case with ribosomal genes. The *gyraseB* gene is relatively conserved but displays more nucleotide variations than the rRNA genes. Therefore, it provides higher resolution even at pathovar level (Yamamoto and Harayama, 1996; Fukushima *et al.*, 2002; Yin *et al.*, 2008; Parkinson *et al.*, 2009). The majority of *Xanthomonas* possess limited *gyraseB* sequence variation, which is consistent with rapid and extensive pathovar diversification that has occurred in relatively recent times, before any mutations could accumulate (Parkinson *et al.*, 2009).

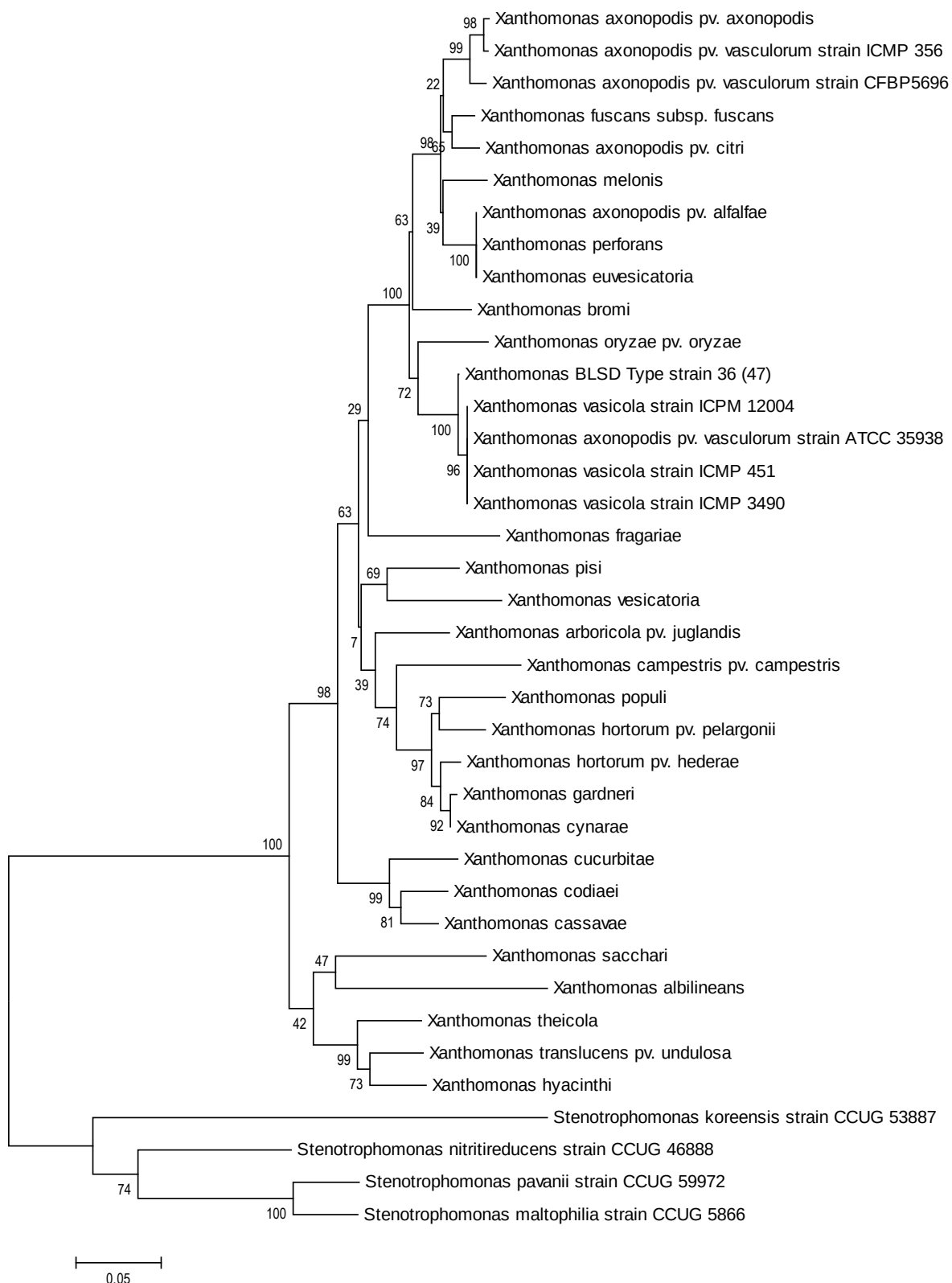


Figure 11 - Phylogeny constructed from partial *gyraseB* sequences for several *Xanthomonas* species and pathovars and the placement of *Xanthomonas* BLSD within the tree diagram.

As it was with the other two genes 100% sequence similarity could not be obtained with this particular pathogen *Xanthomonas* BLSD. However, a query coverage of a 100%, along with an E value score of 0.0 and a 99% sequence similarity was obtained with *Xanthomonas vasicola* strains. Sequences of *Xanthomonas* BLSD were aligned to *gyraseB* sequences of several representative *Xanthomonas* spp. and pathovars obtained from the NCBI data base. The neighbour-joining method with a 1000 replicates was used for phylogenetic analysis (figure 11). Sequences used were 700 bp in length. *Stenotrophomonas* species was selected as the out-group.

No sub-clustering was detected amongst the *Xanthomonas* BLSD isolates based on their *gyraseB* sequences. This finding also suggests that there is no variation amongst the isolates. As with the ribosomal genes, there was a clear differentiation amongst the *Xanthomonas* species, pathovars and strains as seen in figure 11. With regards to their aligned *gyraseB* sequences there was a six nucleotide difference between *Xanthomonas* BLSD Type Strain 36 and *X. axonopodis* pv. *vasculorum* ATCC 35938, a six nucleotide difference between *Xanthomonas* BLSD Type Strain 36 and *X. vasicola* strains ICMP 12004, ICMP 451 and ICMP 3490. In both cases the *Xanthomonas* BLSD Type Strain and *X. vasicola* were grouped together by their 16S rDNA sequences and their *gyraseB* sequences; for the 23S rDNA sequences there was no *X. vasicola* to align with the *Xanthomonas* BLSD Type Strain. For all genes the *Xanthomonas* BLSD Type Strain grouped closely with *X. axonopodis* pv. *vasculorum* strain ATCC 35938.

Previously, the *gyraseB* gene was shown to possess the ability to discriminate between almost all recognised *Xanthomonas* species. However, it's been established that even this gene locus isn't equipped with the necessary resolution power to distinguish between the type strains *X. euvesicatoria*, *X. perforans* and *X. alfalfae*, which form a species complex with identical *gyraseB* sequences (Parkinson *et al.*, 2007). However, Young *et al.* (2008) determined with their studies that these species were not unmistakably differentiable as separate species from *X. axonopodis* and that *X. euvesicatoria* and *X. perforans* were possibly synonyms. Parkinson and his colleagues (2007) highlighted the potential use of *gyraseB* sequencing as a suitable and accurate technique for establishing *Xanthomonas* phylogeny at the species and, possibly, strain or pathovar level.

In a further study conducted by Parkinson *et al.* (2009) it was determined that this gene was capable of differentiating between and constructing phylogenetic clusters amongst a large number of *Xanthomonas* pathovars. The correct allocation of well characterized pathovars to clades comprising their relevant or related reference pathotype strains corroborates the

accuracy of the existing classification and the *gyraseB* phylogeny. *GyraseB* gene sequence analysis was also responsible for transferring pathovars from a one species to another, such as allocating pathovars of *X. campestris* to species related to *X. axonopodis*. *GyraseB* also assigned unidentified species of *Xanthomonas* to an assortment of (novel) species clades. Thus, this locus is more than able to adequately identify and distinguish intraspecies sequence variation. A large amount of diversity was detected within the species *X. translucens*, *X. arboricola*, *X. hortorum* and *X. campestris*; differentiating various key pathovars. The gene also established the lineage between *X. axonopodis* and *X. citri*, *X. fuscans* and the *X. euvesicatoria* species complex. All *X. axonopodis* pathovars clustered in the clades containing these species, thereby revealing the relatedness of the taxa. Additional species/subspecies and pathovars were identified within these main clades, including a related clade to the *X. euvesicatoria* species complex, which consists of six pathovars. Also it distinguished between two taxa which infect the same host, *X. fuscans* and *X. axonopodis* pv. *phaseoli*. The gene differentiated also between two pathovars from the *Poaceae* family, namely *X. axonopodis* pv. *axonopodis* and the pathotype strain of *X. axonopodis* pv. *vasculorum*

In a study lead by Tsushima *et al.* (2006) the ability of *gyraseB* to differentiate amongst geographically distinct strains of *X. albilineans* was assessed. The ability of this gene to distinguish between strains and group them according to their point of origin was confirmed.

Mondal *et al.* (2013) established the capacity of the *gyraseB* locus to discriminate between *X. citri* subsp. *malvacearum* and pathovars of *X. axonopodis* pv. *manihotis* and between these entities and the isolates of *X. axonopodis* pv. *punicae* as a whole. For this gene isolates of *X. axonopodis* pv. *punicae* displayed 100% sequence conservation, thereby, signifying that geographical origin does not necessarily affect genetic variation.

Of all the genes, the *gyraseB* gene provided the best resolution for discriminating between *Xanthomonas* BLSD Type Strain 36 and the other *Xanthomonas* species and pathovars. In two cases (16S rRNA and *gyraseB* gene sequence analysis) *Xanthomonas* BLSD Type Strains and *X. vasicola* were grouped together. For the 23S rDNA sequences there was no *X. vasicola* to align with the *Xanthomonas* BLSD Type Strain. For all three gene sequence analysis the *Xanthomonas* BLSD Type Strain grouped closely with *X. axonopodis* pv. *vasculorum* ATCC 35938.

3.8 Genomic Profiling

For final conformation regarding the relatedness amongst the isolates of *Xanthomonas* BLSD genomic profiling (via rep-PCR) was conducted. This technique is advantageous over sequencing techniques as it surveys the whole genome. As a result the technique may be applied for (i) the identification of bacterial strains, (ii) analysing taxonomic/genetic diversity amongst bacterial groups (such as between different microbial species, pathovars and/or strains) and for (iii) the determination of the phylogenetic structure (classification) of bacterial populations. In the present study BOX-PCR genetic fingerprinting was used. The details are described in Section 1.10.4 and 2.7. *X. axonopodis* pv. *vasculorum* strain ATCC 35938 was selected as ingroup (as no reference strain of the pathovar – *Xanthomonas* BLSD is available) and *P. stewartii* subsp. *stewartii* as outgroup. All of the *Xanthomonas* BLSD isolates were identical in the number of PCR products (12 distinct bands) and overall pattern. The bands ranged in size from approximately 500 bp to over 6 kb (Figure 12). Bands were selected manually and the UPGMA statistical method with Jaccard's coefficient was used for statistical analysis.

Table 3- A presence (1) and absence (0) band matrix for the 47 *Xanthomonas* BLSD isolates and the reference species.

		<i>Xanthomonas</i> BLSD isolates and ingroup and outgroup species																											
		1	2	4	6	7	9	10	13	15	16	19	20	21	22	23	24	25	26	30	32	33	35	36	39	41			
Rf values	0.114	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.134	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.167	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.195	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.219	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.237	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
	0.267	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.299	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.328	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.370	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.589	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.656	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.817	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
		<i>Xanthomonas</i> BLSD isolates and ingroup and outgroup species																											
		43	45	46	48	49	50	53	55	58	59	60	61	62	65	66	67	68	70	72	75	76	78	80	X.a	P			
Rf values	0.114	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1			
	0.134	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0			
	0.167	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.195	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0			
	0.219	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.237	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1			
	0.267	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.299	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0			
	0.328	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.370	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0			
	0.589	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0			
	0.656	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
	0.817	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0			

This matrix illustrated (Table 3) the identical nature of *Xanthomonas* BLSD isolates in terms of the bands which were present and absent. The matrix also highlighted the differences between this pathovar and the *X. axonopodis* pv. *vasculorum* strain ATCC 35938 (X.a) and also the outgroup *Pantoea stewartii* subsp. *stewartii* (P). Therefore, only a number of *Xanthomonas* BLSD isolates were selected in order to be compared with *X. axonopodis* pv. *vasculorum* strain ATCC 35938 and *Pantoea stewartii* subsp. *stewartii* in a dendrogram.

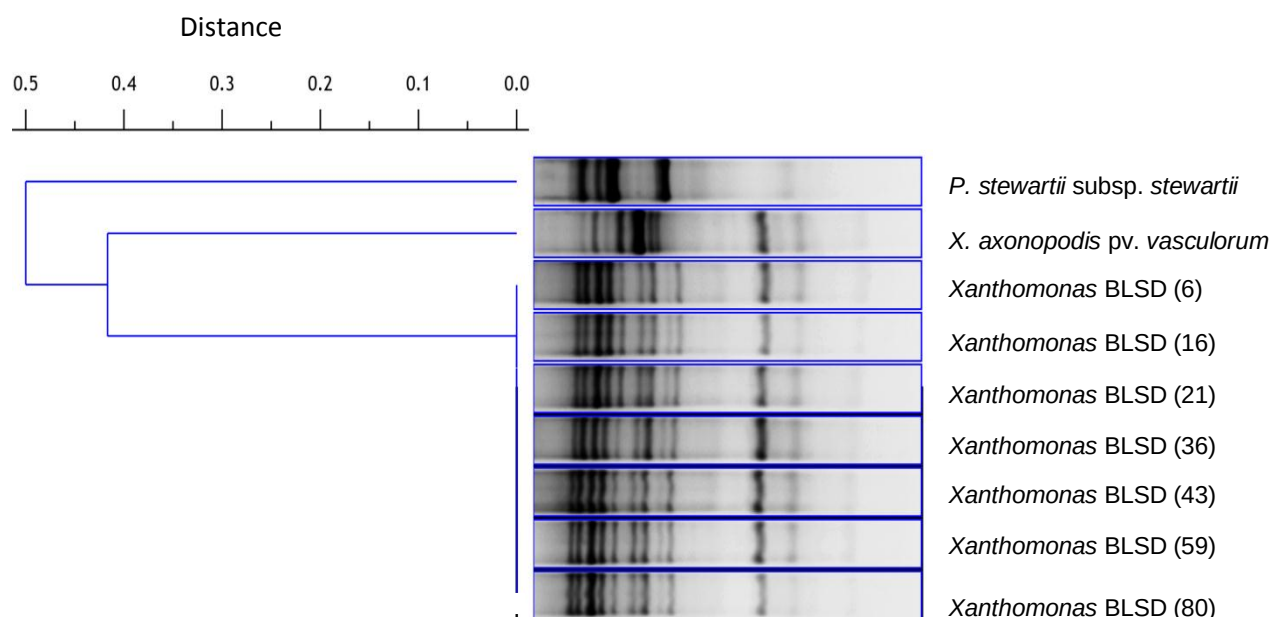


Figure 12 - A dendrogram constructed from BOX generated genomic profiles of a selected number of *Xanthomonas* BLSD isolates and reference species.

This profiling technique detected no variation amongst the *Xanthomonas* BLSD isolates. However, it did differentiate between the isolates and the pathovar *X. axonopodis* pv. *vasculorum* strain ATCC 35839 and *P. stewartii* subsp. *stewartii*. *X. axonopodis* pv. *vasculorum* strain ATCC 35938 had 10 major bands and *P. stewartii* subsp. *stewartii* had 7 clearly distinguishable bands (as seen in figure 12).

This technique may also be useful to identify *Xanthomonas* BLSD in the field according to its unique DNA fingerprint. However, this method needs to be verified by included as many *Xanthomonas* sp. as possible and several representatives of these species.

Previously variation between these two pathovars the causal agent of bacterial leaf streak disease, *Xanthomonas* BLSD (from South Africa) and *X. axonopodis* pv. *vasculorum* (from South Africa, Zimbabwe and Mauritius) had been observed when profiling techniques were used. However, no intrapathovar diversity/polymorphisms were observed amongst the

isolates, suggesting a clonal origin (Qhobela *et al.*, 1990). In the past Coutinho and Wallis (1991) partially distinguished between isolates of *Xanthomonas* BLSD and *X. axonopodis* pv. *vasculorum* (maize strain), however, the biochemical and physiological properties that were used may be subjected to certain limitations. These include, various strains of an organism may have different biochemical profiles, thereby promoting confusion in identification and isolates which exhibit unusual phenotypic profiles are occasionally encountered (Hauben *et al.*, 1997; Kolbert and Persing, 1999; Drancourt *et al.*, 2000; Woo *et al.*, 2003; Mignard and Flandrois, 2006).

Further clarification regarding the relatedness of these two pathogens (*Xanthomonas* BLSD and the maize strain of *X. axonopodis* pv. *vasculorum*) may be needed in order to determine if these are truly different from one another. Possibly *Xanthomonas* BLSD diversified from *X. axonopodis* pv. *vasculorum*, as this pathovar is capable of infecting both maize and sugarcane, whilst *Xanthomonas* BLSD is reported to be only capable of infecting maize (Coutinho and Wallis, 1991; Qhobela *et al.*, 1990) and due to the fact that the hosts of these pathovars had been grown in close proximity in the past. *Xanthomonas* species/pathovars possess the ability and potential to diversify and exploit new plant hosts. Minimal sequence diversity is indicative of rapid and extensive pathovar diversification which has occurred in relatively recent times. Low-intensity farming practices might have provided conditions beneficial to pathovar development (Parkinson *et al.*, 2009).

In the past rep-PCR based genomic profiling played an essential role in the differentiation amongst *Xanthomonas* species and their subsequent reclassification (Vauterin *et al.*, 1995). It had also been used to generate unique genomic profiles of a variety of *Xanthomonas* isolates and to identify and detect pathovars and strains that were formerly not distinguishable by other methods (Louws *et al.*, 1994).

This technique had been applied in numerous studies to evaluate diversity amongst species and bacterial populations, to differentiate between or highlight similarities amongst pathovars/strains and to detect races and intrapathovar diversity (profile variation within a pathovar) (Louws *et al.*, 1994; Valverde *et al.*, 2007; Zamani *et al.*, 2011). It had also been used to group intra-pathovar isolates/strains according to geographical origin (Lopes *et al.*, 2001) even where sequencing techniques failed to do so (Adhikari *et al.*, 2012).

With previous studies this profiling technique also highlighted the highly similar genomic composition of *X. campestris* pv. *campestris* strains/isolates infecting different members of *Brassica oleracea* and clearly differentiated this pathovar from other closely related

Xanthomonas pathovars (Zaccardelli *et al.*, 2008; Popovic *et al.*, 2013). In another study conducted by Zamani and colleagues (2011), rep-PCR revealed a high degree of homogeneity amongst all strains of *Xanthomonas axonopodis* pv. *phaseoli* which were collected from various localities within Iran. Rep-generated profiles were in accordance with results obtained from other profiling techniques. It was concluded that original inoculum introductions might have distributed among the different growing areas of the host.

Chapter 4 - Conclusions and Recommendations

In this chapter the conclusions of the study is briefly discussed and recommendations for future studies made. Section 4.1 contains the conclusions and section 4.2 the recommendations.

4.1 Conclusions

To determine the molecular characteristics of the causal agent of bacterial leaf streak disease, five objectives were set. A brief discussion of each objective is described below.

(i) Incidence and severity of bacterial leaf streak and the distribution range of the disease

It was determined that *Xanthomonas* BLSD has a well-established distribution range within the major and minor maize production regions of South Africa. This distribution pattern is possibly due to the mechanisms by which the pathogen disseminates and the climatic conditions of these areas which favour the development and growth of both the host and the pathogen. As climate change is inevitable, it may impact the distribution range of the host, pathogen and possible insect transmitters, plant-pathogen interactions, resistance and pathogen development stages and the severity of disease epidemics.

The incidence and severity of the disease varied from field to field. Symptoms varied from a few streaks, to entire leaves being covered in longitudinal streaks and even in most severe cases the leaves were hanging in tatters with wilting occurring.

Numerous endophytes were detected and often overgrew the pathogen, even to such an extent that another isolation technique was needed. These endophytes may have several effects on the growth and development of both the host and this pathogen.

(II) Identification of the causal organism using *XgumD*

The molecular marker, *XgumD*, was only able to detect and identify that the causal agent was a member of the genus *Xanthomonas*. No variations amongst the *gumD* sequences of a selected number of *Xanthomonas* BLSD isolates could be detected. Though, minor nucleotide differences existed between the sequenced *XgumD* gene fragment of

Xanthomonas BLSD isolates as a whole and *X. axonopodis* pv. *vasculorum* strain ATCC 35938. However, it still might be valuable in terms of managing the disease, by detecting the presence of *Xanthomonas* in maize fields even before visualisation of any symptoms or perhaps even its presence on/in seed if the pathogen is seedborne.

III) Establishing the potential resolution power of ribosomal genes and a protein encoding gene (*gyraseB*) to differentiate amongst isolates via the construction of phylogenies.

No variations were observed amongst the *Xanthomonas* BLSD isolates based on their aligned ribosomal and *gyraseB* fragments. All of the analysed genes were successful in their ability to distinguish between the type strain of *Xanthomonas* BLSD and a selection of *Xanthomonas* species and pathovars. The fact that these genes did not differentiate between the isolates of *Xanthomonas* BLSD does not necessarily mean the incompetence to do so, however it may just be indicative of the identical nature of the isolates. With both the 16S rDNA and *gyraseB* analyses the *Xanthomonas* BLSD Type Strain and *X. vasicola* grouped together; however for the 23S rDNA sequences there was no *X. vasicola* to align with the *Xanthomonas* BLSD Type Strain. For all genes the *Xanthomonas* BLSD Type Strain grouped closely with *X. axonopodis* pv. *vasculorum* strain ATCC 35938.

(IV) Determine possible variation amongst the population (intrapathovar diversity) by means of genomic profiling (BOX-PCR)

There was no variation amongst the fingerprint profiles of the *Xanthomonas* BLSD isolates; all had the same banding pattern. This technique did also differentiate between the isolates of *Xanthomonas* BLSD, *X. axonopodis* pv. *vasculorum* strain ATCC 35839 and *P. stewartii* subsp. *stewartii*.

These findings suggested that the *Xanthomonas* BLSD isolates had a clonal origin with geographical origin having no effect on the genetic composition of the isolates. This information regarding the pathogen may prove to be beneficial in terms of developing resistant maize varieties as it is necessary to know beforehand whether or not differences in the *Xanthomonas* BLSD population exists.

4.2 Recommendations

- Determine the incidence of the disease in the minor maize production areas of South Africa, including the Limpopo, KwaZulu Natal and Eastern Cape provinces. Genetically compare these to those obtained from the other provinces in order to establish intrapathovar relatedness. Compilation of phylogenies based on a larger selection of *Xanthomonas* species and *Xanthomonas campestris* pathovars is necessary in order to compile the phylogeny of *Xanthomonas* BLSD that is based on interpathovar variation.
- Further analyses, such as PFGE, DNA-DNA hybridisation, fatty-acid analysis and whole genome analysis may be needed to clarify the genetic relationship between *X. axonopodis* pv. *vasculorum*, *X. vasicola* pv. *vasculorum* and *Xanthomonas* BLSD. As all of these are capable of infecting maize.
- Perform field trials to assess the impact that this disease has on maize yield, along with cultivar susceptibility analysis. This is an essential aspect as leaf necrosis of up to 40% has been observed.
- Determine what effects do seasonal and geographical climate variations have on this pathogen. May an increase in temperature result in an increased level of xanthan production to avoid desiccation and thereby increase the virulence of the pathogen.
- Establish whether or not this disease is seedborne and which insects are responsible for its possible transmission. Determine the length of time this pathogen remains viable in crop debris as this will impact control strategies. Also establish whether or not volunteer hosts or alternate weeds are responsible for its spread.
- Design possible pathovar specific molecular markers for the early detection of this particular *Xanthomonas* pathovar. For this as many as possible *Xanthomonas* species, pathovars and strains need to be included to assess variable regions. A pathovar specific marker may be designed from the more variable regions of the operon – the *gumA* and *gumN-gumP* or even from the *hrp* gene cluster.
- Identify the virulence genes of the pathogen and determine if host-pathogen interactions are based on monogenic or polygenic resistance. Commercially this may

be the most crucial aspect of the pathogen to be addressed as there was a withdrawal of a susceptible maize variety in the past.

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