Genetic diversity of Vetiver clones (*Chrysopogon zizanioides* and *Chrysopogon nigritana*) available in South Africa based on sequencing analyses and anatomical structure

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Dissertation submitted in fulfillment of the requirements for the degree *Magister Scientiae* in Botany at the Potchefstroom Campus of the North-West University

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November, 2013

Opsomming

Vetiver gras of *Chrysopogon zizanioides* (L.) Roberty (1960) is ‘n steriele grasspesie wat vegetatief kan voortplant vanaf die wortelstok. Tesame met sy kragtige, diep wortelstelsel en vloed-toleransie, is dit die ideale kandidaat vir grond remediasie en die beheer van erosie. Hydromulch (Pty) Ltd. in Suid-Afrika is deel van die landskap, grondherwinning en erosie-beheer industrie. Hydromulch gebruik Vetiver vir verskeie toepassings en op grootskaal, en het ‘n aantal isolate versamel om as moontlike kiemlyne te dien. As gevolg van verskillende omgewingsbestuursmetodes en omgewingsfaktore, ontwikkel ‘n verskeidenheid ekotipes gedurende die kweking en aanpassing van die gras. *Chrysopogon nigritanus* (Benth.) Veldkamp (1999), is inheems tot Afrika en is naby verwand aan *C. zizanioides*. *C. nigritanus* en *C. zizanioides* verskil minimaal van mekaar op ‘n morfologiese vlak. Die hoof verskil tussen die bogenoemde spesies is dat *C. nigritanus* die vermoë het om vrylik fertiele saad te vorm en as gevolg van hierdie eienskap moet die spesies verkieslik nie gebruik word vir erosiebeheer nie. Die noodsaaklikheid het ontstaan om ander steriele lyne te vind en dus om addisionele genotipiese variteite op te spoor sodat die aanplantingsmateriaal se biodiversiteit behoue bly. Die hoof doel van die studie was om 19 verskillende Vetiver isolate, verkry vanaf Hydromulch (Pty) Ltd., genotipies te karaktiseer met genetiese volgorde analise van drie DNS fragmente, *ITS, ndhF* en *rbcL.* Addisioneel is die wortel anatomie ook ondersoek en vergelyk met die genetiese analise se resultate. Op grond van die resultate kan daar waargeneem word dat daar min of geen genotipiese verskille was tussen die verskillende isolate nie wat dui op plastisiteit. Slegs in die geval van die *ITS* geen-analise het drie van die isolate ‘n verskil getoon. Daar is geen noemenswaardige verskil tussen die verskillende isolate op grond van wortelanatomie nie, met die uitsondering van twee van die isolate, wat styselgranules gevorm het.

Sleutelwoorde:
Vetiver, *Chrysopogon zizanioides, Chrysopogon nigritanus ITS, ndhF, rbcL,* Genetiese diversiteit, wortel anatomie.

ABSTRACT

Vetiver grass or *Chrysopogon zizanioides* (L.) Roberty (1960) is a sterile grass which can regenerate vegetatively from clumps of the rootstock. This, as well as its vigorous and deep root system and flood tolerance makes it an ideal candidate for the use in soil remediation and erosion control. In South Africa, Hydromulch (Pty) Ltd. is part of the landscape, soil reclamation and erosion control industry. The company uses vetiver grass on a wide scale

and has accumulated a collection of isolates to serve as possible germ lines for industrial use. Due to the different approaches in environmental management as well as environmental factors, a variety of ecotypes form during the planting and acclimatisation of this genus. *Chrysopogon nigritanus* (Benth.) Veldkamp (1999), which is a native species to Africa, is closely related to *C. zizanioides* and differs only slightly from *C. zizanioides* on a morphological level. The major difference between the two species is that *C. nigritanus* is able to seed freely and thus the use of this species should be avoided. The need arose to screen other non-fertile plants to uncover additional genotypic variety to enable diversification of vetiver plantings. The aim of this study was to characterise the genotype of 19 isolates of vetiver obtained from Hydromulch (Pty) Ltd. via sequencing analyses of three DNA fragments, *ITS*, *ndhF* and *rbcL*. In addition, the radial root anatomy was also investigated and compared with the genetic analyses. According to the results generated during this study, very little or no genotypical differences exist amongst the different isolates available from the Hydromulch (Pty) Ltd. plant collection. Only in the case of the *ITS* inference were differences observed between three of the studied isolates. There was no significant difference between the different isolates based on the root anatomy, with the exception of two of the studied isolates which formed starch granules.

Keywords:

Vetiver, *Chrysopogon zizanioides, Chrysopogon nigritanus ITS, ndhF, rbcL,* Genetic diversity, root anatomy.

ACKNOWLEDGEMENTS

Foremost, I thank and praise God for providing me with the opportunity to do this study. He gave me the strength and patience to finish this study.

I would like to express my sincere gratitude to my promoter, Prof. Sandra Barnard, for the continuous support of my study and research, for your patience, motivation, enthusiasm, and immense knowledge. Your guidance and support helped me in all the time of research and writing of this paper. I could not have imagined having a better advisor and mentor for my Masters study.

Dr. Karin Conradie and Dr. Wayne Towers, thank you for allowing me to do the lab work in your lab, and also for your guidance and support with the sequencing, alignments and phylogeny. Karin, thank you for being such a helpful and kind co-supervisor, always supporting and helping me with everything. I am forever grateful.

My parents, who helped me emotionally through the study, always encouraging me to do my best. Thanks to all my family who supported me through this.

Thank you, Mr Roley Nöffke from Hydromulch (Pty) Ltd. for your financial support and plant material used during this study. I'm grateful for the inputs and technical help and permission to publish this data, as well as the opportunity to present our findings in India. Thank you Prof. Paul Truong, Dr. Mark Daffron and Dr. Jane Wright for valuable discussions and input. Thank you, Dr. P.A. Christin for the primers used in this study.

Jacques and Monica for letting me stay with you for months in Potchefstroom to finish my dissertation. Thank you for your friendship and support. I also want to thank my Bible study group and friends for all their prayers and support.

Dr. Tiedt, for assisting me with the microscopy. Thank you for your advice and support over there. Madeleen, thank you for helping me with the herbarium examplers and also for your insight with the data analyses. Dr. Jordaan, thank you for your contribution.

Thank you Prof. Leon van Rensburg for the support necessary to finish this project.

I acknowledge with gratitude the Central Analytical Facilities: Stellenbosch University, the North West University and the School of Environmental Sciences and Development for

providing the funds and facilities to make this study possible. To all those who have given advice, showed interest, encouraged, or in some or other way contributed in the completion of this dissertation, I give my sincere thanks.

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CHAPTER1 INTRODUCTION

"Science means progress, and progress means changes, and no list of 'Names in Current Use', as proposed by some, will or should stop that"- Veldkamp (1999)

Vetiver grass (*Vetiveria zizanioides* (L.) Nash, reclassified as *Chrysopogon zizanioides* (L.) Roberty (Roberty, 1960) is a perennial grass of the Poaceae family, which originated from either Indonesia or India (Dong *et al.,* 2003). Other names for Vetiver in Indian include Khus Khus, Ya Faek, Cuscus and Vetivert (Srifah *et al.,* 2010).

C. zizanioides is closely related to *Sorghum spp.* but also shares many morphological characteristics with other fragrant grasses such as lemongrass (*Cymbopogon citratus* (DC.) Stapf), and citronella (*Cymbopogon nardus* (L.) Rendle)*, Cymbopogon winterianus* Jowitt).

Chrysopogon zizanioides has traditionally been used for the essential oils it produces in the plant's root system. It was first used in soil and water conservation in India during the mid-1980s (Truong *et al*., 2008) by planting hedges across a slope to limit rainfall runoff and as a result decrease soil erosion. This application still plays an important role in the management of the environment, but during the past years it has been demonstrated that it can also be successfully used in bioengineering of steep slope stabilization, and as a phyto-mitigator of contaminated land and water (Truong *et al*., 2008). The main reason for *C. zizanioides* being used on such a wide scale is because it is sterile (does not produce fertile seeds), and propagates itself by small offsets instead of underground stolons. This makes it non-invasive and easily controlled. However, even though *C. zizanioides* is sterile, some genotypes do produce flowers and sterile seeds (Truong *et al*., 2008).

C. zizanioides is known to be also cultivated in Africa and in particular in South Africa since at least 1892 (Chippindall, 1955). A close relative of *C. zizanioides* is *Chrysopogon nigritanus* (Benth.) Veldkamp (Veldkamp, 1999), which is a wetland species native to Africa. Traditionally, *C. nigritanus* is used by farmers in Africa as a wind break, to separate fields as well as for mulch. The leaves are also used to make mats in countries such as Senegal (Goudiaby *et al.*, 2010). It is also used as an erosion control agent, building material, water disinfectant, the production of handicrafts out of the roots and leaves, as well as grazing material for cattle (Goudiaby *et al.*, 2010). *C. nigritanus* produces fertile seeds, which makes it unsuitable to use in erosion control globally as it may become a weed (Veldkamp, 1999). According to Veldkamp (1999), the differences between *C. zizanioides* and *C. nigritanus* are

so slight, they may easily be confused. Both species' leaves are similar in shape and colour. The only noticeable differences are observed in the root system and in their size and performance (Adams, 2002). The root system of *C*. *nigritanus* is dense, hardy, but rarely extends beyond 75 cm; whereas the root system of *C. zizanioides* exceeds 75 cm. *C. zizanioides* also has the ability to grow new adaptive adventitious roots on its leaf stalk (Adams, 2002).

Due to the apparent lack of genetic variation in this organism as a result of the fact that cuttings from single clones are distributed, plantings of this grass are very vulnerable to disease and insects. This may lead to millions of erosion control terraces being destroyed (Adams & Dafforn, 1999; Adams, 2002). Different approaches in environmental management as well as environmental factors, causes *C. zizanioides* to form a variety of ecotypes during the planting, acclimatisation and domestication of this genus (Dong *et al.,* 2003).

In South Africa, the major source of planting material is the vegetatively propagated slips of Vetiver (*C. zizanioides*). The company Hydromulch (Pty) Ltd. has established a Vetiver Grass Nursery on its farm which is situated 20km north of the Johannesburg International Airport. The company is able to supply Vetiver Grass slips/plants to any destination worldwide but in particular to African countries (Hydromulch, 2007). It can be used for the rehabilitation of soil in regions outside the grass's native countries, because it has the ability to easily acclimatise to new environments (Truong, 1999). The genus has the ability to withstand extreme temperatures, drought, high humidity and pH changes (Dong *et al.,* 2003). According to Juliard (2006) over 100 countries are currently using Vetiver, such as China, Iran, Vietnam, Brazil, Mexico and the USA. The use of vetiver has undoubtedly become a highly valuable eco-engineering technology in water and soil conservation (Dong *et al.,* 2003) due to low investment costs, short establishment periods and quick effect in the planting and growth of this species (Dong *et al.,* 2003).

Hydromulch (Pty) Ltd. uses Vetiver grass (*C. zizanioides*) on a wide scale and has compiled a collection of isolates to serve as possible germ lines for industrial use. The need arose to screen these other non-fertile plants in order to uncover additional germ lines so as to enable diversification of Vetiver propagation. Furthermore the native genus, *C. nigritanus* is morphologically very closely related to *C. zizanioides* and could therefore be confused for *C. zizanioides.* This may cause that *C. nigritanus* is used for erosion control instead of *C. zizanioides*, which may lead to the plants becoming uncontrollable as this subspecies can from fertile seeds.

This study will focus on the genetic diversity as well as the root anatomy of the probable *C. nigritanus* and *C. zizanioides* types available in South Africa. The genetic data will be compared in conjunction to the anatomical data to investigate the diversity of the germplasm available in South Africa. The results obtained from this study will give a clearer understanding about the relationship of the studied *Chrysopogon* lines to each other. This is the first study of its kind pertaining to the germplasm available in South Africa and the results will not only serve as a base for future genetic and anatomical studies but also play a vital role in the planning and management on the use of Vetiver grass as the differences between these lines are so slight they may be confused with each other, which may lead to *C. nigritanus* becoming a pest when it is mistaken and planted as *C. zizanioides* (Veldkamp, 1999).

The objectives for this study will include:

1. Determination of the genetic diversity of the Vetiver grass isolates available in South Africa obtained from Hydromulch (Pty) Ltd. using by sequencing of the *ITS I, ITS II, 5.8S* rDNA as well as two chloroplast genes: *rbcL* and *ndhF*;
2. The comparison of potential anatomical variations observed in the roots of the different studied isolates of *Chrysopogon* to determine if the roots can be used to distinguish between the different isolates obtained from Hydromulch (Pty) Ltd.

CHAPTER2 LITERATURE REVIEW

Vetiver grass or *Chrysopogon zizanioides* was originally used for its aromatic oil extracted from the roots (Dong *et al.*, 2003). However, since the 1980's the occurrence of Vetiver has increased tremendously throughout the world due to the widespread planting of this species to form hedges for soil stabilization and erosion control (Adams, 2002).

C. zizanioides is economically beneficial on several levels by utilizing the harvested grass in a) non-processed products such as roof thatches, compost, mulch, mushroom medium, animal fodder and bouquets, b) semi-processed products including handicrafts (Dong *et al.,* 2003), botanical pesticides, pots and furniture, and c) fully processed products, including essential oils and its derived products, herbal medicine, pulp and paper, pozzalan cement and industrial products (Dong *et al.,* 2003).

Chrysopogon zizanioides was not traditionally used as an erosion control agent, but during the 1980's the World Bank applied Vetiver as a solution to control erosion and other environmental problems. In 1993 the USA Academy of Science conducted a major study on the understanding of the use of Vetiver worldwide, but specifically focussed on its use in Africa (USNAS/NRC, 1993). The conclusions were that *C. zizanioides* is more widespread globally then *C. nigritanus*. Their study also found that *C. zizanioides* was a sustainable solution to enhance food production of other crops as well as mitigate erosion problems when the plants were planted in simple hedges across slopes (USNAS/NRC, 1993). What makes *C. zizanioides* very promising, according to this study, is that adult plants can survive long periods of drought and can be planted in any soil type and climate in Africa (The World Bank, 1993). However, it was concluded that regional level testing and research was a necessity. Eleven Western African countries were approached to initiate research projects on *C. zizanioides,* but there was limited interest. However, since 2000 the Vetiver system (VS) (see Section 2.1) has been applied in a number of African countries, such as Senegal, Mali, Burkina Faso and Madagascar (Juliard, 2006).

Currently, *C. zizanioides* is cultivated for several different reasons: It stabilizes the soil: it is useful in erosion control, protects fields against pest and weeds and is also used as animal feed. The root extracts are also used for its essential oil used in cosmetics and aromatherapy. Because of the plant's fibrous properties, it is also used by the local communities (in Africa, Thailand and Vietnam) for making ropes and handicrafts (Juliard, 2006).

2.1 The Vetiver System (VS)

The VS was initialized in 2000 by a small group of individuals interested in the application of *Chrysopogon zizanioides*. This was done based on the following assumptions:

1. Vetiver had commercial value;
2. There was sufficient information and experience with the VS in other countries to reduce the risk entrepreneurs would take to invest and apply the technology; and
3. The program would require the active participation of a more diversified group of stakeholders than the traditional partners targeted in agriculture, water and forestry and research departments (Juliard, 2006).

The following topics were included in meetings regarding the VS:

1. Conducting an initial study of existing knowledge, use, distribution and availability of plant material;
2. Establishing demonstration sites;
3. Organizing information transfer days and training cycles;
4. Promoting research;
5. Encourage private nurseries to multiply Vetiver;
6. Formulating a communication strategy and participate in agriculture and environmental fairs;
7. Assist neighbouring countries to develop a Vetiver program; and
8. Identifying at least one major buyer of the technology.

The first major research was done in Senegal, where 10 000 slips of the certified *C. zizanioides* species from South Africa was distributed to promote the VS. A two-part communication strategy was started, which included the development of research and the adaptation to the local context, and an outreach effort that included making presentations and displays for target groups, including the public sector, non-governmental Organisations, professional associations, environmental groups and companies in relevant businesses (USNAS/NRC, 1993).

The application of the VS includes roadside protection against soil erosion, water purification and the enhancement of agriculture, in which *C. zizanioides* is used to enhance water retention in plantations and dune stabilization. Juliard (2006) also made the observation that the VS system was easily accepted by countries where traditional Vetiver already existed, but wasn't known to the public or used for soil and water rehabilitation (Truong *et al.*, 2008).

2.2 The role of Vetiver in ecological remediation

Phytoremediation is the technology of using plants to mitigate pollutants in the environment. The benefits of phytoremediation are that it is economical, energy efficient and environmentally friendly, and can also be applied to large areas. Vetiver is used in phytoremediation and is a practical and inexpensive plant, yet provides an effective method for soil and water conservation because of the following characteristics:

1. It has the ability to grow upright and to form dense hedges (Srifah *et al.*, 2010);
2. It has a vigorous deep root system (Srifah *et al.*, 2010) which can grow mostly downwards for up to 2-4 meters;
3. Plants grow in close clumps that help to block runoff surface water (Truong *et al.*, 2008);
4. Vetiver propagates by small offsets instead of underground stolons or seeds, which makes it non-invasive and controllable (Truong *et al.,* 2008);
5. It is very adaptable to a variety of ecological conditions (Dong *et al.,* 2003);
6. Vetiver is an ecological climax species, making it perennial, surviving for decades (USNAS/NRC, 1993);
7. Vetiver can withstand drought as well as high levels of flooding (Truong *et al.,* 2008);
8. It is tolerant to high levels of pesticides and herbicides (Cull *et al.*, 2010); and
9. It is tolerant to a wide range of toxins as well as heavy metals (Shu & Xia, 2003).

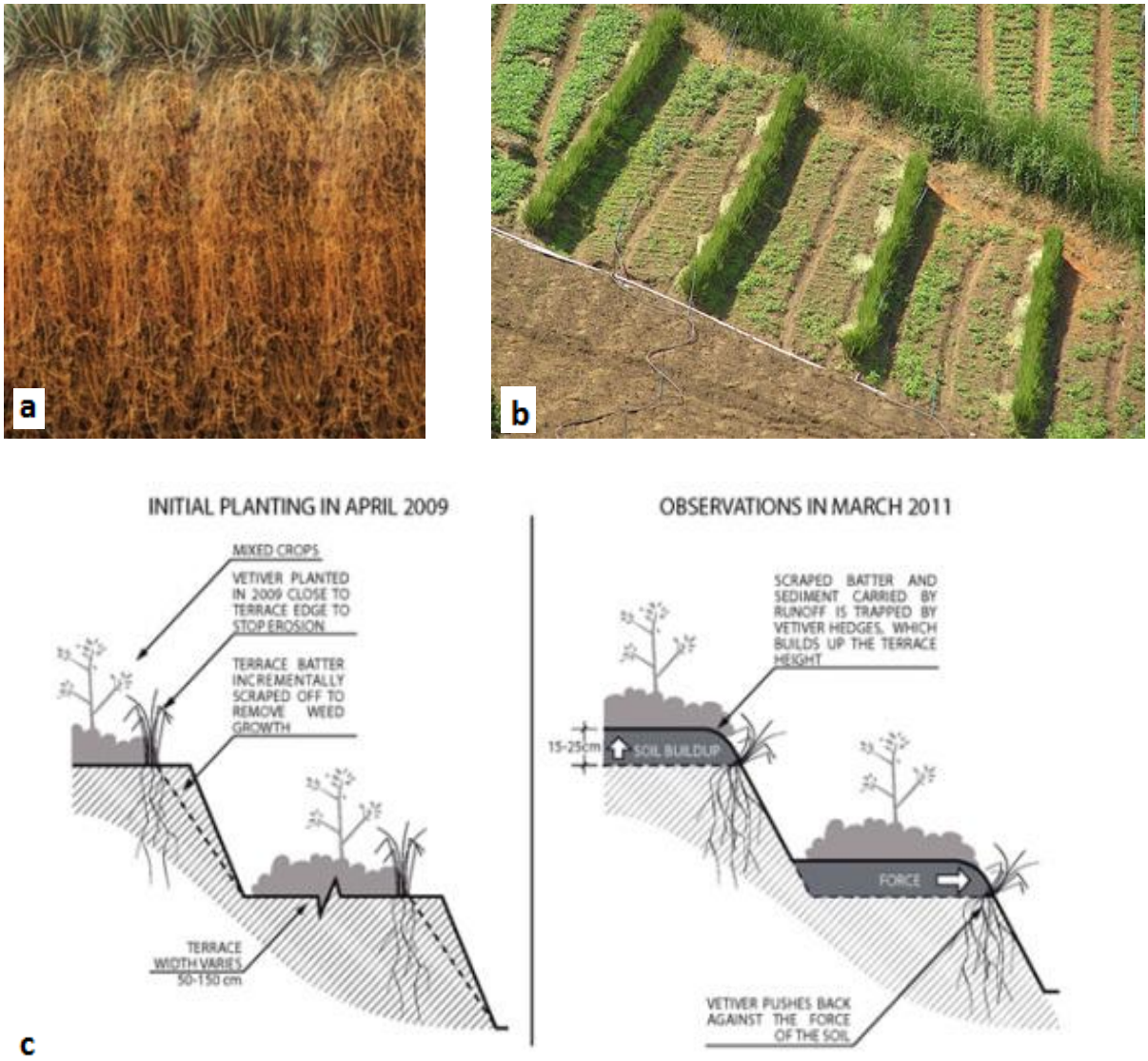

Figure 2.1 *Chrysopogon zizanioides* used for erosion control (a) the root system of *C. zizanioides* grown as a hedge, (b) *C. zizanioides* used for erosion control in Hawaii, (c) The effect of *C. zizanioides* on slope stability (Taken from Calderon & Truong, 2011).

Traditionally, *Chrysopogon nigritanus* is used by farmers in Africa as a wind break, to separate fields (Juliard, 2006) as well as for mulch. It is also used as an erosion control agent. In the region of the Delta River region of Senegal, Vetiver is used as a building material, mixed with cement. The roots of *C. nigritanus* are also used as a water disinfectant agent due to its anti-microbial properties (Juliard, 2006). Other uses include making necklaces from the roots, the production of handicrafts out of the roots and leaves, and the young, odourless leaves provide grazing material for cattle during the dry season (Juliard, 2006). *C. nigritanus* was also traditionally used as a natural antiseptic agent as well as an insect repellent. Because the use of *C. nigritanus* is rooted in the countries of different

African cultures, it is thought that this plant has mystical properties (Juliard, 2006; Goudiaby *et al.*, 2010). In the northern State of Kano in Nigeria, the roots of *C. nigritanus* is used as food (Juliard, 2006). Very little information is available on the usage and availability of the local variety, *C. nigritanus,* which grows in West Africa (Goudiaby *et al.,* 2010; Juliard, 2006). In two studies done on the performance of this species in erosion control (one in Burkina Faso and the other one in Ghana), it performed better than other local gramineae, but not as well as *C. zizanioides* (Juliard, 2006).

2.2.1 *Conclusion*

The use of *C. nigritanus* is solely for traditional purposes, and no known effort has been made to propagate this plant commercially (Juliard, 2006). *C. zizanioides*, on the other hand, has been applied in many phytoremediation projects, and has had a high success rate (Hydromulch, 2007), including in South Africa. Much research has been done on this aspect on Vetiver, especially on the ability of Vetiver to be used in water and soil conservation (Roongtanakiat, 2009; Africa, 2002; Wensheng & Hanping, 2010; Chena *et al.*, 2004).

Currently the Vetiver system is used in more than 100 countries and is propagated by clump subdivision. Mature Vetiver hedges are able to reduce rainfall runoff by 70% and sediment by almost 90%. Because this technology is cost effective and beneficial, the plant has been called the “living Soil Nail” by engineers (Truong *et al.*, 2008).

2.3 Origin and Taxonomical Overview

Table 2.1 The classification on *Chrysopogon* (USDA, 2012)

Subkingdom	**Tracheobionta**	**Vascular plants**
Superdivision	**Spermatophyta**	**Seed plants**
Division	**Magnoliophyta**	**Flowering plants**
Class	**Liliopsida**	**Monocotyledons**
Subclass	**Commelinidae**	
Order	**Cyperales**	
Family	**Poaceae**	**Grass family**
Genus	***Chrysopogon* Trin.**	**false beardgrass**

The Vetiver grass species presently found in South Africa (*C. zizanioides* and *C. nigritanus*) originated in India and Africa respectively. *C. nigritanus* is an indigenous species to Africa and it can also be found in Kwa Zulu Natal South Africa. It is abundant and sometimes dominant in marshes. (ORDPB, n.d.). The problem is, however, that the differences between *C. zizanioides* and *C. nigritanus* are so slight they may be confused with each other, which may lead to *C. nigritanus* becoming a pest when it is mistaken and planted as *C. zizanioides* (Veldkamp, 1999). It is unknown when *C. zizanioides* was first introduced into South Africa from the East. However, this species was grown at Ventersdorp in 1892 (Chippindall, 1955). It was brought from Cape Town by ‘n Voortrekker family, and was used for scenting bags (coffers) (Chippindall, 1955). The aim of the book published by Chippindall was to assist farmers to identify the grass species on their farms and to manage it appropriately. In this volume Chippindall identified, classified, and noted the distribution 952 grasses known to occur in South Africa. According to Grimshaw the *C. zizanioides* present in South Africa is generally genetically identical to the *C. zizanioides* Monto of Queensland, Australia and the *C. zizanioides* Sunshine from Louisiana, USA and is considered to be non-invasive (Grimshaw, n.d.).

2.3.1 *Vetiveria or Chrysopogon*

Chrysopogon zizanioides was first classified as *Phalaris zizanioides* L. Mant (1771) (Veldkamp, 1999). The name *Vetiveria zizanioides* (L.) Nash (1903) is more commonly used today even though it was reclassified as *Chrysopogon zizanioides* (L.) Roberty (1960). Veldkamp (1999) revised the identification and nomenclature of this group to provide clarity and kept it as *C. zizanioides*.

Chrysopogon and *Vetiveria* have traditionally been viewed as two related, but distinct entities, either as subgroups of *Andropogon* L., or as totally different genera (Veldkamp, 1999). However, occasional remarks have been made of the presence of a possible intermediary species. *Vetiveria* and *Chrysopogon* were merged into the group *Chrysopogon* by Roberty (1960), but this grouping did not receive any taxonomic recognition until the revision by Veldkamp (1999). This revision by Veldkamp (1999) and references therein state that: *Vetiveria* and *Chrysopogon* can be grouped together due to the similarity of the spikelet structure (Blake, 1944; Clayton & Renvoize, 1986). *Vetiver zizanioides* is considered as the most primitive form of *Chrysopogon* because of the transverse articulation of the several-noded partial inflorescences with well-developed pedicelled spikelets, and a short and obtuse, glabrous to setulose callus (Veldkamp, 1999). *Sorghum nitidum (Vahl) Pers., Syn. Pl. 1: 101 (1805)* seems to be the most similar taxon to *Chrysopogon* based on their observations. However it is not necessarily the most closely related (Clayton & Renvoize 1986). *Chrysopogon* also shares many morphological characteristics with other fragrant grasses, such as lemongrass (*Cymbopogon citratus*), citronella (*Cymbopogon nardus*, *C. winterianus*), and palmarosa (*Cymbopogon martinii* Roxb. wats. var. motia) (USDA1, n.d.).

Chrysopogon might be derived from *Vetiveria* as they may be joined by an intermediate species. This observation was based on *Chrysopogon* arbitrarily having 1- or 2-jointed racemes and an acute to pungent callus, but observed that *Vetiveria* consists of all stages of reduction from multi-joined racemes and of elongation of the obtuse callus and most species of *Chrysopogon* consists of 1-jointed racemes of 3 spikelets ('triad') and a pungent callus (Veldkamp, 1999).

The species which presumably integrates *Chrysopogon* and *Vetiveria* is *Chrysopogon sylvaticus* C.E.Hubb. (1938), however according to Clayton & Renvoize (1986) the separation of the above two genera are subjective, especially in Australia. It is partially justified by the convenience of treating the compact cluster of species with triads as a single entity, and also, Clayton & Renvoize (1986) suggested that *Vetiveria pauciflora*, with only 2 or 3 spikelet pairs per raceme, links *Vetiveria* to *Chrysopogon* (Veldkamp, 1999; Clayton & Renvoize, 1986).

More recently, the relationship between *V. zizanioides* and other *Chrysopogon* species, as well as *Sorghum* and *Vetiveria* were determined and compared genotypically by Adams *et al.* (1998). Eighteen accessions of *Vetiveria, Chrysopogon* and *Sorghum* were analysed using RAPD analysis. The *V. zizanioides* (as identified by the authors) accessions were

obtained from all over the world, including India, Haiti, Monto, Australia, New Zealand, the USA, Thailand, China, Venezuela, Costa Rica and South Africa.

Results showed that *C. gryllus* and *C. fulvus* are more closely related to *Vetiveria* than to each other, which initiated the further comparative studies on the relationship between *Vetiveria* and *Chrysopogon,* and ultimately led to the confirmation of the name change of *V. zizanioides* to *C. zizanioides* by Veldkamp (1999). Also, almost all the *Chrysopogon* samples analysed was noted to be derived from a single genotype, "Sunshine."

Chrysopogon is divided into informal groups based on the relative length of the pedicel (distinctly less than half as long as the sessile spikelet vs. more than half as long) and whether it is setose or glabrous. According to USDA (2012), there are 8 recorded *Chrysopogon* species, but according to Veldkamp (1999) and references therein, there are 11 species in Thailand and 13 in Malaysia, including *Chrysopogon aciculatus* (Retz.) Trin., Fund. Agrost. 188. 1820, *Chrysopogon borneensis* Henrard., (Blumea 4: 534, 1941), *Chrysopogon celebicus* Veldkamp (Veldkamp, 1999), *Chrysopogon festucoides* (C.Presl) Veldkamp (Veldkamp, 1999), *Chrysopogon filipes* (Benth.) Reeder (Veldkamp, 1999), *Chrysopogon fulvus* (Spreng.) Chiov. (Veldkamp, 1999), *Chrysopogon intercedens* Veldkamp (Veldkamp, 1999), *Chrysopogon lawsonii* (Hook.f.) Veldkamp (Veldkamp, 1999), *Chrysopogon micrantherus* Veldkamp (Veldkamp, 1999), *Chrysopogon nemoralis* (Balansa) Holttum (Holtt., 1947), *Chrysopogon orientalis* (Desv.) A.Camus (Camus, 1925), *Chrysopogon perlaxus* Bor (Larsen, 1965), *Chrysopogon serrulatus* Trin. (Veldkamp, 1999), *Chrysopogon subtilis* (Steud.) Miq. (Veldkamp, 1999), *Chrysopogon tenuiculmis* Henrard. (Blumea 4: 532, 1941), *Chrysopogon zizanioides* (L.) Roberty (Roberty, 1960), *Chrysopogon argutus* (Steud.) Trin. Ex B.D.Jacks. (Veldkamp, 1999), *Chrysopogon benthamianus* Henrard, (Blumea 4: 532, 1941), *Chrysopogon elongatus* (R.Br.) Benth. (Bentham, 1878), *Chrysopogon fulvibarbis* (Trin.) Veldkamp (Veldkamp, 1999), *Chrysopogon gryllus* (L.) Trin. (Trinius, 1820), *Chrysopogon nigritanus* (Benth.) Veldkamp (Veldkamp, 1999), *Chrysopogon oliganthus* Veldkamp (Veldkamp, 1999), *Chrysopogon rigidus* (B.K. Simon) Veldkamp (Veldkamp, 1999), *Chrysopogon fuscus* (Presl) Trin. ex Steud. (Veldkamp, 1999), *Chrysopogon leucotrichus* A. Camus (Camus, 1955), *Chrysopogon strictus* (Nees) B.D. Jacks (Veldkamp, 1999) and *Chrysopogon villosulus* (Steud.) W. Watson (Veldkamp, 1999).

2.3.2 *Chrysopogon zizanioides*

Description according to Veldkamp (1999)

Chrysopogon zizanioides is a perennial grass, which forms culms of about 1.5 to 2.5 meters tall. Its ligule is 0.3–0.75 mm in length, its leaf blades are conduplicate, 23–94 cm by 2.5–7 mm wide, adaxially pilose in the lower part. The panicle is 20–33 by 2.5–6 cm in outline, with many branches and spikelets, purplish; lowermost branches whorled, with longest branch simple, 5.5–12 cm in length. The raceme peduncles are 1–4 cm in length, smooth to scaberulous, with 6–14 spikelet groups per branch. The joints are 3.75–6.75 mm in length, glabrous to setulose. Sessile spikelets are 3.75–6 mm in length (including the callus). The callus is rounded, 0.6–0.8 mm in length, laterally ciliate at the base, especially near the base of the pedicel, with white hairs which are 0.1–1.35 mm long. The lower glume is spinulose, aculeate, especially on the nerves and setulose, with apex acute. The upper glume is aculeate, especially on the midrib and midrib distally setulose, without a dorsal fringe of hairs, with apex muticous. The second lemma is muticous to mucronate, the awn is usually enclosed, straight, 0–1.95(–4.5) mm long, with a glabrous column. Three anthers are present that are 1.65–2.25 mm in length. The pedicel is 2.25–4.3 mm long, more than half as long as the sessile spikelet, and is also scaberulous. The spiklets are pedicelled with 1 male floret, 2.85–4.6 mm long. The lower glume is scaberulous, aculeate, especially on the nerves, and is muticous. The upper glume is muticous. The anthers are 1.65–2 mm long. 2n = 20.

There are two noted forms of *C. zizanioides*. The first type is a wild, flowering and seeding type which originated in North India. It has shallow roots that contain the highly laevorotatory ‘Vetiver oil’, and is widely-cultivated. Then there is the usually non-flowering and sterile type, of which the exact origin is unknown, but it is believed that it may have originated from South India. This type has deep roots that contain the dextrorotatory ‘Oil of Vetiver roots’ (Veldkamp, 1999). Although, this distinction between the two types is made, there are not enough morphological differences to distinguish the two types from each other (Ramanujam & Kumar, 1964). This is because the characteristics are only observed in live clumps, and not in herbarium material. According to Veldkamp (1999), *C. zizanioides* may be confused with *C. nemoralis*, which may lead to the misapplication of the latter, especially in Thailand.

2.3.3 *Chrysopogon nigritanus*

Chrysopogon nigritanus (Benth.) Hack. (1889), Mon. Androp: 544; *Andropogon nigritanus* Benth. (1894) Niger FL 573; *Vetiveria nigritana* (Benth.) Stapf. (1917), Fl. Trop. Afr. 9: 15; *Vetiveria zizanioides* var. *nigritana* (Benth.) A. Camus; *Chrysopogon zizanioides* var. *nigritana* (Benth.) Roberty (1960) Bull. Inst. Franç. Afrique Noire, A 22: 106; *Chrysopogon nigritanus* (Benth.) Veldkamp (Veldkamp, 1999).

Chrysopogon nigritanus is similar to *Chrysopogon festucoides.* This species has been reported in Sri Lanka, Thailand, Malaysia, Philippines, but according to Veldkamp (1999) all these records are probably based on misidentified specimens of *C. festucoides* or *C. zizanioides* (Veldkamp, 1999).

Chrysopogon nigritanus is a perennial grass, 150-300 cm in length. The inflorescence is large; the panicle is up to 40 cm long, with 8-10 whorls of up to 15 slender branches with sessile linear-lanceolate spikelets (Hyde *et al*., 2006).

Chrysopogon zizanioides is almost undistinguishable from *C. nigritanus* as the leaves are similar in shape and colour. The difference between the two species is below ground in the roots, their size and their performance. The root system of *C. nigritanus* is dense, hardy but rarely extends beyond 75 cm in length (Juliard, 2006). According to Juliard (2006), *C. zizanioides* can grow adventitious roots on its leaf stem, while *C. nigritanus* cannot. Juliard (2006) stated that there is little evidence that *C. nigritanus* was cultivated or multiplied, and also that the germline is fertile or can reproduce via seed formation. However, it is widely assumed that the present germline in Western Africa is in fact sterile, and only spreads to different regions via tillers which are detached from larger clumps and float to new areas as a result of floods (Juliard, 2006).

2.3.4 *Conclusion*

According to Veldkamp (1999), Vetiver is the closest related to *Sorghum* but also shares many morphological characteristics with other fragrant grasses, such as lemongrass (*Cymbopogon citratus*), citronella (*Cymbopogon nardus*, *Cymbopogon winterianus*), and palmarosa (*Cymbopogon martinii*). Currently *C. zizanioides* is widely cultivated all over the world, including Haiti, India, Java, Réunion and South Africa. However *C. zizanioides* is known to be cultivated in Africa, and since the differences in comparison with *C. nigritanus*

are so slight, they may easily be confused, and the uses attributed to *C. nigritanus* may well relate to *C. zizanioides* (Veldkamp, 1999). *C. nigritanus* is a wild species that may be expected to seed freely; use of the species for soil binding is to be discouraged, as it could escape and become a pest (USDA, 2012).

2.4 Genetic diversity of *Chrysopogon*

Studies done on the genetic diversity of Vetiver includes RAPD analyses of *Chrysopogon* germlines in India (Dong *et al.*, 2003), Thailand (Srifah *et al.,* 2010; Nakorn, 1993), the USA (Kresovich *et al.,* 1994), and other countries outside South Asia (Adams, 2002; Adams & Dafforn, 1999). All the studies concluded that different *Chrysopogon* accessions tend to form polymorphisms and that the different germlines can be distinguished from each other by using RAPD analyses. Srifah *et al.* (2010) discovered that one of the polymorphism occurs in the non-coding intron region of the ∆9 stearoyl-acyl carrier protein desaturase gene. However, the exact point of mutation (the gene sequence) is not known. Nakorn (1993) distinguished between two groups within the Thailand Vetiver accessions: the upland species (*C. nemoralis*), and a wetland species (*C. zizanioides*). Adams and Dafforn (1999) compared taxons from different places by using DNA fingerprinting, including Haiti, Australia, Venezuela, Panama, Costa Rica, USA, India, Thailand, China, Ethiopia, Peru, Netherlands, New Zealand, Philippines, Kenya, Colombia, Mexico, Mozambique, Malawi and South Africa, and concluded from their results that all the Vetiver cultivars which are used for erosion control outside of South Asia, are derived from a single germline, namely “Sunshine”.

Vetiver is thus widely used over the world and has settled far from its country of origin, in the areas of India, Vietnam and Africa (Adams, 2002). Due to the different approaches in environmental management as well as environmental factors, *Chrysopogon* can form a variety of ecotypes during the planting, adaptation and domestication of this genus (Dong *et al.,* 2003). Genetic variation of Vetiver grass has only been studied via indirect methods using RAPD’s and AFLP analyses. No literature could be found during this study where the direct method of sequencing was employed. In order to ensure the genetic biodiversity of this plant for its protection against pests and to understand the germplasm pool available for the management of propagation and uses of this grass we need to analyse the genetic diversity of these plants currently employed in the Vetiver Grass System more clearly.

2.5 Gene sequencing

Various gene sequencing methods have been developed in the past few years including the following:

1. Maxim-Gilbert method: based on chemical modification of DNA and subsequent cleavage at specific bases (Troy *et al*., 2001);
2. Chain-termination method or Sanger method: this method includes dye – terminator sequencing which is the most dominant and thorough sequencing technique for the past 30 years (Voelkerding *et al.*, 2009), using a DNA sequence trace chromatogram after capillary electrophoresis (Applied Biosystematics, 2011). This method produces more 300-1000 bp fragments and has 97% accuracy (Voelkerding *et al.*, 2009). The drawbacks are, however, that the Sanger method can only sequence relatively short sequences (300-1000 nucleotides), it takes more time to generate the sequences than new generation sequencers and that the first 15-40 bases of the sequence cannot be used due to poor quality and quality trimming is thus necessary (Christin *et al.*, 2008);
3. Amplification and clonal selection: A large-scale sequencing technique that enables the sequencing of long DNA strands such as entire chromosomes. This technique includes Emulsion PCR (Williams *et al.*, 2006) and Bridge PCR (Braslavsky *et al.*, 2003);
4. High-throughput sequencing (next generation sequencing): Sequencing technologies that produces thousands or millions of sequences simultaneously by running parallel sequencing processes (Hall, 2007). These techniques include the Lynx Therapeutics' Massively Parallel Signature Sequencing (MPSS) (Brenner *et al*., 2000), the Polony Sequencing (Porreca *et al.*, 2006), the Illumina (Solexa) sequencing (Mardis, 2008), SOLiD sequencing (Valouev *et al.*, 2008), DNA nano ball sequencing (Drmanac *et al.*, 2010) and Single molecule Real-time DNA sequencing (SMRT) (Eid, *et al*., 2009). These methods are less accurate than the Sanger method, but are faster (Voelkerding *et al.*, 2009).

2.5.1 *Genes previously sequenced from Chrysopogon*

Several genes or gene fragments have been sequenced from *Chrysopogon*. Among the genes analysed (partially as well as completely) in *C. zizanioides* were *matK* (Christin *et al.,*

2008), *trnK* (Christin *et al.,* 2008), *rbcL* (Christin *et al.,* 2008), *ndhF* (Christin *et al.,* 2008), *trnL*, *trnL-trnF*, *trnF* (Neamsuvan *et al.,* 2009), the 18S ribosomal RNA gene, *ITS* and 28S ribosomal RNA gene (Neamsuvan *et al.,* 2009). In contrast, since *C. nigritanus* is not used commercially, only a few gene fragments have been sequenced, including the 18S ribosomal RNA gene, the 5.8S ribosomal RNA gene, with the internal transcribed spacers I and II (Neamsuvan *et al.,* 2009). The genes/DNA fragments used during this study were chosen based on their sequence availability in related groups and their applicability in phylogenetic studies.

2.5.1.1 ITS I and II, and the 5.8S ribosomal gene

According to Alvarez & Wendel (2003), the internal transcribed spacer (*ITS*) region of the 18S–5.8S–26S nuclear ribosomal cistron, is widely used globally for phylogenetic inference at the generic and intra-generic levels in plants: out of 224 published journal papers they analysed, 66% of the papers compared different genus's using *ITS* sequence data, and 34% of all the published phylogenetic hypotheses have been based on only *ITS* sequences. The *ITS* region contains bi-parental inheritance. The 18S-26S rDNA arrays are present in the nuclear genome making this useful to reveal hybridisation, hybrid speciation and parentage of polyploids (Alvarez & Wendel, 2003). Furthermore, the primer sequences are universal (White *et al.,* 1990) which makes this a convenient gene to use. White, *et al*. (1990) published primer sets which could be used to amplify the *ITS* region of most plants as well as fungi. The 18S-5.8S-26S locus has a high copy number as well as a small size (500-700bp according to Baldwin *et al*., 1995), making this DNA fragment relatively easy to isolate and amplify via PCR reactions (Alvarez & Wendel, 2003). Another advantage of the locus is that it is intra-genomically uniform, limiting the mutations in the same genome which may lead to confusing variation and only leaving species- and clade-specific characteristics noticeable (Alvarez & Wendel, 2003). It is inter-genomically variable, making this sequence phylogenetically informative, showing nucleotide polymorphisms as well as insertion–deletion polymorphisms (Alvarez & Wendel, 2003; Baldwin *et al.,* 1995) and it has a low functional constraint. The function of *ITS* is related to the specific cleavage of the primary transcript within *ITS*-1 and *ITS*-2 spacers during maturation of the small subunit (SSU), 5.8S, and the large subunit (LSU) ribosomal RNAs (Veldman *et al.,* 1981; Alvarez & Wendel, 2003). Although this maturation and splicing process is dependent on the secondary structure of the *ITS* region, some degree of conservation at the sequence or at the structural level is noticeable (Alvarez & Wendel, 2003).

Several negative aspects have also been linked to the use of the internal transcribed spacer (*ITS*) region of the 18S–5.8S–26S rDNA, such as multiple rDNA arrays, which may lead to rDNA pseudogenes, which in turn may lead to an inaccurate phylogenetic analysis (Pedersen & Linde-Laursen, 1994). Concerted evolution within the ribosomal genes may also occur, which may lead to hybridization and polymorphisms, influencing the phylogenetic analysis of the *ITS* sequences and if analysed by themselves would not reveal the history of genomic merger, and the topology obtained for the allopolyploids would be misleading (Alvarez & Wendel, 2003). Secondary rDNA structures may occur due to the high GC content of the locus (Alvarez & Wendel, 2003) which may lead to base changes, especially in the conserved regions (Mai & Coleman, 1997; Alvarez & Wendel, 2003). The alignment, accuracy and rooting of this rDNA locus may also be influenced, because the sequence does not code for proteins, making it impossible to make an "alignment and error check" after every three nucleotides. This also may influence the process of scoring the sequence data (Alvarez & Wendel, 2003). The *ITS* sequence also evolves relatively quickly (Baldwin *et al.,* 1995; Alvarez & Wendel, 2003) making it challenging to make confident alignments between in-group and out-group sequences. This leads to situations where some phylogenetic analysis' are performed without out-group rooting (Alvarez & Wendel, 2003). Since the 5.8S and *ITS* genes are universal, the contamination of any plant or fungi may influence the results obtained which may lead to inaccurate phylogenetic trees (Alvarez & Wendel, 2003). The occurrence of homoplasy is also frequent in the analysis of the *ITS* gene which may influence the accuracy of phylogenetic analysis (Alvarez & Wendel, 2003). However, although homoplasy itself does not always influence phylogenetic analyses negatively, high levels of homoplasy may increase the risk of an incorrect inference (Alvarez & Wendel, 2003).

Although there are many negative aspects to the usage of the *ITS* locus, this gene fragment is phylogenetically informative. Due to the fact that it is easily isolated and that the primers are universal there are many sequences available on the databases which are useful for comparisons.

2.5.1.2 NdhF Chloroplast gene

The *ndh* genes in the plastid genome of higher plants were first reported after sequencing the chloroplast genomes of *Marchantia polymorpha* (Ohyama *et al.,* 1986) and tobacco (Shinozaki *et al.,* 1986). The plastid *ndh* genes encode components of the thylakoid *ndh* complex which acts as an electron feeding valve to adjust the redox level of the cyclic

photosynthetic electron transporters (Martín & Sabater, 2010). The *ndh* complex also encodes polypeptides homologous to subunits of the mitochondrial NADH-ubiquinone oxidoreductase complex (Catala *et al.,* 1997). These *ndh* genes are also present in most of the studied plastid genomes of vascular plants (Catala *et al.,* 1997; Hiratsuka *et al.,* 1989).

The NDHF polypeptide regulates the *ndh* complexes through phosphorylation reactions and is therefore an essential subunit of the *ndh* complex (Lascano *et al.,* 2003). NDHF is also encoded by a single gene which is not part of a multi-cistronic transcription unit, i.e. its transcription does not influence the transcription of other, unrelated, genes (Favory *et al.,* 2005). Favory (2005) also suggested that the SIG4 (via *ndh*F transcription), regulates the overall quantity of NDH complexes and, as result, influences the activity of NDH.

Plastid DNA sequences are highly conserved between different plant species, but Favory *et al.* (2005) noted that the upstream sequences of the *ndhF* gene fragment does not show real sequence conservation when the sequences were aligned.

Although only a few publications were found on the function of this gene fragment, many publications used the *ndhF* sequence in phylogenetic studies where the sequences are available in Genbank. This, and the fact that the *ndhF* gene fragment is considered to be less conservative, was the motivation behind the use of the *ndhF* gene in this study along with other genes fragments.

2.5.1.3 rbcL Chloroplast gene

The *rbcL* gene is the most common gene used in phylogenetic analyses (Chase *et al.,* 1993; Donoghue & Baldwin, 1993; Chase & Albert, 1998). It was chosen for generating the first large molecular dataset of angiosperms (Chase *et al.,* 1993) and is still used in large –scale analyses of green plants (Lewis *et al.,* 1997). It is a single copy plastid-encoding gene, approximately 1430 base pairs in length, which does not contain any length mutations except for at the 3' terminal, and has a fairly conservative rate of evolution (Hongping, 2003). The function of the *rbc*L gene is to code for the large subunit of ribulose 1, 5 bisphosphate carboxylase/oxygenase (RUBISCO or RuBPCase) (Hongping, 2003).

Barker *et al.* (1995) found in their research that the *rbcL* gene appears to be well suited for systematic studies in the Poaceae family, even though there was poor support for some relationships in the family. The disadvantage of using the *rbcL* gene according to Gielly &

Taberlet (1994) and Doebley *et al.* (1990) is that it often does not resolve the phylogenetic relationships below the family efficiently.

Although there is the possibility that the *rbcL* gene may show poor support between genotypes in this study, it is universally used to compare different genotypes on a systematic level, and because of this, there are many sequences available on the databases making this gene useful to compare the different germlines in our study by using this locus along with other genes fragments.

2.5.2 *Problems in Grass Systematics*

Although many systematic and evolutionary studies have been done on different genera in the Poaceae family, the following difficulties should be taken into consideration: 1) the simplicity of floral and vegetative morphology; 2) bidirectional character evolution, which makes it difficult to determine the polarity of a character during phylogenetic analyses (Stebbins, 1982; Stebbins, 1987); 3) widespread hybridization and polyploidy with about 80% of the taxa studied for chromosome numbers having undergone polyploidy during their evolutionary histories (Hongping, 2003); and 4) frequent parallel evolution caused by the adaptation to similar environmental conditions and a variety in evolution along the same line (Stebbins, 1987).

2.5.2.1 Conclusion

Because of the difficulties in the systematics in the Poaceae family, this study will compare the phylogenetic results obtained with the anatomical and molecular studies of the germlines used in this study. As stated in Hongping (2003), Poaceae has been studied extensively in the past few decades using morphological, anatomical, cytological, numerical, physiological, biochemical, and molecular approaches. Among these methods, DNA sequencing seems to be very promising.

2.6 ROOT ANATOMY

The root system of *Chrysopogon* has been studied in broad, but most of the studies done concentrated on the effect of different symbiotic relationships which Vetiver may form to improve oil production (Adams *et al.,* 2008; Monteiro *et al.,* 2011; Vollú *et al.,* 2012; Giudice *et al.,* 2008). To the author's knowledge, only a few in depth studies have been done on the anatomical variation of Vetiver roots as well as the effect of waterlogging on it (see section 2.6.2.2).

Studies have been done on the root system of rice, also a grass and therefore a related species to *Chrysopogon*. In 1970, Nicou *et al.* reported significant genetic variation for different rice roots studied, distinguishing between the upland and lowland cultivars of Asia, Africa, and South America. The root systems of different genotypes react differently to environmental conditions, as observed by Amelia *et al.* (2011). They reported a substantial genetic variation among different rice cultivars for root morphological traits, including root diameter (Armenta-Soto *et al*., 1983) and root growth plasticity (O'Toole, 1982; Ingram *et al.*, 1994; Price *et al.,* 2002). In another study, Lafitte *et al.* (2001) investigated the genotypic variation for root traits in different types of rice and reported that the xylem vessel size varied in different species.

The root structure of *C. zizanioides* has similar characteristics to *Oryza sativa* L. (1753), seeing that both genera can be cultivated in both water-logged areas as well as dry land, they both form a similar amount of aerenchyma when exposed to water-logged conditions, the cortextual cell arrangement in both is cuboidal and both have a strong barrier against radial O_2 loss (Goller, 1977; McDonald *et al.,* 2002), which therefor opens the possibility of anatomical variation between the different root systems of the isolates to be investigated during this study.

2.6.1 *Studies done on Vetiver roots*

Putiyanan *et al.* (2006) analyzed the internal characteristics of the roots of *Chrysopogon zizanioides*. The study included 7 cultivars from Thailand. Starch grains, oil granules as well as calcium oxalate crystals were visible in the roots of the different cultivars. The study concluded that the large vessels, fibers, sclereid cells and trichomes are slightly different among the studied cultivars based on the cell shape, detail and size of each tested strain.

Putiyanan, *et al.* (2006) concluded that the cell types have systematic value and could be used in the identification of the roots of different Vetiver strains.

2.6.2 *Phenotypic plasticity*

Phenotypic plasticity is the ability of individual genotypes to produce different phenotypes when exposed to different environmental conditions (Pigliucci *et al.*, 2006). Phenotypic plasticity enhances a plant's ability to survive its environment (Sultan, 2000). This may lead to the morphological and anatomical changes in plant organs, including traits such as root length (Ryser, 1998), as well as the increase of biomass (Bloom *et al.*, 1985; Gedroc *et al.*, 1996; Wahl *et al.,* 2001). This can also be seen in the case of *Chrysopogon* as seen in this study.

Interspecific variation in the root anatomy, such as the proportion of the stele, the proportion of cell walls of the vessels found in the stele, the characteristics of the xylem system, as well as the diameter of roots is closely linked with the ecological requirements of the plant species (Wahl & Ryser, 2000).

The phenotypic plasticity of anatomical characteristics of above ground plant organs has been well studied but few such studies have been done on roots (Wahl *et al.,* 2001).

Wahl *et al.* (2001) analyzed the phenotypic plasticity in the anatomical characteristics of roots in response to light and nutrient conditions. Their findings showed that a high nutrient supply leads to the production of larger xylem vessels, a decrease in root diameter and an increase in root tissue mass density. This may have contrasting effects on specific root length, which has been found to increase, decrease or remain unchanged in response to low nutrient supply (Ryser, 1998).

2.6.2.1 Effect of flooding on root morphology and anatomy in grasses

The lack of an external oxygen supply to support a plant's aerobic root respiration is one of the major stresses for a plant growing in flooded soil. Wetland plants adapt by developing aerenchyma, which provides a continuous, intercellular pathway for the rapid fluctuation of oxygen levels (Armstrong, 1979). According to Armstrong the most successful wetland

species have modified root morphology and anatomy to accommodate internal oxygen movement.

In the study conducted by Armstrong *et al.* (1991), *Typha* and *Cladium* both exhibited an aerenchymatous cortex, which facilitates internal gas transport in tissues growing in an hypoxic or anoxic environment. Also, the root sections showed thickening of hypodermal cell walls in differentiated regions distal to the root apex (Chabbi, *et al.,* 2000).

Justin and Armstrong (1987) conducted a study on the effect of flooding on 91 different plant species, including rice (*O. sativa*). Their findings were that most of the aerenchymatous species in their study had relatively wide roots or the benefit of a thickened (oxygen-impermeable) hypodermis. Graminacean-type aerenchyma was most widespread in the cortical types, such as in *O. sativa* (Justin & Armstrong, 1987).

Numerous studies were also done on the effect of light and nutrient availability on the morphology and anatomy of grass roots (Wahl *et al.,* 2001; Cruz *et al.,* 1992; Sultan, 2000; Grime *et al.,* 1991; Crick & Grime, 1987). These studies concluded that both these parameters have an influence on the cell size as well as the root formation. Wahl *et al.,* (2001) concluded that interspecific variation in ecological behaviour may be better explained using average characteristics of the different plants than by the amount of plasticity in these characteristics.

Because waterlogged soils are usually characterized by the absence of O_2 and a reduced chemical condition (Ponnamperuma 1984; Laanbroek 1990), a plant must be able to depend upon an internal supply of O_2 through the adaptation of the roots, which allows O_2 to move from the atmosphere through the aerenchyma in the plant to the root apex (Armstrong 1979). The efficacy of internal aeration within a root is determined by traits including: (i) anatomical features such as the extent of aerenchyma, stele dimensions, the pattern of cortical cell arrangement, and a barrier to radial O_2 loss; (ii) morphological characteristics such as root diameter and the amount of lateral roots; and (iii) physiological processes such as respiratory demand for O_2 (Armstrong *et al.,* 1991; Armstrong, 1979; McDonald *et al.,* 2002).

Because of this phenomenon, a lot of research has been done on finding a plant species that is suitable to survive the above mentioned conditions. Goller (1977) conducted the largest survey of the anatomical features of adventitious roots of grasses, involving 265 species from 127 genera of five subfamilies. Different root types were distinguished in his

study, namely Festucoid, Panicoid and Intermediate. The Festucoid type is characterized by the hexagonal packing of the cortical cells and a stele diameter ranging from less than 200 µm to a maximum of 400 µm (Poaideae subfamily). The Panicoid type has cuboidal packing of the inner cortical cells and a stele diameter ranging from 200 µm to greater than 400 µm (Panicoideae, Chlorideae, Bambusoideae and Arundinoideae subfamilies). The Intermediate type has cuboidal packing of the inner cortex (similar to the 'Panicoid' root anatomy), but its stele dimensions are similar to the 'Festucoid' root type (McDonald, *et al.,* 2002). The 'Intermediate' type is present in its most discrete form in genera from the Oryzoideae tribe such as *Oryza* (rice), but occurs in various forms in all subfamilies (McDonald *et al.,* 2002). The Intermediate type usually has a potentially greater cortex volume, the tissue in which aerenchyma is usually formed. The cuboidal packing in the cortex of 'Intermediate' root types also has a greater porosity than tissue with cells in a hexagonal arrangement (Kawase, 1981; Justin & Armstrong, 1987) and roots with cuboidal cortical cell packing tend to form extensive aerenchyma (McDonald *et al.,* 2002; Justin & Armstrong, 1987). These characteristics are important because they play a vital role for the plant to survive, and even thrive in O_2 depleted soils such as waterlogged fields (McDonald *et al.,* 2002).

Vetiver was classified as the Intermediate-Panicoid type and has the characteristics of both the Intermediate and Panicoid species in the study and is also considered to be a wetland species. Young Vetiver is easily negatively influenced by waterlogging, but mature Vetiver thrives under waterlogged conditions. (Truong *et al.,* 2008).

Table 2.2 Summary of different root types in the Poaceae with a representative species within each root type, the habitat, constitutive aerenchyma (10 mm below the root–shoot junction), stele size, cortical cell arrangement and Rapid Oxygen Loss characteristics of each representative species. Taken from McDonald *et al* (2002).

Root type	Representative species	Habitat	Constitutive aerenchyma (%)	Stele size	Cortical cell arrangement	Strong barrier to ROL
Festucoid	*Avena Sativa*	Dryland	15–20%	0·165–0·254 mm^2	Hexagonal	No
Intermediate-Festucoid	*Lolium multiflorum*	Wetland	8–13%	0·048–0·069 mm^2	Hexagonal	Yes
Panicoid	*Sorghum Bicolor*	Dryland	26–36%	0·368–0·588 mm^2	Hexagonal/ Cuboidal	No
Intermediate-Panicoid	*Vetiveria zizanioides*	Wetland	29–32%	0·682–0·703 mm^2	Hexagonal/ Cuboidal	Yes
Intermediate	*Oryza sativa*	Wetland	4–5%	0·031–0·033 mm^2	Cuboidal	Yes

In the study done by Truong (2001), *C. zizanioides* demonstrated to be an efficient method for treating effluent and leachate in water (figure 2.4) and is used in wastewater management globally (Truong, 2001; Truong *et al.,* 2008). This is significant because it demonstrates the ability of *C. zizanioides* to survive in flooded areas.

Figure 2.2 *Chrysopogon zizanioides* used for pollution control in the form of pontoons in an effluent pond at Toogoolawah (Truong, 2001).

2.6.2.2 Aerenchyma Formation

Aerenchyma is tissue which contains large gas-filled spaces (lacunae) that interconnect longitudinally to assist in long-distance gas transport along plant organs (Voesenek *et al.,* 2006). Aerenchyma increases the porosity of the tissue resulting from the usual intercellular gas-filled spaces formed as a constitutive part of development.

In roots, aerenchyma usually forms in the cortex (Armstrong, 1979) and the volume of aerenchyma formed depends on the genotype as well as environmental conditions the plant is exposed to (Voesenek *et al.,* 2006).

Two basic forms of aerenchyma have been distinguished (Voesenek *et al.,* 2006) namely 'schizogenous' aerenchyma and 'lysigenous' aerenchyma (fig 2.2). 'Schizogenous' aerenchyma develops when the cortex cells separate, and 'lysigenous' aerenchyma, when the cells in the cortex collapse. Even though there are only 2 main types of aerenchyma, different processes and conditions stimulate the formation of the above (Justin & Armstrong, 1987; Voesenek *et al.,* 2006).

In many wetland plants, aerenchyma forms constitutively, and is further enhanced as a response to flooding. In most non-wetland species, aerenchyma is mostly absent until the

environmental conditions are favourable for aerenchyma formation, like flooding (Armstrong, 1979). Soil compaction or nutrient deficiencies can also contribute to aerenchyma formation in certain species (He *et al*., 1996).

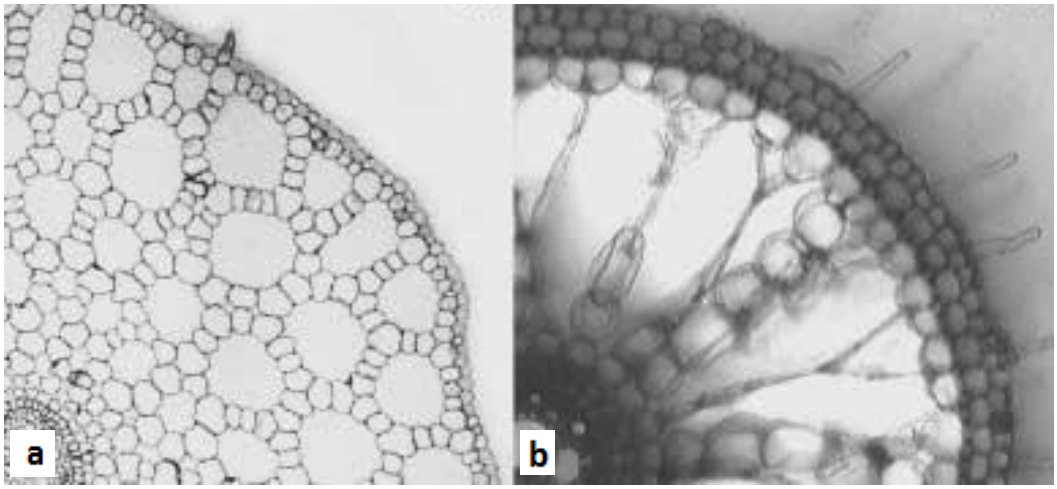

Figure 2.3 Two main types of aerenchyma: schizogenous aerenchyma in a *Rumex palustris* adventitious root (a), and lysigenous aerenchyma in a rice adventitious root (b). Schizogenous aerenchyma in *R. palustris* forms as a result of cells being forced apart because of oblique divisions by some of the cortical cells in radial rows. The lysigenous aerenchyma in rice forms because of the collapse of radial files of cortical cells (taken from Voesenek *et al.,* 2006).

Also, a lack of oxygen can lead to the development of more aerenchyma tissue (He *et al.,* 1999), whereas drought causes an increased deposition of suberin and lignin in the hypodermis and endodermis (Cruz *et al.,* 1992), and also a deposition of hydrophobic substances in epidermal cell walls (Watt *et al.,* 2001).

2.6.2.3 Anatomical studies done on related species

2.6.2.3.1 Xylem vessel size

The Hagen-Poiseuille law states that the conductance of a pipe increases with the fourth power of its diameter (Wahl *et al.,* 2001). The most cost-efficient way to increase the transport capacity of a root system is to increase the vessel diameter rather than producing more vessels in one root or by producing more roots (Wahl *et al.,* 2001). An increased xylem vessel size and total xylem cross sectional area as a result in stressful situations also

correspond well to the known relative consistency of the ratio between conducting cross sectional area and leaf area (Wahl *et al.,* 2001).

2.6.2.3.2 Exodermis and endodermis formation and function

The exodermis and endodermis serve as barriers, restricting the radial flow of water and other solutes through the roots (Hose *et al.,* 2001; Raven *et al.,* 2005) as well as defending the roots against microorganisms (Raven *et al.,* 2005). The primary walls of the exodermis and endodermis consist of Casparian bands (containing lignin and suberin), which direct the flow of material from the surface to the cortex, and from the cortex to the stele. The selectivity of the exodermis and endodermis may change during root development (Clarkson, 1993; Hose *et al.,* 2001) and is also known to vary in structure among species (Kroemer, 1903; Perumalla *et al.*, 1990; Hose *et al.*, 2001).

The majority of plants, with the exception of xerophytes, contain a uniform exodermis. The uniform exodermis may be uniseriate, as in the case of *Zea mays* and *Oryza sativa* (Perumalla *et al.*, 1990; Miyamoto *et al.*, 2001) or multiseriate as in the case of *Iris germanica* (Kroemer, 1903; Shishkoff, 1986; Peterson & Perumalla, 1990; Zeier & Schreiber, 1998), *Typha* spp. (Seago *et al.*, 1999) and *Phragmites australis* (Armstrong *et al.*, 2000; Soukup *et al.*, 2002), forming up to 20 layers (Hose *et al.,* 2001; von Guttenberg, 1968).

The exodermis is well developed in wetland plants and is underlined with a layer of lignified sclerenchyma cells as seen in *Oryza sativa* (Morita & Abe, 1999; Hose *et al.,* 2001) to prevent the outward diffusion of oxygen in this environment while still taking up the sufficient amount of water and nutrients (Colmer *et al.,* 1998; Armstrong *et al.,* 2000; Hose *et al.,* 2001).

The presence of an endodermis is observed in all roots so far tested except for some members of the Lycopodiaceae (Clarkson, 1993). The presence of tertiary lignified cellulosic walls is described in Meyer *et al.* (2009) and Zeier & Schreiber (1998). These wall thickenings are often U-shaped and sometimes it is difficult to identify the Casparian bands and suberin lamellae when these wall thickenings are present in the endodermis (Van Fleet, 1961; Clarkson, 1993). Contrary to the exodermis, the maturation of the endodermis is not influenced by the growth conditions (Meyer *et al.,* 2009).

2.6.2.4 Conclusion

Phenotypic plasticity plays an important role in the adaptation of genotypes in different environmental conditions. Pigliucci *et al.* (2006) stated that plasticity may be expressed at different levels, including behavioural, biochemical, physiological or developmental levels. There is a degree of reversibility depending on the kind of plasticity: typically biochemical and physiological responses can be reversed over short time periods, while developmental plasticity tends to be irreversible or takes longer to be reversed (Pigliucci *et al.*, 2006).

The anatomical as well as genotypical differences will give insight into the degree of plasticity in the different isolates being analysed and also how easily the isolates can adapt to different environmental conditions.

Because the root structure of *C. zizanioides* has a similar root structure to *O. sativa* (Goller, 1977), we hope to find differences in the anatomical structure of the roots of the different isolates. Differences expected include a higher percentage of aerenchyma formation in the roots grown in water, a thickened hypodermis and also a change in the size and distribution of the xylem elements.

In this study the Vetiver isolates obtained from Hydromulch (Pty) Ltd. were grown under two different environmental conditions: 1. in soil and 2. in water. We hope to find differences in the anatomical structure of the roots of the different isolates, and compare it with the genetic differences between them and also to compare the differences of the root structures of the isolates grown in soil to the isolates grown in water.

CHAPTER3 PHYLOGENETIC ANALYSES

3.1 Materials and Methods

3.1.1 *Plant material*

Nineteen isolates of Vetiver grass were used in this study, which were all provided by Mr. Roley Nöffke from the plant collection at Hydromulch (Pty) Ltd., South Africa. The company supplies Vetiver grass slips/plants to any destination worldwide but mostly to African countries (http://hydromulch.co.za). The origins of the collected plants are notated in table 3.1. The sources of accessions 4, 8, 14 and 15 were also verified by Prof Truong (personal communication). These plants were cultivated in a grass nursery at Bapsfontein, situated about 20km north of the Oliver Tambo International Airport in Johannesburg.

Plant material was collected in the form of slips, and planted in pots in the Botanical Garden of the North West University, Potchefstroom, South Africa. All the isolates were planted in the same soil. The pots were kept in a regulated greenhouse with an average daily temperature of 25^0C±2^0C in the summer and 18^0C±2^0 C during the winter. Each pot contained between 2-3 slips of each specimen as collected from Hydromulch (Pty) Ltd. All the plants received a general fertilizer once every 3-4 weeks to sustain the growth of the plants.

The outgroups as well as other accessions (8 for *ITS*, 6 for *ndhF* and 7 for *rbcL*) are listed in Table 3.2 and were obtained from the National Centre for Biotechnology Information (NCBI) Bethesda, Maryland, United States of America. These accessions were selected for this study because they were classified as being closely related to *Chrysopogon* (Adams *et al.,* 1998) as well as on the availability of sequence data. The outgroups included species from the genera *Cymbopogon, Saccharum, Sorghum.*

Table 3.1 Taxa of *Chrysopogon spp* used during this study obtained from the Hydromulch (Pty) Ltd. Vetiver nursery.

Isolate number	Voucher	Country of origin	Location	
1	PUC12173	Congo, DRC	Kinshasa	Selembao south west of Kinshasa
2	PUC12174	Madagascar (South)	Fort Dauphin	Farming area 50 km west of Fort Dauphin
3	PUC12175	Congo, DRC	Kinshasa	About 50 km south of Selembao
4	PUC12176	Australia	Brisbane	Monto as identified by Paul Truong
5	PUC12177	Mozambique	Nampula	North of Nampula
6	PUC12178	Venezuela	Caracas	Southern outskirts of Caracas
7	PUC12179	South Africa	Rustenburg	Commercial farm north of Rustenburg
8	PUC12180	Ethiopia	Addis Ababa	Outskirts of Addis Ababa
9	PUC12181	Madagascar (North)	Antsohiny	Old farming area on the RN5 route
10	PUC12182	Congo, DRC	Kinshasa	Local community close to Selembao
11	PUC12183	Ghana	Gingani	Agricultural fields near the Golinga Dam, 16 km south west of Tamale
12	PUC12184	Ghana	Buleng	In open field in the Buleng North Ghana
13	PUC12185	Ghana	Manga	In open field close to Nalerigu Dam, Ganbaga district North Ghana
14	PUC12186	New Zealand	North Island	Residence of John Greenfield
15	PUC12187	Kenya	Voi, Coastal Plain	Elise Pinners Vetiver Network, Kenya
16	PUC12188	Mozambique	Alto Molocute District	South of Nampula (200 km south of ACC5 location)
17	PUC12189	Puerto Rico	Guyama district	Near Lake Carite, Guyama
18	PUC12190	South Africa	Rustenburg	Commercial farm north of Rustenburg
19	PUC12191	Ghana	Kumasi	In open field

Table 3.2 The GenBank accession numbers of the taxa of outgroups sampled for this study as well as that of *Chrysopogon (Vetiveria) zizanioides* and *Chrysopogon (Vetiveria) nigritanus*.

Taxon	Accessions of *ITS*	Accessions of *ndhF*	Accessions of *rbcL*
Vetiveria zizanioides	DQ005089	AM849196	AM849394.1
Vetiveria nigritanus	GQ856335.1		
Vetiveria fulvibarbis	GQ856324.1		
Saccharum officinarum	DQ005064.1	AF443824	EF125120
Sorghum bicolor subsp. verticilliflorum	GQ121746	U21981	AM849341
Chrysopogon serrulatus	DQ005032.1		
Cymbopogon citratus		AM849141.1	GQ436383.1
Sorghum halepense	GQ121749	AF117424	EF125122
***Zea mays* strain W13A**	DQ683012	U21985	Z11973
Chrysopogon gryllus		AF117399.1	
Chrysopogon festucoides	GQ856339.1		

3.1.2 *Sample collection and preparation for DNA Isolation*

Young leaves were harvested for the purpose of DNA isolation after at least six months of cultivation in the greenhouse since the plants were collected from Hydromulch (Pty) Ltd. at the onset of autumn. Fresh plant material was collected from three different slips of the same isolate. The tips of leaves were cut, avoiding older or degraded parts of the leaves. The

material was then placed in marked plastic bags and put on ice for the duration of the collection period.

The material was washed with distilled water to remove any potential contamination on the day of collection. The midriffs of the young leaves were removed to eliminate xylem and phloem which may influence the final quantity and quality of extracted DNA. This was done due to the insufficient DNA yield from the leaves when the midriff was included during the initial analyses.

3.1.3 *DNA isolation*

The Qiagen DNeasy Mini Plant isolation kit, obtained from Southern Cross Biotechnologies was used to isolate the DNA from the different plants according to instructions of the manufacturer. The following modifications were however made to the prescribed isolation kit protocol: 200 mg fresh plant material was used instead of 100 mg (to ensure that a sufficient amount of DNA was extracted); 450 µl extraction buffer AP1 was used instead of 400 µl (to accommodate the higher amount of sample); the incubation period (cell lyse step) was increased from 10 minutes to 30 minutes; the elution step was modified as follows: 30 µl Buffer AE was pipetted onto the DNeasy membrane (instead of 100 µl) and incubated for 10 minutes at room temperature (instead of 5 minutes). This was repeated a second time with 20 µl buffer AE to ensure that the maximum amount of DNA was eluted from the membrane.

The purity as well as the concentration of the DNA was assessed by using a NANO-DROP spectrophotometer. The software automatically determines the quality of the DNA based on the following equation:

Equation 1

DNA concentration = A(260) x dilution x 50 ug/ml

where A(260) is the absorbance measured at 260 nm

The 260:280 as well as the 230:260 ratios were determined by the software (NanoDrop) which gives an indication of the purity of the isolated DNA (figure 3.1). If the A260:280 ratios are lower than, or equal to 1.8, the DNA is free from protein contaminants. The 230:260 is an indication of phenolate, thiocyanate and other organic compound- contamination. If the 230:260 ratio is in the range of 2-2.2, the sample is free from organic contaminants. The $A_{230:260:280}$ ratio of a pure DNA sample should be around 1:1.8:1 (Thermo Scientific, 2008).

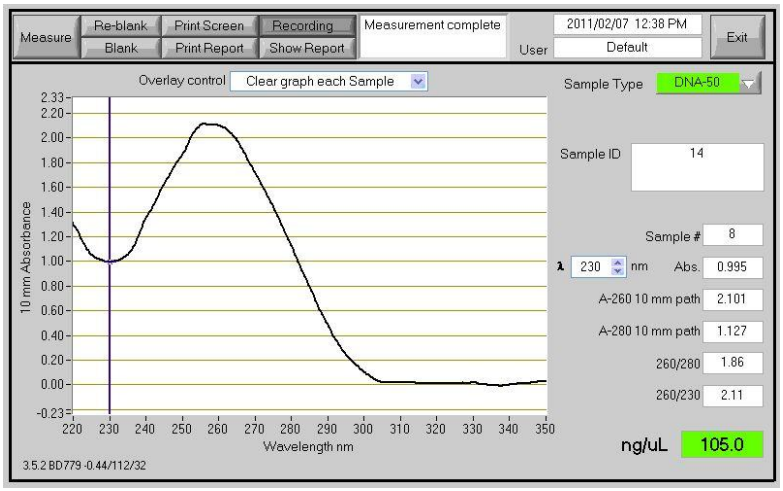

Figure 3.1 The NanoDrop absorbance – graph and ratios of isolate 14. If the A 260:280 ratio was over the value of 2, or the A230:260 ratio, over 2.3, the sample was DNA was discarded and the sample – DNA was re-isolated by using new plant material.

Subsequently, the purified DNA was also evaluated through conventional agarose gel electrophoresis to ensure the intactness of the isolated DNA. A 1% agarose gel and 1x TAE buffer mixture was used to for the electrophoresis reaction. The DNA was stained with ethidium bromide and visualised under ultra-violet light to see if the DNA was isolated successfully (figure 3.2).

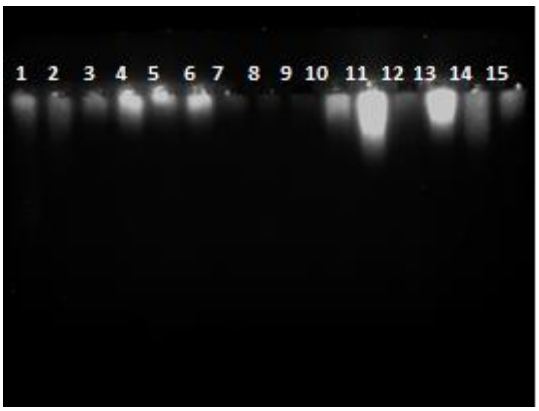

Figure 3.2 Agarose gel electrophoresis of the isolated DNA to determine if the DNA was isolated successfully. The numbers represent the different isolates loaded in the agarose gel.

3.1.4 *Amplification of DNA*

The selected genes were amplified via Polymerase Chain Reaction (PCR). Three genes were selected for this study, the *ITS* regions of the 5.8s ribosomal gene and the two chloroplastic genes, *ndhF* and *rbcL*. Primers by White *et al*. (1990), Stanford *et al*. (2000) and Christin *et al*. (2008) were used for amplifying these fragments (Table 3.3). Amplification of the DNA of all the genes in this study, were done in a 25 µl reaction mixture, using the Bio-rad i-cycler (V4.006). The reaction mixture included: 1x reaction buffer, 1.5 mM MgCl, 0.2 mM dNTP's, 1.25 units (Promega) Taq DNA polymerase, forward primer (0.5 µM), reverse primer (0.5 µM), PCR grade water and 50 ng template DNA. The reactants were added as well as centrifuged in a laminar chamber, under sterile conditions.

An annealing temperature gradient of between 46°C to 65°C was done initially for each of the different primers (table 3.3) to determine which of these primer combinations were the most suitable for this study and at which annealing temperature they would yield the best results. The product sizes of the different gene fragments were between 600 and 800 base pairs. The fragment sizes were then compared to a commercially available DNA size fragment ladder to establish the size of nucleotides of the amplified product (figure 3.3).

Table 3.3 The primers used for the amplification and sequencing of the *ITS, ndhF*, and *rbcL* gene fragments used for the phylogenetic analyses.

Primer	5′ to 3′ primer sequence	Reference	Annealing temperature for PCR
ITS 4F	TCCTCCGCTTATTGATATGC	White *et al.*, 1990	58.0°C
ITS 5A	CCTTATCATTTAGAGGAAGGAG	Stanford *et al.*, 2000	58.0°C
*ndh*F_1F	ATGGAACATACATATCAATA	Christin *et al.,* 2008	50.8°C
*ndhF*_746R	CTTCCATAGCATCNGGYAACC	Christin *et al.,* 2008	50.8°C
*ndhF*_630F	AATAGCTAATAACTGGATTCC	Christin *et al.,* 2008	50.8°C
*ndhF*_1630R	AAAGNARTAATATAAGAAGAGG	Christin *et al.,* 2008	50.8°C
*ndhF*_1417F	TTGYATTCAATATCYTTATGGG	Christin *et al.,* 2008	50.8°C
*ndhF*_2110R	CCCCCTACATATTTGATACCTTCTC	Christin *et al*. 2008	50.8°C
*rbc*L_1F	ATGTCACCACAAACAGAAACTAAAGC	Christin *et al.,* 2008	63.5°C
*rbcL*_826R	TAATRAGNCAAASTAGTATTTGC	Christin *et al.,* 2008	63.5°C
*rbcL*_629F	CATTTATGCGCTGGAGAGACC	Christin *et al.,* 2008	63.5°C
*rbcL*_1388R	TTCCATAYTTCACAAGCTGC	Christin *et al.,* 2008	63.5°C

PCR amplified fragments were separated on a 2% agarose gel via electrophoresis. The presence of particular amplification products was visualised with UV light in a dark chamber. In the case of primer dimer formation, the desired fragment was cut out of the gel and sent for Post-PCR purification before the sequencing was done.

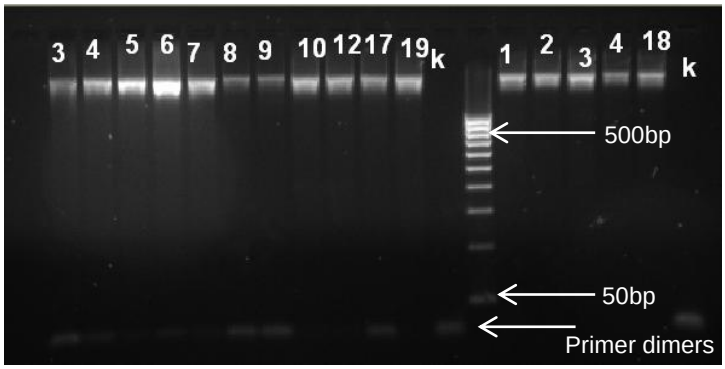

Figure 3.3 Gel image of the *ITS* gene products after the PRC reaction (annealing temperature = 50.8˚C, for 35 cycles). K represents the negative control and numbers 1 to 19 the different isolates. The size maker in lane 13 had a range of 500 bp to 50 base pares (bp). In some cases primer dimers formed. They were eliminated by cutting the desired PCR DNA band out of the gel.

3.1.5 *Sequencing and Sequence analyses*

All sequencing reactions were performed by the Central Analytical Facilities, Stellenbosch University, South Africa. Post-PCR purification was done using the NucleoFast Purification System (obtained from Separations). Sequencing was performed with BigDye Terminator V1.3 (Applied Biosystematics, 2011) followed by electrophoresis on the 3730xl DNA Analyser (Applied Biosystematics, 2011). Sequences were analysed and trimmed using Sequencing Analysis V5.3.1 (Applied Biosystematics, 2011).

3.1.6 *Phylogenetic analyses*

Sequence verifications and alignments were done unambiguously with CLC DNA Workbench 6 (CLC bio, Aarhus, Denmark), using the following settings during alignment: gap open cost (10), gap extension cost (1) and end gap cost (as any other). Alignments were also verified manually and ambiguous bases corrected by visual inspection.

The*, ITS, rbcL* and *ndh*F matrices were analysed separately and in combination using MEGA version 5 (Tamura *et al*., 2011). Gaps were eliminated. A distance method, Neighbour-Joining (Saitou & Nei, 1987), as well as a model based approach, Maximum Likelihood, was used for the analysis. Neighbour-Joining was performed using the Jukes-Cantor model (Jukes & Cantor, 1969). The Best fit nucleotide substitution models for use with the Maximum Likelihood were calculated with the Bayesian Information Criterion available within Mega 5 and evaluated using the Akaike Information Criterion within Mega 5. The heuristic search model used for the Maximum Likelihood was the Close-Neighbour-Interchange. Bootstrap analysis (1000 replicates) was performed to determine internal support (Felsenstein, 1985). A bootstrap percentage of 80% and higher was considered a high bootstrap support, a bootstrap support of 50% and higher as moderate and a bootstrap support of less than 50% as weak. The bootstrap consensus trees of the Neighbour-Joining and Maximum Likelihoods were reported. The congruency index and the P-values for the matrices were calculated (De Vienne *et al*., 2007) before the matrices were combined.

3.2 Results

3.2.1 *Phylogenetic analyses of the ITS rDNA sequence data*

After sequencing and aligning the different genes, the evolutionary relationships were inferred using both the Neighbour –Joining and Maximum Likelihood methods. All positions containing gaps were eliminated according to the program MEGA5 (Tamura *et al*., 2011). In all cases the phylogenies were inferred using all the taxa including outgroups. The phylogenetic tree contained the 19 isolates from Hydromulch as well as the Genbank accessions for *Chrysopogon zizanioides* (Vetziz, accession: DQ005089)*, Chrysopogon nigritanus* (Vetnig, accession: GQ856335.1), *Saccharum officinarum* (Sacof, accession: DQ005064.1), *Sorghum bicolor subsp. verticilliflorum* (Sorgbi, accession: GQ121746), *Sorghum halepense* (Sorghal, accession: GQ121749), *Zea mays* (Zeam, accession: DQ683012), *Chrysopogon serrulatus* (Cryser, accession: DQ005032.1), *Vetiveria festucoides* (Vetfest*,* accession: GQ856339.1*)* and *Vetiveria fulvibarbis* (Vegful, accession: GQ856324.1).

3.2.1.1 ITS Neighbour – Joining approach

The Neighbour Joining approach included the 19 isolates from Hydromulch including all selected outgroups. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor, 1969) and were expressed in the units of the number of base substitutions per site. Also, the percentage of replicate trees in which the associated taxa clustered together in the bootstrap tests (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 28 sequences and 452 positions in the final dataset. The optimal tree with the sum of branch length = 0.69896056 is shown in figure 3.4.

Four main clusters were observed based on the results of the Neighbour-joining analyses. All the selected outgroups (Zeam, Sorgbi, Sorghal and Sacof) grouped separately from the *Chrysopogon* accessions. The *Chrysopogon* accessions grouped together, supported by a bootstrap value of 66%. The accessions of *C. serrulatus* (Chryser) and *V. fulvibarbis* (Vegful) (which both originate from Africa) grouped together with a bootstrap value of 92%. The isolates from the Democratic Republic of the Congo (1 and 10) and Madagascar (9) grouped separately from the other isolates that grouped with *C. nigritanus* (Vetnig)*, V. festucoides*

(Vetfest) and *C. zizanioides* (Vetziz), supported by a bootstrap value of 82%. The two groups of accessions of *C. nigritanus* (Vetnig) and *C. zizanioides* (Vetziz) showed no significant difference (having a low bootstrap value). Isolate 6 from Venezuela grouped with the accession of *C. nigritanus* (Vetnig) with a bootstrap value of 44%. The rest of the isolates clustered with the accession of *C. zizanioides* (Vetziz). The isolates 2, 3, 4, 5, 7, 8, 11, 12, 13, 14, 15, 16, 18 and 19 grouped with *C. zizanioides* (Vetziz), showing no significant difference between these isolates. Isolate 12 and 18 grouped separately from isolate 19 supported by a bootstrap value of 86%. However, because they did not differ statistically significantly from the other studied isolates, they still form part of the *C. zizanioides- C. nigritanus* complex.

A Neighbour- Joining tree was drawn as in figure 3.4 with selected closely related outgroups in an effort to increase the resolution of the groups (figure 3.5). Selected outgroups included *Chrysopogon zizanioides* (Vetziz), *Chrysopogon nigritanus* (Vetnig), *Chrysopogon festucoides* (Vetfest), *Chrysopogon serrulatus* (Chryser) and *Vetiveria fulvibarbis* (Vegful). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 25 nucleotide sequences and there were a total of 532 positions in the final dataset. The optimal tree with the sum of branch length = 0.11278889 is shown in figure 3.5.

Three main clusters were observed on the results of the Neighbour-joining analyses. The outgroup (Sacof) grouped separately from the *Chrysopogon* accessions. Isolate *C. serrulatus* (Chryser) and *V. fulvibarbis* (Vegful) (which both originate from Africa) grouped together. In this analyses, *C. festucoides* (Vetfest) could be distinguished from the *C. nigritanus* (Vetnig) and *C. zizanioides* (Vetziz) complex with a bootstrap support of 95%. Once again the isolates from the Democratic Republic of the Congo (1 and 10) and Madagascar (9) appeared to be different from the other isolates that grouped with *C. nigritanus* (Vetnig) and *C. zizanioides* (Vetziz) with a bootstrap support of 67%. The accessions of *C. nigritanus* and *C. zizanioides* did not differ statistically significantly, with the differences supported by a bootstrap value of 40%. Isolate number 6 from Venezuela was the only isolate that grouped with *C. nigritanus*. There appeared to be no statistical significant difference between the rest of the isolates (2, 3, 4, 5, 7, 8, 11, 13, 14, 15, 16 and 17), which grouped with the GenBank accession of *C. zizanioides*. Isolate 12 (from Ghana) grouped separately with isolates 18 (from South Africa) and 19 (from Ghana). However, they are grouped as part of the *C. zizanioides - C. nigritanus* complex, because the difference was not statistically well supported.

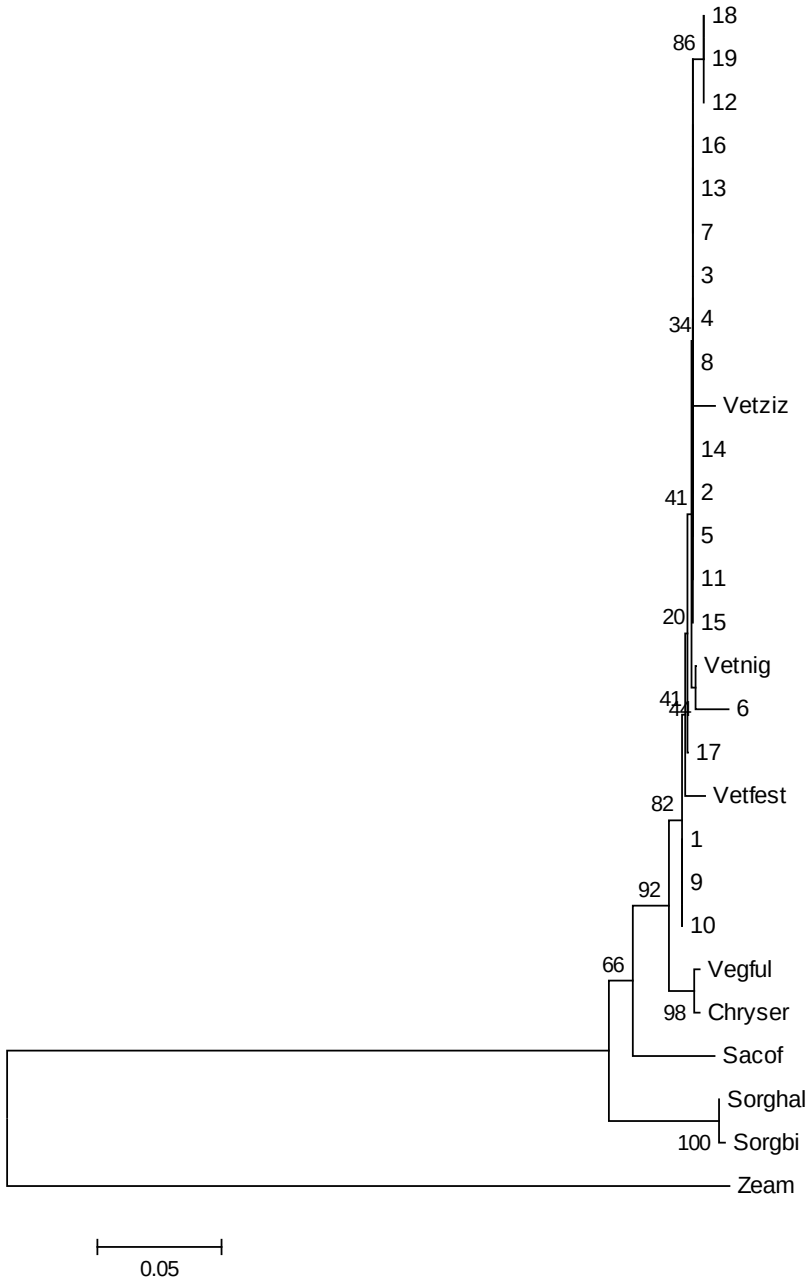

Figure 3.4 The evolutionary history inferred using the Neighbour-Joining method and *ITS* sequence data including all outgroups. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz), *Chrysopogon nigritanus* (Vetnig), *Saccharum officinarum* (Sacof), *Sorghum bicolor* subsp. *verticilliflorum* (Sorgbi), *Sorghum halepense* (Sorghal), *Zea mays* (Zeam), *Chrysopogon. festucoides* (Vetfest), *Chrysopogon serrulatus* (Cryser) and *Vetiveria fulvibarbis* (Vegful).

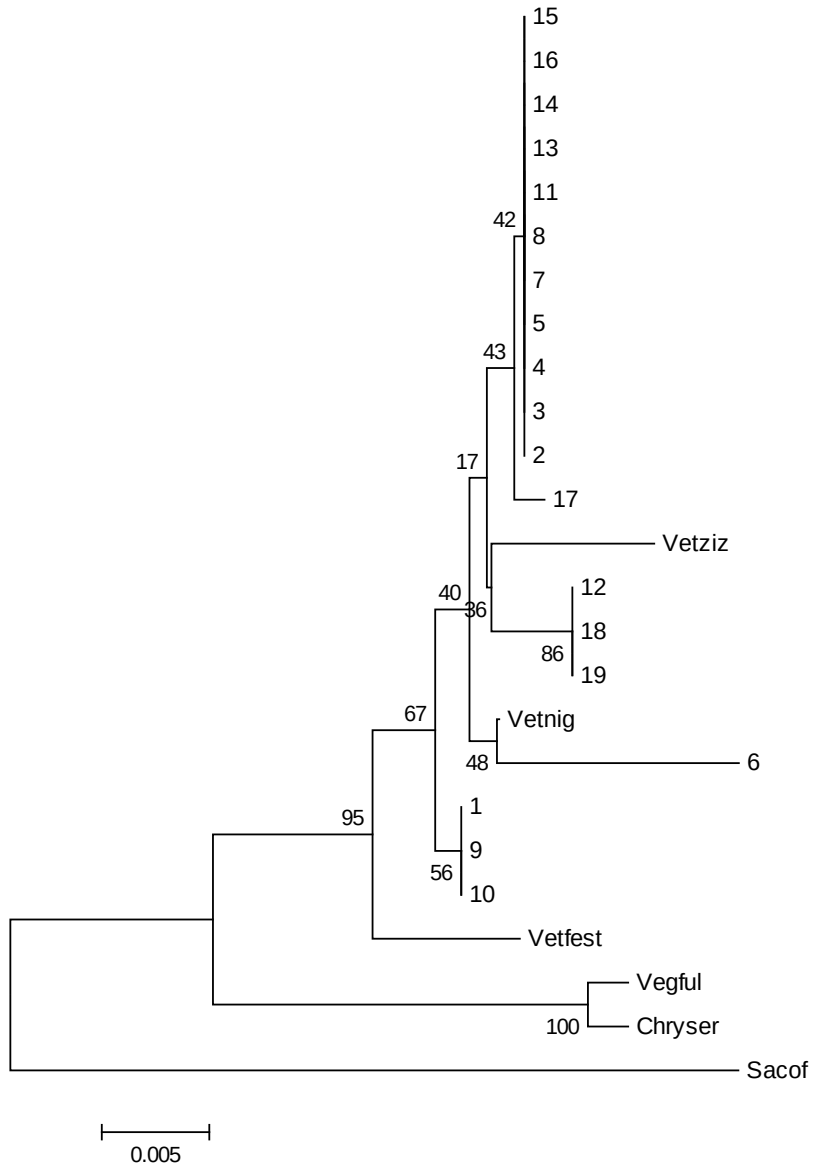

Figure 3.5 The evolutionary history inferred using the Neighbour-Joining method and *ITS* sequence data excluding selected outgroups. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz), *Chrysopogon nigritanus* (Vetnig), *Chrysopogon serrulatus* (Cryser), *Chrysopogon. festucoides* (Vetfest), *Vetiveria fulvibarbis* (Vegful) and *Saccharum officinarum* (Sacof).

3.2.1.2 ITS Maximum Likelihood approach

The Maximum Likelihood approach included the 19 isolates from Hydromulch including all selected outgroups, as in the previous section (section 3.2.1.1). Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). The Kimura 2+I parameter model (Kimura, 1980) was the best fit for the *ITS* alignment (Appendix A). When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 30.9205% sites). The tree is drawn to scale with a total of 28 sequences and 452 positions in the final dataset. The tree with the highest log likelihood (-1529.6942) is shown in figure 3.6.

Two main clusters were observed based on the results of the Maximum Likelihood analyses. All the outgroups (Zeam, Sorgbi, Sorghal and Sacof) grouped separately from the *Chrysopogon* accessions. The *Chrysopogon* accessions grouped together. *C. serrulatus* (Chryser) and *V. fulvibarbis* (Vetful) (which both originate from Africa) grouped together. However, the bootstrap value for this cluster was low (42%) making this result not statistically significant. In this analysis, the accessions of *C. nigritanus* (Vetnig), *C. zizanioides* (Vetziz) and *V. festucoides* (Vetfest), formed a complex, supported by a bootstrap value of 51%. Isolate 1 (Democratic Republic of the Congo) grouped separately from the *C. nigritanus, C. zizanioides* and *V. festucoides* – cluster, supported by a bootstrap value of 51%. The isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19 grouped with *C. nigritanus, C. zizanioides* and *V. festucoides* with low distinguishing bootstrap values.

The second analysis included the 19 isolates from Hydromulch and excluded selected outgroups, as in the case of the Neighbour-Joining approach (figure 3.5). The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura 3- + G parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.0500)) (APPENDIX B). The tree is drawn to scale with a total of 25 sequences and 532 positions in the final dataset. The tree with the highest log likelihood (-1074.0035) is shown in figure 3.7.

Two main clusters were observed based on the results of the Maximum Likelihood analyses. The outgroup *Saccharum officinarum* (Sacof) grouped separately from the *Chrysopogon* accessions. The accessions of *C. serrulatus*, *V. fulvibarbis* and *V. festucoides* grouped together, with the latter separate from *C. serrulatus* and *V. fulvibarbis* with a bootstrap value

of 29%. The accessions of *C. nigritanus,* and *C. zizanioides* did not differ statistically significantly, with the differences supported by a bootstrap value of 30%. The isolates 1, 2, 3, 4, 8, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19 grouped together within the *C. nigritanus- C. zizanioides*-complex, indicating no significant difference between these isolates.

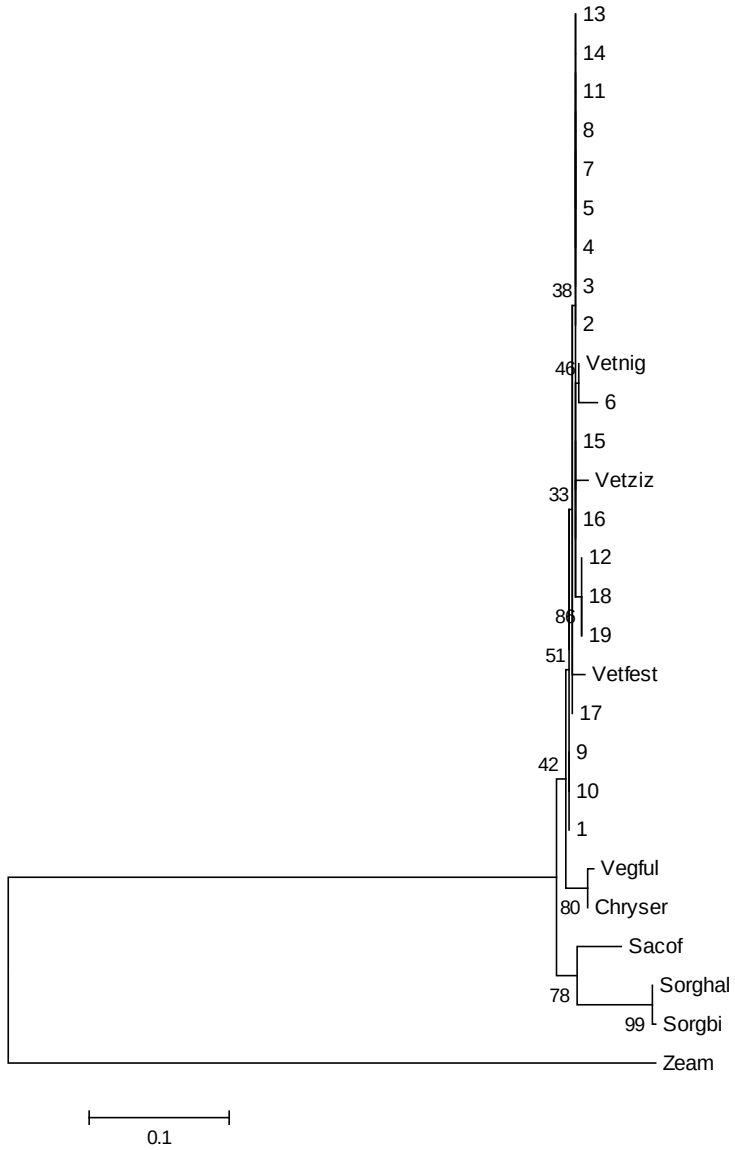

Figure 3.6 The evolutionary history inferred using the Maximum Likelihood method and Kimura 2-+I parameter model, with the *ITS* sequence data, including all selected outgroups. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz), *Chrysopogon nigritanus* (Vetnig), *Saccharum officinarum* (Sacof), *Sorghum bicolor* subsp. *verticilliflorum* (Sorgbi), *Sorghum halepense* (Sorghal), *Zea mays* (Zeam), *Chrysopogon serrulatus* (Cryser), *Chrysopogon. festucoides* (Vetfest) and *Vetiveria fulvibarbis* (Vegful).

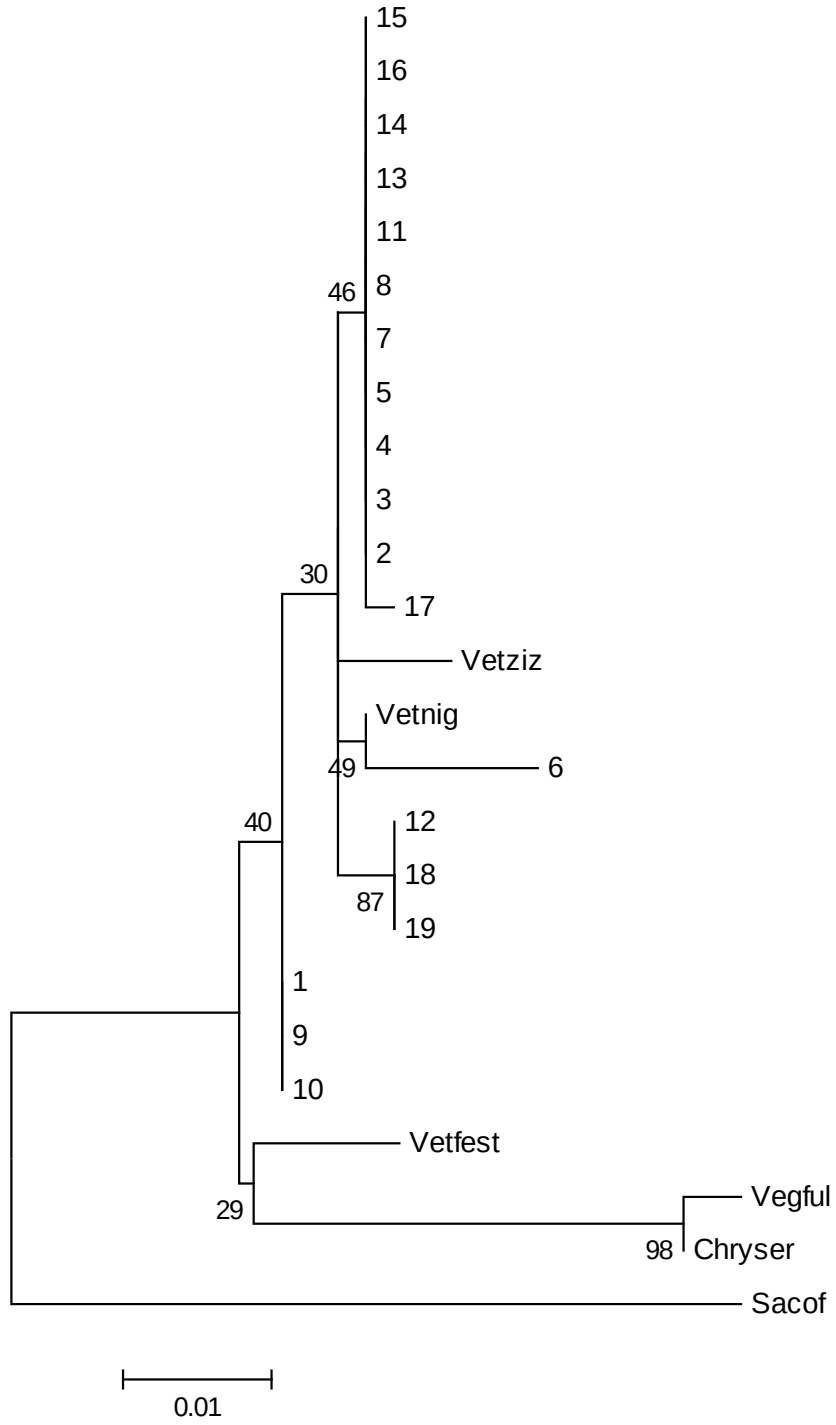

Figure 3.7 The evolutionary history inferred using the Maximum Likelihood method and the Tamura 3-+G parameter model, with the *ITS* sequence data, excluding selected outgroups. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz), *Chrysopogon nigritanus* (Vetnig), *Chrysopogon serrulatus* (Cryser), *Chrysopogon festucoides* (Vetfest), *Vetiveria fulvibarbis* (Vegful) and *Saccharum officinarum* (Sacof).

Both the Neighbour –Joining and Maximum Likelihood methods showed similar results, with the inclusion as well as the exclusion of outgroups. The outgroups clustered separately from the accessions of *Chrysopogon.* The *Chrysopogon* accessions grouped together. *C. serrulatus* and *V. fulvibarbis* (which both originate from Africa) grouped together. The accession of *V. festucoides* grouped with the accessions of *C. nigritanus,* and *C. zizanioides* when all the outgroups were included, but separately from the *C. nigritanus- C. zizanioides* complex, when selected outgroups were excluded. Isolates 1, 9 and 10 (the Democratic Republic of the Congo and Madagascar) grouped separately from the accessions of *C. nigritanus* and *C. zizanioides* in all the above analyses, supported by high bootstrap values. The isolate from Venezuela (isolate 6) grouped with *C. nigritanus*. The rest of the isolates grouped with *C. zizanioides,* exhibiting no significant difference between them. The accessions of *C. nigritanus,* and *C. zizanioides* did not differ significantly in any of the above analyses.

3.2.2 *Phylogenetic analyses of the ndhF sequence data*

As for the *ITS* sequence data the evolutionary relationships were inferred using both the Neighbour –Joining and Maximum Likelihood approaches. All the positions which contained gaps were eliminated according to the program MEGA5 (Tamura *et al*., 2011). In all cases the phylogenies were inferred using all the taxa including all outgroups. The phylogenetic tree contained the 19 isolates from Hydromulch as well as the Genbank accessions for *Chrysopogon zizanioides* (Vetziz, accession AM849196), *Saccharum officinarum* (Sacof, Accession AF443824), *Sorghum bicolor* (Sorgbi, accession U21981), *Sorghum halepense* (Sorghal, accession AF117424), *Zea mays* (Zeam, accession U21985), *Chrysopogon fulvibarbis* (Chryful, accession AF117398.1), *Cymbopogon citratus* (Cymcitr, accession AM849141.1) and *Chrysopogon gryllus* (Chrygry, accession AF117399).

3.2.2.1 ndhF Neighbour – Joining approach

The Neighbour Joining approach included the 19 isolates from Hydromulch including all selected outgroups. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor, 1969) and are expressed in the units of the number of base substitutions per site. Also, the percentage of replicate trees in which the associated taxa clustered together in the bootstrap tests (1000 replicates) are shown next to the branches. The tree is drawn to scale, with

branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 27 sequences and 2009 positions in the final dataset. The optimal tree with the sum of branch length = 0.027 is shown in figure 3.8.

Two main groups were observed in this analysis. All the selected outgroups (Zeam, Sorgbi, Sorghal, Cymcitr and Sacof) grouped separately from the *Chrysopogon* accessions. The *Chrysopogon* accessions grouped together. All the isolates grouped with *C. zizanioides* (Vetziz), showing no significant difference between these isolates.

A Neighbour- Joining tree was drawn as in figure 3.8 with selected closely related outgroups in an effort to increase the resolution of the groups (figure 3.9). Selected outgroups included *Chrysopogon zizanioides* (Vetziz) and *Chrysopogon gryllus* (Chrygry). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 22 nucleotide sequences and there were a total of 2059 positions in the final dataset. The optimal tree with the sum of branch length = 0.00828552 is shown in figure 3.9.

Two main groups were observed in this analysis. The outgroup (Chrygry) grouped separately from the *Chrysopogon* accession. The *ndhF* results indicated that there was no genotypic difference between the 19 isolates and the GenBank accession of *C. zizanioides*, with the exception of isolate 1, which grouped together with all the other isolates, but on its own branch.

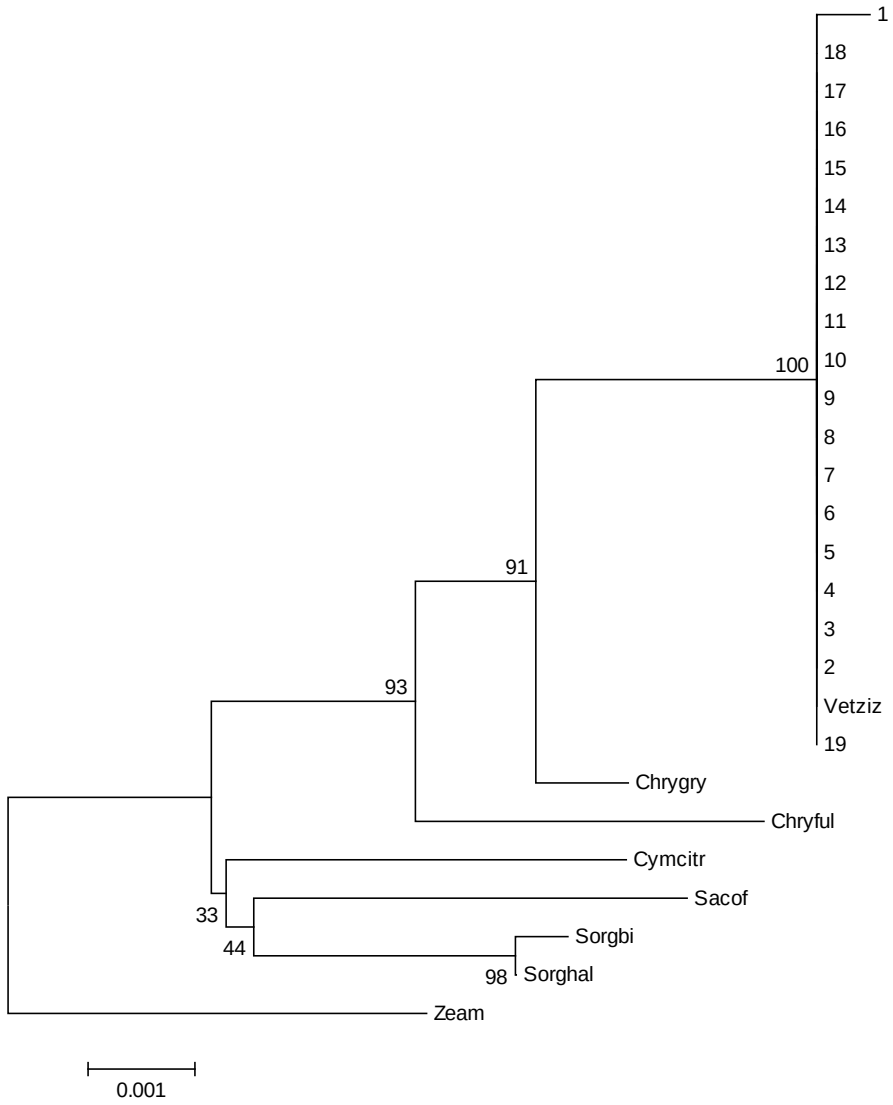

Figure 3.8 The evolutionary history inferred using the Neighbour-Joining method and *ndhF* sequence data, excluding selected outgroups. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz), *Saccharum officinarum* (Sacof), *Sorghum bicolor* (Sorgbi), *Sorghum halepense* (Sorghal), *Zea mays* (Zeam), *Chrysopogon fulvibarbis* (Chryful), *Cymbopogon citratus* (Cymcitr) and *Chrysopogon gryllus* (Chrygry).

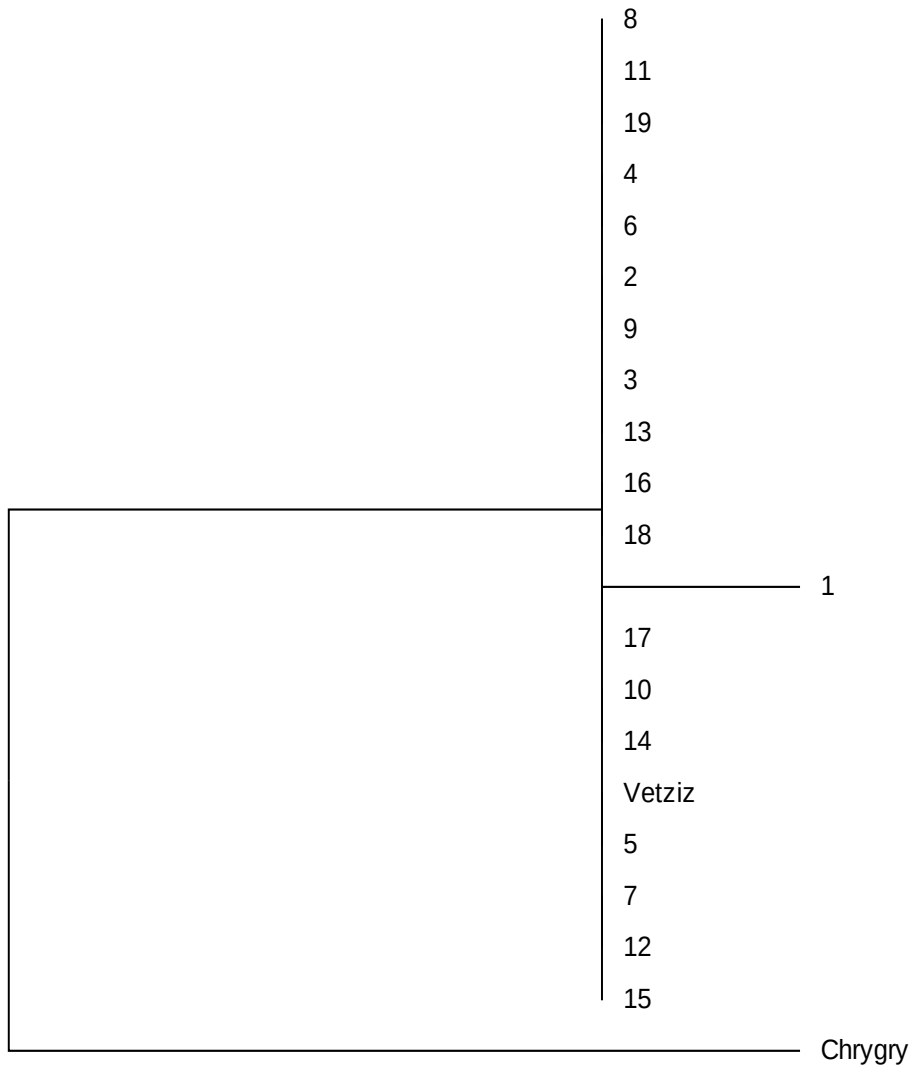

Figure 3.9 The evolutionary history inferred using the Neighbour-Joining method and *ndhF* sequence data, excluding selected outgroups. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz) and *Chrysopogon gryllus* (Chrygry).

3.2.2.2 ndhF Maximum Likelihood approach

The Maximum Likelihood approach included the 19 isolates from Hydromulch including all selected outgroups, as in the case of the Neighbour – Joining approach (figure 3.8). Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). The Hasegawa-Kishino-Yano +G model (Hasegawa *et al.,* 1995) was the best fit for the *ndhF* alignment (APPENDIX C). Initial tree(s) for the heuristic search were obtained automatically as follows: When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.1281)). The tree is drawn to scale with a total of 27 nucleotide sequences and 2009 positions in the final dataset. The tree with the highest log likelihood (-3080.8075) is shown in figure 3.10.

Three main clusters were observed in this analysis. The outgroups *Zea mays* (Zeam) and *Cymbopogon citratus* (Cymcitr) grouped separately from the other accessions. The bootstrap value indicating the separation of the accessions of the remaining outgroups with the accessions of *Chrysopogon* was 38%, indicating that the above formed one cluster. As in the case of the Neighbour Joining results, the studied isolates grouped together with the accession of *C. zizanioides* (having a bootstrap value of 89%). However, the low bootstrap value separating the outgroups from the accessions of *Chryopogon* indicates that there is no statistically significant difference between the isolates and the majority of the outgroups based on the *ndhF* gene fragment analyses.

The second analysis included the 19 isolates from Hydromulch excluding selected outgroups, as in the case of the Neibour- Joining approach (figure 3.9.). The evolutionary history was inferred by using the Maximum Likelihood method and the Hasegawa-Kishino-Yano model. Codon positions included were 1st+2nd+3rd+Noncoding (APPENDIX D). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 21 nucleotide sequences and 2059 positions in the final dataset. The tree with the highest log likelihood (-2781.9198) is shown in figure 3.11.

Two main clusters were observed. The outgroup (Chrygry) grouped separately from the accession of *C. zizanioides.* The isolates from Hydromulch (Pty) Ltd. grouped together with the accession of *C. zizanioides,* showing that there is no significant difference between them based on the *ndhF* gene fragment analysis.

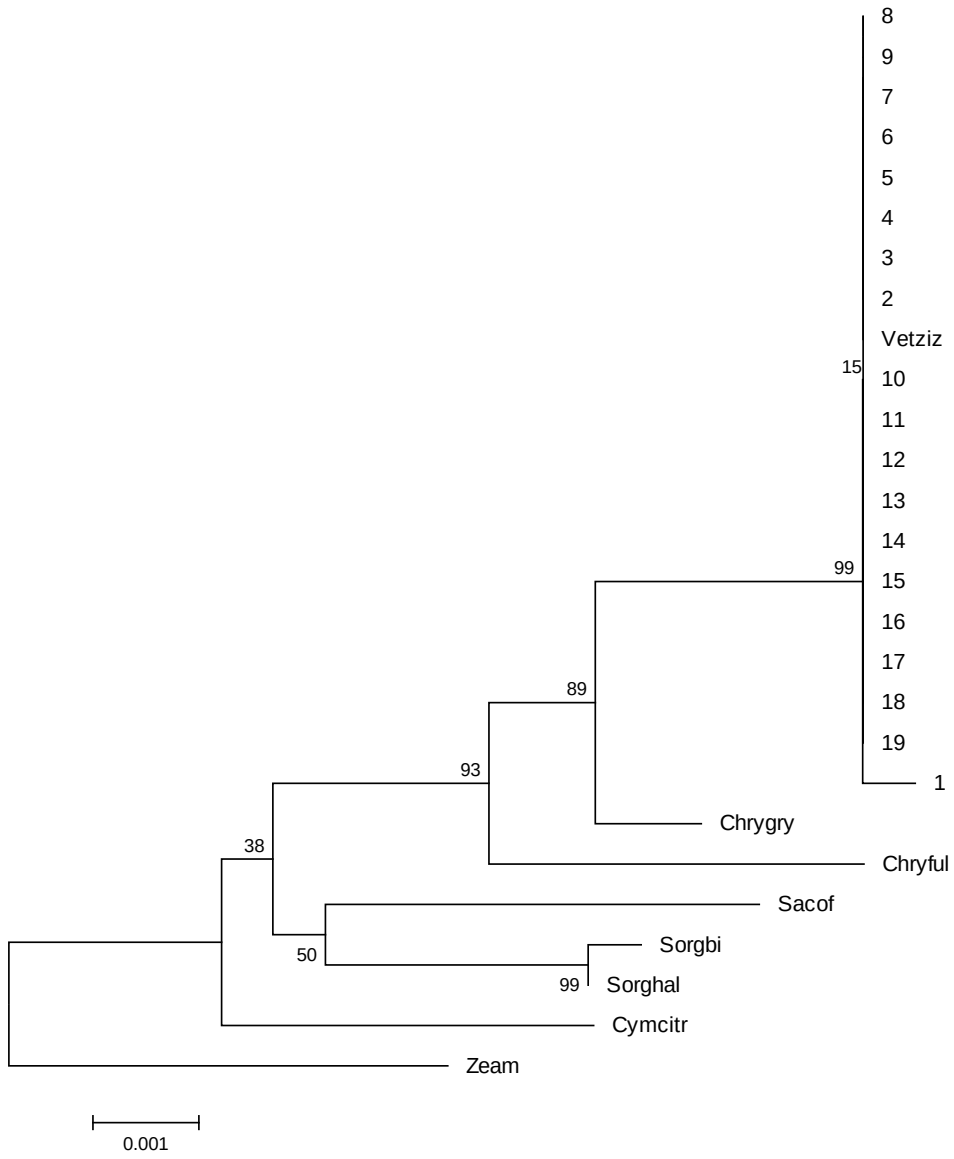

Figure 3.10 The evolutionary history inferred using the Maximum Likelihood method and the Hasegawa-Kishino-model, with the *ndhF* sequence data, including selected outgroups. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz), *Saccharum officinarum* (Sacof), *Sorghum bicolor* (Sorgbi), *Sorghum halepense* (Sorghal), *Zea mays* (Zeam), *Chrysopogon fulvibarbis* (Chryful), *Cymbopogon citratus* (Cymcitr) and *Chrysopogon gryllus* (Chrygry).

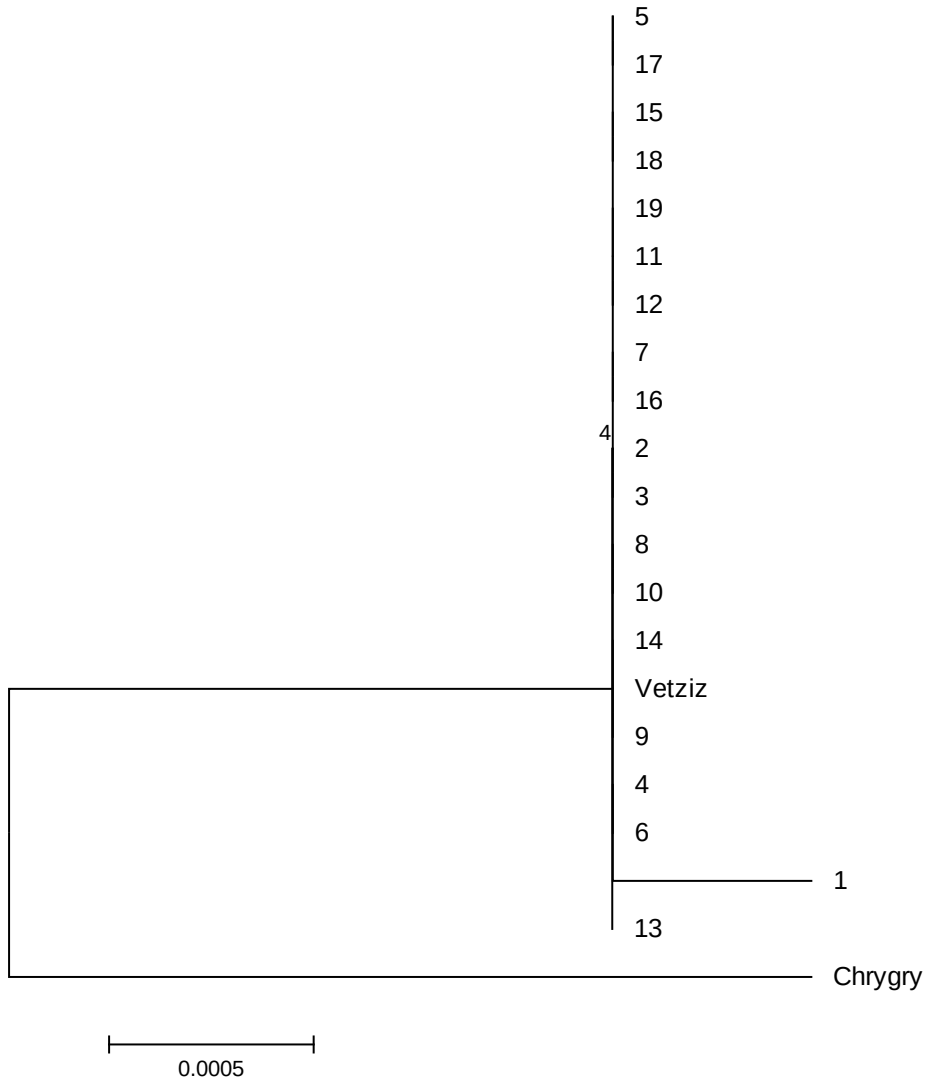

Figure 3.11 The evolutionary history inferred using the Maximum Likelihood method, and the Hasegawa-Kishino-model, with the *ndhF* sequence data, excluding selected outgroups. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz) and *Chrysopogon gryllus* (Chrygry).

Both the Neighbour –Joining and Maximum Likelihood methods, with the inclusion as well as the exclusion of outgroups showed similar results. All the isolates from Hydromulch (Pty) Ltd. formed one cluster with the accession of *C. zizanioides,* supported by a high bootstrap value, indicating that there is no phylogenetic difference between the above mentioned in the *ndhF* gene fragment.

3.2.3 *Phylogenetic analyses of the rbcL sequence data*

In the case of the *rbcL* analyses, sequencing was done on all 19 isolates from Hydromulch. However, not all the sequences yielded good sequencing results after several sequencing attempts. As result, isolates 6, 10, 13, 14 and 15 were excluded from this analysis. The evolutionary relationships of the remaining isolates as well as the outgroups were inferred using both the Neighbour –Joining and Maximum Likelihood approaches. All the positions containing gaps were eliminated according to the program MEGA5 (Tamura *et al*., 2011). In all cases the phylogenies were inferred firstly using all the taxa including outgroups and then without selected outgroups. The phylogenetic tree consisted of 14 isolates from Hydromulch (Pty) Ltd. as well as the Genbank accessions available for *rbcL,* namely *Chrysopogon zizanioides* (Vetziz, accession AM849394), *Sorghum bicolor* (Sorgbi, accession AM849341), *Sorghum halepense* (Sorghal, accession EF125122) and *Zea mays* (Zeam, accession Z11973).

3.2.3.1 rbcL Neighbour Joining approach

The Neighbour Joining approach included the 14 isolates from Hydromulch including all selected outgroups. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor, 1969) and are expressed in the units of the number of base substitutions per site. Also, the percentage of replicate trees in which the associated taxa clustered together in the bootstrap tests (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 18 sequences and 1235 positions in the final dataset. The optimal tree with the sum of branch length = 3.01 is shown in figure 3.12.

Three main clusters were observed. The outgroup *Zea mays* grouped separately from the other accessions. The isolates from Hydromulch (Pty) Ltd. grouped together, but grouped separately from the accession of *C. zizanioides* and the other outgroups (*Sorghum*), supported by a bootstrap value of 53%.

A Neighbour- Joining tree was drawn as in figure 3.12 with selected closely related outgroups in an effort to increase the resolution of the groups. Selected outgroups included *Chrysopogon zizanioides* (Vetziz), *Sorghum bicolor* (Sorgbi) and *Sorghum halepense* (Sorghal). The tree is drawn to scale, with branch lengths in the same units as those of the

evolutionary distances used to infer the phylogenetic tree. The analysis involved 18 nucleotide sequences and there were a total of 841 positions in the final dataset. The optimal tree with the sum of branch length = 0.54724153 is shown in figure 3.13.

Three main clusters were observed. The outgroups grouped separately from the accession of *C. zizanioides* and the isolates. The accession of *C. zizanioides* grouped separately from the isolates from Hydromulch. The results indicated that there was no genotypic difference between the 14 isolates used during this analysis.

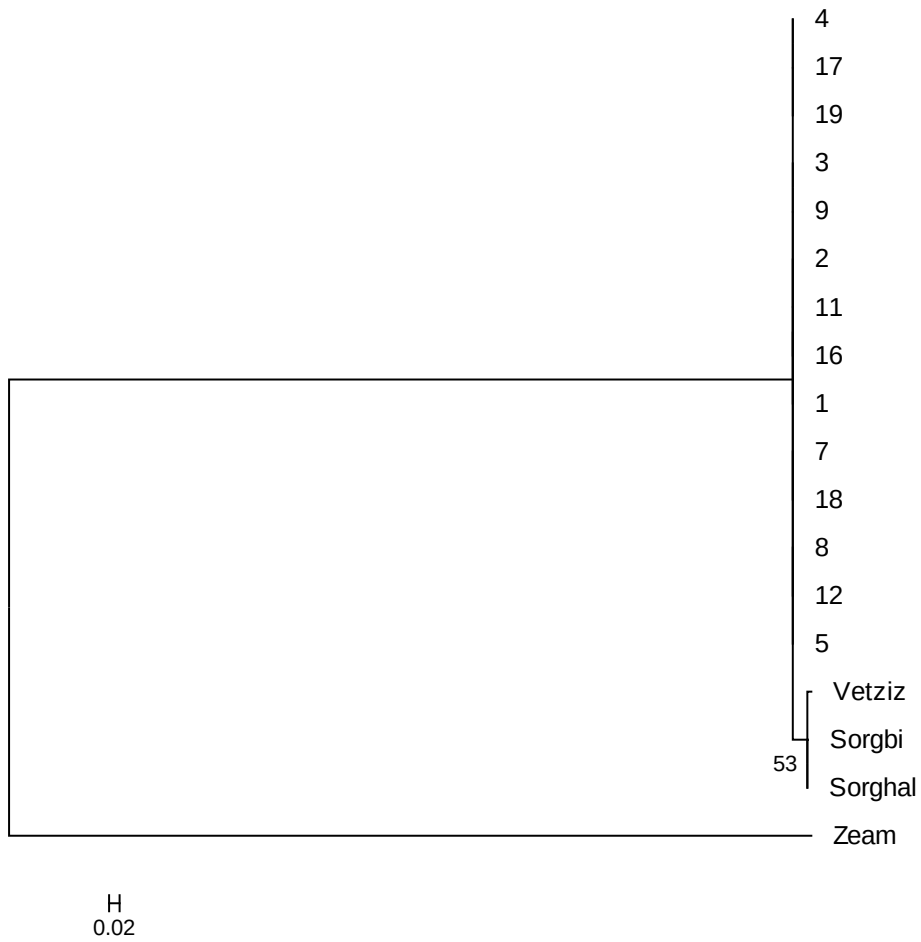

Figure 3.12 The evolutionary history inferred using the Neighbour-Joining method and *rbcL* sequence data, including selected outgroups. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz), *Sorghum bicolor* (Sorgbi), *Sorghum halepense* (Sorghal) and *Zea mays* (Zeam).

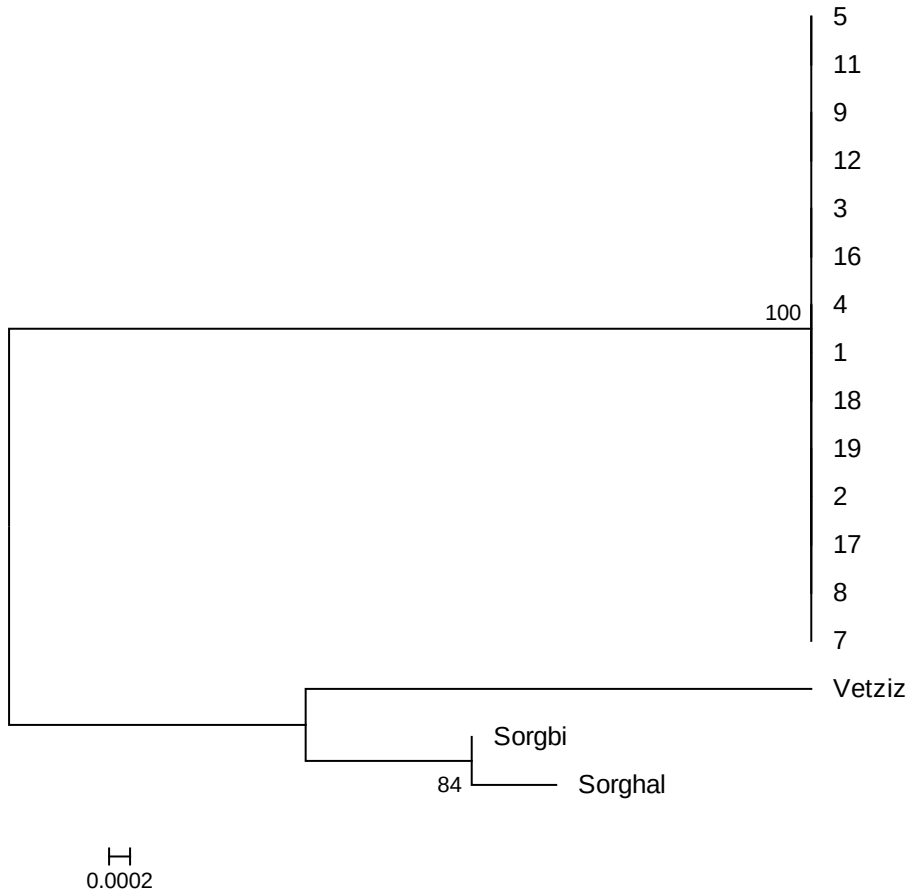

Figure 3.13 The evolutionary history inferred using the Neighbour-Joining method and *rbcL* sequence data, excluding selected outgroups. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz), *Sorghum bicolor* (Sorgbi) and *Sorghum halepense* (Sorghal).

3.2.3.2 rbcL Maximum likelihood approach

The Maximum Likelihood approach included the 14 isolates from Hydromulch including all selected outgroups, as in the case of the Neighbour – Joing approach (figure 3.12). The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura 3-parameter model. (APPENDIX E). There were a total of 18 nucleotide sequences and 1235 positions in the final dataset. The tree with the highest log likelihood (-3548.7917) is shown in figure 3.14.

Three main clusters were observed in this analysis. The outgroup *Zea mays* grouped separately from the rest of the accessions. The accession of *C. zizanioides* grouped separately from the outgroups as well as the isolates from Hydromulch, grouping closer to the two accessions of *Sorghum*. All the isolates from Hydromulch formed a cluster, supported by a bootstrap value of 76%

The second analysis included the 14 isolates from Hydromulch excluding selected outgroups. The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura-Nei model (APPENDIX F). There were a total of 17 nucleotide sequences and 1247 positions in the final dataset. The tree with the highest log likelihood (-1866.5447) is shown in figure 3.15.

Three main clusters were observed. The outgroups clustered separately from the accession of *C. zizanioides* as well as the isolates obtained from Hydromulch. The isolates from Hydromulch (Pty) Ltd. grouped separately from *C. zizanioides,* and were supported by a bootstrap value of 99%.

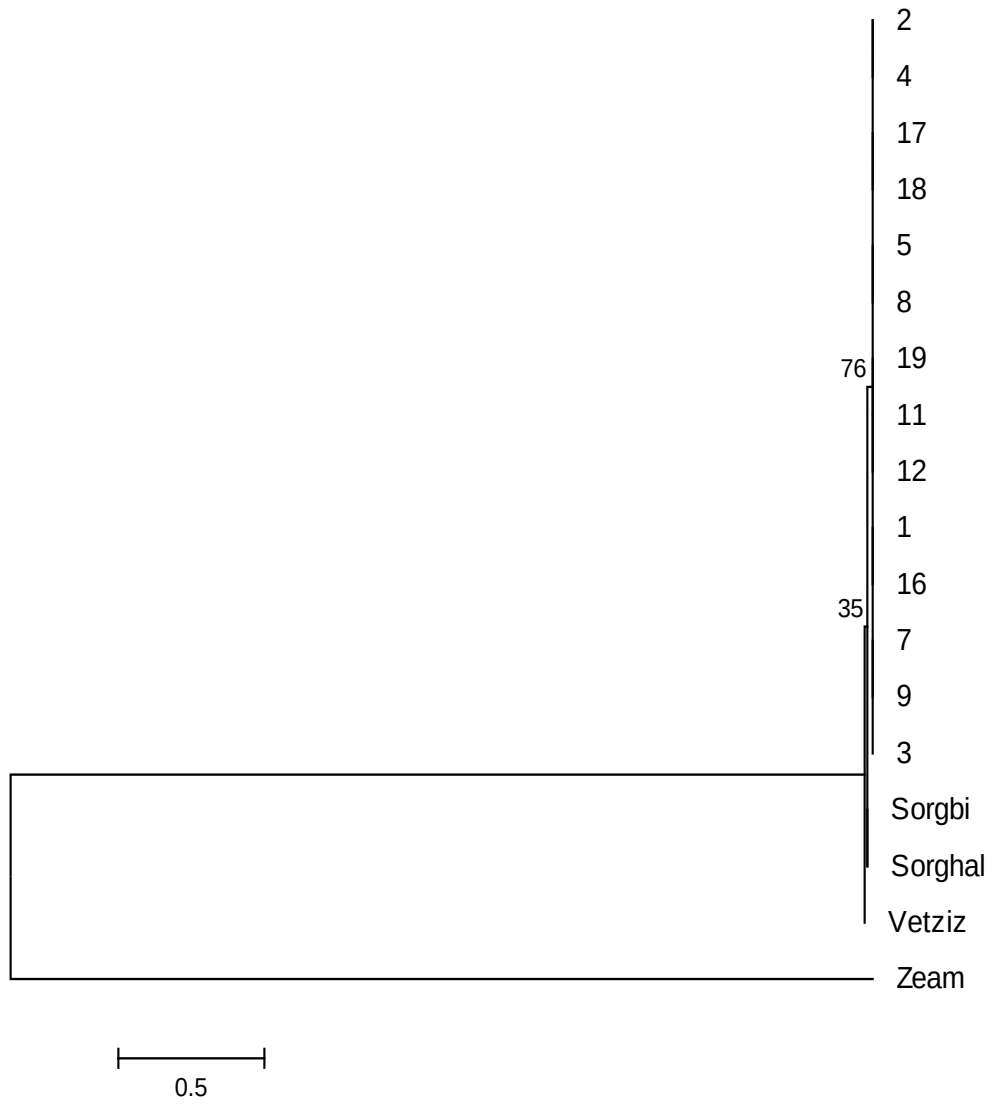

Figure 3.14 The evolutionary history inferred using the Maximum Likelihood method, and the Tamura 3-parameter -model, with the *rbcL* sequence data, including selected outgroups. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz), *Sorghum bicolor* (Sorgbi), *Sorghum halepense* (Sorghal) and *Zea mays* (Zeam).

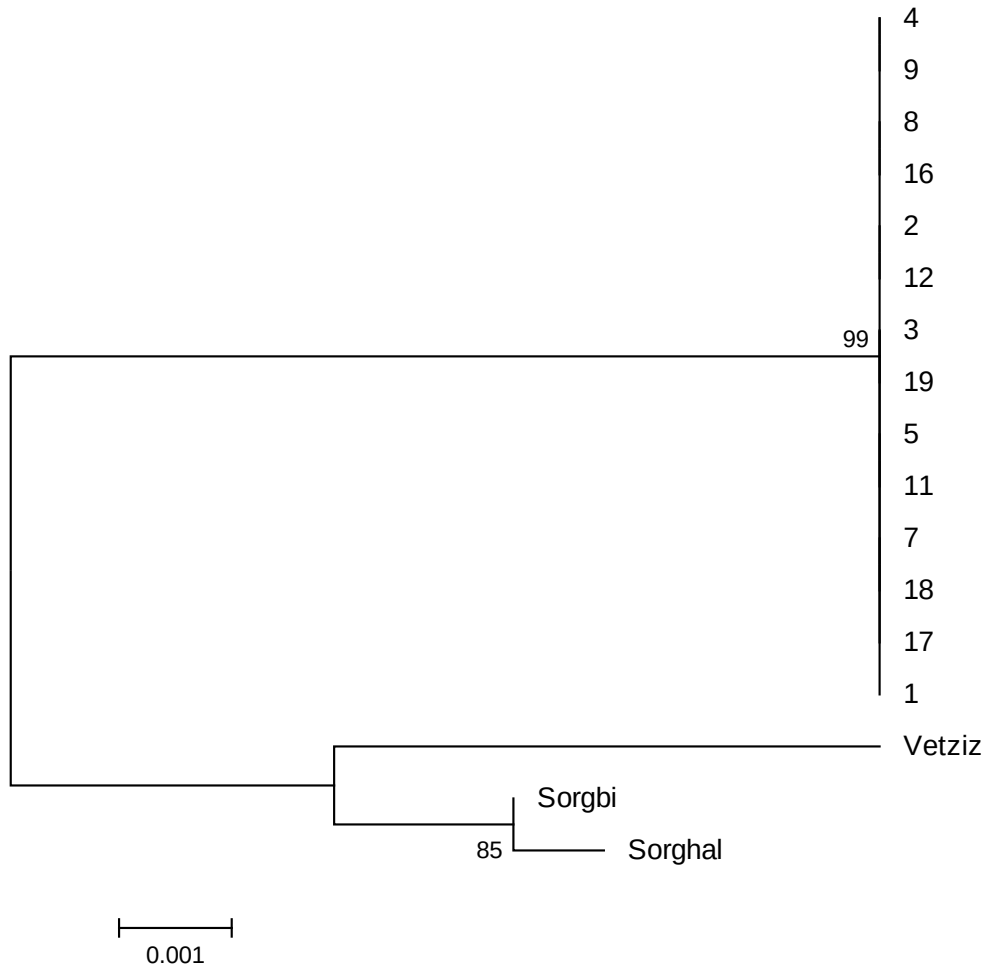

Figure 3.15 The evolutionary history inferred using the Maximum Likelihood method and the Tamura 3-parameter model, with the *rbcL* sequence data, excluding selected outgroups. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz), *Sorghum bicolor* (Sorgbi) and *Sorghum halepense* (Sorghal).

Both the Neighbour –Joining and Maximum Likelihood methods, with the inclusion as well as the exclusion of outgroups showed similar results. All the isolates from Hydromulch (Pty) Ltd. formed one cluster separate from the accession of *C. zizanioides,* supported by a moderate to high bootstrap value, indicating that there is a genetic difference between the isolates from Hydromulch (Pty) Ltd. and the accession from *C. zizanioides* obtained from Genbank regarding the *rbcL* gene fragment. It should be kept in mind that due to low base scaling of isolates 6, 10, 13, 14, and 15 after two attempts of sequencing, they were excluded from this analysis.

3.2.4 *Congruency*

The Neighbour-Joining trees firstly had to be notated using the Newick formulation in order to be tested statistically for congruency (Tables 3.4, 3.5 and 3.6). The results indicated that the trees of the *ndhF* and *rbcL* gene fragments (Table 3.4) are more congruent than expected by chance (*p= 0.00005*), while the *ITS* tree was not more congruent than expected by chance with either the *ndhF* or the *rbcL* trees, *p=191.6144* and *p= 146.76261* respectively (Table 3.5). The evolutionary distances for the congruent Neighbour-Joining tree were computed using the Jukes-Cantor method (Jukes & Cantor, 1969) method and are expressed in the units of the number of base substitutions per site**.** There were a total of 15 nucleotide sequences and 3345 positions in the final dataset. The optimal tree with the sum of branch length = 0.042 is shown in figure 3.16.

The results indicated that there was no genotypic difference between the 19 isolates and the GenBank accession of *C. zizanioides* based on the results obtained from the Neighbour-Joining analyses of the *ndhF* and *rbcL* gene fragments.

Table 3.4 The congruency analysis of the *ndhF* and *rbcL* gene fragments. Analysis showed trees having 24 leaves and the Maximum Agreement SubTree (MAST) having 12 leaves.

TREE 1 (*ndhF*)	((((((SB,SH),SC),ZM),O),B),(G,Q)),(VZ,J),((A,F),(M,R)),((C,I),(E,K)),(H,(D,P)),(L,N),S);	
TREE 2 (*rbcL*)	(((((SB,SH),VeZ),ZM),(B,K)),((A,C),(E,G)),(R,(D,L)),((H,Q),(I,P)),S);	
RESULTS	I_{cong} = 1.68055702161744	P-value = 5.13842807191919 e-05

Table 3.5 The congruency analysis of the *ITS* and *ndhF* gene fragments. The analysis showed trees having 25 leaves and the Maximum Agreement SubTree (MAST) having 5 leaves.

TREE 1 (*ITS*)	((((((((((((((((VZ,P),O),M),G),C),(B,(E,(K,(L,(R,S)))))),D),H),N),Q),F),A),I),J),CF),SC),(SH, SB),ZM);	
TREE 2 (*ndhF*)	((((((SB,SH),SC),ZM),N),P),((VZ,H),(A,K)),((B,Q),(D,E)),(C,R),(F,I),(G,M),(O,(J,L)),S);	
RESULTS	I_{cong} = 0.685871056241427	P-value = 191.614368000113

Table 3.6 The congruency analysis of the ITS and *rbcL* gene fragments. The analysis showed trees having 24 leaves and the Maximum Agreement SubTree (MAST) having 5 leaves.

TREE 1 (*ITS*)	((((((((((((VZ,N),B),O),((E,G),(H,M))),(C,P)),K),D),(R,(L,S))),Q),F),(A,(I,J))),SC),(SH,SB),ZM);	
TREE 2 (*rbcL*)	(((((SB,SH),VeZ),ZM),(B,K)),((A,C),(E,G)),(R,(D,L)),((H,Q),(I,P)),S);	
RESULTS	I_{cong} = 0.700232092340599	P-value = 146.762609777457

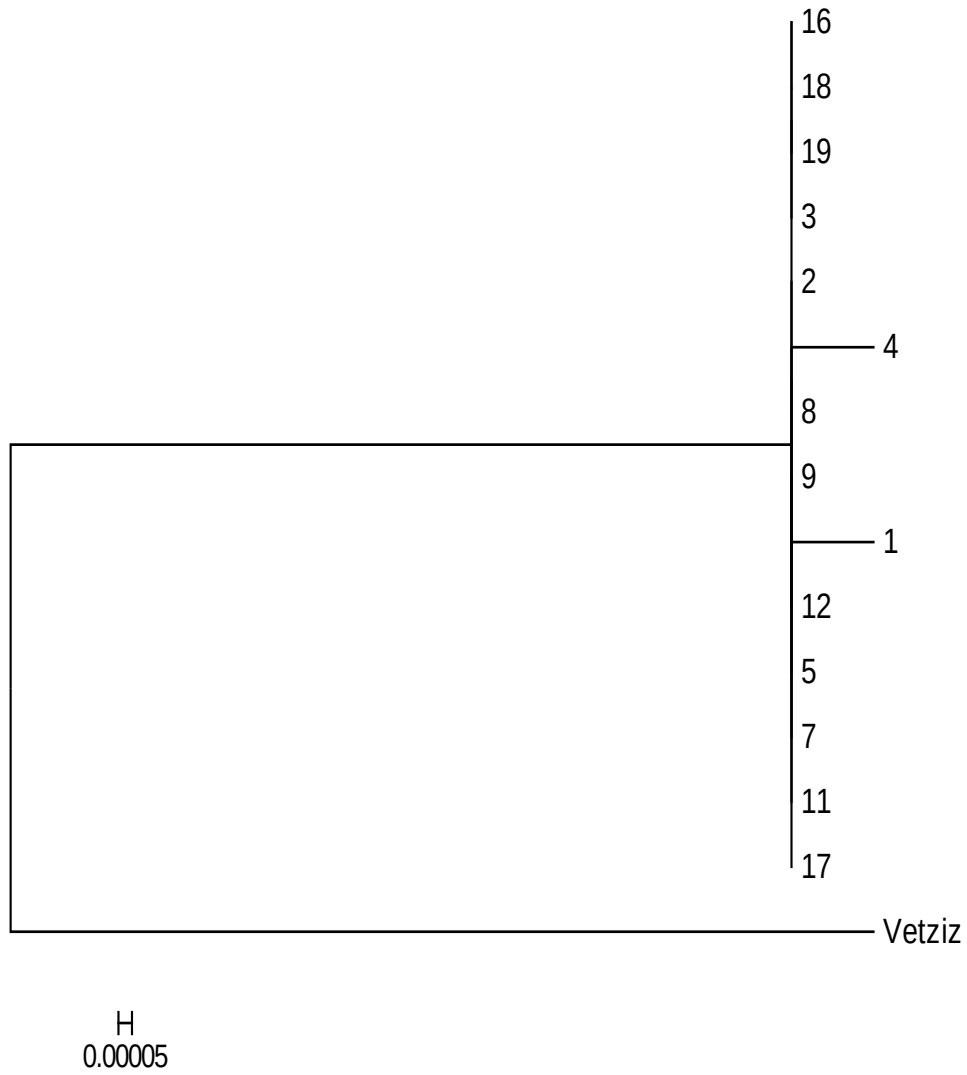

Figure 3.16 The congruency tree of the combined *ndhF* and the *rbcL* genes. The evolutionary history inferred using the Neighbour-Joining method without any outgroups. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes–Cantor method and are in the units of the number of base substitutions per site. GenBank accession was used for *Chrysopogon zizanioides* (Vetziz).

3.3 Discussion

Vetiver is one of the most considered remediation options for erosion control as *C. zizanioides* is non-invasive because of its infertility and the ability to only grow from clumps of rootstocks (Adams, 2002). Therefore, to fully exploit and manage this mediator, it is essential to understand the germplasm pool available of the isolates, for the propagation and use. It is also essential to ensure genetic biodiversity and proper management of this plant. In this section of the study the diversity of 19 different Vetiver isolates available in South Africa was investigated using DNA sequencing.

Three well known gene fragments were investigated in this study, namely the internal transcribed spacer (*ITS*) region of the 18S–5.8S–26S nuclear ribosomal cistron, the chloroplastic *ndhF* gene for NADH dehydrogenase subunit F and the chloroplastic *rbcL* gene for ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit.

Previous studies done on the genetic diversity of Vetiver grass consisted mostly of RAPD analysis (Kresovich, *et al.,* 1994; Srifah*, et al.,* 1996; Dong *et al.,* 2003). These studies reported results distinguishing between the different Vetiver ecotypes tested. Adams *et al.,* (1998), however, showed that all the Vetiver lines that were characterized and used for erosion control outside South Asia, are derived from a single genotype, namely “Sunshine”.

We base our conclusions on both the Neighbour- Joining Method (Jukes & Cantor, 1969) and the Maximum Likelihood methods. The Maximum Likelihood approach attempts to reconstruct a phylogeny using a model of evolution (Kuhner & Felsenstein, 1994), while Neighbour Joining is a clustering method using a distance matrix.

3.3.1 *ITS gene fragment*

According to literature cited in section 2.5.1.1., the nucleic *ITS* fragment can be used to infer the phylogenetic relationship between species and closely related genera (Baldwin *et al*., 1995; Soltis *et al.*, 1998) because it is a highly repeated region in the genome. The region also undergoes rapid concerted evolution and the small size and the highly conserved sequences flanking each of the spacers make this region easy to amplify (Balwin *et al*., 1995; Soltis *et al*., 1998). Because this gene fragment is subjected to concerted evolution, which leads to its intra-genomic uniformity, it thereby limits potential mutations which may

have led to variation. The result is that it is left with only species- and clade-specific characteristics noticeable (Alvarez and Wendel, 2003).

The Neighbour–Joining and Maximum Likelihood methods in this study showed similar results in the analyses of this fragment, with the outgroups such as *Sorghum* and *Saccharum spp*. clustering separately from the accessions of *Chrysopogon spp.* as expected. The accessions of *Chrysopogon* grouped together. Within this cluster the accessions of *C. serrulatus* and *V. fulvibarbis*, which both originate from Africa, grouped separately from the other species investigated as well as from the isolates obtained from Hydromulch in all the analyses.

C. nigritanus was previously identified as a variety of *C. zizanioides* (Veldkamp, 1999). However Veldkamp (1999) listed them as separate species. *C. zizanioides* and *C. nigritanus* are morphologically very similar and both are also similar to *C. festucoides* (Veldkamp, 1999). In all the analyses run during this study there was no significant difference between *C. zizanioides* and *C. nigritanus.* In our analyses the accession of *V. festucoides* grouped with the accessions of *C. nigritanus,* and *C. zizanioides* when all distant outgroups were included, but separately (bootstrap value =95%) from the *C. nigritanus- C. zizanioides* complex, when selected outgroups were excluded, despite the lack of morphological differences. Most isolates (2, 3, 4, 5, 6, 7, 8, 11, 12, 13, 14, 15, 16, 17, 18 and 19) grouped within the *C. zizanioides. -C. nigritanus* complex.

Isolates 1, 9 and 10 (the Democratic Republic of the Congo and Madagascar) grouped separately from the accessions of *C. nigritanus, C. zizanioides* and *V. festucoides* in all of the analyses, supported by high bootstrap values. These three isolates do not differ from the other isolates, based on the study done of the root anatomy, apart from the formation of starch granules in isolates 1 and 10. Furthermore the isolates 1, 9 and 10 do not appear to be as closely related to *C. zizanioides, C. nigritanus* or *C. festucoides* as the other isolates analysed. All the trees also indicated that isolate number 6 from Venezuela was the only isolate that grouped with *C. nigritanus.* During this study no statistically significant difference was observed between the two isolates obtained in Rustenburg, South Africa (accessions 7 and 18) although they did group separately. It is speculated that these two isolates might have originated from different sources of *C. zizanioides* although they were obtained from the same commercial farm, and may thus differ due to limited somatic mutation as a result of environmental factors. However, further studies need to be conducted to confirm the above results.

Only a few statistically significant differences in the *ITS* gene fragment were observed between the 16 isolates from Hydromulch (Pty) Ltd. and the accessions of *C. zizanioides* (Vetziz) and *C. nigritanus* (Vetnig). One of the isolates investigated by Adams *et al.* (1998) is noted as *C. zizanioides* var Monto Australia. Isolate no 4 in this study is also a *C. zizanioides* var Monto Australia. Thus according to Adams *et al.* (1998), the assumption can be made that the isolates which grouped with this accession have the same genotypic origin, namely *C. zizanioides* "Sunshine".

The *ITS* gene fragment was not congruent with either the *rbcL* or *ndhF* gene fragments, thus the results obtained could not be compared in combination with the other gene fragments and therefor the identity of *C. zizanioides* and *C. nigritanus* could not be confirmed. Even when the majority of the accessions of the outgroups was removed, the *ITS* dataset was the most informative.

3.3.2 *The ndhF and rbcL chloroplast gene fragments*

The *ndh*F gene is a rapidly evolving chloroplast gene (Olmstead & Sweere, 1994; Soltis *et al.*, 1998). The 5' region (1380 bp) is different from the 3' region (855 bp) in that the 3' region is A + T rich, has higher levels of non-synonymous base substitutions and higher transversion bias at the codon positions. The different patterns of base substitution at the 5' and 3' region makes this gene ideal for phylogenetic reconstruction as the conserved and variable segments can be used for older and recent groups respectively (Kim & Jansen, 1995). According to the literature reviewed (Adams *et al.,* 1998; Scotland *et al.,* 1995; Backlund, *et al.,* 2000; Olmstead & Sweere, 1994) the combination of the *ndhF* and *rbcL* gene fragments prove to be informative in plant systematics.

The results of this study showed no significant difference between the different isolates obtained from Hydromulch (Pty) Ltd. in the analyses of either of the less conservative chloroplast gene fragments (*ndhF* and *rbcL*), which is contradictory to the findings based on RAPD and AFLP analyses of previous publications (Adams *et al.,* 1998; Backlund *et al.,* 2000). Both the Neighbour–Joining and Maximum likelihood analyses with the inclusion and exclusion of selected outgroups yielded similar results for both of the chloroplastic genes fragments. In the analysis of the *ndhF* gene fragment, the results indicated that there was no genotypic difference between the 19 isolates and the Genbank accession of *C. zizanioides*. For the *rbcL* gene fragment there was no difference between the different isolates of Hydromulch (Pty) Ltd. However, there was a difference between the studied isolates and the

accession of *C. zizanioides.* This may be the result of insufficient taxon sampling since the accession was sequenced. Despite the high bootstrap support for this, only minor differences were visually observed during sequence alignment between the different isolates as well as the outgroups.

Unfortunately, there is currently no data available for *C. fulvibarbis, C. festucoides*, *C. serrulatus* nor *C. nigritanus* for either the *rbcL* or the *ndhF* gene, so the difference between the above could not be analysed. However, because the chloroplast gene fragments showed no significant difference between any of the isolates from Hydromulch (Pty) Ltd. (including isolate number 6) and *C. zizanioides*, it can be hypothesised that *C. nigritanus* would group with the *Chrysopogon* cluster.

The *ndhF* and *rbcL* gene fragments were congruent, with the congruency tree showing similar results to the individual *ndhF* and *rbcL* phylogenetic trees. The data obtained from the congruency analysis also showed that there is no difference between the Hydromulch (Pty) Ltd. isolates when it comes to the chloroplast genes. Thus, despite the use of rapidly evolving chloroplast gene fragments (Kim & Jansen, 1995), the results generated during this study showed no difference amongst the 19 isolates in the above gene fragments.

CHAPTER4 ANATOMICAL ANALYSES

This chapter describes the comparative study done on the root anatomy of the isolates obtained from Hydromulch (Ltd) Pty.

4.1 Materials and Methods

4.1.1 *Cultivation and harvest of sample material*

The roots of the isolates were cultivated as previously described (see section 3.1.1). In order to investigate patterns of growth plasticity in the roots of the different isolates, the effect of waterlogging was induced. Young plant slips (3 of each) were taken from the different isolates and grown in tap water in containers and polystyrene was used to keep the plants afloat. All the plants received a general fertilizer once a week and the water was replaced once every two weeks to limit microbial growth around the roots. This was done to compare the roots of the soil grown isolates to the roots of the isolates grown in water in addition to compare the roots of the different isolates. The water grown isolates were kept in the same greenhouse as the soil grown isolates (see section 3.1.1).

The roots of the isolates grown in water were harvested after a growth period of 8 weeks, while the roots of the soil-grown isolates were harvested after a growth period of 9-11 weeks after new roots were given time to occur and develop. Roots were collected at approximately 2 cm from the rootcaps from three different plants of the same isolate. The roots were rinsed with tap water to remove sand particles. Cross sections of 3 mm were cut in 4% aqueous paraformaldehyde using a surgical blade. The tissue samples were then fixed in 4% aqueous paraformaldehyde in glass vials for anatomical studies. The sample material was left overnight at 4 °C in the 4% paraformaldehyde.

4.1.2 *Tissue preparation for histochemical analysis*

The plant material was rinsed in three changes of 20 ml distilled water, dehydrated in an ethanol series (20%-70%) and embedded and polymerized in LR White™ resin. Sections of 1 µm thickness were cut with a Reichert Ultracut E microtome, collected on glass slides and stained with 0.5% aqueous toluidine blue and 0.05% aqueous Sudan red (see section 4.1.3).

Micrographs were digitally captured using Motic images software with an Olympus OM2 light microscope.

4.1.3 *Histochemical analysis*

For histochemistry 1 µm thick cross-sections were made of the embedded material. Ten cross sections were made from each root sample. The samples were stained with Sudan red for lipid detection (Brundrett *et al.,* 1991) and Toluidine Blue, which stains lignin blue-green and cellulose and polycarboxylic acids red to reddish purple (Wahl *et al*., 2001).

4.1.4 *Cell Measurements*

The diameters of the cells in the different tissue regions were measured under a light microscope using Motic Image Plus07 software. A minimum of 5 cells was measured in each tissue region. The regions measured included the following:

1. The total diameter of the root cross section (in µm)
2. The diameter of the vascular cylinder (all the cells in the vascular cylinder including the endodermis)
3. The width of the cortex area
4. The width of the exodermis area
5. The diameters of the epidermis cells
6. The diameters of the cortex parenchyma cells
7. The diameters of the endodermis cells
8. The diameters of the vascular cambium parenchyma
9. The diameters of the xylem vessels
10. The diameters of the sclerenchyma cells in the vascular cylinder
11. The width of the Casparian strips

The meta-xylem vessels were counted. The presence of starch granules and aerenchyma were also noted. Scale images were taken of the different root tissue regions using Motic Live software.

4.1.5 *Statistical analyses*

Statistica version 10- software was used to determine the differences between the different variables measured for each isolate. The Kolmogorov-Smirnov and Lilliefors test for normality was used to determine if the datasets were distributed parametrically. The data did not meet the assumptions of normality in the distribution of all variables. Therefore the non-parametric Kruskal-Wallis ANOVA for comparing multiple independent samples was used to determine differences between the different isolates grown in both soil and water.

4.2 Results

4.2.1 *Root cross-section*

Vetiver roots, like most monocotyledonous roots, showed no typical secondary growth. Generally no cuticle or root hairs were observed in the radial cross section. The different tissue regions were clearly defined: The epidermis (hypodermis) consisted of 1 cell layer while 2 to 3 cell layers contributed towards the exodermis, the cortex, containing air spaces, the endodermis, the pericycle (which was only visible in younger roots), the vascular cylinder, containing clearly visible metaxylem elements, arranged in a single ring, with primary phloem groups alternating with the protoxylem groups in some of the roots, as well as a parenchymatuous pith.

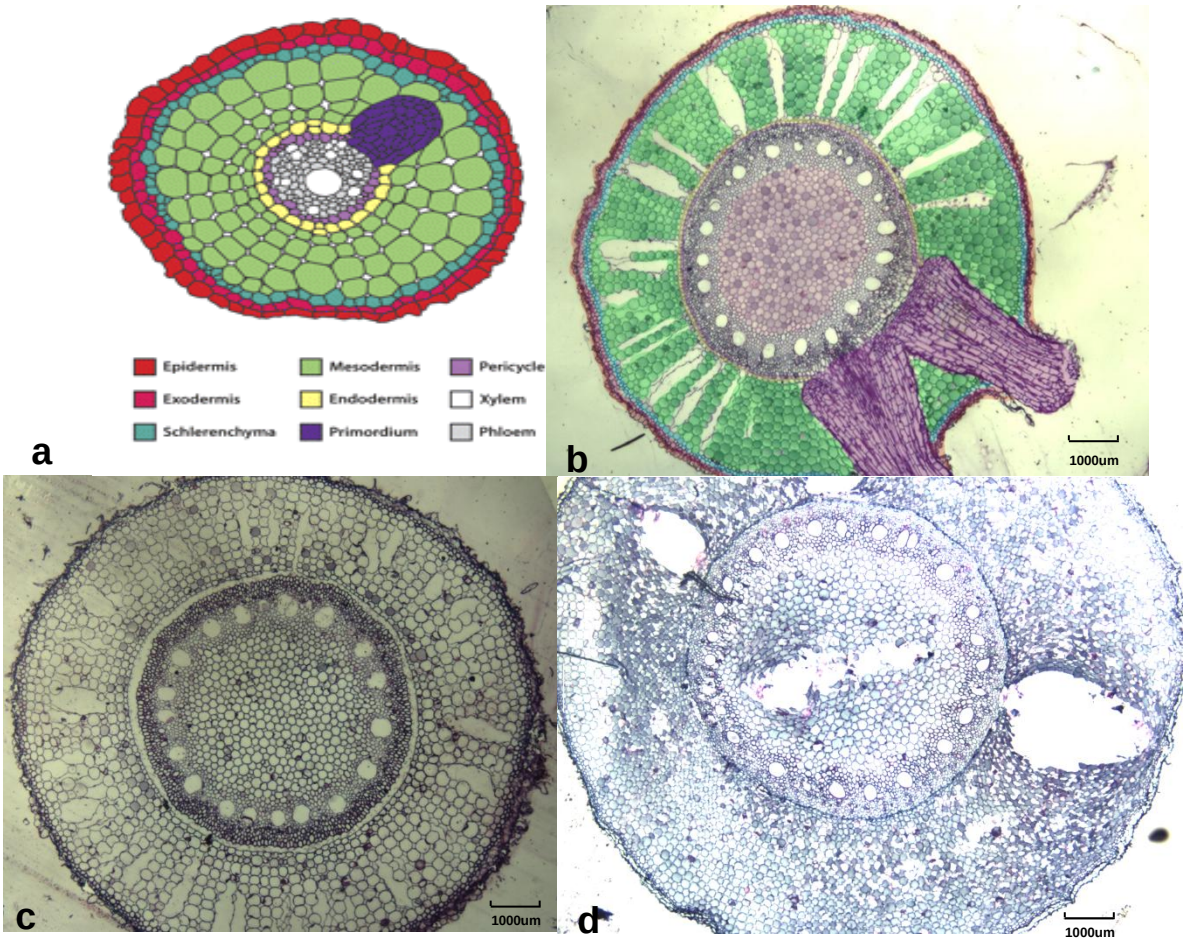

Figure 4.1 A cross section of *O. sativa* (a), and isolate 2 (b) grown in water that allows the identification of the different cell layers. The cross section of isolate 5 (Mozambique, nampula) (c) cultivated in soil, and isolate 11 (Ghana, Gingani) (d) in water taken at a 4x magnification are also shown to compare the average width of the roots grown in soil to the roots grown in water.

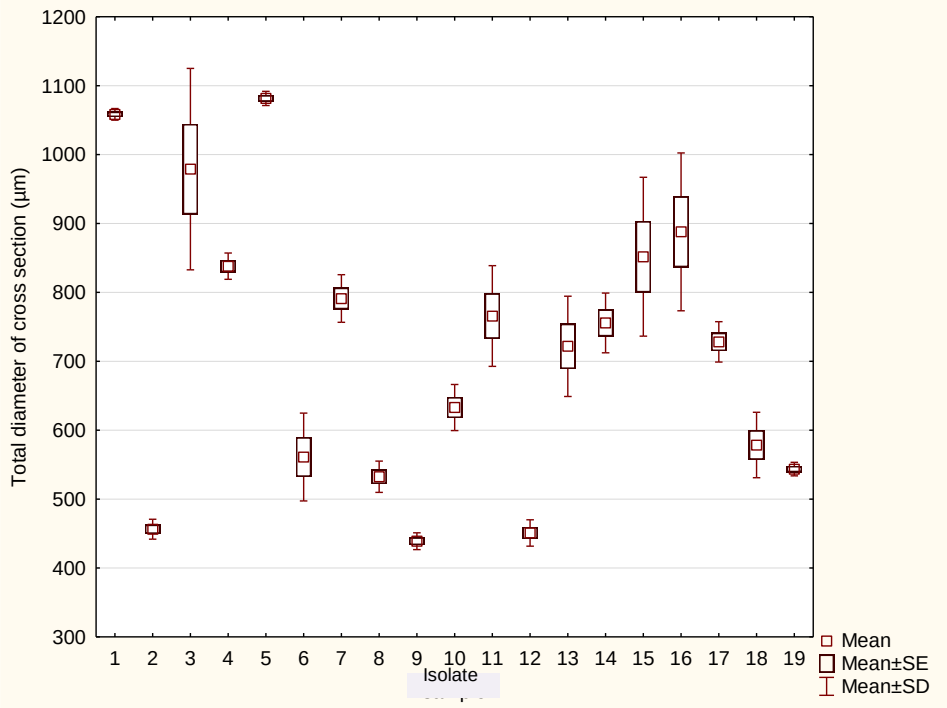

Figure 4.2 The total width of the cross section of roots of the different isolates (numbered 1-19) grown in soil. The widths ranged from isolate 9 (Madagascar South) with the lowest width (~438µm) to isolate 5 (Mozambique, nampula) with the highest (~1135µm).

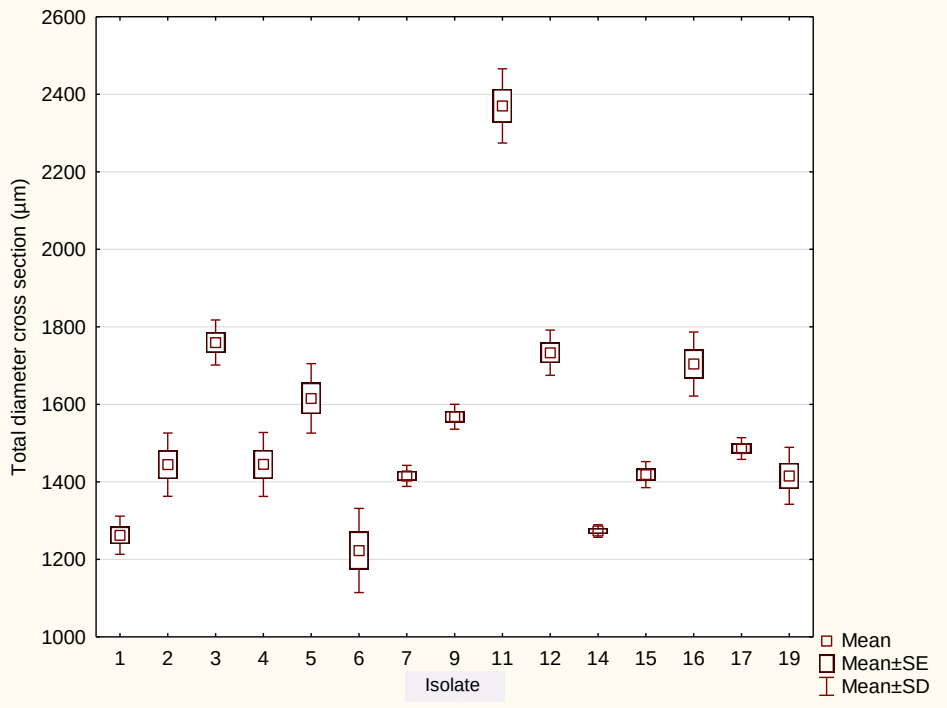

Figure 4.3 The total width of the cross section of roots of the different isolates (numbered 1-19) grown in water. The widths ranged from isolate 9 (Madagascar South) with the lowest width (~1222µm) to isolate 5 (Mozambique, nampula) with the highest (~2370µm).

On average, the total width of the roots grown in water was double the width of roots grown in soil (figures 4.2 and 4.3). The smallest mean width of the roots grown in soil was obtained from isolate 9 (438 µm), and the largest mean width from isolate 5 (1131.8 µm). The average total widths of the roots grown in water were very similar, with isolate 6 having on average the smallest width (1222.94 µm). Isolate 11 exhibited the largest average width (2370.12 µm). This difference in measurement can possibly be accounted for by the presence or absence of mechanical resistance by the different isolates grown under different conditions.

4.2.2 *Epidermis and exodermis*

There were variation between the widths of the different isolates of the roots grown in soil as well as of the roots grown in water (figures 4.4 and 4.5). The epidermal cells of the water grown roots had larger average widths. The lowest average epidermal cell width was measured in isolate 17 (26.20 µm) and the highest in isolate 19 (57.82µm) in the soil grown roots. The lowest average width of the roots grown in water was measured from isolate 19 (38.67 µm) and the highest from isolate 7 (94.12 µm). The exodermal cells varied in shape, but in most cases were isodiametric, and sometimes the cells were flattened. In the roots of the isolates grown in soil, the exodermal layer was well defined and contained cells with lignified cell walls (figures 4.6a and 4.6e).

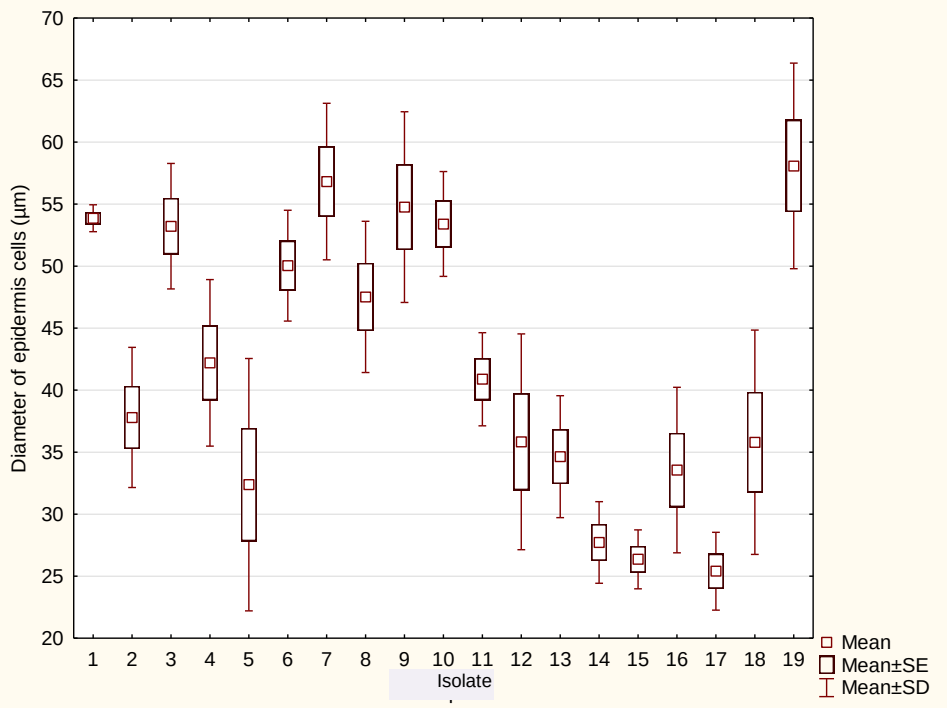

Figure 4.4 The width of the epidermal cells of roots of the different isolates (numbered 1-19) grown in soil. The widths ranged from isolate 17 (Puerto Rico) having the smallest epidermal cells (~26.20 µm) to isolate 19 (Ghana, Kumasi) with the largest (~57.82 µm).

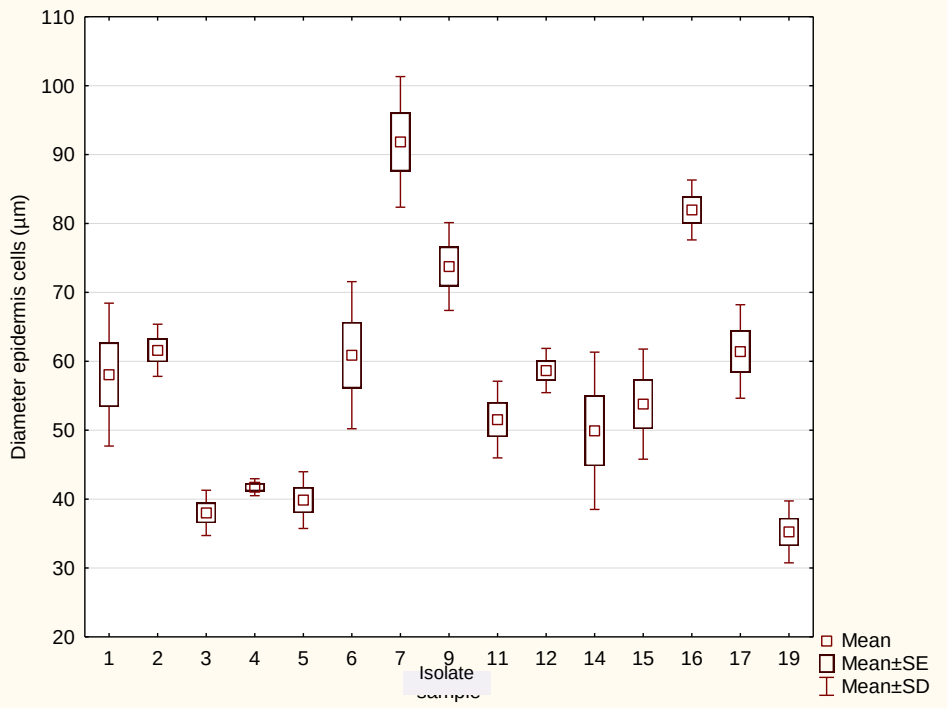

Figure 4.5 The width of the epidermal cells of roots of the different isolates (numbered 1-19) grown in water. The widths ranged from isolate 19 (Ghana, Kumasi) having the smallest epidermal cells (~38.67 µm) to isolate 17 (Puerto Rico) with the largest (~94.12 µm).

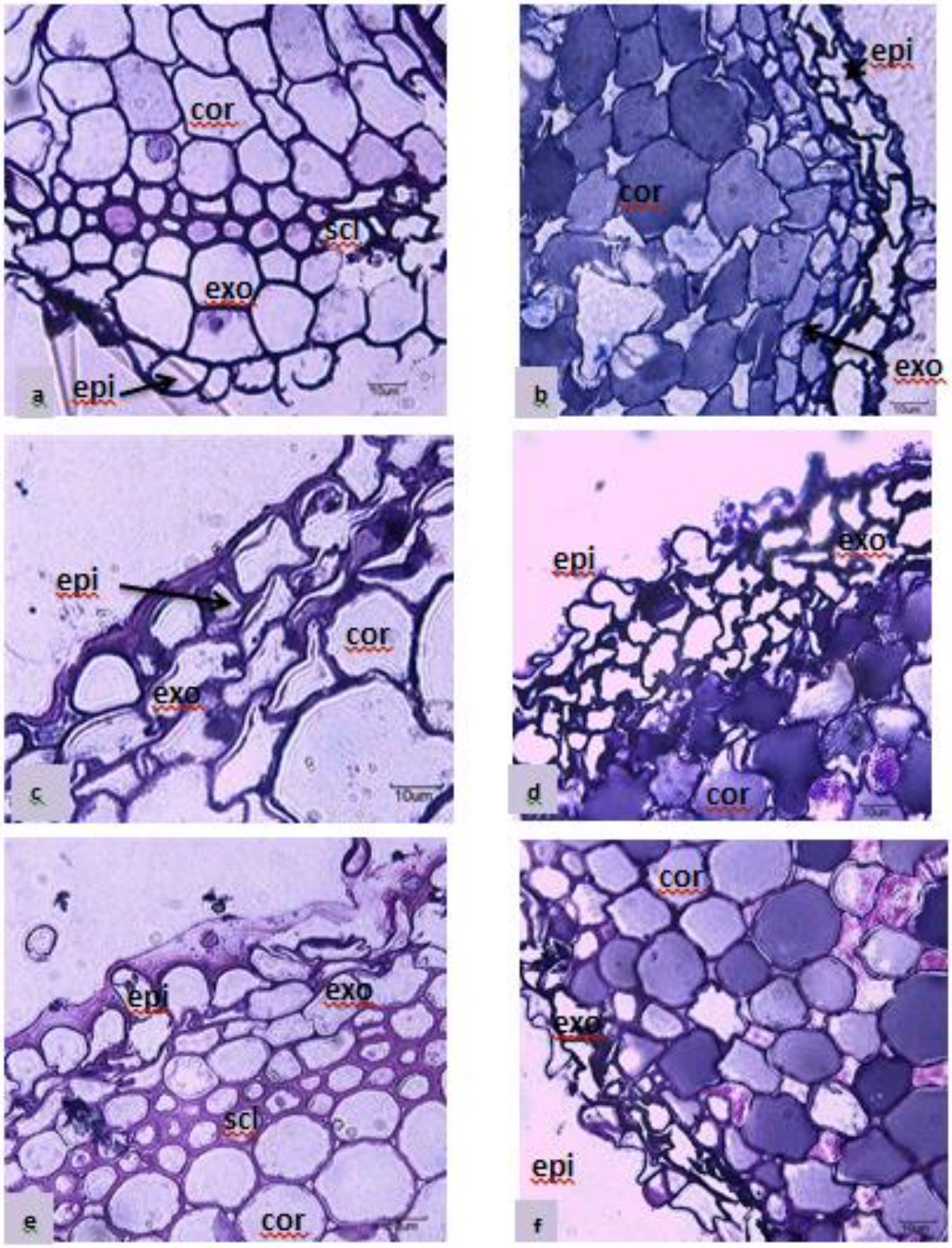

Figure 4.6 Selected isolates chosen to show the epidermis and exodermis in soil-grown isolates (a, c, e) and water- grown isolates (b, d, f): Isolate 11(Ghana, Gingani) (a and b), 17 (Puerto Rico) (c), 16 (Mozambique) (d) and 19 (Ghana, Kumasi) (e and f) taken at a 40x magnification. (epi= epidermis, exo= exodermis, cor= cortex, scl= sclerenchyma).

4.2.3 *Cortex*

In all the vetiver root cross sections studied, the root cortex consisted primarily of radially arranged parenchyma (figure 4.9). Considerable cortical structural variation was noted in the roots grown in water. The cortical layers varied considerably, with the roots grown in water generally comprising of more layers. The cells were mostly periclinal, variously isodiametric, rounded, or irregular and the cell walls were either straight or sinuous. Intercellular spaces were observed in all the isolates, and some exhibited aerenchyma (figure 4.10). The widths of the cortex of the roots of the isolates grown in water were on average 1.5 - 2 times greater than those of the roots grown in soil (figures 4.7 and 4.8). Isolate 9 grown in soil had the lowest average cortex width (120 µm), and isolate 11 grown in water had the highest average width (1300 µm). There was a large variation between the widths of the cortex layers of the different roots grown in soil, with the widths ranging from 120 µm (isolate 9) to 520 µm (isolates 1 and 7). There was little variation between the widths of the cortex layers of the different roots grown in water (600 µm – 800 µm), with the exceptions being isolate 6 (480 µm) and isolate 11 (1300 µm).

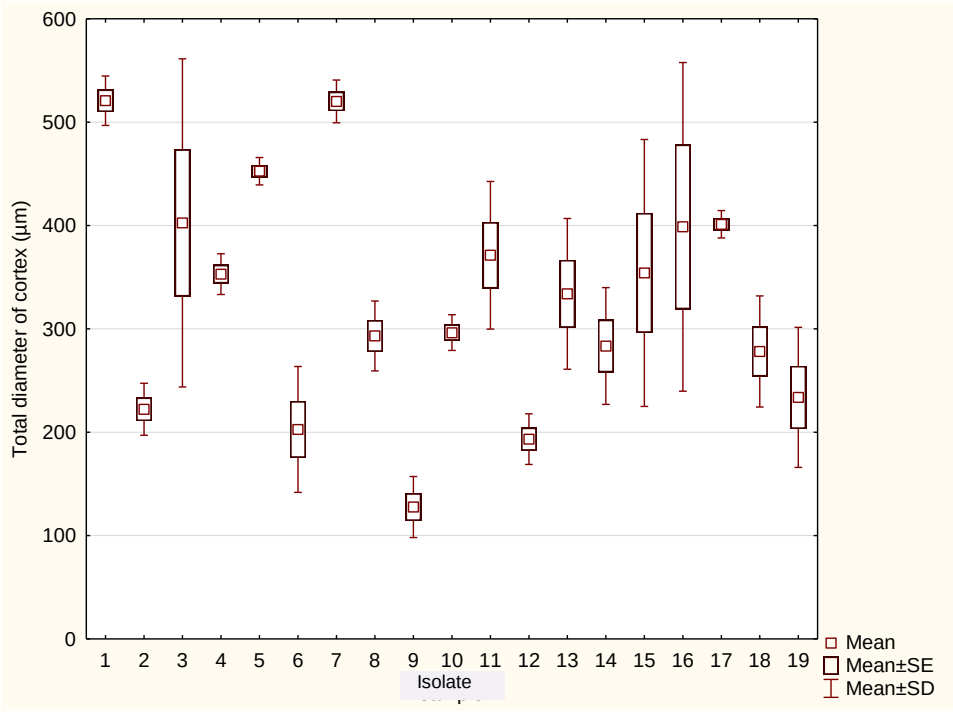

Figure 4.7 The width of the cortex layer of roots of the different isolates (numbered 1-19) grown in soil. The widths ranged from isolate 9 (Madagascar North) with the lowest cortex width (120 µm) to isolates 1 (Congo, DRC) and 7 (South Africa, Rustenburg) with the highest cortex width (520 µm).

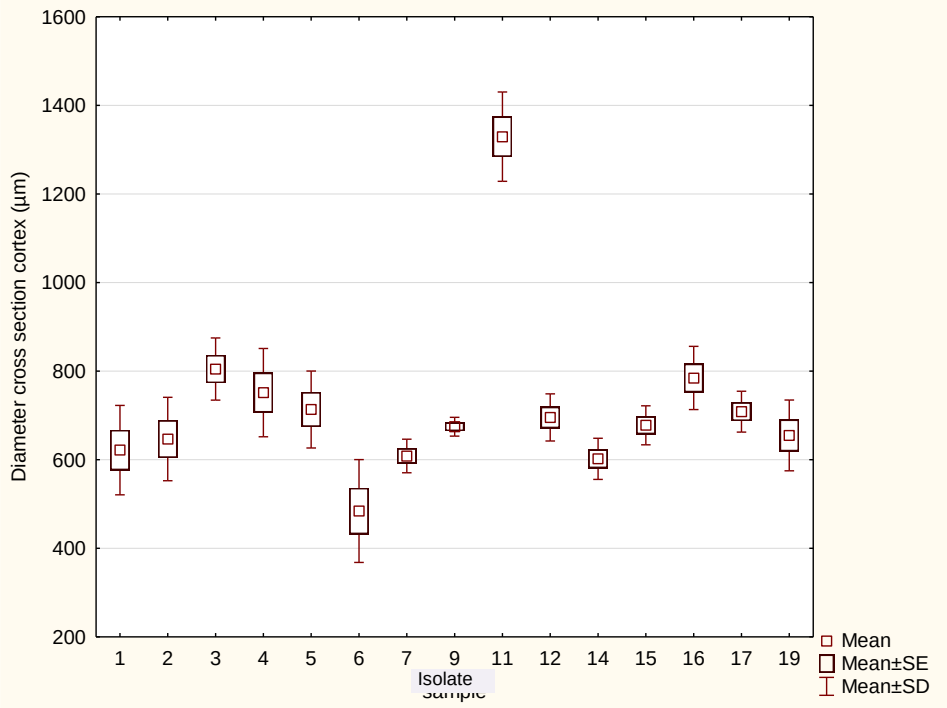

Figure 4.8 The width of the cortex layer of roots of the different isolates (numbered 1-19) grown in water. All the isolates had a similar average cortex width (~500 µm - ~800 µm), with the exception of the cortex of of isolate 11 (Ghana, Gingani), that was more than twice as wide as some of the other isolates (1300 µm).

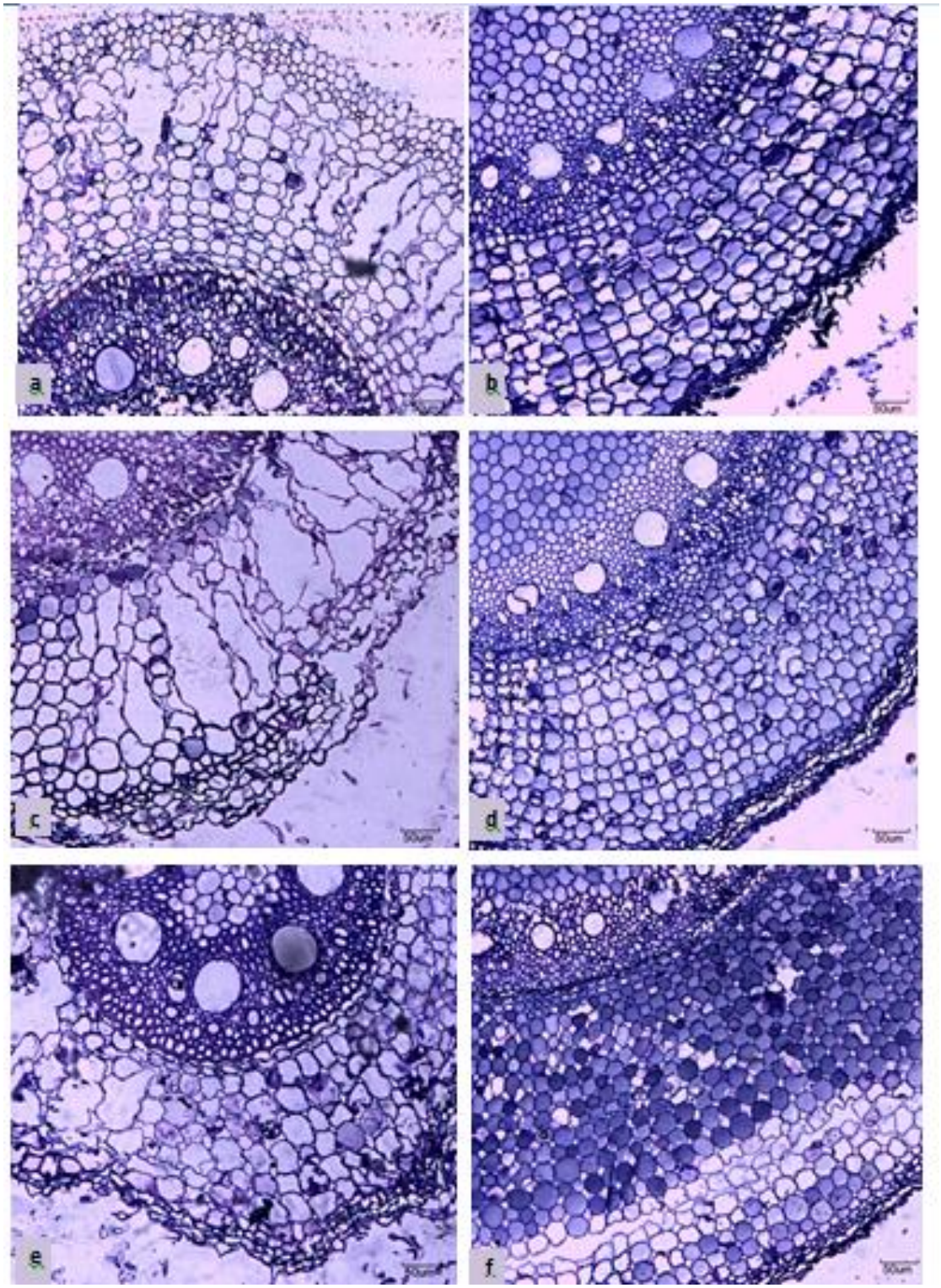

Figure 4.9 Selected isolates chosen to show the cortex cell arrangement in isolates grown in soil (a, c, e) and water (b, d, f) taken at 10x magnification Isolate 1 (Congo, DRC) (a), 7 (South Africa) (b), 9 (Madagascar North) (c), 9 (Madagascar North grown in water) (d), 11 (Ghana, Gingani) (e), 14 (New Zealand) (f). The parenchyma had a radial arrangement in both the roots grown in soil and in water. The overall width of the cortex parenchyma was higher for the roots grown in water (500 µm - 1300 µm) than the roots grown in soil (120 µm – 520 µm).

4.2.4 *Aerenchyma formation*

Lysigenous aerenchyma (see section 2.6.1.1) was present in water grown roots of isolate 2 (Madagascar- South, Fort Dauphin) and 6 (Venezuela, Caracas), whereas no aerenchyma was observed for the same isolates grown in soil (figure 4.10).

Lysigenous aerenchyma was present or was developing in the roots grown in soil (isolates 1, 3, 5, 7, 9, 10, 11, 15 and 16) but was absent in the roots of the same isolates which were grown in water.

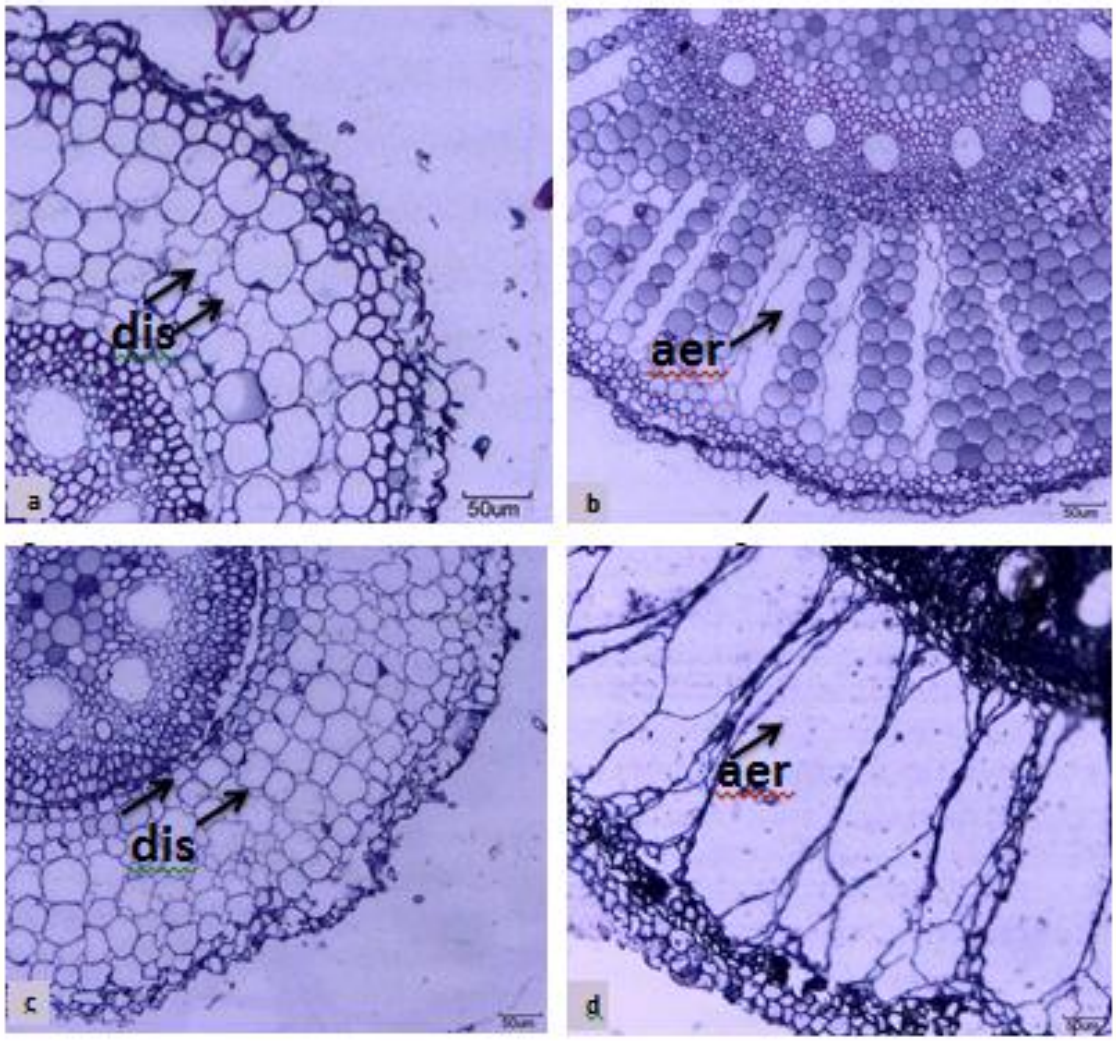

Figure 4.10 The cross-section of Vetiver roots showing the presence of aerenchyma at a 10x magnification. 2a) Isolate 2 (Madagascar South, Fort Dauphin) grown in soil, and 2b) isolate 2 grown in water. 2c) isolate 6 (Venezuela, Caracas) grown in soil, and 2d) isolate 6 grown in water. The cortex cells in the isolates shown in a and c disintegrated (dis), and the developed aerenchyma was observed in b and d (aer).

4.2.5 *Endodermis*

In the Vetiver cross sections studied, one to two layers of endodermal cells were observed, usually arranged in a periclinal pattern, their shape being variously isodiametric-polygonal or rectangular shaped (figure 4.11).

In the soil grown roots the inner layer of the cortex consisted of one layer of mature endodermis cells with Casparian strips. The endodermis layer was well defined with U-shaped tertiary wall thickenings.

In water grown roots no U- shaped tertiary wall thickenings were observed in the mature endodermis. This may be because there was a sufficient water potential gradient to ensure that water diffuses freely into the xylem to be transported to the rest of the plant.

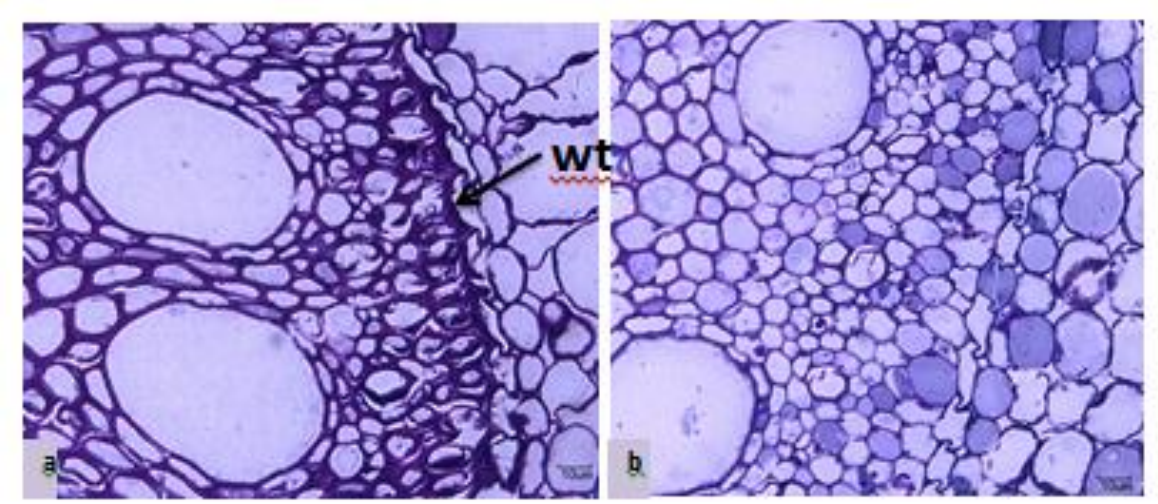

Figure 4.11 Isolate 3 (Congo, DRC, Kinshasa) showing the endodermal cell layer in roots grown in soil (a) and in water (b). In the roots grown in soil well defined U-shaped tertiary wall thickenings was observed (wt).

4.2.6 *Pericycle*

The pericycle was reduced / absent / not well defined in some of the roots analysed. Lateral roots were obsereved in some of the isolates, but it is not clear if the development of the lateral roots originated from the endodermis or the pericycle as seen in figure 4.12.

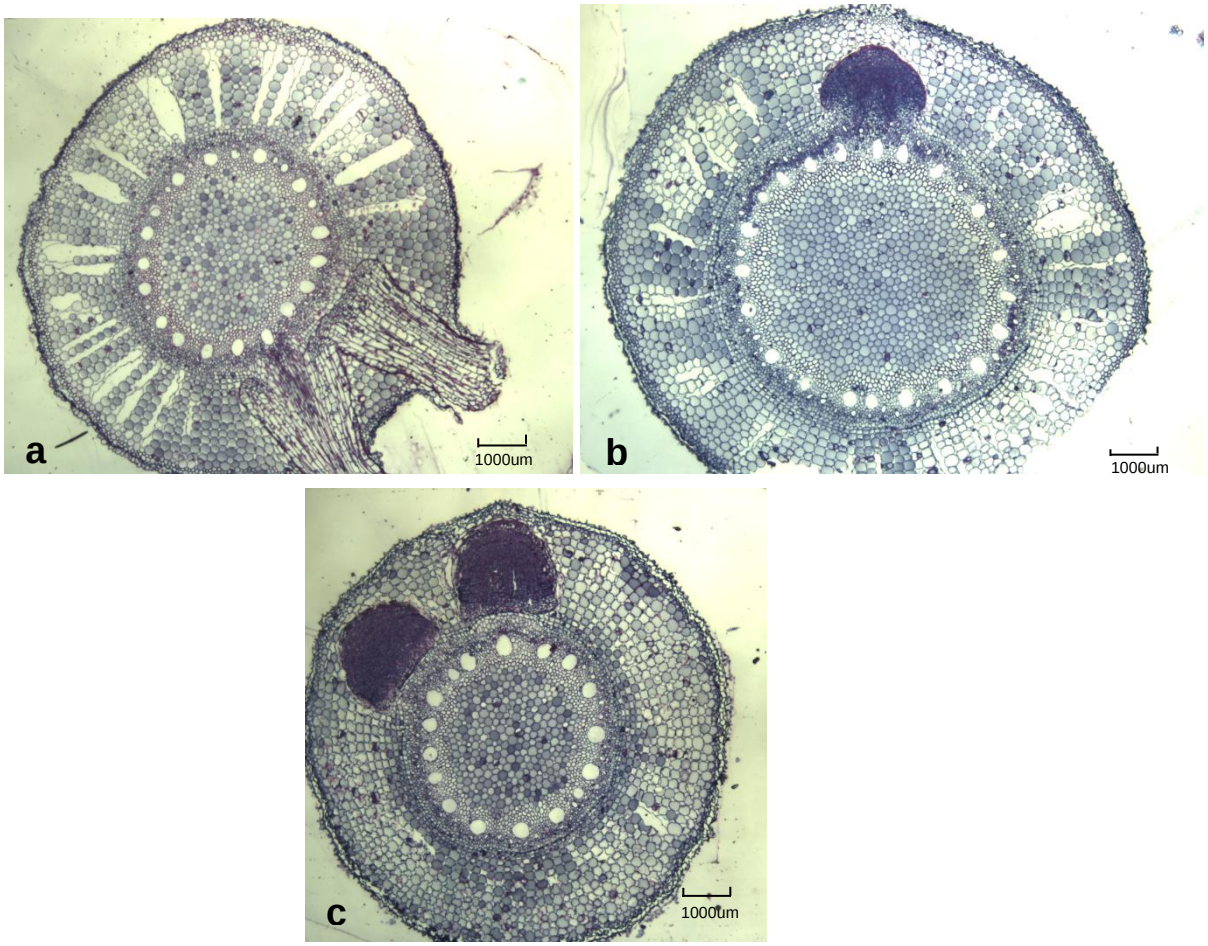

Figure 4.12 The cross section of isolates grown in water: 2 (a) (Madagascar South), 12 (b) (Ghana Buleng) and 17 (c) (Puerto Rico) to show the formation of the lateral roots, taken at a 4x magnification.

4.2.7 *Vascular cylinder*

The widths of the vascular cylinders of the roots grown in water (figure 4.14) were on average twice the width of the vascular cylinders of the roots grown in soil (figure 4.13). In both the soil- and water grown roots, the measurements varied of the different isolates. This may be due to the possibility of the presence or absence of mechanical resistance of the different isolates in this study. Isolate 2 from the roots grown in soil had the smallest vascular cylinder width (184 µm), and the greatest width was obtained from isolate 5 (619 µm). The smallest width of the vascular cylinder of the roots grown in water was from isolate 1 (640.46 µm) and the greatest width from isolate 11 (1040.78 µm).

The studied roots showed a central xylem cylinder (protostele) with phloem groups i.e., sieve tubes and companion cells, alternating with the proto-xylem groups (figure 4.15). The mature soil roots showed an average of 11 meta-xylem elements, which is typical of monocotyledon roots, arranged in a single ring, ranging from 6 (isolates 2, 13 and 14) to 22 elements (isolate 19). The mature water grown roots show an average of 19 meta-xylem elements, ranging from the smallest number being 14 (isolate 14) and the largest number being 24 elements (isolate 9).

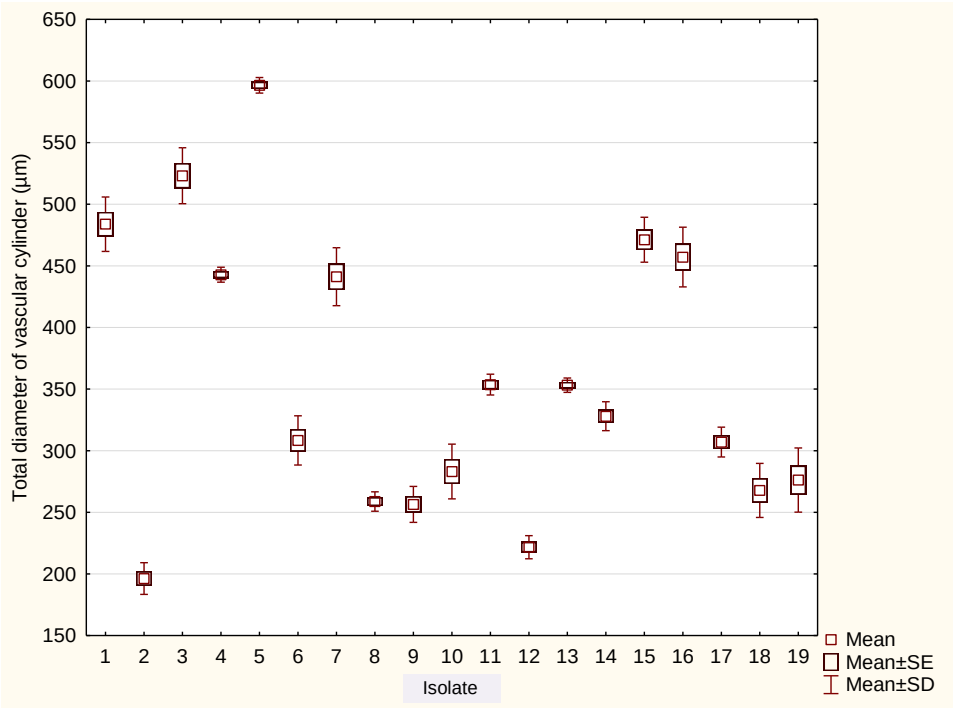

Figure 4.13 The widths of the vascular cylinders of roots of the different isolates (numbered 1 - 19) grown in soil. The widths of the different isolates varied, with isolates 2 (Madagascar South) and 12 (Ghana, Buleng) having the average smaller vascular cylinders (~184 µm), and isolate 5 (Mozambique, nampula) the average bigger vascular cylinder (~619 µm).

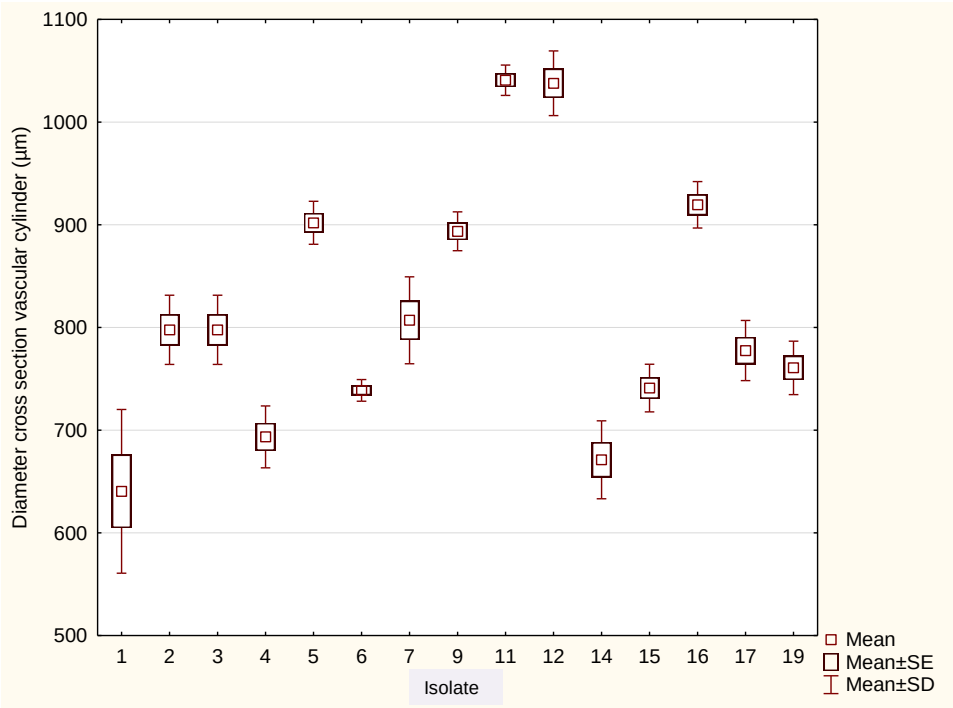

Figure 4.14 The widths of the vascular cylinders of roots of the different isolates (numbered 1 - 19) grown in water. The widths of the different isolates varied, with isolate 1 (Congo, DRC) having on average a smaller vascular cylinder (~640.46 µm) and isolates 11 (Ghana, Gingani) and 12 (Ghana, Buleng) having the average bigger vascular cylinders (~1040.78 µm).

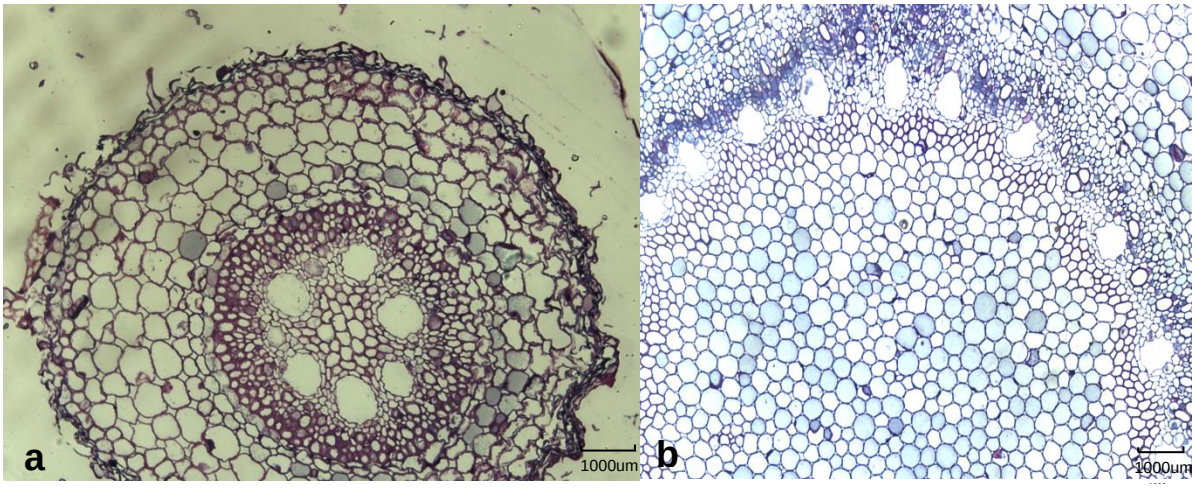

Figure 4.15 The vascular cylinder of the roots of isolate 12 (Ghana, Buleng) grown in a) soil (with an average width of ~224 µm) and b) water (with an average width of ~1045 µm). The vascular cylinder of the roots grown in soil was on average two times smaller and contained fewer xylem elements than the roots grown in water.

A substantial amount of sclerenchyma was observed in both the soil- and water-grown roots that typically provides mechanical support and contributes towards the tensile strength of roots.

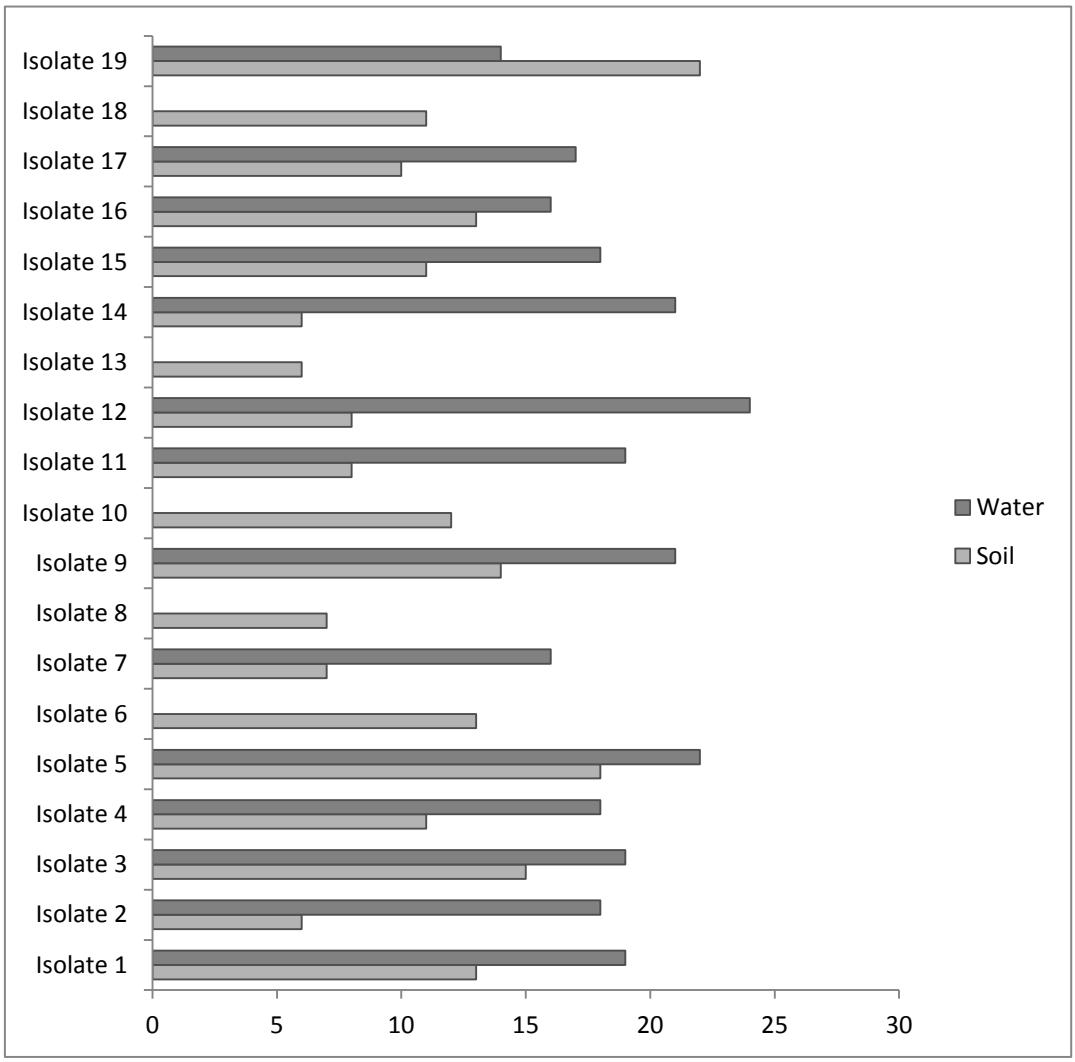

Figure 4.16 The number of meta-xylem elements found in the different roots of the isolates. The dark bars represent the xylem elements of the roots grown in soil and the light bars (ranging from 6 to 22), the xylem elements of the roots grown in water (ranging from 14 to 24).

More xylem elements were present in the roots of isolates grown in water than in soil, with the isolate 19 being the only exception (figure 4.16). The isolates of the roots grown in soil, had between 6 (isolates 1, 13 and 14) and 22 (isolate 19) xylem elements, where the isolates grown in water had between 14 (isolate 19) and 24 (isolate 12) elements.

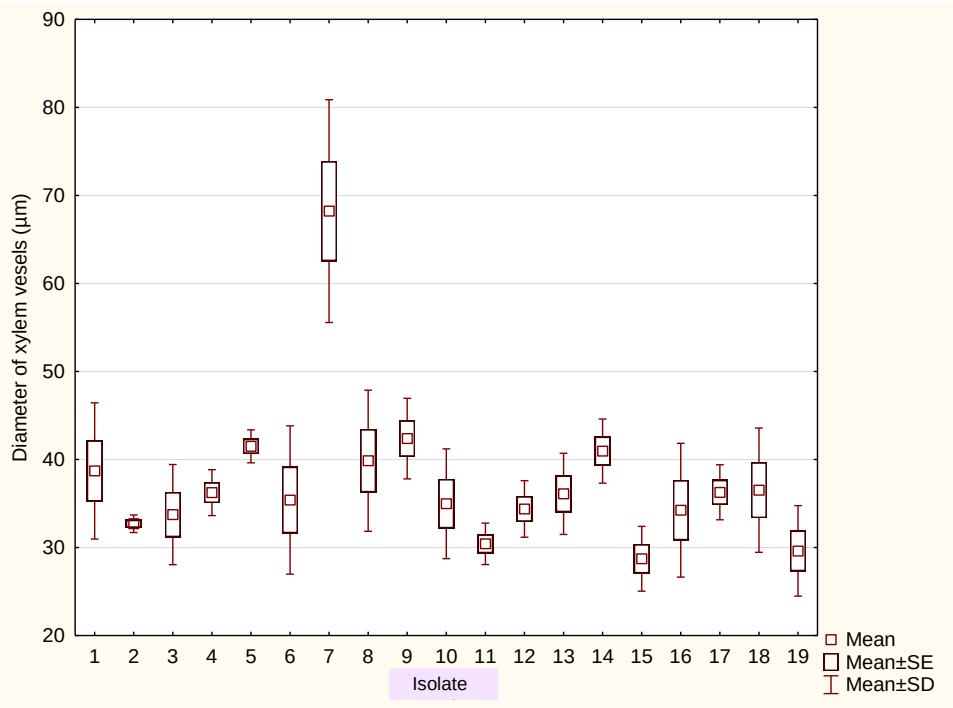

Figure 4.17 The average diameters of the meta-xylem elements of the roots of the 19 isolates (numbered 1 - 19) grown in soil. The roots from the different isolates had meta-xylem elements of a similar diameter (between 28 µm and 42 µm) with the exception of isolate 7 which displayed diameter values that were almost twice as wide as the others (of 67 µm).

The diameter of most of the isolates ranging from 29 µm (isolate 15) to 42 µm (isolate 9), with the exception of isolate 7, which had an average diameter of 67 µm.

4.2.8 *Central vascular cylinder (Pith) of the Vetiver root isolates*

The root piths of the 19 investigated isolates grown in soil and water had a primarily parenchymatuous structure with polygonal, oval or rounded cells (figure 4.18). In the soil grown roots of isolates 1 and 10 (figure 4.18 a-b) as well as the water grown roots of isolate 10 (figure 4.18 c - d) from Congo and DRC Kingshasha respectively, starch granules were present in the parenchyma cells of the central vascular cylinder. Sclerenchyma cells with lignified cell walls were present towards the outside of the parenchymatuous pith, and also between the xylem vessels.

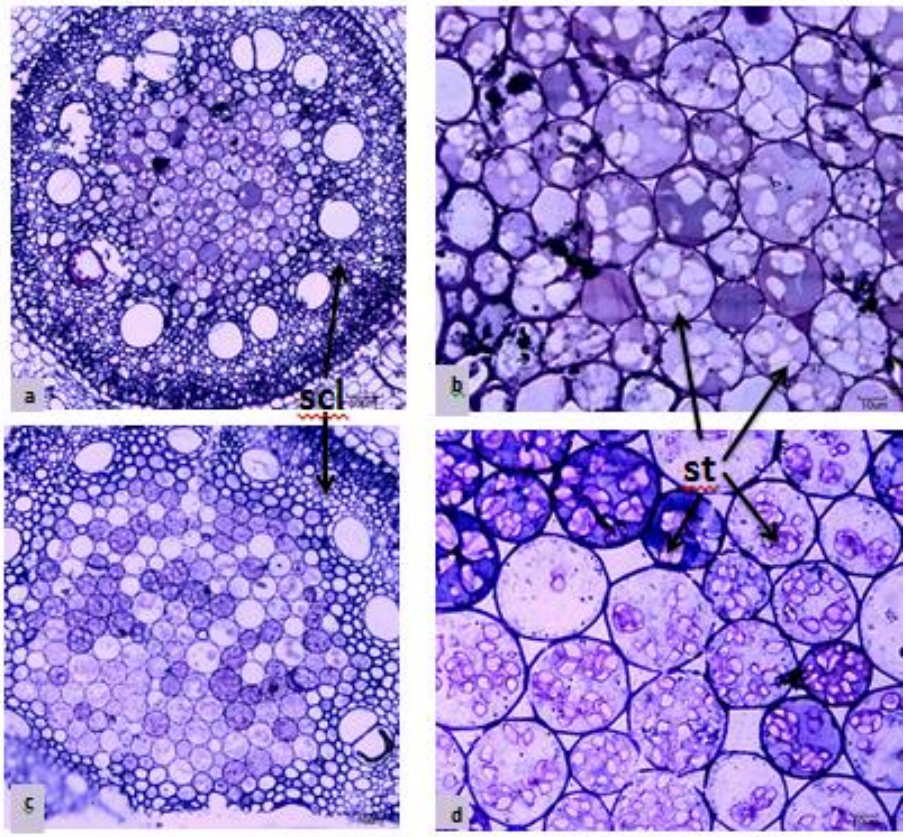

Figure 4.18 The cross-sections of isolates 1 and 10 showing the presence of starch granules in the central paranchymatuous pith. Isolate 1, (a and b, cultivated in soil) and isolate 10 (c, d, cultivated in soil) were both labelled as Congo, DRC Kingshasha (*C. nigritana*), and were the only isolates which formed starch granules (st) during the cultivation period. Sclerenchyma (scl) was present in both isolates to serve as support.

4.2.9 *Ratios of different root areas*

Ratios were calculated of the different root areas in the cross sections of the isolates to determine if the measurements differed from the ratios to get a clearer picture of the root structure.

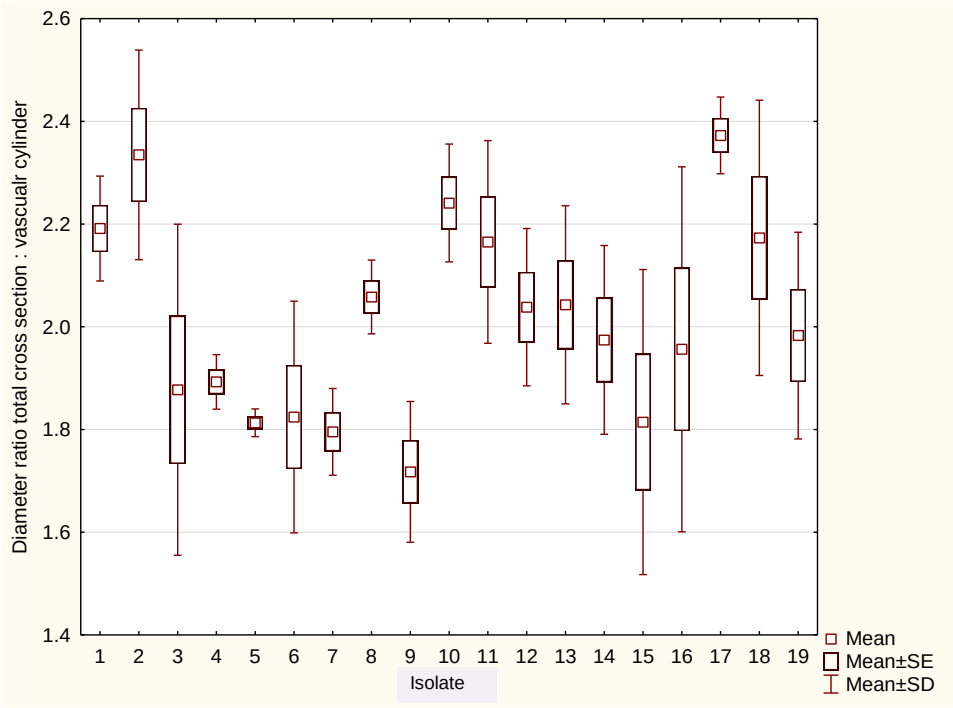

Figure 4.19 The ratios of the diameters of the total cross sections to vascular cylinders of the different isolates (numbered 1 - 19) grown in soil. The lowest cross section: vascular cylinder ratio was measured from isolate 9 (Madagascar North), with a 1:1.7 ratio, and the highest average ratio by isolate 17 (Puerto Rico) (1:2.38).

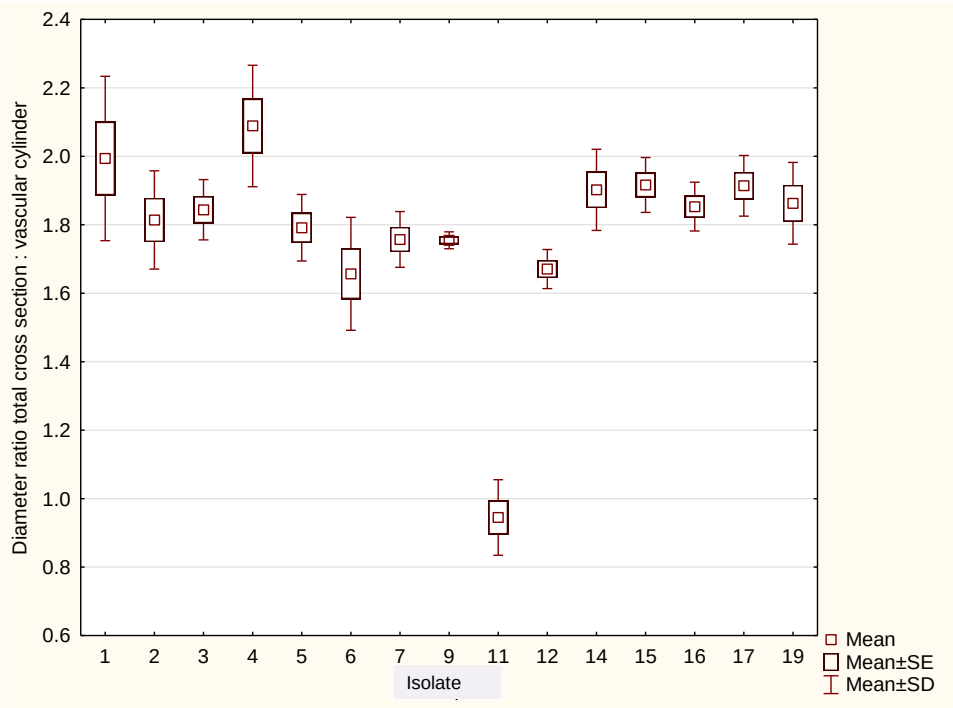

Figure 4.20 The ratio of the diameter of the total cross section to vascular cylinder of the different isolates (numbered 1-19) grown in water.The cross section : vascular cylinder ratio displayed little variation amongst isolates (1: ~1.8) with the exception of isolate 11 (Ghana, Gingani), which had a ratio of 1:0.9.

The total vascular cylinder diameter ratio of the isolates grown in soil (figure 4.19) was more variable than the ratio of the isolates grown in water (figure 4.20). The average ratio of the total root width to the vascular cylinder was 1:2. Isolate 17 had the highest average ratio (1:2.35) and isolate 9 the lowest (1:1:1.7). The roots grown in water had an average total width: vascular cylinder ratio of 1:1.9, with the exception of isolate 11, with a ratio of 1:0.95.

Table 4.1 The summarized qualitative comparison of the different isolates (numbered 1-19). The presence and absence of starch granules, aerenchyma, Casparian bands, the number of xylem elements as well as the development of the exodermis were compared in the roots of the isolates grown in soil and water.

	Starch granules	Aerenchyma	Xylem elements	U-shaped cell walls	Exodermis
Isolate 1 Soil	+	-	13	++	+
Isolate 1 Water	-	-	19	-	-
Isolate 2 Soil	-	-	6	+	+
Isolate 2 Water	-	+	18	-	+
Isolate 3 Soil	-	+	15	++	++
Isolate 3 Water	-	-	19	-	+
Isolate 4 Soil	-	-	11	-	+
Isolate 4 Water	-	-	18	-	D
Isolate 5 Soil	-	+	18	-	+
Isolate 5 Water	-	-	22	-	D
Isolate 6 Soil	-	-	13	+	+
Isolate 6 Water	-	+	21	-	-
Isolate 7 Soil	-	+	7	++	D
Isolate 7 Water	-	-	22	-	D
Isolate 8 Soil	-	-	7	+	D
Isolate 9 Soil	+	-	14	++	+
Isolate 9 Water	-	-	21	-	++
Isolate 10 Soil	+	-	12	+	++
Isolate 10 Water	+	-	-	+	-
Isolate 11 Soil	-	+	8	+	+
Isolate 11 Water	-	-	19	-	D
Isolate 12 Soil	-	-	8	-	+
Isolate 12 Water	-	-	24	-	++
Isolate 13 Soil	-	-	6	+	-
Isolate 14 Soil	-	-	6	++	+
Isolate 14 Water	-	-	21	-	D
Isolate 15 Soil	-	+	11	-	+
Isolate 15 Water	-	-	18	-	+
Isolate 16 Soil	-	+	13	+	D
Isolate 17 Soil	-	-	10	-	+
Isolate 17 Water	-	-	17	-	D
Isolate 19 Soil	-	-	22	-	++
Isolate 19 Water	-	-	14	-	D

Key: Starch granules and aerenchyma: - : absent, + : present
U-shaped cell wall thickenings in endodermis : ++: thick and well defined, + : present, - : absent
Exodermis: ++: present, >2 layers cells with lignified cell walls, +: present, 1-2 layers cells with lignified cell walls, D: denaturizing, -: absent

The exodermis layers as well as the shapes of the cells of the different isolates also varied: Isolates 3, 10 (in soil), 9 and 12 (in water) had a well-developed exodermis, with more than 2 layers of cells with lignified cell walls which serves as support. Isolates 2, 4, 5, 6, 9, 11, 12, 14, 15, 17 (in soil), 2, 3 and 15 (in water) had an exodermis with 1-2 layers of cells with lignified cell walls. The exodermis of the remaining isolates was either disintegrating or absent. This may be because of the exodermis being damaged during the sampling or fixating process.

The endodermal layers as well as the shapes of the cells of the different isolates varied: Isolates 1, 3, 7, 9 and 14 (grown in soil) contained well defined U-shaped wall thickenings, isolates 2, 6, 8, 10, 11, 13 and 16 (grown in soil) contained U-shaped wall thickenings, but wasn't as well developed and thick as the above. All the roots of the isolates grown in water (with the exception of isolate 10), as well as isolates 4, 5, 12, 15 and 19 grown in soil had no observable U-shaped wall thickenings.

As discussed in section 4.2.8, and seen in table 4.1, starch granules were observed in isolates 1 (DRC, soil), 9 (Madagascar North, soil) and 10 (DRC, soil and water). Aerenchyma was observed in isolates 2 (water), 3 (soil), 5 (soil), 6 (water), 7 (soil), 11 (soil), 15 (soil) and 16 (soil). As discussed in section 4.2.7, the number of xylem elements varied. The roots of the soil grown isolates had fewer xylem elements than the roots of the water grown isolates.

4.3 Discussion

4.3.1 *Comparison of the general anatomy of the roots between the isolates of Chrysopogon with Oryza sativa*

According to McDonald *et al. (*2002), the anatomical structure of the roots of *Chrysopogon zizanioides* and *O. sativa* are similar, because both genera can have the same adaptations when grown under the same environmental conditions, such as the ability to form aerenchyma: *O. sativa* being classified as being an intermediate species and *Chrysopogon zizanioides* being an intermediate-Panicoid.

Oryza sativa and *C. zizanioides* have similar cortical parenchyma arrangements. The young roots of both *O. sativa (*Rost, 1997) and *C. zizanioides* have a solid cortex with radiating cortical parenchyma. Older roots of *O. sativa* contain distinguishable cortical fibers *(*Rost, 1997), in contrast with the cortex of the *Chrysopogon* isolates examined. Both *O. sativa* and *C. zizanioides* form 'lysigenous' aerenchyma, from collapsed cortex cells.

The parenchyma observed in the pith of the *Chrysopogon* isolates analysed had thinner cell walls than *O. sativa* as described in Rost (1997). Also, fewer parenchyma cells were noted in the pith of *O. sativa* (Rost, 1997), with more sclerenchyma cells present, which serves as support, compared to the *Chrysopogon* isolates analysed. This may be because the isolates analysed were subjected to less mechanical stress than the *O. sativa* described in Rost, 1997.

The lateral roots originate from the well-defined pericycle of *O. sativa* (as described in Rost, 1997), whereas the lateral roots of the *Chrysopogon* isolates did not originate from a well-defined pericycle. .

There were no significant distinguishable qualitative characteristics observed between the investigated isolates, with the exception of the formation of starch granules (observed in isolates 1, 9, and 10 grown in soil and isolate 10 grown in water) and aerenchyma (observed in isolates 3, 5, 7, 11, 15 and 16 in roots of isolates grown in soil and isolates 2 and 6 in isolates of roots grown in water) as discussed in sections 4.2.4 and 4.2.8. Significant quantitative differences were observed in isolates 11 grown in water (having a larger average width and cortex width, and a smaller total cross section diameter: vascular cylinder

ratio than the other isolates grown in water), and isolate 7 grown in soil (having a larger average meta-xylem diameter than the other isolates grown in soil).

The roots of the isolates grown in soil had a few layers of thickened lignified cells beneath the exodermis. The uniform exodermis (as described in section 4.2.2) formed part of the outer cortex layers and was also multiseriate, contradictory to *O. sativa*. This is needed for support for the roots against the pressure of the soil and also serves as a barrier restricting the diffusion of water and nutrients. The roots grown in water, however, didn't contain layers of sclerenchyma under the exodermis, contradictory to the exodermis of *O. sativa* (Morita & Abe, 1999; Hose *et al.,* 2001) which may indicate that the roots of the isolates examined weren't as well developed to prevent the loss of oxygen under these conditions. The root diameters (the diameter of the cortex as well as the vascular cylinders) of the roots grown in water were also generally larger, seeing that the water did not create as much growth pressure as that of soil.

Generally the roots grown in soil consisted of a mature endodermis with well defined, U-shaped tertiary wall thickenings, contrary to the endodermis of the water grown isolates. According to Naseera *et al.* (2012) the function of Casparian strips is to regulate the flow from water and ions in the plant. Although all the roots of the isolates contained Casparian strips, it was not clearly visible in all the isolates due to the endodermal cells with the U-shaped wall thickenings. In water grown roots there was no U- shaped tertiary wall thickenings observed in the endodermal cells. This may be because there is a sufficient water potential gradient to ensure that water diffuses freely into the xylem to be transported to the rest of the plant body (Hose *et al.,* 2001).

The pericycle was absent in older roots. With the exception of isolate 19 (Ghana, Kumasi), the roots of the isolates grown in water had more xylem elements. This adaptation of the isolates grown in water may have occurred to increase the transportation rates of the water grown isolates due to the elevated amount of water available for transportation.

Statistical analysis indicated that there was no consistant difference between the 19 isolates grown in soil, as there was a lot of variance in the data. This may be due to the plasticity of the isolates in reaction to the growth conditions. The isolates grown in water had less variation, with the exception of isolate 11 (Ghana, Gingani), where the total diameter, the diameter of the cortex, and the total cross section: vascular cylinder ratio was smaller than the other examined isolates. However, further, more in- depth studies need to be conducted

to determine if the differences are significant enough to classify isolate 11 as a different ecotype.

4.3.2 *Starch Formation*

In the study done by Bowen & Pate (1993), the roots of *Stirlingia latifolia* (R.Br.) Steud. (1841), Nomencl. Bot. edn 2, 2:644 and *Stirlingia tenuifolia* (R.Br.) Steud. (1841), Nomencl. Bot. edn 2, 2:644 were analysed to determine the effect of fire on the plants. They concluded that starch formation is a vital process that ensures the resprouting of the analyzed plants, after being exposed to stress (fire). The formation of starch was drastically reduced after the plants were exposed to stress (Bowen & Pate, 1993).

In this study, starch granules were only formed by isolates 1 (Congo, DRC Kingshasha grown in soil) and isolate 10 (Congo, DRC Kingshasha, grown in water as well as soil). This may indicate that DRC Kingshasha did not undergo stress when the slips were grown in soil or in water, or, that these isolates had the ability to produce starch under the different culturing conditions which may contribute to the plants ability to survive under stress in different environments.

4.3.3 *Aerenchyma Formation*

Lysigenous aerenchyma (see section 4.2.4.) was present in the cross-sections of the water grown roots of isolates 2 (Madagascar- South, Fort Dauphin) and 6 (Venezuela, Caracas), in contrast with the roots grown in soil. However, aerenchyma was present in the cross-sections of the roots grown in soil (isolates 3, 5, 7, 11, 15 and 16) in contrast with the roots grown in water. The roots of these isolates did not form any aerenchyma when grown in water.

According to Setter and Waters (2003) as well as Thomson *et al.* (1992), there is a correlation between the flood tolerance and the rate of root aerencyma formation of cereal crops such as oats, which is more tolerant to flooding than other cereals such as wheat and barley. Also, the development of aerenchyma minimizes the loss of oxygen into the surrounding environment (Armstrong, 1979), while also creating a barrier which may impede

the movement of soil-derived toxins and gasses into the roots (Armstrong, 1979; Colmer, 2003). Wetland plant species generally use the above mentioned characteristics to adapt to flooded environments (Colmer, 2003; Malik *et al.,* 2011).

In the study of Suralta & Yamauchi (2008), aerenchyma was also formed in droughted roots of *O. sativa*, regardless of genotypes, but was resistant to internal O_2 transport under O_2 deficiency. They concluded that deep and extensive roots, developed under drought, should immediately enhance its aerenchyma to facilitate O_2 diffusion to the roots when confronted with sudden waterlogging. If the plant is unable to acclimate to changes in soil- water composition, it will affect root growth and functions, and will result in the negative affect of biomass production.

The formation of aerenchyma in roots has many advantages, including that the roots have a lifespan of less than 2 years and also decompose slowly, which contributes to keeping channels open for new generations of roots, providing the grass with a continuous access to water. Also aerenchyma helps the plants to survive during prolonged droughts by having an additional water reservoir (USDA, 1997; Laan *et al.* 1989).

The results of our study regarding aerenchyma formation, supports the findings of the USDA (1997) that Vetiver may form aerenchyma in waterlogged areas. However, only certain isolates grown in soil (isolates 3, 5, 7, 11, 15 and 16) formed aeranchyma and some in water (isolates 2 and 6) which may indicate that these isolates are more flood tolerant than the other isolates examined. Seeing that all the isolates were grown under the same environmental conditions, certain isolates responded faster to stress than others.

4.3.4 *Anatomical and genetic analysis*

When visually comparing the anatomical to the phylogenetic data, no significantly statistical differences were observed between the different isolates. However, based on the *ITS* gene and the presence of starch granules, it is evident that isolates 1 and 10 (DRC) are different, or respond differently from the rest of the group. This may be an indication that these isolates are different ecotypes than the rest of the isolates as well as the accessions of both *C. zizanioides* and *C. nigritana* obtained from Genbank. Further, in depth studies need to be conducted to support these findings.

CHAPTER5 CONCLUSION

Isolates from Hydromulch (Pty) Ltd. were undistinguishable in the less conservative chloroplast genes, but showed variation in the more conservative gene fragment. Even though minor differences in the *ITS* gene fragment were observed, the differences are not significant enough to distinguish between the different isolates in the Hydromulch (Pty) Ltd. collection.

Despite the use of rapidly evolving gene fragments, results generated during this study showed very little or no difference amongst the different isolates. Also, the anatomical data obtained showed little or no difference amongst the different isolates. The formation of aerenchyma was not supported by other results, such as genetic or other anatomical measurements, even though all the isolates were cultured under the same conditions. The formation of root aerenchyma in the analysed isolates may give a basis to understanding the ability of some of the *Chrysopogon zizanioides* and *C. nigritanus* isolates to adapt to flooding. This also gives the possibility to conduct more research on the reason why some of the isolates form aerenchyma, where others do not, and to select the most suitable ecotype to be used under different environmental conditions.

Both the Neighbour- Joining and Maximum Likelihood methods concluded that *Chrysopogon zizanioides* and *Chrysopogon nigritanus* differ from *Chrysopogon serrulatus* and *Vetiveria fulvibarbis*, which are found in Africa. Based on the phylogenetic results there is statistically no significant difference between *C. zizanioides* and *C. nigritanus.* Isolates 1, 9 and 10 differ from the other isolates in the analyses and it seems that they are not related to *C. zizanioides*, *C. nigritanus* or *C. festucoides.* Isolates 12, 18 and 19 also grouped separately, but still grouped within the group of *C. zizanioides.* The Neighbour- Joining method was faster and not as statistically complicated as the Maximum-Likelihood approach and was conclusively chosen to present the results.

The only anatomical results supported by genetic results, were the presence of starch granules in isolates 1 and 10. We therefore conclude that with the possible exceptions of isolates 1 and 10 from the DRC, there is no significant genotypic difference between *C. zizanioides* and the different individuals available from the Hydromulch (Pty) Ltd. plant collection.

Because only 3 genes were analysed in this study and the anatomical results showed more variation between the different isolates, it is important to conduct further studies, such as Cytochrome- and fatty acid analyses, whole genome sequencing, as well as morphological and more in-depth anatomical studies are advisable to support the above findings to fully distinguish between the different isolates. This dissertation supplies a base for these future studies.

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South African Journal of Botany 86 (2013) 63–67

Contents lists available at SciVerse ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Genetic diversity of vetiver isolates (*Chrysopogon zizanioides/nigritanus*) available in South Africa based on *ITS*, *ndh*F and *rbc*L sequencing analyses

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ARTICLE INFO

Article history:
Received 3 August 2012
Received in revised form 1 February 2013
Accepted 2 February 2013

Edited by E Balazs

Keywords:
Vetiver
ITS
ndhF
rbcL
Genetic diversity

ABSTRACT

Vetiver grass (*Chrysopogon zizanioides* (L.) Roberty) is sterile and only regenerates vegetatively from clumps of the rootstock. Together with its vigorous and deep root system this makes it ideal for use in soil remediation and erosion control. In South Africa, Hydromulch (Pty) Ltd is part of the landscape, soil reclamation and erosion control industry. The company uses vetiver grass on a wide scale and has compiled a collection of isolates to serve as possible germ lines for industrial use. Due to the different approaches in environmental management as well as environmental factors, a variety of ecotypes form during the planting, adaptation and domestication of this genus. *Chrysopogon nigritanus* ((Benth.) Veldkamp, 1999), is a close relative native to Africa and differs morphologically only slightly from *C. zizanioides*. It may seed freely and thus use of this species should be avoided. The need arose to screen other non-fertile plants to uncover additional genotypic variety to enable diversification of vetiver plantings. The aim of this study is to characterise the genotype of 19 isolates of vetiver obtained from Hydromulch (Pty) Ltd via sequencing analyses of three DNA fragments, *ITS*, *ndhF* and *rbcL*. According to the results generated during this study very little or no genotypical differences exist amongst the different isolates available from the Hydromulch (Pty) Ltd plant collection. Only in the case of the *ITS* inference were differences observed between 3 isolates.

1. Introduction

Vetiver grass (*Vetiveria zizanioides* (L.) Nash, reclassified as *Chrysopogon zizanioides* (L.) Roberty, 1960) was first applied in soil and water conservation in India during the mid 1980s (Truong et al., accessed, 2012/04/26). This application still plays a vital role in agricultural land management today, but during the past years it has been demonstrated that it can also be successfully used in bioengineering of steep slope stabilization, and as phyto-remediator of contaminated land and water (Truong et al., accessed, 2012/04/26).

C. zizanioides is a perennial grass of the Poaceae, native to India. It is closely related to *Sorghum* but also shares many morphological characteristics with other fragrant grasses such as lemongrass (*Cymbopogon citratus*), and citronella (*Cymbopogon nardus*, *Cymbopogon winterianus*). The most commonly used commercial genotypes of vetiver are sterile (do not produce fertile seeds), and propagate itself by small offsets instead of underground stolons. These genotypes are therefore non-invasive and can easily be controlled by cultivation of the soil at the boundary of the hedge.

C. zizanioides is known to be also cultivated in Africa and in particular in South Africa since at least 1892 (Chippindall, 1955). A close relative, *Chrysopogon nigritanus* ((Benth.) Veldkamp, 1999), is a wetland species native to Africa. It may be expected to seed freely and thus use of this species for soil binding is discouraged, as it may become a weed (Veldkamp, 1999). According to Veldkamp (1999), the differences between *C. zizanioides* and *C. nigritanus* are so slight, they may easily be confused. Both species' leaves are similar in shape and colour. The only noticeable difference is the root system, their size and performance (Adams, 2002). The root system of *C. nigritanus* is dense, hardy, but rarely extends beyond 75 cm; whereas the root system of *C. zizanioides* exceeds 75 cm. *C. zizanioides* also has the ability to grow new adaptive adventitious roots on its leaf stalk (Adams, 2002).

Vetiver is widely used over the world and is cultivated far from its region of origin, namely the area from India to Vietnam (Adams, 2002). Due to the different approaches in environmental management as well as environmental factors, *C. zizanioides* tends to form a variety of ecotypes during the planting, adaptation and domestication of this genus (Dong et al., 2003). However, despite this characteristic of *C. zizanioides* results showed in a study by Adams et al. (1998) indicated that all the vetivers cultivated outside of South Asia have been derived from a single genotype that has been designated as "Sunshine". This, despite the fact that the isolates used were sampled from vastly different geographical regions, namely the Americas, Africa, Australia and Asia.

In South Africa, Hydromulch (Pty) Ltd is part of the landscape, soil reclamation and erosion control industry. The company uses vetiver grass on a wide scale and has compiled a collection of isolates to serve

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0254-6299/$ – see front matter © 2013 Published by Elsevier B.V. on behalf of SAAB.
http://dx.doi.org/10.1016/j.sajb.2013.02.005

as possible different varieties for industrial use. The need arose to screen other non-fertile plants to uncover additional genetic biodiversity to enable diversification of vetiver plantings (Adams et al., 1998). It was therefore important to verify the genotype of these isolates for current and future plantings of vetiver grass. The aim of this study is to characterise the genotype of 19 isolates of vetiver via sequencing analyses of three DNA fragments, *ITS*, *ndhF* and *rbcL*.

2. Material and methods

2.1. Sampling

Sampling of the apparent different genotypes available in South Africa included 19 isolates of vetiver grass. These plants were obtained from the plant collection at Hydromulch (Pty) Ltd, South Africa (from Mr. Roley Nöffke). Hydromulch (Pty) Ltd has established a vetiver grass nursery on its farm situated 20 km north of the Oliver Tambo International Airport in Johannesburg. The company supplies vetiver grass slips/plants to any destination worldwide but in particular to African countries. The origins of the collected plants are notated in Table 1. The sources of accessions 4, 8 14 and 15 were also verified by Prof Paul Truong (personnel communication). Plant material was collected in the form of slips, and planted in pots at the Botanical Garden of the North-West University, Potchefstroom, South Africa. The samples were planted in potting soil and were kept in a regulated greenhouse, with an average daily temperature of 25 °C ± 2 °C in the summer and 18 °C ± 2 °C during winter. Each pot contained between 2 and 3 slips of each specimen. All the plants received a general fertilizer once every 3–4 weeks to sustain the growth of the plants.

Other accessions (8 for *ITS*, 6 for *ndhF* and 7 for *rbcL*) included in this study are listed in Table 2. The relevant sequences were obtained from the National Centre for Biotechnology Information (NCBI), Bethesda, Maryland, United States of America. The outgroups included species from the genera *Cymbopogon*, *Saccharum*, *Sorghum*.

2.2. DNA isolation

Young leaves were harvested for the purpose of DNA isolation after at least six months of cultivation in the greenhouse since the plant was collected from Hydromulch (Pty) Ltd. at the onset of autumn. The midriffs of the leaves were removed before homogenization. The Qiagen DNeasy Mini Plant isolation kit was used to isolate the DNA from the fresh leaf material. The following modifications were made to the prescribed isolation kit protocol to ensure optimal DNA yield: 200 mg fresh plant material was used instead of the recommended 100 mg; 450 µl extraction buffer AP1 was used instead of 400 µl; the incubation period (cell lyse step) was increased from 10 min to 30 min. The elution step was then also modified as follows: 30 µl Buffer AE was added onto the DNeasy membrane (instead of 100 µl) and incubated for 10 min at room temperature (instead of 5 min). The above mentioned steps were repeated with 20 µl buffer AE.

2.3. DNA amplification

The selected genes were amplified via Polymerase Chain Reaction (PCR). Three genes were selected for this study, the *ITS* regions of the 5.8s ribosomal gene and the two chloroplastic genes *ndhF* and *rbcL*. Primers by White et al. (1990), Stanford et al. (2000) and Christin et al. (2008) were used for amplifying these fragments (Table 3). The amplification of the DNA of all the genes in this study was done in a 25 µl reaction mixture, in a Bio-rad i-cycler (V4.006). This included 1× reaction buffer, 1.5 mM MgCl, 0.2 mM dNTPs, 1.25 units (Promega) Taq DNA polymerase, forward primer (0.5 µM), reverse primer (0.5 µM), PCR grade water and 50 ng template DNA. The reactants were added as well as centrifuged in a laminar chamber, under sterile conditions.

2.4. Sequencing and sequencing analysis

All sequencing reactions were performed by the Central Analytical Facilities, Stellenbosch University, South Africa. Post-PCR purification was done using the NucleoFast Purification System (obtained from Separations). Sequencing was performed with BigDye Terminator V1.3 (Applied Biosystems) followed by electrophoresis on the 3730xl DNA Analyser (Applied Biosystems). Sequences were analysed and trimmed using Sequencing Analysis V5.3.1 (Applied Biosystems).

2.5. Phylogenetic analyses

Sequence verifications and alignments were done unambiguously with CLC DNA Workbence 6 (CLC bio, Aarhus, Denmark), using the following settings during alignment: gap open cost (10), gap extension cost (1) and end gap cost (as any other). Alignments were also verified manually and ambiguous bases corrected by visual inspection.

The, *ITS*, *rbcL* and *ndh*F matrices were analysed separately and in combination using MEGA version 5 (Tamura et al., 2011). Gaps were eliminated. A distance method, Neighbour-Joining (Saitou and Nei, 1987), as well as a model based approach, Maximum Likelihood, were used. Neighbour-Joining was performed using the Jukes–Cantor model (Jukes and Cantor, 1969). The best fit nucleotide substitution models for use with the Maximum Likelihood were calculated with the

Table 1
Taxa of *Chrysopogon* spp. used during this study obtained from the Hydromulch (Pty) Ltd vetiver nursery.

Taxon	Country of origin	Location	
ACC01	Congo, DRC	Kinshasa	Selembao south west of Kinshasa
ACC02	Madagascar (South)	Fort Dauphin	Farming area 50 km west of Fort Dauphin
ACC03	Congo, DRC	Kinshasa	About 50 km south of Selembao
ACC04	Australia	Brisbane	Monto as identified by Paul Truong
ACC05	Mozambique	Nampula	North of Nampula
ACC06	Venezuela	Caracas	Southern outskirts of Caracas
ACC07	South Africa	Rustenburg	Commercial farm north of Rustenburg
ACC08	Ethiopia	Addis Ababa	Outskirts of Addis Ababa
ACC09	Madagascar (North)	Antsohiny	Old farming area on the RN5 route
ACC10	Congo, DRC	Kinshasa	Local community close to Selembao
ACC11	Ghana	Gingani	Agricultural fields near the Golinga Dam, 16 km south west of Tamale
ACC12	Ghana	Buleng	In open field in the Buleng North Ghana
ACC13	Ghana	Manga	In open field close to Nalerigu Dam, Ganbaga district North Ghana
ACC14	New Zealand	North Island	Residence of John Greenfield
ACC15	Kenya	Voi, Coastal Plain	Elise Pinners Vetiver Network, Kenya
ACC16	Mozambique	Alto Molocute District	South of Nampula (200 km south of ACC5 location)
ACC17	Puerto Rico	Guyama district	Near Lake Carite, Guyama
ACC18	South Africa	Rustenburg	Commercial farm north of Rustenburg
ACC19	Ghana	Kumasi	In open field

Table 2
The GenBank accession numbers of the taxa of outgroups sampled for this study as well as that of *Chrysopogon (Vetiveria) zizanioides* and *Chrysopogon (Vetiveria) nigritanus*.

Taxon	Accessions of *ITS*	Accessions of *ndhF*	Accessions of *rbcL*
Vetiveria zizanioides	DQ005089	AM849196	AM849394.1
Vetiveria nigritanus	GQ856335.1		
Vetiveria fulvibarbis	GQ856324.1		
Saccharum officinarum	DQ005064.1	AF443824	EF125120
Sorghum bicolor subsp. *verticilliflorum*	GQ121746	U21981	AM849341
Chrysopogon serrulatus	DQ005032.1		
Cymbopogon citratus		AM849141.1	GQ436383.1
Sorghum halepense	GQ121749	AF117424	EF125122
Chrysopogon gryllus		AF117399.1	

Bayesian Information Criterion available within Mega 5 and evaluated using the Akaike Information Criterion within Mega 5. The heuristic search model used for the Maximum Likelihood was the Close-Neighbour-Interchange. Bootstrap analysis (1000 replicates) was performed to determine internal support (Felsenstein, 1985). A bootstrap percentage of 80% and higher is considered a high bootstrap support, a bootstrap support of 50% and higher as moderate and a bootstrap support of less than 50% is considered as a very low bootstrap support. The bootstrap consensus trees of the Neighbour-Joining and Maximum Likelihoods are reported. The congruency index and the P-value for the matrices were calculated (De Vienne et al., 2007) before the matrices were combined.

3. Results

3.1. Phylogenetic analysis of the ITS data

The evolutionary relationships were inferred using both the Neighbour-Joining and Maximum Likelihood methods. All positions containing gaps were eliminated. In all cases the phylogenies were initially inferred using all the taxa and including the outgroups. However, since the differences between the different isolates and *C. zizanioides* and *C. nigritanus* were so small, only the trees with the outgroup *Saccharum officinarum* (Sac of) is shown. The *ITS* dataset was the most informative and contained the 19 isolates from Hydromulch (Pty) Ltd as well as the GenBank accessions for *C. zizanioides* (Vetziz), *C. nigritanus* (Vetnig), *Chrysopogon festucoides* (Vetfest), *Chrysopogon serrulatus* (Chryser) and *Vetiveria fulvibarbis* (Vegful). There were a total of 532 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). The optimal tree (Neighbour-Joining) with the sum of branch length = 0.11278889 is shown (Fig. 1). The analysis involved 25 nucleotide sequences. The optimal Neighbour-Joining tree indicates that *C. festucoides* can be distinguished from the *C. nigritanus* and *C. zizanioides* complex with a bootstrap support of 95%. The isolates (1, 9 and 10) from the Democratic Republic of the Congo and Madagascar differ from the other isolates that grouped with *C. nigritanus* and *C. zizanioides* with a bootstrap support of 67%. The accessions of *C. nigritanus* and *C. zizanioides* clearly formed two separate groups but these two groups were not statistically supported. Isolate number 6 from Venezuela was the only isolate that grouped with *C. nigritanus*. The rest of the 19 isolates grouped with the GenBank accession of *C. zizanioides* and there appeared to be no statistically significant difference between isolates: 2, 3, 4, 5, 7, 8, 11, 13, 14, 15, 16 and 17. The three isolates 12, 18 and 19 (two from Ghana and one from South Africa) grouped separately from the latter group of isolates, however, this grouping also obtained very low statistical support (bootstrap 36%).

3.2. Phylogenetic analysis of the rbcL and ndhF data

Both the Neighbour-Joining and Maximum likelihood analyses with and without the outgroups yielded similar results for both of the chloroplastic gene fragments *ndhF* and *rbcL* that were sequenced. In each case the GenBank accession of *C. zizanioides* (Vetziz) was included in the analyses. In the case of the *ndhF* gene fragment, the optimal Neighbour-Joining tree with the sum of branch length = 0.00828552 is shown (Fig. 2). Analysis involved 22 nucleotide sequences and included the GenBank accessions for *V. fulvibarbis* (Chryful) as well as *Chrysopogon gryllus* (Chrygry). There were a total of 2059 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). The results indicated that there was no genotypic difference between the 19 isolates and the GenBank accession of *C. zizanioides*. For the *rbcL* gene fragment the optimal Neighbour-Joining tree with the sum of branch length = 0.54724153 is shown (Fig. 3). The analysis involved 20 nucleotide sequences. There were a total of 841 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). As outgroups the following GenBank accessions were added: *C. zizanioides* (Vetziz) and *C. citratus* (Cymcit). The 18 isolates used during this analysis did not differ from each other but there was very strong bootstrap support (91%) for distinguishing them from *C. zizanioides*. The *ndhF* trees are congruent with the *rbcL* trees, however, it was not congruent with the *ITS* data presented here. The congruent *ndhF* and *rbcL* trees did not yield different results to what have been shown already by the *rbcL* and *ndhF* analyses, namely that there appear to be no genotypic differences between the different isolates.

4. Discussion

The key characteristic that makes vetiver the most preferred option for soil remediation and erosion control is that it is sterile and only regenerates vegetatively from clumps of rootstock (Adams, 2002). It is therefore necessary to understand the genotypic diversity available, of the plants in the Hydromulch Ltd. collection, for propagation and to ensure the genetic biodiversity of this plant for future management and protection of plantings.

Previous studies done on the genetic diversity of vetiver grass mostly included RAPD analysis (Kresovich et al., 1994; Srifah et al.,

Table 3
The primers used for the amplification and sequencing of the *ITS*, *ndhF*, and *rbcL* gene fragments used for the phylogenetic analyses.

Primer	5′ to 3′ primer sequence	Reference	Ta (annealing temperature) PCR
ITS 4F	TCCTCCGCTTATTGATATGC	White et al. (1990)	58.0 °C
ITS 5A	CCTTATCATTTAGAGGAAGGAG	Stanford et al. (2000)	58.0 °C
*ndhF*_1F	ATGGAACATACATATCAATA	Christin et al. (2008)	50.8 °C
*ndhF*_746R	CTTCCATAGCATCNGGYAACC	Christin et al. (2008)	50.8 °C
*ndhF*_630F	AATAGCTAATAACTGGATTCC	Christin et al. (2008)	50.8 °C
*ndhF*_1630R	AAAGNARTAATATAAGAAGAGG	Christin et al. (2008)	50.8 °C
*ndhF*_1417F	TTGYATTCAATATCYTTATGGG	Christin et al. (2008)	50.8 °C
*ndhF*_2110R	CCCCCTACATATTTGATACCTTCTC	Christin et al. (2008)	50.8 °C
*rbcL*_1F	ATGTCACCACAAACAGAAACTAAAGC	Christin et al. (2008)	63.5 °C
*rbcL*_826R	TAATRAGNCAAASTAGTATTTGC	Christin et al. (2008)	63.5 °C
*rbcL*_629F	CATTTATGCGCTGGAGAGACC	Christin et al. (2008)	63.5 °C
*rbcL*_1388R	TTCCATAYTTCACAAGCTGC	Christin et al. (2008)	63.5 °C

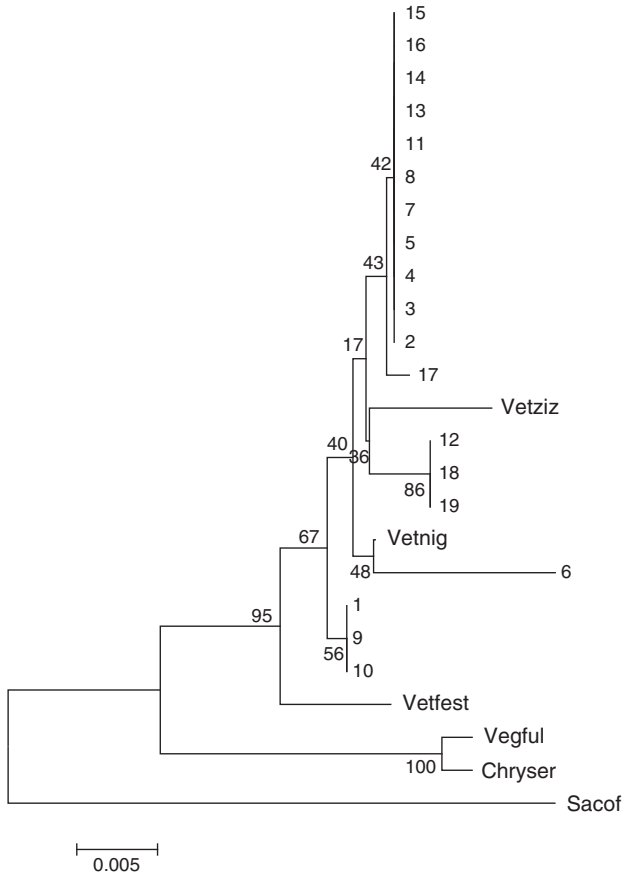

Fig. 1. The evolutionary history inferred using the Neighbour-Joining method and *ITS* sequence data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes–Cantor method and are in the units of the number of base substitutions per site. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz), *Chrysopogon nigritanus* (Vetnig) and *Vetiveria fulvibarbis* (Vegful) *Saccharum officinarum* (Sac of).

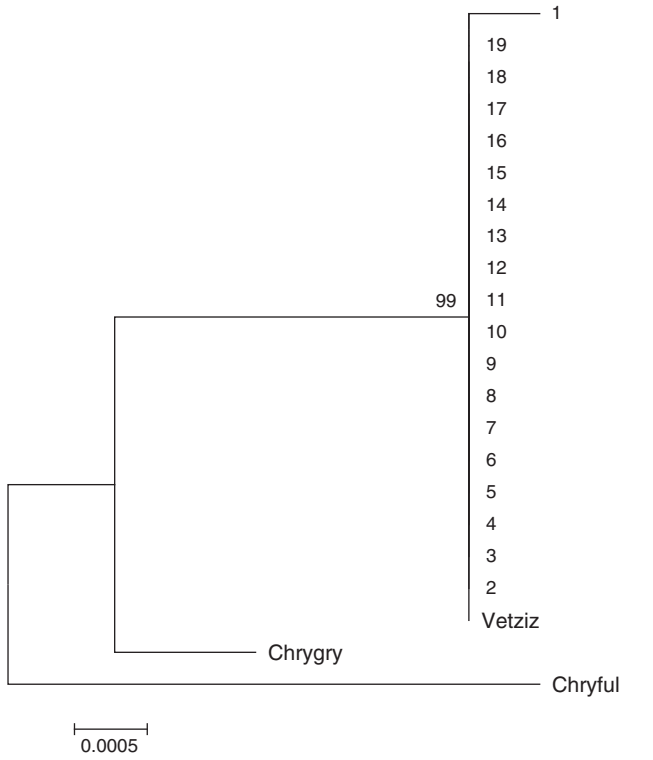

Fig. 2. The evolutionary history inferred using the Neighbour-Joining method with the *ndhF* sequence data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes–Cantor method and are in the units of the number of base substitutions per site. GenBank accessions were used for *Vetiveria fulvibarbis* (Chryful), *Chrysopogon gryllus* (Chrygry).

n.d. accessed 30/07/2012; Dong et al., 2003). These studies reported results distinguishing between the different vetiver ecotypes tested. Adams et al. (1998), however, showed that all the vetiver lines that were characterized and used for erosion control outside South Asia, are derived from a single genotype, namely "Sunshine". Due to different environmental and geographical factors, diversity may exist within these lines.

During this study the diversity between 19 different vetiver isolates available in South Africa was investigated using DNA sequencing which provides a direct means of comparison. For this study three well known gene fragments were employed, namely the internal transcribed spacer (*ITS*) region of the 18S–5.8S–26S nuclear ribosomal cistron, the chloroplastic *ndhF* gene for NADH dehydrogenase subunit F and the chloroplastic *rbcL* gene for ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit. The *ITS* fragment can be used to infer the phylogenetic relationship between species and closely related genera (Baldwin et al., 1995; Soltis and Soltis, 1998) because it is a highly repeated region in the genome. The region undergoes rapid concerted evolution and the small size and the highly conserved sequences flanking each of the spacers make this region easy to amplify (Baldwin et al., 1995). Due to its subjection to concerted evolution, which leads to its intra-genomic uniformity, thereby limiting the mutations in the same genome which may have led to confusing variation, it is left with only species- and clade-specific characteristics noticeable (Alvarez and Wendel, 2003).

According to Veldkamp (1999) and references therein *C. nigritanus* has previously been identified as a variety of *C. zizanioides* and within the *C. zizanioides* complex *C. zizanioides* and *C. nigritanus* are morphologically most similar to *C. festucoides*. Results obtained during this study exhibited a significant difference (bootstrap value 95%) between *C. festucoides* and, *C. zizanioides* and *C. nigritanus* despite the lack of morphological differences. Only in the case of the *ITS* inference could there be distinguished significantly between the different isolates tested. Most isolates still grouped with *C. zizanioides*. The two species, *C. zizanioides* and *C. nigritanus* grouped very close together, with no significantly statistical difference. From this analysis isolates 1, 9 and 10 were significantly different from *C festucoides* as well as from both *C. zizanioides* and *C. nigritanus*. Although, these three isolates do not differ morphologically from the other isolates, according to this *ITS* inference isolates 1, 9 and 10 do not originate from *C. zizanioides*. They are furthermore clearly different from the other *Chrysopogon* species distributed in Africa (Fig. 1) namely: *C. serrulatus* — distributed over Southern Africa, Madagascar, Afghanistan and North India to Burma, Sri Lanka and Thailand; and *Chrysopogon fulvibarbis* — distributed over Western Africa (Veldkamp, 1999) and would need to be further investigated. One of the isolates investigated by Adams et al. (1998), is noted as *C. zizanioides* var Monto Australia. Isolate no 4 in this study is also a *C. zizanioides* var Monto Australia. Thus according to Adams et al. (1998) one can assume that at least the isolates that grouped with this accession (2, 3, 5, 7, 8, 11, 13, 14, 15, 16 and 17) has the same genotypic origin, namely *C. zizanioides* "Sunshine". During this study no significant difference was observed between the two isolates obtained in South Africa (accessions 7 and 18) although they did group separately. It is speculated that these two isolates might have originated from different sources of *C. zizanioides*

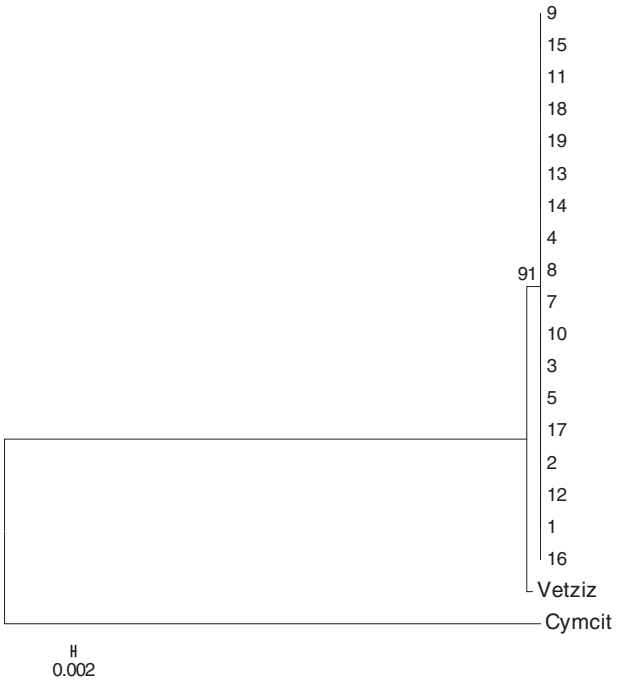

Fig. 3. The evolutionary history inferred using the Neighbour-Joining method using the *rbcL* sequencing data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes–Cantor method and are in the units of the number of base substitutions per site. GenBank accessions were used for *Chrysopogon zizanioides* (Vetziz) and *Cymbopogon citratus* (Cymcit).

although they were obtained from the same commercial farm, and may thus differ due to limited somatic mutation as a result of environmental factors.

The *ndh*F gene is a rapidly evolving chloroplast gene (Olmstead and Sweere, 1994; Soltis and Soltis, 1998). The 5′ region (1380 bp) is different from the 3′ region (855 bp) in that the 3′ region is A + T rich and has higher levels of non-synonymous base substitutions and higher transversion bias at the codon positions. The different patterns of base substitution at the 5′ and 3′ regions makes this gene ideal for phylogenetic reconstruction as the conserved and variable segments can be used for older and recent groups respectively (Kim and Jansen, 1995). Based on the results obtained from the *ndhF* gene analysis these isolates are all *C. zizanioides* with no variation between them. Although, there was no difference between the isolates when compared with the *rbcL* gene analysis, the isolates were significantly different from *C. zizanioides*. This may however, be the result of insufficient taxon sampling since, despite the high bootstrap support, only minor differences were visually observed during sequence alignment between the different isolates as well as the outgroups. Unfortunately there is currently not any DNA data available for neither *C. fulvibarbis*, *C. festucoides*, *C. serrulatus* nor *C. nigritanus* for either the *rbcL* or the *ndhF* gene.

Despite the use of rapidly evolving gene fragments results generated during this study showed very little or no difference amongst the different isolates. We therefore conclude that with the possible exceptions of accessions 1 and 10 from the DRC, and 9 from Madagascar (North) there is no significant genotypical difference between *C. zizanioides* and the different individuals available from the Hydromulch (Pty) Ltd plant collection.

Acknowledgements

We thank Hydromulch (Pty) Ltd for their financial support and plant material used during this study. We acknowledge gratefully the inputs and technical help from Mr Roley Nöffke and his permission to publish this data. We thank Dr Paul Truong, Dr Mark Daffron and Dr Jane Wright for valuable discussions and input. We acknowledge the help of Mr W Towers with the alignments.

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APPENDIX A- Maximum Likelihood fits of 24 different *ITS* nucleotide substitution models tested in MEGA 5 for the alignment including all outgroups.

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor (MEGA 5).

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)	*r*(GA)	*r*(GT)	*r*(GC)
K2+I	55	3581.567	3172.532	-1531.021	0.32	n/a	2.51	0.25	0.25	0.25	0.25	0.036	0.036	0.179	0.036	0.179	0.036	0.036	0.179	0.036	0.179	0.036	0.036
K2+G	55	3582.372	3173.337	-1531.424	n/a	1.57	2.17	0.25	0.25	0.25	0.25	0.039	0.039	0.171	0.039	0.171	0.039	0.039	0.171	0.039	0.171	0.039	0.039
K2	54	3586.269	3184.663	-1538.096	n/a	n/a	1.83	0.25	0.25	0.25	0.25	0.044	0.044	0.162	0.044	0.162	0.044	0.044	0.162	0.044	0.162	0.044	0.044
K2+G+I	56	3587.957	3171.494	-1529.494	0	0.67	2.66	0.25	0.25	0.25	0.25	0.034	0.034	0.182	0.034	0.182	0.034	0.034	0.182	0.034	0.182	0.034	0.034
T92+I	56	3592.2	3175.737	-1531.615	0.33	n/a	2.52	0.182	0.182	0.318	0.025	0.025	0.043	0.232	0.025	0.232	0.043	0.025	0.133	0.043	0.133	0.025	0.043
T92+G	56	3594.346	3177.883	-1532.688	n/a	1.61	2.11	0.182	0.182	0.318	0.318	0.028	0.048	0.221	0.028	0.221	0.048	0.028	0.127	0.048	0.127	0.028	0.048
T92	55	3598.459	3189.424	-1539.468	n/a	n/a	1.77	0.182	0.182	0.318	0.318	0.031	0.055	0.208	0.031	0.208	0.055	0.031	0.12	0.055	0.12	0.031	0.055
T92+G+I	57	3599.408	3175.517	-1530.496	0.02	0.69	2.68	0.182	0.182	0.318	0.318	0.023	0.041	0.236	0.023	0.236	0.041	0.023	0.136	0.041	0.136	0.023	0.041
TN93+I	59	3610.536	3171.79	-1526.614	0.33	n/a	2.92	0.185	0.18	0.309	0.326	0.022	0.037	0.143	0.022	0.339	0.039	0.022	0.197	0.039	0.081	0.022	0.037
HKY+I	58	3611.706	3180.388	-1531.922	0.33	n/a	2.53	0.185	0.18	0.309	0.326	0.024	0.042	0.238	0.025	0.226	0.044	0.025	0.131	0.044	0.136	0.024	0.042
HKY+G	58	3613.873	3182.555	-1533.006	n/a	1.61	2.11	0.185	0.18	0.309	0.326	0.027	0.047	0.226	0.028	0.215	0.05	0.028	0.125	0.05	0.129	0.027	0.047
TN93+G	59	3614.635	3175.889	-1528.664	n/a	1.52	2.24	0.185	0.18	0.309	0.326	0.026	0.045	0.157	0.027	0.292	0.048	0.027	0.17	0.048	0.089	0.026	0.045
HKY	57	3617.986	3194.095	-1539.785	n/a	n/a	1.77	0.185	0.18	0.309	0.326	0.031	0.053	0.214	0.032	0.203	0.056	0.032	0.118	0.056	0.122	0.031	0.053
TN93+G+I	60	3618.883	3172.711	-1526.065	0.25	1.84	2.99	0.185	0.18	0.309	0.326	0.021	0.036	0.148	0.022	0.337	0.038	0.022	0.196	0.038	0.084	0.021	0.036
HKY+G+I	59	3618.977	3180.232	-1530.835	0.03	0.71	2.68	0.185	0.18	0.309	0.326	0.023	0.04	0.242	0.024	0.229	0.042	0.024	0.133	0.042	0.138	0.023	0.04
TN93	58	3620.432	3189.114	-1536.285	n/a	n/a	1.81	0.185	0.18	0.309	0.326	0.03	0.052	0.161	0.031	0.259	0.055	0.031	0.151	0.055	0.092	0.03	0.052
GTR+G	62	3631.939	3170.914	-1523.147	n/a	0.55	3.03	0.185	0.18	0.309	0.326	0.057	0.018	0.149	0.059	0.327	0.029	0.011	0.19	0.03	0.085	0.016	0.029
GTR+I	62	3632.148	3171.124	-1523.252	0.34	n/a	3.06	0.185	0.18	0.309	0.326	0.057	0.015	0.136	0.058	0.341	0.031	0.009	0.198	0.031	0.077	0.017	0.029
GTR+G+I	63	3640.442	3171.992	-1522.676	0.28	2.03	3.21	0.185	0.18	0.309	0.326	0.057	0.014	0.14	0.059	0.342	0.029	0.008	0.199	0.028	0.08	0.016	0.027
GTR	61	3645.244	3191.645	-1534.522	n/a	n/a	1.83	0.185	0.18	0.309	0.326	0.051	0.046	0.16	0.052	0.258	0.04	0.027	0.15	0.053	0.091	0.022	0.051
JC+G	54	3646.313	3244.707	-1568.118	n/a	1.66	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC	53	3646.344	3252.166	-1572.856	n/a	n/a	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+I	54	3646.891	3245.284	-1568.406	0.3	n/a	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+G+I	55	3653.925	3244.891	-1567.201	0	0.76	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083

NOTE.-- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (lnL), and the number of parameters (including branch lengths) are also presented (Nei & Kumar, 2000). Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (R) are shown for each model, as well. They are followed by nucleotide frequencies (f) and rates of base substitutions (r) for each nucleotide pair. Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 25 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 532 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.,* 2011).

APPENDIX B- Maximum Likelihood fits of 24 different *ITS* nucleotide substitution models tested in MEGA 5 for the alignment, excluding outgroups.

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor (MEGA 5).

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)	*r*(GA)	*r*(GT)	*r*(GC)
T92+G	50	2622.846	2248.455	-1074.035	n/a	0.05	2.61	0.176	0.176	0.324	0.324	0.023	0.042	0.24	0.023	0.24	0.042	0.023	0.13	0.042	0.13	0.023	0.042
T92+G+I	51	2633.451	2251.579	-1074.59	0.36	0.19	3.66	0.176	0.176	0.324	0.324	0.018	0.032	0.26	0.018	0.26	0.032	0.018	0.141	0.032	0.141	0.018	0.032
T92	49	2635.499	2268.589	-1085.109	n/a	n/a	2.17	0.176	0.176	0.324	0.324	0.026	0.048	0.228	0.026	0.228	0.048	0.026	0.124	0.048	0.124	0.026	0.048
TN93+G	53	2640.976	2244.145	-1068.857	n/a	0.14	2.54	0.181	0.171	0.326	0.323	0.022	0.043	0.101	0.024	0.381	0.042	0.024	0.2	0.042	0.057	0.022	0.043
HKY+I	52	2641.911	2252.56	-1074.072	0.81	n/a	2.65	0.181	0.171	0.326	0.323	0.022	0.042	0.24	0.023	0.242	0.041	0.023	0.127	0.041	0.135	0.022	0.042
HKY+G	52	2641.951	2252.6	-1074.09	n/a	0.05	2.62	0.181	0.171	0.326	0.323	0.022	0.042	0.239	0.023	0.242	0.042	0.023	0.127	0.042	0.134	0.022	0.026
T92+I	50	2643.235	2268.844	-1084.23	0.09	n/a	2.18	0.176	0.176	0.324	0.324	0.026	0.048	0.228	0.026	0.228	0.048	0.124	0.048	0.124	0.026	0.048	0.048
TN93	52	2650.071	2260.721	-1078.152	n/a	n/a	2.2	0.17	0.1811	0.326	0.323	0.025	0.047	0.101	0.026	0.361	0.047	0.026	0.19	0.047	0.057	0.025	0.047
TN93+G+I	54	2650.196	2245.886	-1068.719	0.49	0.52	2.57	0.181	0.171	0.326	0.323	0.022	0.042	0.1	0.023	0.383	0.042	0.023	0.201	0.042	0.056	0.022	0.042
HKY+G+I	53	2652.526	2255.696	-1074.632	0.33	0.17	3.66	0.164	0.171	0.326	0.323	0.017	0.032	0.258	0.018	0.261	0.032	0.018	0.137	0.032	0.145	0.017	0.032
HKY	51	2654.682	2272.811	-1085.205	n/a	n/a	2.17	0.181	0.171	0.326	0.323	0.025	0.048	0.227	0.027	0.229	0.048	0.027	0.12	0.048	0.127	0.025	0.048
TN93+I	53	2657.03	2260.2	-1076.884	0.13	n/a	2.22	0.181	0.171	0.326	0.323	0.025	0.047	0.101	0.026	0.363	0.047	0.026	0.19	0.047	0.057	0.025	0.047
GTR+G	56	2662.667	2243.4	-1065.459	n/a	0.14	2.62	0.181	0.171	0.326	0.323	0.059	0	0.098	0.062	0.381	0.034	0	0.199	0.047	0.055	0.018	0.047
K2+G	49	2666.317	2299.406	-1100.518	n/a	0.05	2.53	0.25	0.25	0.25	0.25	0.035	0.035	0.179	0.035	0.179	0.035	0.035	0.179	0.035	0.179	0.035	0.035
K2+I	49	2669.964	2303.054	-1102.342	0.68	n/a	2.32	0.25	0.25	0.25	0.25	0.038	0.038	0.175	0.038	0.175	0.038	0.038	0.175	0.038	0.175	0.038	0.038
GTR+G+I	57	2671.93	2245.185	-1065.343	0.39	0.36	2.65	0.181	0.171	0.326	0.323	0.058	0	0.098	0.062	0.383	0.033	0	0.201	0.046	0.055	0.018	0.047
GTR	55	2673.689	2261.9	-1075.718	n/a	n/a	2.23	0.181	0.171	0.326	0.323	0.058	0.011	0.099	0.061	0.359	0.042	0.006	0.188	0.049	0.055	0.022	0.05
K2+G+I	50	2675.509	2301.118	-1100.366	0.56	0.44	2.57	0.25	0.25	0.25	0.25	0.035	0.035	0.18	0.035	0.18	0.035	0.035	0.18	0.035	0.18	0.035	0.035
K2	48	2679.247	2319.817	-1111.73	n/a	n/a	2.15	0.25	0.25	0.25	0.25	0.04	0.04	0.171	0.04	0.171	0.04	0.04	0.171	0.04	0.171	0.04	0.04
GTR+I	56	2680.658	2261.391	-1074.454	0.12	n/a	2.25	0.181	0.171	0.326	0.323	0.058	0.01	0.098	0.062	0.361	0.041	0.006	0.189	0.049	0.055	0.022	0.049
JC+G	48	2690.645	2331.216	-1117.43	n/a	0.1	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+I	48	2690.732	2331.302	-1117.474	0.78	n/a	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+G+I	49	2699.895	2332.984	-1117.307	0.51	0.51	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC	47	2700.703	2348.754	-1127.207	n/a	n/a	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083

NOTE.-- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (lnL), and the number of parameters (including branch lengths) are also presented (Nei & Kumar, 2000). Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (R) are shown for each model, as well. They are followed by nucleotide frequencies (f) and rates of base substitutions (r) for each nucleotide pair. Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 28 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 452 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.,* 2011).

APPENDIX C- Maximum Likelihood fits of 24 different *ndhF* nucleotide substitution models tested in MEGA 5 for the alignment including all outgroups.

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)	*r*(GA)	*r*(GT)	*r*(GC)
HKY+G	56	6774.62	6276.269	-3082.076	n/a	0.05	1.22	0.275	0.392	0.163	0.17	0.083	0.035	0.098	0.058	0.094	0.036	0.058	0.226	0.036	0.158	0.083	0.035
HKY	55	6781.169	6291.715	-3090.801	n/a	n/a	1.23	0.275	0.392	0.163	0.17	0.083	0.034	0.098	0.058	0.094	0.036	0.058	0.227	0.036	0.159	0.083	0.034
HKY+G+I	57	6782.694	6275.446	-3080.662	0.48	0.05	1.21	0.275	0.392	0.163	0.17	0.083	0.035	0.097	0.058	0.094	0.036	0.058	0.225	0.036	0.158	0.083	0.035
TN93+G	57	6785.344	6278.096	-3081.987	n/a	0.05	1.23	0.275	0.392	0.163	0.17	0.083	0.035	0.089	0.058	0.101	0.036	0.058	0.242	0.036	0.144	0.083	0.035
TN93	56	6791.845	6293.494	-3090.688	n/a	n/a	1.23	0.275	0.392	0.163	0.17	0.083	0.034	0.089	0.058	0.102	0.036	0.058	0.244	0.036	0.144	0.083	0.034
HKY+I	56	6791.976	6293.625	-3090.754	0.01	n/a	1.23	0.275	0.392	0.163	0.17	0.083	0.034	0.098	0.058	0.094	0.036	0.058	0.227	0.036	0.159	0.083	0.034
T92+G	54	6793.172	6312.615	-3102.253	n/a	0.05	1.22	0.333	0.333	0.167	0.167	0.07	0.035	0.096	0.07	0.096	0.035	0.07	0.193	0.035	0.193	0.07	0.035
TN93+G+I	58	6793.476	6277.331	-3080.602	0.48	0.05	1.21	0.275	0.392	0.163	0.17	0.083	0.035	0.09	0.058	0.1	0.036	0.058	0.239	0.036	0.146	0.083	0.035
T92	53	6799.868	6328.208	-3111.051	n/a	n/a	1.22	0.333	0.333	0.167	0.167	0.07	0.035	0.096	0.07	0.096	0.035	0.07	0.193	0.035	0.193	0.07	0.035
T92+G+I	55	6801.054	6311.6	-3100.743	0.5	0.05	1.21	0.333	0.333	0.167	0.167	0.07	0.035	0.096	0.07	0.096	0.035	0.07	0.193	0.035	0.193	0.07	0.035
TN93+I	57	6802.628	6295.38	-3090.629	0.01	n/a	1.23	0.275	0.392	0.163	0.17	0.083	0.034	0.089	0.058	0.102	0.036	0.058	0.244	0.036	0.144	0.083	0.034
GTR+G	60	6805.002	6271.063	-3075.464	n/a	0.05	1.25	0.275	0.392	0.163	0.17	0.053	0.071	0.084	0.037	0.097	0.009	0.119	0.234	0.071	0.136	0.022	0.068
T92+I	54	6810.689	6330.132	-3111.011	0.01	n/a	1.22	0.333	0.333	0.167	0.167	0.07	0.035	0.096	0.07	0.096	0.035	0.07	0.193	0.035	0.193	0.07	0.035
GTR	59	6811.249	6286.208	-3084.038	n/a	n/a	1.24	0.275	0.392	0.163	0.17	0.053	0.07	0.084	0.037	0.097	0.01	0.118	0.233	0.071	0.136	0.023	0.068
GTR+G+I	61	6815.827	6272.992	-3075.426	0.01	0.05	1.25	0.275	0.392	0.163	0.17	0.053	0.071	0.084	0.037	0.097	0.009	0.119	0.234	0.071	0.136	0.022	0.068
GTR+I	60	6821.816	6287.877	-3083.871	0.03	n/a	1.24	0.275	0.392	0.163	0.17	0.053	0.07	0.084	0.037	0.097	0.01	0.118	0.233	0.071	0.136	0.023	0.068
JC+G	52	7015.34	6552.578	-3224.238	n/a	0.05	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2+G	53	7015.648	6543.988	-3218.941	n/a	0.05	1.2	0.25	0.25	0.25	0.25	0.057	0.057	0.136	0.057	0.136	0.057	0.057	0.136	0.057	0.136	0.057	0.057
JC+G+I	53	7022.189	6550.53	-3222.212	0.55	0.05	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2+G+I	54	7022.529	6541.972	-3216.931	0.55	0.05	1.21	0.25	0.25	0.25	0.25	0.056	0.056	0.137	0.056	0.137	0.056	0.056	0.137	0.056	0.137	0.056	0.056
JC	51	7022.716	6568.851	-3233.377	n/a	n/a	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2	52	7023.004	6560.241	-3228.07	n/a	n/a	1.19	0.25	0.25	0.25	0.25	0.057	0.057	0.136	0.057	0.136	0.057	0.057	0.136	0.057	0.136	0.057	0.057
JC+I	52	7033.55	6570.788	-3233.343	0.01	n/a	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2+I	53	7033.816	6562.156	-3228.025	0.01	n/a	1.19	0.25	0.25	0.25	0.25	0.057	0.057	0.136	0.057	0.136	0.057	0.057	0.136	0.057	0.136	0.057	0.057

NOTE.-- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) are also presented (Nei & Kumar, 2000). Non-uniformity of evolutionary rates among sites may be modelled by using a discrete Gamma distribution (+*G*) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (*R*) are shown for each model, as well. They are followed by nucleotide frequencies (*f*) and rates of base substitutions (*r*) for each nucleotide pair. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 27 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 2009 positions in the final dataset (MEGA 5).

APPENDIX D- Maximum Likelihood fits of 24 different *ndhF* nucleotide substitution models tested in MEGA 5 for the alignment, excluding outgroups.

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)	*r*(GA)	*r*(GT)	*r*(GC)
HKY	43	6022.815	5649.899	-2781.906	n/a	n/a	1	0.276	0.391	0.166	0.168	0.092	0.039	0.088	0.065	0.087	0.04	0.065	0.206	0.04	0.145	0.092	0.039
TN93	44	6032.998	5651.411	-2781.66	n/a	n/a	1	0.276	0.391	0.166	0.168	0.093	0.04	0.053	0.066	0.114	0.04	0.066	0.268	0.04	0.088	0.093	0.04
HKY+I	44	6033.49	5651.903	-2781.906	0	n/a	1	0.276	0.391	0.166	0.168	0.092	0.039	0.088	0.065	0.087	0.04	0.065	0.206	0.04	0.145	0.092	0.039
HKY+G	44	6033.49	5651.903	-2781.906	n/a	200	1	0.276	0.391	0.166	0.168	0.092	0.039	0.088	0.065	0.087	0.04	0.065	0.206	0.04	0.145	0.092	0.039
T92	41	6041.568	5685.994	-2801.957	n/a	n/a	1	0.333	0.333	0.167	0.167	0.078	0.039	0.088	0.078	0.088	0.039	0.078	0.177	0.039	0.177	0.078	0.039
TN93+G	45	6043.672	5653.416	-2781.66	n/a	200	1	0.276	0.391	0.166	0.168	0.093	0.04	0.053	0.066	0.114	0.04	0.066	0.268	0.04	0.088	0.093	0.04
TN93+I	45	6043.672	5653.416	-2781.66	0	n/a	1	0.276	0.391	0.166	0.168	0.093	0.04	0.053	0.066	0.114	0.04	0.066	0.268	0.04	0.088	0.093	0.04
HKY+G+I	45	6044.165	5653.908	-2781.906	0	200	1	0.276	0.391	0.166	0.168	0.092	0.039	0.088	0.065	0.087	0.04	0.065	0.206	0.04	0.145	0.092	0.039
T92+I	42	6052.243	5687.998	-2801.957	0	n/a	1	0.333	0.333	0.167	0.167	0.078	0.039	0.088	0.078	0.088	0.039	0.078	0.177	0.039	0.177	0.078	0.039
T92+G	42	6052.243	5687.998	-2801.957	n/a	200	1	0.333	0.333	0.167	0.167	0.078	0.039	0.088	0.078	0.088	0.039	0.078	0.177	0.039	0.177	0.078	0.039
TN93+G+I	46	6054.347	5655.42	-2781.66	0	200	1	0.276	0.391	0.166	0.168	0.093	0.04	0.053	0.066	0.114	0.04	0.066	0.268	0.04	0.088	0.093	0.04
GTR	47	6059.575	5651.978	-2778.937	n/a	n/a	1	0.276	0.391	0.166	0.168	0.048	0.048	0.048	0.034	0.102	0	0.08	0.241	0.161	0.079	0	0.159
T92+G+I	43	6062.917	5690.002	-2801.957	0	200	1	0.333	0.333	0.167	0.167	0.078	0.039	0.088	0.078	0.088	0.039	0.078	0.177	0.039	0.177	0.078	0.039
GTR+I	48	6070.249	5653.982	-2778.937	0	n/a	1	0.276	0.391	0.166	0.168	0.048	0.048	0.048	0.034	0.102	0	0.08	0.241	0.161	0.079	0	0.159
GTR+G	48	6070.249	5653.982	-2778.937	n/a	200	1	0.276	0.391	0.166	0.168	0.048	0.048	0.048	0.034	0.102	0	0.08	0.241	0.161	0.079	0	0.159
GTR+G+I	49	6080.924	5655.987	-2778.937	0	200	1	0.276	0.391	0.166	0.168	0.048	0.048	0.048	0.034	0.102	0	0.08	0.241	0.161	0.079	0	0.159
JC	39	6253.456	5915.223	-2918.575	n/a	n/a	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2	40	6263.188	5916.284	-2918.104	n/a	n/a	1	0.25	0.25	0.25	0.25	0.062	0.062	0.125	0.062	0.125	0.062	0.062	0.125	0.062	0.125	0.062	0.062
JC+I	40	6264.13	5917.226	-2918.575	0	n/a	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+G	40	6264.131	5917.227	-2918.576	n/a	200	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2+G	41	6273.862	5918.287	-2918.104	n/a	200	1	0.25	0.25	0.25	0.25	0.062	0.062	0.125	0.062	0.125	0.062	0.062	0.125	0.062	0.125	0.062	0.062
K2+I	41	6273.862	5918.288	-2918.104	0	n/a	1	0.25	0.25	0.25	0.25	0.062	0.062	0.125	0.062	0.125	0.062	0.062	0.125	0.062	0.125	0.062	0.062
JC+G+I	41	6274.805	5919.231	-2918.575	0	200	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2+G+I	42	6284.536	5920.291	-2918.104	0	200	1	0.25	0.25	0.25	0.25	0.062	0.062	0.125	0.062	0.125	0.062	0.062	0.125	0.062	0.125	0.062	0.062

NOTE.-- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) are also presented. Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+*G*) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (*R*) are shown for each model, as well. They are followed by nucleotide frequencies (*f*) and rates of base substitutions (*r*) for each nucleotide pair. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 2059 positions in the final dataset (MEGA 5).

APPENDIX E- Maximum Likelihood fits of 24 different *rbcL* nucleotide substitution models tested in MEGA 5 for the alignment, including outgroups.

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)	*r*(GA)	*r*(GT)	*r*(GC)
T92	35	7463.32	7183.111	-3556.499	n/a	n/a	2.16	0.281	0.281	0.219	0.219	0.044	0.034	0.151	0.044	0.151	0.034	0.044	0.193	0.034	0.193	0.044	0.034
HKY	37	7470.754	7174.54	-3550.207	n/a	n/a	2.16	0.275	0.286	0.195	0.244	0.045	0.03	0.168	0.043	0.134	0.038	0.043	0.197	0.038	0.189	0.045	0.03
T92+I	36	7473.197	7184.986	-3556.433	0.01	n/a	2.16	0.281	0.281	0.219	0.219	0.044	0.034	0.151	0.044	0.151	0.034	0.044	0.193	0.034	0.193	0.044	0.034
T92+G	36	7473.332	7185.121	-3556.501	n/a	200	2.16	0.281	0.281	0.219	0.219	0.044	0.034	0.151	0.044	0.151	0.034	0.044	0.193	0.034	0.193	0.044	0.034
TN93	38	7478.005	7173.789	-3548.828	n/a	n/a	2.16	0.275	0.286	0.195	0.244	0.044	0.03	0.1	0.042	0.194	0.038	0.042	0.285	0.038	0.113	0.044	0.03
HKY+I	38	7480.691	7176.475	-3550.171	0.01	n/a	2.16	0.275	0.286	0.195	0.244	0.045	0.03	0.168	0.043	0.134	0.038	0.043	0.197	0.038	0.189	0.045	0.03
HKY+G	38	7480.764	7176.548	-3550.207	n/a	200	2.16	0.275	0.286	0.195	0.244	0.045	0.03	0.168	0.043	0.134	0.038	0.043	0.197	0.038	0.189	0.045	0.03
TN93+I	39	7487.947	7175.729	-3548.794	0.01	n/a	2.17	0.275	0.286	0.195	0.244	0.044	0.03	0.101	0.042	0.194	0.037	0.042	0.285	0.037	0.114	0.044	0.03
TN93+G	39	7488.019	7175.801	-3548.83	n/a	200	2.16	0.275	0.286	0.195	0.244	0.044	0.03	0.1	0.042	0.194	0.038	0.042	0.285	0.038	0.113	0.044	0.03
HKY+G+I	39	7490.717	7178.499	-3550.179	0.01	15.21	2.17	0.275	0.286	0.195	0.244	0.045	0.03	0.168	0.043	0.134	0.038	0.043	0.197	0.038	0.189	0.045	0.03
T92+G+I	37	7491.252	7195.038	-3560.456	0.01	200	2.17	0.281	0.281	0.219	0.219	0.044	0.034	0.151	0.044	0.151	0.034	0.044	0.193	0.034	0.193	0.044	0.034
K2	34	7491.433	7219.227	-3575.56	n/a	n/a	2.16	0.25	0.25	0.25	0.25	0.04	0.04	0.171	0.04	0.171	0.04	0.04	0.171	0.04	0.171	0.04	0.04
JC	33	7493.334	7229.131	-3581.515	n/a	n/a	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
TN93+G+I	40	7498.125	7177.905	-3548.878	0.01	6.25	2.19	0.275	0.286	0.195	0.244	0.044	0.03	0.101	0.042	0.194	0.037	0.042	0.286	0.037	0.114	0.044	0.03
K2+I	35	7500.921	7220.712	-3575.299	0.02	n/a	2.17	0.25	0.25	0.25	0.25	0.039	0.039	0.171	0.039	0.171	0.039	0.039	0.171	0.039	0.171	0.039	0.039
JC+G	34	7501.728	7229.522	-3580.707	n/a	200	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2+G	35	7501.814	7221.605	-3575.746	n/a	200	2.16	0.25	0.25	0.25	0.25	0.04	0.04	0.171	0.04	0.171	0.04	0.04	0.171	0.04	0.171	0.04	0.04
JC+I	34	7503.343	7231.137	-3581.515	0	n/a	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+G+I	35	7514.17	7233.962	-3581.924	0.02	200	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2+G+I	36	7525.851	7237.64	-3582.76	0.02	200	2.17	0.25	0.25	0.25	0.25	0.039	0.039	0.171	0.039	0.171	0.039	0.039	0.171	0.039	0.171	0.039	0.039
GTR+G	42	7569.519	7233.296	-3574.567	n/a	200	0.71	0.275	0.286	0.195	0.244	0.109	0.038	0.099	0.105	0.086	0.061	0.054	0.126	0.077	0.112	0.072	0.061
GTR	41	7578.315	7250.093	-3583.969	n/a	n/a	0.71	0.275	0.286	0.195	0.244	0.109	0.038	0.099	0.105	0.086	0.061	0.054	0.126	0.077	0.112	0.072	0.061
GTR+G+I	43	7579.529	7235.304	-3574.567	0	200	0.71	0.275	0.286	0.195	0.244	0.109	0.038	0.099	0.105	0.086	0.061	0.054	0.126	0.077	0.112	0.072	0.061
GTR+I	42	7588.324	7252.101	-3583.969	0	n/a	0.71	0.275	0.286	0.195	0.244	0.109	0.038	0.099	0.105	0.086	0.061	0.054	0.126	0.077	0.112	0.072	0.061

NOTE.-- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) are also presented (Nei & Kumar, 2000). Non-uniformity of evolutionary rates among sites may be modelled by using a discrete Gamma distribution (+*G*) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (*R*) are shown for each model, as well. They are followed by nucleotide frequencies (*f*) and rates of base substitutions (*r*) for each nucleotide pair. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1235 positions in the final dataset (MEGA 5).

APPENDIX F- Maximum Likelihood fits of 24 different *rbcL* nucleotide substitution models tested in MEGA 5 for the alignment, excluding outgroups.

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)	*r*(GA)	*r*(GT)	*r*(GC)
T92	33	4071.309	3808.679	-1871.286	n/a	n/a	2.16	0.28	0.28	0.22	0.22	0.044	0.034	0.151	0.044	0.151	0.034	0.044	0.192	0.034	0.192	0.044	0.034
K2	32	4079.793	3825.118	-1880.509	n/a	n/a	2.16	0.25	0.25	0.25	0.25	0.039	0.039	0.171	0.039	0.171	0.039	0.039	0.171	0.039	0.171	0.039	0.039
JC	31	4081.03	3834.311	-1886.108	n/a	n/a	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
T92+I	34	4081.271	3810.685	-1871.286	0	n/a	2.16	0.28	0.28	0.22	0.22	0.044	0.034	0.151	0.044	0.151	0.034	0.044	0.192	0.034	0.192	0.044	0.034
T92+G	34	4081.271	3810.686	-1871.287	n/a	200	2.16	0.28	0.28	0.22	0.22	0.044	0.034	0.151	0.044	0.151	0.034	0.044	0.192	0.034	0.192	0.044	0.034
HKY	35	4084.502	3805.961	-1867.921	n/a	n/a	2.16	0.274	0.286	0.195	0.245	0.045	0.03	0.168	0.043	0.134	0.038	0.043	0.196	0.038	0.189	0.045	0.03
K2+I	33	4089.755	3827.124	-1880.509	0	n/a	2.16	0.25	0.25	0.25	0.25	0.039	0.039	0.171	0.039	0.171	0.039	0.039	0.171	0.039	0.171	0.039	0.039
K2+G	33	4089.755	3827.125	-1880.509	n/a	200	2.16	0.25	0.25	0.25	0.25	0.039	0.039	0.171	0.039	0.171	0.039	0.039	0.171	0.039	0.171	0.039	0.039
JC+I	32	4090.992	3836.317	-1886.108	0	n/a	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+G	32	4090.992	3836.317	-1886.109	n/a	200	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
T92+G+I	35	4091.233	3812.692	-1871.287	0	200	2.16	0.28	0.28	0.22	0.22	0.044	0.034	0.151	0.044	0.151	0.034	0.044	0.192	0.034	0.192	0.044	0.034
TN93	36	4091.711	3805.215	-1866.545	n/a	n/a	2.16	0.274	0.286	0.195	0.245	0.044	0.03	0.101	0.042	0.194	0.038	0.042	0.284	0.038	0.113	0.044	0.03
HKY+I	36	4094.463	3807.968	-1867.921	0	n/a	2.16	0.274	0.286	0.195	0.245	0.045	0.03	0.168	0.043	0.134	0.038	0.043	0.196	0.038	0.189	0.045	0.03
HKY+G	36	4094.464	3807.968	-1867.921	n/a	200	2.16	0.274	0.286	0.195	0.245	0.045	0.03	0.168	0.043	0.134	0.038	0.043	0.196	0.038	0.189	0.045	0.03
K2+G+I	34	4099.717	3829.131	-1880.509	0	200	2.16	0.25	0.25	0.25	0.25	0.039	0.039	0.171	0.039	0.171	0.039	0.039	0.171	0.039	0.171	0.039	0.039
JC+G+I	33	4100.954	3838.324	-1886.109	0	200	0.5	0.25	0.25	0.25	0.25	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
TN93+I	37	4101.673	3807.222	-1866.545	0	n/a	2.16	0.274	0.286	0.195	0.245	0.044	0.03	0.101	0.042	0.194	0.038	0.042	0.284	0.038	0.113	0.044	0.03
TN93+G	37	4101.673	3807.223	-1866.545	n/a	200	2.17	0.274	0.286	0.195	0.245	0.044	0.03	0.101	0.042	0.194	0.038	0.042	0.284	0.038	0.113	0.044	0.03
HKY+G+I	37	4104.425	3809.975	-1867.921	0	200	2.16	0.274	0.286	0.195	0.245	0.045	0.03	0.168	0.043	0.134	0.038	0.043	0.196	0.038	0.189	0.045	0.03
TN93+G+I	38	4111.635	3809.23	-1866.545	0	200	2.17	0.274	0.286	0.195	0.245	0.044	0.03	0.101	0.042	0.194	0.038	0.042	0.284	0.038	0.113	0.044	0.03
GTR	39	4116.322	3805.963	-1863.908	n/a	n/a	2.16	0.274	0.286	0.195	0.245	0	0.039	0.099	0	0.191	0.057	0.055	0.28	0.056	0.111	0.066	0.044
GTR+I	40	4126.284	3807.971	-1863.908	0	n/a	2.16	0.274	0.286	0.195	0.245	0	0.039	0.099	0	0.191	0.057	0.055	0.28	0.056	0.111	0.066	0.044
GTR+G	40	4126.285	3807.972	-1863.908	n/a	200	2.16	0.274	0.286	0.195	0.245	0	0.039	0.099	0	0.191	0.057	0.055	0.28	0.056	0.111	0.066	0.044
GTR+G+I	41	4136.247	3809.979	-1863.908	0	200	2.16	0.274	0.286	0.195	0.245	0	0.039	0.099	0	0.191	0.057	0.055	0.28	0.056	0.111	0.066	0.044

NOTE.-- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) are also presented (Nei & Kumar, 2000). Non-uniformity of evolutionary rates among sites may be modelled by using a discrete Gamma distribution (+*G*) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (*R*) are shown for each model, as well. They are followed by nucleotide frequencies (*f*) and rates of base substitutions (*r*) for each nucleotide pair. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 17 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1247 positions in the final dataset (MEGA 5).