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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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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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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

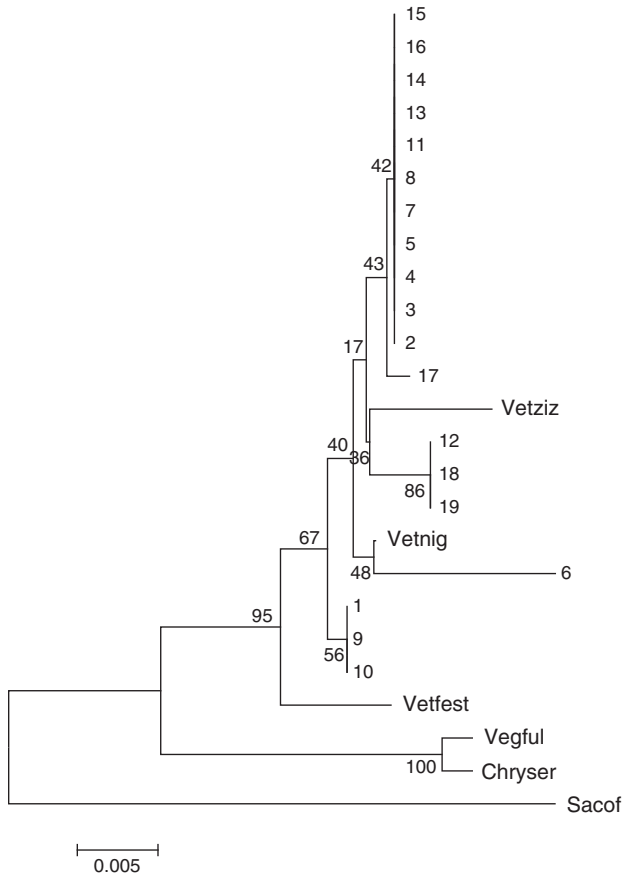

Fig. 1. The evolutionary history inferred using the Neighbour-Joining method and *ITS* sequence data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths 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).

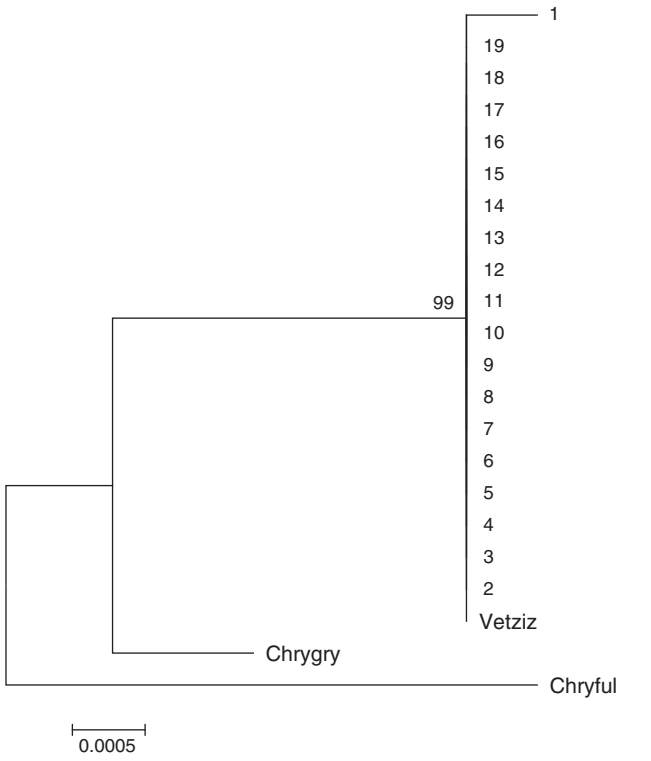

Fig. 2. The evolutionary history inferred using the Neighbour-Joining method with the *ndhF* sequence data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch 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*

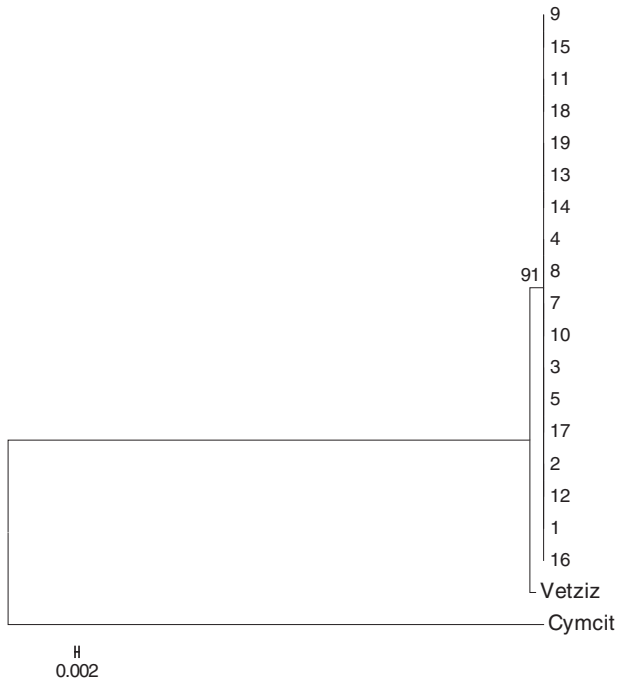

Fig. 3. The evolutionary history inferred using the Neighbour-Joining method using the *rbcL* sequencing data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch 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.

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