ASSESSMENT OF SPECIES BOUNDARIES OF THE *MORINGA OVALIFOLIA* (DINTER & A. BERGER) IN NAMIBIA USING NUCLEAR ITS DNA SEQUENCE DATA

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ABSTRACT

The genus *Moringa* comprises of thirteen (13) species of trees and shrubs, widely distributed in the tropical and subtropical regions. For its size, this genus is one of the phenotypically varied group of angiosperms whose members are mostly sought after for nutritional, nutraceutical and industrial uses. The aim of this study was to assess the species boundaries of *Moringa ovalifolia* in Namibia by using the nuclear Internal Transcribed Spacer (ITS) sequenced data analysis. A total of seventeen (17) edited sequences of *M. ovalifolia* together with thirteen (13) other sequences of *Moringa* retrieved from NCBI were used to construct phylogenetic trees in Molecular Evolutionary Genetic Analysis software version 6.0 (MEGA). The phylogenetic trees were resolved using Neighbor-joining method, Maximum Parsimony method, Maximum Likelihood method based on the Tamura-Nei and the bootstrap consensus tree was determined from 500 replicates that represented the evolutionary history of the taxa analyzed. The nuclear ITS molecular marker successfully delimited species boundaries of *M. ovalifolia* specimens that were investigated in this study. The topology of all three phylogenetic trees generated by the three methods were similar. All sequences of *M. ovalifoli*a under investigation and those retrieved from NCBI) were grouped together and are not nested with other *Moringa* species. The different populations of the Namibian *M. ovalifolia* are not phylogenetically different though geographically isolated. This means that the Namibian *M. ovalifolia* is a taxonomically 'good species'. The study demonstrated the useful role of ITS in determining the species boundaries of populations. It is recommended that efforts be made to initiate domestication and breeding programs of the Namibian *M. ovalifolia.*

Key words: Specimens, Internal Transcribed Spacer, *Moringa ovalifolia*, species boundaries, phylogenetic trees.

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ABBREVIATIONS

BLAST- Basic Local Alignment Search Tool

bp – Base pair

CTAB- cetyl trimethylammonium bromide

EDTA – Ethylenediaminetetra acetic acid

DNA – Deoxyribonucleic acid

ITS – Internal Transcribed Spacer regions

PCR – Polymerase Chain Reaction

RAPD – Random Amplified Polymorphic DNA

RNA - Ribonucleic acid

Taq- Thermophillus aquaticus

^{o}C – Degree Celsius

µl – microliter

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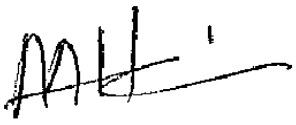
DEDICATIONS

This study is dedicated to my beautiful daughters Rosie and Posie. You girls give a meaning to my life and in you I find the strength to work hard and endure tough times. I appreciate your unconditional love and I take this opportunity to say I deeply love you and appreciate God for your lives.

DECLARATIONS

I, Martha Kasiku Hausiku, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.



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Martha Kasiku Hausiku

CHAPTER 1: INTRODUCTION

1.1. Orientation of the study

So far, *Moringa* is the sole genus of Moringaceae family, comprising of 13 species (Padayachee and Baijnath, 2012). The genus spans a range of phenotypic forms and geographical distribution. The well-known *Moringa oleifera,* along with *Moringa concanensis,* are native to the Indian subcontinent (Brilhante et al., 2017; Lalas et al. 2012). *Moringa ovalifolia* known as Phantom or Ghost tree (Kwaambwa et al., 2012) is indigenous to Namibia and Angola (Shailemo et al., 2016), whereas *M. peregrina* grows around the Red Sea, north to the Dead Sea, and around the southern Arabian Peninsula (Olson et al., 2016; Lalas et al., 2012; Osman and Abohassan, 2012; Olson, 2001). *M. drouhardii* and *M. hildebrandtii* are endemic to Madagascar (Padayachee and Baijnath, 2012) and the rest of *Moringa* species (*M. arborea*, *M. borziana, M. longituba, M. pygmaea*, *M. rivae, M. ruspoliana,* and *M. stenopetala*) occur naturally in Kenya, Ethiopia, and Somalia (Olson et al., 2016). *Moringa* species varies from massive, water-storing 'bottle trees' in southern Africa, to slender trees in Arabia and India, to tiny tuberous herbs in northeastern Africa (Price, 2007).

The genus *Moringa* has gained worldwide status as a miracle tree and a multi-purpose crop due to its versatility in use. The outstanding economic potential of these thirteen species of *Moringa* are of interest due to their role in providing nutritious leaf and fruit vegetables, high-quality seed oil, antibiotics, and as water clarification agents (James and Zikankuba, 2017; Olson and Razafimandimbison, 2000). Members of this genus are known to have nutritional and medicinal use for humans and animals as well as

industrial applications (Lalas et al., 2012; Dhakar et al., 2011; Bey, 2010). *Moringa*'s applications in food, manufacturing and medicinal industries coupled with its characteristic ability to thrive in dry conditions has earned its reputation as a 'miracle plant' (Fuglie, 2001). *Moringa* species have variations in their nutritional content and secondary plant metabolites profile. Even though environmental and agronomical factors are able to affect the concentration of secondary plant metabolites, much of the variations are influenced by genetic factors (Demasi et al. 2018; Sampaio, et al., 2016). Thus, understanding the genetic variation of *Moringa* germplasm resources is of great theoretical and practical value.

Traditionally, genetic variation, identification and classification of plants were mainly done based on morphological characteristics. Morphological characteristic however, may have downfall due to phenotypic plasticity, cryptic species or distorted sample material. When morphology is compromised, molecular methods, based on genomic differences amongst individuals, offer a promising means to circumvent the challenge (Wallinger et al., 2012; Green et al. 2010). Molecular identification based on molecular markers has become a popular tool for evaluating genetic relationships between and within populations beyond what can be ascertained from morphological evaluations (Liu et al. 2018; Lashkari et al., 2014). Thus, the development of DNA barcode technology application, which is an advanced method using the sequence of short DNA strands featured in the genomes of organisms, to identify and distinguish individuals has not only improved the drawbacks of morphological identification but also brought high efficiency in identification within a short period of time (Huan, et al., 2018). Due to the generic nature and universal workflow of the technique, DNA barcoding has been

applied to a wide range of scientific disciplines, from food authenticity to conservation studies (Holmeset al., 2009; Hrcek et al., 2011; Adamowicz, 2015).

As both Internal Transcribed Spacer (ITS) regions are part of the cytoplasmic ribosome genes playing a role in the formation of the mature ribosome, these making them abundant and thus, there are hundreds, or in some cases thousands of tandem copies (Poczai and Hyvonen, 2009; Pinkel et al., 1998). Because of the high copy number, this region is recognized as a multi-copy gene family, which provides easy amplification via PCR. This is an advantage, but on the other hand, it can be a problem in phylogenetic analyses, if paralogous sequences are present. However, the general assumption for phylogenetic studies is that all ribosomal copies present within the genome have identical sequences due to functional constraints (Poczai and Hyvonen, 2009).

Though, the majority of the *Moringa* species are less documented, there is a possibility that they might as well potentially represent valuable commodities in both food and medicine industries. The preservation of the genus *Moringa* is thus of great concern from a biodiversity, ethnobotanical, dietary and pharmacological perspectives. Considering the economic potential of the genus based on the few species that have been documented, there is a need for scientific examination of the less documented species such as *Moringa ovalifolia*. Olson (2002), hypothesized that the genus *Moringa* cannot be divided into comparable monophyletic taxa, due to the non-monophyly coupled with incoherent geographic distribution pattern of the basal grade. Consequently, the alternative terms; “bottle tree clade, slender tree clade, and tuberous clade” should be maintained. (2002). The present study expands the previous work on phylogenetic status of *M. ovalifolia* by including more samples to investigate its intraspecific variation.

1.2. Statement of the problem

Moringa ovalifolia can be challenging to diagnose to species due to their morphological variation that is often subtle across other bottle trees (i.e. *Moringa drouhardii*, *Moringa hildebrandtii* and *Moringa stenopetala*). These together with their parapatric and sometimes allopatric distributional ranges, gaps in sampling, and a general lack of comparative material for assessments of species diagnoses, highlight the need for their assessment of species boundaries. Literature review revealed scanty scientific information on *M. ovalifolia* as a species. Few documentation available on this species focused on its seed propagation (Korsor et al., 2016), antimicrobial activity (Shailemo et al. 2016) nutritional and trace element contents (Huber et al. 2017; Ananias et al., 2016). The paucity in information on the species boundary of *Moringa ovalifolia* might have put limitations on the species' potential to add value to the economy of Namibia, as the species boundaries of the Namibian *M. ovalifolia* populations with possible desirable characteristics from consumers' perspective have not been established. *Moringa ovalifolia*, may have similar economic and / or industrial benefit as other *Moringa* species that have been documented. Unless the species boundaries of *Moringa ovalifolia* is established, its potential to contribute to nutrition and economy in Namibia as well as its conservation will remain a challenge. Clear species circumscription of *Moringa ovalifolia* therefore serves as the baseline not only for the species breeding and genetic improvement but also a guide for the identification of ecotypes with desirable traits for industrial value such as high percentage of seed oil, proteins, medicine and wide adaptability to agro-climate.

1.3. Overall aim of the study

The aim of the study was to provide a clarification of the phylogenetic status of the different Namibian *Moringa ovalifolia* populations and their evolutionary relationships as well as to test the hypothesis of phylogeny proposed by Olson (2002). According to Olson's proposed hypothesis, *M. ovalifolia* groups with the Madagascar species; *M. hildebrandtii* and *M. draouhardtii* and *M. stenopetala* from the western horn of Africa.

1.4. Specific objectives of the study

1.4.1. To investigate the taxonomic value of molecular characters in separating different *Moringa ovalifolia* populations in Namibia.

1.4.2. To reconstruct phylogenetic relationships, among the different *Moringa ovalifolia* populations in Namibia and to test whether the different forms comprise a monoplyletic lineage.

1.4.3. To reconstruct phylogenetic relationships between *Moringa ovalifolia* and other *Moringa* species and test the hypothesis of phylogeny proposed by Olson (2002), where *Moringa ovalifolia* emerged as a sister species to *M. stenopetala, M. drouhardtii* and *M. hildebrandtii*.

1.5. Null Hypotheses of the study

1.5.1. The nuclear ITS does not separate the different Namibian *Moringa ovalifolia* populations.

1.5.2. Different *Moringa ovalifolia* populations in Namibia are not phylogenetically distinct from each other.

1.5.3. Namibian *Moringa ovalifolia* do not form a monophyletic lineage.

1.5.4. Namibian *Moringa ovalifolia* is not phylogenetically distinct from other African *Moringa* species.

1.6. Significance of the study

Moringa ovalifolia is of conservation importance, protected in Namibia under the Nature Conservation Ordinance 4 of 1975, and under the Preservation of Tree and Forests Ordinance of 1952 (Curtis and Mannheimer, 2005). Namibia, being one of the two countries where this species occur holds the responsibility not only to conserve, but also importantly to provide sound scientific information on its endemics and near-endemics, for proper conservation management and development efforts. Sarwat et al. (2011), stated that information on the extent of genetic diversity is essential for development of proper a conservation strategy and management of the gene-bank. The conservation of the *Moringa ovalifolia* is thus of great concern not only from a biodiversity perpective but also from ethnobotanical, dietary and pharmacological perspectives. The present study will explore variation of within *M. ovalifolia* to clarify its species boundaries by using molecular techniques. The information generated would further be used as basis for studies on genetic diversity of *M. ovalifolia* and by potential breeders interested in

identifying and selecting germplasm for breeding programmes based on desired characteristics.

1.7. Limitations of the study

Due to time constraint, investigation of genetic diversity of the different *Moringa ovalifolia* population using genetic markers such as Microsatellites such as simple sequence repeats (SSR) or Random Amplification of Polymorphic DNA (RAPDS) could not be carried out.

CHAPTER 2: LITERATURE REVIEW

2.1. Background

Moringa ovalifolia belongs to the flowering plant family *Moringaceae*, order Capparales, class Magnoliopsida and division Magnoliophyta (Ggandji et al., 2018; Shahzad et al., 2013; Lalas et al., 2012). The genus, *Moringa* consist of 13 species namely; *M. oleifera, M. arborea, M. borziana, M. concanensis, M. douhardii, M. hildebrandtii, M. longituba, M ovalifolia, M. Peregrine, M pygmaea, M. rivae, M. ruspoliana and M stenopetala* (Paliwal, et al., 2011). These species range from small herbs to large trees and are distributed in tropical and subtropical regions as shown in **Figure 1** (Said-Al Ahl, et al., 2017; Padayachee and Baijnath, 2012).

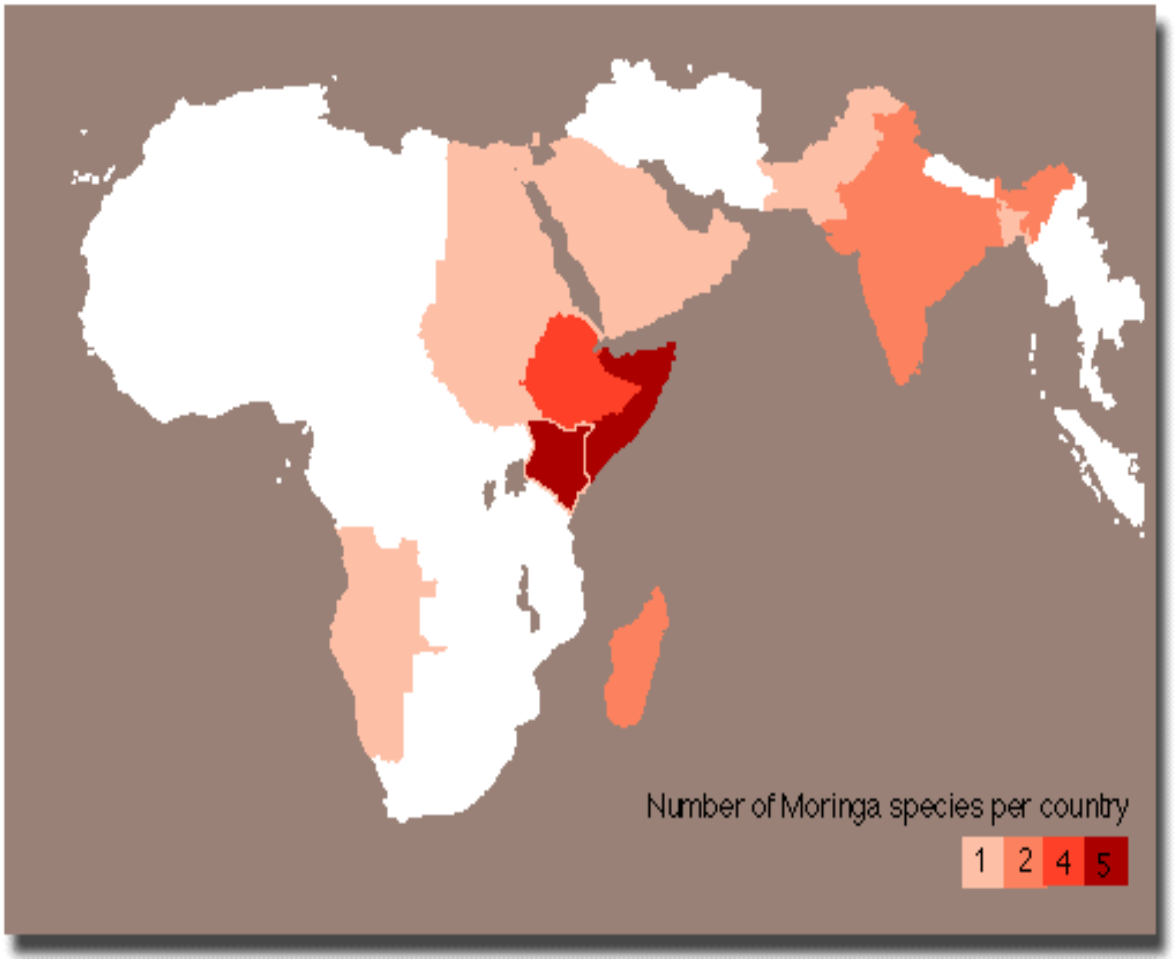
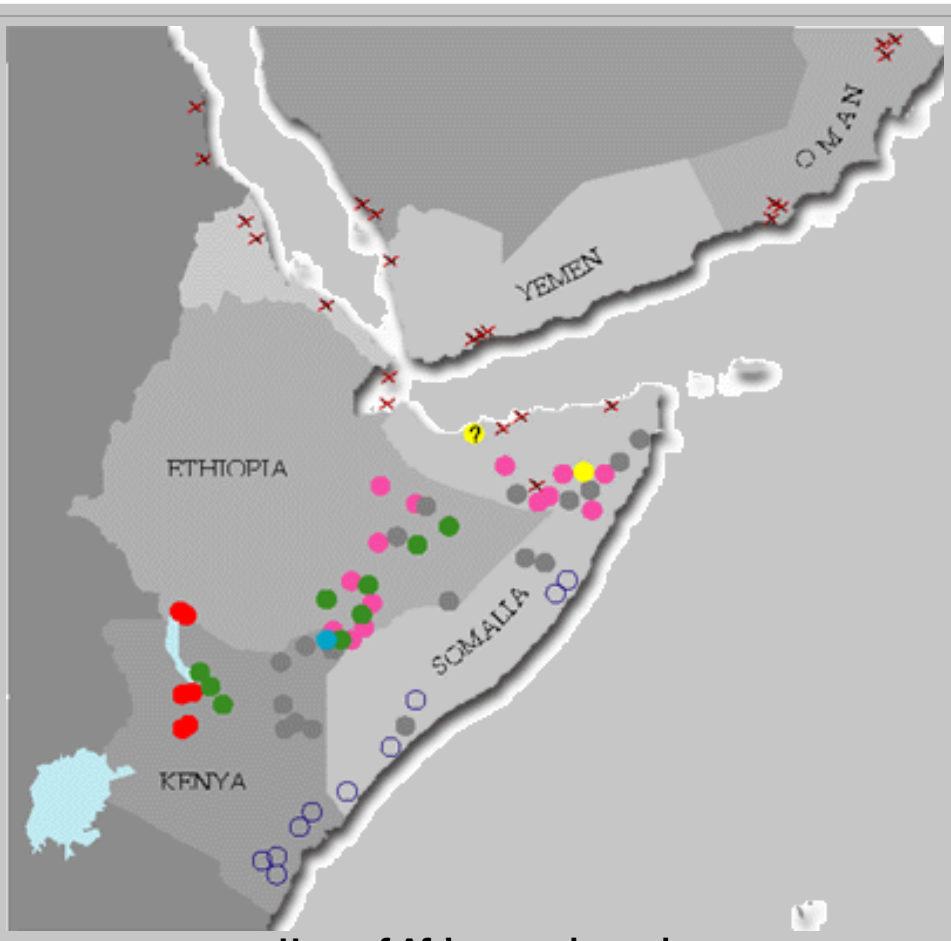


Figure 1: Map shows the global distribution of *Moringa* species adopted from http://www.mobot.org/gradstudents/olson/moringahome.html

Out of the 13 species, only *Moringa ovalifolia* is naturally found distributed from central-southern Namibia to south-western Angola (Ananias, et al. 2016; Shailemo et al., 2016). The area with the highest number of *Moringa* species is the Horn of Africa (**Figure 2**), with seven species endemic to Kenya, Ethiopia, and Somalia (*M. arborea, M. borziana, M. longituba, M. pygmaea, M. rivae, M. ruspoliana,* and *M. stenopetala*) as shown in **Figure 1** (Olson et al., 2016). Madagascar is home to *M. drouhardii* and *M.*

hildebrandtii (Padayachee and Baijnath 2012) while *M. oleifera*, along with *M. concanensis* are natives of India (Brilhante et al., 2017; Lalas et al., 2012).



Horn of Africa map legend

Red x's	*M. peregrina*
Yellow dots	*M. pygmaea*
Gray dots	*M. longtituba*
Pink dots	*M. ruspoliana*
Green dots	*M. rivae*
Blue circles	*M. borziana*
Red dots	*M. stenopetala*
Turquoise dot	*M. arborea*

Figure 2: The map shows that the horn of Africa has more diversity of endemic *Moringa* species are naturally found (adopted from http://www.mobot.org/gradstudents/olson/moringahome.html)

For its size, *Moringa* is one of the phenotypically varied group of angiosperms (Olson, 2002). Based on the physical appearance, the genus of *Moringa* is divided into three classes; bottle trees, slender trees, tuberous shrub trees. The bottle trees (i.e. *M. drouhardii, M. hildebrandtii, M. ovalifolia, M. stenopetala*) are characterized by massive trees with bloated water-storing trunks and small radially symmetrical flowers. The stem of slender trees (i.e. *M. concanensis; M. oleifera; M. peregrina*), is characterized by a preponderance of libriform fibres that show little seasonal variation in shape with little axial parenchyma (Olson and Carlquist, 2001). The sarcorhizal trees (i.e. *M. arborea, M. borziana, M. longituba, M. pygmaea, M. rivae, M. ruspoliana)* are characterized by a slender trunk and fleshy, brittle, tuberous roots while the tuberous trees have slender stem that are often shed during severe drought and massive, soft underground tubers (Olson, 2002).

2.2. Uses of *Moringa*

Members of the genus *Moringa* are mostly sought after for medicine, food, a domestic cleaning agent and water purifier amongst other uses (Hamza and Azmach, 2017; Popoola Oluyisola, and Obembe, 2014; Abd El Baky and El-Baroty, 2013; Adebayo et al., 2011). Every part of *Moringa,* from the roots to flower are nutrient powerhouse with therapeutic properties. The leaves are rich in minerals, amino acids, proteins and multi

vitamins (Leone et al., 2018; Ntila et al., 2018; Gopalakrishnan, et al., 2016). The seeds and leaves of *M. peregrina* are used as food and medicine (Alaklabi et al. 2014; Osman and Abohassan, 2012). Fortification of feed with *Moringa* leaf meal has been shown to not only enhance nutrient digestibility but also increased milk yield as well as positively modify milk fatty acid profile in livestock (Kholif et al, 2015; Sultana et al., 2015). Supplementing the food for pregnant women with *Moringa* in rural Tanzania, reportedly produced similar results (Bisanz et al., 2015). The reports about the *Moringa* leaves being a good resource used as a nutritional supplement may carry additional opportunities with potential for food ingredient innovations, pharmaceutical and cosmetics products (Rodríguez-Pérezab et al., 2015; Asante et al., 2013).

Scavenging activity results confirmed that *Moringa* leaves extract might be a potent source of natural antioxidants with human health benefits (Saleh et al., 2016). Antimicrobial activity results indicate that *Moringa* leaves extracts may be used as an antimicrobial agent with reasonable safety margins to inhibit bacterial growth in pharmaceutical and food applications (Jayawardana et al., 2015; Majali, et al., 2015). Besides the leaves, the bark extracts of *Moringa* have been reported to possess anti-cancer activity that can be used to develop new drugs for treatment of breast and colorectal cancers (Al-Asmari et al., 2016). Various secondary metabolites (e.g. glucosinolate, isothiocyanates, phenolic compounds and carotenoids) are known to be drivers of health-promoting effects, including anti-carcinogenic properties. The main physiological mechanisms underlying the production of secondary plant metabolite are related to defense and plant protection and this is pre-determined by genetics. Variations in their nutritional content and secondary plant metabolites profile of *Moringa* have been

reported. These variations may be a result of environmental and agronomical factors but a great deal of inter and intra population variations are influenced by genetic factors.

Evident from scientific literature, studies have been carried out on genetic diversity of the genus *Moringa* (Alaklabi, 2015; Mgendi et al., 2010; Popoola et al., 2014), however, most of the published studies are on *Moringa oleifera* and *Moringa peregrina*. Documentation on the rest of the genus is rather scanty (El-Hak et al., 2018). This observation proves true for *Moringa ovalifolia*, as literature review did not reveal published information on its genetic diversity. This suggests that *M. ovalifolia* have been understudied.

2.3. Geographic distribution of *Moringa ovalifolia*

Moringa ovalifolia is native to Namibia and Angola. According to Olson (2002), *M. ovalifolia* is naturally distributed from central southern Namibia to southwestern Angola. In Namibia, it is widely distributed from the Escarpment Mountains northwest as shown in **Figure 2**. In the north, it grows in dry mopane woodland *(Colophospermum mopane*). At Epupa Falls, beautiful examples occur on the rocky island and can be seen growing together with the baobab (*Adansonia digitata*), *Commiphora virgata, Euphorbia subsalsa, Adenium oleifolium* and *Pachypodium lealii. Moringa ovalifolia* are often associated with rocky, mountainous terrain or sandy stones. Its habitat consists mainly of desert or arid savannah vegetation as described in **Table 1**.

Table 1: Presents a general description of the habitat of *Moringa ovalifolia* populations in Namibia

POPULATION	DESCRIPTION of GENERAL POPULATION
Etosha- Sprokieswoud	The population grows on a plain and is surrounded by *C. mopani* and *Lyceum bascifolium*. The area is covered with white sand stone. Individuals of this population are big, tall and have a dark and shiny sided bark.
Halali	Could be the healthiest population, growing on loose sedimentary rock. All levels of growth stage are present. The population was not flowering and the trees are not very tall and large. The species distribution include *Aloe* sp., *Gramminoid* family, *Mopani* trees, *Acacia*, *Comifora* sp., and *Bercemia distala*. The area is mountainous.
Sesfontein	Semi- arid, rocky and mountainous environment. Presence of metamorphic rocks. The species diversity consist of C*omiforas, Othoptera balleria*. Most individuals are dwarfs and leaves not well developed. Stem is large, colour is grey and brown for some individuals.
Uis	Very dry environment, rocky outcrops. The population has individuals that are big, tall with red stem colour. Trees observed to be a home for bats. Population appeared to be healthy with juveniles. Population observed to be flowering and containing dry fruits from previous fruiting season. Associated plants include: *Maerua schinzii*, *Acacia sp*., and *Boscia foetida*.
White lady lodge	Very dry environment, rocky out crops. The population has a big, tall, red stem colour. Population observed bear fruit or flower.

The International Union for Conservation of Nature (IUCN) Red List is widely recognized as a robust method of assessing the extinction risk of species, based on assessment of population sizes and population decline rates. The method uses an open

source, browser-based tool called Geospatial Conservation Assessment Tool (GeoCAT). GeoCAT was developed and implemented by a collaboration between Kew Gardens and the European project ViBRANT. The tool performs rapid geospatial analysis to facilitate the process of Red List assessment (Bachman et al., 2011). The tool could also be used to carry out the geospatial analysis of *Moringa ovalifolia* which is only endemic to Namibia and southern part of Angola (Shailemo et al., 2016) as shown in **Figure 3**. Furthermore, Olson (2002), observed that the bottle trees (a group to which *Moringa ovalifolia* belongs) are disjunctly distributed. Three of the four species making up the basal grade in the family have austral distributions with *M. drouhardii* and *M. hildebrandtii* being restricted to Madagascar and *M. ovalifolia* reaching from central southern Namibia to southwestern Angola. *Moringa stenopetala* is found well to the north in the western Horn of Africa just to the west of the area occupied by the tuberous group.

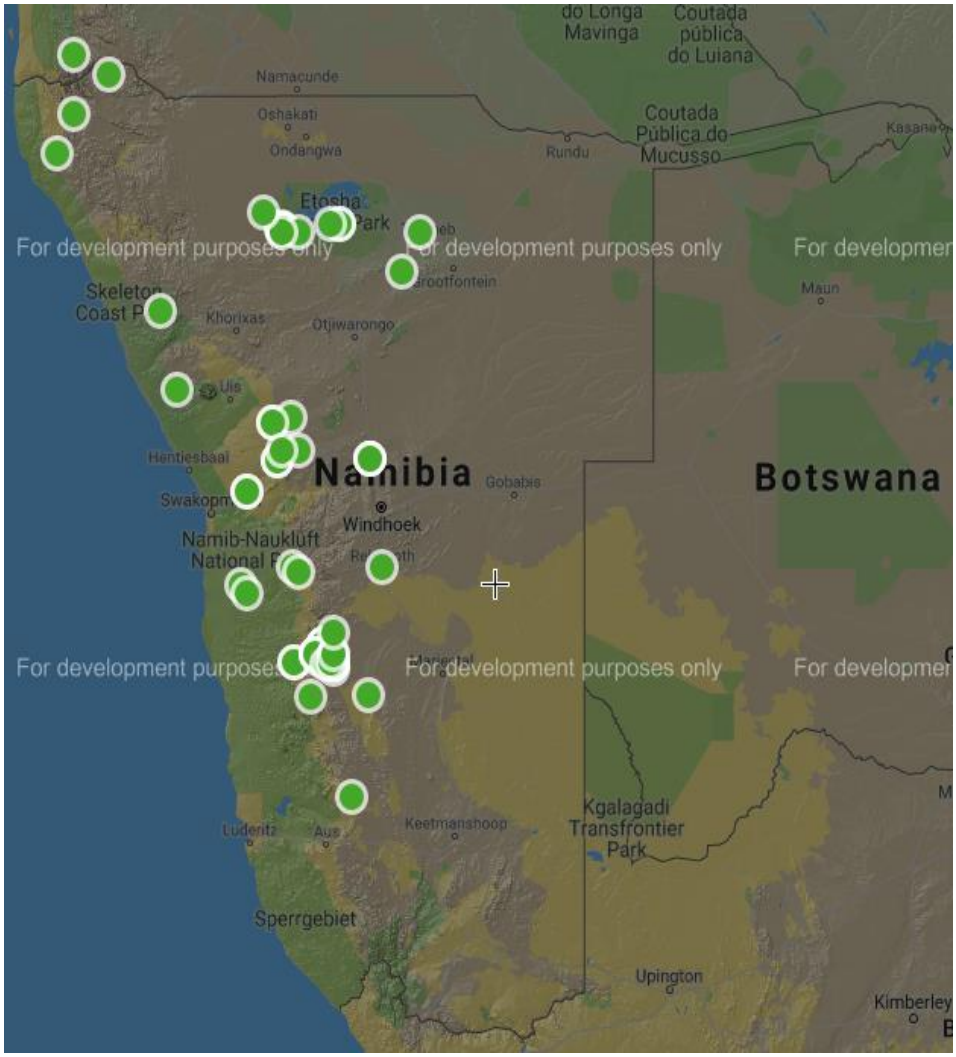


Figure 3: The distribution map of *Moringa ovalifolia* in the north western side of Namibia and in the southern part of Angola was generated using GeoCAT.

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The GeoCAT analysis of *Moringa ovalfolia* per km^2, which uses data from Global Biodiversity Information Facility (GBIF), classifies it as a species of least concern (6,472,409.338 km^2) based on extent of occurrence (EOO) and Area of Occupancy176.000 km^2 (AOO). EOO is a parameter which is used to measure the spatial spread of the areas occupied by the taxon at a point in time, while the AOO is a

parameter that represents the area of suitable habitat occupied by a taxon at a point in time (IUCN Standard and petition Subcommittee 2017) This assessment method alone might not reflect the true status of *M. ovalfolia* because; it only considers its spatial occurrence while neglecting the extent of genetic variation. Without genetic studies, it is impossible to determine the extent of genetic variation between and within populations of *M. ovalifolia*. According to Arif and Khan (2009) populations with narrow genetic variations are at risk of extinction, as they have reduced ability to adapt to environmental changes. Furthermore, Okun et al. (2008) pointed out that knowledge about genetic variation of species is important in sampling strategies and breeding programmes. Muluvi *et al.* (1996) investigated genetic diversity among populations of *Moringa oleifera*. Their finding revealed significant differences among the populations. They emphasized the importance of genetic information in promoting germplasm conservation programs. Genetic studies especially on the least documented species such as *Moringa ovalifolia* are necessary to facilitate the development of breeding strategies that aim at optimizing secondary plant metabolite profile concentrations. The data generated from genetic studies would provide baseline information for developing best-practice strategies for the future. Information on genetic variation is necessary to select suitable breeding materials for obtaining superior hybrid offspring. Furthermore, such information can be used for germplasm improvement and innovation, and for reducing breeding time. Thus, understanding the genetic variation of different *Moringa ovalifolia* populations' germplasm resources is of great theoretical and practical value.

Genetic variation among and within populations have been studied using phenological, morphological, cytological, chemical, and molecular markers (El-Esawi et al., 2018).

Genetic variability assessment of breeding lines based on morphological and cytological approaches are not only time consuming but can also lead into an error at multiple level (Kumar et al., 2017). Molecular applications based on molecular markers has become a popular tool for evaluating genetic studies between and within populations beyond what can be ascertained from morphological evaluations (Liu et al. 2018; Lashkari et al., 2014). Understanding the patterns of population structure is essential for efficient germplasm organization. Characterization of the genetic diversity and phylogenetic relationships provides valuable information needed to broaden the narrow genetic base as well as enhance breeding and conservation strategies for crop improvement.

2.4. Species concepts

Species are one of the basic units to compare in almost all fields of biology. For taxon delimitation, a species concept and the criteria to define the species boundaries are necessary. Various concepts of species are in use today but many of them are notably inconsistent in terms of biological diversity (Mayden, 1997). Apart from the morphological species concept, there is biological species concept (Danta-Torres, 2018), the evolutionary species concept (Wiley, 1978), and the phylogenetic species concept (Cracraft et al., 2000; Cracraft, 1997).

For instance, according to the lineage species concept, species diverge from each other due to multiple mechanisms, such as a disrupted gene flow, local adaptation, intrinsic reproductive isolation, and/ or introgression (De Queiroz, 2007). On the other hand, the phylogenetic species concept considers species that survived natural selection and descent to be identified based on their reciprocal monophyly and/or diagnosability

(Leliaert et al., 2014; Mayden, 1997). An attempted to reconcile those concepts and proposed that most contemporary species concepts share a common element in the conceptualization of what constitutes a species and their incompatibilities are often in the criteria used to determine species boundaries (De Queiroz, 2007).

Thus, the term "plant species" may carry different meanings for different botanists and it could be based on phenetic or phylogenetic concept, it is imperative for the authors to spell out which concept is being used (Aldhebiani, 2018). The term phenetic is applied to a classification system which relies on similarities between present properties of organisms with no consideration or references in how they possess them. Morphology, cytology, phytochemistry, anatomy, embryology and even some generic features are considered to be source data for phenetic analysis. On the other hand, when the relationship describes the pathways of ancestry (how the characters of organisms arose in evolution regardless their present day state) it is called cladistic which is the same as phylogenetics.

Every species spans a certain geographical range thus the ecological and evolutionary processes that limit the geographical range may be crucial for creating new species, particularly allopatric species (Wiens, 2007). Delimitating species based on morphological or breeding data is often problematic, and population genetics and phylogenetics should be ideally incorporated (Ma et al., 2017).

2.5. Delimitation of species boundary

Traditionally, species delimitation and taxonomic inquiry in the plant kingdom has predominantly been based on the use of morphology (Ruiz-Sanchez and Sosa, 2010). According to Steenkamp et al. (2018) the morphological character and character state discontinuities separating one species from another denote species boundaries. Most of our knowledge regarding the species boundaries is thus based on morphological data generated by using morphological characters, as phenotypic similarity has been the criterion used historically by taxonomists to group individuals into species (Duminil et al., 2012). These types of markers are subject to phenotypic plasticity and genetic variability. They can also fail to discriminate between morphologically similar species such as in cryptic species, which can be distinguished based only on molecular markers (Chen and Lui, 2014; Edwards et al., 2012; Yang and Rannala, 2010; Bickford et al., 2007; Whittall et al., 2004). Morphological analyses can also improperly subdivide species through inaccurate interpretation of natural phenotypic diversity over their distribution area such as ecotypes, and varieties; (Duminil and Di Michele, 2009). Furthermore, phylo-geographical studies have shown that species with wide distribution ranges often have genetically distinct groups that cannot be discriminated by morphology (Rastorgueff et al., 2014; Zhou et al., 2012). Thus, dependency on morphological characters may ultimately mislead phylogenetic inference.

However, the use of molecular applications together with morphological studies in recent years has provided more evidence for delimiting species (Ruiz-Sanchez and Sosa, 2010). Molecular markers provide insights into the genealogical descent of lineages and represent alternative and powerful tools for species delimitation (Sass et al. 2007). Hebert et al., (2003) introduced the concept of “DNA barcode” as a new approach to

taxon recognition. Genetic barcoding has been used as a method to assign unknown samples into existing species using a single-locus (Tautz et al., 2003). Ideally, each species would be identified by a single, unique Deoxyribonucleic acid (DNA)-tag also known as DNA barcode, which is defined as "short segments of DNA that can be used to uniquely identify an unknown specimen to species" (Sass et al., 2007). Ever since the introduction of DNA barcoding, several barcode regions have been used in molecular studies. These studies focus on coding and non-coding regions located primarily in the plastid genome (Fazekas et al., 2008).

When molecular markers are used, species delimitation could be assessed by the presence of a DNA barcoding gap between intra- and inter-specific genetic variation (Lahaye et al., 2008) and gene tree methods are used to infer phylogeny, whereby monophyly and branch support represent the diagnostic criteria for species delimitation (Fazekas et al., 2008). Although DNA barcoding represents a powerful approach (Hollingsworth et al., 2009; Lahaye et al., 2008), it has its own limitations such as the lack of DNA barcode divergence among closely related species (Muellner et al., 2011; Seberg and Petersen, 2009), inadequate taxonomy, incomplete lineage sorting (i.e. a gene tree does not always reflect the species tree) (Devos et al., 2003 Seberg and Petersen, 2009), hybridization (Dobes, Mitchell-Olds and Koch, 2004), and cytoplasmic capture (Tsitrone, Kirkpatrick and Levin, 2003).according to Ballardini et al. (2013), no locus (alone or in combination), has proven to be 100% efficient as universal DNA barcode in plants at the species level.

Delimiting species and clarifying the phylogenetic relationship among taxa remain necessary for enumerating and understanding the origins of biodiversity and

implementing conservation management strategies (Meyer et al., 2013; Fujita et al., 2012; O'Meara, 2010; Sites and Marshall, 2004; Wilson, 2003). Among procedures for establishing species boundaries is the novel method proposed by Wiens and Penkrot (WP) (2002), which uses morphological and molecular data and tree-based and character-based analyses simultaneously. The tree-based morphological analysis uses populations as terminals rather than individuals to avoid a biased treatment of the polymorphisms shared between populations as homoplasies rather than synapomorphies. The WP method utilizes DNA haplotypes in parsimony analysis assuming a phylogeny of non-recombining haplotypes which may show the focal species to be either exclusive or not exclusive. Furthermore, the character-based analysis in the WP method involves finding diagnostic character states that represent differences among the putative species. Sites and Marshall (2004) designated two broad categories for delimiting species boundaries: tree-based and non-tree-based method.

2.6. Molecular phylogenetics

The similarity of biological functions and molecular mechanisms in living organisms studied strongly suggests that all organisms on earth descended from a common ancestor i.e. the species are related to each other by virtue of having evolved from the same common (now extinct) ancestor (Singh, 2015; Dowell, 2009). Such a relationship of species is called phylogeny and its graphical representation is called a phylogenetic tree. Computational methods infer these ancestor-descended relationships from presently thriving species and reconstruct what their course of evolution might have been (Singh, 2015).

Molecular phylogenetics emerged in the early 20th century but did not begin in earnest until the 1960s, with the advent of protein sequencing, PCR, electrophoresis, and other molecular biology techniques. It has become an indispensable tool for genome comparisons. (Dowell, 2009; Yang and Rannala, 2012). It is used to classify meta-genomic sequences (Yang and Rannala, 2012; Brady and Salzberg, 2011) to identify genes, regulatory elements and non-coding RNAs in newly sequenced genomes (Yang and Rannala, 2012; Lindblad-Toh et al., 2011; Pedersen et al., 2006; Kellis et al., 2003); to interpret modern and ancient individual genomes (Gronau et al., 2011; Li and Durbin, 2011; Green et al., 2010); and to reconstruct ancestral genomes (Paten et al., 2008). The primary objective of molecular phylogenetic studies is to recover the order of evolutionary events and represent them in evolutionary trees that graphically depict relationships among species or genes over time (Dowell, 2009). This is an extremely complex process, further complicated by the fact that there is no one right way to approach all phylogenetic problems. Phylogenetic data sets can consist of hundreds of different species or genes, each of which may have varying mutation rates and patterns that influence evolutionary change (Dowell, 2009). Consequently, there are numerous evolutionary models and stochastic methods available. The optimal methods for a phylogenetic analysis depend on the nature of the study and data used.

Molecular phylogenetic methods have been practical for identification of independent evolutionary lineages especially the general lineage concept where species are defined as independently evolving metapopulation lineages (De Queiroz, 2007). The application of evolutionary species concept is one of the approaches to using the general lineage concept which often relies on molecular phylogenetics to identify species-level

divergences and linking them to criteria such as ecological and morphological differences (Engelbrecht et al., 2018).

Earlier classifications of the genus *Moringa* by Engler (1902) proposed that the 13 species be divided into infrageneric taxa to reflect their remarkable morphological diversity. As a result, seven of the eight species where placed into *EuMoringa* Section. That classification gave birth to a new section, which was called *Dysmoringa* to emphasize the unique flowers of *M. longituba*. After sometime, Verdcourt (1985; 1958) transferred the four bottle trees to Section *Donaldsonia* based on their morphological integration. Later, phylogenetic reconstructions of both *Donaldsonia* and *EuMoringa* by Olson (2002) resulted into the two sections being paraphyletic, while the *Dysmoringa* Section happened to be nested within the Section *EuMoringa* clade. Prior to Olson's study, Verdcourt (1985) had proposed the only phylogenetic hypothesis for the family *Moringa* based on his study of herbarium specimens. He hypothesized a monophyletic bottle tree clade which formed a sister group to the rest of the family, while the clade of slender trees formed a sister group to a clade of the tuberous species.

Due to the paraphyletic assemblage, the sectional division of the bottle trees was done away with. Consequently, the informal terms; bottle tree clade, slender tree clade and tuberous clade were adopted. Life form based grouping major groupings and the grouping based on wood anatomy that were done by Olson (2002) were reportedly congruent to the grouping recovered in phylogenetic analyses. Again, when life form groupings were superimposed on the phylogeny, the bottle trees formed a paraphyletic clade despite monophyly phylogeny observed by Verdcourt's (1985) earlier. The paraphyly was still observed by Olson (2002) when he reconstructed a phylogenetic

hypothesis of the genus *Moringa* based on the enzyme phosphoenolpyruvate carboxylase (PEPC), chloroplast locus (trnG), and the tandemly-repeated internal transcribed spacer ITS region, as well as a data set based on morphological characters.

2.7. Phylogenetic tree reconstruction

The phylogenetic tree construction helps us go back in time and develop a "hypothesis" of how life evolved from the single common ancestor. This hypothesis (a phylogenetic tree) is represented as a cladogram, a branching diagram (Singh, 2015). A phylogeny is a tree containing nodes that are connected by branches. Each branch represents the persistence of a genetic lineage through time, and each node represents the birth of a new lineage (Yang and Rannala, 2012). When a phylogenetic tree represents the relationship among species, the nodes represent speciation events. In a gene tree however, the nodes represent birth events of individuals who are ancestral to the sample, whereas in a tree of paralogous gene families, the nodes might represent gene duplication events (Yang and Rannala, 2012). Phylogenetic trees are not directly observed and are instead inferred from sequence or other data. Phylogeny reconstruction methods are either distance-based or character-based (Yang and Rannala, 2012).

Below is an outline of a step-by-step guide to phylogeny reconstruction as discussed by Harrison and Langdale (2006).

i) *Choice of data*:

No type of sequence is perfect for all purposes and the decision should be based on objective criteria (Barton et al., 2007). The dominant data source used in molecular phylogenetic reconstruction is sequence data, which can be collected in large amounts at low cost (Zhou et al., 2017; Felsenstein, 2004). Both amino acid and nucleotide data can be analyzed and used to generate a phylogeny (Simmons and Freudenstein, 2002). Some researchers prefer to use amino acid data to infer phylogeny due to easiness of aligning the sequence data and due to the fact that there are more possible character states for amino acids than for nucleotides (20 versus 4). It is further believed that the increased number of characters in nucleotide sequences can lead to better resolution of the tree. The DNA sequences are however, used for phylogenetic analyses of closely related species, because of more information at the DNA level when compared with the protein level, while the amino acid sequences are used for phylogenetic analyses of more distant relationships (Michu, 2007; Simmons and Freudenstein, 2002).

ii) Obtaining homologous Sequences:

Homologous sequences could be obtained by either sequence database searching or sequencing. Sequences of interest could be retrieved from databases such as GenBank nucleotide sequence database, The European Molecular Biology Laboratory (EMBL) Nucleotide Sequence database and DNA Data Bank of Japan (DDBJ) (Benson et al., 2018; Mashima et al., 2016; Barton et al., 2007). What is more, sequences retrieved from basic local alignment search tool (BLAST) searches could be used alongside generated sequences from one's own data to be used for phylogeny reconstruction. One however, has to be realistic when choosing the number of sequences retrieved from BLAST searches. It is advisable that one pays attention to an allocated e-score of each

retrieved sequence, which is an indication of the degree of similarity between the query sequence and the sequence retrieved. The closer the e-value is to 0, the higher the degree of similarity between the two sequences. The e-scores of those sequences can then be used as a guide for the cut-off point (Hall, 2001).

iii) Multiple Sequence Alignment:

In order to compare individual homologous positions found in different homologous sequences, sequence alignments needs to be made. Multiple sequence alignment is an essential tool in biological studies such as phylogenetic inference (Hall, 2008). Sequence alignment is achieved by assigning each sequence to a separate row in a matrix, and lining up homologous positions in different sequences in columns (Barton et al., 2007). The sequences in rows corresponding to the operational taxonomic units (OTUs), the columns corresponding to homologous traits, and the specific nucleotide base in each sequence being the character states (Barton et al., 2007). Sequence alignment can be achieved automatically or manually both of which has its own limitation. Automatic alignments may fail to correctly identify regions of conservation within a gene, whereas manual alignments allow this but are more labor-intensive. If one chooses to use manual alignment, Se-Al (Rambaut, 1996) and BioEdit (Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA) are examples of such programs, and the added advantage is that they both are freely available on the web. It is worth noting that alignments (either multiple sequence alignment or a pairwise alignment) between a query and a database represent a hypothesis and not truth (Barton et al., 2007).

2.8. Methods used to infer phylogenetic trees

After alignment, the data could then be subjected to phylogenetic analysis. Phylogenetic reconstruction methods are classified into four main categories based on their overall scheme as shown in **Table 2**. The type of phylogenetic analysis used will be determined by the length of computational time and the degree of rigor applied. Main analysis techniques are distance, parsimony and likelihood (including Bayesian analysis). All three can be performed using Phylogenetic Analysis Using Parsimony (PAUP) software (Swofford, 2003) that is available from Sinauer Associates Inc. Publishers, Sunderland, MA, USA.

Table 2: Presents molecular phylogenetic methods adopted from Barton et al. (2007)

Method	Description
Distance	The optimal tree is generated by first calculating the estimated evolutionary distance between all pairs of sequences. These distances are then used to generate a tree in which the branch patterns and lengths best represent the distance matrix.
Parsimony	Possible trees are compared and each is given a score that is a reflection of the minimum number of character state changes (e.g., amino acid substitutions) that would be required over evolutionary time to fit the sequences into that tree. The optimal tree is considered to be the one requiring the fewest changes (the most parsimonious tree).
Maximum likelihood	This method is similar to parsimony methods in that possible trees are compared and given a score. The score is based on how likely the given sequences are to have evolved in a particular tree given a model of amino

	acid or nucleotide substitution probabilities. The optimal tree is considered to be the one that has the highest probability of producing the observed data
Bayesian	Related to maximum likelihood methods, but attempts to infer the probability of trees themselves and not just the probability that a tree could produce the observed data. Current implementations of these methods search for distributions of trees with high probability by first starting with some assumptions about "prior" distributions of possible trees.

Distance Methods:

Distance-based methods rely on the calculation of genetic distances between each pair of sequences in a data set to generate a distance matrix from which phylogenetic trees could be constructed using a clustering algorithm (De Bruyn et al., 2014). Barton et al., (2007), earlier mentioned that, the principle that underlying the various distance methods of phylogenetic reconstruction is that on, organisms sharing a recent common ancestor should are more similar to each other than organisms whose last common ancestor was more ancient. Therefore, it should be possible to infer evolutionary relationships from the patterns of similarity among organisms. Distance methods such as neighbor joining (NJ) and minimum evolution are used to calculate pairwise distances between sequences, and group sequences that are most similar. The distance approach is quite versatile, computationally efficient and remarkably accurate (Pardi et al., 2016; De Bruyn et al., 2014). The method however, does not allow an analysis of which characters contribute to particular groupings. Thus, caution should be exercised when using the method for a final tree. Parsimony and likelihood could be used to supplement the

distance method as they have the potential to rigorously explore the relationship between the tree and the entities included.

Parsimony Methods: The principle known as Occam's (or Ockham's) razor states that, other things being equal, the simplest explanation—the most parsimonious one—should be chosen (Barton et al., 2007). Parsimony assumes that shared characters in different entities result from common descent and groups are built based on such shared characters. The simplest explanation for the evolution of characters is taken to be the correct or most parsimonious one. With multiple characters, different groupings may be equally plausible, or equally parsimonious, and therefore multiple trees are generated from which a consensus tree is derived that shows only topologies that are not contradicted in any of the initial trees. A majority rule consensus tree shows nodes that are consistent in half to all the most parsimonious trees and the percentage of trees in which a given topology exists is shown on the branches.

All parsimonious trees are considered equally good; however, if any tree contradicts the others the node in question is collapsed. Majority rule trees are not informative about phylogeny because all parsimonious trees reconstructed are considered equally good and the nodes of any one of them that contradicts the others should be collapsed. If the consensus tree is however unresolved, it means there is incongruence between initial trees, and thus it is likely that the data used to build the tree are phylogenetically uninformative.

Likelihood methods: In contrast to parsimony, maximum likelihood (ML) analysis compute the probability that a data set fits a tree derived from that data set, given a

specified model of sequence evolution. The analysis starts with a specified tree derived from the input dataset (for example a NJ tree) and swaps the branches on the starting tree until the tree with the highest likelihood score (i.e. the best probability of fitting the data) is gained. This score is a function both of the tree topology and the branch lengths. Likelihood analysis therefore allows an explicit examination of the assumptions made about sequence evolution. These methods are the most computationally demanding techniques for phylogenetic analysis. Likelihood methods produce a number of trees, one of which is usually found to be the most likely tree.

Phylogenic tree reconstruction methods can be evaluated objectively by using five (5) criteria (Barton et al., 2007):

(i) *Efficiency*: how fast each method performs remains a major concern because some of these methods are very computationally demanding. Both parsimony and likelihood methods need to scan through tree space; thus, they take much longer to process the same dataset than a distance method would. Likelihood methods are often slower due to their typically more complex calculations.

(ii) *Consistency*: Reliably to generate the correct tree from sufficient data is another criterion. An inconsistent method will frequently produce the wrong tree even if given infinite amounts of data.

(iii) *Chances of rejecting a wrong null hypothesis:* Statistical power of a method, relates to the chance that the null hypothesis will be rejected when that hypothesis is wrong.

(iv) *Robustness*: All phylogenetic methods make assumptions about the evolutionary processes that underlie the character changes being studied. Because the accuracy of

these assumptions is not always known, methods are also evaluated by comparing their degree of dependency on these assumptions (i.e., their robustness).

(v) *Falsifiability*: i.e., whether the results produced will allow determination of the objectives.

(vi) *Violation of evolutionary assumptions*: A method is evaluated whether it has violated the underlying evolutionary assumptions or not. This is especially important for methods that are not very robust.

Rooting phylogenetic trees

Generally, knowledge of evolutionary direction of change is fundamental to our understanding of evolutionary processes. Phylogenetic methods usually give rise to branching diagrams from which it is impossible to examine the direction in which traits change when they are not rooted. Thus, rooting trees are used to infer the direction of change when direct evidence of ancestor–descendant relationships is absent. Outgroup rooting (Maddison, Donoghue and Maddison, 1984) compares the character states in the group of interest (the in-group) with those in a group that is closely related to (the out-group). These differences are used to infer the direction of character change in the resultant tree.

There are two good ways to root molecular trees:

(i) Outgroup rooting (Maddison et al., 1984) compares the character states in the in-group with those in a group that is closely related to which is in the out-group. The differences are used to infer the direction of character change in the resultant tree.

(ii) Another way of rooting is to use duplicated genes, where sequences from one gene clade are used to root another (Simmons et al. 2000). Duplicate gene can reveal unexpected relationships amongst the species or genes in the main clades where there has previously been ambiguous rooting (Fournier and Gogarten, 2010; Mathews and Donoghue, 1999; Brown and Doolittle, 1995; Iwabe et al., 1989).

Statistical support for phylogenetic trees

To test the robustness of the data used for making phylogenetic trees, the bootstrap and jack-knife support values are used as quantifiers. Jackknife randomly delete half of the sites from the original sequences so that the new sequences will be half as long as the original. This resampling procedure typically will be repeated many times to generate numerous new samples. The jack-knife value shows the percentage of times that a clade appears when a specified percentage of characters are randomly removed from the data set and the analysis performed again (Farris et al., 1996). Reasonable support is roughly 70% and high support 95% (Felsenstein, 1985; Hillis and Bull, 1993). Common interpretation of bootstrap is the measures the probability of recovering a given data set for the same group if the population was to be re-sampled from scratch (Goloboff et al., 200).

The bootstrap value shows the percentage of times that a clade appears when individual characters in the data set are randomly removed and replaced with data from another character from the same data set, and the analysis performed again for a specified number of replications (Felsenstein, 1985). In ML analyses, tree support can be evaluated by bootstrapping (which is more time-consuming than with parsimony

analyses), and conflict between alternative tree topologies can be examined with less likely trees. In contrast, Bayesian analysis outputs both a tree and the support for that tree together. This is conceptually equivalent to ML with bootstrapping.

CHAPTER 3: MATERIALS AND METHODS

3.1. Research design

A total of 21 samples of *Moringa ovalifolia* leaves were collected from Halali, Uis, Sesfontein, Sprokieswoud (Etosha) and White lady lodge and were used in the present study. Genomic DNA was extracted from the sampled leaves and analyzed.

3.2. POPULATION

The main population of interest for the present study is the *Moringa ovalifolia* individuals in Namibia. This includes habitats such as Uis, Sesfontein, Etosha amongst others were *Moringa ovalifolia* occurs naturally. The different habitats have sub-populations (hereafter referred to as populations) of *M. ovalifolia* with varying morphological characters from each other.

3.3. SAMPLE

The 21 samples of *Moringa ovalifolia* were used in the present study. The samples were collected from Halali Resort area (HA1, HA2, HA5 and HA6), Uis (U1, U4, U5, U6, U7 and U11), White lady lodge (W1), Sprokieswoud, (Sp7) and Sesfontein (S1, S3, S4, S5, S6, S7 and S11). Samples were collected from different areas across their geographic ranges in order to address their morphological variations.

3.4 Genomic DNA extraction

Chemical materials: The chemicals used to isolate the total DNA from leaf samples: CTAB

(/chloroform-isoamyl alcohol protocol as described by Doyle (1991)

Chemicals for electrophoresis on Agarose gel: 2% Agarose, 1 kb DNA ladder, ethidium bromide.

The primers: The ITS4 (5´ TCCTCCGCTTATTGATATGC 3´) and ITS (5 ´ GGAAGTAAAAGTCGTAACAAGG 3´) primers used in the present study were sourced from Inqaba Biotechnical Industries (Pty) Ltd.

Symbols of these samples were taken in accordance with their abbreviations and localities from which they were collected from (i.e. HA-Halali Resort area, SES-Sesfontein, U- Uis and W-White lady lodge).

To lyse the cell wall, individual silica gel dried leaf tissues sample of *Moringa ovalifolia* was ground to fine powder using a pestle and a mortar that had been decontaminated (by autoclaving at 121 °C, 1.5psi for 20 minutes) prior to each use. Nuclear genomic DNA was extracted from ground tissue using the CTAB (cetyl trimethylammonium bromide)/chloroform-isoamyl protocol as described by Doyle (1991). For each sample, about 50 mg of ground tissue was transferred to 1.5 ml Eppendorf tube. An amount of 500µl CTAB buffer [1M Tris, pH8.0, 5M NACLl 0.5M EDTA and CTAB buffer (concentration of 20g/l)] was added to the content in the Eppendorf to solubilize the plant cell wall and lipid membranes of the internal organelles, to denature proteins and bind the polysaccharides. After addition of CTAB buffer, the solutions were incubated at 55 °C for 60 minutes to lysis cells in the suspension, digest proteins and suspends lipids. Thereafter, 500µl of 24:1 chloroform-isoamyl alcohol was added to each tube containing the lysates to denature the proteins and facilitates the proper separation of aqueous and

organic phase then mixed by hand shaking the tube gently to form an emulsion. The Eppendorf tubes were then centrifuged for 10 minutes at 13 000 revolutions per minute (rpm) for the content to form three layers. The aqueous phase (top layer) containing DNA was collected and transferred into 1.5 ml Eppendorf tubes, leaving behind the lower layers containing the cell debris. 0.08 volumes of cold 7.5M ammonium acetate was added to precipitate the DNA and mixed by inverting. The tubes were then kept in the freezer overnight and centrifuged the next morning at 13 000 rpm for 3 min. The supernatant was pipetted off from the tubes leaving the DNA pellet in the tube. A volume of 700µl of cold 70% ethanol was added to precipitate the DNA, the tubes were inverted once to mix and centrifuged at 13 000 rpm for 1 minute. The DNA pellet was collected by pipetting the supernatant out and left to dry by inverting the tube on a towel paper. Upon drying, the DNA pellet was re-suspended in 100µl double distilled water and stored in the freezer at -20 °C until use. Prior to storage, the concentration, purity and integrity levels of total DNA were determined by using A NanoDrop 2000c spectrophotometer (Thermo Scientific Massachusetts, USA) at a wavelength of 260 nm and electrophoresis methods on 2% agarose gel.

3.5 ITS amplification protocol

The extracted genomic DNA was diluted accordingly to a ratio of 1:100 with TE buffer in micro cuvettes. The diluted DNA samples were amplified in 50µl Polymerase chain reaction (PCR) volumes using ITS4 and ITS5 primers. PCR reaction mixture contained 4µl template DNA, 2µl of 0.5 µM concentration of ITS4, 2µl of 0.5 µM concentration of ITS5 primer, 17 µl of nuclease free water and 25 µl of 2x Dream Taq master mix (the Dream Taq master mix is made of Taq DNA polymerase, 2x Dream Taq buffer, dATP, dCTP, dGTP, dTTP of 0.4 mM each and 0.4 mM of MgCl2). The PCR reaction profile

consisted of initial denaturation temperature of 95°C for 3 minutes followed by 35 cycles of denaturation temperature at 95°C for 30 seconds, annealing temperature of 60°C for 30 seconds, and extension temperature at 72°C for 60 seconds. Final extension was carried out at 72°C for 10 minutes followed by holding of PCR products at 4°C. A 2% agarose gel was then prepared in order to visualize the PCR products and determine the success of the amplification. About 10 μg/ML of ITS amplicons from each sample were sent to Inqaba Biotechnical Industries (Pty) Ltd for sequencing.

3.6. Data collection

The raw sequences were manually cleaned by editing the chromatograms using Chromas version 2.6.2. Editing was done by double clicking on respective chromatogram file to open the file in Chromas. The chromatogram were trimmed by deleting the nucleotides in the unclean region. The unclean region had baseline noise that looks like many peaks all jumbled together and occurred at the beginning and the end of each chromatogram. All chromatogram files were edited individually by making the edits as lower case bases. Once editing was completed, the sequences were saved as FASTA format. The FASTA formatted files were further edited in BioEdit (Biological Sequence Alignment editor for Windows 99/98/NT/2K/XP/7 sequence alignment editor software (Hall 1999)). Sequence alignment was performed using the on-line a multiple sequence alignment program, Multiple Alignment using Fast Fourier Transform (MAFFT) version 7 and phylogenetic analyses carried out by Molecular Evolutionary Genetics Analysis (MEGA 6) software.

3.7. Data analysis

3.7.1. DNA sequence generation:

The DNA sequence data from the Namibian *Moringa ovalifolia* were gathered experimentally. A targeted sequencing approach was done by using primers that targeted the nuclear ITS was during the PCR amplification. In addition, ITS sequences of thirteen (13) other species of *Moringa*'s ITS sequences were retrieved from National Center for biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/) using basic local alignment search tool (BLAST) and used alongside the 17 generated sequences for phylogeny reconstruction.

3.8. Multiple Sequence Alignment:

Multiple sequence alignment enables molecular analyses, especially in inferring phylogenetic relationships (Katoh et al., 2002). Multiple sequence alignment were done using the on-line MAFFT (Multiple Alignment using Fast Fourier Transform), a high speed multiple sequence alignment program.

3.9. Methods of sequence analysis: distance, parsimony and ML

The 17 edited sequences were then used to construct a phylogenetic tree in MEGA (Molecular Evolutionary Genetic Analysis) software version 6.0 (Tamura et al., 2013). The phylogenetic tree was resolved by using Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) and the bootstrap consensus tree was determined from 500 replicates which represented the evolutionary history of the taxa analyzed. A numerical cut-off (≥70%) was used to define support for "successful" resolution as a monophyletic species as suggested by Fazekas et al. (2008) and Hillis and Bull (1993). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed and initial trees for the heuristic search were obtained by

applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. All site function was used in the missing data treatment and the $1^{st}+2^{nd}+3^{rd}$ + Non-coding codons were included in the analysis.

3.10. Rooting tree

A single sequence of *Cylicomorpha parviflora* retrieved from (NCBI) Website with accession number (AF378579.1) was included in the analysis and used as the out-group to root the phylogenetic tree. *Cylicomorpha parviflora* belongs to family Caricaceae, a sister family to Moringaceae and the two families share many morphological features (Olson and Carlquist 2001). In addition, thirteen (13) other species of *Moringa*'s ITS sequences were retrieved from NCBI and used in combination with the 17 sample sequences. This was done in order to assess the degree of relatedness based on the ITS sequences amongst the *Moringa ovalifolia* samples and other germplasms.

3.11. Research Ethics

A clearance certificate from the Centre of Postgraduate Studies was obtained to clear the proposed study. An Ethical Clearance was obtained from the Centre for Research and Publications. The study was part of a project that was being carried out by the University of Namibia, Department of Biological Sciences. Thus, the Department was already in possession of a permit from the Ministry of Environment and Tourism to carry out research on *Moringa ovalifolia*.

CHAPTER 4: RESULTS

Species boundaries using nuclear sequences (ITS markers) in Namibian *Moringa ovalifolia* was investigated using samples from various populations where distinct morphospecies were found. A morphospecies is defined in this study as a taxonomic entity with particular diagnostic morphological characters (that enable its identification and classification), but for which reproductive isolation has not been demonstrated. The definition of phylogenetic species was adopted to delimit species boundaries. The results of the phylogenetic analyses of the samples (both sampled and retrieved) investigated in the present study as described below.

4.1. **Genomic DNA extraction**

Genomic DNA was extracted from silica gel dried *Moringa ovalifolia* leaf samples collected from five populations in the Namibian germ plasm. The CTAB/chloroform-isoamyl alcohol protocol as described by Doyle (1991) was followed for extraction. The concentration of the resulting genomic DNA was quantified using a nanodrop as shown in **Table 3**. After quantification, electrophoresis was carried out on 2% agarose gel stained with ethidium bromide. The DNA samples were then diluted accordingly to 10ng/ul.

Table 3: Shows the concentration of genomic DNA of Namibian *Moringa ovalifolia* samples measured quantified using a nanodrop at A260.

SAMPLE NO	SAMPLE IDENTITY	CONCENTRATION OF DNA (MG/ML)
1	Ha1	57.3

2	Ha2	50.4
3	Ha5	54.5
4	Ha6	50.8
5	Ses 1	78.6
6	Ses 3	105.8
7	Ses 4	35.9
8	Ses 5	46.7
9	Sp 7	35.0
10	U1	25.5
11	U4	27.8
12	U5	76.9
13	U6	81.6
14	U7	105.8
15	U11	97.9
16	U13	64.7
17	W1	78.6
18	OK1	13.4
19	Positive control	Not determined

4.2. ITS PCR amplification

A total of 26 samples were available and of these, only 18 were successfully amplified alongside the control, the rest were discarded due to contamination or failure to amplify with primers ITS4 and ITS5 using the previously mentioned reaction conditions. The

concentration of total DNA was determined by using A NanoDrop 2000c spectrophotometer at a wavelength of 260nm as shown in Table 3. The PCR products sizes ranged between 700 and 1000 base pairs. The amplicons were electrophoresed on 2% agarose gel.

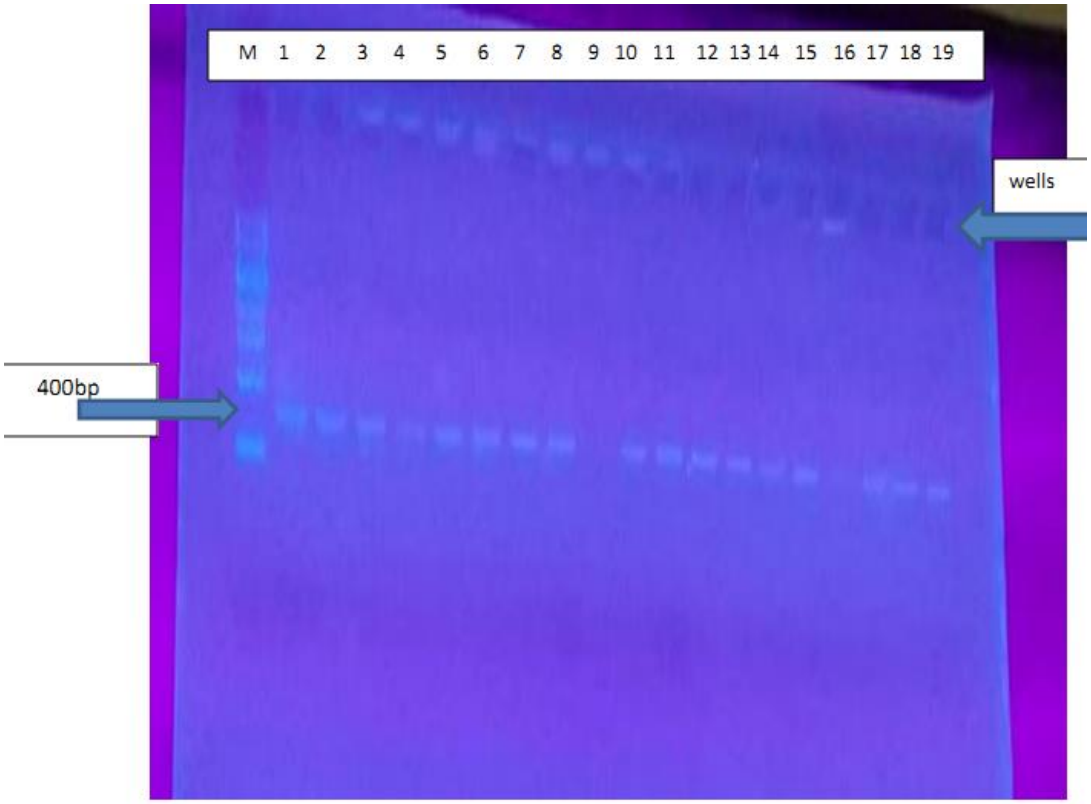

Figure 4: Shows electrophoresis 2% w/v gel showing PCR bands of Namibian *Moringa ovalifolia* samples amplified by ITS4 and ITS5.

4.3. Molecular phylogenetic analyses

Out of the 18 PCR samples under investigation were sent to Inqaba Biotechnical Industries (Pty) Ltd for sequencing, 17 reliable sequences were obtained and the identity of the sequence for 18th sample could not be resolved. The seventeen (17) *M. ovalifolia* genotypes where combined with thirteen (13) other *Moringa* species retrieved from

NCBI were used in the phylogenetic analysis. *Cylicomorpha parviflora* AF378579.1, also retrieved from NCBI was selected and used as an out group to root the phylogenetic trees. Overall character congruence was estimated by the consistency (CI) and the retention index (RI). Nodal support was estimated using 500 bootstrap pseudo replicates to infer the robustness of clades (Felsenstein, 1985) were calculated by performing a heuristic search using the TBR option with Multree on.

4.3.1 Maximum Parsimony method

The Maximum Parsimony phylogenetic analysis generated three large clades referred herewith in as Group I and Group II as shown in **Figure 5**. These groups represented samples under investigation and those retrieved from NCBI.

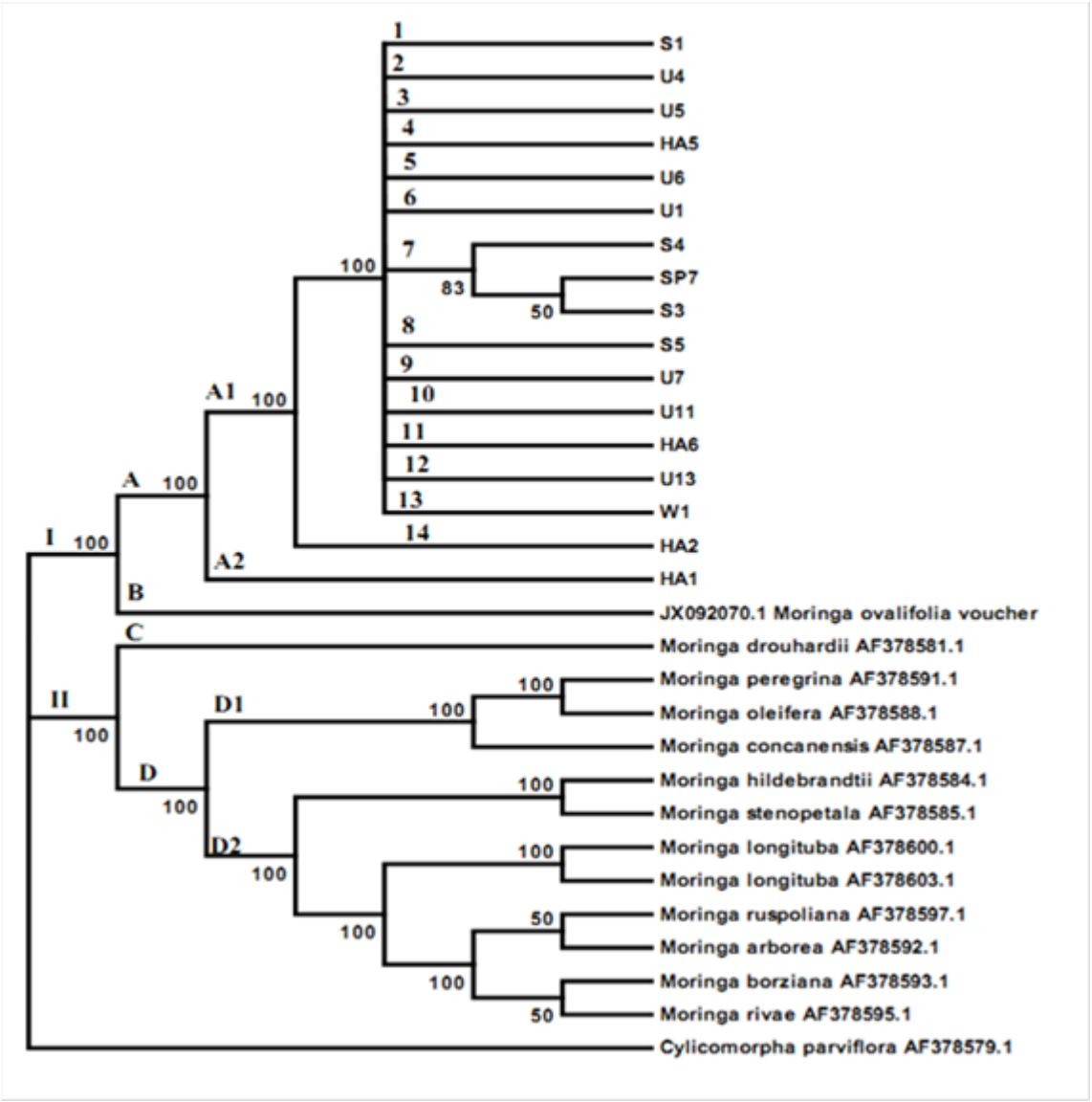

Figure 5: Shows the evolutionary history of the Namibian *Moringa ovalifolia* inferred using the Maximum Parsimony method. The consensus tree inferred from 6 most parsimonious trees is shown. Branches corresponding to partitions reproduced in less than 50% trees were collapsed. The consistency index is 0.867380 (0.820809), the retention index is0.965324 (0.965324), and the composite index is 0.837303 (0.792347) for all sites and parsimony-informative sites (in parentheses). The percentage of parsimonious trees in which the associated taxa clustered together are shown next to the

branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar, 2000) with search level 0 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 31 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were 1127 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

The samples under investigation together with the NCBI retrieved *Moringa ovalifolia* voucher JX092070.1 (Group I) formed a monophyletic clade. Two major divisions appear within group I referred to as sub-groups and labeled A and B with strong support (bootstrap value of 100%). Sub-group A was made up of all the samples under investigation which formed a sister clade to sub-group B (*Moringa ovalifolia* voucher JX092070.1). Within the samples under investigation (subgroup A), another split (bootstrap value of 100%) which formed sister clades A1 and A2 was observed whereby A1 nested all the other samples but HA1 which was then nested under A2. Sub-group A was further split into two clusters labeled A1 and A2 (bootstrap value of 100%). A2 clade was formed by only one sample (designated 15) which was a sister to clade A1 under which the rest of the samples (designated 1-14). A sub-cluster (designated 7) was further split into two smaller clusters with fair support (bootstrap value 83%) made up of two Sesfontein samples (S3 and S4) and the Sprokieswoud sample (SP7).

Group II was made up of two strongly supported monophyletic clades (bootstrap of 100%) comprising of all the other NCBI retrieved *Moringa* species. The major division within group II are referred to as Sub-group C and sub-group D ((bootstrap of 100%). Sub-group C was made up of only one sample (*M. drouhardii* AF378581.1) while sub-

group D split into two clusters (bootstrap of 100%) labelled D1 and D2. Cluster D1 was made up of the slender trees (*M. peregrina* AF378591.1, *M. oleifera* AF378588.1 and *M. concanensis* AF378587.1.).

Cluster D2 was made up of the bottle trees together with the tuberous shrub trees (*M. stenopetala* AF378585.1 *M. hildebrandtii* AF378584.1 *M. longituba* AF378600.1, *M. longituba* AF378603.1, *M. ruspoliana* AF378597.1, *M. arborea* AF378592.1, *M. borziana* AF378593.1 and *M. rivae* AF378595.1). The NCBI retrieved out group, *Cylicomorpha parviflora* AF378579.1 was nested on its own.

4.3.2. Maximum Likelihood method

The Maximum Likelihood method also grouped the samples into two main groups labelled group I and II ass shown in **Figure 6**. Group I was formed by a monophyletic clade of all the samples under investigation only. Sub-group A was further split into two clades labeled cluster A1 and A2. Cluster A1 nested all the 16 samples under investigation but HA1, which was the only sample under cluster A2.

Sub-group B nested the *M. ovalifolia* JX092070.1 which formed a sister clade with the samples under investigations. Group II nested only the NCBI retrieved sample whereby sub-group D1 was made up of only the slender trees (*M. peregrina* AF378591.1, *M. oleifera* AF378588.1 and *M. concanensis* AF378587.1), that formed a sister clade to a combination of bottle and tuberous shrub trees. One sub-cluster within D2 nested two monophyletic clades of the bottle (*M. stenopetala* AF378585.1 and *M. hildebrandtii* AF378584.1) and tuberous shrub trees (M. *longituba* AF378600.1, *M. longituba*

AF378603.1, *M. ruspoliana* AF378597.1, *M. arborea* AF378592.1, *M. borziana* AF378593.1 and *M. rivae AF378595.1*).

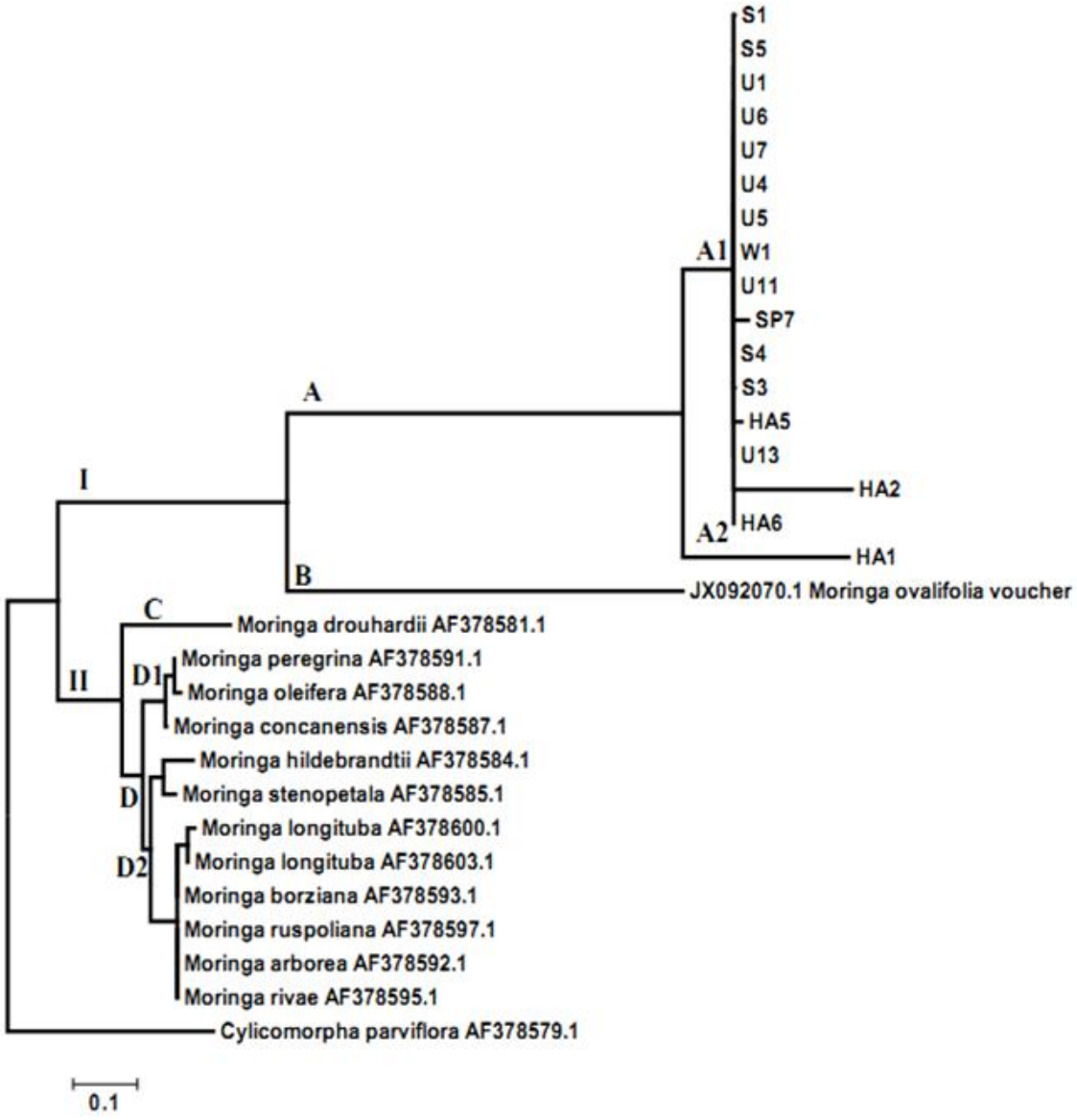

Figure 6: Shows the evolutionary history of Namibian *Moringa ovalifolia* inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-16381.6128) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join

and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 31 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were 1127 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

4.3.3. Neighbor-joining method

Similar to the Maximum Parsimony method, the Neighbor-joining method also divided the samples into two main clades labelled Group I nested only the monophyletic *Moringa ovalifolia* accessions (those under investigation and the NCBI retrieved *M. ovalifolia* JX092070.1). Group II nested all the other NCBI retrieved *Moringa* samples. Within the samples under investigation (Sub-group A, a further division (A1 and A2) was observed with strong support (bootstrap value 98%) where by one sample from Halali (HA1) formed a separate cluster, which was a sister clade (A1) to the rest of the samples that were nested in one clade (A1). Sub-cluster A1 was further split into two monophyletic clades of which one clade was made up of 15 samples while the other clade was only made up of once sample from Halali (HA2).

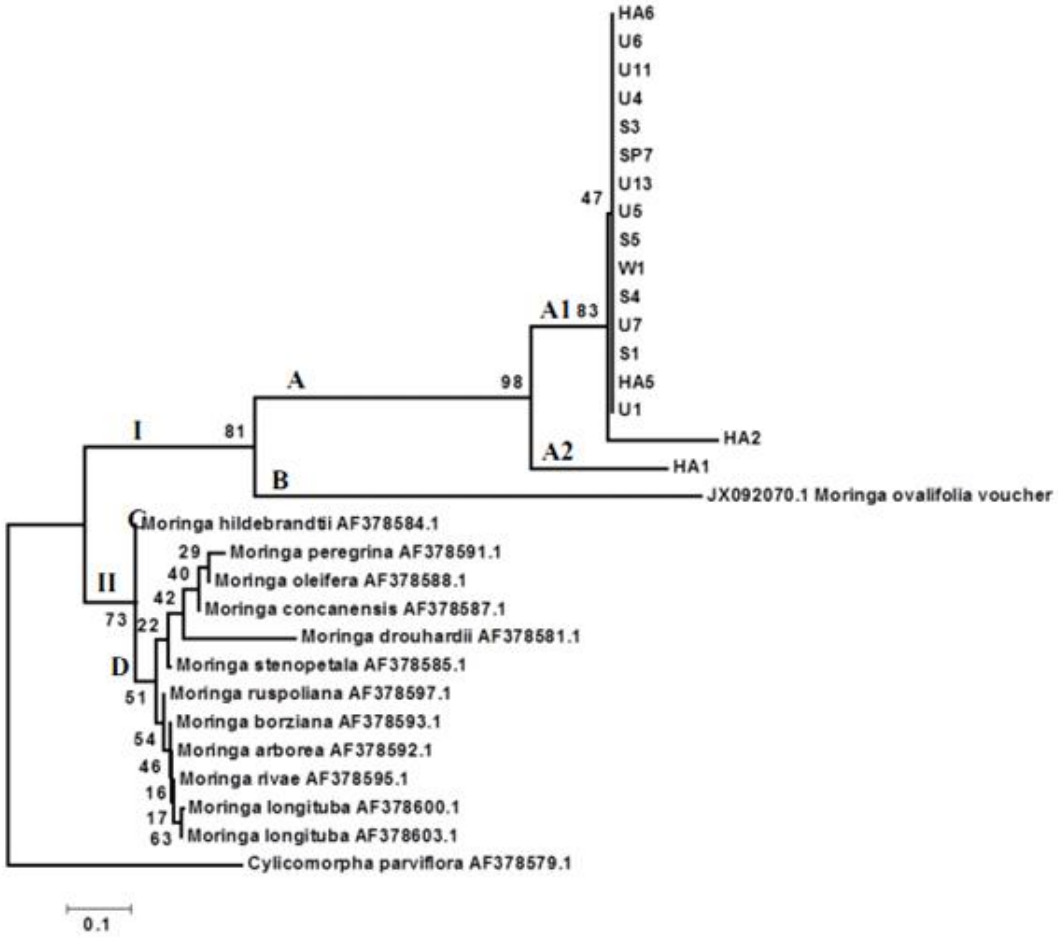

Figure 7: shows the evolutionary history of the Namibian *Moringa ovalifolia* inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 2.30639062 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). 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 Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number

of base substitutions per site. The analysis involved 31 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were 258 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

CHAPTER 5: DISCUSSION

In the present study, the nuclear ITS was used to assess the species boundaries of the *Moringa ovalifolia* using 17 samples collected from various populations in Namibia. The data that were collected from the samples used during this study together with other samples retrieved from NCBI were analyzed phylogenetically by using maximum parsimony, maximum likelihood and neighbor-joining methods. All the three methods that were used to carry out phylogenetic analyses resolved essentially identical topologies. The nuclear ITS phylogenetic trees that resulted from all the three methods of analysis provided strong evidence of phylogenetic relationships among the samples (both investigated and retrieved) investigated in the present study. The samples that were being investigated consistently grouped together with the NCBI retrieved *Moringa ovalifolia* voucher JX092070.1 to form a monophyletic clade with very strong support, suggesting that the investigated samples and *M. ovalifolia* voucher JX092070.1 evolved from a recent common ancestor. This observation further suggests that ITS region in *Moringa ovalifolia* is phylogenetically informative at the species level. Some of the slightly deeper nodes were poorly resolved on the Neighbor joining and Maximum Parsimony trees. Despite the monophyly of all the samples under investigation, paraphyly was particularly evident between the samples under investigation and the other *Moringa ovalifolia* sample which were retrieved from NCBI. The *M. ovalifolia* that was retrieved from NCBI was not nested together with other bottle trees but the Namibian samples under investigation. This observation was congruent with findings from a previous phylogeny reported by Olson (2002) where *Moringa ovalifolia* with other bottle trees formed a paraphyletic group. Olson (2002) reported these findings after

carrying out analysis on the genus *Moringa* using the ITS and the enzyme phosphoenolpyruvate carboxylase (PEPC). In Olson's observation, the bottle formed a non-monophyletic clade. The inconsistency in the observations between the present study and Verdcourt's (1985) could be attributed to the number of samples used for analysis. On the contrary, Verdcourt (1985), earlier observed monophyly in the grouping of bottles trees. Less coherence and incongruous was observed within Halali. Some samples from Halali formed sister clades with the rest of the samples under investigation while others from the same habitat were located on different branches displaying a paraphyly. Similarly, inconsistency was observed between the morphology and nesting of samples as some individuals samples from Sesfontein whose population was mostly dwarfs were nested with either those from Sprokieswoud or Uis whose population were made up of huge tall individuals. Adaptation to the respective environment and micro climate may have given rise to what Grandcolas et al. (2001) referred to as geographical taxonomic characters. Geographical taxonomic characters' development may occur during individual growth and development process and causes populations with similar genetic material may have different character appearance. The differences might not only be observed in different populations but also in different individual or even parts of the same individual (Laimeheriwa et al., 2018; Grandcolas et al., 2001). The incoherence in nesting of the various samples from different populations could also be a result of variations in individual sequences of respective samples resulting from insertion, substitution and/or deletion. Similar variations in ITS repeats amongst individuals were reportedly observed in Bromus (Poaceae) by Ainouche and Bayer (1997).One difference observed among the phylogenetic trees generated during the present was the swapping placement of *M. drouhardii* AF378581.1 and *M. hildebrandtii* AF378584.1 on the

Maximum Parsimony and Neighbor-Joining trees. On the Maximum Parsimony tree, *M. drouhardii* AF378581.1 was placed as a sister to the other sample retrieved from online while on the Neighbor-Joining tree, the place was held by *M. hildebrandtii* AF378584.1.

CHAPTER 6: CONCLUSION

The species boundaries of Namibian *Moringa ovalifolia* was determined by analyzing the nuclear ITS sequences. The present study has demonstrated that the Namibian *M. ovalifolia* populations group together with the NCBI retrieved *M. ovalifolia* voucher JX092070.1 sample as one species.

The nuclear ITS as a molecular character served as a good indicator of kinship amongst the Namibian populations of *Moringa ovalifolia.* It however did not demonstrate the ability of separating the different populations, thus having no taxonomic value in this regard. Understanding the limitations of the ITS as a molecular character is essential and fundamental for its wider use as a molecular taxonomic character. It is thus worth considering exploration of other molecular characters that can address the relationship between the different morphological characteristics relative to the different populations. Despite the inability of the nuclear ITS in separating the Namibian *Moringa ovalifolia* populations from each other, it was able to distinguish it from other African *Moringa* species. Because of its ability to delimit species boundaries, the ITS molecular marker could be considered when carrying out similar studies on other plant general. The nuclear ITS region might have potential as universal plant barcodes as alluded to by Kress et al. (2005). The present study successfully reconstructed the phylogenetic relationships among the different *Moringa ovalifolia* populations in Namibia by inferring to a relatively common ancestor.

The Namibian *Moringa ovalifolia* forms in the present study comprise a monophyletic lineage regardless of their morphological differences that could have arisen from environmental adaptation. The morphological differences observed among different

population implies potential adaptation to the environmental variable changes. The present study successfully reconstructed phylogenetic relationships between *Moringa ovalifolia* and other *Moringa* species. Furthermore, the hypothesis of phylogeny proposed by Olson (2002), was accepted as it also emerged in the present study that *Moringa ovalifolia* is as sister species to *M. stenopetala, M. drouhardtii* and *M. hildebrandtii*. From the results of this study, it can be concluded that the Namibian *Moringa ovalifolia* forms a 'good' taxonomic species.

CHAPTER 7: RECOMMENDATIONS

Since the species boundaries of the Namibian *Moringa ovalifolia* population that were investigated in the present study have been delimited using the nuclear ITS marker, further studies are required using other molecular characters. Further studies that pursue the use of other molecular markers that can link phenotypic traits related to yield parameters are needed to be integrated into a molecular map of *Moringa ovalifolia*. This present study could also guide breeding programmes that could help maintain genetic diversity and sustain long term selection of population with desired characteristics. Furthermore, this study could also be used as a baseline by other researchers to explore the genetic variability within and amongst the different populations that. Domestication and breeding programmes are required to fully reap the benefits of *M. ovalifolia*.

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CHAPTER 9: APPENDICES

APPENDIX A: Main ingredients for the CTAB protocol

Main ingredients for the CTAB protocol

CTAB: for 1L of CTAB buffer

100ml of 1 M Tris, pH 8.0

280 ml of 5 M NaC1

40 ml of 0.5 M EDTA

20 g of CTAB (Cety1 Trimethy1 Ammonium Bromide) bring to 1L with H2O

1M Tris, pH 8.0: for 1 L

121.1 g Tris

700 ml ddH2O

Preparation of CTAB Buffer

Dissolve Tris and bring to 900 ml.

PH to 8.0 with concentrated HC1 (will need – 50ml)

Bring to 1 L

0.5 M EDTA pH 8.0: for 1 L

186.12 g of EDTA

750 ml ddH2O

Add about 20 g of NaOH pellets

Slowly add more NaOH until pH 8.0; EDTA will not dissolve until the pH is near 8.0.

5 M NaC1: for 1 L

292.2 g of NaC1

700 ml ddH2O

Dissolve and bring to 1 L

7.5 M ammonium acetate: for 250 ml

144.5 g ammonium acetate

Bring to volume with ddH2O

APPENDIX B: Microwave instructions for agarose preparation of gel concentrations ≤2% w/v for electrophoresis

1. Choose a conical flask that is 2-4 times the volume of the solution.

2. Add room temperature 1X or 0.5X buffer to the beaker

3. Sprinkle the premeasured agarose powder

4. Heat the beaker in the microwave oven on HIGH power until bubbles appear.

CAUTION: Any microwaved solution may become superheated and foam over when agitated

APPENDIX C: *M. ovalifolia* ITS sequence alignments

Moringa ovalifolia internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: AY461551.1Length: 625Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
455 bits(246)	1e-123	350/401(87%)	3/401(0%)	+/_

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Query  15   CGCGTCTCGATCGATCGATCATGGGTCCATGAGCCACGAGCGAGCGACGCGCGCACGGTG  74
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  625  CGCGTCTCGATCGATCGATCATGGGTCCATGAGCCACGAGCGAGCGACGCGCGCACGGTG  566

Query  75   GAGCACGAGGCtttttttACCCCCGCCAAACCGTGACGCGGGGTCGCTCGGGGGTCGTT  134
            |||||||||||||||||||| ||||||| |||||||||| |||||||||| |||||
Sbjct  565  GAGCACGAGGCTTTTTTTTACCACCGCCAAGCCGTGACGCGGCGTCGCTCGGGGCTCGTT  506

Query  135  TTTAGGGCCACCGCGAGCCCAAAGGGCACGGGAGGGCAAAATccccccccgaggggccc  194
            |||||| | |||||||||| || | |||||||||| ||| |||| |||| |||||  ||
Sbjct  505  TTTAGGCCAACCGCGAGCCGAAGGCGCACGGGAGGCCAATATCCGCCCCGCGAGGTCCCT  446

Query  195  ccccacccccaaagggtgggggggaacgggggggacggggCTTTACACCCCAGG-AGAAGT  253
            |||| |||| || || |||||||| |||||| ||||| |||| |||||| ||| ||| ||
Sbjct  445  CCCCTACCCGAAGGGATGGGGGGGATCGGGGGCGACGGTGCTTGACACCC-AGGCAGACGT  387

Query  254  GCCCCCGGGCTGATGGGTTTGGGGGCAAATTGCGTTCAAAAAATCCATGGGTCACGGGAA  313
            |||| ||| ||||||| |||||| |||| |||||||||| | || |||| ||||||||
Sbjct  386  GCCCTCGGCCTGATGGCTTTGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGAT  327

Query  314  TCTGCAAATTACACCCAGGATCGCATTTCCCTACCTTCTTCATCGATGCGAAAGCCCAGA  373
            ||||||| | ||||| || |||||||||| |||| ||||||||||||||| |||| |||
Sbjct  326  TCTGCAATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGA  267

Query  374  TATCCGTTGTCCAAAATCATTTTAAAAATCATTTAAGGGAC  414
            |||||||||| | | |||||||| | |||||||| |||||
Sbjct  266  TATCCGTTGTCGAGAGTCATTTTAGACATCATTTA-GGGAC  227
```

Moringa ovalifolia internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: AY461551.1Length: 625Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1144 bits (619)	0.0	623/625(99%)	0/625(0%)	+/_

```
Query  2    CGCGTCTCGATCGATCGATCATGGGTCCATGAGCCACGAGCGAGCGACGCGCGCACGGTG  61
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  625  CGCGTCTCGATCGATCGATCATGGGTCCATGAGCCACGAGCGAGCGACGCGCGCACGGTG  566

Query  62   GAGCACGAGGCtttttttttACCACCGCCAAGCCGTGACGCGGCGTCGCTCGGGGCTCGTT  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  565  GAGCACGAGGCTTTTTTTTTACCACCGCCAAGCCGTGACGCGGCGTCGCTCGGGGCTCGTT  506

Query  122  TTTAGGCCAACCGCGAGCCGAAGGCGCACGGGAGGCCAATATCCGCCCCGCGAGGTCCCT  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  505  TTTAGGCCAACCGCGAGCCGAAGGCGCACGGGAGGCCAATATCCGCCCCGCGAGGTCCCT  446

Query  182  CCCCTACCCAAAGGGATGGGGGGATCGGGGGCGACGGTGCTTGACACCCAGGCAGACGTG  241
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  445  CCCCTACCCGAAGGGATGGGGGGATCGGGGGCGACGGTGCTTGACACCCAGGCAGACGTG  386

Query  242  CCCTCGGCCTGATGGCTTTGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGATT  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  385  CCCTCGGCCTGATGGCTTTGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGATT  326

Query  302  CTGCAATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGAT  361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  325  CTGCAATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGAT  266

Query  362  ATCCGTTGTCGAGAGTCATTTTAGACATCATTTAGGGACGACACGGCCACCCGTCGCGCA  421
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  265  ATCCGTTGTCGAGAGTCATTTTAGACATCATTTAGGGACGACACGGCCACCCGTCGCGCA  206

Query  422  CCGAGATCGGGGGCGACGAGGGCGTGCTCGTTCATTCGAGTAACTTGGGGCATCCCGCGCT  481
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  205  CCGAGATCGGGGGCGACGAGGGCGTGCTCGTTCATTCGAGTAACTTGGCGCATCCCGCGCT  146

Query  482  GTGGGTTTTGTTATTTCGCGCGACGAGGGGCTTGGACGTGAGCCCGCGCGCCCTTGCCGC  541
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  145  GTGGGTTTTGTTATTTCGCGCGACGAGGGGCTTGGACGTGAGCCCGCGCGCCCTTGCCGC  86

Query  542  GCGAGAGACGAGGGGCTCGAGAGCCCTCCGCCCCTCTAGTGTGTTTGAAACGAGTTCGCG  601
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  85   GCGAGAGACGAGGGGCTCGAGAGCCCTCCGCCCCTCTAGTGTGTTTGAAACGAGTTCGCG  26

Query  602  GGTCTGTTTCGCTGAGCGAGTTTTC  626
            |||||||||||||||||||||||||
Sbjct  25   GGTCTGTTTCGCTGAGCGAGTTTTC  1
```

Moringa ovalifolia internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: AY461551.1Length: 625Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1144 bits(619)	0.0	623/625(99%)	0/625(0%)	+/_

```
Query  57   CGCGTCTCGATCGATCGATCATGGGTCCATGAGCCACGAGCGAGCGACGCGCGCACGGTG  116
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  625  CGCGTCTCGATCGATCGATCATGGGTCCATGAGCCACGAGCGAGCGACGCGCGCACGGTG  566

Query  117  GAGCACGAGGCtttttttACCACCGCCAAGCCGTGACGCGGCGTCGCTCGGGGCTCGTT  176
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  565  GAGCACGAGGCTTTTTTTTACCACCGCCAAGCCGTGACGCGGCGTCGCTCGGGGCTCGTT  506

Query  177  TTTAGGCCAACCGCGAGCCGAAGGCGCACGGGAGGCCAATATCCGCCCCGCGAGGTCCCT  236
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  505  TTTAGGCCAACCGCGAGCCGAAGGCGCACGGGAGGCCAATATCCGCCCCGCGAGGTCCCT  446
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Query  237  CCCCTACCCAAAGGGATGGGGGGATCGGGGGCGACGGTGCTTGACACCCAGGCAGACGTG  296
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  445  CCCCTACCCGAAGGGATGGGGGGATCGGGGGCGACGGTGCTTGACACCCAGGCAGACGTG  386

Query  297  CCCTCGGCCTGATGGCTTTGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGATT  356
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  385  CCCTCGGCCTGATGGCTTTGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGATT  326

Query  357  CTGCAATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGAT  416
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  325  CTGCAATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGAT  266

Query  417  ATCCGTTGTCGAGAGTCATTTTAGACATCATTTAGGGACGACACGGCCACCCGTCGCGCA  476
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265  ATCCGTTGTCGAGAGTCATTTTAGACATCATTTAGGGACGACACGGCCACCCGTCGCGCA  206

Query  477  CCGAGATCGGGGCGACGAGGGCGTGCTCGTTCATTCGAGTAACTTGGGGCATCCCGCGCT  536
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  205  CCGAGATCGGGGCGACGAGGGCGTGCTCGTTCATTCGAGTAACTTGGCGCATCCCGCGCT  146

Query  537  GTGGGTTTTGTTATTTCGCGCGACGAGGGGCTTGGACGTGAGCCCGCGCGCCCTTGCCGC  596
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  145  GTGGGTTTTGTTATTTCGCGCGACGAGGGGCTTGGACGTGAGCCCGCGCGCCCTTGCCGC  86

Query  597  GCGAGAGACGAGGGGCTCGAGAGCCCTCCGCCCCTCTAGTGTGTTTGAAACGAGTTCGCG  656
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  85   GCGAGAGACGAGGGGCTCGAGAGCCCTCCGCCCCTCTAGTGTGTTTGAAACGAGTTCGCG  26

Query  657  GGTCTGTTTCGCTGAGCGAGTTTTC  681
            |||||||||||||||||||||||||
Sbjct  25   GGTCTGTTTCGCTGAGCGAGTTTTC  1
```

Moringa ovalifolia internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: AY461551.1Length: 625Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1136 bits(615)	0.0	620/625(99%)	0/625(0%)	+/_

```
Query  48   CGCGTCTCGATCGATCGATCATGGGTCCATGAGCCACGAGCGAGCGACGCGCGCACGGTG  107
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  625  CGCGTCTCGATCGATCGATCATGGGTCCATGAGCCACGAGCGAGCGACGCGCGCACGGTG  566

Query  108  GAGCACGAGGCttttttttACCACCGCCAAGCCGTGACGCGGCGTCGCTCGGGGCTCGTT  167
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  565  GAGCACGAGGCTTTTTTTTACCACCGCCAAGCCGTGACGCGGCGTCGCTCGGGGCTCGTT  506

Query  168  TTTAGGCCAACCGCGAGCCGAAGGCGCACGGGAGGCCAATATCCGCCCCGCGAGGKSCCT  227
            ||||||||||||||||||||||||||||||||||||||||||||||||||||  |||
Sbjct  505  TTTAGGCCAACCGCGAGCCGAAGGCGCACGGGAGGCCAATATCCGCCCCGCGAGGTCCCT  446

Query  228  CCCCTACCCGAAGGGATGGGGGGRTCGGGGGCGACGGTGCTTGACACCCAGGCAGACGTG  287
            |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  445  CCCCTACCCGAAGGGATGGGGGGATCGGGGGCGACGGTGCTTGACACCCAGGCAGACGTG  386

Query  288  CCCTCGGCCTGATGGCTTTGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGATT  347
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  385  CCCTCGGCCTGATGGCTTTGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGATT  326

Query  348  CTGCAATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGAT  407
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  325  CTGCAATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGAT  266

Query  408  ATCCGTTGTCGAGAGTCATTTTAGACATCATTTAGGGACGACACGGCCACCCGTCGCGCA  467
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265  ATCCGTTGTCGAGAGTCATTTTAGACATCATTTAGGGACGACACGGCCACCCGTCGCGCA  206

Query  468  CCGAGATCGGGGCGACGAGGGCGTGCTCGTTCATTCGAGTAACTTGGCGCATCCCGCGCT  527
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  205  CCGAGATCGGGGGCGACGAGGGCGTGCTCGTTCATTCGAGTAACTTGGCGCATCCCGCGCT  146

Query  528  GTGGGTTTTGTTATTTCGCGCGACGAGGGGCTTGGACGTGAGCCCGCGCGCCCTTGCCGC  587
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  145  GTGGGTTTTGTTATTTCGCGCGACGAGGGGCTTGGACGTGAGCCCGCGCGCCCTTGCCGC  86

Query  588  GCGAGAGACGAGGGGCTCGAGAGCCCTCCGCCCCTCTAGTGTGTTTGAAACGAGYTCGCG  647
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  85   GCGAGAGACGAGGGGCTCGAGAGCCCTCCGCCCCTCTAGTGTGTTTGAAACGAGTTCGCG  26

Query  648  GGTCTGTTTCGCTGAGCGAGTKTTC  672
            |||||||||||||||||||| |||
Sbjct  25   GGTCTGTTTCGCTGAGCGAGTTTTC  1
```

Moringa ovalifolia internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: AY461551.1Length: 625Number of Matches: 2

Score	Expect	Identities	Gaps	Strand
1147 bits(621)	0.0	623/625(99%)	0/625(0%)	+/_

```
ery  43   CGCGTCTCGATCGATCGATCATGGGTCCATGAGCCACGAGCGAGCGACGCGCGCACGGTG  102
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  625  CGCGTCTCGATCGATCGATCATGGGTCCATGAGCCACGAGCGAGCGACGCGCGCACGGTG  566

Query  103  GAGCACGAGGCtttttttACCACCGCCAAGCCGTGACGCGGCGTCGCTCGGGGCTCGTT  162
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  565  GAGCACGAGGCTTTTTTTTACCACCGCCAAGCCGTGACGCGGCGTCGCTCGGGGCTCGTT  506

Query  163  TTTAGGCCAACCGCGAGCCGAAGGCGCACGGGAGGCCAATATCCGCCCCGCGAGGTCCCT  222
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  505  TTTAGGCCAACCGCGAGCCGAAGGCGCACGGGAGGCCAATATCCGCCCCGCGAGGTCCCT  446

Query  223  CCCCTACCCGAAGGGATGGGGGGATCGGGGGCGACGGTGCTTGACACCCAGGCAGACGTG  282
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  445  CCCCTACCCGAAGGGATGGGGGGATCGGGGGCGACGGTGCTTGACACCCAGGCAGACGTG  386

Query  283  CCCTCGGCCTGATGGCTTTGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGATT  342
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  385  CCCTCGGCCTGATGGCTTTGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGATT  326

Query  343  CTGCAATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGAT  402
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  325  CTGCAATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGAT  266

Query  403  ATCCGTTGTCGAGAGTCATTTTAGACATCATTTAGGGACGACACGGCCACCCGTCGCGCA  462
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265  ATCCGTTGTCGAGAGTCATTTTAGACATCATTTAGGGACGACACGGCCACCCGTCGCGCA  206

Query  463  CCGAGATCGGGGCGACGAGGGCGTGCTCGTTCATTCGAGTAACTTGGCGCATCCCGCGCT  522
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  205  CCGAGATCGGGGCGACGAGGGCGTGCTCGTTCATTCGAGTAACTTGGCGCATCCCGCGCT  146

Query  523  GTGGGTTTTGTTATTTCGCGCGACGAGGGGCTTGGACGTGAGCCCGCGCGCCCTTSCCGC  582
            |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  145  GTGGGTTTTGTTATTTCGCGCGACGAGGGGCTTGGACGTGAGCCCGCGCGCCCTTGCCGC  86

Query  583  GCGAGAGACGAGGGGCTCGAGAGCCCTCCGCCCCTCTAGTGTGTTTGAAACGAGWTCGCG  642
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  85   GCGAGAGACGAGGGGCTCGAGAGCCCTCCGCCCCTCTAGTGTGTTTGAAACGAGTTCGCG  26

Query  643  GGTCTGTTTCGCTGAGCGAGTTTTC  667
            |||||||||||||||||||||||||
Sbjct  25   GGTCTGTTTCGCTGAGCGAGTTTTC  1
```

Moringa ovalifolia internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: AY461551.1Length: 625Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1155 bits(625)	0.0	625/625(100%)	0/625(0%)	+/_

```
Query  43   CGCGTCTCGATCGATCGATCATGGGTCCATGAGCCACGAGCGAGCGACGCGCGCACGGTG  102
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  625  CGCGTCTCGATCGATCGATCATGGGTCCATGAGCCACGAGCGAGCGACGCGCGCACGGTG  566

Query  103  GAGCACGAGGCtttttttACCACCGCCAAGCCGTGACGCGGCGTCGCTCGGGGCTCGTT  162
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  565  GAGCACGAGGCTTTTTTTTACCACCGCCAAGCCGTGACGCGGCGTCGCTCGGGGCTCGTT  506

Query  163  TTTAGGCCAACCGCGAGCCGAAGGCGCACGGGAGGCCAATATCCGCCCCGCGAGGTCCCT  222
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  505  TTTAGGCCAACCGCGAGCCGAAGGCGCACGGGAGGCCAATATCCGCCCCGCGAGGTCCCT  446

Query  223  CCCCTACCCGAAGGGATGGGGGGATCGGGGGCGACGGTGCTTGACACCCAGGCAGACGTG  282
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  445  CCCCTACCCGAAGGGATGGGGGGATCGGGGGCGACGGTGCTTGACACCCAGGCAGACGTG  386

Query  283  CCCTCGGCCTGATGGCTTTGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGATT  342
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  385  CCCTCGGCCTGATGGCTTTGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGATT  326

Query  343  CTGCAATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGAT  402
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  325  CTGCAATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGAT  266

Query  403  ATCCGTTGTCGAGAGTCATTTTAGACATCATTTAGGGACGACACGGCCACCCGTCGCGCA  462
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265  ATCCGTTGTCGAGAGTCATTTTAGACATCATTTAGGGACGACACGGCCACCCGTCGCGCA  206

Query  463  CCGAGATCGGGGCGACGAGGGCGTGCTCGTTCATTCGAGTAACTTGGCGCATCCCGCGCT  522
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  205  CCGAGATCGGGGGCGACGAGGGCGTGCTCGTTCATTCGAGTAACTTGGCGCATCCCGCGCT  146

Query  523  GTGGGTTTTGTTATTTCGCGCGACGAGGGGCTTGGACGTGAGCCCGCGCGCCCTTGCCGC  582
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  145  GTGGGTTTTGTTATTTCGCGCGACGAGGGGCTTGGACGTGAGCCCGCGCGCCCTTGCCGC  86

Query  583  GCGAGAGACGAGGGGCTCGAGAGCCCTCCGCCCCTCTAGTGTGTTTGAAACGAGTTCGCG  642
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  85   GCGAGAGACGAGGGGCTCGAGAGCCCTCCGCCCCTCTAGTGTGTTTGAAACGAGTTCGCG  26

Query  643  GGTCTGTTTCGCTGAGCGAGTTTTC  667
            |||||||||||||||||||||||||
Sbjct  25   GGTCTGTTTCGCTGAGCGAGTTTTC  1
```