# Genetic diversity of some *Moringa oleifera* Lam. cultivars available in South Africa

**by**

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# PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Genetics, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Durban, South Africa. The research was financially supported by the Agricultural Research Council.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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# ABSTRACT

*Moringa oleifera* Lam. (Moringaceae) is a nutritious and high medicinal value tree. The species is native to India but grown globally due to its properties. The nutritional content of the species includes high levels of vitamins, minerals, calcium, magnesium and iron.

It is now known that different cultivars of *M. oleifera* are grown throughout the world depending on the intended products. However, in South Africa, there is limited knowledge on the genetic variation of the cultivars most farmers and researchers are growing. In this study, a partial sequence of chloroplast *atpB* gene was used to determine genetic diversity of *M. oleifera* cultivars from different regions across the world but grown for research at the Agricultural Research Council, Vegetable and Ornamental Plants campus in Roodeplaat, Gauteng Province, South Africa. Thirteen cultivars were collected, of which three were domesticated cultivars from South Africa, eight were from Thailand, and one cultivar each was from Taiwan and the United States of America. Each cultivar consisted of six replicates. DNA extraction, PCR and gel electrophoresis were performed at the University of KwaZulu-Natal whereas DNA sequencing was done at Inqaba Biotec laboratory. DNA sequence analysis was done using BioEdit, MegaX, POPArT and DnaSP softwares.

Based on the phylogenetic analysis, the average total length of the sequences was 404 nucleotides with the number of polymorphic segregating sites and mutation of 21 and 24, respectively. From the maximum likelihood tree and neighbour-joining tree, South African domesticated cultivars SH and Limpopo were more related to each other than to the domesticated cultivar, CHM (South African). The cluster which was well supported with 84% bootstrap value was between cultivars TOT 5077 (Thailand) and TOT 4100 (Taiwan). The polymorphic data indicated a nucleotide diversity of 0.01654 and average number of nucleotide difference (k) of 6.58095. The haplotype network also showed less genetic differences between the cultivars with some cultivars recorded as similar.

The low genetic diversity observed in these cultivars suggests that the cultivars might have originated from a common ancestor. However, further study is necessary by collecting more cultivars of *M. oleifera* South Africa and other parts of the world to get a clear view on the genetic diversity. The results from this study will be an addition to the already existing knowledge of *M. oleifera* available in the world and new knowledge on genetic variation

among South African cultivars, which may be relevant in cultivar development and conservation.

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# CHAPTER 1: Introduction

## 1.1 Background

*Moringa oleifera* Lam. is a tree found in many parts of the world but originated in India. It is rich in protein, calcium, potassium, magnesium, vitamins A, B6 and C as well as iron (Valdez-Solana et al., 2015, Isitua *et al*., 2015, Gupta *et al.*, 2018). Therefore, people use it for food as well as animal feed (Kholif *et al*., 2015, Sultana *et al*., 2015, Agashe *et al*., 2017, Voemesse *et al*., 2018). Other research revealed the success of using *M. oleifera* in fuel production (Fernandes *et al*., 2015), cosmetics (Adegbe *et al*., 2016), water purification (Tunggolou and Payus, 2017), and plant growth enhancement (Bashir *et al*., 2017, Muthalagu *et al*., 2018). One other important property of *M. oleifera* is its medicinal properties. Research reviews by Dixit *et al*. (2016) , Maizuwo *et al*. (2017) and Mishra *et al*. (2017), outline some of the well-known medicinal uses of *M. oleifera*, which include anticancer, antidiabetic, skin and eye infection treatment, reduction of cholesterol level, and fever among others. Cultivation of *M. oleifera* offers a solution for dealing with the increasing human population in the South Africa and the world. In South Africa, the tree is grown in Limpopo and Gauteng and KwaZulu-Natal (Pakade *et al*., 2013, Tiloke *et al*., 2013).

Genetic diversity studies are important for determining the variation within a species or between species or cultivars and also provides information for breeding programs to improve or develop new varieties. Genetic variation occur as a result of insertions, deletions, duplications or mutations in the DNA sequences (McClellan and King, 2010). The variation can be detected using molecular markers also referred to as genetic markers. Molecular markers, which are derived from chloroplast, mitochondria or nuclear DNA regions, have been designed to study genetic diversity between and within species. This include markers such as internal transcribed spacer (ITS), adenosine triphosphate synthase beta-subunit (*atpB*), restriction fragment length polymorphism (RLFP), simple sequence repeats (SSR), random amplified polymorphism DNA (RAPD) and amplified fragment length polymorphism (AFLP) (Govindaraj *et al*., 2015, Nadeem *et al*., 2018).

The genetic diversity of *M. oleifera* has been reported in several works. The first report of *M. oleifera* genetic diversity using SSR was by Wu *et al*. (2010), in which they developed 20 SSR markers and used them to study genetic diversity in *M. oleifera*. Shahzad *et al*. (2013) investigated the genetic diversity of *M. oleifera* cultivated in Pakistan using 19 SSR and partial

sequence of chloroplast *atpB* markers. The results showed a higher genetic diversity among the *M. oleifera* accessions. Ganesan *et al*. (2014) observed the same results when they investigated genetic diversity on Indian cultivars using 19 SSR markers. On the other hand, Rufai et al. (2013) and Popoola (2014) used 12 and five RAPD markers to determine the genetic diversity among *M. oleifera* accessions. The accessions used in Rufai et al. (2013) were collected from six different areas around the world while the five RAPD markers were based on accessions from nine states in Nigeria (Popoola, 2014). Both studies reported a high level of genetic diversity in the species.

Different cultivars or accessions are used to investigate genetic diversity that exists within *M. oleifera*. Some of the cultivars includes PKM-1, PKM-2,GKVK-1 from India (Saini et al., 2013), Tumu, , Pakistan Black, Pakistan White from Pakistan (Nouman *et al*., 2016), Lombardia, Michoacán, Coahuila from Mexico (Valdez-Solana *et al*., 2015) and TOT 4893, TOT 4951, TOT 4977 from Thailand as well as SH and CHM from South Africa (Makita *et al*., 2017). Other cultivars are shared among countries due to research efforts (Makita et al., 2017).

*Moringa oleifera* in South Africa, particularly Limpopo Province, is cultivated by both commercial and smallholder famers (Mabapa *et al*., 2017). Farmers in these regions grow *M. oleifera* plants to sell the seeds and leaves in order to generate income and also use it as food for animals and human beings as well as for medical purposes. However, farmers' complaints about the crop included infestation by spiders and presence of termites on the tree, which reduces the quality and yield of the crop. In particular, such pests have been reported to reduce production of leaves and seeds (Saha *et al*., 2014). It is therefore of utmost importance to have knowledge of the genetic diversity between South African *M. oleifera* cultivars to allow researchers and scientists to know of the variations among cultivars available in South Africa and in future enable them to improve the plant characteristics such as plant pest resistance.

## 1.2 Justification

*Moringa oleifera* could be a significant crop for South Africa due to the scarce medical accessibility and malnutrition that exists in the country. There is potential for commercial and smallholder famers in parts of South Africa cultivating the tree to increase production. This will also allow other provinces that do not grow the plant in South Africa to grow it. Even though, more research studies in South Africa focuses on the metabolites and the nutritional

content of the *M. oleifera* (Moyo *et al*., 2011, Fitri *et al*., 2015), it is also important that farmers are able to identify different cultivars of *M. oleifera* morphologically. Hassanein and Al-Soqeer (2018) and Islam *et al*. (2020) outlined some of the characters they used in their studies to determine the morphological difference between *M. oleifera* cultivars. Additionally, genetic diversity studies will be crucial for plant improvement and development of new cultivars. Hence the study focused on determining the genetic diversity of *M. oleifera* cultivars (Table 1) available in South Africa. The study will be contributing to closing the gap in knowledge about South African cultivars and building on the genetic diversity that already exists on *M. oleifera* elsewhere in the world.

## 1.3 Aims and objectives

The study aimed to evaluate the genetic diversity of *M. oleifera* cultivars available in South Africa and reconstruct their phylogenetic relationships with other selected cultivars. The study focused on cultivars available at the Agricultural Research Council's (ARC) Roodeplaat farm in Pretoria, and sourced locally, and from Thailand, Taiwan and the United States of America.

The objective of the study was:

- To determine the genetic diversity and phylogenetic relationships of *M. oleifera* cultivars.

Table 1: Origin of *M. oleifera* cultivars used in the study and grown at the ARC for research purposes

| Country of origin | Cultivar(s) |
|---|---|
| Thailand [World Vegetable Centre (AVRDC)] | TOT 4893, TOT 4951, TOT 4977, TOT 5028, TOT 5077, TOT 5169, TOT 5330 and TOT 7266 |
| South Africa | Silver Hill (SH), CHM and Limpopo |
| Taiwan | TOT 4100 |
| USA | TOT 4880 |

## 1.4 Outline of the dissertation

Chapter 1 contains the introduction of the dissertation. It has the background information, the justification, aims, and objectives and provides information on *M. oleifera* cultivars used for the study.

Chapter 2 presents the literature review of the study. It outlines the importance of the plant, its distribution and ecology, molecular markers and its application on plant breeding and lastly describe previous genetic and morphological studies on *M. oleifera*.

Chapter 3 focuses on the genetic diversity and phylogenetic study of *M. oleifera* from different ecological regions but available in South Africa. The *atp*B markers were used to determine the genetic diversity among *M. oleifera* cultivars.

## 1.5 References


ADEGBE, A., LARAYETAN, R. & OMOJUWA, T. 2016. Proximate analysis, physicochemical properties and chemical constituents characterization of *Moringa oleifera* (Moringaceae) seed oil using GC-MS analysis. *American Journal of Chemistry,* 6**,** 23-28.

AGASHE, J. L., MANWAR, S., KHOSE, K. & WADE, M. 2017. Effect of supplementation of *Moringa oleifera* leaf powder on growth performance of broilers. *Journal of Poultry Science and Technology,* 5**,** 28-34.

BASHIR, K., MUSA, D. & MOHAMMED, I. 2017. Exploring the potential of drumstick (*Moringa oleifera*) leaf extract as vegetative growth enhancer of guinea corn (*Sorghum bicolor* L.). *International Journal of Current Science and Studies,* 1**,** 9-12.

DIXIT, S., TRIPATHI, A. & KUMAR, P. 2016. Medicinal properties of *Moringa oleifera*: A review. *International Journal of Education and Science Research Review,* 3**,** 175-185.

FERNANDES, D. M., SOUSA, R. M., DE OLIVEIRA, A., MORAIS, S. A., RICHTER, E. M. & MUÑOZ, R. A. 2015. *Moringa oleifera*: A potential source for production of biodiesel and antioxidant additives. *Fuel,* 146**,** 75-80.

FITRI, A., TOHARMAT, T., ASTUTI, D. & TAMURA, H. 2015. The potential use of secondary metabolites in *Moringa oleifera* as an antioxidant source. *Media Peternakan,* 38**,** 169-175.

GANESAN, K. S., SINGH, R., CHOUDHURY, D. R., BHARADWAJ, J., GUPTA, V. & SINGODE, A. 2014. Genetic diversity and population structure study of drumstick (*Moringa oleifera* Lam.) using morphological and SSR markers. *Industrial Crops and Products,* 60**,** 316-325.

GOVINDARAJ, M., VETRIVENTHAN, M. & SRINIVASAN, M. 2015. Importance of genetic diversity assessment in crop plants and its recent advances: An overview of its analytical perspectives. *Genetics Research International,* 2015, 431487.

GUPTA, S., JAIN, R., KACHHWAHA, S. & KOTHARI, S. 2018. Nutritional and medicinal applications of *Moringa oleifera* Lam.—Review of current status and future possibilities. *Journal of Herbal Medicine,* 11**,** 1-11.

HASSANEIN, A. M. A. & AL-SOQEER, A. A. 2018. Morphological and genetic diversity of *Moringa oleifera* and *Moringa peregrina* genotypes. *Journal of Horticulture, Environment, and Biotechnology* 59**,** 251-261.

ISITUA, C. C., LOZANO, M., JARAMILLO, C. & DUTAN, F. 2015. Phytochemical and nutritional properties of dried leaf powder of *Moringa oleifera* Lam. from machala el oro province of Ecuador. *Asian Journal of Plant Science and Research,* 5**,** 8-16.

ISLAM, M., SHEIKH, A., WATERMAN, C. & HOSENUZZAMAN, M. 2020. Morphology, pod yield and nutritional quality of two cultivars of Moringa (Moringa oleifera) in Bangladesh. Indian Journal of Science and Technology, 13, 3725-3735.

KHOLIF, A., GOUDA, G., MORSY, T., SALEM, A., LOPEZ, S. & KHOLIF, A. 2015. *Moringa oleifera* leaf meal as a protein source in lactating goat's diets: Feed intake,

digestibility, ruminal fermentation, milk yield and composition, and its fatty acids profile. *Small Ruminant Research,* 129**,** 129-137.

MABAPA, M. P., AYISI, K. K., MARIGA, I. K., MOHLABI, R. C. & CHUENE, R. S. 2017. Production and utilization of Moringa by farmers in Limpopo province, South Africa. *International Journal of Agricultural Research,* 12**,** 160-171.

MAIZUWO, A. I., HASSAN, A. S., MOMOH, H. & MUHAMMAD, J. A. 2017. Phytochemical constituents, biological activities, therapeutic potentials and nutritional values of *Moringa oleifera* (Zogale): A review. *Journal of Drug Design and Medicinal Chemistry,* 3**,** 60.

MAKITA, C., MADALA, N., CUKROWSKA, E., ABDELGADIR, H., CHIMUKA, L., STEENKAMP, P. & NDHLALA, A. 2017. Variation in pharmacologically potent rutinoside-bearing flavonoids amongst twelve *Moringa oleifera* Lam. cultivars. *South African Journal of Botany,* 112**,** 270-274.

MCCLELLAN, J. & KING, M.-C. 2010. Genetic heterogeneity in human disease. *Cell,* 141**,** 210-217.

MISHRA, G., SINGH, P., SRIVASTAV, S. & VERMA, R. K. 2017. *Moringa oleifera*-An important medicinal plant: A review of its traditional uses, phytochemistry and pharmacological properties. *Der Pharmacia Lettre* 3**,** 141-164.

MOYO, B., MASIKA, P. J., HUGO, A. & MUCHENJE, V. 2011. Nutritional characterization of Moringa (*Moringa oleifera* Lam.) leaves. *African Journal of Biotechnology,* 10**,** 12925-12933.

MUTHALAGU, A., ANKEGOWDA, M., HOSAHALLI, J., BALAJI, R. & NARENDRA, C. 2018. Effect of natural growth enhancer on growth, physiological and biochemical attributes in black pepper (*Piper nigrum* L.). *International Journal of Current Microbiology and Applied Sciences,* Special Issue 6, 2857-2866.

NADEEM, M. A., NAWAZ, M. A., SHAHID, M. Q., DOĞAN, Y., COMERTPAY, G., YıLDıZ, M., HATIPOĞLU, R., AHMAD, F., ALSALEH, A. & LABHANE, N. 2018. DNA molecular markers in plant breeding: current status and recent advancements in genomic selection and genome editing. *Biotechnology & Biotechnological Equipment,* 32**,** 261-285.

NOUMAN, W., ANWAR, F., GULL, T., NEWTON, A., ROSA, E. & DOMÍNGUEZ-PERLES, R. 2016. Profiling of polyphenolics, nutrients and antioxidant potential of germplasm's leaves from seven cultivars of *Moringa oleifera* Lam. *Industrial Crops and Products,* 83**,** 166-176.

PAKADE, V., CUKROWSKA, E. & CHIMUKA, L. 2013. Metal and flavonol contents of *Moringa oleifera* grown in South Africa. *South African Journal of Science,* 109**,** 01-07.

POPOOLA, J. O. 2014. Genetic diversity in *Moringa oleifera* from nigeria using fruit morpho-metric characters & random amplified polymorphic DNA (RAPD) markers. *Covenant Journal of Physical and Life Sciences,* 1**,** 43-50.

RUFAI, S., HANAFI, M., RAFII, M., AHMAD, S., AROLU, I. & FERDOUS, J. 2013. Genetic dissection of new genotypes of drumstick tree (*Moringa oleifera* Lam.) using random amplified polymorphic DNA marker. *BioMed Research International,*2013,604598.

SAHA, T., NITHYA, C. & RAY, S. 2014. Integrated pest management approaches for the insect pests of moringa (*Moringa oleifera* L.). *Rashtriya Krishi,* 9 (2)**,** 33-35.

SAINI, R. K., SAAD, K. R., RAVISHANKAR, G. A., GIRIDHAR, P. & SHETTY, N. P. 2013. Genetic diversity of commercially grown *Moringa oleifera* Lam. cultivars from India by RAPD, ISSR and cytochrome P450-based markers. *Plant Systematics and Evolution,* 299**,** 1205-1213.

SHAHZAD, U., KHAN, M. A., JASKANI, M. J., KHAN, I. A. & KORBAN, S. S. 2013. Genetic diversity and population structure of *Moringa oleifera*. *Conservation Genetics,* 14**,** 1161-1172.

SULTANA, N., ALIMON, A., HUQUE, K., BABA, M. & HOSSAIN, J. 2015. Evaluation of Moringa foliage (*Moringa oleifera*) as goat feed. *Iranian Journal of Applied Animal Science,* 5**,** 865-871.

TILOKE, C., PHULUKDAREE, A. & CHUTURGOON, A. A. 2013. The antiproliferative effect of *Moringa oleifera* crude aqueous leaf extract on cancerous human alveolar epithelial cells. *BMC Complementary and Alternative Medicine,* 13**,** 226.

TUNGGOLOU, J. & PAYUS, C. 2017. *Moringa oleifera* as coagulant used in water purification process for consumption. *Earth Sciences Pakistan,* 1**,** 1-3.

VALDEZ-SOLANA, M. A., MEJÍA-GARCÍA, V. Y., TÉLLEZ-VALENCIA, A., GARCÍA-ARENAS, G., SALAS-PACHECO, J., ALBA-ROMERO, J. J. & SIERRA-CAMPOS, E. 2015. Nutritional content and elemental and phytochemical analyses of *Moringa oleifera* grown in Mexico. *Journal of Chemistry*, 2015.

VOEMESSE, K., TETEH, A., NIDEOU, D., N'NANLE, O., GBEASSOR, M., DECUYPERE, E. & TONA, K. 2018. Effect of *Moringa oleifera* leaf meal on growth performance and blood parameters of egg type chicken during juvenile growth. *International Journal of Poultry Science*, 17**,** 154-159.

WU, J.C., YANG, J., GU, Z.J. & ZHANG, Y.P. 2010. Isolation and characterization of twenty polymorphic microsatellites loci for *Moringa oleifera* (Moringaceae). *Horticultural Science,* 45**,** 690-692.

# CHAPTER 2: Literature review

## 2.1 Introduction

This chapter focuses on reviewing literature on the diversity of *Moringa oleifera*. *Moringa oleifera* is one of the most important species in the Moringaceae family because it can be grown under several conditions as compared to the other 13 species in the family. In Limpopo, South Africa, the farmers use *M. oleifera* to boost their immune system, to purify water and as feed for livestock (Mabapa et al., 2017). In Guinea Bissau, West Africa, the locals use the plant to improve breast feeding in women as well as to treat ailments such as eye infections, fever, asthma and regulating blood pressure (Bancessi *et al*., 2019). These uses indicate the importance of *M. oleifera* to human beings and livestock. Therefore, this chapter will outline the importance of *M. oleifera* in South Africa and beyond as well as the phylogenetic studies that have been carried out on the species, including the morphological diversity of the species.

## 2.2 Taxonomy

*Moringa oleifera* (Figure 1) is a deciduous tree that belongs to the Order Brassicales formerly known as Capparales, and monophyletic family Moringaceae (Fahey, 2005). The family Moringaceae consists of 13 species (Padayachee & Baijnath, 2012). The species include *Moringa oleifera*, *M. arborea*, *M. borziana*, *M. concanensis*, *M. drouhardii*, *M. hildebrandtii*, *M. longituba*, *M. ovalifolia*, *M. pygmaea*, *M. peregrina*, *M. ruspoliana*, *M. rivae* and *M. stenopetala*. *Moringa oleifera* is the most well-known species in the genus and family. Some of the scientific names that were used for this species are *M. pterygosperma* Gaertn, *Guilandina moringa* L., and *M. moringa* (L.). The species is commonly known as horseradish tree or drumstick tree, but the common name differs among countries. Periyakulum 1 and 2 (PKM 1,2), Bombay, Jaffna, Chavakacheri and Saijan are some of the common cultivars of *M. oleifera*. PKM 1,PKM 2, Bombay, Jaffna and Chavakacheri cultivars are known for quality and higher pod yield while Saijan is a good source of wood used in the pulp and paper (Ciju, 2019). *Moringa oleifera* has been reported to be phylogenetically related to *M. concanensis* and *M. peregrina* than other Moringa species. Olson (2002) showed that the three species were related using three molecular loci and morphological characters. Hausiku *et al*. (2020) observed the same result when he used the nuclear ribosomal DNA data. *Moringa peregrina*, just like *M. oleifera*, is also one of the most studied species belonging to the Moringaceae family.

Figure 1: One of the 13 *Moringa oleifera* cultivars grown at the Agricultural Research Council's Vegetable and Ornamental Plants Institute at Roodeplaat Farm in Pretoria, South Africa. Source: T. Makgolane

## 2.3 Distribution and habitat

*Moringa oleifera* is native to sub-India (Usman *et al*., 2012), but has been introduced to many countries due its medicinal and nutritional properties. The plant is now tolerated in many countries as shown on Figure 2 (Leone *et al*., 2015). In Africa, the tree is widespread and many of the countries that cultivate it are poverty stricken (Lea, 2010). Therefore, growing this tree will help to reduce malnutrition as well as eradicate poverty when farmers sell its products for income (Omotesho *et al*., 2013). The cultivation of *M. oleifera* has been reported in all provinces of South Africa but largely in Limpopo and North West provinces (Pakade *et al*., 2013, Tiloke *et al*., 2013).

Figure 2: The global distribution of *M. oleifera*. Although native to India, the tree is introduced and naturalised in other countries as shown on the map (http://bioweb.uwlax.edu/bio203/s2012/moldenha_kat2/habitat.htm).

*Moringa oleifera* can withstand high temperatures and it can be grown in a variety of soils (Nouman *et al*., 2014) . Moreover, it is resistant to drought and tolerant to pH between 5 and 9. Poor drained and waterlogged soils cause the roots of the plant to rot easily (Fuglie and Sreeja, 1999).

## 2.4 Cultivation and harvesting

Although *M. oleifera* grows in different climatic conditions, it still needs soils that are good for their growth and development to produce higher yields of leaves, seeds, flowers and fruits. *Moringa oleifera* can be grown from either stem cuttings, seedlings or direct seeding (Roloff et al., 2009). Stem cuttings are dried before they can be put into soil to avoid infection by micro- organisms. The seeds are grown in a way that seeds-eating animals such as mice cannot access them. The seeds and young seedlings are put directly into moist soil to stimulate good germination.

Eight months to one year after planting or seed sowing, a mature *M. oleifera* plant will produce flowers. The flowers are bisexual, having both female and male organs. This allows self- and cross pollination to occur. Pods or fruits will form a month or two months after pollination. Subsequently, mature pods will be harvested before the seeds inside fall out of the pods. Young leaves of *M. oleifera* are mostly used to obtain leaf extract to use as plant growth stimulator (Elzaawely *et al*., 2017) whereas older leaves are dried for powder, which is used as a tea or

spice (Kumssa *et al*., 2017b). All plant parts are used after harvesting and therefore it can be considered a highly beneficial crop.

## 2.5 Importance of *Moringa oleifera* as food and in medicine

*Moringa oleifera* is used worldwide for both food and for medicinal purposes. The plant has been reported to have high amounts of various minerals and vitamins (Yaméogo *et al*., 2011, Kwenin *et al*., 2011). All plant parts are used as either food and animal feed. In India, the leaves are used as vegetables (Kakengi *et al*., 2005), as a herbal infusion like tea, added to cookies or bread to increase the nutrition levels (Dachana et al., 2010) and in production of juices and beverages (Fahey, 2005). Feed made from leaves was reported to be more nutritious than other animal feeds. A study done in Tanzania showed that the use of *M. oleifera* leaves as goat feed increases the digestibility in goats compared to the sunflower seed-cake (Foidl *et al*., 2001, Sarwatt *et al*., 2002, Kakengi *et al*., 2005). The goat feed was reported to have high protein content, carotene and ascorbic acid. Contradictory results were observed by Moyo *et al*. (2012) when Xhosa lop-eared goats fed with dried leaves of *M. oleifera* were compared with those feed with sunflower cake animal feed. They observed no difference in the animal growth and the carcass weight. This might suggest that effect of the feed on the animal depends on the proportion of feed that is fed since the proportions used in the two examples above were different. The effect might also depend on the breed or the type of animal used.

All plant parts of *M. oleifera* have been reported to have been used for different purposes (Fahey, 2005, Mbikay, 2012, Jung, 2014). A review by Biswas *et al*. (2012) outlined some of the importance of *M. oleifera* leaf extract in reducing ailments such as gastric ulcer, edema and more. The rural areas in Uganda uses the leaves for treatment of ailments such as HIV-related symptoms, ulcers, flu and many other ailments (Verma *et al*., 2009). The leaves are also reported to have anti-inflammatory, anticancer and antimicrobial properties (Farooq *et al*., 2012). Extract from the roots are used to prevent damage to the liver in animals and human beings (Mishra *et al*., 2011). The extracts from this plant are still tested on laboratory rats to determine if there are more potential effects they may have and also on controlling microbes.

Other functions of the plant include; use of the leaf extract as a growth promoter (Phiri, 2010, Phiri and Mbewe, 2010, Basra *et al*., 2011), use of seed extract in water purification processes (Ali et al., 2010, Sotheeswaran et al., 2011, Mangale *et al*., 2012), cosmetics (Dubey *et al*., 2013) and as antimicrobial agent and biofungicide (Donli and Dauda, 2003), flower extracts used to make tea and cosmetics (Price, 2007) and more (Figure 3).

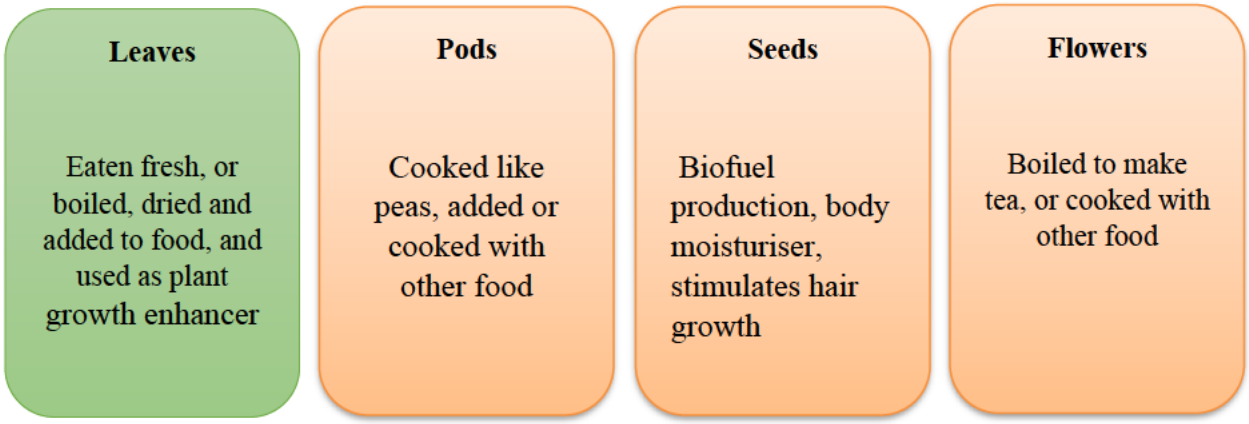


Figure 3: Uses of different parts of *M. oleifera* tree (Sandeep et al., 2019)

## 2.6 Morphological diversity

Morphology of the plant is important for identification of the species. The morphology of *M. oleifera* has been described before and some of the parts are shown on Figure 4. The leaflet of *M. oleifera* is ovate with reticulate venation and entire margins. The compound leaf of the plant is alternate and discolourous, green on the upper surfaces and whitish or discoloured on the bottom surface. The flowers are fragrant, white in colour and consist of five stamens and one ovary. The bark of young trees is green whereas old trees are whitish-grey.

Studies have been done to compare the morphology of *M. oleifera* grown in different geographical regions of same country. One such study was done by Zhigila *et al*. (2015) in Kwara State, Nigeria and it was observed that *M. oleifera* cultivars from the different regions differed morphologically. The leaves were either bipinnate or tripinnate, the seed shape ovate or isodiametric with a wing form which is prominent or less prominent and hairy or smooth leaf surface. Although differences were found between the cultivars, there were similarities observed such as entire leaflet margins, broader terminal leaflet and alternate leaves.

In another study done in India, morphological variations were observed in cultivars of *M. oleifera* across ecological regions (Ganesan *et al*., 2014). These morphological variations might be as a result of natural selection. Additionally, plants can change their phenotype to increase their chances of survival. This is termed phenotypic plasticity. Phenotypic plasticity is caused by environmental conditions such as water availability and temperature (Gratani, 2014).

A.  B  .C. 

Figure 4: Parts of *M. oleifera*; leaf (A), flower (B) and pods (C). Source: T. Makgolane

Another investigation by Abubakar et al. (2011a) on the morphological and anatomical diversity of *M. oleifera* cultivars in northern Nigeria showed variation between different cultivars. This including variations in leaf length, leaflet length and width, stomatal length and width, and epidermal wall pattern. This shows that there are high possibilities that *M. oleifera* cultivars grown in different region will show variation. Although studies have been successful in identifying species using their morphology and anatomy, researchers have found a better way to differentiate species using their molecular markers.

## 2.7 Molecular markers and their applications

Molecular markers also known as genetic markers are defined as a specific region of DNA that can be used to trace specific regions in the genome of a species. They are classified into three different groups; hybridisation, DNA chips/sequence based and PCR-based molecular markers (Kumar *et al*., 2009). The types of markers used in each category are shown in Table 2. The classification of the molecular markers is according to the dominance, detection and inheritance method (Nadeem *et al*., 2018).

Table 2: Some molecular markers used in plant breeding

| Categories of genetic/ molecular markers | Types of genetic/molecular marker |
|---|---|
| Hybridisation-based | Restriction Fragment Length Polymorphism (RFLP) |
| Biochemical | Protein markers |
| DNA chip (microarray)/Sequence-based | Single Nucleotide Polymorphism (SNP), Sanger sequencing, Next generation sequencing (NGS), Diversity Array Technology (DArT) |
| PCR-based | Random Amplified Polymorphism DNA (RAPD), Single Sequence Repeat (SSR), Amplified Fragment Length Polymorphism (AFLP) |

*2.7.1 Hybridisation-based markers*

Hybridisation-based marker, RFLP, was the first marker to be developed out of all markers known (Singh and Singh, 2015). It involves the use of restriction enzymes specific to a region in the DNA to differentiate between two DNA sequences from two species (Botstein et al., 1980). Restriction enzymes were first discovered in the 1980s in bacteria, where they were used to digest foreign DNA such as virus DNA. Some of the most well-known RFLP restriction enzymes includes *EcoRI*, *Hind III, Pst I, BamHI* etc (Zhanjiang, 2007). These restriction enzymes target different sites on the DNA and therefore produce different product size of the DNA fragments (Rasmussen, 2012). RFLP markers have been used successfully in research focusing on genetic diversity, and in comparison of performance in different cultivar crops belonging to the same species (Benchimol *et al*., 2000, Duval *et al*., 2001, Huang *et al*., 2002, Jordan *et al*., 2003, Sun *et al*., 2001). Advantages of the markers include their high stability as well as the ability to differentiate between co-dominance and dominance in diploid individuals. The disadvantage includes the requirement for sufficient and high quality DNA, which is sometimes difficult to obtain during DNA extraction (Asmelash *et al*., 2017, Salisu *et al*., 2018). Moreover, they do not record variations at all sites in the genome.

### *2.7.2 Biochemical markers*

Biochemical markers are markers that are able to detect variations between proteins or amino acids in different species. The markers are protein based. Some examples of protein-based markers include the isozymes and allozymes (Ismail *et al*., 2020). A review by Kumar *et al*. (2018) discusses some of the phylogenetic and genetic diversity studies on Chrysanthemum species using biochemical markers. Biochemical markers are co-dominant and cost effective but they are disadvantageous because they depend on the quality of the extraction method and the type of tissue the proteins are from (Nadeem *et al*., 2018). The less effective the extraction method, the more contamination is expected on the final product.

### *2.7.3 DNA chips/microarray*

DNA chips also known as DNA microarrays involve use of arrays to determine the concentration of nucleic acid in the solution and/or the expression levels of genes in a genome (Bumgarner, 2013). The method includes having probes specific to target DNA/gene/nucleotides being represented by each spot on the microarray. The DNA or RNA sample, which will be labelled with a fluorescent dye, will be added to the microarray and only nucleotides or sequence complementary to the probe will bind to the probe on the microarray. The scanner will detect the patterns of hybridisation by sensing the fluorescent on the array. The two markers that follow this method are the SNP and the DArT.

#### *2.7.3.1 Single Nucleotide Polymorphism (SNP)*

Single nucleotide polymorphism are single nucleotide variations that are observed between two or more species (Qi *et al*., 2018). These variations are caused by mutations, substitutions, deletions or insertions. Single nucleotide polymorphism has been used in genome diversity studies (Wang *et al*., 2014), and diseases and diagnosis (Mitchell and Mitchell, 2010, Wallentin *et al*., 2010). The marker is advantageous because it allows detection of all the base variation within the genome but this needs prior knowledge of the organism's genome. This restricts the use of the marker to only species that have already been sequenced.

#### *2.7.3.2 Diversity Array Technology (DArT)*

Similar to Single Nucleotide Polymorphisms, Diversity Array Technology allows detection of insertions, deletions and base changes (Abdel-Mawgood, 2012). It is different from SNP on the grounds that it does not require prior knowledge of the genome sequence. The markers can be used in large and complex genomes of crops such as sugarcane (Heller-Uszynska *et al*., 2011, Ovesná *et al*., 2013). DArT markers are frequently used in whole-genome genotyping, linkage mapping and genetic diversity studies. The technique have been used in species such

as rapeseed (Raman *et al*., 2011), *Eucalyptus* (Sansaloni *et al*., 2011), potatoes (Traini *et al*., 2013) and Nile tilapia (Lind *et al*., 2017).

### *2.7.4 PCR-based markers*

Polymerase Chain Reaction(PCR) is the process that involves amplification of a short DNA segment into thousands or millions of copies. The first step in the process is the denaturation process, where DNA is separated from the helix. The second step is the annealing of primers to the single strands and then finally is the extension of the strands. All steps are temperature sensitive so each step is done at a different temperature. The primers also referred to as markers are specific to a specific region of the DNA that is amplified. Most PCR- based markers commonly used in plant research are; AFLP, RAPD and SSR.

#### *2.7.4.1 Amplified Fragment Length Polymorphism (AFLP)*

Amplified Fragment Length Polymorphism is a PCR-based technique which involves DNA extraction, DNA fragmentation using restriction enzymes, ligation of adaptors to the restriction site, and lastly amplification of the adaptor sequence which will serve as primers for the PCR process that follows . The technique is used frequently in DNA fingerprinting and genetic diversity studies (Chial, 2008). Pecina-Quintero *et al*. (2014) used AFLP markers to determine the genetic diversity and population structure of physic nut (*Jatropha curcas*) plants in Mexico whereas Hoda *et al*. (2010) used the AFLP markers specific to albanian sheep to investigated the genetic diversity in three local sheep breeds in Albania . Different markers are designed for different species. Although AFLP markers have been reported successful in animal, plants and microbe studies, it still has drawbacks, which include the high cost of adaptors and difficulty to differentiate between heterozygotes and dominant homozygotes.

#### *2.7.4.2 Random Amplified Polymorphism DNA (RAPD)*

RAPD markers are DNA fragments that amplify randomly on the template sequence. They are dominant markers made up of approximately 10 nucleotides which can differentiate between species (Abubakar *et al*., 2011a). Although the markers can differentiate species, they cannot distinguish between dominant homozygotes and heterozygotes, and have low reproducibility (Bardakci, 2001) . RAPD markers are advantageous because no prior knowledge of the genome is required. This allows researchers to study the diversity of animals and plants of understudied species as well as the relatedness between those species.

#### *2.7.4.3 Single Sequence Repeats (SSR)*

Single Sequence Repeats (SSR) also known as microsatellites are repetitive tandem sequence repeats made of four to six repetitive bases in the genome. SSRs are mostly found in the

untranslated regions of the genome (Miyao *et al*., 1996, Gupta *et al*., 2010) but not the gene regions. This is because the repeats result from either strand slippage during replication or recombination errors (Marín-García, 2014, Vieira *et al*., 2016). Strand slippage occurs when there are two sequence repeats at different positions in the genome whereas recombination errors occur when there is either duplication or deletion during crossing over in the cell. On the hand, there has been reports of the presence of repeats in translated regions in the genome of some species but the repeats were in small quantities (Wang *et al*., 1994, Miyao *et al*., 1996). The markers are considered useful because of their conservation in related species (Asp et al., 2007).

Simple sequence repeats markers have been used in population genetic diversity, forensic genetics and phylogenetic relationship studies. This includes studies in medicinal plants such as *Chrysanthemum morifolium* (Feng *et al*., 2016), *Centella asiatica* (Rohini *et al*., 2019), and crops such as maize *Zea mays* (Synrem *et al*., 2017), rice *Oryza sativa* (Hue *et al*., 2018) and *M. oleifera* (Wu *et al*,. 2010).

## 2.8 Morphological and population genetic diversity of *Moringa oleifera*

The discovery of the qualities that *M. oleifera* possess opened a platform for studies on the plant. It is important that researchers know the different cultivars present in their own countries and around the world as well as variation between the cultivars. Both morphological and genetic studies were reported successful in diversity studied. The studies help in cultivar identification and breeding programs.

### *2.8.1 Morphological diversity of Moringa oleifera cultivars*

Morphological diversity of *M. oleifera* cultivars arises as a result of out-crossing or environmental conditions. The variability is observed between character states such as pods/fruit length, leaf size, seeds per fruit and flower colour among others. A few articles have been published on the morphological variations within *M. oleifera* cultivars. Mgendi *et al*. (2010) investigated the morphological variability between and within cultivated and non-cultivated *M. oleifera* from Tanzania. From their data, they were able to conclude that there were variations between the character states they investigated. Similar results were observed when Hassanein and Al-Soqeer (2018) researched the morphological diversity in seven genotypes or cultivars of *M. oleifera* grown in Saudi Arabia. Abubakar *et al*. (2011b) carried out another study to determine the variation between *M. oleifera* plants collected in different

states of the northern Nigeria and they observed variation as well. The morphological diversity studies are considered useful as they allow breeders or farmers to develop new varieties.

*2.8.2 Molecular markers used in population genetic diversity of* Moringa oleifera

Population genetic diversity studies involve two or three molecular techniques; DNA extraction, polymerase chain reaction (PCR) and/ or sequencing. DNA can be extracted from plants using DNA extraction kits or Cetyltrimethyl ammonium bromide (CTAB) method among other techniques. DNA extraction is followed by PCR, which entails amplifying a region of DNA through heating and cooling process (Lorenz, 2012). The amplification is achieved through three processes; the denaturation of double stranded DNA, annealing of primers and extension of the single strand DNA; which occurs at different temperatures (Clark and Pazdernik, 2013). The PCR products can then be viewed using gel electrophoresis or sequenced to determine relatedness or diversity between species (Hamza et al., 2004). All these techniques can be modified for high yield and quality DNA or PCR products (Schlink and Reski, 2002, Tilwari *et al*., 2016).

Genetic diversity of *M. oleifera* has been investigated around the world using markers made from nuclear, mitochondrial and chloroplast genes. One of the most investigated marker is the SSR markers. Twenty SSR markers have been developed by Wu *et al*. (2010) to study the genetic diversity in *M. oleifera* cultivars, genotypes or accessions. This includes the study by Shahzad *et al*. (2013) in Pakistan, Ganesan *et al*. (2014) and Rajalakshmi *et al*. (2017) in India, Popoola and Obembe (2013) in Nigeria among other published work. SSR markers are better markers to estimate the genetic diversity in *M. oleifera* because they are co-dominant and have high polymorphism and reproducibility compared to other markers.

Additionally, other chloroplast, nuclear or mitochondrial DNA can be used to infer genetic variations needed to construct phylogenetic relationship. DNA from the organelles have slow mutation rate and are conserved, therefore, they are reliable for phylogenetic studies. In plants, plastids and mitochondrial genes are used to determine genetic diversity between species. Some examples of the genes include *rbcL* (ribulose-1,5 bisphosphate large subunit), *matK* (maturase K), *atpB* (ATP synthase subunit beta), intergenic spacer *trnH-psbA* and ITS (internal transcriber spacer) gene (Alaklabi, 2015, Lee *et al*., 2018, Zhang *et a*l., 2018). The genes *rbcL*, *matK* and *atpB* are from chloroplast DNA. Ribulose-1,5 bisphosphate large subunit (*rbcL*) gene plays a role in carbon fixation and oxygenation processes (Xu and Tabita, 1996), *matK* is responsible for the splicing of group II intron (Selvaraj *et al*., 2008) and *atpB* gene synthesizes

ATP with the help of the membrane proton gradient. Unlike *rbc*L and *mat* K gene, *atp*B gene markers have been specifically developed for genetic diversity studies of *M. oleifera* cultivars (Kar *et al*., 2015). Hence, *atp*B gene marker would be a good choice for determining the genetic diversity of *M. oleifera*.

## 2.9 Methods used in phylogenetic reconstruction

The first step in phylogenetic reconstruction is to obtain sequence data, followed by alignment of the sequence to obtain a matrix that can be used for further examination. Phylogeny can be reconstructed using either distance-based or character-based methods (De Bruyn *et al*., 2014). Distance-based methods measure the pairwise distance between aligned sequence data whereas, character-based methods measure the differences between the sequences in the data (Sohpal *et al*., 2013). Examples of distance-based methods include the Unweighted Pair Group Method with Arithmetic Mean and the Neighbour-Joining whereas the character-based methods include Maximum Likelihood (ML) and Maximum Parsimony (MP).

All these methods have been used successfully in determining phylogenetic relationships in plants but for the current research, the ML will be used. The ML has been used in determining phylogenetic relationships in various plant species (Cron *et al*., 2012, Bird *et al*., 2017, Liede-Schumann *et al*., 2020). Cron *et al*. (2012) used the chloroplast DNA markers *matK, trnK* and *psb* to construct phylogenetic relationship in species belonging to the family Strelitziacea. Olson (2002) was successful in determining the phylogenetic relationship between species belonging to Moringaceae using the chloroplast sequence of trnG and DNA sequences derived from PEPC (Phosphoenolpyruvate carboxylase) and ITS. Patwardhan *et al*. (2014) reviewed various chloroplast and mitochondrial DNA markers used in both phylogenetic and genetic diversity studies. All these studies show the success of using chloroplast and mitochondrial DNA in genetic studies and phylogenetic reconstruction.

## 2.10 Conclusion

The review highlights the importance of *M. oleifera*, the molecular markers used in genetic diversity as well as previous studies on the morphological and genetic diversity of *M. oleifera*. *Moringa oleifera* is among some of the most important nutritional medicinal plants in South Africa. The high nutritional content and medicinal properties of the plant makes it valuable to conserve as it reduces the malnutrition and improve the health of the people in the country. Knowledge about the genetic diversity of *M. oleifera* will be useful in understanding variation of *M. oleifera* available in South Africa and will be useful in conservation strategies, and

breeding programs to improve characteristics of the plant. Hence, this study was done to extend the knowledge on the diversity of *M. oleifera* that already exists around the world, but the focus is on cultivars available in South Africa. At the time of this research, there was no published research on the diversity of *M. oleifera* of these cultivars available in South Africa.

## 2.11 References


ABDEL-MAWGOOD, A. L. 2012. DNA based techniques for studying genetic diversity. *Genetic Diversity in Microorganisms.* IntechOpen.

ABUBAKAR, B., MUARSQUO, S., KHAN, A. & ADAMU, A. 2011a. Morpho-anatomical variation in some accessions of *Moringa oleifera* Lam. from Northern Nigeria. *African Journal of Plant Science,* 5**,** 742-748.

ABUBAKAR, B., WUSIRIKA, R., MUAZU, S., KHAN, A. & ADAMU, A. 2011b. Detection of genetic variability using random amplified polymorphic DNA markers in some accessions of *Moringa oleifera* Lam. from northern Nigeria. *International Journal of Botany,* 7**,** 237-242.

ALAKLABI, A. 2015. Genetic diversity of *Moringa peregrina* species in Saudi Arabia with ITS sequences. *Saudi Journal of Biological Sciences,* 22**,** 186-190.

ALI, E. N., MUYIBI, S. A., SALLEH, H. M., ALAM, M. Z. & SALLEH, M. R. M. 2010. Production of natural coagulant from *Moringa oleifera* seed for application in treatment of low turbidity water. *Journal of Water Resource and Protection,* 2**,** 259-266.

ASMELASH, B., DIRIBA, S. & PAL, S. K. 2017. Molecular markers based characterization and conservation of wild animals. *Research Journal of Recent Sciences* 6**,** 53-62.

ASP, T., FREI, U. K., DIDION, T., NIELSEN, K. K. & LÜBBERSTEDT, T. 2007. Frequency, type, and distribution of EST-SSRs from three genotypes of *Lolium perenne,* and their conservation across orthologous sequences of *Festuca arundinacea, Brachypodium distachyon,* and *Oryza sativa*. *BMC plant biology,* 7.

BANCESSI, A., BANCESSI, Q., BALDÉ, A. & CATARINO, L. 2019. Present and potential uses of *Moringa oleifera* as a multipurpose plant in Guinea-Bissau. *South African Journal of Botany*,129,206-208.

BARDAKCI, F. 2001. Random amplified polymorphic DNA (RAPD) markers. *Turkish Journal of Biology,* 25**,** 185-196.

BASRA, S., IFTIKHAR, M. & IRFAN, A. 2011. Potential of *Moringa* (*Moringa oleifera*) leaf extract as priming agent for hybrid maize seeds. *International Journal of Agriculture and Biology,* 13**,** 1006-1010.

BENCHIMOL, L., DE SOUZA JR, C., GARCIA, A., KONO, P., MANGOLIN, C., BARBOSA, A., COELHO, A. & DE SOUZA, A. 2000. Genetic diversity in tropical maize inbred lines: Heterotic group assignment and hybrid performance determined by RFLP markers. *Plant Breeding,* 119**,** 491-496.

BIRD, K. A., AN, H., GAZAVE, E., GORE, M. A., PIRES, J. C., ROBERTSON, L. D. & LABATE, J. A. 2017. Population structure and phylogenetic relationships in a diverse panel of *Brassica rapa* L. *Frontiers in Plant Science,* 8, 1-12.

BISWAS, S. K., CHOWDHURY, A., DAS, J., ROY, A. & HOSEN, S. Z. 2012. Pharmacological potentials of *Moringa oleifera* Lam.: A review. *International Journal of Pharmaceutical Sciences and Research,* 3**,** 305-310.

BOTSTEIN, D., WHITE, R. L., SKOLNICK, M. & DAVIS, R. W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics,* 32**,** 314-331.

BUMGARNER, R. 2013. Overview of DNA microarrays: Types, applications, and their future. *Current Protocols in Molecular Biology,* 101**,** 1-17.

CHIAL, H. 2008. DNA fingerprinting using amplified fragment length polymorphisms (AFLP): No genome sequence required. *Nature Education,* 1**,** 176.

CIJU, R. J. 2019. *Moringa, the drumstick tree,* Agrihortico: Redefining food and agriculture.

CLARK, D. P. & PAZDERNIK, N. J. 2013. Chapter e6 - Polymerase Chain Reaction. *In:* CLARK, D. P. & PAZDERNIK, N. J. (eds.) *Molecular Biology (Second Edition).* Boston: Academic Press.

CRON, G. V., PIRONE, C., BARTLETT, M., KRESS, W. J. & SPECHT, C. 2012. Phylogenetic relationships and evolution in the Strelitziaceae (Zingiberales). Systematic Botany, 37, 606-619.

DACHANA, K., RAJIV, J., INDRANI, D. & PRAKASH, J. 2010. Effect of dried *Moring*a (*Moringa oleifera* Lam) leaves on rheological, microstructural, nutritional, textural and organoleptic characteristics of cookies. *Journal of Food Quality,* 33**,** 660-677.

DE BRUYN, A., MARTIN, D. P. & LEFEUVRE, P. 2014. Phylogenetic reconstruction methods: An overview. *Molecular Plant Taxonomy*. Springer.

DONLI, P. O. & DAUDA, H. 2003. Evaluation of aqueous *Moringa* seed extract as a seed treatment biofungicide for groundnuts. *Pest Management Science,* 59**,** 1060-1062.

DUBEY, D. K., DORA, J., KUMAR, A. & GULSAN, R. K. 2013. A multipurpose tree—*Moringa oleifera*. *International Journal of Pharmaceutical and Chemical Sciences,* 2**,** 415-423.

DUVAL, M.-F., NOYER, J.-L., PERRIER, X., D'EECKENBRUGGE, C. & HAMON, P. 2001. Molecular diversity in pineapple assessed by RFLP markers. *Theoretical and Applied Genetics,* 102**,** 83-90.

ELZAAWELY, A. A., AHMED, M. E., MASWADA, H. F. & XUAN, T. D. 2017. Enhancing growth, yield, biochemical, and hormonal contents of snap bean (*Phaseolus vulgaris* L.) sprayed with *Moringa* leaf extract. *Archives of Agronomy and Soil Science,* 63**,** 687-699.

FAHEY, J. W. 2005. *Moringa oleifera*: A review of the medical evidence for its nutritional, therapeutic, and prophylactic properties. Part 1. *Trees for Life Journal,* 1.

FAROOQ, F., RAI, M., TIWARI, A., KHAN, A. A. & FAROOQ, S. 2012. Medicinal properties of *Moringa oleifera*: An overview of promising healer. *Journal of Medicinal Plants Research,* 6**,** 4368-4374.

FENG, S., HE, R., LU, J., JIANG, M., SHEN, X., JIANG, Y., WANG, Z. A. & WANG, H. 2016. Development of SSR markers and assessment of genetic diversity in medicinal *Chrysanthemum morifolium* cultivars. *Frontiers in Genetics,* 7**,** 113.

FOIDL, N., MAKKAR, H. P. S. & BECKER, K. The potential of *Moringa oleifera* for agricultural and industrial uses. *In:* DE SAINT SAUVEUR ARMELLE, A. V. B. F., ed. International workshop on development potential for Moringa Products, 2001-10-29 / 2001-11-02 2001 Dar es Salam, Tanzanie. None: CIRAD, 1 Cd-Rom.

FOROUGHBAKHCH, R., FERRY SR, R., HERNÁNDEZ-PIÑERO, J., ALVARADO VAZQUEZ, M. & ROCHA ESTRADA, A. 2008. Quantitative measures of leaf epidermal cells as a taxonomic and phylogenetic tool for the identification of *Stanhopea* species (Orchidaceae). *Phyton (Buenos Aires),* 113.

FUGLIE, L. J. & SREEJA, K. 1999. Cultivation of moringa. *In:* FUGLIE, L. J. (ed.) *The Miracle tree. Moringa oleifera: Natural nutrition for the tropics.* Darkar, Senegal: Church World Service.

GANESAN, K. S., SINGH, R., CHOUDHURY, D. R., BHARADWAJ, J., GUPTA, V. & SINGODE, A. 2014. Genetic diversity and population structure study of drumstick (*Moringa oleifera* Lam.) using morphological and SSR markers. *Industrial Crops and Products,* 60**,** 316-325.

GRATANI, L. 2014. Plant phenotypic plasticity in response to environmental factors. *Advances in Botany,* 2014**,** 17.

GUPTA, S., SHUKLA, R., ROY, S., SEN, N. & SHARMA, A. 2010. *In silico* SSR and FDM analysis through EST sequences in *Ocimum Basilicum*. *Plant Omics,* 3**,** 121-128.

HAMZA, S., HAMIDA, W. B., REBAÏ, A. & HARRABI, M. 2004. SSR-based genetic diversity assessment among Tunisian winter barley and relationship with morphological traits. *Euphytica* 135**,** 107-118.

HAUSIKU, M. K., KWEMBEYA, E. G., CHIMWAMUROMBE, P. M., & MBANGU, A. 2020. Assessment of species boundaries of the *Moringa ovalifolia* in Namibia using nuclear its DNA sequence data. *South African Journal of Botany*, 131, 335-341.

HASSANEIN, A. M. A. & AL-SOQEER, A. A. 2018. Morphological and genetic diversity of *Moringa oleifera* and *Moringa peregrina* genotypes. *Journal of Horticulture, Environment, and Biotechnology* 59**,** 251-261.

HELLER-USZYNSKA, K., USZYNSKI, G., HUTTNER, E., EVERS, M., CARLIG, J., CAIG, V., AITKEN, K., JACKSON, P., PIPERIDIS, G. & COX, M. 2011. Diversity arrays technology effectively reveals DNA polymorphism in a large and complex genome of sugarcane. *Molecular Breeding,* 28**,** 37-55.

HODA, A., AJMONE-MARSAN, P., DOBI, P., BOZGO, V. & C ONSORTIUM, E. 2010. Genetic diversity in albanian sheep breeds estimated by AFLP markers. *Albanian Journal of Agricultural Sciences,* 9**,** 23-29.

HUANG, X., BÖRNER, A., RÖDER, M. & GANAL, M. 2002. Assessing genetic diversity of wheat (*Triticum aestivum* L.) germplasm using microsatellite markers. *Theoretical and Applied Genetics,* 105**,** 699-707.

HUE, H., NGHIA, L. T., MINH, H. T., ANH, L. H., TRANG, L. T. T. & KHANH, T. D. 2018. Evaluation of genetic diversity of local-colored rice landraces using SSR markers. *International Letters of Natural Sciences,* 67**,** 24-34.

JORDAN, D., TAO, Y., GODWIN, I., HENZELL, R., COOPER, M. & MCINTYRE, C. 2003. Prediction of hybrid performance in grain sorghum using RFLP markers. *Theoretical and Applied Genetics,* 106**,** 559-567.

JUNG, I. L. 2014. Soluble extract from *Moringa oleifera* leaves with a new anticancer activity. *PLoS One,* 9**,** e95492.

ISMAIL, A., MOSA, K. A., ALI, M. A. & HELMY, M. 2020. Biochemical and molecular markers: Unraveling their potential role in screening germplasm for thermotolerance. In: SHABIR, H. W. & VINAY, K. (eds.) *Heat Stress Tolerance in Plants: Physiological, Molecular and Genetic Perspectives*. John Wiley & Sons Ltd.

KAKENGI, A., SHEM, M., SARWATT, S. & FUJIHARA, T. 2005. Can Moringa oleifera be used as a protein supplement to ruminants? *Asian-Australasian Journal of Animal Sciences,* 18**,** 42-47.

KAR, P., GOYAL, A. & SEN, A. 2015. Maturase K gene in plant DNA barcoding and phylogenetics. *Plant DNA Barcoding and Phylogenetics***,** 75-86.

KUMAR, P., GUPTA, V., MISRA, A., MODI, D. & PANDEY, B. 2009. Potential of molecular markers in plant biotechnology. *Plant Omics,* 2**,** 141-162.

KUMAR, M., CHAUDHARY, V., SIROHI, U., SINGH, M. K., MALIK, S. & NARESH, R. 2018. Biochemical and molecular markers for characterization of *Chrysanthemum* germplasm: A review. *Journal of Pharmacognosy and Phytochemistry,* 7, 2641-2652.

KUMSSA, D. B., JOY, E. J., YOUNG, S. D., ODEE, D. W., ANDER, E. L., MAGARE, C., GITU, J. & BROADLEY, M. R. 2017. Challenges and opportunities for Moringa growers in southern Ethiopia and Kenya. *PLoS One,* 12**,** e0187651.

KWENIN, W., WOLLI, M. & DZOMEKU, B. 2011. Assessing the nutritional value of some African indigenous green leafy vegetables in Ghana. *Journal of Animal and Plant Sciences,* 10**,** 1300-1305.

LEA, M. 2010. Bioremediation of turbid surface water using seed extract from *Moringa oleifera* Lam.(drumstick) tree. *Current Protocols in Microbiology,* 16**,** 1-10.

LEE, S. R., CHOI, J. E., LEE, B. Y., YU, J. N. & LIM, C. E. 2018. Genetic diversity and structure of an endangered medicinal herb: implications for conservation. *AoB Plants,* 10**,** ply021.

LEGLAND, D., DEVAUX, M.-F. & GUILLON, F. 2018. Quantitative imaging of plants: Multi-scale data for better plant anatomy. *Journal of Experimental Botany,* 69**,** 343-347.

LEONE, A., SPADA, A., BATTEZZATI, A., SCHIRALDI, A., ARISTIL, J. & BERTOLI, S. 2015. Cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of *Moringa oleifera* leaves: An overview. *International Journal of Molecular Sciences,* 16**,** 12791-12835.

LIEDE-SCHUMANN, S., GRIMM, G. W., NÜRK, N. M., POTTS, A. J., MEVE, U. & HARTMANN, H. E. 2020. Phylogenetic relationships in the southern African genus *Drosanthemum* (Ruschioideae, Aizoaceae). *PeerJ- Life and Environment,* 8, 1-44.

LIND, C., KILIAN, A. & BENZIE, J. 2017. Development of diversity arrays technology markers as a tool for rapid genomic assessment in Nile tilapia, *Oreochromis niloticus*. *Animal Genetics,* 48**,** 362-364.

LORENZ, T. C. 2012. Polymerase chain reaction: Basic protocol plus troubleshooting and optimization strategies. *Journal of Visualized Experiments* 1-14.

MABAPA, M. P., AYISI, K. K., MARIGA, I. K., MOHLABI, R. C. & CHUENE, R. S. 2017. Production and utilization of Moringa by farmers in Limpopo Province, South Africa. *Agricultural Research,* 12**,** 160-171.

MANGALE, S. M., CHONDE, S. G. & RAUT, P. 2012. Use of *Moringa oleifera* (drumstick) seed as natural absorbent and an antimicrobial agent for ground water treatment. *Research Journal of Recent Sciences,* 1**,** 31-40.

MARÍN-GARCÍA, J. 2014. Chapter 19 - The Aging Heart: A Post-Genomic Appraisal. *In:* MARÍN-GARCÍA, J. (ed.) *Post-Genomic Cardiology (Second Edition)*. Boston: Academic Press.

MBIKAY, M. 2012. Therapeutic potential of *Moringa oleifera* leaves in chronic hyperglycemia and dyslipidemia: A review. *Frontiers in Pharmacology,* 3**,** 1-12.

MGENDI, M. G., MANOKO, M. L. & NYOMORA, A. M. 2010. Genetic diversity between cultivated and non-cultivated *Moringa oleifera* Lam. provenances assessed by RAPD markers. *Journal of Cell and Molecular Biology,* 8**,** 95-102.

MISHRA, G., SINGH, P., VERMA, R., KUMAR, S., SRIVASTAV, S., JHA, K. & KHOSA, R. 2011. Traditional uses, phytochemistry and pharmacological properties of *Moringa oleifera* plant: An overview. *Der Pharmacia Lettre,* 3**,** 141-164.

MITCHELL, A. T. & MITCHELL, M. 2010. Detecting fetal chromosomal abnormalities using tandem single nucleotide polymorphisms. Google Patents.

MIYAO, A., ZHONG, H. S., MONNA, L., YANO, M., YAMAMOTO, K., HAVUKKALA, I., MINOBE, Y. & SASAKI, T. 1996. Characterization and genetic mapping of simple sequence repeats in the rice genome. *DNA Research,* 3**,** 233-238.

MOYO, B., MASIKA, P. J. & MUCHENJE, V. 2012. Effect of supplementing crossbred Xhosa lop-eared goat castrates with *Moringa oleifera* leaves on growth performance, carcass and non-carcass characteristics. *Tropical Animal Health and Production,* 44**,** 801-809.

NADEEM, M. A., NAWAZ, M. A., SHAHID, M. Q., DOĞAN, Y., COMERTPAY, G., YıLDıZ, M., HATIPOĞLU, R., AHMAD, F., ALSALEH, A. & LABHANE, N. 2018. DNA molecular markers in plant breeding: Current status and recent advancements in genomic selection and genome editing. *Biotechnology & Biotechnological Equipment,* 32**,** 261-285.

NOGUERA-SAVELLI, E. & JÁUREGUI, D. 2011. Comparative leaf anatomy and phylogenetic relationships of 11 species of Laeliinae with emphasis on Brassavola (Orchidaceae). *Revista de Biologia Tropical,* 59**,** 1047-1059.

NOUMAN, W., BASRA, S. M. A., SIDDIQUI, M. T., YASMEEN, A., GULL, T. & ALCAYDE, M. A. C. 2014. Potential of *Moringa oleifera* L. as livestock fodder crop: A review. *Turkish Journal of Agriculture and Forestry,* 38**,** 1-14.

OLSON, M. E. 2002. Combining data from DNA sequences and morphology for a phylogeny of Moringaceae (Brassicales). *Systematic Botany* 27, 55–73.

OMOTESHO, K. F., SOLA-OJO, F. E., FAYEYE, T. R., O, B., OTUNOLA, G. & ALIYU, T. 2013. The potential of Moringa tree for poverty alleviation and rural development: Review of evidences on usage and efficacy. *International Journal of Development and Sustainability,* 2**,** 799-813.

OVESNÁ, J., KUČERA, L., VACULOVÁ, K., MILOTOVÁ, J., SNAPE, J., WENZL, P., HUTTNER, E., KILIAN, A., MARTELLI, G. & MILELLA, L. 2013. Analysis of the genetic structure of a barley collection using DNA Diversity Array Technology (DArT). *Plant Molecular Biology Reporter,* 31**,** 280-288.

PADAYACHEE, B., & BAIJNATH, H. 2012. An overview of the medicinal importance of Moringaceae. *Journal of Medicinal Plants Research,* 6, 5831-5839.

PAKADE, V., CUKROWSKA, E. & CHIMUKA, L. 2013. Metal and flavonol contents of *Moringa oleifera* grown in South Africa. *South African Journal of Science,* 109**,** 01-07.

PATWARDHAN, A., RAY, S. & ROY, A. 2014. Molecular markers in phylogenetic studies-A review. *Journal of Phylogenetics & Evolutionary Biology,* 2, 1-9.

PECINA-QUINTERO, V., ANAYA-LÓPEZ, J. L., ZAMARRIPA-COLMENERO, A., NÚÑEZ-COLÍN, C. A., MONTES-GARCÍA, N., SOLÍS-BONILLA, J. L. & JIMÉNEZ-BECERRIL, M. F. 2014. Genetic structure of *Jatropha curcas* L. in Mexico and probable centre of origin. *Biomass and Bioenergy,* 60**,** 147-155.

PHIRI, C. 2010. Influence of *Moringa oleifera* leaf extracts on germination and early seedling development of major cereals. *Agriculture and Biology Journal of North America,* 1**,** 774-777.

PHIRI, C. & MBEWE, D. 2010. Influence of *Moringa oleifera* leaf extracts on germination and seedling survival of three common legumes. *International Journal of Agriculture and Biology,* 12**,** 315-317.

POPOOLA, J. O. & OBEMBE, O. O. 2013. Local knowledge, use pattern and geographical distribution of *Moringa oleifera* Lam.(Moringaceae) in Nigeria. *Journal of Ethnopharmacology,* 150**,** 682-691.

PRICE, M. L. 2007. The moringa tree. *Echo technical note***,** 1-16.

QI, W. H., JIANG, X. M., YAN, C. C., ZHANG, W. Q., XIAO, G. S., YUE, B. S. & ZHOU, C. Q. 2018. Distribution patterns and variation analysis of simple sequence repeats in different genomic regions of bovid genomes. *Scientific Reports,* 8**,** 14407.

RAJALAKSHMI, R., RAJALAKSHMI, S. & PARIDA, A. 2017. Evaluation of the genetic diversity and population structure in drumstick (*Moringa oleifera* L.) using SSR markers. *Current Science,* 112**,** 1250-1256.

RAMAN, H., RAMAN, R., NELSON, M. N., ASLAM, M. N., RAJASEKARAN, R., WRATTEN, N., COWLING, W. A., KILIAN, A., SHARPE, A. G. & SCHONDELMAIER, J. 2011. Diversity Array Technology Markers: Genetic diversity analyses and linkage map construction in rapeseed (*Brassica napus* L.). *DNA Research,* 19**,** 51-65.

RASMUSSEN, H. B. 2012. Restriction fragment length polymorphism analysis of PCR-amplified fragments (PCR-RFLP) and gel electrophoresis-valuable tool for genotyping and genetic fingerprinting. *Gel electrophoresis-principles and basics.* InTechopen.

ROHINI, M., ANURADHA, S., RAVISH, C. & HIMABINDU, K. 2019. Molecular characterization and DNA fingerprinting of *Centella asiatica* using SSR markers. *International Journal of Chemical Studies,* 7**,** 705-710.

ROLOFF, A., WEISGERBER, H., LANG, U. & STIMM, B. 2009. *Moringa oleifera* LAM., 1785. *Sea,* 10.

RONSE DECRAENE, L. P., DE LAET, J. & SMETS, E. F. 1998. Floral development and anatomy of *Moringa oleifera* (Moringaceae): What is the evidence for a Capparalean or Sapindalean affinity? *Annals of Botany,* 82**,** 273-284.

SALISU, I., OLAWALE, A., JABBAR, B., KOLOKO, B., ABDURRAHAMAN, S., AMIN, A. & ALI, Q. 2018. Molecular markers and their potentials in animal breeding and genetics. *Nigerian Journal of Animal Science,* 20**,** 29-48.

SANDEEP, G., ANITHA, T., VIJAYALATHA, K. & SADASAKTHI, A. 2019. Moringa for nutritional security (*Moringa oleifera* Lam.). *International Journal of Botany Studies,* 4**,** 21-24.

SANSALONI, C., PETROLI, C., JACCOUD, D., CARLING, J., DETERING, F., GRATTAPAGLIA, D. & KILIAN, A. 2011. Diversity Arrays Technology (DArT) and Next-Generation Sequencing combined: Genome-wide, high throughput, highly informative genotyping for molecular breeding of *Eucalyptus*. *BMC Proceedings,* 5**,** P54.

SARWATT, S., KAPANGE, S. & KAKENGI, A. 2002. Substituting sunflower seed-cake with *Moringa oleifera* leaves as a supplemental goat feed in Tanzania. *Agroforestry Systems,* 56**,** 241-247.

SCHLINK, K. & RESKI, R. 2002. Preparing high-quality DNA from moss (*Physcomitrella patens*). *Plant Molecular Biology Reporter,* 20**,** 423-423.

SELVARAJ, D., SARMA, R. K. & SATHISHKUMAR, R. 2008. Phylogenetic analysis of chloroplast *matK* gene from Zingiberaceae for plant DNA barcoding. *Bioinformation,* 3**,** 24.

SHAHZAD, U., KHAN, M. A., JASKANI, M. J., KHAN, I. A. & KORBAN, S. S. 2013. Genetic diversity and population structure of *Moringa oleifera*. *Conservation Genetics,* 14**,** 1161-1172.

SINGH, B. & SINGH, A. 2015. Hybridization-based markers. *Marker-Assisted Plant Breeding: Principles and Practices.* Springer.

SOHPAL, V., DEY, A. & SINGH, A. 2013. Computational analysis of distance and character based phylogenetic tree for capsid proteins of human herpes virus. *Data Mining Genomics Proteomics,* 4, 1-8.

SOTHEESWARAN, S., NAND, V., MAATA, M. & KOSHY, K. 2011. *Moringa oleifera* and other local seeds in water purification in developing countries. *Research Journal of Chemistry and Environment,* 15**,** 135-138.

SUN, C., WANG, X., LI, Z., YOSHIMURA, A. & IWATA, N. 2001. Comparison of the genetic diversity of common wild rice (*Oryza rufipogon* Griff.) and cultivated rice (*O. sativa* L.) using RFLP markers. *Theoretical and Applied Genetics,* 102**,** 157-162.

SYNREM, G. J., MARKER, S., RAMTEKE, P. & ALEXANDER, A. 2017. Simple sequence repeat (SSR) markers for molecular diversity and heterozygosity analysis in maize (*Zea mays* L.) inbred lines. *Journal of Pharmacognosy and Phytochemistry,* 6**,** 732-737.

TILOKE, C., PHULUKDAREE, A. & CHUTURGOON, A. A. 2013. The antiproliferative effect of *Moringa oleifera* crude aqueous leaf extract on cancerous human alveolar epithelial cells. *BMC Complementary and Alternative Medicine,* 13**,** 226.

TILWARI, A., CHAUHAN, D., SHARMA, R. & SINGH, R. 2016. Assessment of genetic variations among medicinal plant *Cassia tora* from different geographic regions of Central India using RAPD markers. *Medicinal and Aromatic Plants (Los Angeleles),* 5**,** 276.

TRAINI, A., IORIZZO, M., MANN, H., BRADEEN, J. M., CARPUTO, D., FRUSCIANTE, L. & CHIUSANO, M. L. 2013. Genome microscale heterogeneity among wild potatoes revealed by diversity arrays technology marker sequences. *International Journal of Genomics,* 2013.

USMAN, M. R. M., BARHATE, S., ABULLAIS, M. & USMAN, M. 2012. A review on drumstick tree (*Moringa pterygosperma* Gaertn): Multiuse tree with higher economical values. *International Journal of Current Pharmaceutical Review and Research,* 3**,** 15-22.

VERMA, A., VIJAYAKUMAR, M., MATHELA, C. & RAO, C. 2009. In vitro and in vivo antioxidant properties of different fractions of *Moringa oleifera* leaves. *Food and Chemical Toxicology,* 47**,** 2196-2201.

VIEIRA, M. L. C., SANTINI, L., DINIZ, A. L. & MUNHOZ, C. D. F. 2016. Microsatellite markers: What they mean and why they are so useful. *Genetics and Molecular Biology,* 39**,** 312-328.

VOS, P., HOGERS, R., BLEEKER, M., REIJANS, M., LEE, T. V. D., HORNES, M., FRITERS, A., POT, J., PALEMAN, J., KUIPER, M. & ZABEAU, M. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Research,* 23**,** 4407-4414.

WALLENTIN, L., JAMES, S., STOREY, R. F., ARMSTRONG, M., BARRATT, B. J., HORROW, J., HUSTED, S., KATUS, H., STEG, P. G. & SHAH, S. H. 2010. Effect of CYP2C19 and ABCB1 single nucleotide polymorphisms on outcomes of treatment with

ticagrelor versus clopidogrel for acute coronary syndromes: A genetic substudy of the PLATO trial. *The Lancet,* 376**,** 1320-1328.

WANG, S., WONG, D., FORREST, K., ALLEN, A., CHAO, S., HUANG, B. E., MACCAFERRI, M., SALVI, S., MILNER, S. G. & CATTIVELLI, L. 2014. Characterization of polyploid wheat genomic diversity using a high-density 90 000 single nucleotide polymorphism array. *Plant Biotechnology* 12**,** 787-796.

WANG, Z., WEBER, J. L., ZHONG, G. & TANKSLEY, S. 1994. Survey of plant short tandem DNA repeats. *Theoretical and Applied Genetics,* 88**,** 1-6.

WU, J.-C., YANG, J., GU, Z.-J. & ZHANG, Y.-P. 2010. Isolation and characterization of twenty polymorphic microsatellites loci for *Moringa oleifera* (Moringaceae). *Horticultural Science,* 45**,** 690-692.

XU, H. H. & TABITA, F. R. 1996. Ribulose-1, 5-bisphosphate carboxylase/oxygenase gene expression and diversity of Lake Erie planktonic microorganisms. *Applied and Environmental Microbiology,* 62**,** 1913-1921.

YAMÉOGO, C. W., BENGALY, M. D., SAVADOGO, A., NIKIEMA, P. A. & TRAORE, S. A. 2011. Determination of chemical composition and nutritional values of *Moringa oleifera* leaves. *Pakistan Journal of Nutrition,* 10**,** 264-268.

ZHANG, H., WU, F., GUO, W., BAI, R., YAN, Z., MUVUNYI, B. P., YAN, Q., ZHANG, Y., YI, X. & ZHANG, J. 2018. Genetic variation and diversity in 199 Melilotus accessions based on a combination of 5 DNA sequences. *PLOS One,* 13**,** e0194172.

ZHANJIANG, J. L. 2007. Restriction Fragment Length Polymorphism (RFLP). *In:* TECHNOLOGIES, A. G. (ed.) *Marking Genomes.* Blackwell Publishing.

ZHIGILA, D. A., MOHAMMED, S., OLADELE, F. A. & SAWA, F. B. 2015. Numerical analyses of leaf and fruit external morphology in *Moringa oleifera* Lam. *Jurnal Teknologi,* 77**,** 123-131.

# CHAPTER 3: Phylogenetic and genetic diversity analysis of *Moringa oleifera* Lam. cultivars available at the ARC-VOPI, Pretoria, South Africa

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## Abstract

*Moringa oleifera* is a tree species belonging to the family Moringaceae and originating from India. It is well-known for its medicinal and nutritional properties as well as water purification qualities. In this study, a partial sequence of the chloroplast *atpB* gene was used to determine genetic diversity of *M. oleifera* cultivars from different regions across the world but grown for research purposes in South Africa. Of the thirteen cultivars collected, eight were from the World Vegetable Centre (AVRDC) in Thailand, three were from South Africa, and one cultivar each was from Taiwan and the United States of America. Chloroplast *atpB* gene was sequenced successfully for all thirteen cultivars tested. The maximum likelihood and Bayesian trees showed similar topology. The nucleotide diversity was 0.01654 with the average number of nucleotide difference (k) of 6,58095. Based on the phylogenetic analysis, two cultivars, TOT 5077 of Thailand and TOT 4100 of Taiwan had the highest bootstrap value indicating that they are more related than other cultivars investigated. A cluster that had the lowest bootstrap value consisted of cultivars CHM of South Africa, TOT 5028 and TOT 5330 of Thailand. Although clusters were formed between the cultivars, haplotype network recorded low nucleotide diversity which suggests that the cultivars originate from a common ancestor or same population. However, further study is necessary by collecting more cultivars of *M. oleifera* in the South African provinces from both farms and household gardens to gain a better understanding of its genetic diversity.

**Keywords**: chloroplast *atpB*; cultivar; diversity; *Moringa oleifera*; nutritional; phylogenetic.

## 3.1 Introduction

Moringaceae is one of the families under the order Brassicales (Kumssa *et al*., 2017a). It consists of only one genus, Moringa, which has 13 species (Senthilkumar *et al*., 2018). One of the species gaining attention in the family is *Moringa oleifera*. The species is well known for its nutritional and medicinal properties as well as industrial purposes (Dhakad *et al*., 2019). The leaves of *M. oleifera* are reported to contain high levels of protein, vitamin C and B6, magnesium, iron, calcium and phosphorus (Moyo *et al*., 2011). Fresh or dried leaves and stems of *M. oleifera* have been used as an alternative for most animal feed (Sun *et al*., 2018). Most studies have been undertaken and were focused on *M. oleifera* leaf meal as an alternative feed for poultry (Cui *et al*., 2018), goats (Kholif *et al*., 2016) and dairy cattle (Mendieta-Araica *et al*., 2011b). The studies reported that there was an improvement in egg quality, weight gain and milk quality (Mendieta-Araica *et al*., 2011a, Briones *et al*., 2015). Leaf extract of *M. oleifera* has been used as a crop enhancer or promoter to increase growth and yields of tomato, beans and maize (Culver *et al*., 2012, Mvumi *et al*., 2013). Furthermore, the extract was reported to prevent fungi and bacterial infections in crops.

Beyond its use as food for human and feed for animals, *M. oleifera* is used for medicinal purposes. In a survey done in Ugandan communities, the majority of the people used *M. oleifera* leaves to alleviate symptoms of diabetes, hypertension, HIV/AIDS related symptoms and treat worms in both cattle and people (Kasolo *et al*., 2010). Traditional healers in some districts of Limpopo province, South Africa, also prescribed *M. oleifera* leaves for treatment of diabetes symptoms (Semenya *et al*., 2012). Other medicinal properties of *M. oleifera* include anti-inflammatory, anti-depression, cell proliferation inhibition and anti-cancer and also cure stomach aches and ulcers (Abdull Razis *et al*., 2014, Kumar, 2017, Popoola and Obembe, 2013). In addition to medicinal purpose, *M. oleifera* is used in water purification and cosmetics (Baldisserotto *et al*., 2018, Delelegn *et al*., 2018).

Thus far, there is limited knowledge on the genetic diversity of *M. oleifera* in South Africa. However, there are published articles that report on genetic diversity in other countries. The majority of the studies used DNA-based markers to determine the genetic diversity of the species. Simple sequence repeats (SSRs) designed by Wu *et al*. (2010) have been used in assessing genetic diversity of *M. oleifera*. Shahzad *et al*. (2013), Ganesan *et al*. (2014) and Popoola *et al*. (2017) used SSR markers to assess genetic diversity of the species in Pakistan,

India and Nigeria, respectively. All these studies reported large genetic diversity within the populations in each of the countries. Swati *et al*. (2020) investigated genetic diversity of *M. oleifera* using amplified fragment length polymorphism (AFLP) whereas Muluvu *et al*. (2004) used random amplified polymorphic DNA (RAPD). All these markers have their own advantages and disadvantages, however, they provide information on the genetic diversity of *M. oleifera* that can be used in conservation strategies or breeding programs.

Organelle genes have been used successfully to determine the genetic diversity in plants. Mitochondrial and chloroplast genes are conserved and have slow rate of nucleotide substitution thereby making them the best genes for genetic and population diversity studies in plants (Smith, 2015). Some of genes include *matK*, *nadH*, ITS, *atpB*, 16S RNA and *cox1* (Skuza *et al*., 2013). In *M. oleifera* genetic diversity studies, there are only few studies that used the mitochondrial or chloroplast genes. One of the gene that was previously and extensively used is the *atpB* (ATP synthase subunit beta) gene (Shahzad *et al*., 2013), which is involved in ATP synthesis. Shahzad *et al*. (2013) study focused on determining the genetic diversity of *M. oleifera* collected from five continents. The gene was successfully in determining the variation between the cultivars or accession of *M. oleifera*. Other studies that successfully used *atpB* gene for phylogenetic studies include that by Hoot *et al*. (1995) on species belonging to the Lardizabalaceae family, Guillon (2007) on horsetails, Mahmood *et al*. (2016) on varieties of apples and Iqbal *et al*. (2019) on species belonging to the Rhamnaceae family. This background showed that *atpB* gene might be a suitable gene to study the genetic diversity in plants species. Therefore, the current study was aimed at investigating the genetic diversity of thirteen cultivars of *M. oleifera* present at the ARC, South Africa.

## 3.2 Materials and methods

### *3.2.1 Plant material collection*

Seeds of thirteen *M. oleifera* Lam. cultivars collected from several countries were sown in small pots in the green house by Moringa Agro-processing team in 2011 at the Agricultural Research Council (ARC) experimental farm in Roodeplaat, Pretoria (25°36’1.85”S ; 28°21’54.78”E) (Figure 5). The seedlings were later transplanted to the field to allow for growth into mature plants. The plants were given the same the management practices and watered thrice a week with no fertilizers application. After every twelve months, the leaves and seeds were harvested and the remaining plant parts were cut down to allow regrowth from the roots/stems left. Of the thirteen cultivars (Table 1), eight were from the World Vegetable Centre (AVRDC) in Thailand, three were from South Africa and one cultivar each were from

Taiwan and USA. Each cultivar was represented by six replicate plants. Mature leaves from each replicate of all cultivars were collected and immediately stored at -80° C until use.

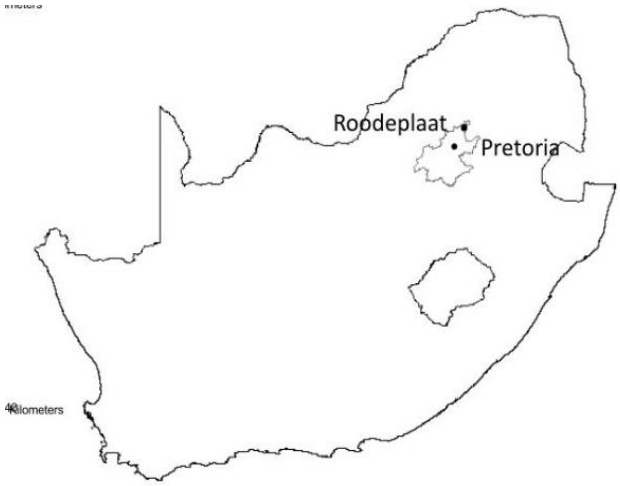



Figure 5: Locality where *M. oleifera* cultivars were grown

*3.2.2 DNA extraction and quantification*

DNA was extracted following the protocol on Quick-DNA™ Plant/Seed Miniprep Kit from Zymo Research. Leaves of *M. oleifera* were cut into small pieces and weighed to the total mass of 0,150g for each cultivar replicate. DNA was then extracted following the manual (Appendix 1) from Quick-DNA™ Plant/Seed Miniprep Kit

DNA purity and concentration was quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) at the absorbance of 260 and 280 nm and DNA Elution buffer was used as a blank. The presence of DNA was further analysed using 1.5% agarose gel stained with ethidium bromide.

*3.2.3 PCR amplification and sequencing*

The *atpB* primers (Table 3), successfully amplified all thirteen cultivars. Gradient PCR was performed to determine the best annealing temperature (Table 4). All PCR reactions were set up to a total volume of 25µL consisting of 12.5uL of DreamTaq PCR Green Master mix (Thermo Scientific, USA), 1.0µL of each primer, 5.0µL genomic DNA and 10.5µL of distilled water. The PCR cycling conditions for *atpB* primer were set up according to Shahzad et al. (2013) with modifications on the annealing temperature according to the results from the gradient PCR. The unpurified PCR products were then quantified on 1.5% gel before it was sent to Inqaba Biotec (Pty) Ltd for sequencing.

Table 3: Primers used in PCR

| Primer Sequence ID (Forward, reverse) | Primers sequences | References |
|---|---|---|
| Chloroplast *atpB* gene | **F** GGCCGTATTGCTCAAATCAT<br>**R** TTTCCTCCACGACGATAAGG | (Shahzad *et al*., 2013) |

Table 4: Gradient PCR annealing temperature set up

| Different temperatures labels | *atpB* (°C) |
|---|---|
| A | 66 |
| B | 64.3 |
| C | 63 |
| D | 61.1 |
| E | 58.8 |
| F | 56.9 |
| G | 55.7 |
| H | 55 |

*3.2.4 Sequence and phylogeny analysis*

The sequences were edited using FinchTV and sequence identity was confirmed by Basic Local Alignment Search Tool in NCBI (https://www.ncbi.nlm.nih.gov/). The sequences were aligned together with *atpB* partial sequences of *Staphylea bumalda* and *S. colchica* using ClustalW in Molecular Evolutionary Genetics Analysis (MEGA) X. The best fit substitution model for *atpB* was found to be Jukes Cantor model with discrete Gamma distribution (+G) and assuming that some sites are evolutionarily invariable (+I). The DnaSP software was used to generate information on nucleotide diversity, haplotype diversity, average number of nucleotide differences, and Tajima's D statistics. Nucleotide diversity, also as referred to nucleotide difference, is the difference between two sequences at a specific region in a population (Nei and Jin, 1989). Nucleotide diversity was introduced by Nei and Li (1979), and it determines

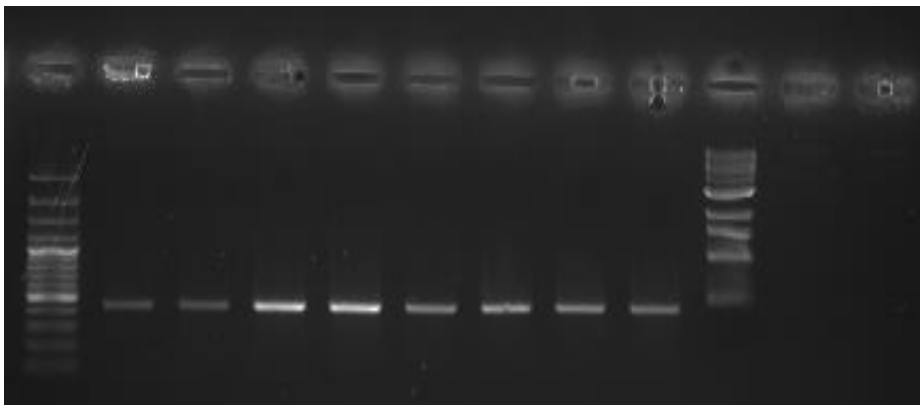
the evolutionary relationships and genetic variation within the sequences or population. All the parameters from the DnaSP would help determine the genetic diversity among the cultivars.

For phylogeny analysis, a maximum likelihood tree was constructed using bootstrap method on 1000 replicates with Heuristic search model of the nearest-neighbour interchange (Kumar *et al*., 2018). POPArT software was used to construct haplotype network which was used to support some of the data from DnaSP such as haplotype diversity and the number of haplotypes between the cultivars as well as determine the relationship between the cultivars (Leigh and Bryant, 2015, Rozas *et al*., 2017).

## 3.3 Results

### *3.3.1 PCR amplification*

The gradient PCR gave different results (band intensity) for the *atpB* primer (Figure 6). The annealing temperature chosen for PCR of the cultivars was 51.2 °C for *atpB* primer. The primer was able to amplify the gene in all thirteen cultivars. The samples amplified from the primer was then sent for sequencing




M-MOLECULAR MARKER

A- 66
B- 64.3
C- 63
D- 61.1
E- 58.8
F- 56.9
G- 55.7
H- 55.0

N- negative control

EXPECTED LENGTH- 400bp

Figure 6: Gradient PCR results using different temperatures

### *3.3.2 Genetic diversity*

The *atpB* gene was successfully sequenced for *M. oleifera* cultivars and two species of *Staphylea* were used as outgroups. Evolutionary analysis conducted using MEGA X involved 15 nucleotide sequences with the total of 404 positions in the final dataset. The sequence identity of all cultivar sequences was greater than 98% for *M. oleifera*. DNA polymorphism data (Table 5) were constructed using DnaSP v.5.10.01. DNA polymorphism data included

statistics such as nucleotide diversity, haplotype diversity, number of haplotypes and Tajima's D values.

In this study, the nucleotide diversity and average number of nucleotide difference was estimated to be 0.01654 and 6.58095, respectively. One other parameter used in DNA polymorphism is the haplotype diversity and number of haplotypes. Haplotype diversity was 0.943 whereas the number of haplotypes was 10 out of the 13 cultivars. The haplotype network constructed using POPArT in Figure 7b was able to support the data on the number of haplotypes.

Under neutrality, the value of Tajima's D is expected to be zero. Positive or balancing selection is acquired when the value is positive and negative or expansion selection is acquired when the value is negative (Ahmed et al., 2018). In the study, Tajima's D statistic was estimated to be -0.959055, which indicates negative or expansion selection.

Table 5: DNA polymorphism based on *atpB* gene of *M. oleifera* cultivars

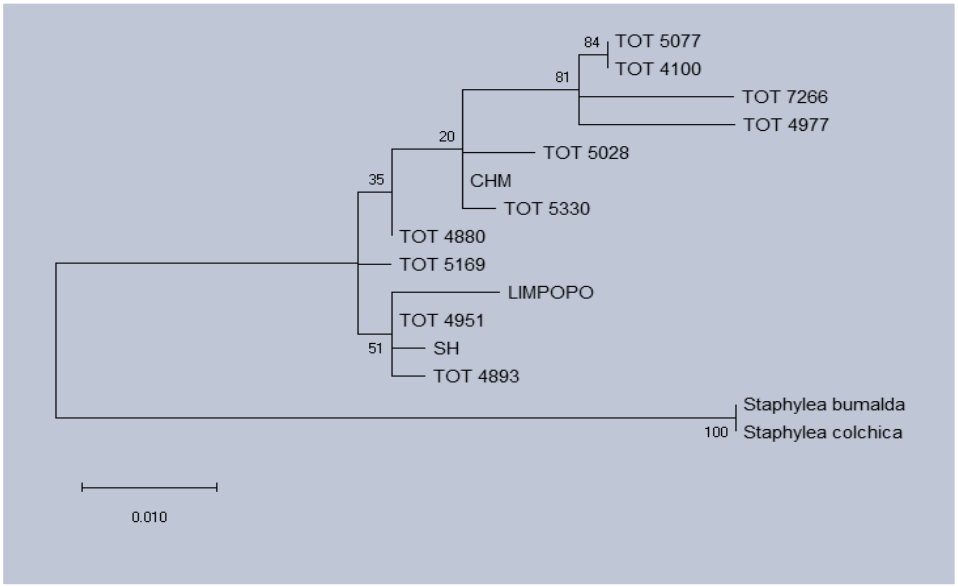
| Nucleotide analysis | Parameter estimate |
|---|---|
| Number of polymorphic (segregating) sites (S) | 21 |
| Total number of mutations (Eta) | 24 |
| Number of haplotypes | 10 |
| Haplotype diversity (Hd) | 0,943 |
| Nucleotide diversity | 0,01654 |
| Theta (per sequence) from Eta | 7,38107 |
| Variance of theta (with no recombination) | 7,140 |
| *Tajima's D | -0,44999 (p>0.10) |

*P value greater than 0.10 was considered not significant

### *3.3.3 Population diversity*

Based on the phylogenetic analysis, there are four clusters that were observed when Maximum Likelihood and Bayesian trees (Figure 7a) were constructed. From Maximum Likelihood phylogenetic tree, it was observed that the cluster with the highest bootstrap value of 84% and well supported was observed between the cultivars TOT 5077 of Thailand and TOT 4100 of Taiwan. The two cultivars seemed to be related to two more cultivars of Thailand which is TOT 4977 and TOT 7266. The cluster that consisted of South African cultivar CHM and Thailand cultivars TOT 5028 and TOT 5330 was poorly supported with the bootstrap value of

20%. The USA cultivar TOT 4880 was closely related to cultivar TOT 5169 of Thailand. Cultivars collected from SA, LIMPOPO and SH, were more related to each but distantly related to CHM. The haplotype network (Figure 7b) showed sequences that were more identical to each other. The network supported the results observed from the two phylogenetic trees in Figure 7a. The cultivars from Taiwan, SA and USA were more related to the Thailand cultivars than to each other. This shows that there was no geographical isolation between the cultivars.

A. 

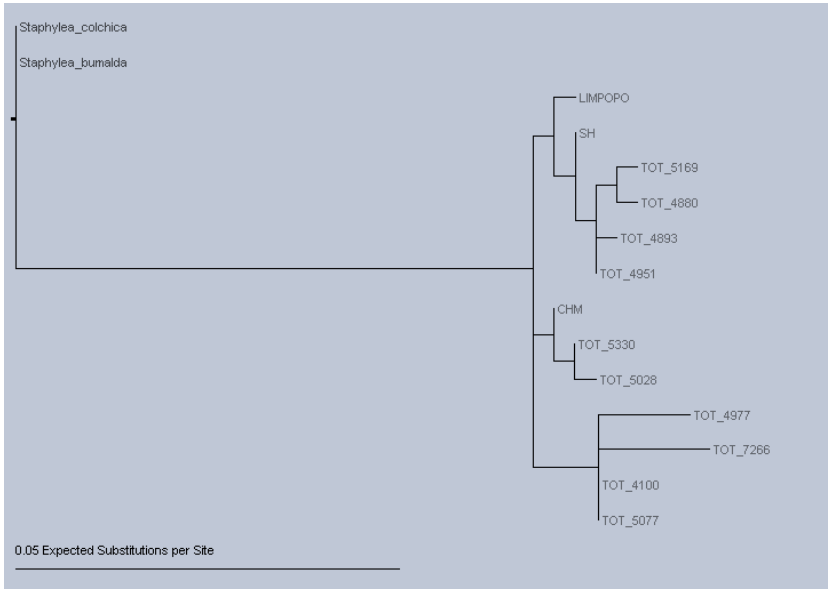
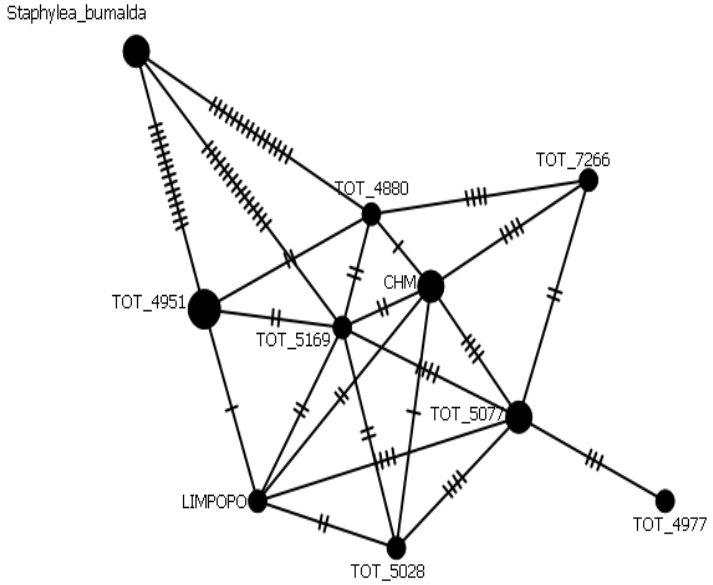
B. 


Figure 7a: Maximum likelihood (A) and Bayesian (B) phylogenetic trees based on the *atpB* gene. Maximum likelihood was constructed using Mega X whereas Bayesian tree was constructed using TOPALI software




A.

| Node label | Matching Sequences |
|---|---|
| TOT_4951 | TOT 4951<br>SH<br>TOT 4893 |
| S._bumalda | S. bumalda<br>S. colchica |
| CHM | CHM<br>TOT 5330 |
| TOT 5077 | TOT 5077<br>TOT 4100 |

B.

Figure 7b: Haplotype network (A) and identical sequences (B) representing the relatedness between *M. oleifera* cultivars with two *Staphylea* species as outgroup. Table labelled B are identical sequences represented by nodes but not shown on the haplotype network. The network was constructed using with POPArT

## 3.4 Discussion

In this study, chloroplast gene *atpB* was used to determine genetic diversity among thirteen cultivars of *M. oleifera* available in South Africa. Previous studies done on genetic diversity of *M oleifera* cultivars focused on using PCR-based markers such as RAPD (Ojuederie *et al*., 2013, Mgendi *et al*., 2010) and SSR (Ganesan *et al*., 2014, Popoola *et al*., 2017, Rajalakshmi *et al*., 2017). All the studies reported high genetic diversity between the cultivars tested. From the polymorphic data (Table 5), a low nucleotide diversity of 0,01654 was observed. The results were further supported by a haplotype network observed in Figure 7b. These results were not comparable with the values reported by Shahzad *et al*. (2013), who recorded the nucleotide diversity of 0,957 between cultivars collected from other parts of the world. The results of the two studies follow the neutral theory which states that higher population size allows for gene flow and therefore increases genetic diversity (Hague and Routman, 2016). Our study had a population size of 13 while Shahzad *et al*. (2013) had a population size of 161 thus low genetic diversity is expected in our study.

From the phylogenetic trees, it was observed that each cluster, except the outgroup cluster, consisted of at least one cultivar from Thailand. Cultivar TOT 4100 from USA had the highest bootstrap value of 84% with the Thailand cultivar TOT 5077 while the cluster made of CHM, TOT 5028 and TOT 5330 had the lowest bootstrap value of 20%. The relationship between these cultivars were further illustrated on the haplotype network. Another clade that showed a higher bootstrap value of 81% was between the Taiwan cultivar TOT 4100 and Thailand cultivars TOT 5077, TOT 7266 and TOT 4977. This showed a higher level of relatedness between these cultivars. On the hand, the South African cultivars CHM, SH and Limpopo did not cluster together but they were rather scattered in the phylogenetic tree. This indicated that these cultivars are not necessarily more related to each other than the other cultivars. Higher bootstrap value between cultivars in the phylogenetic tree illustrates that the cluster or relationship is highly supported (Wiesemüller and Rothe, 2006). The bootstrap values higher than 50% is considered a good support for relatedness between species.

The gene used in our study was designed by Shahzad *et al*. (2013). The focus of Shahzad *et al*. (2013) ‘s study was among cultivars collected from Pakistan and Florida (Educational Concerns for Hunger Organization, ECHO). The cultivars from ECHO were obtained from 10 countries around the world (Shahzad *et al*., 2013). Although the cultivars were different from those used in our study, similar results were observed in Shahzad *et al.* (2013) ‘s study as more cultivars had similar sequences therefore forming one clade. This could have been as a result

of common ancestry between the cultivars or rather the cultivars were collected from one location but cultivated in different countries. On the hand, Stancheva *et al*. (2013) investigated variation between *Spirogyra* species in California and around the world using the *atpB* gene. The authors observed that genetic variation occurred at a taxonomic level lower than that of species (Stancheva *et al*., 2013). This is in contrast with the results observed in our study, which recorded less variation at the cultivar level. The differences in population sizes of 13 cultivars with their study of 1200 cultivars could have also played a role. The other factor that could have contributed to the results is location difference. In our study, seeds were collected from different regions and cultivated in the same area whereas in Stancheva *et al*. (2013) plant material was collected from plants grown in different regions. In this instance, the environment and adaptation by a species might have played a role in altering the genetic difference of the species.

Cultivar variation is affected by various factors. Hyten *et al*. (2006) investigated the effect of bottleneck events on genetic variation between soybean cultivars. From the results obtained, the authors concluded that the introduction of bottlenecks as well as intensive breeding decreased the genetic diversity between the cultivars tested. Selective breeding of cultivars for adaptation to climatic conditions, high yield and herbicide resistance could lead to low genetic diversity (from inbreeding) between cultivars and in turn makes the cultivars susceptible to diseases (Mathews and Campbell, 2000) . A review by Bhandari *et al*. (2017) discussed some more factors (e.g mutation, migration and selection) that may lead to genetic variation in plants.

The results in this study expressed the genetic diversity between *M. oleifera* cultivars present in South Africa. Although the population might be considered small, the results can still be used in breeding or conservation strategies in South Africa. Future research can focus on the genetic diversity on *M. oleifera* cultivars present in South Africa using PCR- based markers such RAPD and SSR that have already been designed. This will allow comparison between genetic diversity present in South Africa and southern Africa or beyond the region. Morphological variation between cultivars of *M. oleifera* available in South Africa can also be investigated with more cultivars added to the study.

## 3.5 Conclusion

In this study, the genetic diversity of thirteen cultivars of *M. oleifera* were tested using *atpB* primers. The low genetic diversity and high haplotype diversity observed among the cultivars illustrated that the cultivars are closely related to each other. They further illustrated that there

was no geographical isolation among the cultivars, which might mean that seeds collected from the different regions could have evolved from one population. The results will help researchers in improving the characteristics of the cultivars that have traits preferred by farmers especially those struggling with pest attack which reduces their yield and product (crop) quality. On the hand, policy makers can be able to use this data to decide which cultivars can be approved to be grown by farmers. However, more cultivars available in South Africa could be included in future research, new markers could also be designed and existing markers such as SSR and RAPD could be used to determine the genetic diversity in the cultivars.

### 3.6 Limitations of the study and future research recommendations

Although the research project was successful, there were few limitations encountered through the study. This include insufficient sample size, and previous studies as well as constraint time. The results of the study would have been more accurate if more cultivars were included in the study. This would have added even more information on other cultivars that are available in South Africa. If ever cultivars similar to those collected in other countries are found, they would be compared to see how similar or different they are to the ones growing in South Africa. Secondly, the absence of previous research studies on gene markers used in *M. oleifera* was a huge limitation to this study. Most *M. oleifera* genetic diversity studies focused on SSR and RAPD markers. Only one published study by Shahzad *et al*. (2013) was able to develop a gene marker specific to *M. oleifera*. This leaves a gap further research on developing gene markers specific to *M. oleifera*. The last limitation encountered was the constraint time given for completion of the project. The research project together with the dissertation must be finished within two years. The amount of time given is not enough for students to write proposal, collect data, perform experiments, analyse data and write thesis.

Overall, there is still knowledge gaps on the genetic diversity of *M. oleifera* cultivars present in South Africa. Future research can focus on finding other cultivars outside of the Agricultural Research Council's Vegetable and Ornamental Institute, using SSR and RAPD already developed to determine if same results as those published are observed, develop new gene markers for studying genetic diversity of *M. oleifera* cultivars in South Africa, and determine morphological diversity among the cultivars.

## 3.7 References


ABDULL RAZIS, A. F., IBRAHIM, M. D. & KNTAYYA, S. B. 2014. Health benefits of *Moringa oleifera*. *Asian Pacific Journal Cancer Prevention,* 15**,** 8571-8576.

AHMED, M. A., LAU, Y. L. & QUAN, F.-S. 2018. Diversity and natural selection on the thrombospondin-related adhesive protein (TRAP) gene of *Plasmodium knowlesi* in Malaysia. *Malaria Journal,* 17**,** 274-274.

AL-SOBEAIA, S. M., ALAMERB, K. H. & A.M., A. S. 2015. Molecular identification of cultivated *Moringa oleifera* in Saudi Arabia. *International Journal of Plant, Animal and Environmental Sciences,* 5, 1-6.

BALDISSEROTTO, A., BUSO, P., RADICE, M., DISSETTE, V., LAMPRONTI, I., GAMBARI, R., MANFREDINI, S. & VERTUANI, S. 2018. *Moringa oleifera* leaf extracts as multifunctional ingredients for "natural and organic" sunscreens and photoprotective preparations. *Molecules,* 23**,** 664.

BHANDARI, H., NISHANT BHANU, A., SRIVASTAVA, K., SINGH, M. & SHREYA, H. A. 2017. Assessment of genetic diversity in crop plants- An overview. *Advances in Plants & Agriculture Research* 7**,** 279-286.

BRIONES, J., LEUNG, A., BAUTISTA, N., GOLIN, S., CALIWAG, N., CARLOS, M., GUEVARRA, J., MIRANDA, J., GUEVARRA, J. & PILI, N. Utilization of *Moringa oleifera* Lam. in animal production. I International Symposium on Moringa 1158, 2015. 467-474.

CUI, Y. M., WANG, J., LU, W., ZHANG, H. J., WU, S. G. & QI, G. H. 2018. Effect of dietary supplementation with *Moringa oleifera* leaf on performance, meat quality, and oxidative stability of meat in broilers. *Poultry Science,* 97**,** 2836-2844.

CULVER, M., FANUEL, T. & CHITEKA, A. Z. 2012. Effect of *Moringa* extract on growth and yield of tomato. *Greener Journal of Agricultural Sciences,* 2**,** 207-211.

DHAKAD, A. K., IKRAM, M., SHARMA, S., KHAN, S., PANDEY, V. V. & SINGH, A. 2019. Biological, nutritional, and therapeutic significance of *Moringa oleifera* Lam. *Phytotherapy Research,* 33**,** 2870-2903.

GANESAN, K. S., SINGH, R., CHOUDHURY, D. R., BHARADWAJ, J., GUPTA, V. & SINGODE, A. 2014. Genetic diversity and population structure study of drumstick (*Moringa oleifera* Lam.) using morphological and SSR markers. *Industrial Crops and Products,* 60**,** 316-325.

GUILLON, J.M. 2007. Molecular phylogeny of horsetails (*Equisetum*) including chloroplast *atpB* sequences. *Journal of Plant Research,* 120**,** 569-574.

HAGUE, M. T. J. & ROUTMAN, E. J. 2016. Does population size affect genetic diversity? A test with sympatric lizard species. *Heredity,* 116**,** 92-98.

HOOT, S. B., CULHAM, A. & CRANE, P. R. 1995. The utility of *atpB* gene sequences in resolving phylogenetic relationships: Comparison with *rbcL* and 18S ribosomal DNA sequences in the Lardizabalaceae. *Annals of the Missouri Botanical Garden,* 82**,** 194-207.

HYTEN, D. L., SONG, Q., ZHU, Y., CHOI, I.-Y., NELSON, R. L., COSTA, J. M., SPECHT, J. E., SHOEMAKER, R. C. & CREGAN, P. B. 2006. Impacts of genetic bottlenecks on soybean genome diversity. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 16666-16671.

IQBAL, J., SHINWARI, Z. K. & MAHMOOD, T. 2019. Phylogenetic relationships within the cosmopolitan family rhamnaceae using *atpB* gene promoter. *Pakistan Journal of Botany,* 51**,** 1027-1040.

KASOLO, J. N., BIMENYA, G. S., OJOK, L., OCHIENG, J. & OGWAL-OKENG, J. W. 2010. Phytochemicals and uses of *Moringa oleifera* leaves in Ugandan rural communities. *Journal of Medicinal Plants Research,* 4**,** 753-757.

KHOLIF, A. E., MORSY, T. A., GOUDA, G. A., ANELE, U. Y. & GALYEAN, M. L. 2016. Effect of feeding diets with processed *Moringa oleifera* meal as protein source in lactating Anglo-Nubian goats. *Animal Feed Science and Technology,* 217**,** 45-55.

KUMAR, S. 2017. Medicinal importance of *Moringa oleifera*: Drumstick plant. *Indian Journal of Scientific Research,* 16**,** 129-32.

KUMSSA, D. B., JOY, E. J., YOUNG, S. D., ODEE, D. W., ANDER, E. L. & BROADLEY, M. R. 2017a. Variation in the mineral element concentration of *Moringa oleifera* Lam. and *M. stenopetala* (Bak. f.) Cuf.: Role in human nutrition. *PLoS One,* 12**,** 1-26.

MAHMOOD, T., BAKHT, N. U. & AZIZ, E. 2016. Computational analysis of *atpB* gene promoter from different Pakistani apple varieties. *Computational Biology and Chemistry,* 64**,** 1-8.

MAIZUWO, A. I., HASSAN, A. S., MOMOH, H. & MUHAMMAD, J. A. 2017. Phytochemical constituents, biological activities, therapeutic potentials and nutritional values of *Moringa oleifera* (Zogale): A review. *Journal of Drug Design and Medicinal Chemistry,* 3**,** 60.

MAKITA, C., MADALA, N., CUKROWSKA, E., ABDELGADIR, H., CHIMUKA, L., STEENKAMP, P. & NDHLALA, A. 2017. Variation in pharmacologically potent rutinoside-bearing flavonoids amongst twelve *Moringa oleifera* Lam. cultivars. *South African Journal of Botany,* 112**,** 270-274.

MANGALE, S. M., CHONDE, S. G. & RAUT, P. 2012. Use of *Moringa oleifera* (drumstick) seed as natural absorbent and an antimicrobial agent for ground water treatment. *Research Journal of Recent Sciences,* 1**,** 31-40.

MATHEWS, J. & CAMPBELL, M. 2000. The advantages and disadvantages of the application of genetic engineering to forest trees: A discussion. *Forestry: An International Journal of Forest Research,* 73**,** 371-380.

MENDIETA-ARAICA, B., SPÖRNDLY, E., REYES-SÁNCHEZ, N. & SPÖRNDLY, R. 2011a. Feeding *Moringa oleifera* fresh or ensiled to dairy cows—effects on milk yield and milk flavor. *Tropical Animal Health and Production,* 43**,** 1039-1047.

MENDIETA-ARAICA, B., SPÖRNDLY, R., REYES-SÁNCHEZ, N. & SPÖRNDLY, E. 2011b. *Moringa* (*Moringa oleifera*) leaf meal as a source of protein in locally produced

concentrates for dairy cows fed low protein diets in tropical areas. *Livestock Science,* 137**,** 10-17.

MGENDI, M. G., MANOKO, M. L. & NYOMORA, A. 2010. Genetic diversity between cultivated and non-cultivated Moringa oleifera Lam. provenances assessed by RAPD markers. *Journal of Cell and Molecular Biology,* 8**,** 95-102.

MIYAO, A., ZHONG, H. S., MONNA, L., YANO, M., YAMAMOTO, K., HAVUKKALA, I., MINOBE, Y. & SASAKI, T. 1996. Characterization and genetic mapping of simple sequence repeats in the rice genome. *DNA Research,* 3**,** 233-238.

MOYO, B., MASIKA, P. J., HUGO, A. & MUCHENJE, V. 2011. Nutritional characterization of *Moringa* (*Moringa oleifera* Lam.) leaves. *African Journal of Biotechnology,* 10**,** 12925-12933.

MVUMI, C., TAGWIRA, F. & CHITEKA, A. Z. 2013. Effect of *Moringa* extract on growth and yield of maize and common beans. *Greener Journal of Agricultural Sciences,* 3**,** 55-62.

NEI, M. & JIN, L. 1989. Variances of the average number of nucleotide substitutions within and between populations. *Molecular Biology and Evolution,* 6**,** 290-300.

NEI, M. & LI, W.-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences,* 76**,** 5269-5273.

OJUEDERIE, O. B., IGWE, D. O., OKUOFU, S. I. & FALOYE, B. 2013. Assessment of genetic diversity in some *Moringa oleifera* Lam. landraces from Western Nigeria using RAPD markers. *African Journal of Plant Science and Biotechnology,* 7**,** 15-20.

POPOOLA, J. O. 2014. Genetic diversity in *Moringa oleifera* from nigeria using fruit morpho-metric characters & random amplified polymorphic DNA (RAPD) markers. *Covenant Journal of Physical and Life Sciences,* 1**,** 43-50.

POPOOLA, J. O., BELLO, O. A., OLUGBUYIRO, J. & OBEMBE, O. O. 2017. Simple sequence repeats (SSR) analysis of genetic intraspecific relationships of *Moringa oleifera* populations from Nigeria. *Science International (Lahore),* 29**,** 645-657.

POPOOLA, J. O. & OBEMBE, O. O. 2013. Local knowledge, use pattern and geographical distribution of *Moringa oleifera* Lam.(Moringaceae) in Nigeria. *Journal of Ethnopharmacology,* 150**,** 682-691.

RAJALAKSHMI, R., RAJALAKSHMI, S. & PARIDA, A. 2017. Evaluation of the genetic diversity and population structure in drumstick (*Moringa oleifera* L.) using SSR markers. *Current Science,* 112**,** 1250.

RAMAN, H., RAMAN, R., NELSON, M. N., ASLAM, M. N., RAJASEKARAN, R., WRATTEN, N., COWLING, W. A., KILIAN, A., SHARPE, A. G. & SCHONDELMAIER, J. 2011. Diversity Array Technology markers: Genetic diversity analyses and linkage map construction in rapeseed (*Brassica napus* L.). *DNA Research,* 19**,** 51-65.

SELVARAJ, D., SARMA, R. K. & SATHISHKUMAR, R. 2008. Phylogenetic analysis of chloroplast *matK* gene from Zingiberaceae for plant DNA barcoding. *Bioinformation,* 3**,** 24.

SEMENYA, S., POTGIETER, M. & ERASMUS, L. 2012. Ethnobotanical survey of medicinal plants used by Bapedi healers to treat diabetes mellitus in the Limpopo Province, South Africa. *Journal of Ethnopharmacology,* 141**,** 440-445.

SENTHILKUMAR, A., KARUVANTEVIDA, N., RASTRELLI, L., KURUP, S. S. & CHERUTH, A. J. 2018. Traditional uses, pharmacological efficacy, and phytochemistry of *Moringa peregrina* (Forssk.) Fiori. -A review. *Frontiers in Pharmacology,* 9**,** 1-17.

SHAHZAD, U., KHAN, M. A., JASKANI, M. J., KHAN, I. A. & KORBAN, S. S. 2013. Genetic diversity and population structure of *Moringa oleifera*. *Conservation Genetics,* 14**,** 1161-1172.

SKUZA, L., FILIP, E. & SZUĆKO, I. 2013. Use of organelle markers to study genetic diversity in soybean. *A Comprehensive Survey of International Soybean Research-Genetics, Physiology, Agronomy and Nitrogen Relationships.* IntechOpen.

SMITH, D. R. 2015. Mutation rates in plastid genomes: They are lower than you might think. *Genome Biology and Evolution,* 7**,** 1227-1234.

STANCHEVA, R., HALL, J. D., MCCOURT, R. M. & SHEATH, R. G. 2013. Identity and phylogenetic placement of *Spirogyra pirogyra* species (Zygnematophyceae, Charophyta) from California streams and elsewhere. *Journal of Phycology,* 49**,** 588-607.

SULTANA, N., ALIMON, A., HUQUE, K., BABA, M. & HOSSAIN, J. 2015. Evaluation of Moringa foliage (*Moringa oleifera*) as goat feed. *Iranian Journal of Applied Animal Science,* 5**,** 865-871.

SUN, B., ZHANG, Y., DING, M., XI, Q., LIU, G., LI, Y., LIU, D. & CHEN, X. 2018. Effects of *Moringa oleifera* leaves as a substitute for alfalfa meal on nutrient digestibility, growth performance, carcass trait, meat quality, antioxidant capacity and biochemical parameters of rabbits. *Journal of Animal Physiology and Animal Nutrition,* 102**,** 194-203.

SWATI, A. B., ATTRI, C. & KULSHRESTHA, S. 2020. Evaluating the genetic diversity of *Moringa oleifera* obtained from South-central and Northern States of India using RAPD markers technique. *Asian Journal of Plant Sciences,* 19**,** 1-7.

WIESEMÜLLER, B. & ROTHE, H. 2006. Interpretation of bootstrap values in phylogenetic analysis. *Anthropologischer Anzeiger,* 64**,** 161-165.

WU, J.-C., YANG, J., GU, Z.-J. & ZHANG, Y.-P. 2010. Isolation and characterization of twenty polymorphic microsatellites loci for *Moringa oleifera* (Moringaceae). *Horticultural Science,* 45**,** 690-692.

XU, H. H. & TABITA, F. R. 1996. Ribulose-1, 5-bisphosphate carboxylase/oxygenase gene expression and diversity of Lake Erie planktonic microorganisms. *Applied and Environmental Microbiology,* 62**,** 1913-1921.

YAMÉOGO, C. W., BENGALY, M. D., SAVADOGO, A., NIKIEMA, P. A. & TRAORE, S. A. 2011. Determination of chemical composition and nutritional values of *Moringa oleifera* leaves. *Pakistan Journal of Nutrition,* 10**,** 264-268.

ZHANG, H., WU, F., GUO, W., BAI, R., YAN, Z., MUVUNYI, B. P., YAN, Q., ZHANG, Y., YI, X. & ZHANG, J. 2018. Genetic variation and diversity in 199 Melilotus accessions based on a combination of 5 DNA sequences. *PLOS One,* 13**,** e0194172.

# APPENDIX

Appendix 1: Method used for DNA extraction from *Moringa oleifera* leaves as stated on Quick-DNA™ Plant/Seed Miniprep Kit manual from Zymo Research

The leaves of *M. oleifera* were cut into small pieces and transferred into a ZR BashingBead™ Lysis Tube and 750 µL of BashingBead™ buffer was added to the tube. The tube was placed in Disruptor Genie® and run at maximum speed for 20 min to disrupt the plant tissue and then micro-centrifuged for 60 s at a speed of 10000 x g. An amount of 400 µL of the supernatant from the tube was transferred to a Zymo-Spin™ III-F Filter in a collection tube and centrifuged for 60 s at 8 000 x g. The filter tube was discarded and 1200 µL of genomic lysis buffer was added to the contents in the collection tube and mixed well. Some 800 µL of the mixture was transferred to the Zymo-Spin™ IICR Column in a collection tube and centrifuged for 60 s at 10 000 x g. The contents of the collection tube were discarded and the previous step was repeated. The column was transferred to a new collection tube and 200 µL of DNA pre-wash buffer was added to the column and centrifuged at 10 000 x g for 60 s. This was followed by 500 µL g-DNA wash buffer addition and centrifugation at 10 000 x g for 60 s. The Zymo-Spin™ IICR column was transferred to a new collection tube, 100 µL of DNA elution buffer was added and all contents were centrifuged at 10 000 x g for 30 seconds. On the side, 600 µL of prep solution was added to Zymo-Spin™ III-HRC filter in a collection tube and centrifuged at 8 000 x g for 3 min. Finally, the eluted DNA was transferred to the Zymo-Spin™ III-HRC in a new collection tube and centrifuged for 3 min at 16 000 x g. The DNA extracted from the leaves was in the collection and ready to be used for the next step. These procedures were repeated for all 13 cultivars.