



DIVERSITY, DISTRIBUTION, TISSUE CULTURE AND VALUE ADDITION OF
Strychnos SPECIES IN CENTRAL AND NORTH-EAST DISTRICTS OF BOTSWANA

by

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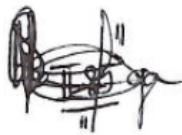
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Appendix J. Declaration and Copyright

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A handwritten signature in dark ink, appearing to read 'Willie Marenga', with a stylized flourish at the end.

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CERTIFICATION

The undersigned certifies that he/she has read and hereby recommends for acceptance by the College of Sciences a dissertation/thesis titled:

Diversity, distribution, tissue culture and value addition of *Strychnos* species in Central and North-East Districts of Botswana, in fulfilment of the requirements for the degree of Master of Science in (*Biological Sciences and Biotechnology*) of the BIUST.



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DEDICATION

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ABBREVIATIONS

ANOVA	Analysis of Variance
Bp	Base pairs
BLAST	Basic Local Alignment Search Tool
cm	Centimetre
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
g	grams
%	Percentage
<i>ITS 2</i>	Internal Transcribed Spacer 2
Kb	Kilobase pairs
km	kilometre
km ²	Square kilometre
kg	kilograms
L	Litre
M	Molar
m	metre
<i>matK</i>	maturase K
mg/g	milligrams per grams
m/v	mass per volume
ml	millilitre
mm	millimetre
mM	milliMolar
mg	milligrams
mg/ 100 g	milligram per 100 grams
MS	Murashige & Skoog medium
mRNA	messenger Ribonucleic acid
ng/ml	nanogram per millilitre
<i>rbcL</i>	Ribulose-1,5-biphosphate carboxylase oxygenase Large subunit
RNA	Ribonucleic acid
s	seconds
Sc	<i>Strychnos cocculoides</i>
Si	<i>Strychnos innocua</i>
Sm	<i>Strychnos madagascariensis</i>

Sp	<i>Strychnos pungens</i>
Ss	<i>Strychnos spinosa</i>
<i>subsp</i>	Subspecies
°C	Centigrade/ Degrees Celsius
<i>trnS-trnG IGS</i>	<i>trnS-trnG Intergeneric Spacer</i>
µg/L	microgram per Litre
µg/µL	microgram per microlitre
µl	microlitre
UV	Ultraviolet
w/v	weight per volume
v/v	volume per volume

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ABSTRACT

Strychnos is a widely varied genus from the *Loganiaceae* family with over 200 species identified worldwide. Most of these species are drought tolerant and used for many things such as food, medicine, tools, and ornaments. In Botswana, 7 *Strychnos* species have been reported, two of them (*S. cocculoides* and *S. spinosa*) have been declared as endangered. In this study, five *Strychnos* species were identified morphologically and through molecular genetics. Their distribution and abundance were studied in the Central District and North-East Districts of Botswana. A fruit pulp-based product was also developed, on which sensory and preference tests were performed. Sterile and non-sterile propagation methods for the *Strychnos* species were also developed.

This study revealed that the morphological and molecular classification using four molecular markers; a nuclear ribosomal marker (*ITS 2*) and three plastid markers (*trnS-trnG IGS*, *matK*, and *rbcL*) were consistent. They both revealed that *S. cocculoides* and *S. spinosa* are more closely related to each other than all the other members. *S. madagascariensis* was more closely related to *S. innocua*. *S. pungens* was more related to the latter group than the former. Therefore, both morphology and molecular markers can be used to distinguish the species. This study also revealed that all the 5 *Strychnos* species are found in the Central District, while only 3 were recorded in the North-East District. The *Strychnos* species in both districts are facing similar challenges such as climate change, elephants, urbanization, village expansion and threats from other anthropogenic activities. Five fruit jams were produced using fruit pulp from the species, and panelists did not show significant differences ($P>0.05$) in preference of the *Strychnos* jams compared amongst each other. However, *S. cocculoides* and *S. spinosa* jams performed better in the sensory evaluations, in attributes such as spreadability and lack of aftertaste. A preference test that included *S. cocculoides* and *S. spinosa* jams along with 4 commercially produced fruit jams revealed that the 2 *Strychnos* jams were amongst the number 1 preferred by the panelists. Therefore, these jams have market potential. In this study additionally, sterile *Strychnos* seedlings of all the species were propagated using embryo culture. This method had a significantly ($P<0.05$) faster germination time (7-9 days) than the conventional seed method (58.65 ± 1.67 days). Embryo culture had a germination percentage of 92-100 %, while 76 % of seeds germinated through the conventional method. Therefore, embryo culture may be a more efficient method of propagation for *Strychnos* species.

KEYWORDS: *Strychnos*, *Loganiaceae*, dicotyledons, molecular genetics, molecular markers, embryo culture, distribution, fruit jams, and germination.

1.0 INTRODUCTION

Strychnos is a very diverse genus that consists of dicotyledonous plants from the *Loganiaceae* family, which has about 200 plant species. This includes shrubs, climbers, and large trees that produce edible and non-edible fruits. *Strychnos* is named so because of its poisonous properties that resemble the poisonous nightshade known as *strukhnos* in Greek. *Strychnos* species are mainly found in semi-arid and dry regions of Central America, Australia, Asia, and Africa (Van Wyk and Gericke, 2000; Philippe *et al.*, 2004; Mwamba, 2006; Van Wyk and Van Wyk, 2013; Adebawale, 2014; Ngadze *et al.*, 2017a). In Botswana, the presence of *Strychnos* species have been reported in all districts. They are found in some parts of North-West, Chobe, Central, North-East, Kgatleng, Southern, South-East, Kweneng, Kgalagadi, and Gantsi Districts (Setshogo and Venter, 2003; Oppelt *et al.*, 2005; Mwamba, 2006; Adebawale *et al.*, 2012; Adebawale, 2014).

Traditionally, *Strychnos* species are mainly used as food, medicine, shade, for making tools, and making ornaments such as beads and necklaces. There are 75 *Strychnos* species identified in Africa. Only 20 of these species produce edible fruits. The edible *Strychnos* species are abundant and mostly found in all countries in Southern Africa (except Lesotho), Eastern Africa, Central Africa and West Africa (Leeuwenberg, 1963; Mwamba, 2006). According to Adebawale, (2014), there are 7 *Strychnos* species in Botswana and 6 of them produce edible fruits. The edible *Strychnos* species found in the Botswana region are *S. cocculoides*, *S. spinosa*, *S. pungens*, *S. innocua*, *S. madagascariensis*, and *S. potatorum* (Setshogo and Venter, 2003; Mwamba, 2006; Motlhanka *et al.*, 2008; Adebawale, 2014).

Based on their taste, the most preferred *Strychnos* species are *S. cocculoides*, *S. spinosa*, *S. innocua*, *S. madagascariensis*, and *S. pungens* (Mwamba, 2006; Ngadze *et al.*, 2017a). These species are commonly found in the woodlands of Southern Africa. Despite their historical use in folk traditional medicine and nourishment, there are few *Strychnos* commercial products available in the local market of Southern Africa. In India and China, however, there are *S. ignatii* and *S. nux-vomica* products available (Mwamba, 2006). These products include Nux vom.30c and Ignatia30c homeopathic medical products. The lack of utilization and commercialization of *Strychnos* species in Africa is thought to be due to limited knowledge and information on value addition and lack of intensive propagation (Ngadze *et al.*, 2017a; Ngemakwe *et al.*, 2017; Ngadze *et al.*, 2018).

A single *Strychnos* tree can produce about 300–700 fruits per year, each weighing between 145–383 g. These fruits are seasonal and are only available for harvest between August and December. This is the period during which there is usually a shortage of cultivated food in most parts of Southern Africa (Mwamba, 2006; Ngadze *et al.*, 2017a). *Strychnos* species can bear fruits during periods of famine and droughts (Mwamba, 2006; Ngadze *et al.*, 2017a; Ngadze *et al.*, 2017b; Ngadze *et al.*, 2018; Ngadze, 2018; van Rayne *et al.*, 2020). They can also thrive in other harsh conditions such as very low soil fertility. Because of this, they are extremely vital to the survival of humans and animals during years of drought and in low soil fertility areas (Council, 2008; Legwaila *et al.*, 2011). Fruit pulp can be sweet or sour and is the only part of the fruit known to be consumed by humans. Animals consume the entire fruit, including the shell and seeds. The pulp of the different species also varies in colour and viscosity. It may be bright yellow or brown. It may also be juicy, slimy, or viscous with few to numerous seeds (Mwamba, 2006; Ngadze *et al.*, 2017a). The pulps of the *Strychnos* species are normally cooked with maize meal to sweeten and enrich it (Corrigan *et al.*, 2011; Ngadze *et al.*, 2017a). It may also be processed into various products such as fruit rolls, jams, pounded into flours, juices, wines, fritters, muffins, and beer (Mkonda *et al.*, 2002; Mwamba, 2006; Ngadze *et al.*, 2017a; Ngadze, 2018).

There are various medicinal uses of the different plant parts of the different edible *Strychnos* species. Seeds of *S. madagascariensis* are used as a remedy for hypertension (De Wet *et al.*, 2016). Additionally, seeds of *S. pungens* and *S. madagascariensis* are used to induce vomiting, whereas seeds of *S. innocua* are used as an emetic (Loeb *et al.*, 1956; Ruffo *et al.*, 2002; Mwamba, 2006; Orwa *et al.*, 2009; Adebawale, 2014).

Other plant organs that are used in traditional medicine are the leaves, barks, and roots. These may be used fresh or dried. They may be used as paste, crushed powder, or boiled depending on the medicinal use. Root decoction or infusion of *S. innocua*, *S. cocculoides*, and *S. spinosa* are taken as a remedy for venereal diseases, fever, wounds, stomachache, and snakebites whereas stem bark of *S. cocculoides*, *S. spinosa*, *S. innocua*, *S. pungens*, and *S. madagascariensis* have been used as a remedy for fever, low libido, and venereal diseases (Ruffo *et al.*, 2002; Mwamba, 2006). Leaves of *S. spinosa*, *S. pungens*, *S. madagascariensis*, and *S. cocculoides* are used medicinally as a remedy for snakebites, malaria, diabetes mellitus, cough, and wounds (Mwamba, 2006; Corrigan *et al.*, 2011). For making tool handles and local construction, the wood of *S. cocculoides*, *S. pungens*, and *S. spinosa* are

usually used. These include handles for axes and hoes, fighting sticks, shafts, hut poles, joinery, walking sticks, and building poles (Maundu and Tengnäs, 2005; Mwamba, 2006; Council, 2008; Orwa *et al.*, 2009; Corrigan *et al.*, 2011). Ornaments made from *Strychnos* include necklaces, handbags, and drinking vessels; these are made from glossy and attractive seeds and shells. The glossy and attractive seeds of *S. cocculoides*, *S. madagascariensis*, *S. innocua*, *S. spinosa*, and *S. pungens* are used to make necklaces. Other uses of the leaves and unripe fruits of *S. spinosa*, *S. madagascariensis*, and *S. innocua* include making anthelmintics, pesticides, and acaricides (Ruffo *et al.*, 2002; Mwamba, 2006; Orwa *et al.*, 2009; Mawela, 2009; Kandungu *et al.*, 2013; Isa *et al.*, 2014). Due to their compact and overspreading canopy that is deciduous and semi-deciduous, *Strychnos* species are a good source of cool shades (Mwamba, 2006; Council, 2008; Van Wyk and Van Wyk, 2013). In Botswana, the fruit pulp of *S. cocculoides* and *S. madagascariensis* are edible (Motlhanka *et al.*, 2008). *S. cocculoides* fruit pulp is mixed with honey to make a remedy for cough (Motlhanka *et al.*, 2008).

Domestication and propagation of *Strychnos* species can be achieved by propagation techniques using seeds, grafting, and plant tissue culture (Ruffo *et al.*, 2002; Mkonda *et al.*, 2002; Somashekhar and Manju, 2002; Mwamba, 2006). Propagation of the *Strychnos* species through air layering, budding, and cuttings has proven unsuccessful so far (Kipkemoi *et al.*, 2013). However, the species have been successfully propagated through grafting of seedlings with a rootstock of unspecified plants through a technique developed by Veld Products Research and Development of Botswana (Taylor *et al.*, 1996; Mwamba, 2006). The growth of the *Strychnos* species seedlings has been shown to be enhanced and boosted by the symbiotic relationship with mycorrhizal fungi that helps with elevated nutrients uptake (particularly phosphorus) and the increases tolerance to drought, pest, and diseases (Mwamba, 2006). The mycorrhizal fungi can be introduced into a seedling by collecting soil from the parent tree into the pit where the seedling is grown.

1.1 PROBLEM STATEMENT

Even though some of the *Strychnos* species are found in human settlements (either planted or naturally occurring), most of them are still in the wild. They are at the risk of extinction due to exposure to rapid climate changes, unregulated harvesting and use by humans

(Mwamba, 2006; Ngemakwe *et al.*, 2017; Ngadze *et al.*, 2017b). To address the concerns of the possible extinction of *Strychnos* species, some countries such as Botswana have initiated domestication programs. These programs are promising as they were able to increase the number of fruits per plant, fruit size, and weight, under field experiments (Council, 2008). However, *S. cocculoides* and *S. spinosa* are still listed among endangered species (and harvested commercially with a permit), therefore, more efforts are required for these species to be saved (Agricultural Resources Conservation Regulations, 2006). There is also limited information regarding their diversity, distribution, and abundance, which are necessary to support and inform conservation strategies. Propagation techniques are also necessary since monitoring harvesting in the wild has been a challenge. Effective propagation would alleviate the pressure of over harvesting of the natural populations. There are limited *Strychnos* commercial products available in the Southern African markets such as Mogorogorwane (*S. cocculoides*) flavoured low fat yoghurt produced by White Angels (Pty) Ltd trading as Dream Flavours at Molepolole, Botswana and Vigo drinks (flavoured with *S. cocculoides*) produced by Namibia Breweries at Windhoek, Namibia. The combination of indigenous knowledge and scientific knowledge generated may be used to make new *Strychnos* commercial products.

1.2 AIMS

This study aims to encourage additional efforts into the conservation, sustainable use, and commercial exploration of the *Strychnos* species. To achieve this, it is fundamental to also highlight the importance of these species traditionally and in modern times to humankind, especially in areas prone to droughts and poverty.

1.3 OBJECTIVES

The objectives of the study are:

- (1) To study the diversity of edible *Strychnos* species in Central and North-East Districts of Botswana using morphology and molecular markers.
- (2) To generate baseline data on the diversity, distribution, and abundance of edible *Strychnos* species in Central and North-East Districts of Botswana.
- (3) To produce market grade fruit jam from the pulp of edible *Strychnos* species found in Central and North-East Districts of Botswana.
- (4) To generate a protocol for sustainable propagation of sterile and non-sterile seedlings of the *Strychnos* species.

2.0 LITERATURE REVIEW

The genus *Strychnos* is divided into 12 sections based on unique morphological attributes of different parts (branches, fruits, barks, leaves, and flowers) and distribution (Leeuwenberg, 1963; Adebowale, 2014). These sections are *Aculeatae*, *Spinosa*, *Phaeotrichae*, *Densiflorae*, *Scyphostrychnos*, *Dolichanthae* (all native to Africa), *Strychnos* (only found in America and Asia), *Rouhamon*, *Breviflorae* (both native to America and Africa), *Penicillatae*, *Brevitubae* and *Lanigerae* (all native to Africa and Asia) (Leeuwenberg, 1963; Adebowale, 2014). In Botswana, the 7 *Strychnos* species were found to belong to 3 sections. These are *Densiflorae* (*S. pungens*, *S. innocua*, and *S. madagascariensis*), *Spinosa* (*S. cocculoides* and *S. spinosa*), and *Rouhamon* (*S. potatorum* and *S. usambarensis*) (Setshogo and Venter, 2003; Mwamba, 2006; Adebowale *et al.*, 2012; Adebowale, 2014). Of the 7 Botswana species, only 6 of them are edible (Leeuwenberg, 1963; Mwamba, 2006). The species are listed in Table 1, along with their vernacular names.

Table 1: *Strychnos* species and their vernacular names

Species	Language	Vernacular name
<i>Strychnos cocculoides</i> Baker.	English	Corky-bark Monkey Orange, Wild/bush Orange
	Tswana	Mogorogorwane
	North Sotho	Morapa
	Shona	Muhumi, Muzumwi
	Ovambo	Maguni
	Khwe	X'oana
	Afrikaans	Geelklapper, Suurklapper, Kurkbasklapper
	Otjimbanderu/ Otjiherero	Omazu
<i>Strychnos spinosa</i> Lam.	English	Spiny Monkey Orange, Green Monkey Orange, Elephant Orange
	Tswana	Mogorogoro, Moruda, Morutlwa, Nsala
	Sotho	Morapa
	Ndebele	umhahli

	IsiZulu	iHlala
	Shona	Mutamba
	Venda	Muramba
	Swahili	umKwakwa
	Afrikaans	Doringklapper, Bobbejaanklapper, Klapper
	French	Oranger de brousse
<i>Strychnos pungens</i> Solered.	English	Spiny- leaved Monkey Orange
	Tswana	Mogwagwa, Tshunya, Mozipe
	Otjimbanderu/ Otjiherero	Omuhuruhuru, Omataa
	Shona	Mudo, Mugati, Mumbumi
	Venda	Mukubudu
	Ndebele	Umgwai
	Khwe	Qoye
	Afrikaans	Kaffir orange, Stekelblaarklapper, Botterklapperboom
<i>Strychnos innocua</i> Dellile.	English	Dull-leaved Monkey Orange
	Tswana	Mogwagwa, Mokuwakwa, Morithwane
	Chindau	Umquaqua
	Swahili	Mkwakwa, mgulungungulu, mtonga
<i>Strychnos madagascariensis</i> Poiret.	English	Black Monkey Orange, Hairy-leaved monkey-orange, Shiny-leaved monkey- orange
	Tswana	Mogwagwa, Mokuwakwa, Morithwane
	Pedi	Morapa/ Mookwane
	Sotho	Morapa
	Venda	Mukwakwa
	IsiZulu	umGluza
<i>Strychnos potatorum</i> Linn.	English	Black Bitterberry, Grape <i>Strychnos</i> , Clearing nut
	Afrikaans	Swartbitterbessie
	Tswana	Morumbarunde, Mulombelombe

	Venda	Mutupa
	Hindi	Nirmali
<i>Strychnos usambarensis</i> Gilg.	English	Stripe-fruited <i>Strychnos</i>
	Afrikaans	Kaapse Kiaat
	Setswana	Motla-o-budule

Information compiled from Ruffo *et al.* (2002), Setshogo and Venter (2003), Mwamba (2006), LLHRC (2007) and Orwa *et al.* (2009).

Molecular systematic reconstruction of the *Strychnos* phylogenetic tree shows that the sectional assemblages are not natural; therefore, further research is required (Frasier, 2008; Adebawale, 2014; Adebawale *et al.*, 2016a). The section *Spinosa* is the only monophyletic clade supported by both morphology and molecular systematic analysis. There are taxonomic challenges to *S. madagascariensis* / *S. innocua* and *S. spinosa* complexes because of their wide distribution all over Africa and variations in morphology based on their geographical location. This has defied the unsupported theory that no single species can be widely dispersed. The species of *S. madagascariensis* / *S. innocua* possess leaf variations in terms of shape and size; glabrous to densely pubescent, orbicular to narrowly oblong, and acuminate or circular at the apex. The branches are usually lenticellate or not, pale brown, grey, black, and sometimes powdery or not. The fruits also vary in terms of size, may be thin or thick-walled, and bear few to numerous seeds (Leeuwenberg, 1963; Adebawale *et al.*, 2012; Adebawale, 2014). An attempt was made to solve the *S. madagascariensis* / *S. innocua* complex by dividing it into five taxa; *S. burtonii*, *S. dysophylla*, *S. engleri*, *S. gerrardii*, and *S. madagascariensis*. The first four species were collected from the continent of Africa (mainland) whilst the latter was collected from the island of Madagascar. These were further separated into three taxa after thorough examination of the samples from mainland Africa and the island of Madagascar; *S. burtonii* was renamed as *S. innocua*, both *S. dysophylla* and *S. engleri* were reduced to *S. madagascariensis* (Leeuwenberg, 1963; Adebawale *et al.*, 2012). The morphological affinity makes it hard to distinguish between *S. innocua* and *S. madagascariensis* and this has led to misidentification in some areas. However, there are slight differences. For example, the leaves of *S. madagascariensis* are glossy, dark-green, largely obovate, circular apex and wedge shaped whereas the leaves of *S. innocua* are dull green, hairy on both sides and bear prominent pale green venation on both sides (Adebawale *et al.*, 2012; Adebawale, 2014). The misidentification of the

Strychnos species can lead to skewed and distorted information on their diversity and distribution.

S. spinosa has been divided into three subspecies; *S. spinosa subsp spinosa*, *S. spinosa subsp volkensii*, and *S. spinosa subsp lokua* which also differ in morphology based on their geographical location (Mwamba, 2006). However, this subdivision of *S. spinosa* species was rejected by Adebawale, (2014) who stated that phenotypical variation due to geographical location cannot be used to partition them into infra-specific categories. The molecular analysis of the phylogeny using the nuclear ribosomal *internal transcribed spacer 2 (ITS 2)* and *trnS-trnG IGS* markers was conducted but was not conclusive on the *S. spinosa* complex. In Africa, the last new taxa descriptions were achieved through the work of Leeuwenberg, (1969). The use of DNA barcoding will lead to a high possibility of finding new taxa (Frasier, 2008; Adebawale *et al.*, 2016a).

S. cocculoides is easily distinguishable from *S. spinosa* due to the presence of a corky and deeply fissured bark. However, both species possess spines (Van Wyk and Gericke, 2000; Mwamba, 2006; Van Wyk, 2013; Adebawale, 2014). *S. spinosa* species have apple green unripe fruits compared to the dark-green mottled unripe fruits of *S. cocculoides* (Mwamba, 2006; Van Wyk, 2013; Adebawale, 2014). The most distinguishable among the *Strychnos* species is *S. pungens*. It has tough, elliptical, smooth leaves with a spiny apex (Van Wyk and Gericke, 2000; Mwamba, 2006; Van Wyk, 2013; Adebawale, 2014) .

Recent molecular studies conducted on *Strychnos* species across Southern Africa involved the use of the nuclear ribosomal *internal transcribed spacer 2 (ITS 2)* and *trnS-trnG IGS* markers (Adebawale, 2014; Adebawale *et al.*, 2016a). However, there were shortfalls and omission of critical information about the diversity, abundance, and distribution of *Strychnos* species specifically in Botswana. The use of *ITS* has been the missing link in solving evolutionary relationships of closely related *Strychnos* species as it partly solved *S. madagascariensis* / *S. innocua* and *S. spinosa* complex (Adebawale *et al.*, 2016a).

2.1 DISTRIBUTION OF THE EDIBLE *Strychnos* SPECIES. IN SOUTHERN AFRICA

Strychnos species are randomly distributed among the Miombo and Savannah woodlands of tropical Southern Africa and Malagasy (Mwamba, 2006; Orwa *et al.*, 2009). *Strychnos* species grow best in warm to hot tropical savannah, rocky hills, riverine forests, deep

valleys, coastal bushlands, and other woodland regions that receive rainfall of approximately 300-1500 mm per annum. *Strychnos* species grows best at an altitude of around 0 -2000 m above sea level. They are native to Botswana, Angola, Malawi, Eswatini, Sudan, South Africa, Mozambique, Madagascar, Seychelles, Comoros Islands, Mauritius, Namibia, Malawi, Zambia, and Zimbabwe (Setshogo and Venter, 2003; Mwamba, 2006; Orwa *et al.*, 2009; Van Wyk, 2013; Adebowale, 2014). Table 2 below depicts the specific distribution, habitat, and altitude of the different *Strychnos* species that exist in different countries of the Southern Africa region.

Table 2: Distribution and Habitat of *Strychnos* species in Southern Africa

Species	Section	Distribution	Habitat	Altitude
<i>S. cocculoides</i>	Spinosae	Angola, Namibia, Botswana, Zambia, Malawi, Zimbabwe, South Africa, and Mozambique	Savanna (dry and open woodlands), bushveld, gallery, and sand forests.	400-2000 m
<i>S. spinosa</i>	Spinosae	Angola, Namibia, Botswana, Zambia, Malawi, Mozambique, Zimbabwe, South Africa, Comoro Islands, Madagascar, Seychelles, and Mauritius.	Savanna (dry and open woodlands), bushveld, gallery, and sand forests.	0-2200 m
<i>S. pungens</i>	Densiflorae	Angola, Namibia, Botswana, Zambia, Malawi, Zimbabwe, South Africa, and Mozambique	Savanna (dry and open woodlands), rocky slopes and rocky kopjes	0-2000 m
<i>S. innocua</i>	Densiflorae	Angola, Botswana, Zambia, Malawi	Dry and open evergreen	0-1600 m

		Zimbabwe, and Mozambique	woodlands and rocky kopjes	
<i>S. madagascariensis</i>	Densiflorae	Angola, Botswana, Zambia, Malawi Zimbabwe, Madagascar, and Mozambique	Open evergreen woodlands, rocky kopjes, and coastal bush	0-1800 m
<i>S. gerrardii</i>	Densiflorae	Madagascar, Mozambique, Eswatini (Swaziland) and South Africa.	Dry coastal or dune forests sometimes on wet coastal forests	0-500 m
<i>S. lucens</i>	Densiflorae	Angola and Zambia	Gallery forests and rocky hills in woodlands	0-1700 m
<i>S. decussata</i>	Rouhamon	Zambia, Zimbabwe, Mozambique, Malawi, Madagascar, Eswatini (Swaziland), and South Africa	Wet coastal forests	0-1600 m
<i>S. potatorum</i>	Rouhamon	Zambia, Malawi, Zimbabwe, Mozambique, Namibia, Botswana, South Africa and Madagascar.	Gallery forests, dry forests, and woodlands	0-1500 m
<i>S. gossweileri</i>	Dolichanthae	Angola and Zambia	Gallery forests, groves on granitic rocks, or woodlands	0-700 m

Information compiled from Ruffo *et al.* (2002), Setshogo and Venter (2003), Mwamba (2006), LLHRC (2007) and Orwa *et al.* (2009)

Edible *Strychnos* species are reported to be present in all districts of Botswana. Table 3 below depicts distribution of the different *Strychnos* species by districts in Botswana.

Table 3: Edible *Strychnos* species distribution in Botswana

Species	Section	Distribution
<i>S. cocculoides</i>	Spinosae	North-West, Chobe, Gantsi, Central, Kgatleng, Southern, Kweneng and Kgalagadi.
<i>S. spinosa</i>	Spinosae	Chobe and Central
<i>S. pungens</i>	Densiflorae	North-West, Chobe, Central, Kgatleng, Southern, Kweneng, and South-East
<i>S. innocua</i>	Densiflorae	Central and North-East
<i>S. madagascariensis</i>	Densiflorae	Chobe, Central, Southern, South-East, and North-East
<i>S. potatorum</i>	Rouhamon	North-West and Chobe

Information compiled from Setshogo and Venter (2003), Oppelt *et al.* (2005), Mwamba (2006), Adebawale *et al.* (2012) and Adebawale (2014).

2.2 BOTANICAL DESCRIPTIONS OF THE EDIBLE *Strychnos* SPECIES FOUND IN BOTSWANA

In Botswana, there are 6 edible species of *Strychnos* with close morphological features. Out of the 6 *Strychnos* species, 4 are unarmed or do not have spines whilst the remaining got curved or straight spines. The *Strychnos* species can have smooth or longitudinally fissured grey, cream to brownish stem barks. Almost all of them possess fruits with hard woody shells whilst some species produce fruits with soft shells. However, the differences in morphological features in some species are not noticeable hence it is necessary to identify them correctly because misidentification can lead to the ingestion of poisonous species which may result in death.

2.2.1 *Strychnos cocculoides* Baker: spiny and corky deciduous tree that grows up to a height of approximately 1-8 m (Figure 1A). It has a creamy brown, thick, rough, corky, and ridged bark (Figure 1B). The stem of *S. cocculoides* has spreading branches and a compact rounded crown. It can have one or several trunks that develop suckers and the trunk ranges from 4-25 cm in diameter. Branchlets of *S. cocculoides* are dark brown while young branchlets are reddish-brown or blackish-purple, smooth, and can also be hairy. The branches of this tree are terminated by spines that are approximately 1.0-15cm in length. These spines are tough, thick, pointed, curved or straight, axillary, and paired. Leaves of *S. cocculoides* are dark green, 6 cm long, 4 cm wide, leathery, smooth, heart-shaped, and oblong-elliptic to broadly ovate below the middle (Figure 1C). Leaves are arranged opposite each other and are hairy on both sides with 3-7 nerves at or slightly above the base. The venation of the leaves is compressed on top and visible underneath and the petiole is approximately 2-6 mm long (Figure 1C). The inflorescence is cymose, almost globose, hairy, overcrowded, and located at the end. The peduncle is also hairy and approximately 6-22 mm in length whilst the pedicels are scattered and hairy in texture. Flowers are usually pentamerous but can be hexamerous and the calyx is 5-lobed and pale green to greenish-yellow (Figure 1D). The calyx is closely linear-lanceolate, huge, and cognate at the base. It is also hairy and shorter than the corolla at approximately 3-6 mm long and 1-1.5 mm wide while the corolla of the flowers of *S. cocculoides* is 5-lobed, pale green to greenish-yellow, scantily hairy or smooth without some hair on the throat. The lobe is lanceolated with a short concave tapering apex and the tube is approximately 1-1.5 mm in length. Stamens are located at the base of the corolla tube, and the filament is smooth, and hairless while the anthers are elliptical at 1.2-1.8 mm long, and 0.8-1.2 mm wide. It is cordate, ciliated, and located far at the base. The ovary is roughly spherical, ovate, lanceolate, covered with papillae in the upper part, 2-celled and acute at approximately 0.4-1 mm. Male and female flowers are borne separately on the same plant. Fruits are huge, smooth, round with a hard-woody shell that is approximately 6-12 cm in diameter. Unripe fruits (Figure 1E) are dark green mottled white and turn green mottled yellow to yellowish orange after ripening (Figure 1F) between October and December. Each fruit contains many seeds (10-100) that bear a hard coat and the seed is 2 cm in diameter, flat, irregular, and curved (Leeuwenberg, 1963; Ruffo *et al.*, 2002; Setshogo and Venter, 2003; Mwamba, 2006; Motlhanka *et al.*, 2008; Orwa *et al.*, 2009; Adebawale, 2014).



Figure 1: *S. cocculoides* tree (A), trunk (B), leaves (C), flowers (D), unripe fruit (E) and ripe fruit (F). Scale bars = 125.7 cm in A, 6.6 cm in B, 4.8 cm in C, 1.9 cm in D, 2.7 cm in E and 3.2 cm in F.

2.2.2 *Strychnos innocua* Dellile: straight, multi-stemmed, and deciduous shrub or tree with a height of approximately 3-16 m and a closely compacted crown with a lot of twigs (Figure 2A). The trunk ranges from 7-40 cm in diameter, and the bark of *S. innocua* is greyish brown, smooth, and powdery with flakes near the base of the trunk (Figure 2B). The branchlets of *S. innocua* are sturdy, thick, and smooth, but can be hairy. Its leaves are dull, light green, leathery, simple, and alternate with a short petiole of about 2-7 mm (Figure 2C). The leaves are obovate to elliptic at long approximately 4-16 cm long, and 2-9 cm wide, round margins at the apex, and narrow at the base (Figure 2C). They can be hairy or not and have 3-7 veins that are prominent on both surfaces and arise from the base. Inflorescences (Figure 2D) arise from an axil that forms multiple cymes and can be acute with fewer flowers, and the peduncle is approximately 6-12 mm long, hairy or hairless. The pedicel is acute and hairy, and the bracts are tiny, resemble the sepal, and are almost smooth. Flowers of *S. innocua* are 4-merous. The calyx is pale green, loose, spherical, and approximately 1.7-3.5 mm long, and 1.5-2.5 mm wide. It can be hairy or smooth, and has a circular apex, and the corolla is greenish, white, or yellowish, about 2-3.5 times as large as the calyx in terms of size. The corolla is smooth on the outside, and hairy on the throat and the inner base of the lobes. Lobes are short, solid, and thinly oblong. Stamens are attached to the mouth of the corolla tube while the filaments are not fully developed and are smooth in texture. Anthers are thinly egg-shaped, 1.2-2 mm long, 0.6-1 mm wide, smooth, and heart-shaped to sagittate at the base. The ovaries are thinly ovoid to triangular with circular bases. They are covered with hair in the upper part and can also be smooth and 2-celled. Fruits of *S. innocua* are smooth, dull, and oval-shaped with a hard-woody shell that is approximately 5-10 cm in diameter. Unripe fruits (Figure 2E) are bluish-green and turn yellow or orange after ripening (Figure 2F) between October and December. Each fruit contain many yellowish-white seeds (8-50) that are 1.5-1.8 cm in diameter, stony hard flat, irregular, ovoid, and curved (Ruffo *et al.*, 2002; Mwamba, 2006; Orwa *et al.*, 2009; Adebawale, 2014; Hassan *et al.*, 2014).

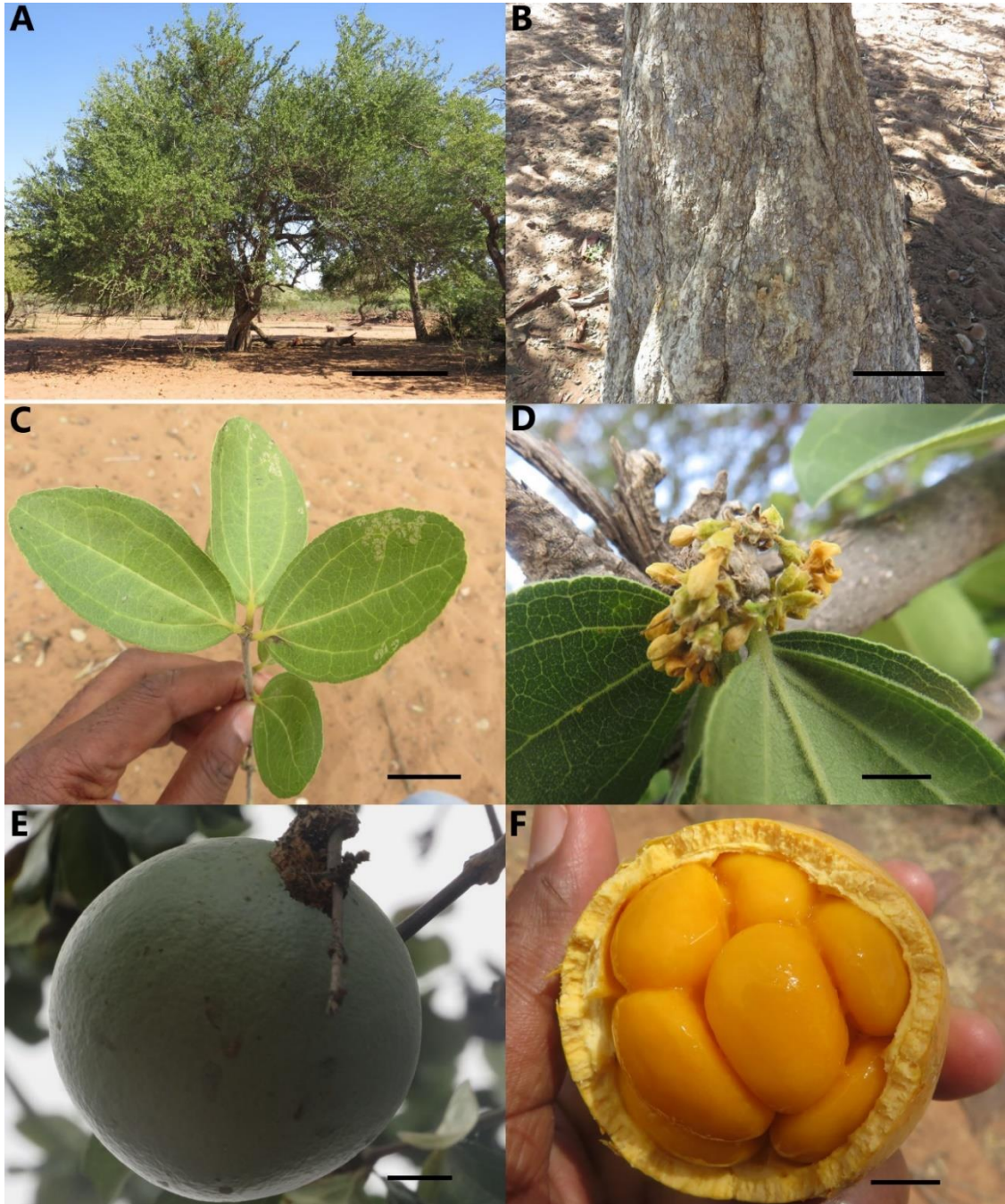


Figure 2: *S. innocua* tree (A), trunk (B), leaves (C), flowers (D), unripe fruit (E) and ripe fruit (F). Scale bars = 316.9 cm in A, 8.4 cm in B, 3.8 cm in C, 0.53 cm in D, 2.9 cm in E and 3.3 cm in F.

2.2.3 *Strychnos madagascariensis* Poiret: multi-branched, multi-stemmed deciduous tree or shrub with an approximate height of 2-20 m and a rounded crown (Figure 3A). The trunk (Figure 3B) ranges from 7-60 cm in diameter and the bark is smooth, pale grey with white patches that darken as the tree ages. Its branches are sometimes powdery, and its leaves are simple, shiny, dark green, hairless and smooth or hairy, leathery and oppositely arranged with smooth margins. The leaves are clustered on the far ends of short thick twigs and not attached by leaf stalks. The leaves are also elliptic, obovate (2-10 cm x 1-4 cm), and have short petioles (1-5 mm). It has 3-7 veins that are prominent on the upper surface (abaxial) and not visible below the leaf (adaxial). Leaves (Figure 3C) bear 2 pairs of secondary veins that arise from the base and curve along the margins and tertiary reticulate venation that is not visible from the abaxial side of the leaf. Inflorescences (Figure 3D) arise from an axil that forms cymes which occur as bundles, are acute, and contain fewer flowers. Flowers of *S. madagascariensis* are 4-merous and the calyx is pale green, loose, almost equal but the inner is smaller in size. The calyx is spherical, ovoid, shaped like a rhombus, 2.5-.3.5 mm long, 2-3 mm wide, hairy or smooth with a circular or obtuse apex while the corolla is whitish or yellowish, about 2-3.3 times as large as the calyx. The corolla is also smooth on the outside with white hair on the throat that resembles a brush. Lobes are short, solid, and thinly oblong and the stamens are attached to the mouth of the corolla tube while the filaments are not fully developed and are smooth in texture. Anthers are thinly egg-shaped, 1.2-2 mm long, 0.8-1 mm wide, smooth, and heart-shaped at the base. The ovary is thinly ovoid to triangular with a circular base. It is covered with hair in the upper part and can also be smooth and 2 celled. Style is solid, dense, 1.5-4.0 mm long, and pilose at the base. Fruits of *S. madagascariensis* are smooth, shiny, oval-shaped, and have a hard-woody shell that is approximately 2-8 cm in diameter. Unripe fruits (Figure 3E) are bluish-green and turn yellow or orange after ripening (Figure 3F) between October and December. Each fruit contains about 15-75 seeds that are yellowish-white, stony hard, and enclosed in an aril (7-12 mm) (Leeuwenberg, 1963; Ruffo *et al.*, 2002; Mwamba, 2006; Motlhanka *et al.*, 2008; Orwa *et al.*, 2009; Van Wyk, 2013; Adebawale, 2014).



Figure 3: *S. madagascariensis* tree (A), trunk (B), leaves (C), flowers (D), unripe fruit (E) and ripe fruit (F). Scale bars = 150.9 cm in A, 15.3 cm in B, 3.1 cm in C, 3.1 cm in D, 3.1 cm in E and 2.6 cm in F.

2.2.4 *Strychnos pungens* Solered: short deciduous tree or shrub, sometimes an evergreen tree with a height of approximately 2-16 m (Figure 4A). Bark is slightly corky, grey to brown, and tends to flake into squares on older trunks (Figure 4B). The trunk ranges from 10-20 cm in diameter. However, younger trees are usually smooth and grey with corky branches that are not deeply fissured. They have clear visible lenticels. Leaves of *S. pungens* (Figure 4C) are oppositely arranged, smooth, leathery, tough, dark green on top and slightly pale underneath. They are narrowly elliptic in shape with a hard-spiny apical point and are 3-8 cm long. They have short (1-4 mm) thick petioles and bear 3 veins. Inflorescences (Figure 4D) arise from an axil that forms cymes which occur as bundles, are acute, contain fewer flowers with very acute branches and pedicels. Peduncles are 2-4 mm long with flowers that are 4 merous. The calyx is green, and the sepals are almost loose and are unequal. It is spherical in shape, ovoid, shaped like a rhombus, 2-4 mm long, 2-3 mm wide, hairy or smooth with a circular or obtuse apex. The corolla is greenish-cream, blunt and diminishes in thickness at the apex, and about 2.4-3.2 times as large as the calyx. It is smooth on the outside with white hair on the throat and the inner base of the lobes. Lobes are short, solid, and thinly oblong. Stamens are projected while the filaments are smooth and placed at the mouth of the corolla tube. Anthers are thinly egg-shaped, 1-2 mm long, 0.5-1mm wide, smooth, and heart-shaped at the base. The ovary is thinly ovoid to triangular with a circular base and covered with hair at the apex but can also be smooth. It is thinly inserted in the style and 2 cells. Style is solid, dense, 3-5 mm long, pilose at the base. Fruits of *S. pungens* are smooth and round with a hard-woody shell that is approximately 5-15 cm in diameter. Unripe fruits (Figure 4E) are bluish-green and turn yellow or orange after ripening (Figure 4F) between October and December. Each fruit contains many (20-100) yellowish-white seeds that bear a hard coat and are usually flat, glossy, irregular, curved, and always embedded in fleshy yellowish pulp (Leeuwenberg, 1963; Mwamba, 2006; Van Wyk, 2013).

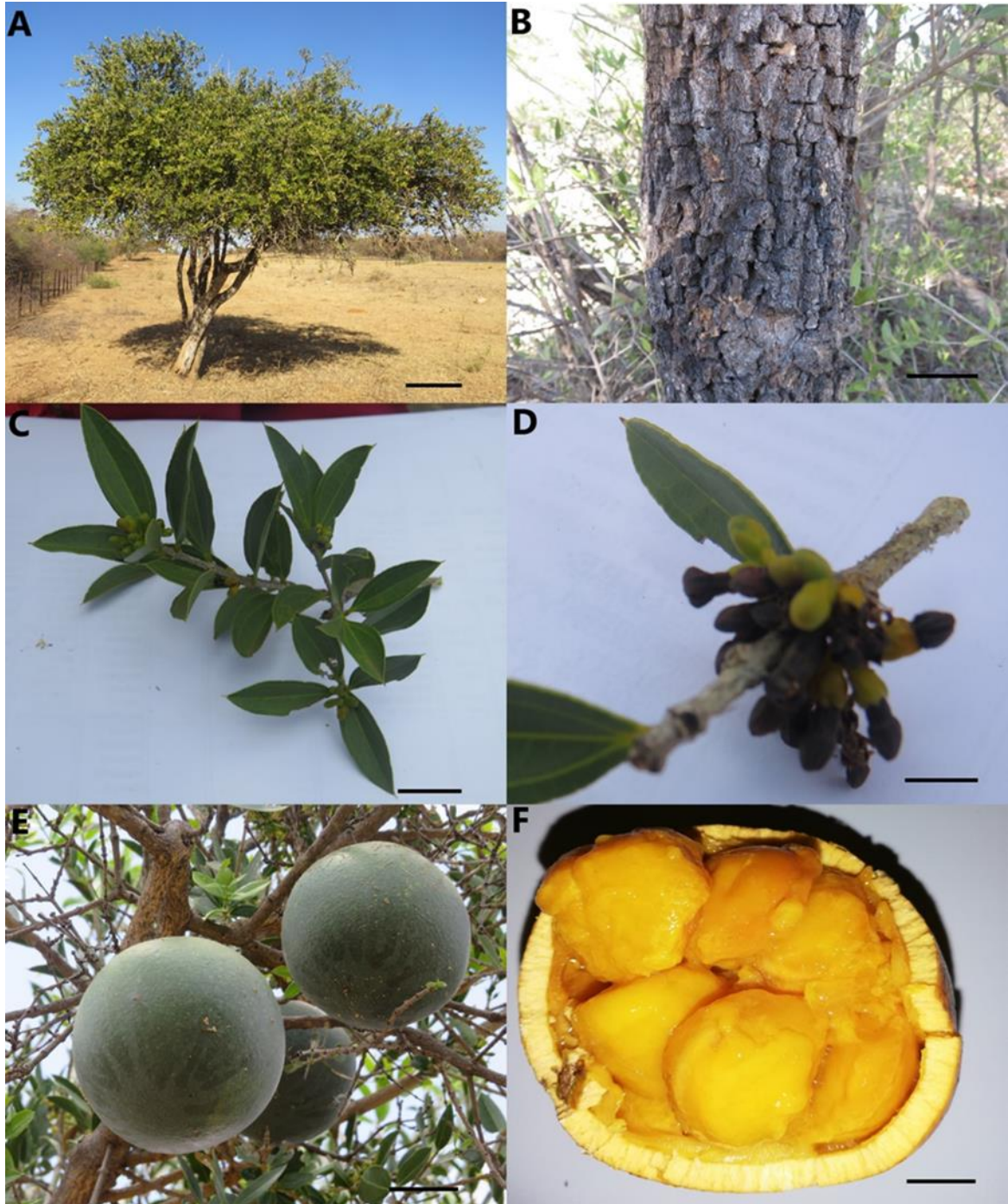


Figure 4: *S. pungens* tree (A), bark (B), leaves (C), flowers (D), unripe fruits (E) and ripe fruit (F). Scale bars = 42.9 cm in A, 3.7 cm in B, 3.5 cm in C, 1.1 cm in D, 5.5 cm in E and 3.0 cm in F.

2.2.5 *Strychnos spinosa* Lam.: round-crowned, thorny deciduous tree (Figure 5A) or shrub that is approximately 1-10 m in height with one or more trunks. The trunk ranges from 4-25 cm in diameter and frequently develops suckers. Bark of *S. spinosa* is rough, not fissured, scally and grey-buff (Figure 5B). Branchlets are usually smooth or hairy, yellowish with green margins, and have straight or hooked axillary spines or thorns. Leaves of *S. spinosa* (Figure 5C) are light to dark green, shiny at the base, and hairy or glabrous. It can be elliptic, ovate to almost circular, 1.5-10 cm long, and about 1.2-7.5 cm wide. They have 1-3 pairs of pale green secondary veins that curve from the base and go along the margin. The apex of the leaves is sometimes pointed, round, or notched with smooth margins and the petiole is approximately 2-10 mm long. The inflorescence (Figure 5D) is usually terminal or axillary at times, almost globose, hairy, overcrowded, and located at the end. Peduncle and pedicels are also sparsely hairy; the peduncle is 4-10.5 mm in length. Bracts are almost linear and sparsely hairy on the outside. Flowers of *S. spinosa* are pentamerous and the calyx is pale green, thinly triangular to linear, 3.5-6 mm long, and 0.8-1.2 wide. The calyx is cognate at the base, less hairy with a pointed apex, sparsely hairy on the base and hairless on the inner part and at the base. The corolla is pale green to greenish-white, about 0.7-2.6 times longer than the calyx, smooth inside but it is hairy on the throat with a triangular lobe. Stamens are located at the base of the tube while the filament is smooth and hairless and about the same size as the anthers. Anthers are located near the base of the corolla, elliptical or oblong, 1-1.2 mm long, 0.7-1 mm wide, cordate, and ciliated at the base. The ovary is roughly spherical, largely ovate, covered with short hair, and 1 cell. Stigma is oblong while the style is acute and approximately 0.3-0.7 mm long. Male and female flowers are borne separately on the same plant. Fruits of *S. spinosa* are smooth, shiny, warty, hard, and circular with a woody shell that is approximately 5-15 cm in diameter. Unripe fruits (Figure 5E) are apple-green with yellow spots and turn deep yellow to yellowish-brown after ripening (Figure 5F) between October and December. Each fruit contains many (10-100) seeds that are 1.5-1.8 cm in diameter, bear a hard coat, flat, irregular, curved, and usually embedded in a fleshy yellowish pulp (Leeuwenberg, 1963; Mwamba, 2006; Orwa *et al.*, 2009; Van Wyk, 2013; Kandungu *et al.*, 2013). Figure 5 below depicts the different parts of the *S. spinosa* species.



Figure 5: *S. spinosa* tree (A), bark (B), leaves (C), flowers (D), unripe fruits (E) and ripe fruit (F). Scale bars = 221.5 cm in A, 10.4 cm in B, 2.3 cm in C, 0.8 cm in D, 5.0 cm in E and 5.3 cm in F.

2.3 PROCESSING AND NUTRITIONAL IMPORTANCE OF THE EDIBLE *Strychnos* SPECIES OF SOUTHERN AFRICA

The *Strychnos* species are a source of food for the various communities around the world especially during drought periods from their fruition under harsh climatic conditions (Mwamba, 2006; Ngadze *et al.*, 2017a; Ngemakwe *et al.*, 2017; Ngadze, 2018; van Rayne *et al.*, 2020). Their fruits are highly nutritious and possess health benefits due to their high content of energy, fiber, vitamin C, and minerals, especially iron which is estimated at an average content of up to 140 mg/100 g (Mwamba, 2006; Ngemakwe *et al.*, 2017; Ngadze *et al.*, 2017a; Ngadze, 2018). Vitamin C content on the other hand ranges from 34-88 mg/100 g, which is relatively high when compared to oranges and strawberries with a content of 50 mg/100 g and 59 mg/100 g respectively (Bille *et al.*, 2013; Ngemakwe *et al.*, 2017). Therefore, they can be a good source of iron for expecting and lactating mothers who are highly susceptible to anemia as well as a source of vitamins (Ngadze *et al.*, 2017a). Many studies have shown that *Strychnos* species are nutritionally and organoleptically better than exotic fruits sold in the local market (Mizrahi *et al.*, 2002; Saka *et al.*, 2007; Bello *et al.*, 2008; Nhukarume *et al.*, 2010; Bille *et al.*, 2013; Hassan *et al.*, 2014; Ngadze *et al.*, 2017a; Ngadze, 2018; Ngadze *et al.*, 2019). The highly valued *Strychnos* species with delicious fruits in Southern Africa includes *S. cocculoides*, *S. spinosa*, *S. pungens*, *S. innocua*, *S. madagascariensis*, *S. decussata*, *S. lucens*, and *S. gerrardii* (Mwamba, 2006; Adebawale, 2014; Ngadze *et al.*, 2017a; Ngadze, 2018). They can be consumed fresh or dried to be eaten at a later stage. In Southern Africa, the fresh fruits of the *Strychnos* species are usually sun-dried, smoked, or dried using fire to produce fruit rolls and leathers. The dried fruit rolls can be pounded into flour that is used to make porridge known as Bozo in Mozambique. The dried *Strychnos* species fruits have a shelf life that ranges from 2 months to five 5 years (Mwamba, 2006; Ngadze *et al.*, 2017a; Ngadze, 2018). There are health benefits associated with the consumption of dried and fresh fruits which are highly linked to reduced cardiovascular disease incidences. Dried fruits have increased total energy content, nutrient density, and fiber content, as compared to fresh fruits (Bennett *et al.*, 2011). Tables 4 and 5 show the nutritional information of the edible *Strychnos* species. These fruits have a moisture content of about 60-91%. However, the moisture content is lowered to 18-24% by various drying methods used to dry the fruits. Some nutrients such as vitamin C are sensitive

to oxygen, light, and heat (Ngadze *et al.*, 2017a; Ngadze, 2018). Therefore, there is a need to develop drying methods that preserve nutrients.

Table 4: Proximate nutritional analysis of edible *Strychnos* fruit pulp

SPECIES	CRUDE PROTEIN (g/100g)	ASH (g/100g)	DRY MATTER (%)	FAT (g/100g)	FIBRE (g/100g)	TOTAL CARBOHYDRATES (g/100g)	MOISTURE (%)	ENERGY (kJ/100g)
<i>S. cocculoides</i>	3.5	2.2	19.6	0.4	4.6	18.2	78.2	1477
<i>S. spinosa</i>	9.0	3.5	22.7	15.8	9.2	28.7	78.8	1681
<i>S. pungens</i>	4.3	2.0	27.9	1.8	13.0	21.3	68.2	1363
<i>S. innocua</i>	5.9	3.3	ND	6.9	9.4	32.5	74.1	1628
<i>S. mdagascariensis</i>	8.27	ND	ND	0.95	53	89.85	ND	790.90

ND = No Data. Information compiled from Mwamba (2006), Amartefio and Mosase (2006), Hassan *et al.* (2014), Ngadze *et al.* (2017) and van Rayne *et al.* (2020).

Table 5: Elemental nutritional analysis of edible *Strychnos* fruit pulp

SPECIES	Ca	P	Fe	Mg	K	Na	Zn	Cu	Vitamin C
<i>S. cocculoides</i>	46.5	116.5	70.5	137.2	959	4.5	0.4	0.36	34.2
<i>S. spinosa</i>	73.1	60.3	3.3	81.2	1342.4	23.3	0.5	0.62	69
<i>S. pungens</i>	67.5	133.6	6.1	136.6	1718	7.2	1.22	1.28	38.6
<i>S. innocua</i>	22.6	77.7	11.4	87	1561.7	99.6	28.7	2.4	45.2
<i>S. mdagascariensis</i>	148.03	94.20	15.78	79.25	594.36	16.45	1.36	0.72	ND

ND = No Data. Information compiled from Mwamba (2006), Amartefio and Mosase (2006), Hassan *et al.* (2014), Ngadze *et al.* (2017) and van Rayne *et al.* (2020).

The fruits of *S. cocculoides* are used by communities in both rural and urban areas. The pulp is edible and has a sweet taste. It can also be processed into fruit jam, fruit rolls, leather, and

non-alcoholic beverage. In Namibia, the pulp of *S. cocculoides* is fermented and distilled to produce a strong alcoholic beverage known as kashipembe (Mwamba, 2006; Bille *et al.*, 2013; Elago and Tjaveondja, 2015). In 1988, the juice was produced from the pulp of *S. cocculoides* fruits by a commercial enterprise in the Okavango region of Namibia. This juice was used as a flavourant in cane and wine-based liquor. The ethnobotany of *Strychnos* species in Botswana is not yet documented. The governments of Malawi and Zambia have considered the prospects of using the *S. cocculoides* for wine production as well as utilizing other fruits such as *Uapaca kirkiana* and *Ziziphus mauritiana* (Mwamba, 2006; Saka *et al.*, 2007).

Fruits of *S. innocua* are consumed fresh or can be processed into jam (Ruffo *et al.*, 2002; Mwamba, 2006). According to Ruffo *et al.* (2002), *S. innocua* fruit pulp should only be consumed in small amounts to avoid stomach upsets caused by strychnine. *S. innocua* seeds are a good source of galactomannan, a polysaccharide that has industrial applications. Galactomannan can be used to make biofilms, thickeners, emulsifiers, textile, pharmaceuticals, and cosmetics (Mwamba, 2006). The fruits of *S. madagascariensis* are consumed fresh (Mwamba, 2006). The fruit pulp is used to produce oil that has high oleic acid content (Mwamba, 2006). The pulp of *S. pungens* is edible and rich in citric acid but less tasty compared to that of other species (Mwamba, 2006; LLHRC, 2007; Van Wyk, 2013). The pulp of *S. spinosa* is edible and has an acidic-sweet taste whilst the seeds are poisonous when ingested. It is usually processed into various products such as jam, fruit leather, alcoholic and non-alcoholic beverages (Mizrahi *et al.*, 2002; Amarteifio and Mosase, 2006; Mwamba, 2006; Kalinganire *et al.*, 2007; Orwa *et al.*, 2009; Bille *et al.*, 2013; Van Wyk, 2013). The leaves of *S. spinosa* are used to make a special dish known as couscous in the Sahel region (Mwamba, 2006). The pulp of immature and mature fruits *S. potatorum* can only be consumed after cooking. Cooking reduces toxicity levels. The pulp can also be processed into different products such as Raja's cup, a coffee substitute in Indian folk medicine (Mwamba, 2006; Adebowale, 2014). Seeds of *S. potatorum* plants are a good source of a polysaccharide known as Galactomannan (Mwamba, 2006).

3.0 METHODOLOGY

3.1 *Strychnos* DIVERSITY

Diversity of different *Strychnos* plants found in different parts of Botswana was determined using morphological attributes and molecular markers.

3.1.1 DETERMINING *Strychnos* DIVERSITY USING MORPHOLOGY

Strychnos species survey was conducted following the method by Heard and Channon, (1997). Species diversity was determined through studying herbarium samples (Voucher: Vadense Herbarium (*S. cocculoides*) collected at Saisi Valley, Mbala District, Zambia on 10 October 1970, Voucher: University of South Florida Herbarium 154623 (*S. spinosa*) collected at Desoto city on 26 March 1990, Voucher: Vadense Herbarium (*S. pungens*) collected in Angola on 01 November 1967, University of Khartoum Herbarium 03162015 (*S. innocua*) collected in Kamoja, Uganda, on 09 March 1958 and Voucher: Harare (Salisbury) Herbarium- K000489701 (*S. madagascariensis*) collected in Francistown, Botswana on 09 February 1964 and field specimen using morphology (Heard and Channon, 1997). The morphological classification was performed using a key (Appendix A) designed using morphological information available in the literature (Leeuwenberg, 1963; Ruffo *et al.*, 2002; Mwamba, 2006; Adebowale, 2014) about each of the five species. Morphological attributes used for classification include leaf shape, colour, size, vein arrangements, fruit colour, presence/absence of spines, the shape of the spines, and trunk colour.

3.1.2 DETERMINING *Strychnos* DIVERSITY USING MOLECULAR MARKERS

Identifying species through morphological examination is sometimes complex, especially between species such as *S. madagascariensis* and *S. innocua* (Adebowale *et al.*, 2012; Adebowale, 2014). Therefore, in addition to morphological characterization, molecular classification of the species was performed. Genomic DNA was extracted from each of the species and 4 molecular markers were amplified from the genomic DNA using Polymerase Chain Reaction (PCR). These molecular markers were Internal Transcribed Spacer 2 (*ITS* 2) of the nuclear ribosomal DNA and three plastid markers (*trnS-trnG* IGS, *rbcL* and *matK*). Consortium for the Barcode of Life (CBOL) Plant Working Group recommended the use of *rbcL* and *matK* from the plastid region as core barcodes and be supplemented with additional

regions (Saarela et al., 2013; Patwardhan et al., 2014; Moorhouse-Gann et al., 2018). The ITS 2 region is regarded as the gold standard barcode due to its high taxonomic resolution (Moorhouse-Gann et al., 2018). Amplified molecular marker fragments were then sequenced and used for phylogenetic studies to determine species diversity.

3.1.2.1. DNA EXTRACTION

Young emerging leaves from each species (except *S. madagascariensis*) were used for DNA extraction. In *S. madagascariensis*, embryos from seeds were used since leaves gave poor quality DNA. The young leaves (from 4 species) and embryos (from *S. madagascariensis*) were collected and preserved at -196 °C in liquid nitrogen. They were collected from several geographical locations within the Central District. *S. cocculoides*, *S. spinosa* and *S. madagascariensis* were collected at Palapye. *S. cocculoides* were collected at Pota post office (S 22°32'34.3" E 027°06'00.0"), *S. spinosa* leaves were collected at District Health Management Team offices (S 22°32'41.0" E 027°06'19.5"), and *S. madagascariensis* within the Botswana International University of Science and Technology (BIUST) campus (S 22°35'41.40" E 027°7'27.90"). *S. pungens* was collected at Makabi cattle post 12 km west of Thabala (S 22°27'51.8" E 026°23'07.5") and *S. innocua* was collected in Lecheng (S 22°40'38.3" E 027°13'12.4").

Genomic DNA extraction was performed according to a protocol described by Turaki et al. (2017), with some modifications. The modifications included using a smaller amount (1 % instead of 2 %) of Polyvinylpyrrolidone (PVP) and no addition of 1 % (w/v) sodium sulfite (Na_2SO_3). Another modification was a one-step clarification that used chloroform:isoamyl alcohol (24:1) instead of two-step clarification that used phenol:chloroform:isoamylalcohol 25:24:1 (v/v) and chloroform:isoamyl alcohol (24:1). In addition, 5 M Sodium chloride was not used; it was replaced by 7.5 M Ammonium acetate. Isopropanol was also replaced by absolute ethanol. After storage (or immersion) in liquid nitrogen, leaves were crushed into powder using a porcelain mortar and pestle. About 500 µl of CTAB extraction buffer was added to 200 mg of leaf powder and they were homogenized. The CTAB extraction buffer (Hexadecyl trimethyl-ammonium bromide) was prepared by dissolving 2.0 g of CTAB (Sigma-Aldrich), 10.0 ml of 1 M Tris (trisaminomethane) pH 8.0 (Sigma-Aldrich), 4.0 ml of 0.5 M EDTA (Ethylenediaminetetracetic acid) pH 8.0 (Sigma-Aldrich), 28.0 ml of 5 M NaCl (Rochelle Chemicals), 40.0 ml of distilled water, and 1 g of Polyvinylpyrrolidone

(Vinylpyrrolidone Homopolymer Mw 40,000; PVP 40; Sigma-Aldrich) into 100 mL of distilled water (Turaki *et al.*, 2017). It was then autoclaved at 121 °C for 15 minutes and allowed to cool at room temperature. The homogenate was incubated in a recirculating water bath at 55 °C for 15 minutes. It was then transferred into a sterile microfuge tube and centrifuged at 9900 rpm for 5 minutes. After centrifuging, the supernatant was transferred into a sterile microfuge tube. To the tube with the supernatant, 250 µl of chloroform: isoamyl alcohol (24:1) was added. The mixture was mixed gently by inversion about 6 times, placed on ice for 5 minutes, and then centrifuged at 13 000 rpm for 1 minute. The centrifuged mixture was separated into three layers (DNA, RNA, and proteins respectively). The upper layer (DNA) was transferred into a clean microfuge tube. Fifty µl of 7.5 M ammonium acetate and 500 µl of ice-cold absolute ethanol were added to the tube. The tube was inverted 6 times to precipitate the DNA. The tube was stored in a freezer at -20 °C for 1 hour and then centrifuged at 13 000 rpm for 1 minute to form a DNA pellet. Ethanol was decanted, leaving a DNA pellet. The pellet was washed twice with 500 µl of ice-cold 70 % ethanol to remove other residues and centrifuged at 13 000 rpm for 1 minute to remove excess ethanol. The pellet was air-dried for 15 minutes and resuspended in 100 µl sterile DNase-free water. The resuspended DNA was incubated at 65 °C for 20 minutes to deactivate DNases and then stored at -20 °C.

Ten µl of the DNA was separated using 1.0 % agarose gel (Bio-Rad 161-310). The gel was stained using 2.5 µl ethidium bromide (Sigma E1510) solution (20 % v/v). Either a 1 kb DNA molecular marker (New England Biolabs) or a 766 bp low molecular weight DNA molecular marker (New England Biolabs) was run along with the DNA. Each mixture loaded into the gel was mixed with 2.0 µl (6X strength) of loading dye (New England Biolabs). The gel was run for 90 minutes at 100 Volts in TBE-EDTA (1X TBE) buffer (VWR International, AMRESCO, LLC). A UV transilluminator (GelDoc-It TS3 315 Imaging System) was used to view and photograph the gel.

DNA quantification was performed using a Nanodrop ONE^c (Thermo Fisher Scientific). The Nanodrop was blanked using DNA elute buffer (Machery-Nagel) and the DNA sample was then loaded to measure the absorbance.

3.1.2.2 PCR AMPLIFICATION

The PCR reaction was carried out in sterile thin wall 0.2 ml PCR tubes in which 4.0 µl of PCR buffer (5X; New England Biolabs), 1.0 µl of dNTP (deoxynucleotide triphosphate; 10 µM), 0.35 µl of Taq polymerase (5 U; New England Biolabs), 3.5 µl of DNA template, 1.0 µl of each primer and 9.15 µl of DNase free water (BioConcept) were added. The primers used for the PCR are listed in Table 6. A thermocycler (HangZhou Jingle Scientific Instrument Co. Ltd) was used to run the following cycling programme: initial denaturation at 94 °C for 30 s followed by 40 cycles of denaturation at 94 °C for 30 s, primer specific annealing temperatures specified in Table 6 for 1 minute and elongation at 68 °C for 2 minutes. The PCR was ended with a final extension at 68 °C for 5 minutes and then cooled down to 4 °C.

Table 6: Primer sequences, annealing temperatures, and expected product sizes for the four molecular markers.

Primer	Sequence	Annealing temperature (°C)	Expected amplicon size (bp)
<i>ITS 2</i>	5'-GCATCGATGAAGAACGCAGC-3' forward 5'-TCCTCCGCTTATTGATATGC-3' reverse	51.5	400
<i>trnS-trnG IGS</i>	5'-CGCTTTAGTCCACTCAGCCA-3' forward 5'-TCCAACAGGGGAAATTGGGCA-3' reverse	51.5	330-450
<i>rbcL</i>	5'-ATCTTGGCAGCATTCCGAGT-3' forward 5'-CCAACAGGGGTCGACCATAC-3' reverse	53.0	380
<i>matK</i>	5'-CGATCAACATCCTTTGAGGTCC-3' forward 5'-TCAGTCCAAACCGGCTTACT-3' reverse	41.5	900

3.1.2.3 GEL ELECTROPHORESIS AND EXTRACTION

About 10 µl of PCR product, 2.0 µl of 6X loading buffer (New England Biolabs) and 8 µl of 1 kb DNA molecular marker (New England Biolabs) or a 766 bp low molecular weight DNA molecular marker (New England Biolabs) were run on a 1% agarose gel stained with Ethidium bromide (Sigma E1510) using 1% TBE buffer for 90 minutes at 100 Volts. The

gel was visualized using the UV transilluminator (GelDoc-It TS3 315 Imaging System). Expected size bands for the five *Strychnos* species were excised from the gels and purified using a MACHEREY- NAGEL Nucleospin Gel and PCR Clean-up kit, following manufacturer's instructions. For each species, three purified DNA samples per molecular marker were sent to Inqaba Biotech (Pretoria, South Africa) for sequencing.

3.1.2.4 SEQUENCE ANALYSIS

BioEdit (version 7.2.5) was used to trim low quality portions of the DNA sequences and to generate a consensus sequence for each sample. Nucleotide sequences were then compared with National Center for Biotechnology Information (NCBI) nucleotide collection (blastn). BioEdit was used to calculate nucleotide sequence percentage identities. The sequences were deposited on the Gene Bank database.

3.1.2.5 PHYLOGENETIC ANALYSIS

The consensus sequences of the five *Strychnos* species were aligned with MUSCLE software using Mega-X-10.1.8. Prior to phylogenetic analysis, all sequences were trimmed to equal and corresponding lengths. Phylogenetic trees were constructed using the Maximum Parsimony method using Mega-X-10.1.8 (Kumar *et al.*, 2018), with the *Gardenia hansemanii* and *Anthocleista grandiflora* sequences designated as outgroups. Branch strength within the resulting tree was calculated using 1000 replicates in a nonparametric bootstrap test (Saitou *et al.*, 1987; Kumar *et al.*, 2018).

3.2 *Strychnos* DISTRIBUTION

The distribution experiments of the *Strychnos* species were conducted through the studying of herbarium samples and traveling around Central and North-East Districts of Botswana from February 2018 to October 2020 (Figure 6) to gather information using a questionnaire administered between August 2018 to October 2020 and through road and on-foot surveys. The Central District is divided into 5 Sub-districts, which are Boteti, Serowe-Palapye, Mahalapye, Bobonong, and Tutume (Figure 7). Upon identification of a *Strychnos* species

population, species diversity within the population was determined through road and foot surveys. Information about the abundance of each species was also recorded. The position of some individuals (except for the Phakwe population, where all individuals were recorded) within the population was marked through coordinates, using a GPS (Garmin GPSmap 62s). From the coordinates, a central individual was selected; its coordinates were used to map the location of the population within the Central and North-East Districts of Botswana. The distribution maps were designed using ArcGIS (2019).

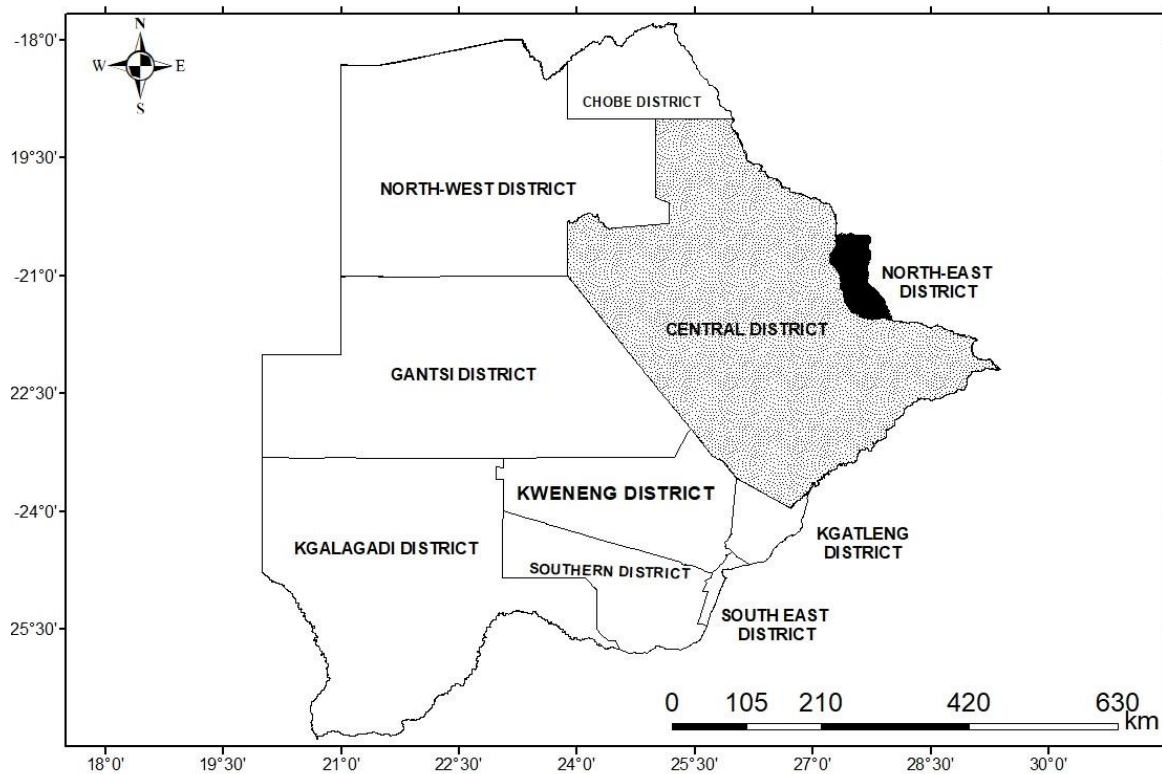


Figure 6: Map of Botswana showing different Districts including Central and North-East Districts.

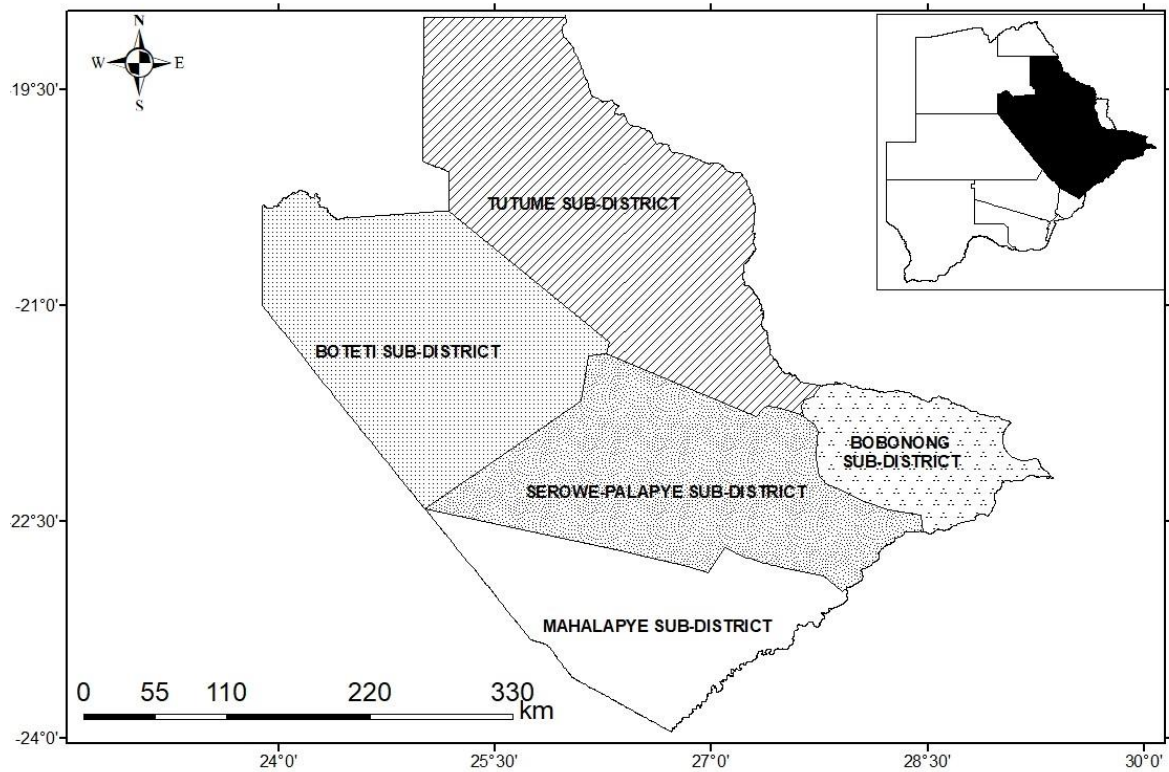


Figure 7: Map of Central District showing Sub-districts.

3.2.1 SPECIES DIVERSITY, ABUNDANCE, AND DISTRIBUTION WITHIN A POPULATION

Strychnos species diversity, abundance, and distribution within the population at Phakwe hill 33.5 km west of Tsetsebjwe village was studied through road and on-foot surveys on the 25th of October 2020. The population is secluded and covered a small area of about 0.561 km² which included the Phakwe hills. The position of all individuals was marked through coordinates, using a GPS. These coordinates were used to map *Strychnos* species distribution and diversity within the Phakwe population.

3.3 PRODUCTION OF *Strychnos* FRUIT JAM

Fruit jam was made from fruits of all the five different species. Healthy ripe fruits were collected from different recorded locations, pulped, and then processed into jam.

3.3.1 FRUIT COLLECTION

Ripe fruits of five *Strychnos* species were collected at different locations within Botswana. *S. cocculoides* fruits were collected at Xaudum (S 18°32'37.5" E 21°18'47.20"), Serowe-Palapye tarred road (S 22°28' 42.4" E 026°54'34.4"), Makabi cattle post 12 km west of Thabala village (S 22°27'10.3" E 026°22'23.1"), and Setekwane (S 22°20'02.9" E 026°33'29.6"). *S. spinosa* fruits were collected from Thune dam (S 22°09'53.1" E 028°42'09.3"), BIUST campus (S 22°36'21.4" E 027°07'32.9"), and Lecheng (S 22°38'04.9" E 027°10'58.9"). *S. pungens* fruits were collected at Makabi cattle post 12 km west of Thabala village (S 22°27'51.8" E 026°23'07.5"), and Old Palapye (S 22°37'51.5" E 027°20'59.3"). *S. innocua* fruits were collected within BIUST campus (S 22°35'41.4" E 027°07'27.9"), Lecheng (S 22°40'38.3" E 027°13'12.4"), Thune dam (S 22°09'46.6" E 028°41'04.2"), and Matsitama junction (S 20°48'44.1" E 027°02'36.6"). *S. madagascariensis* fruits were collected from Lecheng (S 22°40'38.3" E 027°13'11.7"), Mhalapitsa (S 22°48'18.2" E 027°14'08.5"), Thune dam (S 22°09'53.2" E 028°41'37.6"), near Marapong (S 20°51'37.4" E 027°06'32.2"), along the Tutume-Nkange road (S 20°27'44.9" E 027°02'32.0"), and in Dagwi (S 20°14'13.8" E 027°15'30.1").

Strychnos fruit preparation was conducted using a protocol described by Bille *et al.* (2013) with some modifications. The fruits were washed for 10 minutes using running tap water to remove debris and to avoid contamination of pulp and juice. Washed fruits were cracked and pulped. The juice was then extracted using a blender. The pulp and juice were filtered using 20.0 cm fine mesh kitchen sieves to separate the extracted pulp from seeds, fibers, and other seed materials. Cleaned fruits or extracted pulp and juice were stored in sterile plastic bags at -20 °C for future use.

3.3.2 JAM PRODUCTION

Jam production was carried out in a secluded laboratory area with restricted access using a modified protocol (not specified for Intellectual Property (IP) reasons) described by Bille *et*

al. (2013). The processing area was regularly cleaned and disinfected. Processing equipment, utensils, packaging materials (i.e. glass jars and lids) were sterilized using boiling water before use to denature pathogenic and spoilage microorganisms. *Strychnos* fruit pulp and juice blend was heated to a temperature of 100 °C for 30 minutes on a stove. This process of pasteurization was done to deactivate enzymes and denature microorganisms. Then gelling agent (donated by the Botswana University of Agriculture and Natural Resources, and National Food Technology Research Centre) and acidity regulators were mixed with white sugar and dissolved into the pulp mixture. The initial amount of sugar was used at this stage to avoid lumping. An additional white sugar was then added and thoroughly mixed until it was dissolved. The mixture was pasteurised at 100 °C for 10 minutes and hot-filled at 90 °C in 500 ml jars. It was then sealed, tilted to sterilize the headspace, and stored overnight at room temperature to jell. The procedure for jam production is summarized in Figure 8.

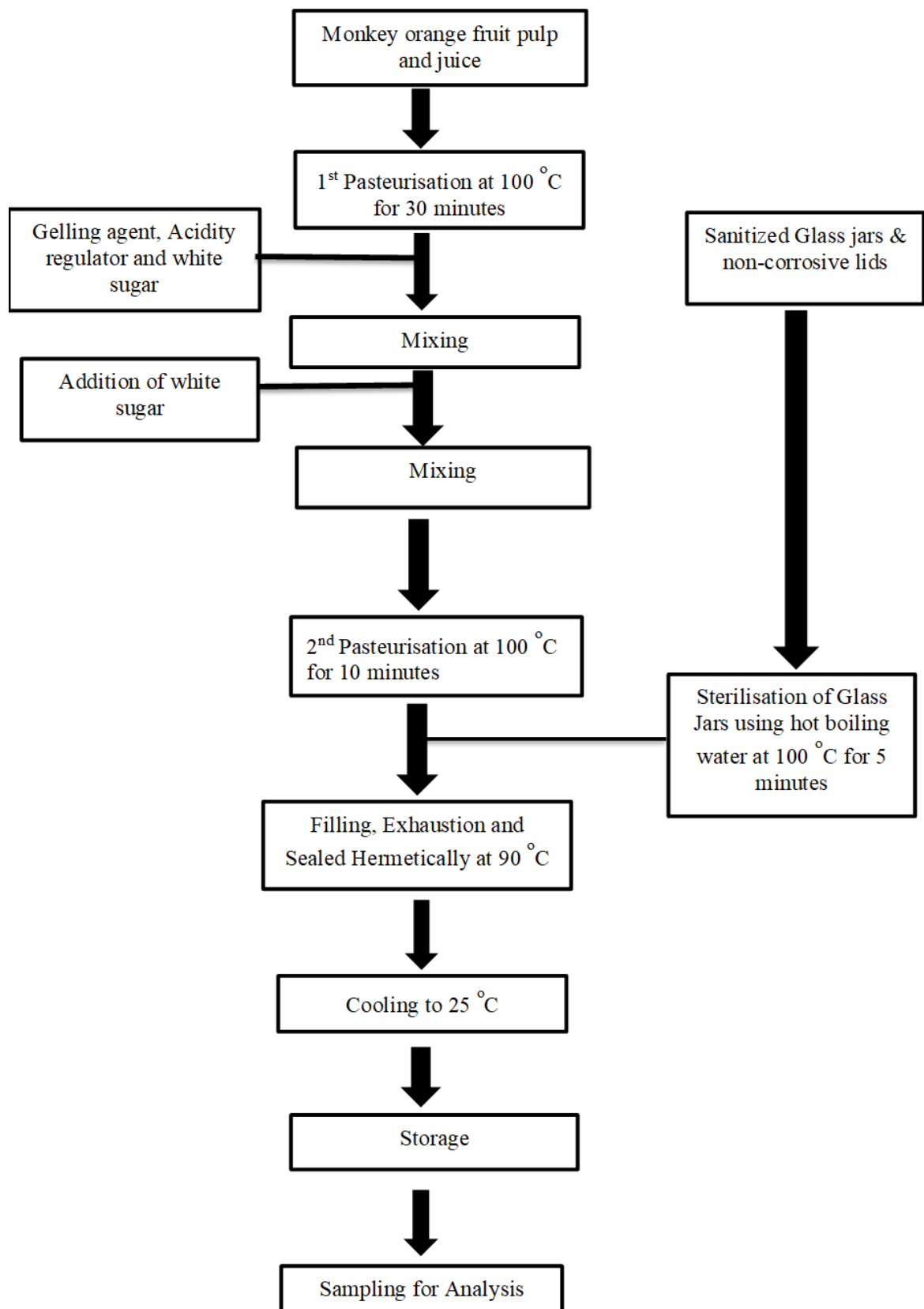


Figure 8: Flow chart for processing of fruit jam from Monkey Orange

3.3.3 JAM SENSORY EVALUATION

Two sensory evaluations were conducted. The first sensory evaluation used two tests (descriptive and preference test) to rank our five *Strychnos* species fruit jams. In the second sensory evaluation, two high ranking jams from the first test were compared with four jams that are already in the retail market.

3.3.3.1 DESCRIPTIVE AND PREFERENCE ANALYSIS OF *Strychnos* JAMS

The descriptive sensory analysis and preference ranking tests were conducted using a sensory evaluation form (Appendix C and D) and 22 untrained assessors in a Seminar room at BIUST. The process was performed as described by Gindi *et al.* (2019), Eke-Ejiofor *et al.* (2013), and Ajenifujah-Solebo *et al.* (2011). The assessors evaluated the attributes of the five *Strychnos* species jam samples (assigned random three-digit codes) based on their colour/appearance, sweetness, taste, aftertaste, viscosity (thin/thick), spreadability, aroma, flavour, and texture using a 9-point hedonic rating scale. The hedonic scale ranged from 1 (dislike extremely/least/weak/not detectable) to 9 (like extremely/strongly detectable/most/dark). The preference ranking was assigned a different rating scale where 1 represented most preferred/liked extremely and 5 represented least preferred/disliked extremely. The coded five *Strychnos* species jams were arranged in 25 possible combinations to reduce biasness and the test was done from left to right of the tray. The assessors were provided with water and a cracker to cleanse their palates before tasting each jam sample to prevent carry-over taste.

3.3.3.2 PREFERENCE TEST OF *Strychnos* JAMS AGAINST DIFFERENT COMMERCIAL JAMS

Two *Strychnos* jams selected based on the results from the above descriptive and preference analysis and four jams bought from the retail market were used. From the commercial jams, two were made from local fruits, one from a cultivated species (Lerotse: *Citrullus lanatus*) and the other from local wild fruit (Marula: *Scelocarya birrea*). The lerotse jam was produced by Brendon's Golden Investments (Molepolole, Botswana) while the marula jam was produced by Len Bakeries PTY, LTD (Gaborone, Botswana). The other two jams were strawberry produced by ALL GOLD (Sandton, South Africa) and pineapple produced by Rhodes Food group (Pniel Road, Groot Drakenstein, 7680 Western Cape, South Africa)

jams. The preference ranking test was conducted by 37 untrained assessors in a seminar room at BIUST. The setup of the sensory room layout is depicted in Appendix E. The preference ranking was assigned a different rating scale where 1 represented most preferred/liked extremely and 6 represented least preferred/disliked extremely. The coded six jams were arranged in 36 possible combinations to reduce biasness and the test was done from left to right of the tray. The assessors were provided with water and a cracker to cleanse their palates before tasting each jam sample to prevent carry-over taste. The data was analysed using GraphPad prism 9.0.2 and presented as Mean $\pm$ Standard Error of Means (S.E.M). Means of individual treatments were compared using a one-way ANOVA. Data was determined to be statistically significant if $P < 0.05$.

3.4 PRODUCTION OF STERILE SEEDLINGS THROUGH PLANT TISSUE CULTURE

Sterile seedlings from the five *Strychnos* species were produced using tissue culture techniques. Leaves, buds, and embryos were used as explants. The explants were collected from different locations. *S. spinosa*, *S. innocua* and *S. madagascariensis* were collected within BIUST campus at S 22°35'39.7" E 027°7'15.8" S 22°35'25.3" E 027°7'12.2" and S 22°35'41.4" E 027°7'27.9" respectively. *S. cocculoides* was collected at Pota Post office (S 22°32'34.3" E 027°06' 00.0") and *S. pungens* was collected in Serowe (S 22°22'31.5" E 026°39'42.9").

3.4.1 PRODUCTION OF STERILE SEEDLINGS USING LEAVES AND BUDS

The leaves and buds from all five *Strychnos* species were harvested from mature plants (Figure 9). They were excised from tree branchlets using secateurs and then surface sterilized. The samples were washed with tap water containing liquid soap to remove dust particles and rinsed with running tap water for 5 minutes. They were treated with a broad-spectrum fungicide and bactericide mixture (5 g of Dithane M-45 and 5 g of virikop dissolved in 1 L of sterile distilled water) in sterile beakers for 10 minutes. The fungicide was used to protect explants from developing fungal growth and bactericide protects explants against bacteria. The explants were then rinsed with sterile distilled water for 10 minutes using sterile gloves to avoid skin irritation. They were then sterilized using 0.6 % commercial bleach (3.5 % m/v Sodium Hypochlorite) (with 0.2 % of tween-20) with

agitation at every 2 minutes for 10 minutes. They were then rinsed 5 times with sterile distilled water. The sterile explants were dried on a sterile paper towel under sterile conditions. Using a sterile scalpel, the leaves were cut into smaller sections of 1.0 by 2.0 cm whilst the buds were cut into smaller sections of 1.0 cm by 0.2 cm (Figure 8). Full strength Murashige and Skoog (MS) Media was prepared by dissolving 4.43 g of MS powder (Duchefa Biochemie), 10 g of agar (Duchefa Biochemie), and 20 g of sucrose (Rochelle Chemicals) and distilled water was added to make up 1 L. Half-strength MS media was prepared by dissolving 2.215 g of MS powder (Duchefa Biochemie), 10 g of agar (Duchefa Biochemie) and 20 g of sucrose (Rochelle Chemicals) and distilled water was added to make up 1 L. The pH of the MS media was adjusted to 5.8 using 1 M NaOH and 1M HCL. The media was autoclaved at 121 °C for 15 minutes, allowed to cool, and then 500 µL of 2 mg/ml Cyclohexamide was added to the media before it solidifies under aseptic conditions. The sterile explants for all five *Strychnos* species were cultured on full strength MS (Murashige and Skoog, 1962) and half-strength MS, with some containing cytokinins (BAP and BA). Five leaves and buds for all five *Strychnos* species were cultured in each Magenta box and the boxes were closed and sealed with parafilm to prevent contamination. For each of the six media treatments, 5 replicates were prepared. Magenta boxes with the leaves and buds were placed in a growth chamber at 25 °C for 12 hours photoperiod with 60 % humidity, and 11 % maximum light intensity. The leaf and bud sections were then monitored daily for signs of contamination and callus development (de-differentiation). This experiment was repeated three times, with 150 leaves and 150 buds from each species.

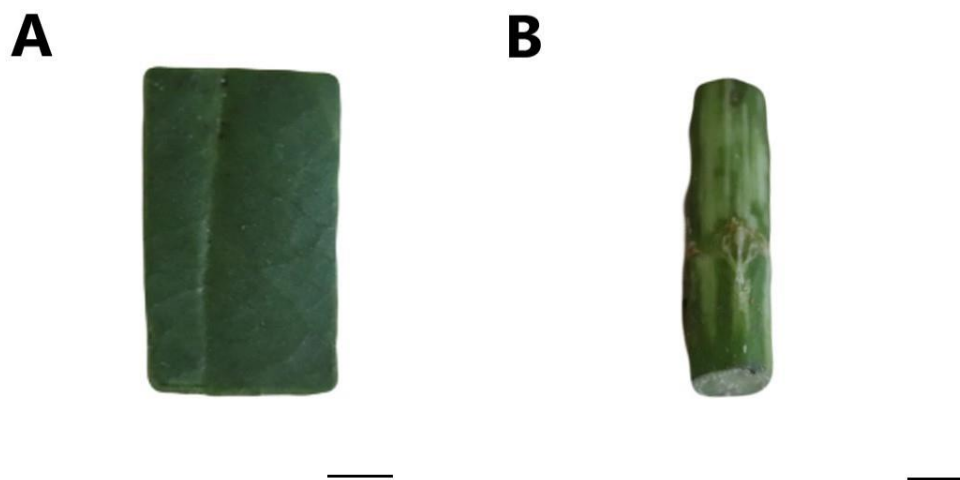


Figure 9: Leaves (A), and buds (B) used as explants for plant tissue culture. Scale bars = 0.3 cm in A, and 0.2 cm in B.

3.4.2 PRODUCTION OF STERILE SEEDLINGS THROUGH EMBRYO CULTURE

Embryos were harvested from fresh immature and mature seeds of five *Strychnos* fruits species. The fruits were collected from Palapye within the BIUST campus, DHMT and Pota post office, Makabi cattlepost near Thabala, Lecheng, and Old Palapye. Mature fruits were placed in the freezer at -20 °C for three months for storage. The immature fruits selected were about 6-7 cm in diameter. Embryo culture was performed following a method described by Kagithoju *et al.* (2013), with some modifications. Surface sterilization of the fruits was started by washing in running tap water containing liquid soap and rinsed for 5 minutes. They were treated with a broad-spectrum fungicide and bactericide mixture (5 g of Dithane M-45 and 5 g of virikop dissolved in 1 L of sterile distilled water) in sterile beakers for 5 minutes and then washed with 70 % ethanol for 2 minutes. The fruits were opened using a sterile scalpel inside the laminar flow. The seeds from each fruit were transferred into sterile beakers and then surface-sterilized for 20 minutes with 0.3 % commercial bleach (3.5 % m/v Sodium Hypochlorite) containing 0.2 % of Tween-20, with regular agitation at every 5 minutes for 20 minutes. After 20 minutes, the seeds were washed with 70% ethanol for 30 seconds and rinsed five times with sterile distilled water. The seeds were placed into sterile Petri dishes. They were dissected by cutting through the seed coat using a sterile scalpel and the embryos were carefully separated from the cotyledons (Figure 10).

The embryo was carefully scooped using the scalpel and put into a sterile Petri dish containing sterile distilled water. The embryos were transferred into Magenta GA7 boxes containing $\frac{1}{2}$ MS media. The media was slightly poked with the forceps to create an embryo insertion well. The embryo was carefully inserted into the well, facing upwards. In each Magenta box, 5 embryos were cultured, and the Magenta box was sealed with a parafilm. The cultures were placed inside a cupboard (in darkness) under room temperature for 5 days. After 5 days of darkness, they were placed in a plant growth chamber at 25 °C for 12 hours photoperiod with 60 % humidity and 11 % maximum light intensity. The germination period and germination percentage were recorded. A total of 25 embryos were cultured per species. The experiment was done in 5 replicates and 3 trials were done per species.

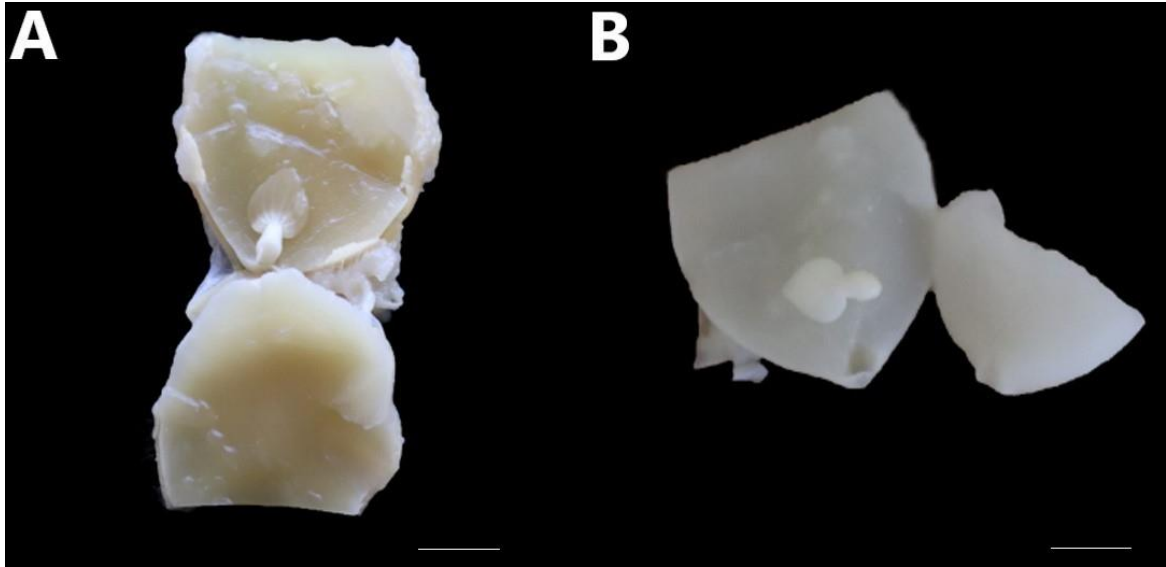


Figure 10: *Strychnos* embryos used for tissue culture. Embryo within the pocket (A) and outside the pocket (B). The cotyledons were cut open to expose and pick out the embryos. Scale bars = 0.4 cm in A, and 0.4 cm in B.

3.5 PRODUCTION OF NON-STERILE SEEDLINGS THROUGH CONVENTIONAL METHOD

For the establishment of seedlings for *Strychnos* species, *S. spinosa* seeds were collected from Lecheng (S 22°40'54.1" E 027°13'08.2") together with the soil sample (0.5 m radius from the plant). About 3 kg of the soil was transferred into a 25 cm diameter flowerpot. Before sowing, seeds were soaked in warm water for 24 hours. In each flowerpot, 14 seeds were sown at a depth of 3 cm using a method described by Mwamba, (2006). The pots were watered 3 times a week until germination. The germination data was recorded. The experiment was conducted in a net shade at the BIUST campus. The experiment was performed in triplicate.

3.6 STATISTICAL ANALYSIS

GraphPad Prism version 9.0.2 (San Diego, California, USA) was used to analyze the germination period and germination percentage data. The data was presented as mean $\pm$ S.E.M. Means of individual treatments were compared using a one-way ANOVA. Data was determined to be statistically significant if $P < 0.05$.

4.0 RESULTS

4.1 *Strychnos* SPECIES DIVERSITY

4.1.1 *Strychnos* SPECIES DIVERSITY BASED ON MORPHOLOGY

A total of 5 *Strychnos* species were identified morphologically using an identification key (Appendix A). These were *S. cocculoides*, *S. spinosa*, *S. pungens*, *S. innocua*, and *S. madagascariensis*.

4.2 *Strychnos* SPECIES DIVERSITY BASED ON MOLECULAR MARKERS

Genomic DNA extracted from each of the *Strychnos* species and 4 molecular markers (*ITS 2*, *trnS-trnG IGS*, *rbcL* and *matK*) were amplified using PCR. Amplified molecular marker fragments were sequenced and the size ranged from 246-790 bp. A homology search of the sequences was done using blastn search on NCBI database and the similarities ranged from 95-100 %. Phylogenetic trees of the *Strychnos* species were generated using all the four molecular markers. Phylogenetic trees generated using *ITS 2* sequences showed that the five *Strychnos* species studied are closely related to similar *Strychnos* species from other regions deposited on NCBI database.

4.2.1 GENOMIC DNA EXTRACTION

After extraction, genomic DNA of the five *Strychnos* species was run using agarose gel, viewed and imaged (Figure 11) through a UV transilluminator (GelDoc-It TS3 315 Imaging System). The DNA was then quantified using a nanodrop to determine the concentration and purity. Its concentration ranged from 5.0-96.4 µg/µL.

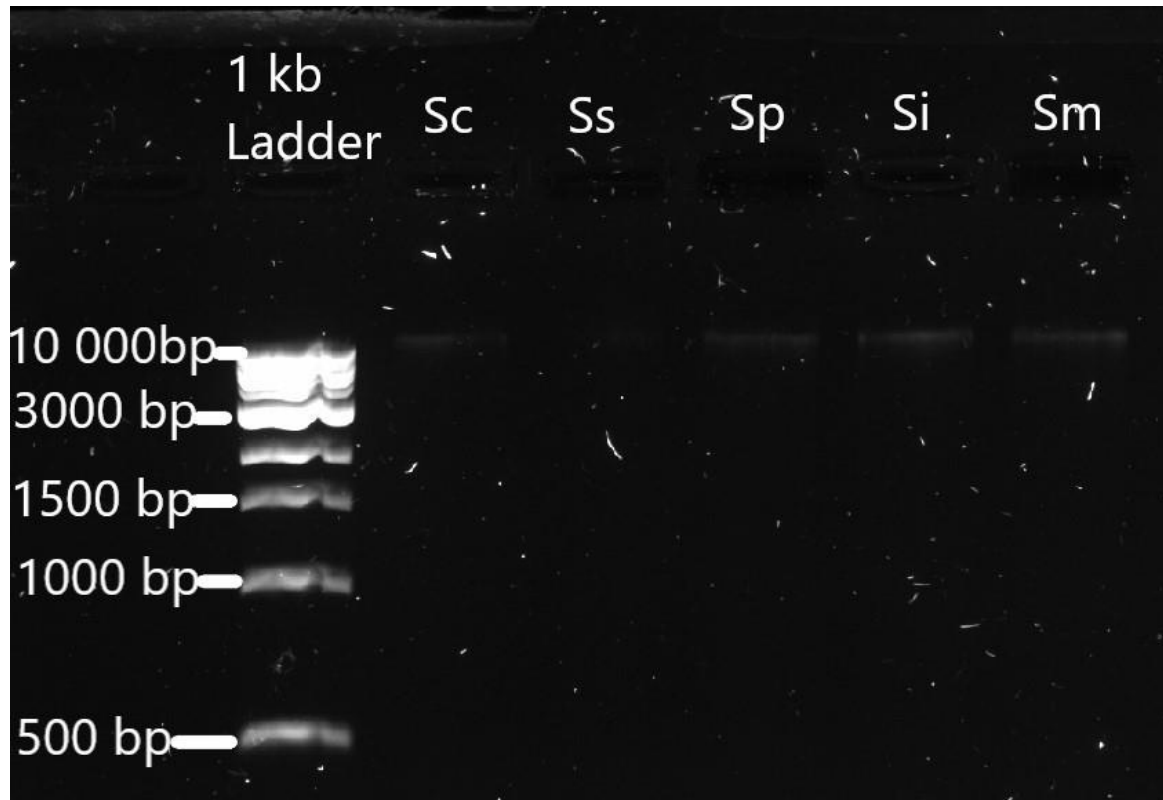


Figure 11: Genomic DNA of *Strychnos* species run the gel electrophoresis. Sc = *S. cocculoides*, Ss = *S. spinosa*, Sp = *S. pungens*, Si = *S. innocua*, and Sm = *S. madagascariensis*.

4.2.2 PCR AMPLIFICATION

From the genomic DNA, four molecular markers (*ITS 2*, *trnS-trnG IGS*, *rbcL*, and *matK*) were amplified in all the 5 species through PCR (Figure 12). The *ITS 2* primers used amplified a product of about 400 bp in all the 5 species (Figure 12A). The *trnS-trnG IGS* primers amplified a 450 bp fragment in 2 species (*S. cocculoides* and *S. spinosa*) and a 350 bp fragment in 3 species (*S. pungens*, *S. innocua* and *S. madagascariensis*) (Figure 12B). The *rbcL* and *matK* primers amplified 400 bp and 900 bp fragments respectively in all species (Figure 12C and 12D respectively).

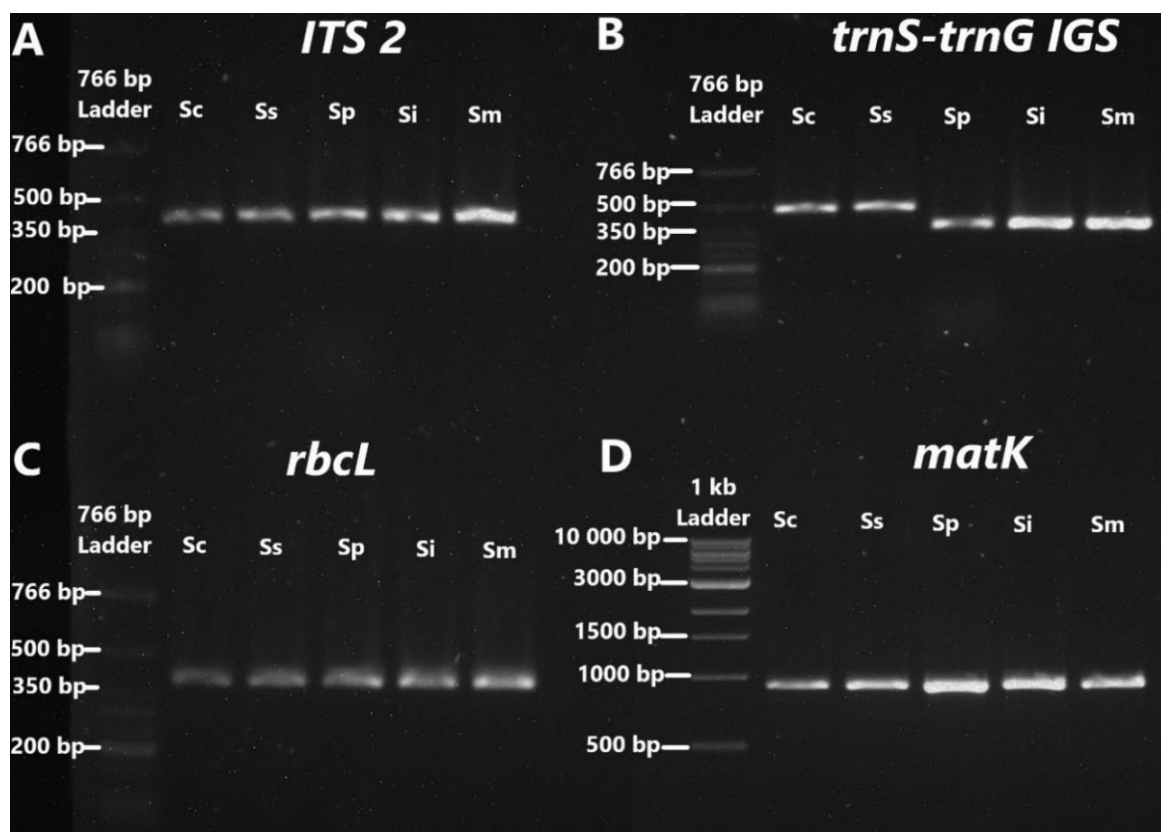


Figure 12: *ITS 2* (A), *trnS-trnG IGS* (B), *rbcL* (C), and *matK* (D) fragments amplified in *Strychnos* species through PCR.

4.2.3 *Strychnos* MOLECULAR MARKER SEQUENCES

The DNA fragments amplified from all the species using the *ITS 2*, *trnS-trnG IGS*, *rbcL* and *matK* primers were sequenced and the sequences were processed and uploaded to NCBI database. A total of 20 sequences were generated for the Botswana *Strychnos* species, of which 19 were new sequences and only 1 (*ITS 2* marker from *S. pungens*; MW381785) had already been generated by Adebowale *et al.* (2014) prior KC609313). The *ITS 2* primers produced 281 bp sequence in *S. cocculoides* (Accession number MW380872), 256 bp sequence in *S. spinosa* (Accession number MW381784), 299 bp sequence in *S. pungens* (Accession number MW381785), 318 bp sequence in *S. innocua* (Accession number MW381786), and 246 bp sequence in *S. madagascariensis* (Accession number MW381787). The *trnS-trnG IGS* primers produced 425 bp sequence in *S. cocculoides* (Accession number MW459102), 427 bp sequence in *S. spinosa* (Accession number MW459103), 321 bp sequence in *S. pungens* (Accession number MW459104), 321 bp sequence in *S. innocua* (Accession number MW459105), and 319 bp sequence in *S.*

madagascariensis (Accession number MW459106). The *rbcL* primers produced 354 bp sequence in *S. cocculoides* (Accession number MW504167), 354 bp sequence in *S. spinosa* (Accession number MW504168), 354 bp sequence in *S. pungens* (Accession number MW504168), 354 bp sequence in *S. innocua* (Accession number MW504169), and 354 bp sequence in *S. madagascariensis* (Accession number MW504170). The *matK* primers produced 745 bp sequence in *S. cocculoides* (Accession number MW504162), 745 bp sequence in *S. spinosa* (Accession number MW504163), 682 bp sequence in *S. pungens* (Accession number MW504164), 790 bp sequence in *S. innocua* (Accession number MW504165), and 745 bp sequence in *S. madagascariensis* (Accession number MW504166).

4.2.4 PERCENTAGE IDENTITY

All sequences from the five *Strychnos* species were analysed using NCBI nucleotide blast (BLASTn) which performed pairwise sequence alignments and calculated percentage identities. The comparison was done using all the four molecular markers. Table 7 summarizes the top hits (from NCBI database) and percentage identities of their alignment with the sequences generated in this study. The query coverage was 100% and percentage identity range was 95-100%.

Table 7: Percentage identity of the *ITS 2*, *trnS-trnG* IGS, *rbcL* and *matK* sequences in the *Strychnos* species with their NCBI matches

Gene	Species and Accession number	Percentage identity	Top hit	Reference
<i>ITS 2</i>	<i>S. cocculoides</i> (MW380872)	100	<i>S. cocculoides</i> (KC609290)	(Adebowale <i>et al.</i> , 2016b)
	<i>S. spinosa</i> (MW381784)	99.61	<i>S. spinosa</i> var <i>lokua</i> (KC609316)	(Adebowale <i>et al.</i> , 2016b)
	<i>S. pungens</i> (MW381785)	100	<i>S. pungens</i> (KC609313)	(Adebowale <i>et al.</i> , 2016b)
	<i>S. innocua</i> (MW381786)	95.59	<i>S. innocua</i> (JF937987)	(Frasier, 2008)
	<i>S. madagascariensis</i> (MW381787)	97.56	<i>S. madagascariensis</i> (KC609307)	(Adebowale <i>et al.</i> , 2016b)

<i>trnS-trnG</i> IGS	<i>S. cocculoides</i> (MW459102)	99.06	<i>S. cocculoides</i> (KM365179)	(Adebowale <i>et al.</i> , 2016a)
	<i>S. spinosa</i> (MW459103)	98.61	<i>S. spinosa</i> (KM365178)	(Adebowale <i>et al.</i> , 2016a)
	<i>S. pungens</i> (MW459104)	99.07	<i>S. pungens</i> (KM365166)	(Adebowale <i>et al.</i> , 2016a)
	<i>S. innocua</i> (MW459105)	97.51	<i>S. innocua</i> (KM365164)	(Adebowale <i>et al.</i> , 2016a)
	<i>S. madagascariensis</i> (MW459106)	96.88	<i>S. madagascariensis</i> (KM365163)	(Adebowale <i>et al.</i> , 2016a)
<i>rbcL</i>	<i>S. cocculoides</i> (MW504167)	100	<i>S. cocculoides</i> (JX573001)	(Maurina <i>et al.</i> , 2013)
	<i>S. spinosa</i> (MW504168)	100	<i>S. spinosa</i> (EU213526)	(Lahaye <i>et al.</i> , 2008)
	<i>S. pungens</i> (MW504169)	99.72	<i>S. pungens</i> (JF265612.1)	(Yessoufou <i>et al.</i> , 2013)
	<i>S. innocua</i> (MW504170)	99.72	<i>S. innocua</i> (KU568140)	(Charles-Dominique <i>et al.</i> , 2016)
	<i>S. madagascariensis</i> (MW504171)	99.72	<i>S. madagascariensis</i> (EU213525)	(Lahaye <i>et al.</i> , 2008)
<i>matK</i>	<i>S. cocculoides</i> (MW504162)	99.61	<i>S. cocculoides</i> (JX517336)	(Maurina <i>et al.</i> , 2013)
	<i>S. spinosa</i> (MW504163)	100	<i>S. spinosa</i> (EU214300)	(Lahaye <i>et al.</i> , 2008)
	<i>S. pungens</i> (MW504164)	100	<i>S. pungens</i> (KX911136)	(Yang <i>et al.</i> , 2016)
	<i>S. innocua</i> (MW504165)	99.75	<i>S. innocua</i> (KX146355)	(Charles-Dominique <i>et al.</i> , 2016)
	<i>S. madagascariensis</i> (MW504166)	100	<i>S. madagascariensis</i> (JF270952)	(Yessoufou <i>et al.</i> , 2013)

4.2.5 PAIRWISE PERCENTAGE IDENTITY OF THE FOUR MOLECULAR MARKERS AMONGST THE FIVE LOCAL *Strychnos* SPECIES

All the molecular marker sequences generated in this study were analysed using BioEdit which performed pairwise sequence alignments and calculated pairwise sequence identities.

Sequences from each molecular marker were compared amongst the different species. Table 8 summarizes the pairwise percentage identities, which ranged between 38 and 100 %. The percentage identities of the *matK* fragments ranged from 99.26 to 100 %. Fragments of *rbcl* were 99.15-100 % identical. *ITS 2* had a percentage identity range of 38.07-97.96 %. The percentage identity of the *trnS-trnG* IGS fragments was 67.39 to 100 %.

Table 8: Pairwise percentage identity of the four molecular markers among local five *Strychnos* species

Molecular marker		<i>S. cocculoides</i>	<i>S. spinosa</i>	<i>S. pungens</i>	<i>S. innocua</i>	<i>S. madagascariensis</i>
<i>matK</i>	Sc	100	99.85	99.26	99.26	99.26
	Ss	99.85	100	99.41	99.41	99.41
	Sp	99.26	99.41	100	100	100
	Si	99.26	99.41	100	100	100
	Sm	99.26	99.41	100	100	100
<i>rbcl</i>	Sc	100	100	99.15	99.15	99.15
	Ss	100	100	99.15	99.15	99.15
	Sp	99.15	99.15	100	100	100
	Si	99.15	99.15	100	100	100
	Sm	99.15	99.15	100	100	100
<i>ITS 2</i>	Sc	100	49.74	38.07	42.13	40.60
	Ss	49.74	100	38.07	40.10	40.60
	Sp	38.07	38.07	100	93.40	95.43
	Si	42.13	40.10	93.40	100	97.96
	Sm	40.60	40.60	95.43	97.96	100
<i>trnS-trnG</i> IGS	Sc	100	100	99.27	97.10	67.39
	Ss	100	100	99.27	97.10	67.39
	Sp	99.27	99.27	100	97.82	68.11
	Si	97.10	97.10	97.82	100	70.28
	Sm	67.39	67.39	68.11	70.28	100

4.2.6 PHYLOGENETIC ANALYSIS

Phylogenetic trees were constructed using the Maximum Parsimony method using Mega-X-10.1.8 (Kumar *et al.*, 2018). A phylogenetic analysis using the *ITS 2* sequences from the different species revealed that *S. cocculoides* and *S. spinosa* are more closely related than all the other species (Figure 13). *S. innocua* and *S. madagascariensis* are more closely related to each other than all the other species as well. *S. pungens* is closely related to *S.*

innocua and *S. madagascariensis* than *S. cocculoides* and *S. spinosa*. *ITS 2* fragments of *G. hansemanii* (FM204691.1) and *A. grandiflora* (DQ449916.1) were used as outgroups.

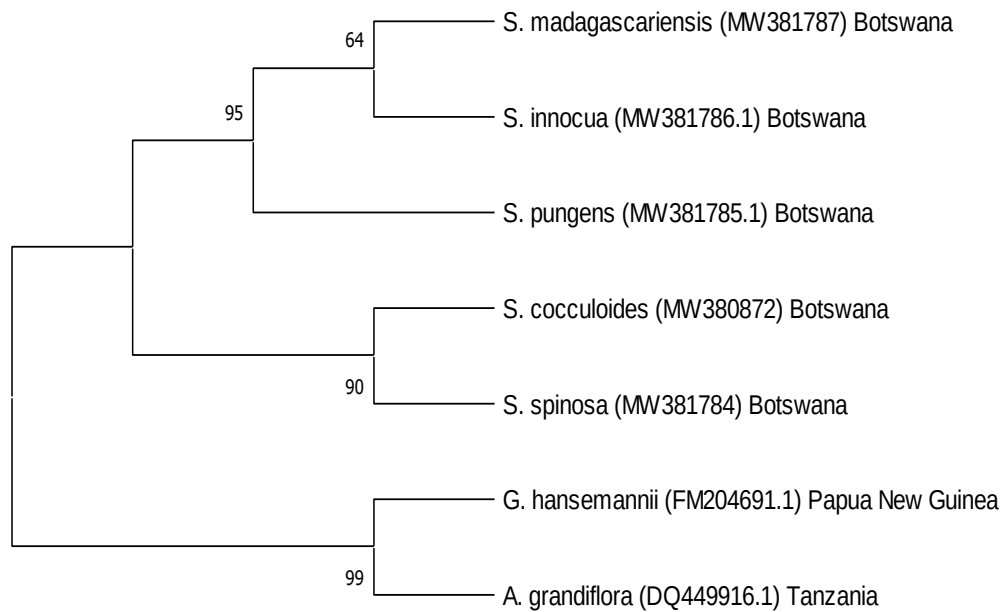


Figure 13: Phylogenetic tree composed of *ITS 2* fragments from five *Strychnos* species, *G. hansemanii* and *A. grandiflora*

Phylogenetic analysis for *trnS-trnG* *IGS* sequences from the different species revealed that *S. cocculoides* and *S. spinosa* are more closely related than all the other species (Figure 14). The *trnS-trnG* *IGS* fragments in *S. innocua* and *S. madagascariensis* are more closely related to each other than all the other species. The *trnS-trnG* *IGS* fragments of *G. hansemanii* (FM204763), and *A. grandiflora* (DQ449916) were used as outgroups.

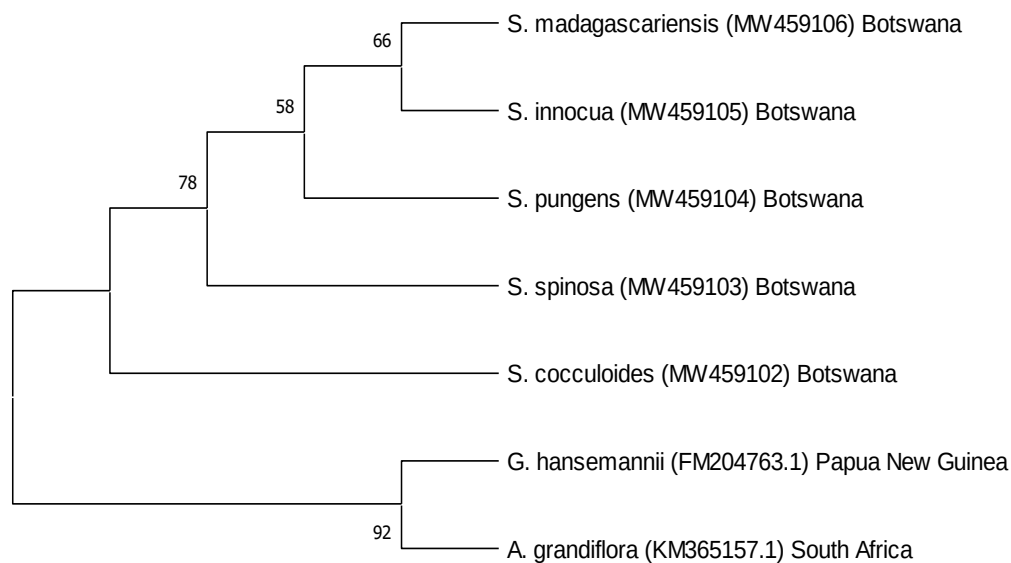


Figure 14: Phylogenetic tree composed of *trnS-trnG* IGS fragments from five *Strychnos* species, *G. hansemanii* and *A. grandiflora*

Phylogenetic analysis for *rbcL* sequences from the different species revealed that *S. madagascariensis* and *S. pungens* are more closely related than all the other species (Figure 15). *S. innocua* *rbcL* fragment is more closely related to *S. pungens* and *S. madagascariensis* than to *S. cocculoides* and *S. spinosa*. The *rbcL* fragments in *S. cocculoides* and *S. spinosa* are more closely related to each other than all the other species as well. The *rbcL* fragments of *G. hansemanii* (AJ318446) and *A. grandiflora* (JX572306) were used as outgroups.

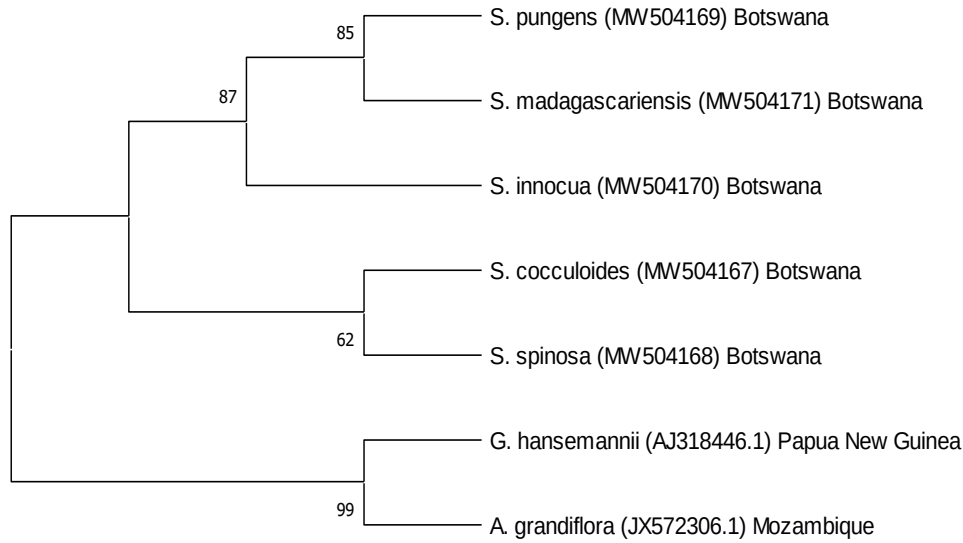


Figure 15: Phylogenetic tree composed of *rbcL* fragments from five *Strychnos* species, *G. hansemannii* and *A. grandiflora*

Phylogenetic analysis using the *matK* sequences from the different species revealed that *S. innocua* and *S. madagascariensis* are more closely related than all the other species (Figure 16). *S. pungens matK* is more closely related to *S. innocua* and *S. madagascariensis* than *S. cocculoides* and *S. spinosa*. *S. cocculoides* and *S. spinosa* are more closely related to each other than all the other species as well. The *matK* fragments of *G. hansemannii* (HM119537) and *A. grandiflora* (JX518238) were used as outgroups.

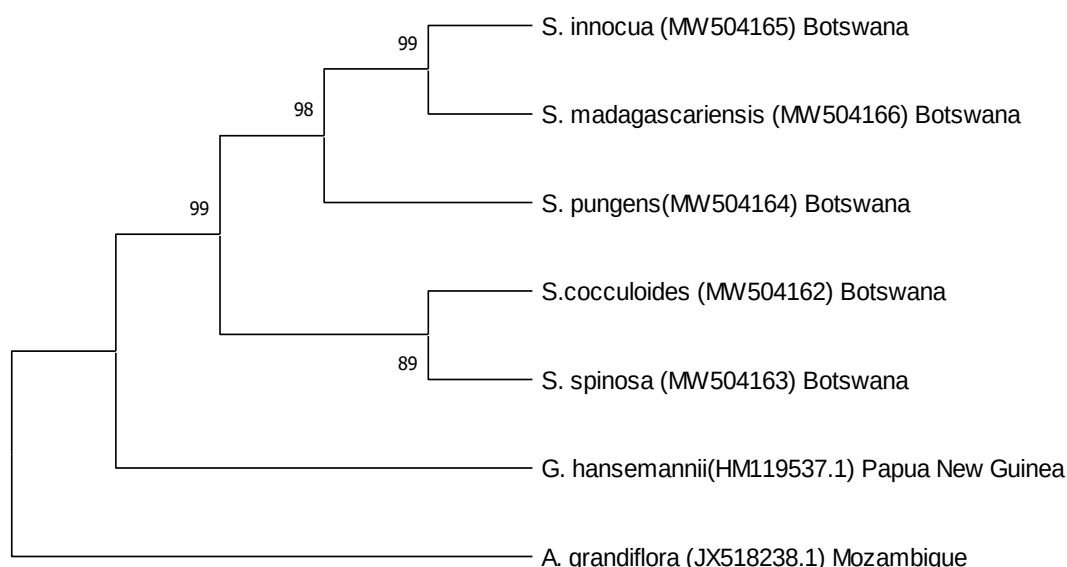


Figure 16: Phylogenetic tree composed of *matK* fragments from five *Strychnos* species, *G. hansemannii* and *A. grandiflora*

Phylogenetic analysis using 194 bp of *ITS 2* sequences from the different *Strychnos* species deposited on the NCBI database revealed *S. pungens* sequence (MW381785) is closely related to another *S. pungens* (KC609315.1) collected from South Africa (Figure 17). *S. cocculoides* (MW380872) is closely related to *S. cocculoides* (KC609290.1) from Namibia. *S. spinosa* (MW381784) is closely related to *S. spinosa* var. *lokua* (KC609316.1) collected from South Africa. *S. innocua* (MW381786) sequence is closely related to *S. innocua* (MN257667.1) collected from Tanzania. *S. madagascariensis* (MW381787) sequence is closely related to *S. madagascariensis* (KC609306.1) collected from South Africa. The *ITS 2* fragments of *G. hansemannii* (FM204691.1) and *A. grandiflora* (DQ449916.1) were used as outgroups.

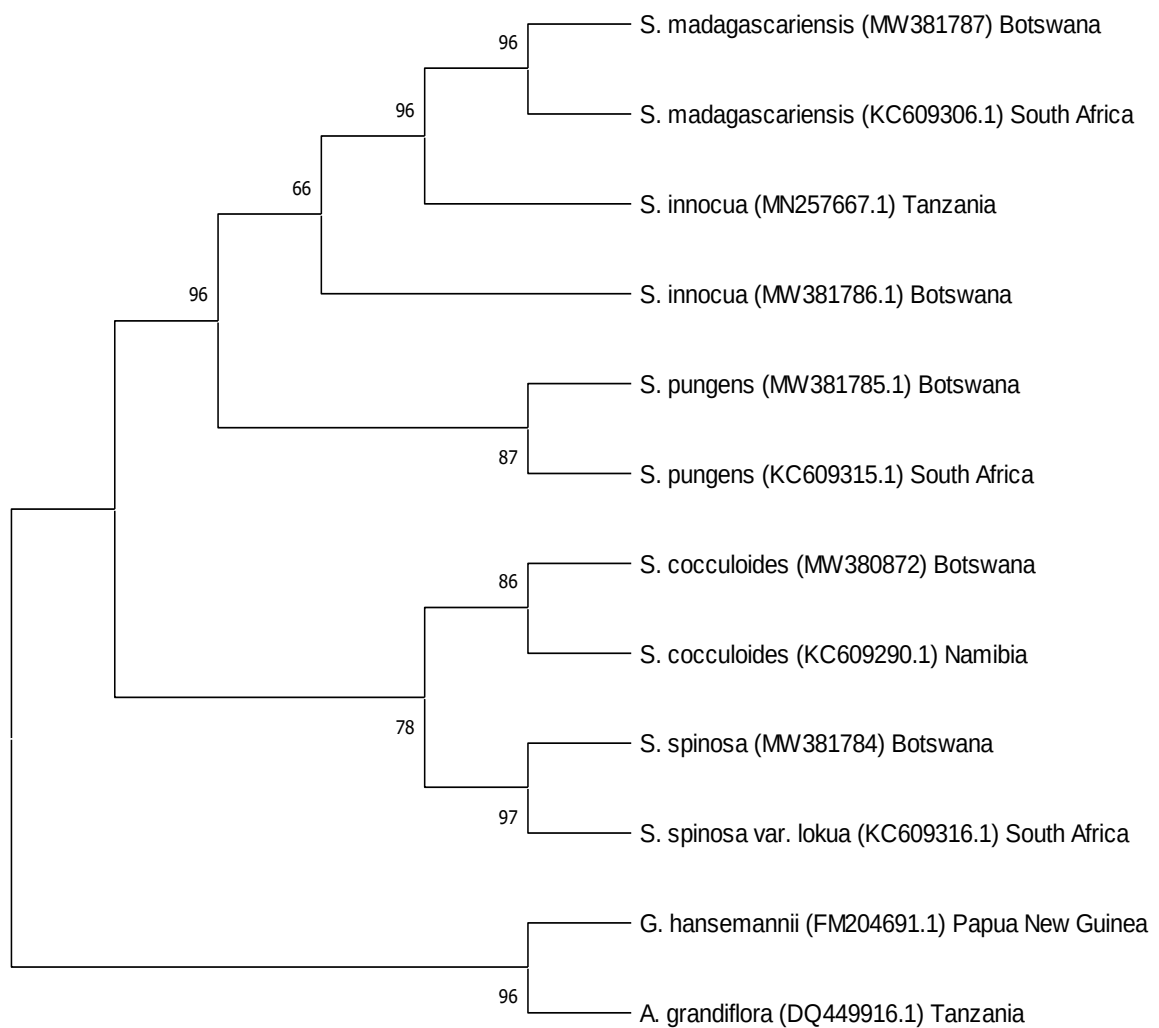


Figure 17: The *ITS* 2 fragment was compared to the available *ITS* 2 sequences from across the world, *G. hansemanii* and *A. grandiflora*.

4.3 *Strychnos* SPECIES DISTRIBUTION

All the 5 *Strychnos* species were identified in the Central District (Figure 18), while only 3 species were identified in the North-East District. These were *S. cocculoides*, *S. innocua* and *S. madagascariensis*.

In the Serowe-Palapye Sub-district, all the five *Strychnos* species were found (Table 9 and Figure 18). The species found were *S. cocculoides*, *S. spinosa*, *S. pungens*, *S. innocua*, and *S. madagascariensis*. Table 9 below indicates the location and coordinates of *Strychnos* species found in the Serowe-Palapye Sub-district.

Table 9: Distribution of *Strychnos* species in Serowe-Palapye Sub-district

SPECIES	LOCATION	COORDINATES
<i>S. cocculoides</i>	Pota post office, Palapye	S 22°32'34.3" E 027°06'00.0"
	BIUST campus	S 22°35'43.2" E 027°07'27.9"
	Along Serowe-Palapye tarred road	S 22°28'42.4" E 026°54'34.4"
	Serowe village	S 22°22'33.6" E 026°39'52.5"
	Serowe-Mogorosi road	S 22°22'17.4" E 026°38'05.4"
	Makabi cattle post, 12 km west of Thabala village	S 22°27'10.3" E 026°22'23.1"
	Setekwane cattle post, 20 km north of Mogorosi	S 22°27'10.3" E 026°22'23.1"
	Near Khama Rhino Sanctuary	S 22°06'09.6" E 026°35'06.1"
	Near Makoba gate	S 21°46'26.9" E 025°58'56.3"
	Near Mmashoro	S 22°03'14.1" E 026°32'53.7"
	Paje village	S 22°15'54.3" E 026°45'12.0"
<i>S. spinosa</i>	BIUST campus	S 22°36'21.4" E 027°07'32.9"
	Along Serowe-Palapye tarred road	S 22°29'39.2" E 026°56'26.0"
	Mogorosi	S 22°25'11.8" E 026°34'06.9"
	Paje	S 22°15'58.0" E 026°47'31.9"
	Mokgware	S 22°45'36.2" E 26°47'50.9"
	Old Palapye	S 22°38'55.1" E 027°21'07.6"
	Lecheng	S 22°38'04.9" E 027°10'58.9"
	Seolwane	S 22°39'14.3" E 027°41'56.7"
	Near Moeng College	S 22°41'26.9" E 027°35'48.3"
<i>S. pungens</i>	Makabi cattle post, 12 km west of Thabala village	S 22°27'51.8" E 026°23'07.5"
	Serowe	S 22°22'31.5" E 026°39'42.9"
	Old Palapye	S 22°37'51.5" E 027°20'59.3"

	Along Paje-Khama Sanctuary Road	S 22°09'28.9" E 026°39'23.5"
	Paje village	S 22°15'48.8" E 026°45'12.7"
<i>S. innocua</i>	BIUST campus	S 22°35'41.4" E 027°07'27.9"
	Serowe	S 22°22'30.8" E 026°39'41.6"
	Seolwane	S 22°39'14.7" E 027°41'55.0"
	Lecheng	S 22°40'38.3" E 027°13'12.4"
<i>S. madagascariensis</i>	BIUST campus	S 22°35'41.7" E 027°07'27.9"
	Lotsane ward, Palapye	S 22°34'12.1", E 027°07'20.7"
	Mokgware	S 22°45'32.9" E 26°47'50.4"
	10 km north-west of Radisele	S 22°44'32.2", E 026°58'45.3"
	Malaka	S 22°36'15.8" E 027°17'46.4"
	Lecheng	S 22°40'38.3" E 027°13'11.7"
	Mokungwane	S 22°42'30.8" E 027°16'34.4"
	Matlhakola	S 22°43'01.8" E 027°18'51.5"
	Seolwane	S 22°42'08.1" E 027°39'11.5"
	Near Moeng College	S 22°41'09.4" E 027°34'48.7"
	Near Paje village	S 22°16'01.2" E 026°45'10.6"
	Paje village	S 22°15'59.0" E 026°47'31.2"

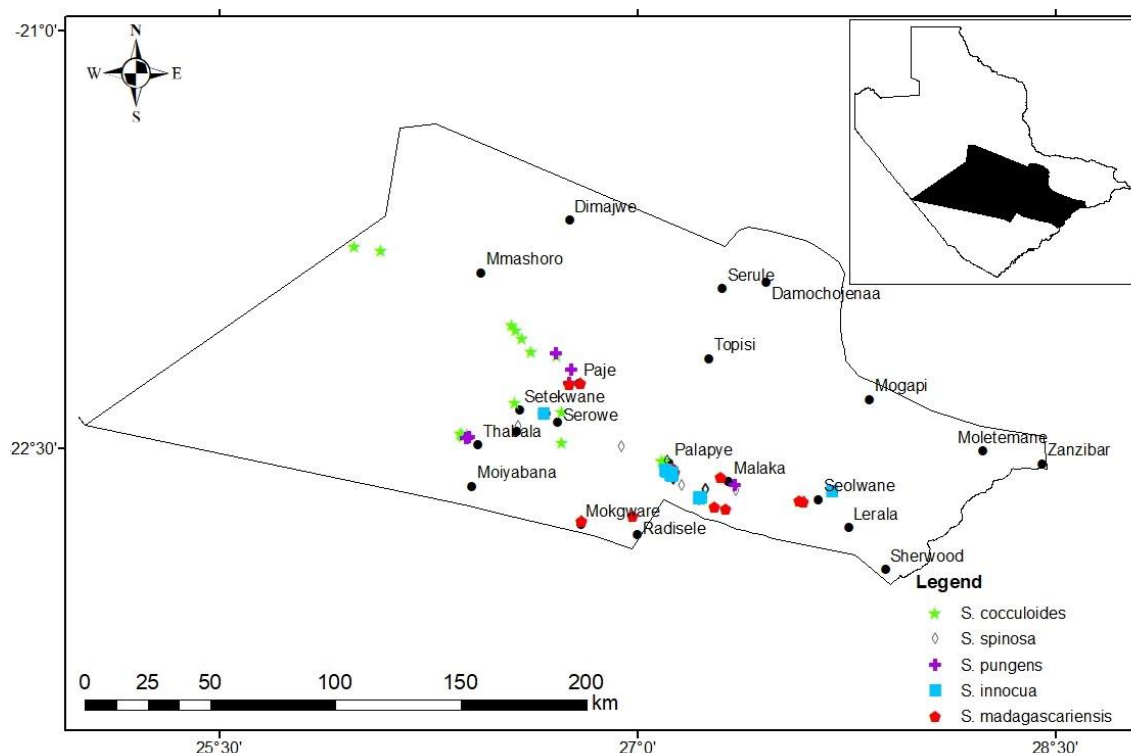


Figure 18: *Strychnos* species distribution in the Serowe-Palapye Sub-district, Central District, Botswana

In the Bobonong Sub-district, three *Strychnos* species were found (Table 10 and Figure 19). These are *S. spinosa*, *S. innocua*, and *S. madagascariensis*. Table 10 below shows the location and coordinates of the *Strychnos* species at Bobonong Sub-district.

Table 10: Distribution of *Strychnos* species in Bobonong Sub-district

SPECIES	LOCATION	COORDINATES
<i>S. spinosa</i>	Makwale fields 17.6 km south of Molalatau,	S 22°09'05.1" E 028°35'49.4"
	Thune dam	S 21°55'46.2" E 027°48'31.9"
	Matshekge hills	S 22°11'33.1" E 028°52'43.5"
<i>S. innocua</i>	Mothajwa cattle post 13 km north-west of Molalatau village	S 22°10'10.1" E 028°35'03.7"

	Makwale lands 12 km south-east of Molalatau	S 22°08'56.7" E 028°35'44.1"
	Molalatau-Tsetsejwe Gravel road	S 22°07'33.5" E 028°35'15.9"
	Seleptswe riverbank	S 22°12'27.9" E 028°40'44.4"
	Along Thune-Mathathane old, tarred road	S 22°11'08.4" E 028°41'48.4"
	Mathathane village	S 22°15'53.0" E 028°44'27.4"
	Phakwe hills 33.5 km north-west of Tsetsebjwe village	S 22°16'57.0" E 028°17'01.7"
	Thune dam	S 22°09'46.6" E 028°41'04.2"
	Kopje 5.2 km north-west of Mathathane	S 22°13'38.2" E 028°45'03.2"
	Mokakatedi	S 22°10'32.2" E 028°42'59.8"
	Lentswelemoriti	S 22°12'08.8" E 029°02'16.0"
	Mothabaneng	S 22°11'15.5" E 028°52'47.4"
<i>S. madagascariensis</i>	Mmadinare	S 21°55'46.2" E 027°48'31.9"
	Mmadiphala lands near Bobonong	S 22°03'50.3" E 028°16'56.8"
	Mabolwe village	S 21°49'17.8" E 028°50'08.3"
	Mmadale fields 15.2 km north-west of Mathathane village	S 22°09'21.6" E 028°43'02.7"
	Thune dam	S 22°09'53.2" E 028°41'37.6"
	Mathathane village	S 22°15'53.0" E 028°44'27.4"
	Along Seleptswe river 13.1 km near Mathathane village	S 22°12'27.9" E 028°40'44.4"
	Mothabaneng village	S 22°10'54.8" E 028°52'02.6"
	Mashatu Game Reserve	S 22°12'20.6" E 029°06'52.9"
	Phakwe hill 33.5 km north-west of Tsetsebjwe village	S 22°16'44.2" E 028°16'54"

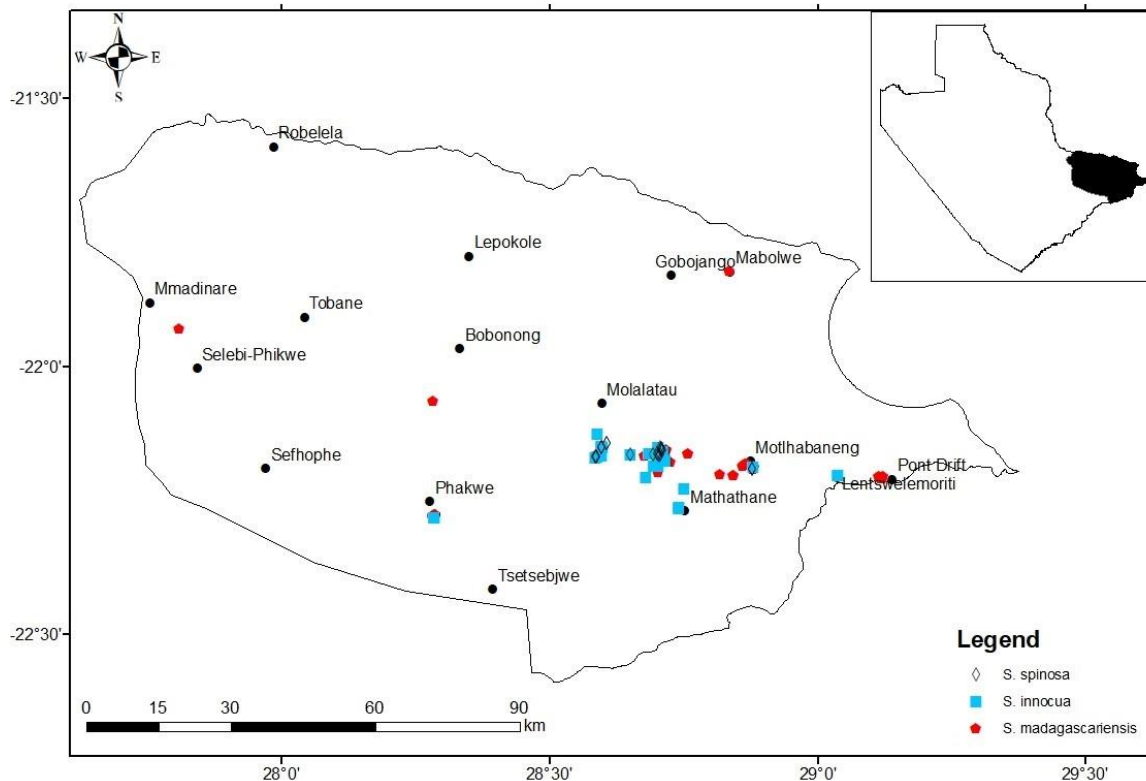


Figure 19: *Strychnos* species distribution in the Bobonong Sub-district, Central District, Botswana.

In the Tutume Sub-district, only two species (*S. innocua* and *S. madagascariensis*) were found (Table 11 and Figure 20). Table 11 below shows the specific location and coordinates of *Strychnos* species in Tutume Sub-district.

Table 11: Distribution of *Strychnos* species in Tutume Sub-District

SPECIES	LOCATION	COORDINATES
<i>S. innocua</i>	Matsitama junction	S 20°48'44.1" E 027°02'36.6"
	Along Tutume-Nkange road	S 20°25'45.7" E 027°03'24.7"
	Changate	S 20°18'58.5" E 027°11'52.5"
	Nswaswi junction	S 20°39'51.1" E 027°06'36.4"
<i>S. madagascariensis</i>	Matsitama junction	S 20°48'49.1" E 027°02'34.7"

	Near Tati Siding	S 21°19'21.5" E 027°28'30.9"
	Near Marapong	S 20°51'37.4" E 027°06'32.2"
	Sebina junction	S 20°51'32.5" E 027°11'38.1"
	Along Tutume-Nkange road	S 20°27'44.9" E 027°02'32.0"
	Changate	S 20°18'58.5" E 027°11'52.4"
	Nswaswi junction	S 20°39'50.6" E 027°06'43.3"
	Dagwi	S 20°14'13.8" E 027°15'30.1"

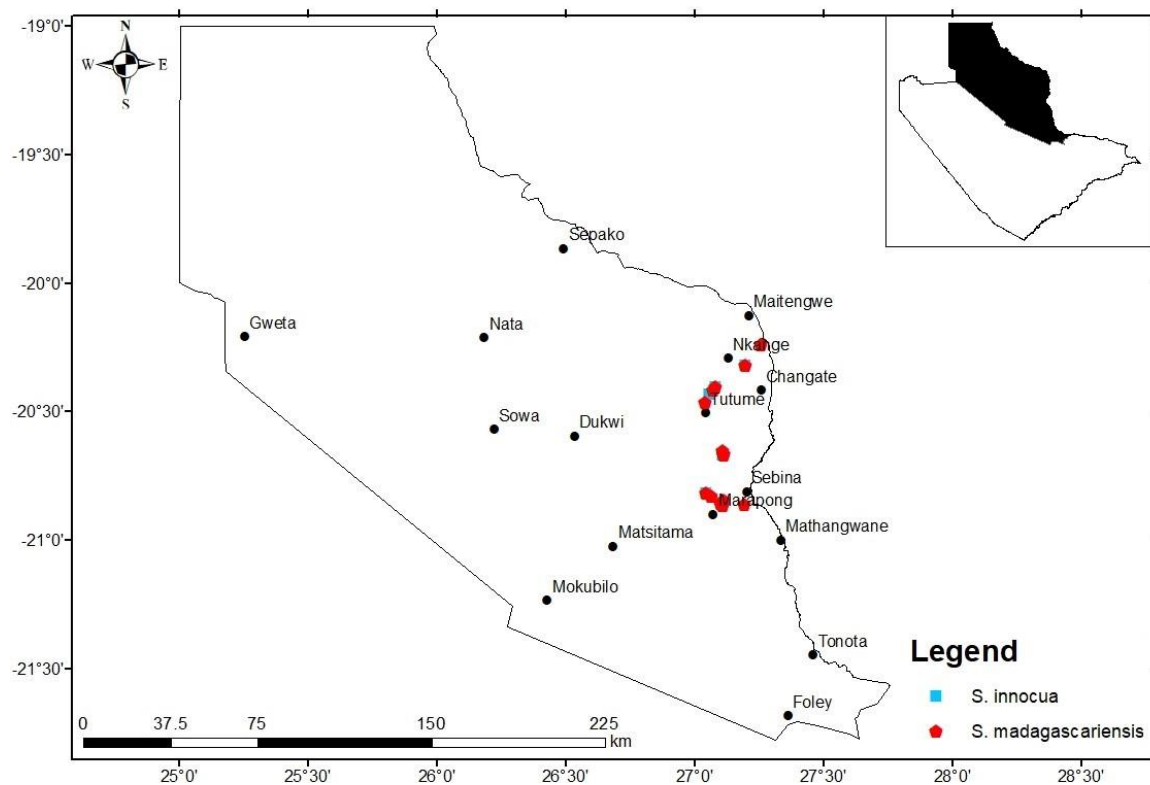


Figure 20: *Strychnos* species distribution in the Tutume Sub-district, Central District, Botswana.

In the Boteti Sub-district, only *S. cocculoides* was found (Table 12 and Figure 21). The location and coordinates are depicted by table 12 below.

Table 12: Distribution of *Strychnos* species in Boteti Sub-District

SPECIES	LOCATION	COORDINATES
<i>S. cocculoides</i>	Ditawana cattle post, 39.3 km near Letlhakane village	S 21°25'47.4" E 025°53'19.6"
	Khwee junction	S 21°33'58.5" E 025°41'15.7"
	Mokoba gate	S 21°45'24.3" E 025°56'37.9"

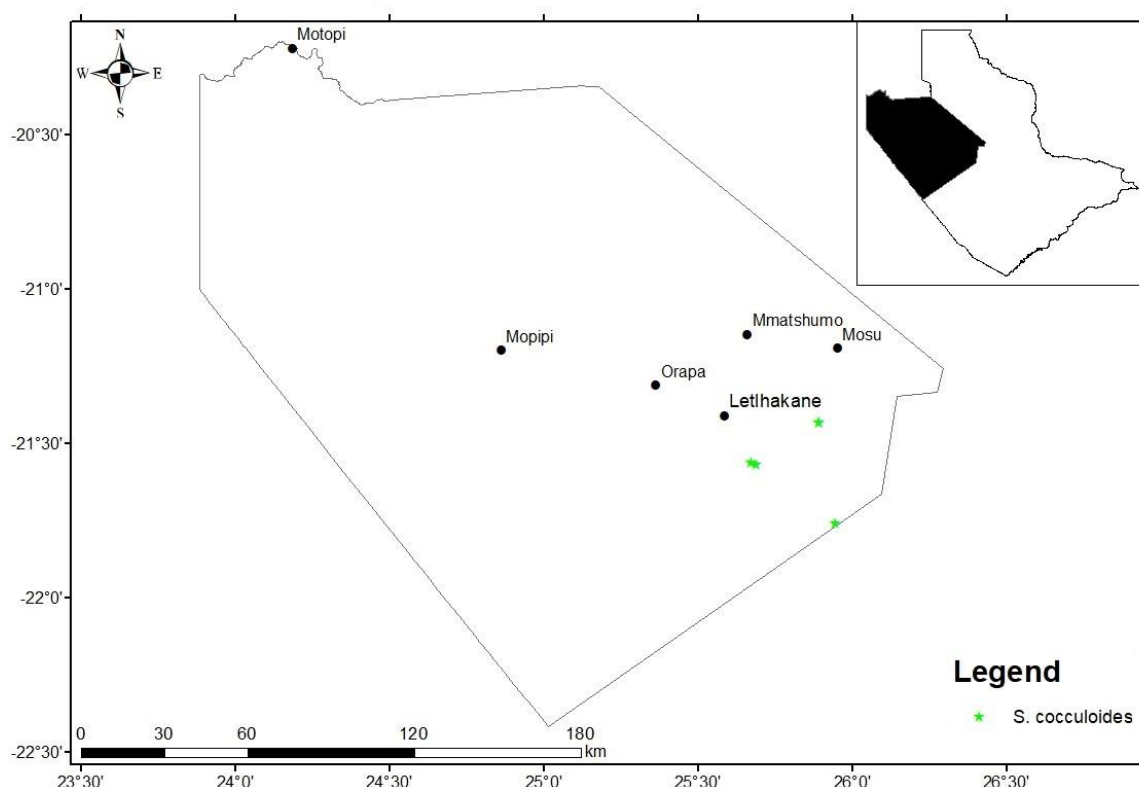


Figure 21: *Strychnos* species distribution in the Boteti Sub-district, Central District, Botswana.

In the Mahalapye Sub-district, three *Strychnos* species were found (Table 13 and Figure 22). These are *S. spinosa*, *S. innocua* and *S. madagascariensis*. Table 13 below shows the location and coordinates of *Strychnos* species in Mahalapye Sub-district.

Table 13: Distribution of *Strychnos* species in Mahalapye Sub-District

SPECIES	LOCATION	COORDINATES
<i>S. spinosa</i>	Mhalapitsa village	S 22°47'38.0" E 027°13'48.0"

	Ramokgonami	S 22°52'2.1" E 027°24'53.9"
	Tumasera-Seleka	S 22°52'57.6" E 027°31'44.5"
	Maapelo	S 22°50'01.7" E 027°23'10.6"
	Along Ramokgonami-Sefhare tarred road 2.35 km from Ramokgonami village	S 22°52'38.2" E 027°24'13.3"
	Sefhare	S 23°01'02.7" E 027°31'39.5"
<i>S. innocua</i>	Along Ramokgonami-Sefhare tarred road 3.30 km from Ramokgonami village	S 22°53'10.4" E 027°24'08.3"
	Along Ramokgonami-Sefhare tarred road 2.28 km from Moshopha village	S 22°56'32.3" E 27°23'32.3"
	Sefhare	S 23°00'37.8" E 27°31'28.8"
<i>S. madagascariensis</i>	Mhalapitsa	S 22°48'18.2" E 027°14'08.0"
	Ramokgonami village	S 22°52'0.3" E 027°24'55.9"
	Along Ramokgonami-Sefhare tarred road 3.58 km from Ramokgonami village	S 22°53'10.4" E 27°24'08.3"
	Along Ramokgonami-Sefhare tarred road 2.98 km from Ramokgonami village	S 22°53'08.9" E 27°24'08.4"
	Sefhare	S 23°00'54.1" E 27°31'32.8"

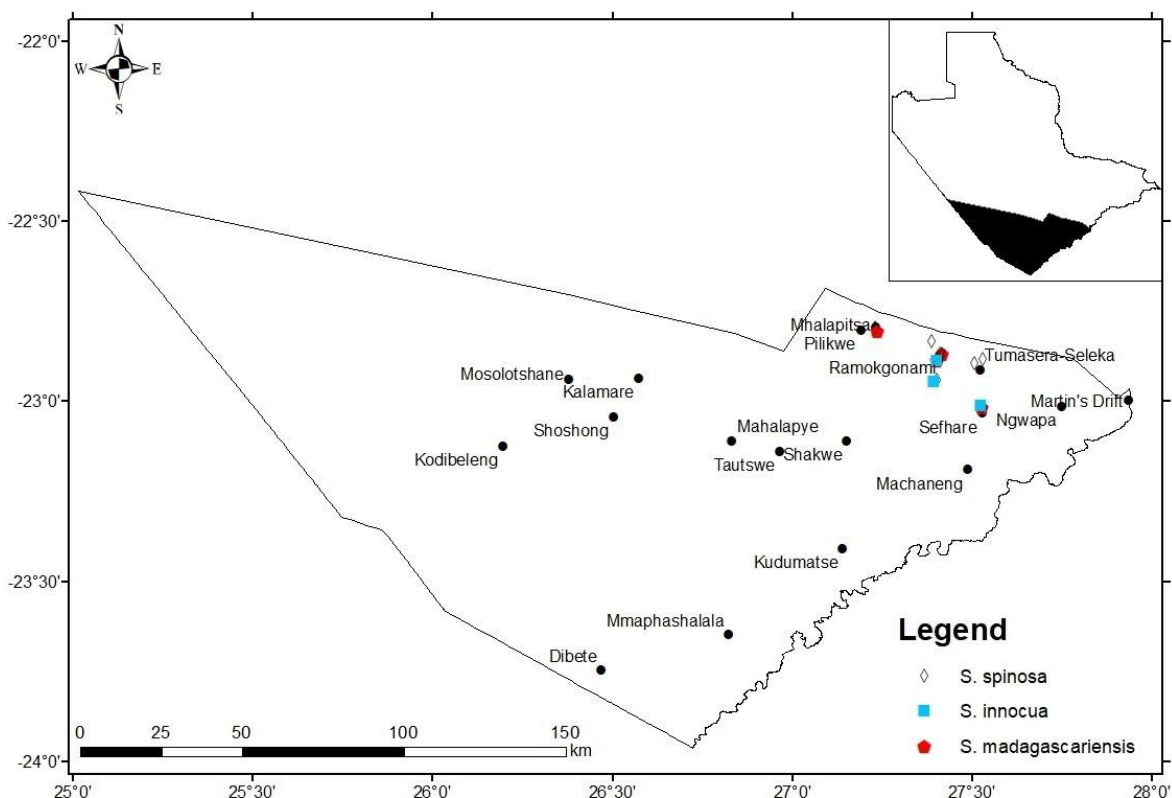


Figure 22: *Strychnos* species distribution in the Mahalapye Sub-district, Central District, Botswana.

The North-East District had 3 species, and these were *S. cocculoides*, *S. innocua* and *S. madagascariensis* (Table 14 and Figure 23). Table 14 below depicts the location and coordinates of *Strychnos* species in North-East District.

Table 14: Distribution of *Strychnos* species in North-East District

SPECIES	LOCATION	COORDINATES
<i>S. cocculoides</i>	Tshesebe	S 20°46'07.3" E 027°35'39.8"
	Mosojane	S 20°37'20.0" E 027°30'50.8"
	Mapoka	S 20°30'06.9" E 027°35'14.8"
<i>S. innocua</i>	Mabudzani	S 20°54'42.7" E 027°37'28.0"
	Butale	S 20°40'57.0" E 027°41'36.6"
	Tshesebe	S 20°42'53.8" E 027°35'23.5"
	Kalakamati	S 20°37'36.9" E 027°18'29.2"

	Mbalambi	S 20°30'09.5" E 027°20'49.8"
<i>S. madagascariensis</i>	Near Tati Siding	S 21°19'21.5" E 027°28'30.9"
	Mabduzani	S 20°54'42.8" E 027°37'28.2"
	Mowana	S 20°48'36.8" E 027°37'12.8"
	Butale	S 20°41'09.6" E 027°41'53.1"
	Kalakamati	S 20°37'54.5" E 027°18'37.6"
	Domboshaba	S 20°36'02.7" E 027°21'22.8"
	Moroka	S 20°31'47.9" E 027°37'52.0"
	Mapoka	S 20°30'04.4" E 027°35'13.0"
	Mbalambi	S 20°29'41.3" E 027°19'39.6"
	Nlapkhwani	S 20°30'33.0" E 027°30'08.1"

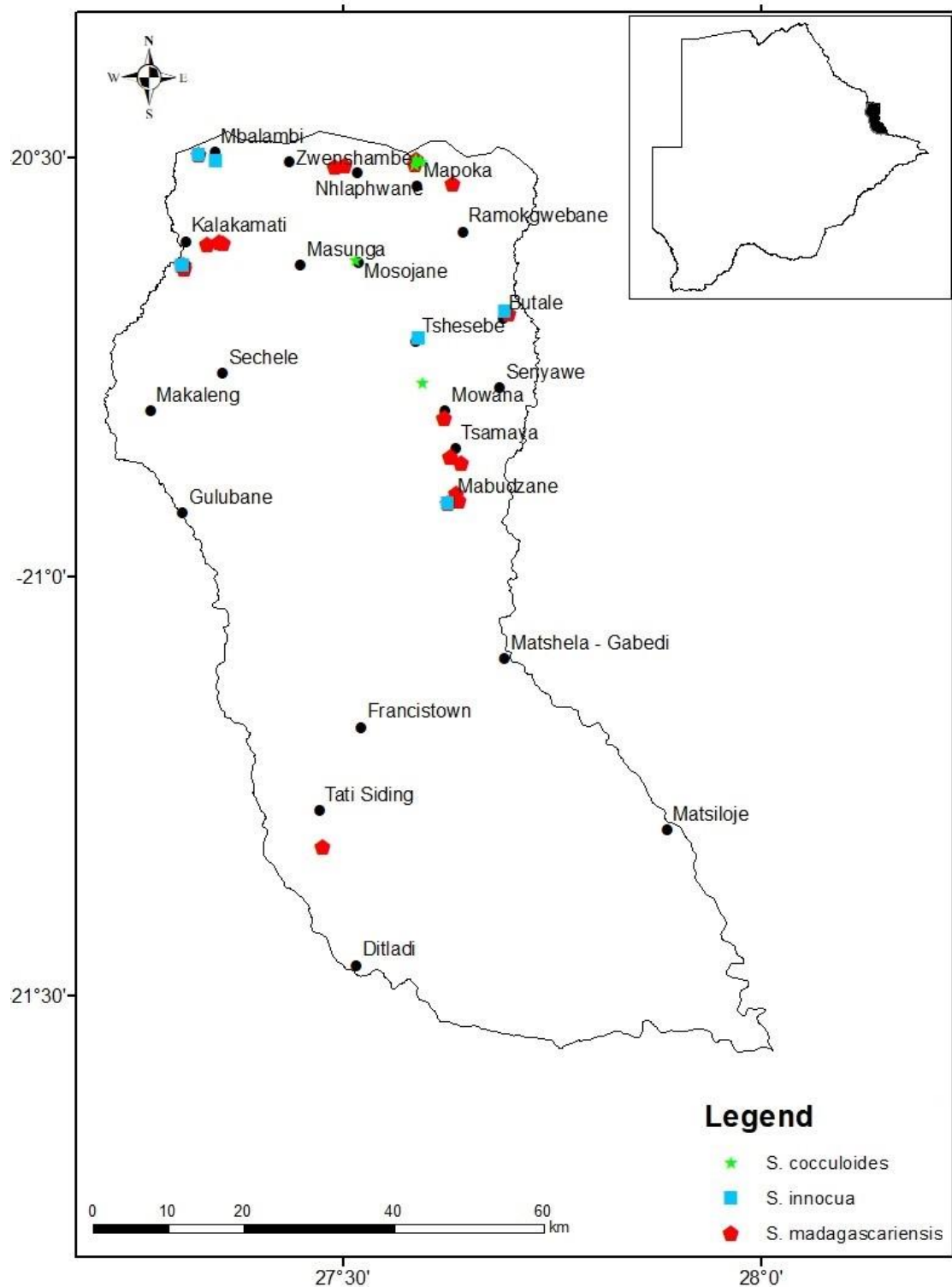


Figure 23: *Strychnos* species distribution in the North-East District, Botswana

4.3.1 SPECIES DIVERSITY, ABUNDANCE, AND DISTRIBUTION WITHIN A POPULATION

Phakwe hill had 2 species and was *S. madagascariensis* and *S. innocua*. A total of 114 trees of *S. madagascariensis* and 3 trees of *S. innocua* were identified and marked with a GPS device (Figure 24). The population is secluded and covered a small area of about 0.561 km². The population consists of 3 large trees of *S. innocua* with an approximate height of 5-8 m. The population consisted of a healthy and flourishing mixed population of *S. madagascariensis* which is made up of seedlings, medium and large trees with an approximate height of 12 m.



Figure 24: *Strychnos* species distribution at Phakwe hill, Bobonong Sub-district, Central District.

4.4 PRODUCT DEVELOPMENT

Five fruit jams were produced from five *Strychnos* species. Assessors showed differential preference of two selected *Strychnos* jams when compared with some of the selected commercially produced jams.

4.4.1 JAM PRODUCTION

Five *Strychnos* species fruit jams were produced and packaged (Figure 25). *S. cocculoides* produced a dark brown jam (Figure 25A) and *S. spinosa* jam (Figure 25B) was light brown to orange. *S. pungens* (Figure 25C), *S. innocua* (Figure 25D), and *S. madagascariensis* (Figure 25E) jams were orange to yellowish.



Figure 25: Jam from *S. cocculoides* (A), *S. spinosa* (B), *S. pungens* (C), *S. innocua* (D), and *S. madagascariensis* (E) respectively. Scale bars = 1.4 cm in A, 01.4 cm in B, 1.4 cm in C, 1.4 cm in D, and 01.4 cm in E.

4.4.2 JAM SENSORY EVALUATION

Descriptive sensory analysis of the fruit jams processed from five *Strychnos* species was done (Figure 26). Assessors significantly (0.2823) preferred the appearance of *S. cocculoides* (4.77 ± 0.49), and *S. spinosa* (4.50 ± 0.52), over that of *S. pungens* (4.09 ± 0.46), *S. innocua* (4.00 ± 0.44), and *S. madagascariensis* (3.54 ± 0.52). There is no significant difference ($P = 0.2823$, $R^2 = 0.05851$) in the appearance of jams from the five species. Assessors significantly (0.5454) preferred the aroma of *S. pungens* (6.05 ± 0.53) and followed by *S. innocua* (5.91 ± 0.63) over that of *S. madagascariensis* with (5.72 ± 0.61), *S. cocculoides* (5.50 ± 0.56), and lastly *S. spinosa*. There is no significant difference ($P = 0.5454$, $R^2 = 0.02859$) in the aroma of jams from the five species (4.77 ± 0.51). The assessors significantly (0.2200) preferred the taste of *S. cocculoides* (6.86 ± 0.40), and *S. madagascariensis* with 5.91 ± 0.39 , over that of *S. spinosa* (5.77 ± 0.45), *S. pungens* (5.77 ± 0.45), and *S. innocua* (5.27 ± 0.56). There is no significant difference ($P = 0.2200$, $R^2 = 0.05265$) in the taste of jams from the five species. Assessors evaluated the sweetness of the jams and significantly (0.0255) detected high sweetness levels in *S. cocculoides* (6.82 ± 0.45), and *S. spinosa* (6.50 ± 0.45) over than of *S. madagascariensis* (6.32 ± 0.45), *S.*

innocua (6.01 ± 0.43), and *S. pungens* (4.59 ± 0.42). There is a significant difference ($P = 0.0255$, $R^2 = 0.1003$) in the sweetness of jams from the five species. Assessors significantly (0.0049) detected the aftertaste of *S. innocua* (5.05 ± 0.60), and *S. madagascariensis* (3.91 ± 0.56) over that of *S. pungens* (3.77 ± 0.65), *S. spinosa* (2.86 ± 0.47), and *S. cocculoides* (2.41 ± 0.37). There is a significant difference ($P = 0.049$, $R^2 = 0.1322$) in the aftertaste of jams from the five species. Assessors significantly (0.2779) detected the high levels of viscosity (thin/thick), of *S. pungens* (6.01 ± 0.54), and *S. cocculoides* (5.95 ± 0.41) over that of *S. innocua* (5.77 ± 0.37), then *S. madagascariensis* (5.00 ± 0.49), and *S. spinosa* (4.95 ± 0.50). There is no significant difference ($P = 0.2799$, $R^2 = 0.04871$) in the viscosity (thin/thick) of jams from the five species. Assessors significantly preferred the spreadability of *S. spinosa* (8.69 ± 0.17) and *S. cocculoides* (7.62 ± 0.36) over that of *S. madagascariensis* (6.77 ± 0.45), *S. innocua* (6.31 ± 0.45), and *S. pungens* (4.62 ± 0.61). There is a significant difference ($P = 0.001$, $R^2 = 0.4453$) in the spreadability of jams from the five species. Error bars represent Standard Error of Means (S.E.M) ($n \geq 22$). Means with the same letters are not significantly different at ($P > 0.05$).

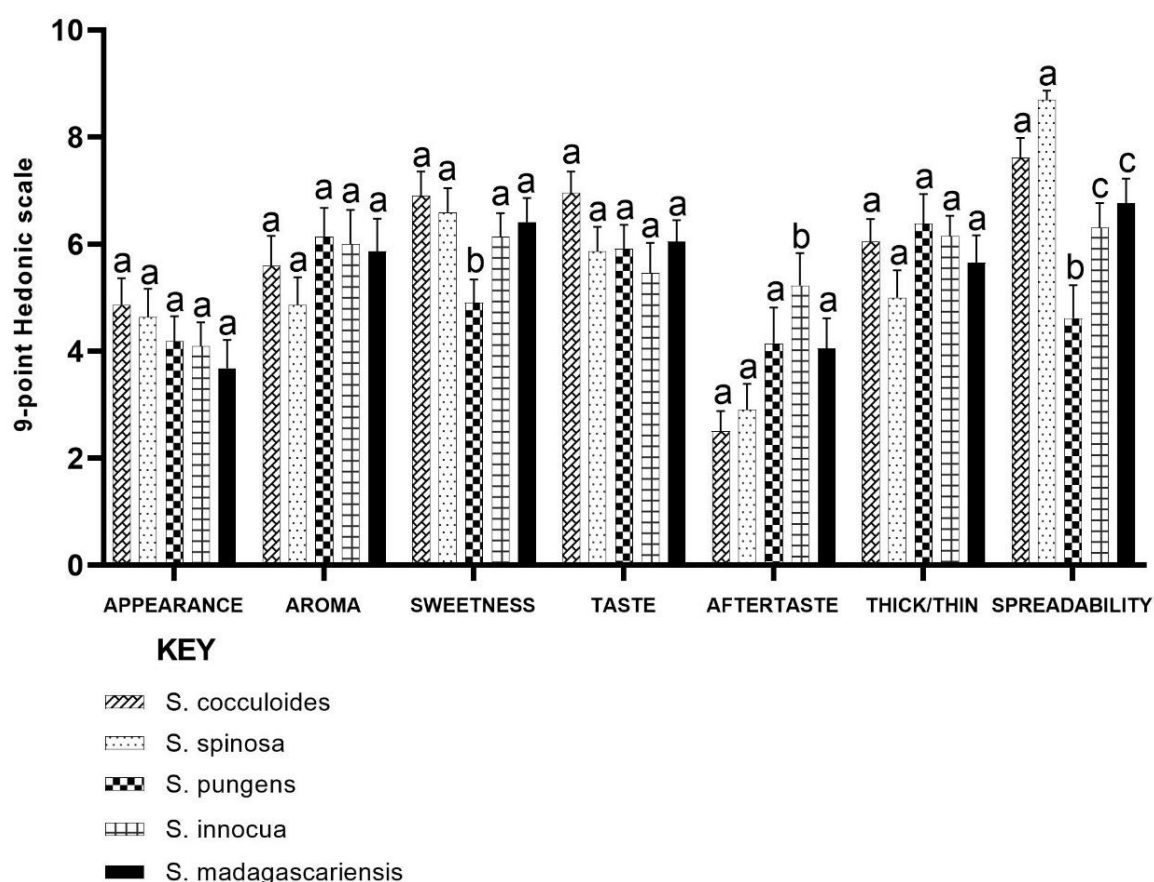


Figure 26: Descriptive Sensory analysis of the five *Strychnos* jams.

There was no significant difference ($P = 0.5759$, $R^2 = 0.03346$) in the preference ranking of the *Strychnos* jams. However, *S. spinosa* jam scored 2.68 ± 0.30 , *S. cocculoides* 2.77 ± 0.36 , *S. madagascariensis* 3.09 ± 0.25 , *S. innocua* 3.23 ± 0.28 and *S. pungens* scored 3.36 ± 0.31 (Figure 27A).

The two *Strychnos* jams which performed better in the descriptive sensory analysis (*S. cocculoides* and *S. spinosa*) were ranked against commercial jams on the retail market. There was no significant difference ($P > 0.05$) in preference of *S. cocculoides* (2.72 ± 0.25), *S. spinosa* (2.91 ± 0.27), Strawberry (3.00 ± 0.26) and Pineapple (3.59 ± 0.29) jams (Figure 27B). Lerotse (4.18 ± 0.26) and Marula (4.51 ± 0.24) were significantly ($P = 0.001$) less preferred by assessors than all the other jam types. However, there was no significant difference ($P > 0.05$) between preference of Lerotse and Marula jams.

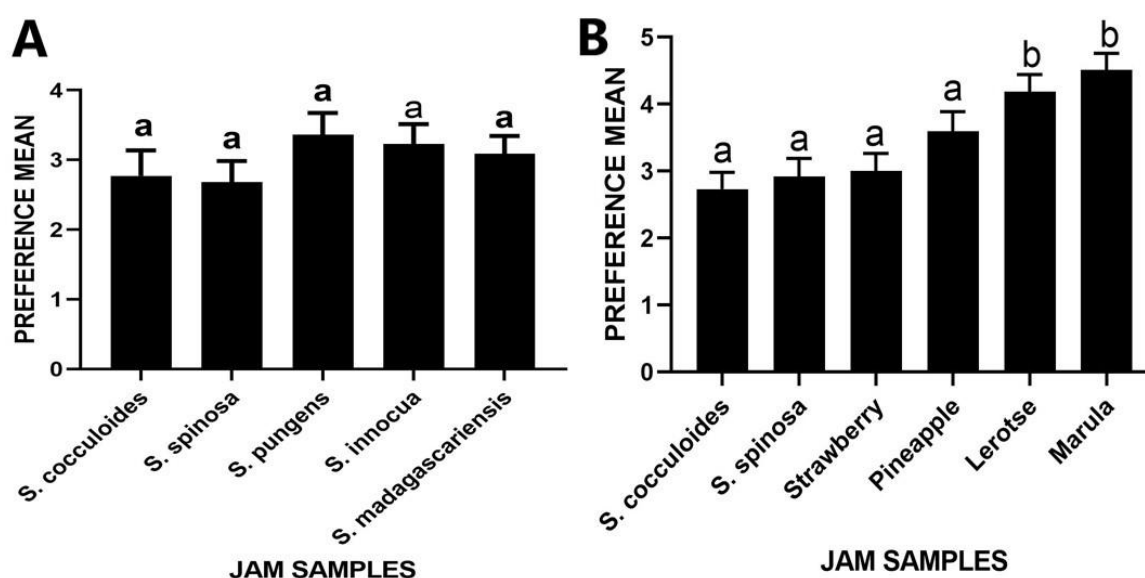


Figure 27: Preference ranking test of jams from five *Strychnos* species (A) and two *Strychnos* species against four commercial jams on the retail market (B). Lower number (1) represents most preferred and large number (5) means least preferred.

4.5 PRODUCTION OF STERILE SEEDLING USING LEAVES AND BUDS

Efforts to make sterile plants from leaves and buds were unsuccessful due to microbial contamination (Figure 28). All the explants showed some microbial growth at around 5 days, and these were persistent to even after 40 days. This was observed in all species.



Figure 28: Fresh leaves and buds of the five *Strychnos* species, before contamination (A & C and after 5 days of planting (B & D) displaying signs of contamination. Scale bars = 1.3 cm in A, 1.3 cm in B, 1.3 cm in C, and 1.3 cm.

4.6 PRODUCTION OF STERILE SEEDLING USING EMBRYOS

Embryos from mature fruits of the five *Strychnos* species did not grow after 40 days of culturing (Figure 29).

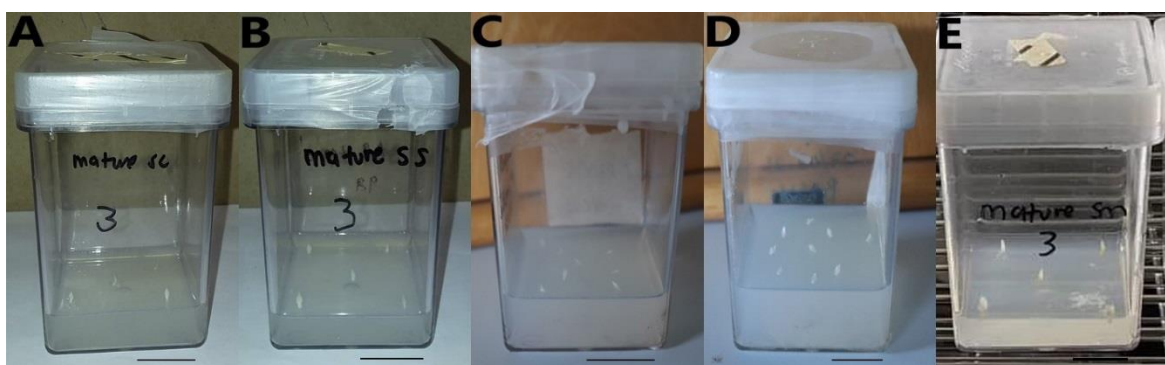


Figure 29: Sterile embryonic cultures using mature fruits of *S. cocculoides* (A), *S. spinosa* (B), *S. pungens* (C), *S. innocua* (D), and *S. madagascariensis* (E) at day 40 of culturing. Scale bars = 1.7 cm in A, 1.7 cm in B, 1.4 cm in C, 11.7 cm in D and 1.8 cm in E.

The embryos from the immature fruits started to show signs of growth at 3 days. These signs included elongation of the radicle at 3-4 days and greening of the epicotyl from 7-9 days. The embryos continued to develop into small healthy plants at 30 days (Figure 30).

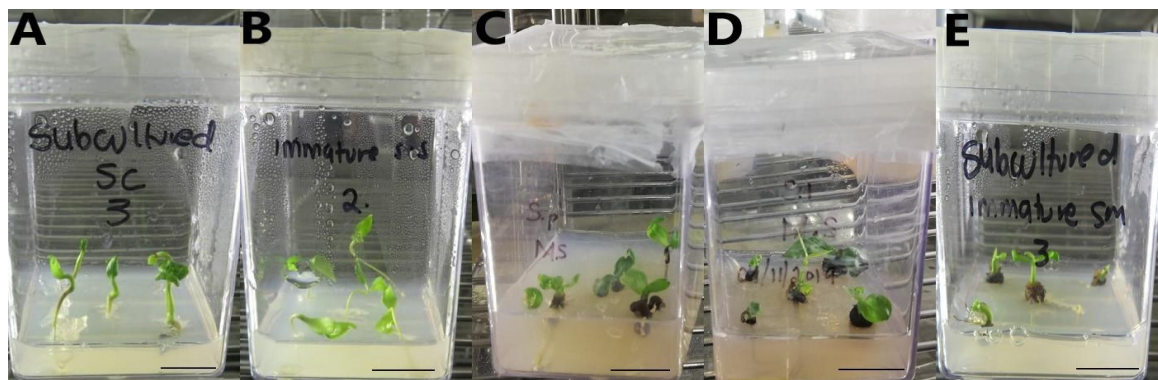


Figure 30: Embryonic cultures of the Immature fruits, *S. cocculoides* (A), *S. spinosa* (B), *S. pungens* (C), *S. innocua* (D), and *S. madagascariensis* (E). Scale bars = 1.4 cm in A, 1.4 cm in B, 1.6 cm in C, 1.6 cm in D, and 1.6 cm in E.

Sterile *Strychnos* seedlings were produced from the embryos of immature fruits of five species through embryo tissue culture on full strength MS medium after 40 days. The germination period of the five *Strychnos* species ranged from 7-9 days (Figure 31A). Both *S. cocculoides* and *S. spinosa* germinated after a period of 7.20 ± 0.37 days whilst *S. pungens* and *S. innocua* took 6.60 ± 0.51 days to germinate and lastly *S. madagascariensis* germinated after 8.40 ± 0.51 days. The germination percentages of the five *Strychnos* species ranged from 92-100% (Figure 31B). Both *S. pungens* and *S. innocua* had germination percentage of 92 ± 2.00 . *S. cocculoides*, *S. spinosa* and *S. madagascariensis* had germination percentage of 100 ± 2.00 . Bars represent S.E.M ($n \geq 150$). Means with same letters are not significantly different at ($P > 0.05$).

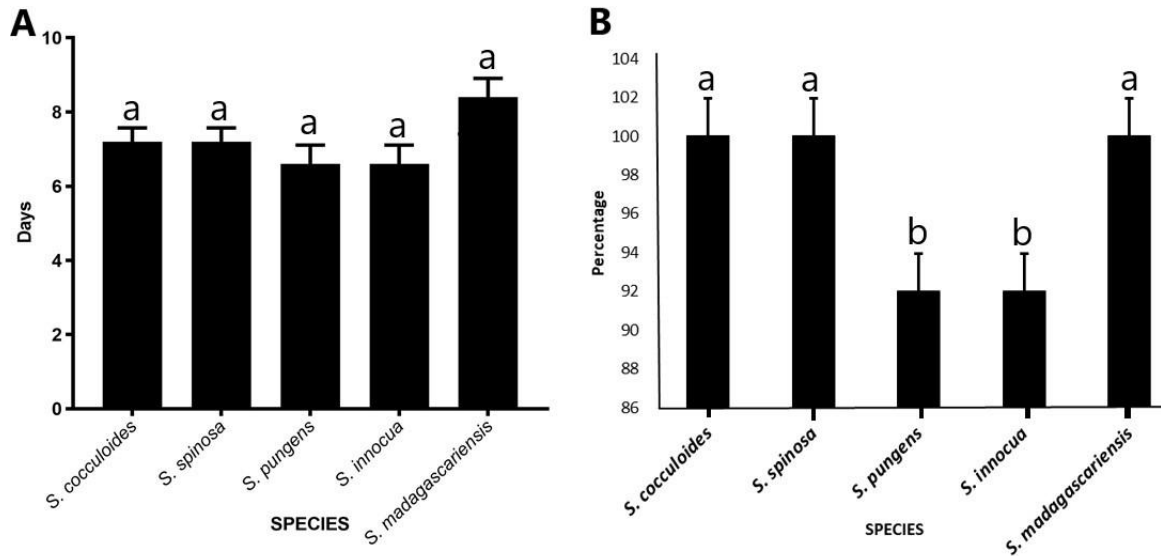


Figure 31: Germination time (A) and percentage (B) of embryos from the immature fruits of the five *Strychnos* species.

4.7 PRODUCTION OF NON-STERILE SEEDLINGS THROUGH CONVENTIONAL METHOD

Seeds from *S. spinosa* produced non-sterile seedlings after 58.65 ± 1.67 days (Figure 32). The germination percentage of *S. spinosa* was 76.19 ± 1.14 .



Figure 32: *S. spinosa* seedlings grown from the seed. Scale bar = 8.1 cm.

5.0 DISCUSSION

5.1 *Strychnos* DIVERSITY, DISTRIBUTION, AND ABUNDANCE IN CENTRAL AND NORTH-EAST DISTRICTS

All the five edible *Strychnos* species are present within the Central District. This was consistent with the findings of Setshogo and Venter (2003), Adebawale (2014), and Adebawale *et al.* (2012) who reported the presence of all the 5 species in the Central District. Of the five species, *S. madagascariensis* is the most widely distributed (Figures 18-22). It was found in all the Sub-districts within Central District, except Boteti. It also had healthy populations in most of the areas where it was recorded, except Mmadinare, Mabolwe, Lentswelemoriti and Phakwe, where only a few individuals were found. Near Mmadinare, only a single individual was found, and it is not known whether this individual was introduced by humans or is native to the area. However, it is a big tree over 10 m tall and was found near a hill that is presently not inhabited by people. In Mabolwe, only 1 individual was spotted as well. Many of the locals in Mabolwe did not know that this plant (or any *Strychnos* plant) existed in the area and this plant was in the village. It is a tall tree, of approximately 7 meters. Mabolwe area is heavily populated by elephants, therefore, it was not feasible to do on-foot surveys of the area. Using small roads around the area, no additional plants were identified. However, this does not necessarily mean other *S. madagascariensis* trees are not present in this area, they may be deeper where they were not visible from the roads. It is also likely that they do not exist as tall trees, especially outside the village since elephants are known to heavily browse on them and leave them shrubby and stunted. *S. madagascariensis* plants may also be depleted in this area due to the high population of elephants. Only the individual identified may remain since it is within the village and may be protected by human presence. In Mashatu Game Reserve, near Lentswelemoriti village, less than 20 individual plants were found. About 15 live individuals were found in a rocky area along the Lentswelemoriti-Mashatu road and were all short (less than 2.5 m) and shrubby. They were heavily browsed-on by elephants and near them were dead individuals who were uprooted by elephants (Appendix F). Two trees were found in Lentswelemoriti village and were tall (about 4 m). They showed signs of being browsed on by elephants, but not as heavily as those along the Lentswelemoriti-Mashatu road. This is

likely because elephants do not come to the village as frequently. All the *S. madagascariensis* plants observed around the Lentswelemoriti area seemed to be struggling to produce fruits. Only 2 small fruits (less than 3 cm in diameter) were recorded from one individual, tucked deep within the shrubby plant. There were also no shells of old fruits on the ground unlike in other places, and this may suggest that plants may not adequately produce fruits and not often. Mashatu game reserve area was only surveyed through roads due to the presence of dangerous animals. Therefore, it is likely that some individuals exist, however, they are likely to experience the same fate as the identified individuals. Overall, the Mashatu *S. madagascariensis* population seems to be under serious threat and may require an in-depth study on its progression over several years.

A total of 114 *S. madagascariensis* plants were identified near Phakwe settlement (Figure 24) on a rocky area of about 0.561 km². The population was made of mixed individuals ranging from small seedlings to large trees above 20 m tall. They produced many fruits, most of which were lying on the ground. Even though located in a small area, this population seemed to thrive well due to a high number of seedlings, and tall trees which seemed to be flourishing. However, it seemed to be limited to the small rocky area of the Phakwe hills, consistent with the *S. madagascariensis* preference for rocky areas (Mwamba, 2006; Adebawale, 2014). The nearest identified population was 24.90 km away in Mmadipahala lands, near Bobonong.

Other flourishing populations were found in Seolwane, Ratholo, Lecheng-Malaka, Palapye, Mokgware, Paje, Mothabaneng, Thune dam (near Mathathane), and Mmadipahala lands between Bobonong and Sefophe villages, in Marapong, Sebina junction, Nswaswi junction, Tutume-Nkange tarred road, Dagwi, Changate, Sefhare, Ramokgonami, and Mhalapitsa (Figures 18, 19, 20, and 22). Even though these populations seemed healthy, each one of them had its risk factors. For example, the population around Palapye was mostly concentrated within the BIUST campus (Figure 18). The campus is most likely to be developed and most of these trees will be cut to make way for developments. The same applies to the plants between Palapye and Lecheng, which is the expansion area for the fast-growing town of Palapye. These plants are under threat from urbanization and may be depleted or highly reduced (probably within the next 10 years) as the town expands. The Thune dam population is the highest in terms of *S. madagascariensis* abundance, but these

plants are located within a prime area most likely to be used for establishing tourist or farming areas in future. Therefore, this population may be under threat as well.

S. madagascariensis was also found in the North-East District. This concurred with Setshogo and Venter (2003) and Adebawale *et al.* (2012) who reported it to be found in the North-East District. It was mostly found in the upper part of the District (Figure 23). Flourishing and healthy populations with a height of approximately 6-12 m were found in Mabudzani, Tsamaya, Mowana, Butale, Mapoka, Nlapkhwani, near Zwenshambe, Mbalambi, Kalakamati and Domboshamba ruins. Even though the populations were healthy and flourishing, most of them are at high risk as they are likely to be cut to make way for development when the above-mentioned villages expand. Some trees are located along the tarred roads, and they are at high risk of overharvesting and can also be cut whenever there is a need to expand the road.

S. innocua is the second widely distributed species. It was found in four Sub-districts of Central District, these are Serowe-Palapye, Mahalapye, Tutume, and Bobonong Sub-districts (Figures 18-22). Similarly, Adebawale *et al.* (2012) reported the presence of *S. innocua* in the Central District. Healthy populations of *S. innocua* were recorded within most of the areas, except Lentswelemoriti and Phakwe. In Lentswelemoriti only four individuals were found. However, there may be more trees present since no on-foot surveys were carried out due to high elephant presence in the area. They are also likely to be over-browsed, like *S. madagascariensis* trees that were observed in the same area. The healthy populations of *S. innocua* in Central District were found in Seolwane, Sefhare, Ramokgonami, Mhalapitsa Lecheng-Malaka, Palapye, Serowe, Mothabaneng, around Thune dam (near Mathathane), Mathathane village, near Matsitama junction, Nswaswi junction, Tutume-Nkange road, and Changate (Figures 18, 19, 20, and 22). In the North-East District, *S. innocua* was found in the upper part of the District (Figure 16). This concurred with Adebawale *et al.* (2012) whose findings indicated the presence of *S. innocua* within North-East District. All the populations seemed to be healthy with a height of 6-12 m and were found in Mabudzani, Tsamaya, Mowana, Butale, Mapoka, Nlapkhwani, near Zwenshambe, Mbalambi, Kalakamati, and Domboshaba ruins. These populations face the same challenges as the *S. madagascariensis* populations reported above since they were usually found in the same areas.

S. spinosa is the third widely distributed species. It was found in three Sub-districts (Serowe-Palapye, Bobonong and Mahalapye) within Central District. All populations recorded seemed to be healthy, except near Mothabaneng where only few individuals were found. These individuals were shrubby. The largest population of *S. spinosa* was recorded at Thune dam (Figure 19), a key area which may be used for tourism and farming. The trees in this population could reach up to 9 m tall. Other seemingly healthy populations were found in Seolwane, Sefhare, Tumasera-Seleka, Ratholo, Ramokgonami, Mhalapitsa, Lecheng-Malaka, Palapye, Paje and Mokgware (Figures 18 and 22). All these populations are found within human settlements and face challenges associated with expansion of settlements, changes in land use and over harvesting. *S. spinosa* was not found within the North-East District. It is not known whether it used to be present within this District but was wiped out, or it never inhabited the area.

S. cocculoides is the fourth widely distributed species and was found in two Sub-districts (Serowe-Palapye and Boteti) within Central District. Its largest population was recorded at Makabi cattlepost near Thabala (Figure 18). Trees here could reach up to 12 m tall. Other healthy populations were found in Palapye, Serowe, Paje, Khama Rhino Sancutuary, near Khwee junction, Makoba gate, and Ditawana cattle post near Lethakane (Figures 18 and 20). *S. cocculoides* was also found in the North-East District, mostly in the upper part of the District (Figure 23). The populations were recorded at Tshesebe, Mosojane, and Mapoka, with heights of up to 8 m. The populations found within towns and villages seemed to have fewer and isolated individuals compared to those found outside settlements. However, those in human settlements tended to be taller.

S. pungens was the least widely distributed and only found in Serowe-Palapye Sub-district within Central District and not found in the North-East District. Its largest population was recorded at Makabi cattlepost near Thabala (Figure 18). The trees were up to 4 m tall. Other populations were recorded in Palapye, Serowe and Paje (Figure 18). Apart from the Thabala population, the other population face all the challenges associated with being found within human settlements.

Strychnos species are at risk of extinction due to climate change, urbanization, village expansion, and other anthropogenic activities, hence, more efforts should be invested into their conservation. This concurred with Heita (2018) who stated that *S. cocculoides* is at

high risk of extinction due to overuse and unsustainable harvesting. There are natural factors such as long droughts, fire, pests and disease that causes a gradual reduction of *Strychnos* species (Heita, 2018). There is a high rate of deforestation in the miombo woodlands of Southern Africa and this pose a big threat to these species (Mkonda *et al.*, 2002).

There were some co-existence patterns of the *Strychnos* species that were noticed, and they varied per district, sub-district, and per location. In Serowe-Palapye, Mahalapye, and Bobirwa sub-districts, *S. spinosa* was found in the same habitat with *S. madagascariensis* and *S. innocua*. In Serowe-Palapye sub-district, *S. cocculoides* and *S. pungens* co-existed especially at Makabi cattlepost near Thabala, in Serowe, and Paje. However, in Lecheng and Old Palapye, *S. pungens* co-existed with *S. spinosa*, *S. innocua*, and *S. madagascariensis*. In the Boteti Sub-district, *S. cocculoides* did not co-exist with any other species. In Tutume sub district, *S. innocua* co-existed with *S. madagascariensis*. In North-East District, *S. cocculoides* co-existed with *S. innocua* and *S. madagascariensis* in Mapoka.

Different *Strychnos* species were found to coexist with plants from other species. *S. cocculoides* and *S. pungens* were found in the same habitat as *Terminalia sericea* (Mogono), *Vangueria infausta* (Mmilo), *Peltophorum africanum* (Mosetlha), *Commiphora africana* (Seroka), *Ximenia americana* (Moretologa wa Pudi) *Schinziophyton rautanenii* (Mungongo). *S. spinosa* was found in the same habitat as *Commiphora africana* (Seroka), *Azanza garckeana* (Morojwa), *Combretum apiculatum* (Mohudiri), *Ximenia caffra* (Moretologa wa kgomo) *Sclerocarya birrea* (Morula) *Ficus burkei* (Moumo), *Ficus cordata* (Motlhatswa), and *Ficus salicifolia* (Motlhatswa). *S. innocua* and *S. madagascariensis* were found in the same habitat as *Azanza garckeana* (Morojwa), *Colophospermum mopane* (Mophane), *Combretum apiculatum* (Mohudiri), *Ximenia caffra* (Moretologa wa kgomo), *Ficus cordata* (Motlhatswa), and *Ficus salicifolia* (Motlhatswa).

5.2 MOLECULAR IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF THE *Strychnos* SPECIES

Percentage identity and phylogenetic analysis suggest that all the sequences were obtained from the target five *Strychnos* species that were identified morphologically. All their top hits from the NCBI Blastn search (Table 7) corresponded to assigned species based on

morphological identification. This suggests that all the species were correctly identified. According to pairwise alignments of the molecular marker fragments between the different species (Table 8), *matK* and *rbcL* showed the least differences between the species, with percentage identities ranges of 99.26-100 % and 99.15-100% respectively. *ITS 2* and *trnS-trnG IGS* showed the highest variation between species at identity ranges of 38.07-97.96 % and 67.39-100 % respectively. Therefore, both *rbcL* and *matK* have low discriminatory power between all the species. This concurred with Moorhouse-Gann *et al.* (2018) and Daniell *et al.* (2016) who stated that *rbcL* and *matK* are useful in inferring phylogeny at high taxonomic levels (family level). The *rbcL* sequences have low evolutionary rates that cannot distinguish closely related species (Saarela *et al.*, 2013; Patwardhan *et al.*, 2014; Daniell *et al.*, 2016; Qin *et al.*, 2017; Moorhouse-Gann *et al.*, 2018). The plant working group of the Consortium for the Barcode of Life recommended the use of *rbcL* and *matK* as a two-locus approach for plant barcodes and successfully discriminated 907 samples from 550 species at the species level with a probability of 72% (Chen *et al.*, 2010). However, it is not reliable due to low probability (72 %) and need to be supplemented with other molecular markers such as nuclear ribosomal markers such as *ITS 2* with higher probability (92.7 %) of successful discrimination at species level (Chen *et al.*, 2010; Moorhouse-Gann *et al.*, 2018). This is also supported here by this study, where *ITS2* and *trnS-trnG IGS* showed more variation and seemed more reliable.

All molecular markers showed higher percentage identities between *S. cocculoides* and *S. spinosa* compared to the other species, suggesting that these species are more closely related to each other based on the percentage identities of the 4 molecular markers. The molecular markers also suggested that *S. madagascariensis* and *S. innocua* are more closely related to each other than they are to the other species. *S. pungens* appears to be more related to *S. madagascariensis* and *S. innocua* than to *S. cocculoides* and *S. spinosa*, based on the percentage identities of all the molecular markers. In terms of sequence length, the fragments were almost the same lengths in all molecular markers except *trnS-trnG IGS*. For *trnS-trnG IGS*, the sequence of *S. cocculoides* and *S. spinosa* had lengths of 425 bp and 427 bp respectively, while *S. pungens*, *S. innocua* and *S. madagascariensis* had 321 bp, 321 bp, and 319 bp respectively. The variation in the length of the sequences of *S. pungens*, *S. innocua*, and *S. madagascariensis* is due to the 105 bp deletion. The large deletion event in the *Densiflorae* concurred with Adebawale *et al.* (2016) whose findings observed 104 nucleotide base pairs deletions. Therefore, *trnS-trnG IGS* has a higher discriminatory power

as compared to *rbcL* and *matK* and this is congruent with Saarela *et al.* (2018) who recommended the use of non-coding loci (*trnS-trnG* IGS and *ITS* 2) as supplementary markers to *rbcL* and *matK* to improve identification success. Other studies confirmed that *trnS-trnG* IGS is efficient and performs better than both *rbcL* and *matK* at resolving phylogenies (Adebowale *et al.*, 2016a). The *ITS* 2 regions have high discriminatory power between closely related species as compared to *trnS-trnG* IGS. The *ITS* 2 regions are regarded as gold standard barcodes, and it is the only nuclear barcode available for plant identification (Moorhouse-Gann *et al.*, 2018). The variation within the sequences of *ITS* 2 regions was six times greater than sequences of *rbcL* (Qin *et al.*, 2017). The results on *ITS* 2 fragments correctly identified all the individual species and seemed to be the best molecular marker to use. However, a combination of nuclear barcodes and numerous plastid barcodes usually offers a robust and better understanding of phylogeny. This concurs with Adebowale *et al.*, (2016) who stated that usage of the multi-locus approach of combining nuclear barcode (*ITS* 2) and several plastid barcodes (*trnS-trnG* IGS, *rbcL*, and *matK*) offered a vigorous and clear understanding of the evolutionary relationships of the *Strychnos* species.

Phylogenetic analysis (Figures 13-17) further suggested that *S. cocculoides* is more related to *S. spinosa* based on all molecular markers. All the markers grouped these 2 species together. Based on 3 molecular markers (*ITS*2, *trnS-trnG* IGS and *matK*), *S. innocua* is more related to *S. madagascariensis* and the two species are grouped together (Figure 13, 14, & 16). One molecular marker (*rbcL*; Figure 15) seemed to suggest that *S. pungens* is more related to *S. madagascariensis*, however, the three *rbcL* sequences from these species were 100 % identical so could not produce useful phylogeny between these species. All the 4 molecular markers revealed that *S. pungens* is more related to *S. innocua* and *S. madagascariensis* than *S. cocculoides* and *S. spinosa*.

Phylogenetic tree analysis (Figure 17) consisting of the five *Strychnos* species from Botswana and same species on NCBI database, using *ITS* 2 fragments, shows that the sequences from the same species clustered together. This further suggests that the species were correctly identified using the *ITS* 2 fragments. The fragments were able to distinguish the species and could be used along with morphological identification of the 5 species in Botswana.

5.3 PRODUCT DEVELOPMENT

Descriptive sensory evaluation of the five *Strychnos* jams (Figure 26) shows that assessors preferred the appearance, aroma, and taste of all jams at the same rate. Food appearance is mostly determined by colour, it plays a role in the choice of a consumer to accept or reject food (Lanza et al., 2011; Eke-Ejiofor et al., 2013). *S. pungens* fruit jam had a rich fruity and honey aroma with an aftertaste, while *S. innocua* and *S. madagascariensis* fruit jams had a rich fruity and oily aroma. *S. cocculoides* fruit jam had a rich honey aroma and taste, while *S. spinosa* fruit jam had a rich aroma and tasted like pineapple, apricot, clove, citrus, and banana (Mizrahi et al., 2002; Sitrit et al., 2003). Assessors detected same levels of sweetness in four jams (*S. cocculoides*, *S. spinosa*, *S. innocua*, and *S. madagascariensis*). Higher sweetness on *S. cocculoides*, *S. spinosa*, *S. innocua*, and *S. madagascariensis* is attributable to the fact that their fruits have a sweeter taste (Mwamba, 2006; Ngadze et al., 2017a; Ngadze, 2018) whilst lower level of sweetness on *S. pungens* is due to their fruits being less sweet and being tart, sour, and bitter (Ngadze et al., 2017a; Ngadze, 2018). Assessors detected higher levels of aftertaste on *S. innocua* compared to the other jams. The bitter aftertaste on *S. innocua* fruit jams is attributable to high levels of ascorbic acid content (274.73 mg/100 g) on *S. innocua* pulp and juice (Bello et al., 2008). However, with a mean of 5.05 ± 0.60 out of 9, this aftertaste did not seem to be too high. Assessors detected same levels of viscosity on all jams. The difference in viscosity of the jams is dependent on the pH of the fruits and the amount of acidity regulator (citric acid) and pectin added. The pH is a vital factor to be considered in obtaining an optimum gel condition (Eke-Ejiofor et al., 2013). The fruit jams of *S. spinosa* and *S. cocculoides* were more spreadable as compared to *S. innocua*, *S. madagascariensis*, and *S. pungens*. The fruit jam of *S. pungens* was the least spreadable. With means of 8.69 ± 0.17 and 7.62 ± 0.36 respectively, the *S. spinosa* and *S. cocculoides* were highly spreadable. Spreadability is a very important attribute in jam processing and consumers prefer jams that are easier to spread (Gindi et al., 2019).

The preference ranking test of *Strychnos* fruit jams (Figure 27A) shows that assessors preferred the jams from all species indifferently. The preference ranking test of the two *Strychnos* jams that performed the best in the descriptive sensory analysis (*S. cocculoides* and *S. spinosa* jams) against jams sold on the retail market (Strawberry, Pineapple, Lerotse and Marula) shows that assessors equally preferred *S. cocculoides*, *S. spinosa*, Strawberry,

and Pineapple jams (Figure 27B). The results show that *S. cocculoides* and *S. spinosa* may be as accepted in the market as Strawberry and Pineapple jams. *S. cocculoides* and *S. spinosa* jams would probably be accepted better than Lerotse and Marula jams since the assessors showed higher preference to the two *Strychnos* jams as opposed to the Lerotse and Marula jams.

5.4 PRODUCTION OF STERILE AND NON-STERILE SEEDLINGS OF *Strychnos* SPECIES USING EMBRYO CULTURE

The production of sterile *Strychnos* plants using leaves and nodes was not successful due to microbial contamination (fungi and bacteria) which was observed after five days of culturing (Figure 28). The microbial contamination of explants might be due to contamination during tissue culturing, the inefficiency of the broad-spectrum fungicide and bactericide and surface sterilizing agents (Cobrado and Fernandez, 2016). It may also be due to endophytes, which may be imbedded within the explant tissue, therefore, will not be targeted by surface sterilization. The microbial contamination may be prevented by increasing the time of immersion of the explants in the fungicide, bactericide, and surface sterilizing agents, using different bactericide and fungicide, or targeting endophytes as well.

The lack of germination in mature embryo cultures may be due to dormancy (Figure 29). The last stage of seed development is seed dormancy (Finch-Savage and Leubner-Metzger, 2006), this is often already activated in mature seeds within ripe fruits because they are fully developed. Dormancy is triggered by activation of the abscisic acid and its action can be counteracted by production of gibberellic acid (GA), which stimulates seed germination. To induce germination of the embryos from mature seeds, GA may be incorporated into the media used. GA is known to improve seed germination in many species (Swain et al., 1997; Debeaujon and Koornneef, 2000; y Aké et al., 2007). The germination period of embryo cultures of immature *Strychnos* species was 7-9 days. There was no significant difference in the germination period for all five species ($P= 0.1336$, $R^2= 0.4340$, Figure 31). The germination times concurred with a similar study conducted by Kagithoju *et al.* (2013) on *S. potatorum*, which germinated after 7-11 days. Previous embryonic micro propagation studies conducted on *S. cocculoides* reported a germination period of 6 days and a 90 % germination rate (Heita, 2018). Species with short germination times emerge earlier than

other species and can benefit from early access to scarce resources like water, space and reduced competition in the semi-arid regions (Gioria *et al.*, 2018; Duncan *et al.*, 2019). Microbial contamination did not pose a challenge in the embryo culture experiments. This is mostly because embryos within the seeds are sterile and therefore do not transfer any microbes to the growth media. The plants produced through embryo culture remained sterile for more than 365 days. Sterile plants produced can be used for fast multiplication of large number of high-quality plants that are free from diseases and pests, and possess same genetic make-up (Cobrado and Fernandez, 2016). It can also be used for downstream stream processes such as genetic transformation and biotechnological studies. However, microbial contamination remains the main challenge in embryo culture (Cobrado and Fernandez, 2016).

Seeds of *S. spinosa* grown using conventional method germinated after 58.65 ± 1.67 days. This is comparable to germination times observed in a similar study conducted by Mwamba, (2006) and Mkonda *et al.* (2002) using *S. cocculoides*, who reported germination times of 21-28 days in summer and 63 days in winter. The germination percentage of the conventional method was 76.19 %. This concurred with studies by Taylor *et al.* (1996), Mwamba, (2006) and Mkonda *et al.* (2002) who reported germination percentages of 54-80 %. In arid ecosystems, 85 % of the plants produce dormant seeds (Duncan *et al.*, 2019). The use of immature seeds through embryo culture can help in the propagation of the plants by by-passing seed dormancy. The germination percentage using embryo culture was 92-100 %, which was higher than germination percentages obtained in the conventional method (76.19 %). This is consistent with the findings of Kagithoju *et al.* (2013). The higher germination percentage during embryo culture suggests that embryos in immature fruits are highly viable and have not reached dormancy. However, this method may be labor intensive and more costly. It could however still be used for fast multiplication of *Strychnos* species given its shorter germination time (7-9 days as compared to 58 days of conventional method) as well. The results show that *Strychnos* species can be effectively propagated using both methods, however, both methods are dependent on presence seeds, which may be a challenge for endangered species. Also, both methods produce only one plant per seed/embryo. Methods to produce multiple plants per seed or embryo would be more effective.

The protocol for production of sterile *Strychnos* plants generated in this study will play a major role in the selection, domestication, and genetic improvement of *Strychnos* species.

Seeds from high performing plants from the wild can be selected and their embryos can be used to produce sterile seedlings. The sterile plants produced will be studied and potentially used for genetic modification experiments. They may also be exploited through cell cultures and for their medicinal properties. Their sterile tissue may also be used to generate callus, which will allow for the generation of multiple clones.

6.0 CONCLUSION

This study focused on the diversity, distribution, product development, and embryo culture of five *Strychnos* species found in the Central and North-East Districts of Botswana. It showed that a combination of morphological characteristics (such as leaf shape, leaf colour, leaf size, vein arrangements, fruit colour, presence/absence of spines, the shape of the spines, and trunk colour) and molecular markers (especially *ITS 2* and *trnS-trnG IGS*) can be used to differentiate the species. The study also showed that the molecular markers can be used to infer relatedness of the species amongst each other. Phylogenetic analysis using fragments of the molecular markers confirmed that the species can be divided into 2 main groups, one with *S. cocculoides* and *S. spinosa*, and the other consisting of *S. pungens*, *S. innocua*, and *S. madagascariensis*). In the later evolutionary group, *S. innocua*, and *S. madagascariensis* are more closely related to each other than they are to *S. pungens*. All the species were recorded in several places within the Central and North-East Districts of Botswana. Within the Central District, all the 5 species were found, while only 3 (*S. cocculoides*, *S. innocua* and *S. madagascariensis*) were found in the North-East district. A few of the populations seemed to have healthy numbers of individuals, however, many of them seemed to face similar challenges that if not addressed will cause their numbers to plummet. In some areas (such as Lentswelemoriti and Mabolwe), elephants seem to have reduced *Strychnos* populations to the brink of depletion. Generally, the most abundant and mostly wide-spread species seems to be *S. madagascariensis* and the least seems to be *S. pungens*. Both propagation through seeds and embryo culture seem to be viable techniques to produce *Strychnos* plants. However, embryo culture has a shorter germination time, higher germination percentage and produces sterile plants that may be used for downstream metabolic and biotechnological processes (e.g. genetic manipulation). Commercial grade jams were produced from fruit pulp of the 5 species and 2 of these jams were highly accepted by assessors, who ranked them similar to some of the best jams in the local retail stores. Based on all these findings of the study and review of existing literature, *Strychnos* species have a great potential to be domesticated, exploited medicinally, nutritionally, and commercially. The baseline data generated on their diversity and distribution in the 2 districts could also be used to monitor changes in the diversity, distribution, and abundance of the species overtime as well as inform conservation strategies.

6.1 FUTUREWORKS

Strychnos species have been traditionally used for both medicinal and nourishment purposes for centuries. However, the indigenous knowledge on the uses of the *Strychnos* species is not documented. There is need to document this knowledge and explore it through scientific research. Further studies can be done on individual population characteristics such as abundance, co-existence, soil preferences and species progression patterns. There is a need for future studies to be conducted on individual populations within conserved (Forest Reserves, Game Reserves and National Parks) and non-conserved areas. *Strychnos* distribution, abundance, and diversity could also be studied in all the Districts of Botswana. Similar data on the other 2 species not recorded in this study (*S. potatorum* and *S. usambarensis*) may also be generated. Further studies can be done by including both *S. potatorum* and *S. usambarensis* in phylogenetic analysis and elucidating how they relate to the other *Strychnos* species. The metabolomics of the *Strychnos* species is not adequately studied, there is a need to characterize and identify the different secondary metabolites, many of which may have medicinal and other purposes. Medicinal products may be generated using these species and their effectiveness in treating different ailments evaluated. *Strychnos* cell cultures may also be generated to improve production of useful secondary metabolites. The jams produced in this study could also be analysed for their nutritional composition, safety, and shelf-life. More efficient propagation methods that produce multiple plants per tissue also need to be developed. These methods will improve the capacity increase propagation efficiency and produce useful clones. These methods may include callus culture using sterile leaves and nodes. There is also a need to use hormones such as GA on mature embryos to break their dormancy and allow production of sterile plants from ripe fruits. This will allow for propagation of plants even from stored seeds (or seeds obtained after processing fruits into jam), which may be more abundant than seeds from immature fruits. Seedlings produced during embryo culture need be acclimatized to the outside environment and maintained until maturity. The effectiveness of conventional propagation using seeds also need to be studied in *S. pungens*, *S. innocua*, and *S. madagascariensis*. Selection of plants with desirable characteristics such as large fruit size and sweet taste could also be performed to create superior germplasm collections. Improving plant and fruit qualities may also be performed using breeding and biotechnological approaches.

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APPENDIX A

Table 16: Morphological identification tool for *Strychnos* species

SPECIES	COLOUR & OF BARK & BRANCHES	HABIT, CANOPY SHAPE	BRANCHES	SPINES PRESENT	LEAVES OUTLINE SHAPE, COLOR	HAIRY LEAVES	NUMBER OF LEAF VEINS	FLOWER	FRUIT SIZE
<i>S. cocculoides</i>	TEXTURE Rough, corky & ridged COLOUR: creamy brown	Tree, shrubs; Open (1-8m height)	Young branch is reddish or blackish purple. Branchlets are often armed with pairs of recurved or straight spines, sometimes branches terminate in a straight spine.	Yes, thick, sharp, curved downwards (can be straight at times), axillary and paired.	COLOR: Dark green SHAPE: oblong-elliptic to Ovate, orbicular (rounded) TEXTURE : coriaceous (leathery) SIZE: 6cm long and 4 cm wide	Yes, on both sides	3-7 nerved at or just above the base and matt or shiny above; venation compressed above, prominent and conspicuous beneath	Terminal inflorescences that are thyrsoid borne in dense terminal cymes, up to 3.5 cm in length, Sepals are greenish-white, hairy about 2 mm long. Corolla is a tube, green to orange, always glabrous at the base but can be	Diameter: 6-12 cm COLOUR : smooth, woody shell Immature: dark green with paler spots Mature: yellow SEEDS: seeds with a hard coat, contain many (10-100) irregular curved and flattened seeds, compressed

								hairy above, lobed with a white penicillate corona at the mouth.	d, up to 2 cm in diameter
<i>S. spinosa</i>	TEXTURE: Rough, shallow, tends to flake in rectangular segments, not deeply fissured or corky. COLOUR: Pale, Scally grey-buff or brown.	Tree, shrubs; Open and spreading (1-10m)	Branchlets are rather pale and thin, with or without short hairs, with straight or hooked axillary spines or thorns; slash yellowish with green margin	Yes, recurved and straight spines.	COLOUR: light to dark green and glossy at the base SHAPE: curving along the margin; apex tapering to rounded or slightly lobed; sometimes notched rounded TEXTURE: Leathery, likely to be waxed. SIZE: 1.5-10cm long and about 1.2-7.5 cm wide, petiole 2-10 mm long.	Sometimes	3-7 veins. 1-3 pairs of secondary veins curved from the base.	Flowers are greenish-white, up to 6 mm long, in compact heads about 3.5-4 cm, terminal on short lateral twigs, densely crowded together on short stalks about 10 mm long. Corolla has a tube with lobes hardly split and hardly spreading with	DIAMETER: 5-15 cm in diameter, COLOUR: shinning, hard woody shell (shell about 0.8-8mm wide), IMMATURE: apple-green, knobbly often with yellow spots when young, MATURE: deep yellow to yellowish-brown

					ARRANGEMENT: opposite			white penicillate, corona at the mouth, lobes of tube triangular	SEEDS: pale brown, obliquely ovate or elliptic, flattened, plano-convex, about 1.0–3.0 cm across (10-100 flattened seeds).
S. <i>pungens</i>	TEXTURE: Thick rough, fissured or corky with swollen nodes and conspicuous lenticels COLOUR: grey or brown	Tree, shrubs (2-16 m)		No	COLOUR: dark green above, slightly paler beneath, SHAPE: spiny apical point; Elliptic, short and thick petiole (1-4 mm), TEXTURE : leathery SIZE: 3-8 cm long and 2 cm wide.	No	3-veined from (near) the base,	Flowers are greenish-white, up to 9 mm long. Corolla greenish-cream. Style thick, 3 – 5 mm long, hairy at the base like the ovary apex; stigma capitate	DIAMETER: 5-12 cm in diameter, COLOUR: shinning, hard woody shell IMMATURE: bluish-green MATURE: yellow to orange. SEEDS: flattened, plano-convex, oblique

					ARRANGEMENT: opposite				y ovate, elliptic, up to 3.0 cm across with thick, very short, erect hairs (20-100 seeds).
<i>S. innocua</i>	TEXTURE: nearly smooth, flaking at the base COLOUR: Bark pale grey or brownish, cream, orange-green, or yellowish-white	Tree, shrubs; open, much branched (3-14m)	Branchlets are stout and smooth but can be hairy.	No	COLOUR: blue green SHAPE: shortly petiole (2-7mm), obovate to elliptic (4-16cm) x (2-9cm) coriaceous; rounded marginate or subacute at the apex; widely to very narrowly cuneate or rarely rounded at the base.	Yes	3-7 nerves arising from the leaf bases that are prominent beneath. 5, prominent on both surfaces	Flowers are greenish-white or yellowish, up to 8 mm long, produced in axillary cymes; stalks short, 2-5 mm long; calyx lobes are triangular, short, and broad. Corolla is cream-green with a ring of	DIAMETER: 5-10 cm in diameter, COLOUR: thick and hard woody shell IMMATURE: blue-green MATURE: yellow or orange SEEDS: yellowish-white, obliquely ovate, Elliptic, tetrahedral, stony

					Petiole 2-6 mm long TEXTURE : leathery, dull and smooth and can be hairy SIZE: 3-8 cm long and 2 cm wide. ARRANGEMENT: alternate			hair on the throat	hard (1.5-1.8 cm in diameter).
S. <i>madagascariensis</i>	TEXTURE: nearly smooth: COLOUR: Pale grey; white patches which darken with age.	Tree, shrubs; open, much branched (2-20 m)	Branches can be powdery or not and hairy or not. Branchlets are lenticellate (spongy), glabrous to pubescent, thick, and bearing	No	COLOUR: Dark green SHAPE: lamina elliptic to obovate (2-10cm x 1-4cm). Leaves are not attached by an obvious leaf stalk and are clustered on the ends of short thick twigs. Short petiole (1-5 mm),	Yes, to various degrees	3-5 veins, not prominent on the adaxial surface Venation includes 2 pairs of secondary veins from the base curved along the margins and not	Flowers are trumpet-like and clustered at the base of the leaves, are greenish-yellow, and often appear after heavy rains.	DIAMETER: 2-8 cm in diameter, COLOUR: smooth, hard woody shell IMMATURE: blue-green MATURE: yellow or orange SEEDS: flattened or not, elliptic, or tetrahedral

			contracted laterally branchlets with congested prominent leaf scars		TEXTURE : Shiny and leathery SIZE: 3-8 cm long and 2 cm wide. ARRANGEMENT: opposite		very prominent tertiary reticulate venation especially above it.		, tightly packed and surrounded by a fleshy, edible covering (1.0 – 2.5 cm).
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APPENDIX B

Table 17: GenBank accession numbers of *Strychnos* species and outgroup taxa sequences used for phylogenetic analysis

SPECIES	MOLECULAR MARKERS	ACCESSION NUMBERS
<i>S. cocculoides</i> Baker	<i>ITS 2</i>	MW380872
<i>S. spinosa</i> var <i>lokua</i> Lam.	<i>ITS 2</i>	MW381784
<i>S. pungens</i> Soler.	<i>ITS 2</i>	MW381785
<i>S. innocua</i> Delile.	<i>ITS 2</i>	MW381786
<i>S. madagascariensis</i> Poir.	<i>ITS 2</i>	MW381787
<i>S. cocculoides</i> Baker	<i>trnS-trnG</i> IGS	MW459102
<i>S. spinosa</i> var <i>lokua</i> Lam.	<i>trnS-trnG</i> IGS	MW459103
<i>S. pungens</i> Soler.	<i>trnS-trnG</i> IGS	MW459104
<i>S. innocua</i> Delile.	<i>trnS-trnG</i> IGS	MW459105
<i>S. madagascariensis</i> Poir.	<i>trnS-trnG</i> IGS	MW459106
<i>S. cocculoides</i> Baker	<i>rbcL</i>	MW504167
<i>S. spinosa</i> var <i>lokua</i> Lam.	<i>rbcL</i>	MW504168
<i>S. pungens</i> Soler.	<i>rbcL</i>	MW504169
<i>S. innocua</i> Delile.	<i>rbcL</i>	MW504170
<i>S. madagascariensis</i> Poir.	<i>rbcL</i>	MW504171
<i>S. cocculoides</i> Baker	<i>matK</i>	MW504162
<i>S. spinosa</i> var <i>lokua</i> Lam.	<i>matK</i>	MW504163
<i>S. pungens</i> Soler.	<i>matK</i>	MW504164
<i>S. innocua</i> Delile.	<i>matK</i>	MW504165
<i>S. madagascariensis</i> Poir.	<i>matK</i>	MW504166
<i>S. spinosa</i> var <i>lokua</i>	<i>ITS 2</i>	KC609316.1
<i>S. pungens</i> Soler.	<i>ITS 2</i>	KC609315.1
<i>S. innocua</i> Delile.	<i>ITS 2</i>	MN257667.1
<i>S. madagascariensis</i> Poir.	<i>ITS 2</i>	KC609306.1
<i>G. hansemanii</i>	<i>ITS 2</i>	FM204691.1
<i>A. grandiflora</i>	<i>ITS 2</i>	DQ449916.1
<i>G. hansemanii</i>	<i>trnS-trnG</i> IGS	FM204763.1

<i>A. grandiflora</i>	<i>trnS-trnG</i> IGS	KM365157.1
<i>G. hansemanii</i>	<i>rbcL</i>	AJ318446.1
<i>A. grandiflora</i>	<i>rbcL</i>	JX572306.1
<i>G. hansemanii</i>	<i>matK</i>	HM119537.1
<i>A. grandiflora</i>	<i>matK</i>	JX518238.1

APPENDIX C

DESCRIPTIVE SENSORY EVALUATION TEST

You have received five samples of Monkey Oranges (*Strychnos* species) fruit jam; RATE the intensity of their colour/appearance, aroma, flavour and texture using the rating scale of 1 – 9 provided below. 1 = not detectable /least/light/weak etc. and 9 = strongly detectable/most/dark etc. **Tick** the number in the scale that best describes the colour/appearance, aroma, flavour and texture of the Monkey Oranges fruit jam. REMEMBER TO DRINK WATER BEFORE TASTING THE SAMPLES.

1. COLOUR/APPEARANCE

Assess the samples provided and check the box that BEST represents the relative intensity of the COLOUR and APPEARANCE of the samples.

1.1 APPEARANCE

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

SMOOTH

ROUGH

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

SMOOTH

ROUGH

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

SMOOTH

ROUGH

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

SMOOTH

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

ROUGH

SMOOTH

ROUGH

2. AROMA

Assess the samples provided and check the box that BEST represents the relative intensity of the AROMA of the samples.

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

LESS FRUITY

STRONG
FRUITY

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

LESS FRUITY

STRONG
FRUITY

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

LESS FRUITY

STRONG
FRUITY

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

LESS FRUITY

STRONG
FRUITY

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

STRONG

LESS FRUITY

FRUITY

3. TASTE/FLAVOUR

Assess the samples provided and check the box that BEST represents the relative intensity of the TASTE and FLAVOUR of the samples. REMEMBER TO DRINK WATER BEFORE TASTING THE SAMPLES.

3.1 SWEETNESS

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

Code_____

1	2	3	4	5	6	7	8	9
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WEAK

STRONG

3.2 TASTE

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

3.3 AFTERTASTE

Code _____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

Code _____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

Code _____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

Code _____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

Code _____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

WEAK

STRONG

4. TEXTURE

Assess the samples provided and check the box that BEST represents the relative intensity of the TEXTURE of the samples.

4.1 THIN/THICK

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

THIN

THICK

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

THIN

THICK

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

THIN

THICK

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

THIN

THICK

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

THIN

THICK

4.2 SPREADABILITY

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

LESS

MOST

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

LESS

MOST

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

LESS

MOST

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

LESS

MOST

Code_____

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

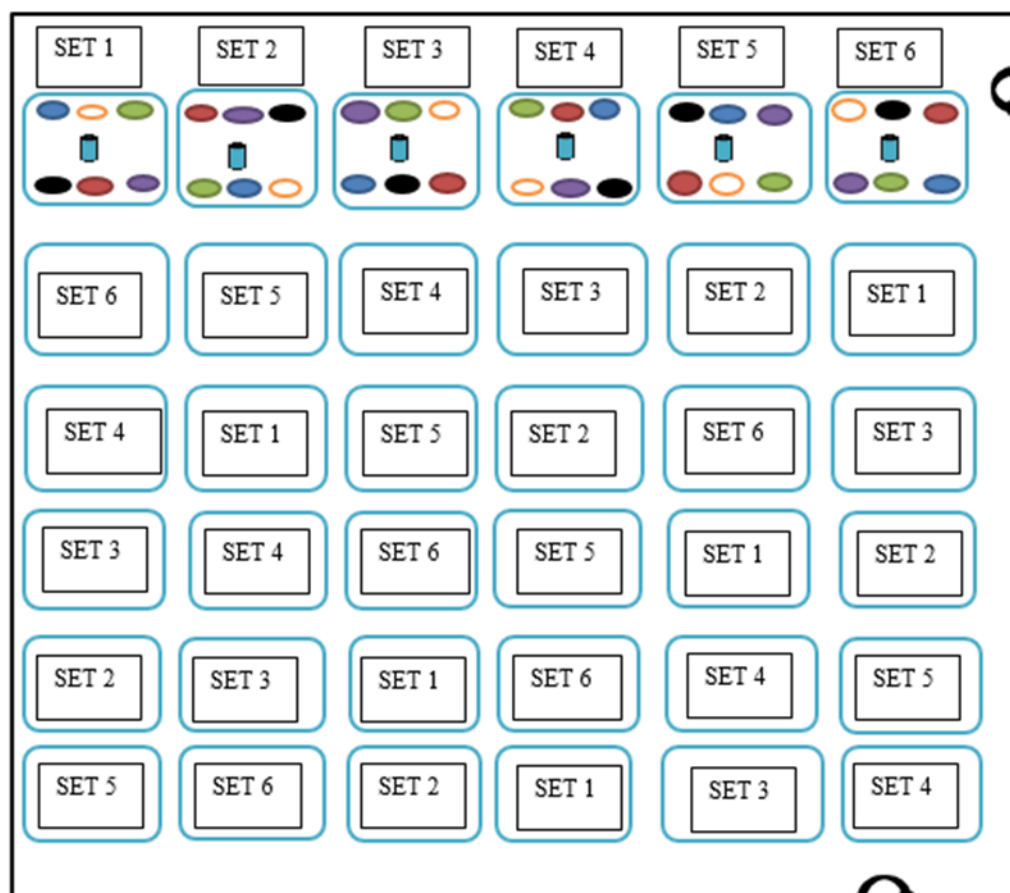
LESS

MOST

APPENDIX D

Preference Ranking test	Test code: <u>8</u>
Assessor no:<u>2</u>..... Name:..... Date: <u>7/08/2020</u>	
Instructions Taste the samples on the tray from left to right. Rank the five samples based on the order of preference. Assign 1 to the most preferred sample and 5 to the least preferred sample	
1 2 3 4 5 Sample: <u>436</u> Sample: <u>289</u> Sample: <u>327</u> Sample: <u>330</u> Sample: <u>338</u>	
Comments: <u>I found 338 to be rather overwhelming in scent/ Aroma. It would be unpleasant for sensitive women.</u> 	

APPENDIX E



APPENDIX F

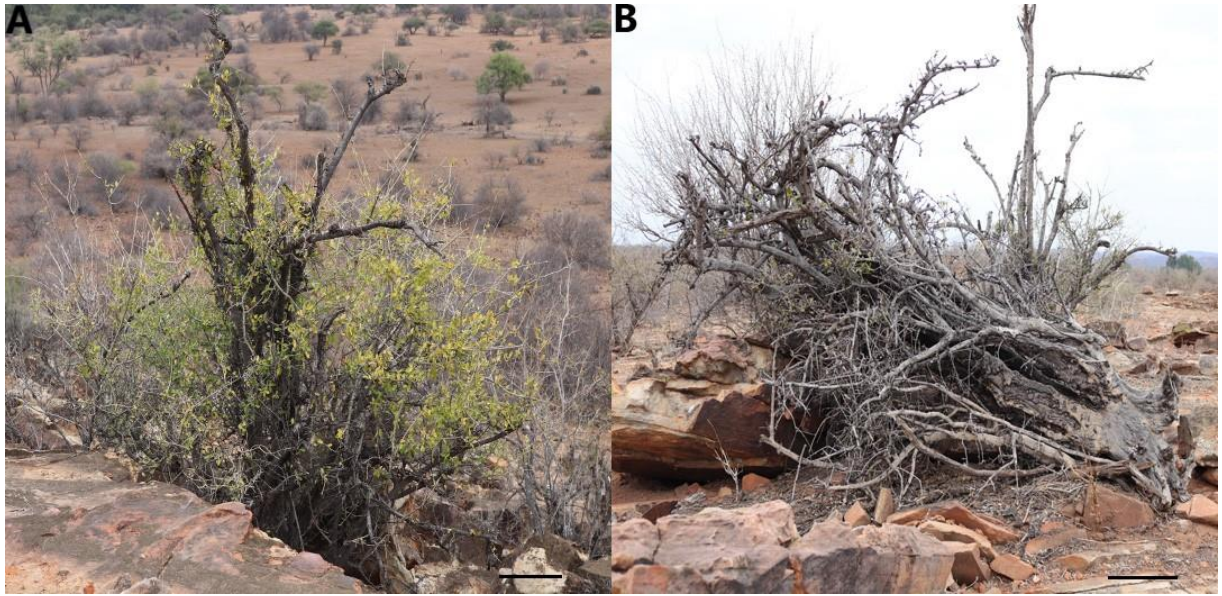


Figure 29: *S. madagascariensis* at Mashatu Game Park, Shrubs (A) and (B) trees uprooted by elephants. Scale bars = 11.7 cm in A, and 13.3 cm in B.