AMPLIFICATION AND CROSS-SPECIES TRANSFERABILITY OF TWO SSR MARKERS SET IN PTEROCARPUS POPULATIONS IN SOUTHWESTERN NIGERIA

Abstract

Pterocarpus is an important nutritional and timber plant in Nigeria. The increasing rate of forest clearing created the need for the measurement of the species' genetic diversities for conservation purposes. This study assessed amplification and cross-species transferability of universal chloroplast (*cpSSR*) and specific nuclear (*nSSR*) microsatellite markers in four species of *Pterocarpus* from Southwestern Nigeria. Ten cpSSR (*ccmp* series) and eight nSSR *(mpo/mph* series) markers from *Pterocrapus offcilinalis* were amplified in the four species from eight populations. Amplified products from the Genomic DNA - Polymerase Chain Reaction was confirmed by Gel electrophoresis, followed by the scoring of amplification and cross-transferability of the markers. The amplification scores of *cpSSR* and *nSSR* were 53% and 42% respectively whereas the cross-transferability scores were 40% and 30% respectively. Only transferrable markers were selected and used to finally sequence the plants' samples to assess the genetic diversity of the four *Pterocarpus* species. The high scores of *cpSSR* marker indicated low specificity of the chloroplast genes but high applicability in the genomic system. Overall, *cpSSR* showed high cross-transferability and produced information on the species population diversity and intra-generic relationships in the plants even when population size was relatively small better than the *nSSR.*

Keywords: *Pterocarpus* species, Cross-species, Makers transferability, Microsatellite markers, Conservation

Background

Pterocarpus belongs to the family Fabaceae and category of Papilionoideae commonly called "winged plant". The plants are important forest tree good for for timber production, light construction, dye production for textile making and good source of phytochemicals for treatment of ailments including malaria, gastro-intestinal disturbances and skin infections (Witness a d Admus, 2015). However, the plants are threatened by increased overexploitation and deforestation including practices of illegal logging and harvesting for foreign trade with little or no regeneration effort, specifically, *P. erinaceus* which was known as African Rosewood, and currently on the red list of the International Union for Conservation of Nature since 2017, and the species was reportedly overexploited for export into Asian countries due its wood quality and coloration (CITES, 2016). Moreover, the plant genetic resources are eroding as the population status decreasing in our forests. Several studies had been carried out on phyto-chemical screening to uncover the species usefulness for healthcare services, thereby, leaving research gaps on species diversity for population management and proper conservation strategies.

Since existence of life, researches had shown that over 95% of the biotypes had gone into extinction while the new development of overexploitation and pressure of natural ecosystems due to rapid population growth and related anthropogenic activities and their effects such as pollution, global warming and climate change had continue to signal threats to the plants species and every element of biodiversity as reflected in the reports of the United Nations World Population Project (2019) and the Global Forest Watch Project (2019). The primary focus on the measurement of and extent of genetic diversity in plant species are more than relevant now, for effective conservation objectives and usage. Plant genetic diversity over time changes and the extent and distribution depends on its evolution, breeding system, ecological factors, history of bottlenecks, and often anthropogenic (Garnier *et al.,* 2018). Diversity of a species vastly are found within individual populations, or partitioned among members of plants community in an ecosystem.

The best approach is to be aware of native plant populations and genetic diversity system including the species distribution for proper managent and sustainable utilisation. Thse would assist in taking actions on what and how to conserve as well as improving on the taxonomical approaches that recount on origin, evolution and phylogeography of plant species. Knowledge of plants collection and utilization from the wild relatives would be in order to manage and conserve genetic resources better and such would require understanding of the genetic diversity in the native plant populations.

The Nigerian ecosysyem and phytotypes species are affected by various anthropogenic activities like settlement expansion, deforestation, chemical-based farming, urbanisation and industrialization (Aju and Ezeibekwe, 2010; Alamu and Agbeja, 2011; Borokini, 2014). A 2015 Country report of Global Forest Resources Assessment submitted to the FAO, revealed that the country forest (areas of 0.5 and above covered by trees with minimum height 5 meters) has depleted by over 64% between 1990 and 2015. The report established a sharp reduction in the forest cover since independent, invariably proportional to the human population increase. Globally, threats to populations of plant species are complex and multidimentional as noted under globalization, industrialization and climate related influences due to increased development indices including expansion of infrastructure, settlements and food production to cater for human needs and well-being (Dai *et al*., 2013; UN, 2015).

Conventional markers such as phenotypic morphological characters, biomolecules, phyto-chemical and molecular markers like Rapid Amplified Polymorphic DNA (RAPD), Randomized Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and Inter Simple Sequence Repeats (ISSR) in the time past had been usedextensively for genrtic diversity assessment and provided information on the species variability at different levels (Fribourg *et al*., 2009). However, the use of these markers had generated some concerns on peculiar limitations, especially, the cost, speed and time, specificity, complex applicability and reproducibility. Microsatellite also referred to as Simple Sequence

Repeat (SSR) markers have therefore been used in recent times owing to many of its advantages above the conventional listed molecular markers in respect to more locus specificity with high reproducibility, high information content, expression of codominance and can be found in the nuclear and in the chloroplast genomes (Jiang *et al.,* 2020). Therefore, the use of microsatellite markers for genetic diversity assessment of *Pterocarpus* species for conservation strategies and sustainable population management is relevant for the protection of natural biodiversity, particularly, the native plant species that are prone to threats within the country of origin.

Materials and Methods

Plant Species Survey and Location

A total of 73 samples of four *Pterocarpus* species were collected from eight (8) of the 25 locations identified (Table 1, Plate 1) based on interview in the communities from botanists (20), foresters (50), taxonomist (5) and farmers (100). These represented *P. erinaceus* (9%), *P. osun* (47%), *P. santalinoides* (26%) and *P. mildbraedii* (18%) in the Southwest Nigeria, within the geographical boundary of Longitude 3°31’1’’E to 5°38’45’’E and Latitude 7°23’2’’N to 7°44’32’’N) (Table 1). Each sample was collected with proper record of the respective coordinate with Global Positioning System (GPS) Smart Android mobile App.

Table 1: *Pterocarpus* populations' survey and sampling locations in the Southwest Nigeria

Locations	Species	Status	N	Longitude	Latitude
Adaba	*Pterocarpus mildbreadii, Harms*	+	6	5°7'0''E	7°19'9''N
Eda	*P. osun, Craib*	-	8	5°38'45''E	7°44'32''N
FUTA	*P. erinaceus, Poir*	-	1	5°8'15''E	7°18'3''N
Ife OAU	*P. mildbreadii*	-	5	4°31'30''E	7°31'4''N
	P. osun	-	5	4°31'30''E	7°31'4''N
	P. santalinoides, L., Henrit	-	3	4°31'36''E	7°31'18''N
Ikire	*P. osun*	+	16	4°12'55''E	7°23'2''N
	P. santalinoides	+	4	4°12'42''E	7°23'25''N
FRIN	*P. osun*	+	3	3°52'6''E	7°23'29''N
	P. santalinoides	+	1	3°51'45''E	7°23'36''N
Olokemeji	*P. erinaceus*	+	6	3°31'1''E	7°24'45''N
	P. santalinoides	+	5	3°31'14''E	7°25'36''N
Unibadan	*P. mildbreadii*	-	2	3°53'47''E	7°27'29''N
	P. osun	-	2	3°53'47''E	7°27'29''N
	P. santalinoides	-	6	3°53'47''E	7°27'29''N
	Total		**73**		

NB: "+" indicates plant stands are many or more than 10; "-"indicates plans stands are few or less than 10

Species: *Pterocarpus erinaceus*
Location: Olokemeji
Year: 2018
Habitat: Forest reserve
Population size: > 50
Type: Flaking dark stem, hard leaf
Information: Taxonomist, Forester

Species: *Pterocarpus osun*
Location: Ikire
Year: 2018
Habitat: Farmland
Population size: > 50
Type: Hard leaf and arrangement
Information: Farmer, Taxonomist

Species: *Pterocarpus santalinoides*
Location: Ikire
Year: 2018
Habitat: Farmland
Population size: < 50
Type: Young stem, hard leaf
Information: Farmer, Taxonomist

Species: *Pterocarpus mildbraedii*
Location: UI Botanical Garden
Year: 2018
Habitat: Botanical garden
Population size: < 50
Type: Plant maturity stage
Information: Botanist, Taxonomist

Plate 1. Selected photographs of four species representatives of the *Ptreocarpus* from collection gallery

DNA Extraction and PCR Amplification

Genomic DNA was extracted from was extracted from leaf tissue with a size of about 1 cm^2 following the DNeasy96 Plant Kit protocol of QIAGEN (Hilden, Germany). DNA quantity and quality were assessed on 1.5% agarose gels after staining with Gelred, followed by serial dilution (in ratio of 1:10, 1:6, 1:60 and 1:100) to standard concentration and volume required for PCR reaction to produce clear amplified products.

***cpSSR* and n*SSR* PCR Reactions and Genotyping**

Ten (10) universal chloroplast microsatellite markers (*ccmps*) adopted from Weising and Gardner (1999) and eight nSSR *(mpo/mph* series) markers adopted from *Pterocrapus offcilinalis* (Muller *et al.* 2006) were used for the amplification and transferability tests, followed by selection of four (ccmp 2, 5, 7, 10) and three (mPoCIRH07, mPoCIRF08 mPoCIRH08) successful markers, respectively, for the final sequencing of the whole samples and genotyping. *cpSSR* PCR mixture included contents in a separate primer multiplex reactions containing 1µL DNA, 1.5 µL 10x reaction buffer, 1.5 µL MgCl2, 1 µL dNTPs, 0.2 µL Taq DNA polymerase, 1 µL forward primer, 1 µL dye labeled (6- FAM or 6-HEX), 1 µL reverse primer and 6.8 µL H2O with a PCR cycle set up as: 5 min at 95°C, 25 cycles of 1 min denaturation at 94°C, 1 min annealing step at 50°C and 1 min elongation at 72°C, and final 20 min elongation at 72 ◦C. Similarly, *nSSR* PCR multiplex reactions contained 1µL DNA, 1.5 µL 10x reaction buffer, 1.5 µL MgCl2, 1 µL dNTPs, 0.2 µL *Taq* DNA polymerase, 0.2 µL tailed forward primer, 0.5 µL PIG-tailed reverse primer, 1 µL dye labeled (6-FAM or HEX) M13 primer and 7.1 µL H2O with a PCR touch-down reaction cycle of 15 min at 95°C, 10 touch-down cycles of 1 min denaturation at 94°C, 1 min annealing step at 55°C (decreasing 1 °C each cycle) and 1 min elongation at 72°C., 25 cycles of 1 min

denaturation at 94°C, a 1 min annealing at 45°C and 1 min elongation at 72°C, and final 20 min elongation at 72 °C. The sizes of DNA fragments were separated on an ABI 3130xl Genetic Analyzer and Microsatellite genotyping and allele scoring were conducted with the GeneMapper 4.0 software (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

After genotyping, the successful identified alleles as positive amplifications and transference were computed as percentage markers amplification and transference products using descriptive analysis. In order to validate the applicability of the transferred SSRs markers, we carried out genetic diversity analyses to construct phylogenetic trees and cluster plots using unweighted pair group method with arithmetic mean (UPGMA) and neighbor joining (NJ) methods with varied bootstrap values to generate best fit trees using POPTREE2 software and Structure 2.3.4 Software respectively (Takezaki *et al*., 2010; Tamura *et al*., 2011).

Results and Discussion

Amplification and Cross-species Transferability of *cpSSR* and *nSSR*

Seven among the 10 Concensus Chloroplast Microsatellite Primer series (Table 2) screened passed to produce amplified products with score values range between 40 % to 60 % whereas only four markers: *ccmp2, ccmp6, ccmp7, ccmp10* were successfully transferred with 100% score values while three markers: *ccmp1, ccmp3* and *ccmp4* exhibited partial transferability in one to three species and the score values range between 25 % to 75 %. Three other markers *ccmp5, ccmp8* and *ccmp9* produced null amplification. This was an indication that the markers were unable to establish allelic matches to produce amplified products and also an interpretation of high level of genomic differentiations exhibited in the genus. Similarly, three of the eight *nSSR (mpo/mph* series) markers adopted from *Pterocrapus offcilinalis* (Table 3) screened passed to produce amplified products with score values range between 38 % to 50 % whereas only three markers: *MP07, MP08, MPH8* were successfully transferred with 75% to 100% score

values while two markers: *MP02, MP03* exhibited partial transferability in one to three species and the score values were 25 % to 75 %. Three other markers: *MP01, MP04, MP09* produced null amplification. More so, the markers produced different but close amplified regions (base pairs) in the species including *ccmp2 (232 – 240 bps), ccmp6 (115 – 121 bps), ccmp7 (149 – 153 bps), ccmp10 (100 – 103 bps) and P. erinaceus* (101 – 232 bps), *P. osun* (103 – 240 bps) *P. santalinoides* and *P. mildbraedii* (100 – 236 bps), similarly, *MP07 (198 – 258 bps), MP08 (188 – 203 bps), MPH8 (230 – 241 bps) and and P. erinaceus* (188 - 258 bps), *P. osun* (198 - 241 bps) *P. santalinoides (187 – 254 bps)* and *P. mildbraedii* (185 – 244 bps). The null and varied amplification and transferability score values were an indication that the markers were unable to establish allelic matches to produce amplified products and also an indication of high level of genomic differentiations exhibited in the markers-genus relationships respectively. Hence, 40% total amplification and transferability result was within what has been reported in many literature as success value. The findings on high transferability of the *cpSSR* and high specificity of *nSSR* from genetic diversity assessment conform with reports of Ekué *et al.* (2009) on markers developed in *Litchi chinensis* to *Blighia sapida* (Sapindaceae); and consistent with the report of Yoshida *et al.,* (2014) on microsatellite screening in *Piper solmsianum* (Piperaceae). The ccmp series has high resolutions with better allelic variation sizes suitable for delimiting boundaries among the species for revealing genetic diversity in *Pterocarpus.* This finding was a deviation from the report of Doulaty *et al.* (2007) with high resolution performance on ccmp3 and 10 for Iraninan wild and cultivated grapevines. Therefore, it could be deduced that *ccmp* series resolutions in the plant genomes varied from one plant genu to another, hence, it provided as good basis for selection of chloroplast microsatellites suitable for assessing genetic diversity of larger population of diverse species.

Table 2 Cross-species amplification and transferability of the 10 cpSSR loci in *Pterocarpus*

Markers	*P. osun*	*P. santalinoides*	*P. erinaceus*	*P. mildbraedii*	Transferability %
CCMP1	+	-	-	-	25
CCMP2	+	+	+	+	100
CCMP3	+	+	-	+	75
CCMP4	+	-	-	-	25
CCMP5	-	-	-	-	0
CCMP6	+	+	+	+	100
CCMP7	+	+	+	+	100
CCMP8	-	-	-	-	0
CCMP9	-	-	-	-	0
CCMP10	+	+	+	+	100
Amplification %	70	50	40	50	

NB: "+" indicates that producs with proper sizes, "-" indicates no products or products with improper sizes

Table 3 Cross-species amplification and transferability of the eight nSSR loci in *Pterocarpus*

Markers	*P. osun*	*P. santalinoides*	*P. erinaceus*	*P. mildbraedii*	Transferability %
MP01	-	-	-	-	0
MP02	-	-	-	+	25
MP03	+	-	+	+	75
MPO4	-	-	-	-	0
MP07	+	+	+	-	75
MP08	+	+	+	+	100
MP09	-	-	-		0
MPH8	+	+	+	+	100
Amplification %	50	38	50	50	

NB: "+" indicates that producs with proper sizes, "-" indicates no products or products with improper sizes

cpSSR* and n*SSR* Markers Applicability in *Pterocarpus

The computed results from the SSRs markers for validating applicability of the transferred markers generated phylogenetic relationship tree (Figure 1) and structure band plot (Figure 2). The phylogenetic tree revealed two distinct groups for the *Pterocarpus* species that included T1,1: *P. mildbraedii* and populations of *P. santalinoides* from Ikire and Olokemeji, and not too distance T1,2 comprising of *P. santalinoides* populations from Olokemeji, Ife, FRIN and Ibadan while the second group with two subgroups T2,1: *P. osun* and *P. erinaceus*. The structure analysis band plot had three (3) distinct bands comprising B1: *P. mildbraediii* and *P. santalinoides* at a very close band frequencies, B2: *P. osun* and B3: *P. erinaceous.* The patterns of banding adopted were from clear and consistent two levels of probability/replication (K: 4 and 5) for the run program. The phylogenetic tree dendogram revealed different levels of nuclear genomic similarities among the species based on genetic distances and there were indications that species populations were sorted genetically along locations, particularly, *P mildbraedii* and *P santalinoides*. Microsatellite markers despite their suitability in assessing species genetic diversities are not easy to produce, therefore, the available series requires to screening for adaptation in different genera and species. From this study, the members of the two transferable markers sets used were found applicable in the four *Pterocarpus* species, although at different but close relationship levels. Also, members of the *cpSSR* markers show high resolution for more selections but the *nSSR* provided clear boundaries among the taxa and in other case sorted the species along locations and geographical boundaries. These results confirmed the general applicability of both markers and the specific function of each of the set in assessing the genetic diversity.

Figure 2 UPGMA dendogram constructed among 72 individuals of four *Ptreocarpus* from eight populations based on eight nSSR markers at Bootstrap = 1000

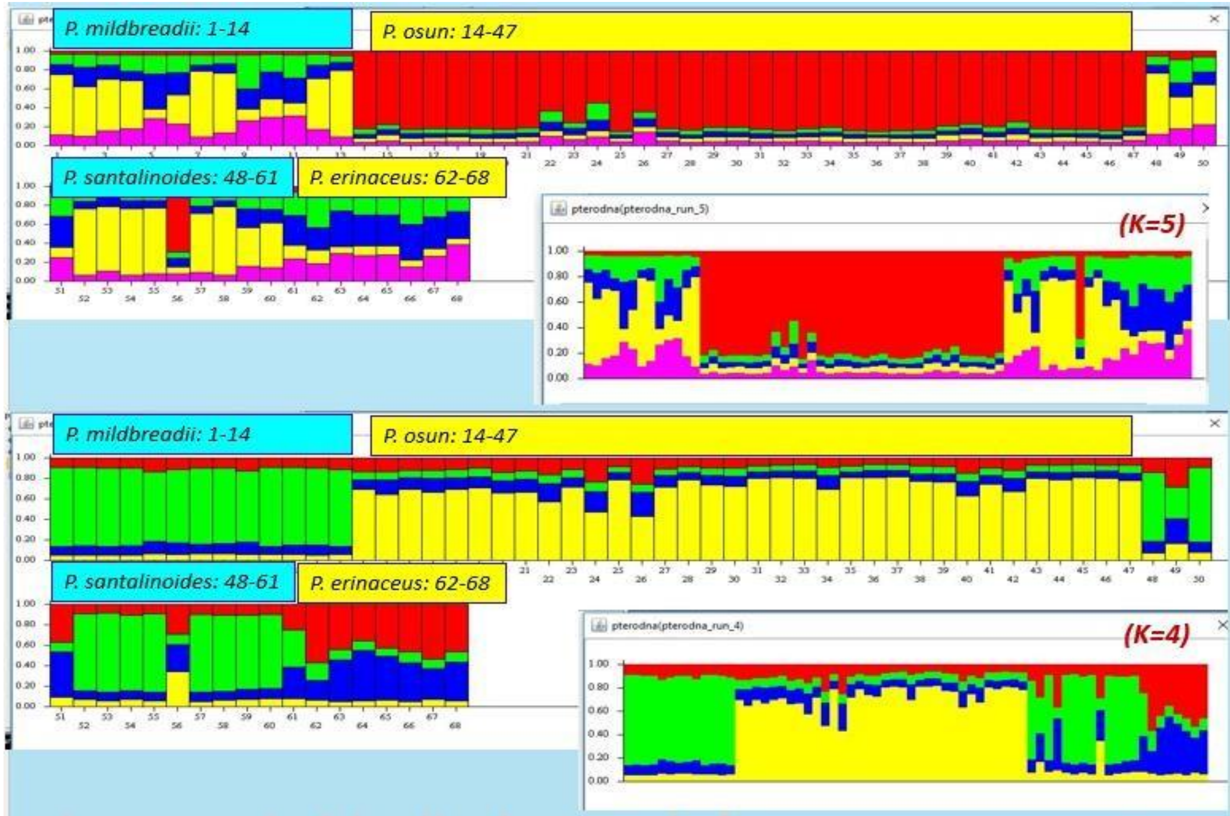

Figure 3. Structure analysis of 72 individuals of four *Ptreocarpus* from eight populations band plots based on *nSSR* markers at program probability/replication levels K=4 and 5

Conclusion

Markers transfer among species of a genus is an opportunity currently been explored by molecular geneticists to use molecular techniques and tools to develop markers associated with conserved regions on the genes of related species for the purpose of carrying out diversity studies and assessing phylogenetic relationships. Also, such could be used to identify populations for *in situ* and ex situ conservation. . This has generally cut cost and proven successful in many plant species.

The markers sets had successfully provided members of the markers series applicable for assessing genetic diversity and other phylogeograpgphic functions in *Pterocarpus,* and also established close phylogenetic relationship between *Pterocarpus mildbraedii* and *Pterocarpus santalinoides*. Hence, the identified markers would prove to be viable tool for measuring genetic in larger populations of Pterocarpus across other ecosystems for conservation purposes. Overall, *cpSSR* showed high cross-transferability and produced information on the species population diversity and intra-generic relationships in the plants even when population size was relatively small better than the *nSSR.*

Acknowledgements

I would like to thank my laboratory works host at the Department of Forest Tree Breeding and Forest Genetics at the University of Gottingen, Germany for the material and training support during the course of this work. Also, thanks to the DAAD, Germany for providing me with Visiting Research Fellowship to Germany for this work.

References

Aju, P C and Ezeibekwe, I O 2010. Understanding and appreciating the need for biodiversity conservation in Nigeria. *Journal of Medicinal Plants Research* 4.24: 2605-2608.

Alamu, L. O., and Agbeja. 2011. Deforestation and endangered indigenous tree species in South- West Nigeria. *International Journal of Biodiversity and Conservation 3.7*:291–297. http://www.academicjournals.org/ijbc

Borokini T. I. 2014. A systematic compilation of endemic flora in Nigeria for conservation management. *Journal of Threatened Taxa* 6.11:6406-6426.

CITES (2016). Convention on International Trade on Endangered Species of Wild Fuana and Flora. Retrieved October 25, 2016 http://www.cites.org

Dai, Z., Si, C., Zhai, D., Huan, P., Qi, S., Zhong, Q., Hu, X., Li, H. and Du, D. 2013. Human impacts on genetic diversity and differentiation in six natural populations of *Madhula hainanensis*, an endemic and endangered timber species in China. *Biochemical Systematics and Ecology* 50:212-219.

Doulaty Baneh, H., Mohammadi, S. A., Labra, M., Nazemieh, A., De Mattia, F., & Mardi, M. (2007). Chloroplast microsatellites markers to assess genetic diversity in wild and cultivated grapevines of Iran. *Pakistan journal of biological sciences : PJBS*, *10*(11), 1855–1859. https://doi.org/10.3923/pjbs.2007.1855.1859

Ekué, Marius R.M., Gailing, O. and Finkeldey, R. 2009. Transferability of simple sequence repeat (SSR) markers developed in *Litchi chinensis* to *Blighia sapida (*Sapindaceae). *Plant Molecular Biology Reporter* 27:570.

FAO 2015. Food and Agricultural Organisation – Global Forest Resources Assessments. Retrieved January 2, 2019 http://www.fao.org/forest-resources- assessment/en/

Fribourg, H. A., D. B. Hannaway, and C. P. West. 2009. Genomic Tools for Improvement. Chapter 21. In: H.A. Fribourg, D.B. Hannaway, and C.P. West (ed.) Tall Fescue for the Twenty-first Century. Agronomy Monographs 53. ASA, CSSA, SSSA, Madison, WI. Also (http://forages.oregonstate.edu/tallfescuemonograph/future_considerations).

Garnier, Eric; Adeline Fayolle,Marie-Laure Navas, Christian Damgaard, Pablo Cruz, Daniel Hubert,Jean

Richarte,Paul Autran,Corentin Leurent, Cyrille Violle. 018. Plant demographic and functional responses to management intensification: A long-term study in a Mediterranean rangeland. *Journal of Ecology* 106.4: 1363 – 1376. https://doi.org/10.1111/1365-2745.12996

Global Forest Watch Project 2019. Retreived February 1, 2019. https://www.globalforestwatch.org/

IUCN 2016. International Union for Conservation of Nature and Natural Resources, Red list of threatened species. Retreived October 25, 2017 http://iucnredlist.org

Muller, F., Voccia, M., Bâ, A. and Bouvet, J. M. 2009. Genetic diversity and gene flow in a Caribbean tree *Pterocarpus officinalis* Jacq.: A study based on chloroplast and nuclear microsatellites. *Genetica* 135.2:185-198. DOI: 10.1007/s10709-008-9268-4.

Takezaki, N., Nei, M., & Tamura, K. 2010. POPTREE2: Software for constructing population trees from allele frequency data and computing other population statistics with Windows interface. *Molecular biology and evolution* 27.4:747–752. https://doi.org/10.1093/molbev/msp312

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology Evolution* 28:2731–2739

United Nations World Population Project (2019). Retreived on February 1, 2019. https://population.un.org/wpp/

UN 2015. United Nations Development of Economic and Social Affairs/ Population Division. World Population Prospects: The 2015 Revision, key findings and advance tables. 66.

Weising K. and Gardner R. C. 1999. A set of conserved PCR Primers for the analysis of Simple Sequence Repeat polymorphism in chloroplast genomes of dicotyledonous angiosperms. *Genome* 42:9-19

Witness Mojeremane and Admus Uyapo Lumbile A. U. 2016. A Review of Pterocarpus angolensis DC. (Mukwa) an Important and Threatened Timber Species of the Miombo Woodlands. *Research Journal of Forestry* 10:1:8-14. https://scialert.net/abstract/?doi=rjf.2016.8.14

Yoshida, Nídia C., Lima, Paula F., Priolli, Regina H. G., Kato, Massuo J. and Colombo, Carlos A. 2014. Isolation and Characterization of Nine Polymorphic Microsatellite Loci in Piper solmsianum

(Piperaceae). *Applications in Plant Sciences* 2:4. 1300092.

Jiang, Y., Xu, S., Wang, R. *et al.* Characterization, validation, and cross-species transferability of EST-SSR markers developed from *Lycori*s *aurea* and their application in genetic evaluation of *Lycoris* species. *BMC Plant Biol* **20,** 522 (2020). https://doi.org/10.1186/s12870-020-02727-3