SYSTEMATIC IDENTIFICATION OF AFRICAN SAPINDACEAE USING DNA BARCODING

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ABSTRACT

Sapindaceae Jussieu is a family of flowering plants in the Order Sapindales. Members of the family exist as trees and shrubs, and tendril-bearing vines with about 140-150 genera and 1400-2000 species worldwide. They are economically, medicinally and aesthetically useful. This research aimed at exploring the diversity of Sapindaceae in West and Central Africa with particular emphasis on identification of the plant samples as well as generation of DNA barcodes with a view to sharing the DNA barcode sequence(s) in a public database. These were achieved following standard protocols. Extracted DNA samples (119) were deposited at the DNA Bank of the Royal Botanic Gardens, Kew, Richmond and voucher specmens were deposited at the University of Lagos Herbarium, Lagos, Nigeria. Silica gel dried specimens yielded good quality DNA unlike the old dried herbarium leaf samples. DNA samples were sent to International Barcode of Life (IBOL) Centre in Guelph, Canada for analysis of the barcode region and sixty-nine (69) DNA barcodes were generated. Barcode data which was hinged on matK and rbcL sequence data have been deposited at the Barcode of Life database (BOLD) website and GenBank for public use. This research therefore forms a basis for further taxonomic work on the family Sapindaceae especially in Africa.

Keywords: Africa, Bio-conservation, DNA barcodes, Identification, Sapindaceae.

INTRODUCTION

The biological diversity of each country is a valuable but vulnerable natural resource while knowledge about biodiversity and the ability to identify organisms that come with it are global public goods e.g. controlling Agricultural pest; identifying disease vectors, ensuring environmental sustainability and many other Millennium Development goals. Africa's biodiversity is one of the most extraordinary in the world, but also one of the most threatened by human activities (population growth, over-exploitation, logging) and global change (desertification, climatic warming). Among the first steps towards protecting and benefiting from biodiversity are sampling, identifying and studying biological specimens. While biodiversity is disappearing alarmingly fast, there have been fantastic technological developments that can help reverse biodiversity loss, one of these is DNA barcoding which provides information on the precise identity of biodiversity for proper conservation. 'DNA barcoding' is a revolutionary diagnostic technique in which short DNA sequence is used for species identification (Powell *et al.,* 2008). It is increasingly being tested in many areas as a cost-effective tool for identifying and regulating agricultural pests, invasive and disease-carrying species, trade and sale of endangered species, and many other species of concern to governments and society. The goal of barcoding is that anyone, anywhere, anytime be able to identify quickly and accurately any species whatever its condition (Stoeckle *et al.,* 2005). This technology is being promoted by the International Consortium for Barcoding of Life (CBOL) to enable the rapid and inexpensive identification of the estimated 10 million species of organisms on earth. It has enormous benefits and brings huge gain to countries rich in biodiversity including: rapid species identification at any life stage or fragment, providing insight into the diversity of life, quick and cheap identification of specimens as well as ability to control the movement of species across national borders (Stoeckle *et al.,* 2005).

Sapindaceae is a family of flowering plants in the order Sapindales. They exist as trees and shrubs, and tendril-bearing vines widely distributed throughout the warm sub-tropical and tropical regions of the world. Sapindaceae is economically, medicinally and aesthetically useful (Odugbemi and Akinsulire, 2006; Sofidiya *et al.,* 2007, 2008; Adesegun *et al.,* 2008; Muanya and Odukoya, 2008;

Pendota *et al.,* 2008; Antwi *et al.,* 2009; Ripa *et al.*, 2010). Although members of the family Sapindaceae have been recorded to be widely distributed in Africa, their occurrence is threatened by high rate of deforestation and agricultural practices leading to loss of forest and thus the threatened status of the family as recorded in the IUCN R.L. (2008). Members of Sapindaceae are known to be difficult to identify, particularly when they are sterile, which makes them an ideal model group to test DNA barcoding techniques. Moreover, the family has problems of synonymy, taxa misidentification, doubtful specific status and grouping dissimilar taxa in the same higher taxonomic rank (Buerki *et al.,* 2009). Hence, the aim of this research was to explore the diversity of Sapindaceae in western and central Africa with particular emphasis on identification of the plant samples using DNA barcode sequences which can be shared in a public database.

METHODS

Study Area

Five African countries were visited during this study namely: Nigeria, Ghana, Ethiopia, Cameroon and Madagascar.

Taxon Sampling

The sampling strategy encompassed the majority of subfamilies, tribes and genera of the family as recognized by the existing classifications of Radlkofer (1933), Müller and Leenhouts (1976) and Thorne (2007). In-group sampling comprised 120 specimens representing 40% of the generic diversity while the family Anacardiaceae (*Sorindeia* sp.) and Simaroubaceae (*Harrisonia abyssinica* Oliv.) were included as out-groups as suggested by Savolainen *et al.*, (2000a) and Muellner *et al.* (2007).

Herbarium, fresh and silica-gel dried samples were used for the study. The national herbaria as well as local herbaria in the study areas were visited to examine and collect samples. Silica-gel dried samples (Chase and Hills, 1991) were collected in the field by the authors and complemented with materials from the DNA banks of the Royal Botanic Gardens, Kew, London. Preliminary identification in the field was achieved with the aid of Floras including those of Hutchinson and Daziel (1958), Fouilloy and Hallé (1973) and Cheek *et al.* (2000). Voucher specimen(s) were deposited at the University of Lagos herbarium while further authentication was carried out at the Forestry Herbarium, Ibadan.

DNA Extraction and Quantification

Total DNA was extracted from herbarium or silica dried leaf material (0.1–0.3 g) using the 2_ cetyltrimethylammonium bromide (CTAB) procedure of Doyle and Doyle (1987) with minor modifications (see Muellner *et al.*, 2005) followed by additional purification using a caesium chloride/ethidium bromide gradient (1.55 g/ml) and a dialysis procedure. The quality of the DNA samples obtained was checked on 1% agarose gel stained with 10mg/ml ethidium bromide and run on 0.5x TBE (Tris Borate EDTA) buffer at 75 volts for 1 h 30 mins. This was then viewed with ultraviolet (UV) trans-illuminator and photographed with Polaroid film. Furthermore, the concentration and absorbance of the DNA samples were measured using an Eppendorf biophotometer.

PCR Amplification

Two coding plastid DNA regions (matK and rbcL) were amplified. Primer(s) used include 390F> and 1326R<for maturase K (matK) (specific primer for the Dodonaeoideae was designed by Harrington *et al.*, 2005) as well as 1F> and 1460R< for ribulose 1, 5, biphosphate carboxylase large subunit (rbcL) (Savolainen *et al.,* 2000b). Also the fragment size amplified was between 870 – 910 bp for matK and 1436 – 1460 bp for rbcL. Amplification of selected regions was achieved in a 25 µl reaction mixtures containing 22.5 µl PCR premix, 0.5 µl BSA (bovine serum albumin), 0.5 µl forward primer, 0.5 µl reverse primer and 1.0-2.0 µl total genomic DNA. The amplification of the matK region was improved by the addition of 4% DMSO in the total volume of the PCR mix. Polymerase Chain Reaction (PCR) amplification was carried out in a Gene Amp® PCR System 9700 thermal cycler (Applied Biosystems Inc. (ABI), Foster City, U.S.A.) using the following programme: initial denaturation for 3 min at 94° C followed by one cycle of denaturation for 1.00 min at 94° C, followed by 35 cycles of annealing for 45 s at 52° C and extension for 2 min 30 s at 72° C. The amplification was completed by holding the reaction mixture for 7

min at 72° C to allow complete extension of the PCR products and a final hold of 4° C. PCR products were visualized on Agarose gel followed by purification on QIA quick silica column (QIAGEN Ltd) following manufacturers protocol.

Sequence of Barcode Region

DNA sequencing was carried out following a modification of dideoxy cycle sequencing with dye terminators (Sanger *et al.,* 1997). Cycle sequencing reactions was achieved in a 10 µl reaction mixtures containing 0.5 µl pink juice (Big Dye Terminator, Applied Biosystems Inc.), 3.0 µl 5X sequencing buffer (Bioloine), 0.75 µl primer (1:10 dilution: forward or reverse for each primer pair) and 40 ng cleaned PCR product, made up to 10 µl with sterile distilled water. The amplification of the matK region was improved by the addition of 4% dimethyl sulfoxide (DMSO) in the total volume of the sequencing mix. Cycle sequencing was carried out in a Gene Amp® PCR System 9700 thermocycler (Applied Biosystems Inc.) using the following programme: initial denaturation for 30 s at 95° C followed by one cycle of denaturation for 1.00 min at 95° C, annealing for 30 s at 55° C and extension for 60 s at 72° C this was run for 30 cycles and the cycle was completed by holding the reaction mixture for 7 min at 72° C to allow complete extension of the PCR products with a final hold of 4° C. The products were purified using ethanol precipitation to remove any excess dye terminator. Cleaned cycle sequencing products were then directly sequenced on a 3130 *xl* Genetic Analyzer (Applied Biosystems Inc.). Purified cycle sequencing products were sequenced in an automated sequencer (ABI PRISM® 3730 DNA Analyzer) following the manufacturer's instructions.

Sequence Alignment and Analyses

The program Sequencer version 4.5 was used to assemble complementary strands and verify software base-calling and the bases were aligned manually in PAUP* v. 4.0bl (Swofford 1998). Cladistic analyses were performed using the parsimony algorithm of the software package PAUP* version 4.0b10. The partition homogeneity test was performed using heuristic search methods and 1000 replications, simple stepwise addition, tree bisection-reconnection (TBR) branch swapping, and MULTREES on (Keeping multiple shortest trees), but holding only 10 trees per replicate to reduce the time spent in swapping on large numbers of suboptimal trees. After the 1000 replicates, the shortest trees from the first round were used as starting trees for a search with a tree limit of 10,000. Robustness of clades was estimated using the bootstrap (Felsenstain, 1985) involving the use of 1000 replicates with simple sequence addition and SPR branch swapping. For visual assessment of the data sets, the bootstrap trees were considered incongruent only if they displayed "hard" (i.e. with high bootstrap support > 85%) rather than "soft" (with low bootstrap support < 85%) incongruence (Seelanan *et al.,* 1997; Wiens, 1998). The following arbitrary scale for describing bootstrap support was applied: 50 - 74% weak, 75 - 84% moderate and 85 - 100% high. DNA barcoding of samples was carried out at the International Barcode of Life (IBOL) Centre in Guelph, Canada using matK and rbcL primers.

RESULTS

Species names, voucher information, and GenBank accession numbers for all sequences are provided in Table 1. Extracted DNA samples (119) were deposited in the DNA bank at the Royal Botanic Gardens Kew. A few taxa could not be amplified for matK and *rbcL* due to low DNA yield during extraction; however seventy (70) samples were successfully amplified during PCR. Of the seventy (70) DNA samples, thirty-four (34) DNA barcode sequences were generated from the matK region while thirty-five (35) barcode sequences were generated from the rbcL region. In total, sixty-nine (69) DNA barcode sequences were generated and deposited at the Genebank. The DNA barcode sequences were 833bp long in the matK region and 555bp in the rbcL region.

Table 1: Voucher information and GenBank accession number of taxa used in the phylogenetic analysis of family Sapindaceae s.l.

Abbreviations: FHI – Forestry Research Institute, Ibadan, Nigeria; LUH – University of Lagos, Lagos, Nigeria; SFRK/HNC – National Herbarium Yaounde, Cameroon; ABU – Ahmadu Bello University, Zaria, Nigeria, GCH – University of Ghana, Legon, Ghana.

S/No	Species	Voucher	Country	Herbarium	Gen Bank Accession Nos.	
					matK	rbcL
1.	*Allophylus africanus*	Adeyemi, T.O 1194	Cameroon	LUH	JN191100	JN191136
2.	*Allophylus bullatus*	Adeyemi, T.O 1185	Cameroon	LUH	JN191101	JN191137
3.	*Allophylus conraui*	Chapman, 78107	Nigeria	FHI	JN191102	JN191138
4.	*Allophylus griseotomentosus*	ATO 043	Cameroon	FHI	JN191103	JN191139
5.	*Allophylus hirtellus*	Adeyemi, T.O 1190	Cameroon	LUH	-	JN191140
6.	*Allophylus sp*	Adeyemi, T.O 3441	Cameroon	LUH	-	JN191135
7.	*Allophylus spicatus*	Adeyemi, T.O 3442	Nigeria	LUH	JN191104	JN191141
8.	*Allophylus welwitschii*	Adeyemi, T.O 1192	Cameroon	LUH	JN191105	JN191142
9.	*Blighia sapida*	Adeyemi, T.O 1196	Nigeria	LUH	JN191106	-
10.	*Blighia welwitschii*	Adeyemi, T.O 3315	Cameroon	LUH	JN191107	JN191143
11.	*Cardiospermum corindium*	Daramola, B.O 049		FHI	JN191108	JN191144
12.	*Cardiospermum grandiflorum*	Adeyemi, T.O 1189	Nigeria	LUH	JN191109	JN191145
13.	*Chytranthus carneus*	Abbiw & Hall, J.B. 4650	Ghana	GCH	-	JN191148
14.	*Chytranthus macrobotrys*	Adeyemi, T.O 1187	Cameroon	LUH	JN191110	JN191149
15.	*Chytranthus setosus*	Adeyemi, T.O 3444	Cameroon	LUH	JN191111	JN191150
16.	*Chytranthus sp1*	Adeyemi, T.O 3445	Cameroon	LUH	JN191112	JN191147
17.	*Chytranthus sp2*	Adeyemi, T.O 3446	Cameroon	LUH	JN191113	JN191146
18.	*Chytranthus talbotii*	Adeyemi, T.O 3447	Nigeria	LUH	JN191114	JN191151
19.	*Deinbollia grandifolia*	Hall, J.B. 47068	Ghana	GCH	JN191115	JN191153
20.	*Deinbollia kilimandscharia*	De WILDE, J.J & De WILDE, B.E 7781.	Ethiopia	GCH	JN191116	JN191154
21.	*Deinbollia sp*	Adeyemi, T.O 3448	Cameroon	LUH	JN191117	JN191152
22.	*Dodonaea viscosa*	Adeyemi, T.O 037	Nigeria	LUH	JN191118	-
23.	*Eriocoelum kertstingii*	Ibhanesebhor 77683	Nigeria	FHI	JN191119	-
24.	*Eriocoelum macrocarpum*	Adeyemi, T.O 1195	Cameroon	LUH	JN191121	-
25.	*Eriocoelum microspermum*	Adeyemi, T.O 069	Cameroon	FHI	JN191120	JN191155
26.	*Laccodiscus ferrugineus*	Adeyemi, T.O 1183	Cameroon	LUH	-	JN191156
27.	*Laccodiscus pseudostipularis*	Florey, J.J. 39252	Cameroon	FHI	JN191122	JN191157
28.	*Lecaniodiscus cupanioides*	Adeyemi, T.O 3451	Nigeria	LUH	JN191123	JN191158
29.	*Litchi chinensis*	Adeyemi, T.O 3452	Madagascar	LUH	JN191125	JN191160
30.	*Majidea fosterii*	Adeyemi, T.O 1718	Cameroon	LUH	JN191124	JN191159
31.	*Pancovia atroviolaceus*	Adeyemi, T.O 1182	Cameroon	LUH	JN191126	JN191162
32.	*Pancovia sp1*	Adeyemi, T.O 1188	Cameroon	LUH	JN191127	-
33.	*Pancovia sp2*	Adeyemi, T.O 1186	Cameroon	LUH	JN191128	JN191161
34.	*Paullinia pinnata*	Adeyemi, T.O 1193	Cameroon	LUH	JN191129	JN191163
35.	*Placodiscus leptostachys*	Adeyemi, T.O 3454	Cameroon	LUH	JN191130	JN191165
36.	*Placodiscus sp1*	Adeyemi, T.O 3455	Cameroon	LUH	JN191131	JN191164
37.	*Radlkofera sp2*	Adeyemi, T.O 3459	Nigeria	LUH	JN191132	JN191167
38.	*Radlkofera sp3*	Adeyemi, T.O 3460	Cameroon	LUH	JN191133	JN191166
39.	*Zanha golungensis*	Adeyemi, T.O 3462	Nigeria	LUH	JN191134	JN191168

From these sequences, trees were generated to show the phylogenetic relationships existing between taxa in the family Sapindaceae and closely related taxa of family the Fabaceae. Phylogenetic analysis of the samples with closely related taxa of family Fabaceae revealed that *Allophylus conraui, Allophylus griseotomentosus, Cardiospermum corindum, Chytranthus carneus* and *Deinbollia kilimandscharia* were more distantly related to *Lessertia* species than other members of the family Sapindaceae (Fig. 1 and 2). Also, phylogenetic analyses revealed that Sapindaceae was monophyletic but paraphyly and polyphyly were shown at subfamilial and tribal levels. The recent suggestion on the taxonomic position of *Xanthoceras sorbifolia*, Aceraceae and Hippocastanaceae as belonging to the family Sapindaceae is corroborated (Fig 3). The family can be subdivided into four (4) subfamilies: Sapindoideae, Dodonaeoideae, (57% bp) Hippocastanoideae (66% bp) (including Aceraceae) and a monotypic Xanthoceroideae. *Xanthoceras* is sister to the rest of the family while Hippocastanoideae is sister to subfamily Sapindoideae and Dodonaeoideae. A high degree of paraphyly and polyphyly is also highlighted at subfamilial level, especially in subfamilies Dodonaeoideae and Sapindoideae. This relationship was the same irrespective of the barcode region i.e. either matK or rbcL.

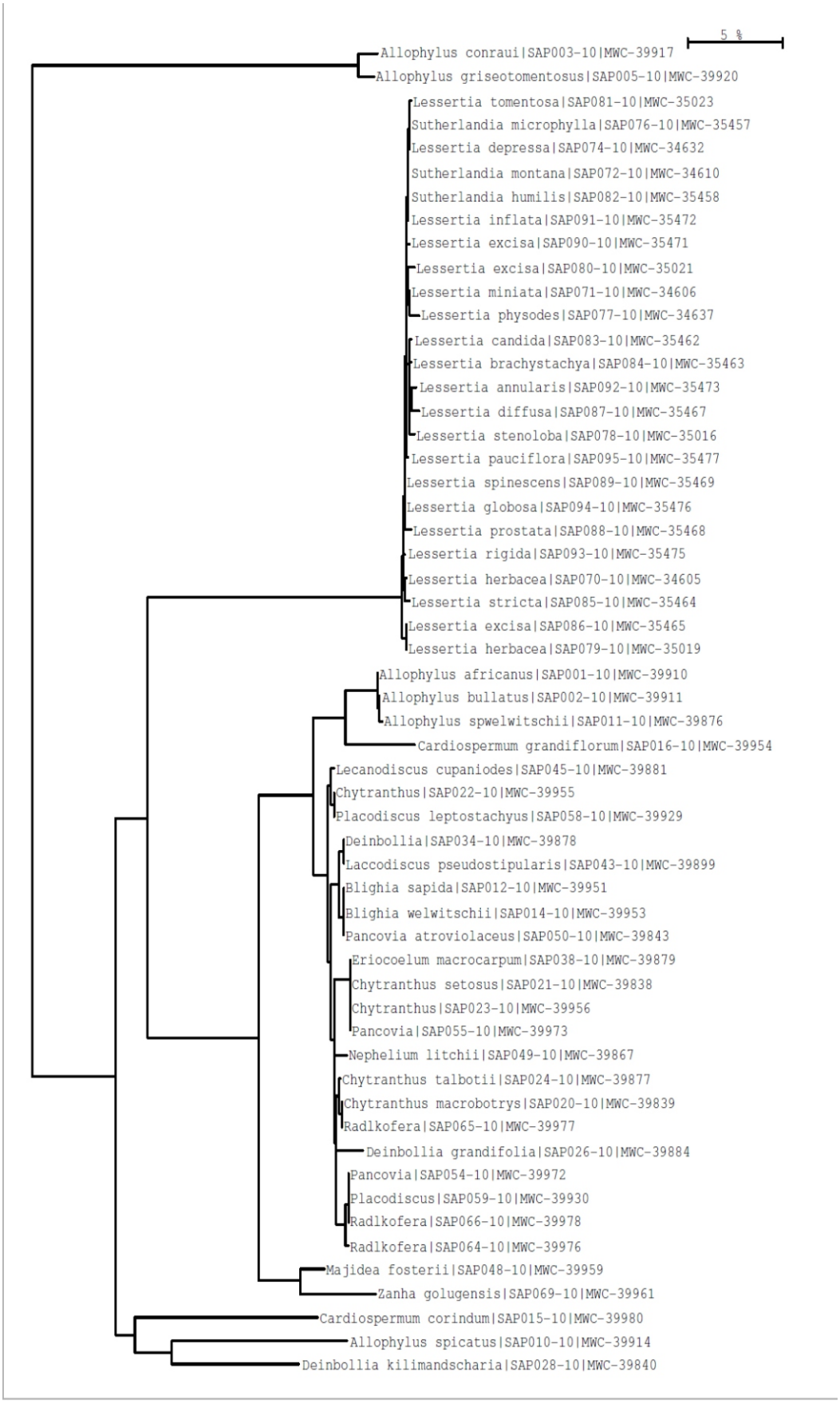

Figure 1: Phylogenetic relationships within Sapindaceae based on matK barcode sequence data

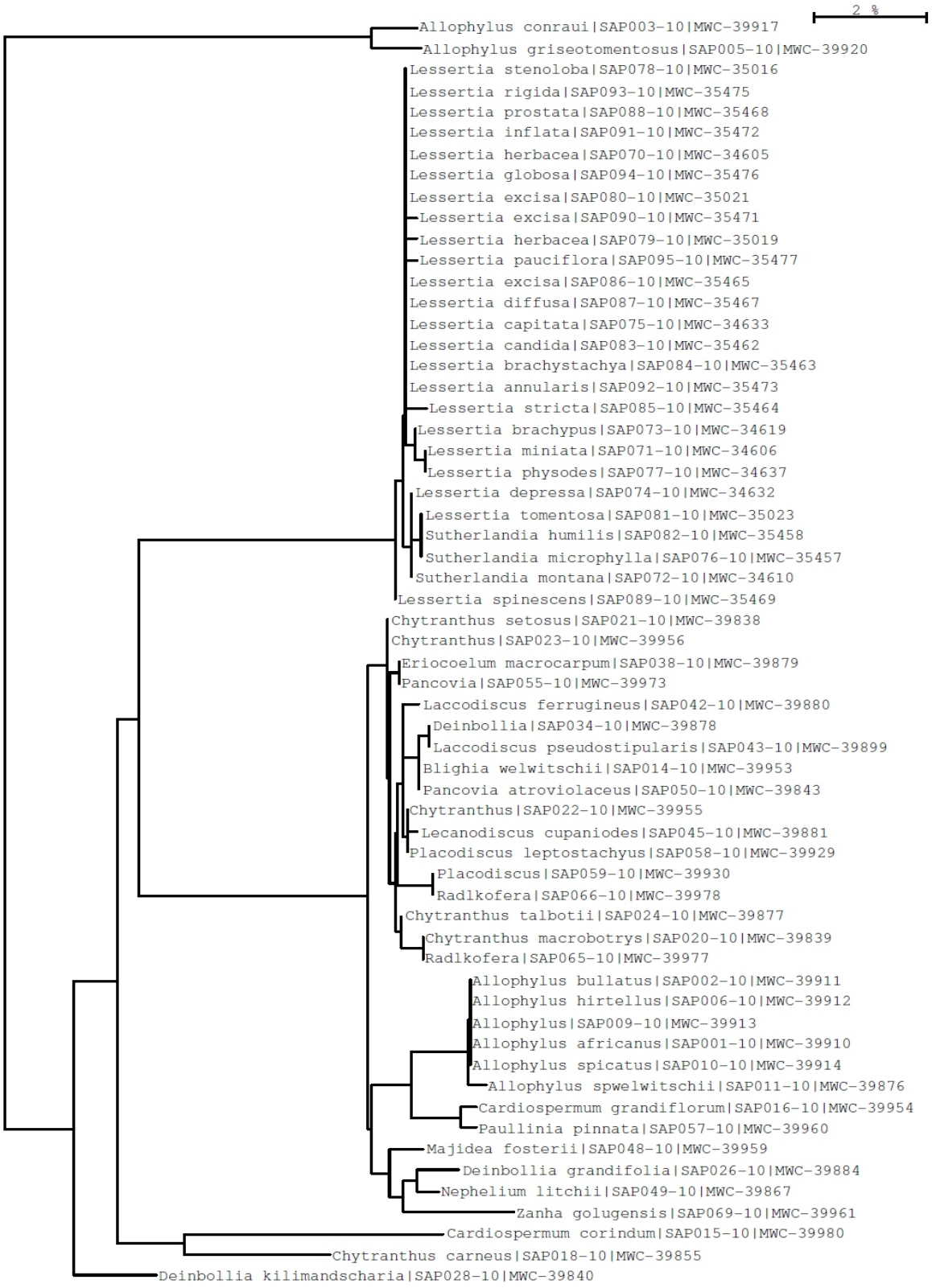

Figure 2: Phylogenetic relationships within Sapindaceae based on rbcL barcode sequence data

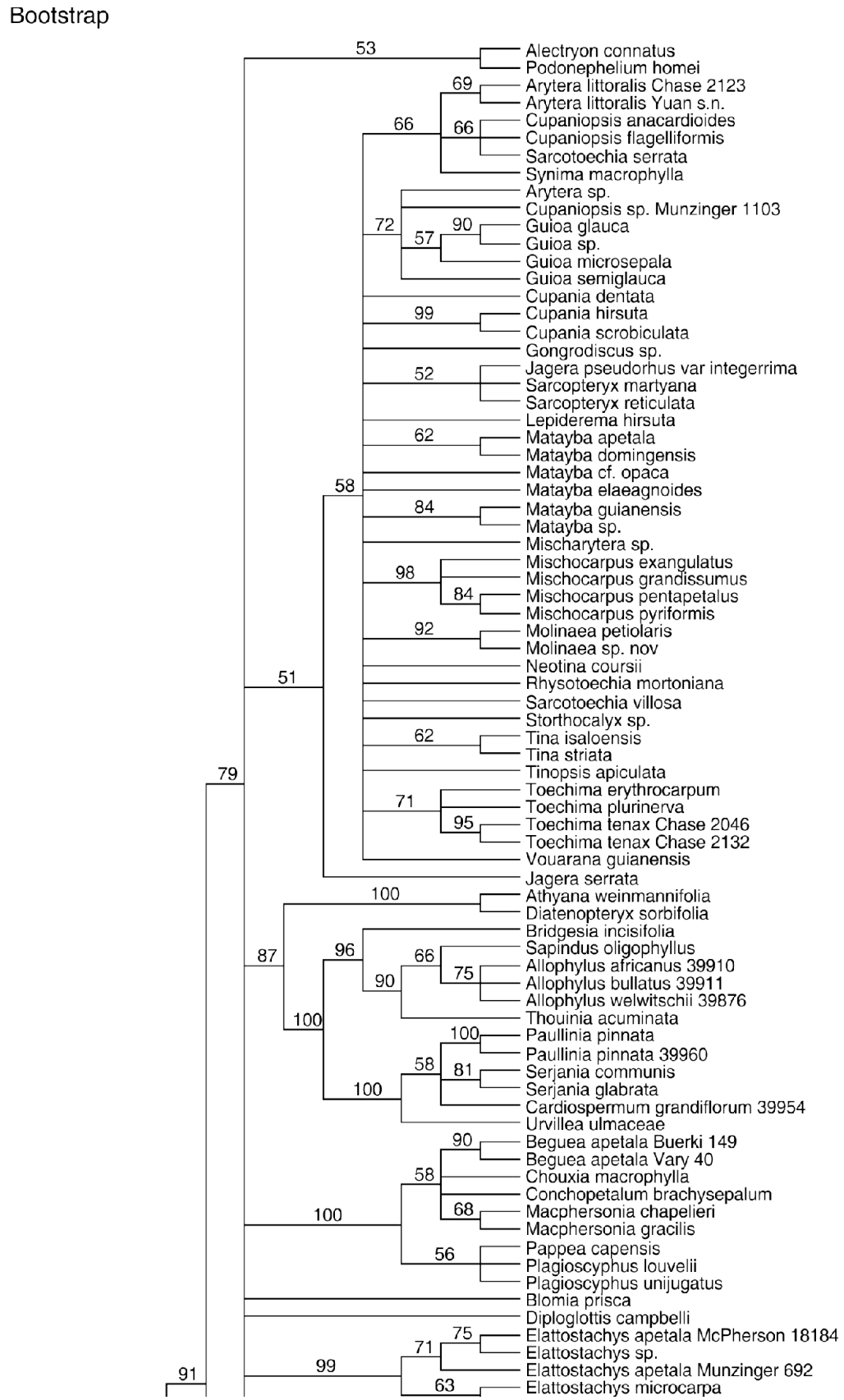

Figure 3: Phylogenetic Relationships within Sapindaceae based on matK data. Bootstrap supports are indicated above branches.

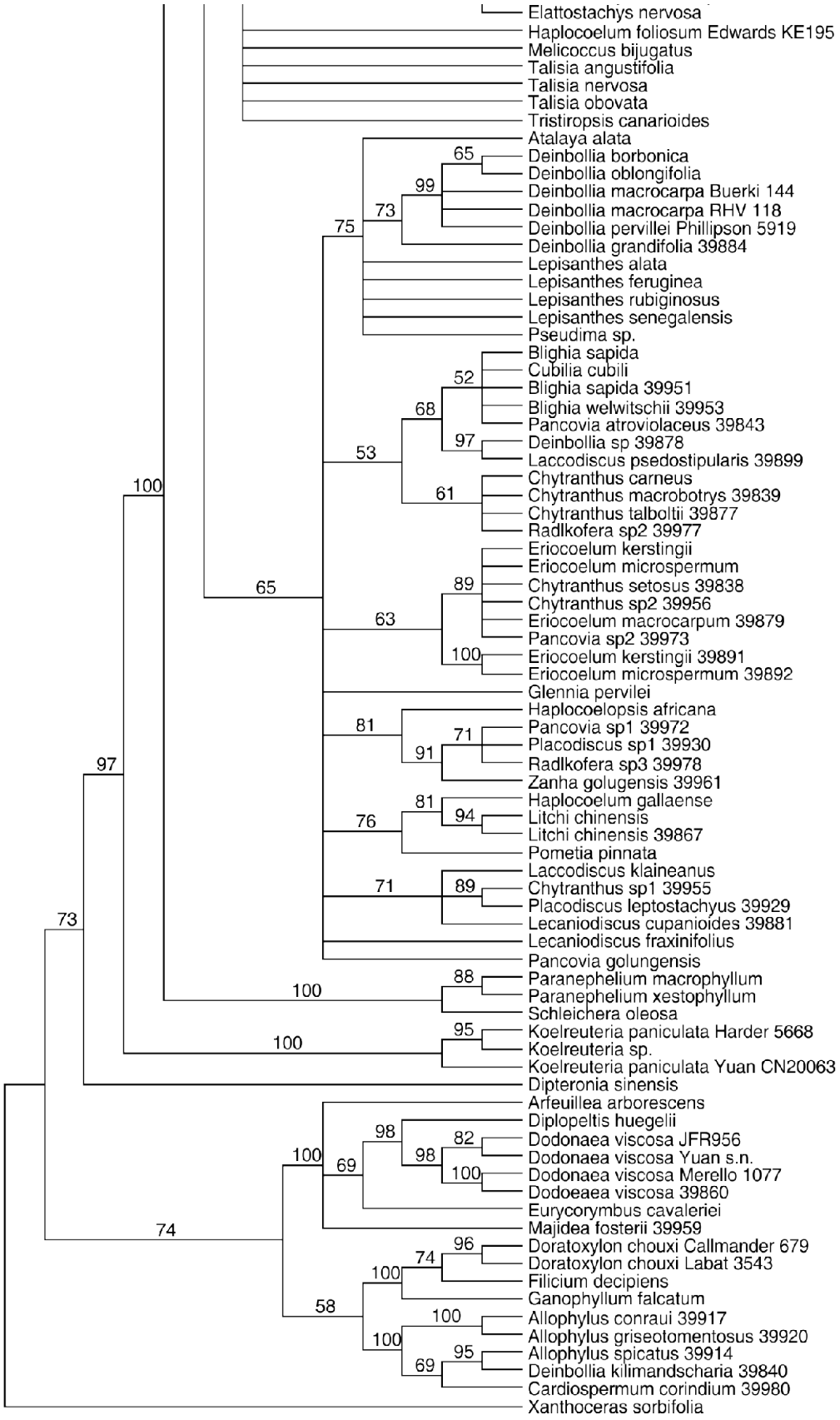

Figure 3: Phylogenetic Relationships within Sapindaceae based on matK data. Bootstrap supports are indicated above branches (cont'd).

DISCUSSION

Although members of the family Sapindaceae have been recorded to be widely distributed in Africa, their occurrence is threatened by a high rate of deforestation and agricultural practices leading to loss of forest and thus the threatened status of the family as recorded in the IUCN R.L. (2008). However, our sampling revealed that there are 28 genera and 119 species in Africa in contrast to the twenty two (22) genera recorded by Burkhill (2000). The other six genera include *Aphania, Atalaya, Ganophyllum, Haplocoelum, Laccodiscus* and *Litchi*. Taxa sampling within the Sapindaceae was difficult especially as DNA was extracted from herbarium materials. Though the herbarium materials were readily available, the problem was how they were preserved and what kinds of chemicals were present in the samples. The method of plant collection and duration of drying the material are important for the survival of the DNA. Some of the materials were oven-dried and the old material had been exposed to pesticides, these could have degraded the quality of DNA. Nevertheless, good quality DNA was successfully extracted from 48% of the samples most of which were silica gel dried samples.

This study supports previous studies by Harrington *et al.* (2005) and Buerki *et al.* (2009) on a broadly defined Sapindaceae including the family Aceraceae and Hippocastanaceae. Also in the matK gene analysis, *Xanthoceras* was found to be sister to all other Sapindaceae. While the Sapindaceae occurs as a monophyletic family, paraphyly was observed at subfamilial level and this supports previous reports by Harrington *et al.* (2005) and Buerki *et al.* (2009). Also, *Laccodiscus* sp. was shown to be more closely related to *Sorindea* sp. one of the out-group taxa from the family Anacardiaceae suggesting a mis-identification and this was supported with 56% bp. Generally, the matK gene gave a better resolution in identification of members of Sapindaceae and this supports the report of Kress and Erickson (2007) that *matK* is one of the most rapidly evolving plastid coding regions which consistently show high levels of discrimination among angiosperms. A particular strength of the barcoding approach is that the identifications can be made with small amounts of tissue from sterile, juvenile or fragmentary materials from which morphological identifications are difficult or impossible (Little and Stevenson, 2007). In addition, it is important to emphasize that the discriminatory power of this standard barcode will be higher in situations that involve geographically restricted sample sets, such as studies focusing on the plant biodiversity of a given region or local area (Valentini *et al.,* 2008). A future challenge for DNA barcoding in plants is to increase the proportion of cases in which unique species identifications are achieved.

CONCLUSIONS

This work is probably the first record of DNA Barcoding of Sapindaceae in Africa and this can be seen as a major contribution to the TREEBOL Africa and Genbank.

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