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The Efficiency of DNA Barcoding in the Identification of Afromontane Forest Tree Species

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Abstract: The identification of flowering plants using DNA barcoding proposed in last decades has slowly gained ground in Africa, where it has been successfully used to elucidate the systematics and ecology of several plant groups, and to understand their evolutionary history. Existing inferences on the effectiveness of DNA barcoding to identify African trees are mostly based on lowland forests, whereas adjacent montane forests significantly differ from the latter floristically and structurally. Here, we tested the efficiency of chloroplast DNA barcodes (*rbcLa*, *matK*, and *trnH-psbA*) to identify Afromontane Forest tree species in a 20.28 ha permanent plot in Ngel Nyaki, Taraba state, Nigeria. We collected, identified, and vouchered 274 individuals with diameter at breast height $\geq$ 1 cm belonging to 101 morphospecies, 92 genera, and 48 families. *rbcLa* and *matK* used alone or in combination performed better than in lowland forests, with the best species discrimination obtained with the two-locus combination of *matK* + *rbcLa*. The intragenic spacer *trnH-psbA* was too variable to align and could not be tested using the genetic distance method employed. Classic DNA barcode can be a powerful tool to identify Afromontane tree species, mainly due to the non-prevalence in these communities of species—rich genera (low species-to-genus ratio) that constitute the biggest challenge of DNA barcoding of flowering plants.

Keywords: DNA barcoding; ForestGEO; montane forest; Ngel Nyaki; species identification

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1. Introduction

Africa includes the second largest tropical forest block in the world, considered as one of the most important pool of biological diversity [1]. Yet, African forests are threatened by expanding human activities such as industrial logging, mining, agriculture, and road networks [2,3], but are also highly susceptible to the impact of climate change [4]. Despite the growing international concern about the future of these forests, the diversity, the ecology and the evolutionary processes that have shaped African forests remain relatively poorly understood, compared to the Amazon forest block [5]. In this regard, there is an urgent need to increase our efforts in documenting and describing the diversity of these forests as many of the species might go extinct before they are discovered. Therefore, large-scale biodiversity inventories of African forests will be critical to develop sound conservation strategies for these forests [6]. During the past decades, significant progress has been made in the study of the biodiversity of African forests using classic floristic inventories and long-term monitoring plots grouped into two main networks, the African Tropical Rainforest Observation Network (AfriTRON, http://www.afritron.org/) (accessed on 10 February 2022)and the Africa program of the Forest Global Earth Observatory Network (ForestGEO, https://forestgeo.si.edu/, accessed on 10 February 2022). In forest inventories, the species are identified merely on the basis of morphological characters, and this is challenging even for expert botanists. Often, up to 30% of the individuals in the plots remain unidentified

for years [7] due to the absence during field surveys of flowers and fruits that are needed to achieve accurate identifications [8].

Biological identification through "DNA barcode" was proposed, first in the animal kingdom [9,10] and later on for land plants [11,12] as a molecular method that could supplement morphological identifications. DNA barcodes are short and standardized fragments of DNA that should be easy to amplify and to sequence, and that can rapidly and reliably distinguish species from each other. DNA barcoding slowly gained ground in Africa, with over 21,000 vascular plants and 3000 animal records in the Barcode of Life Data System in 2019 [13,14], and has been used to elucidate the systematics and ecology of several plant groups, e.g., [15–17]. Existing African DNA barcodes for plants have been concentrated in forest ecosystems in Southern and West Africa [14,18] and more recently in savanna ecosystems [13]. Furthermore, inferences on the effectiveness of DNA barcode to identify African forest trees have been mostly based on lowlands. Whereas montane forests significantly differ floristically and structurally from lowland forests, the effectiveness of DNA barcoding in identifying tree species in these forests is still lacking.

We constructed a local DNA barcode database to aid the identification of tree species and reconstruct their community phylogeny in a 20.28 ha plot located in montane forest in Northeastern Nigeria. Here, we test the ability of this DNA barcode to identify the plot species and genera.

2. Materials and Methods

2.1. Study Site and Sampling

The tissue samples for DNA extraction were collected from the 20.28 hectares (260 × 780 m) Ngel Nyaki Forest Dynamics plot, where all trees with diameter at breast height (dbh) > 1 cm had previously been measured, mapped tagged and morphologically identified [19]. The plot (07°04005″ N, 11°03024″ E) is located within the Ngel Nyaki Forest Reserve on the Mambilla Plateau, Taraba State, Nigeria, with elevation ranging from 1588 m to 1690 m, and is part of the Forest Global Earth Observatory (ForestGEO) network [20]. The mean annual rainfall is 1800 mm while the mean annual temperature is 19 °C. The vegetation of the area is a mosaic of grassland and montane forest [21].

The morphological identifications of the trees were performed in the field by non-professional taxonomists, but were partially checked by the first author. The resulting checklist comprised 105 morphospecies including 74 (71%) identified to species level, 22 (21%) to genus, and 9 (9%) unidentified, even to family level. Of the 105 species (with dbh > 1 cm) recorded in the Ngel Nyaki plot, we sampled 99 belonging to 90 genera and 47 families. Two additional woody species growing in the vicinity of the plot, *Dracaena* cf. *deisteliana* Engl. (Asparagaceae) and *Pittosporum viridiflorum* Sims (Pittosporaceae), were added to our sample, making a total of 101 species in 92 genera and 48 families. We collected leaf tissue from 1 (for species that were represented by a single individual in the plot) to 4 individuals per species. The samples were collected in the field and were immediately dried in silica-gel. They consisted of 5–50 cm^2 of leaf tissue. Voucher specimens accompanying the leaf tissue were also collected and are deposited at the National Museum of Natural History in Washington.

2.2. DNA Extraction and Sequencing

All laboratory work was carried out at the Canadian Centre for DNA Barcoding (CCDB) (https://ccdb.ca/, accessed on 10 February 2022) and following their protocols. Total genomic DNA was extracted from silica dried leaf material using the CCDB protocol (https://ccdb.ca/site/wp-content/uploads/2016/09/CCDB_DNA_Extraction-Plants.pdf, accessed on 10 February 2022). DNA barcode regions *rbcLa*, *matK* and the *trnH-psbA* intergenic spacer were amplified using CCDB standard PCR primers and protocols (https://ccdb.ca/site/wp-content/uploads/2016/09/CCDB_Amplification-Plants.pdf, accessed on 10 February 2022) with the primers available at https://ccdb.ca/site/wp-content/uploads/2016/09/CCDB_PrimerSets-Plants.pdf (accessed on 10 February 2022)

Voucher details and GenBank accession numbers for all sequences are listed in BOLD (http://www.boldsystems.org/) (accessed on 10 February 2022).

2.3. Testing the DNA Barcode Accuracy

Prior to evaluating the identification success of the two barcode regions, we used the Basic Local Alignment Search Tool (BLAST) [22] to compare our sequences to those available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed on 10 February 2022), with the aim of confirming our identifications and identifying our unknowns. After matching our sequences in GenBank, the morphospecies names were updated only after comparison of their voucher specimens to either the type specimens available online or to other herbarium specimens and photographs in Tropicos (https://www.tropicos.org/, accessed on 10 February 2022).

To test the barcode efficiency, we followed [18]. Our DNA barcoding refence database (assumed to be exhaustive in terms of species) had 274 individuals and was used to assign individual trees to species or genera. The test was performed on species represented by at least two individuals in the database, so that we could have a query and a matching sample. The coding genes *matK* and *rbcLa* were aligned and manually adjusted using ClustalW in the Molecular Evolutionary Genetic Analysis software version 7.0.26 [23]. After the global alignment, we computed pairwise genetic distances among all sequences in the dataset using the Kimura's 2-parameter model [24]. The analysis was also performed in Mega7. In the resulting matrix, each sample (query) was assigned to a species or a genus of the sample (matching) from which it is separated with the least genetic distance (excluding itself). The identification was (1) correct when the query sample matched only the samples of its species of genus; (2) multiple if the query sample matched several species or genera including its correct one; and (3) wrong when the query sample matched species or genera different from its own [18]. We were not able to align *trnH-psbA* because it was too variable among the 48 plant families in the study. Hence this locus was not used in the test of DNA barcode accuracy analyses.

3. Results

3.1. Sequencing Success

DNA sequencing success was tested on 274 individual trees, representing 101 species. Sequencing success was lowest for *matK* and highest for *rbcLa*. Reliable contigs were obtained for only 78.9% of all individuals sequenced for *matK*, 95.3% *trnH-psbA*, but 97.5% for *rbcLa*, which corresponded to all the species represented in the database for *rbcLa* and *trnH-psbA*, but only to 93.1% of the species for *matK* (Table 1). The percentages of species represented by at least two individuals for *matK*, *rbcLa* and *trnH-psbA* in the database were 70.3%, 87.2% and 84.2% respectively.

Table 1. Sequencing success of montane forest trees from Ngel Nyaki for *rbcLa*, *matK* and *trnH-psbA* barcode regions.

	matK	***rbcLa***	***trnH-psbA***
Number of individuals tested	274	274	274
Sequencing success: N ind. (% ind.)	216 (78.9)	267 (97.5)	261 (95.3)
Sequencing success: N sp. (% sp.)	94 (93.1)	101 (100)	101 (100)
N sp. with sequences $\geq$ 2 samples	71 (70.3)	88 (87.2)	85 (84.2)

3.2. Taxonomic Update Using BLAST

The identification of 13 morphospecies was updated using the heuristic search in GenBank. Of the nine morphospecies for which the family was unknown, seven were identified to species level and two placed in different plant families. Furthermore, the identification of four other morphospecies was updated. The first morphospecies placed in the *Argocoffeopsis* Lebrun (Rubiaceae) Lebrun was updated to *Psilanthus mannii* Hook. within the same family. A morphospecies thought to belong to the genus *Beilschmiedia* Nees

(Lauraceae) was transferred to the family Sapotaceae. The identification of a morphospecies thought to be *Lannea barteri* (Oliv.) Engl. (Anacardiaceae) was updated to *Brucea antidysenterica* J.F. Mill. (Simaroubaceae). Finally, the morphospecies *Hannoa klaineana* Pierre ex Engl. (Simaroubaceae) was updated to *Ekebergia capensis* Sparrm.

3.3. Barcode Accuracy

The accuracy of two of the three barcode markers (*matK* and *rbcLa*) in identifying montane forest trees is presented in Table 2. The analyses were performed on all available samples for each marker. When used individually, highest success for the identification of species was obtained with *matK* (98.3%). The two makers performed slightly better when combined. At genus level, the same trend was maintained, but with even better performances. Here, *matK* and the *rbcLa* + *matK* combination successfully identified all the samples to the genus, while *rbcLa* alone was successful to identify 98.4% of samples to genus (Table 2).

Table 2. Barcoding accuracy in identifying Ngel Nyaki Afromontane forest trees at species and genus level.

	Barcoding Accuracy			**Query Samples**		
	Correct ID	**Multiple ID**	**Wrong ID**	**N. ind.**	**N. sp.**	**N. Gen.**
Species identification						
rbcLa	93.8	6.15	0	244	92	92
matK	98.3	1.1	0.55	181	67	59
matK + *rbcLa*	98.9	0.5	0.54	186	72	63
Genus identification						
rbcLa	98.4	1.6	0	244	92	92
matK	100	0	0	181	67	59
matK + *rbcLa*	100	0	0	186	72	63

4. Discussion

4.1. Recoverability of DNA Barcode Used

The two DNA barcodes *rbcLa* and *matK* used in this study have long been recognized having sufficient variation to discriminate among land plant species [11,25,26]. Among the three barcodes, *matK* had the lowest rate of recovery (79%), consistent with prior studies [18,27,28]. In contrast, *rbcLa* and *trnH-psbA* had higher rates of recovery (above 95%). However, it is worth pointing out that the rates of recovery were in general higher than in prior studies, probably due to the efficiency of the Canadian Centre for DNA Barcoding that has optimized protocols for higher rates of recovery. For example, recovery rates around 70% have been reported for *matK* in several studies [8,27,29], while sequencing and amplification success for *rbcLa* and *trnH-psbA* is often below 94% e.g., [8,27,30].

4.2. Tree Species Identification Using DNA Barcode in Ngel Nyaki Montane Forest

The morphological identification of the trees in the Ngel Nyaki plot was almost entirely performed by non-professional taxonomists who however accurately identified to species 69% of all tree species occurring in the plot. Only four species were wrongly identified. The DNA barcode was instrumental in updating the identification of 12% of the species in the plot for which prior sequences were available in Genbank. Due to the lack of adequate library in Genbank, 21% of the species in the plot for which good quality barcode sequences were generated could still not be identified to species level. Hence, molecular techniques such as DNA barcode may not replace traditional taxonomic techniques as suggested by some studies [31], but can only supplement it.

This study showed the efficiency of the two barcode loci *rbcLa* and *matK* in accurately assigning Afromontane forest tree species to a correct species or genera. When

used alone, best results for species identification were obtained with *matK* (98%) compared to *rbcLa* (94%). These values are slightly higher than those reported in most lowland forests [8,18,27,30]. The combination of the two markers *matK* + *rbcLa* improved the barcoding success to 99%, a result consistent with those in most lowland forests. Barcoding success was even better at genus level, *rbcLa* alone identifying 98% of all genera, while *matK* and the combination *matK* + *rbcLa* accurately identified all the samples to genus.

The genetic distance method that we used did not allow us to test the accuracy of the intergenic spacer *trnH-psbA*. This locus, easy to amplify and short, is known to be very variable among angiosperms and thus is widely used in plant species identification [32]. In general, *trnH-psbA* locus is more variable than *matK* and *rbcL* and we assume its performance in the identification of montane forest species would even be greater. *matK* and *rbcLa* were variable enough that their combination to *trnH-psbA* was no more relevant.

4.3. The Efficiency of DNA Barcoding in the Context of the Afromontane Flora

DNA barcode is a powerful tool for identifying tree species to genus level. However the identification to species level is not always reliable, especially in plant communities with speciose genera [18]. For example, the identification of tree species (with dbh $\geq$ 1 cm) in a 50-ha plot in the highly diverse Korup National Park, Cameroon using three DNA barcode markers showed a significant decrease in their performance with increasing number of species per clade (genus) [18]. In fact, the five most speciose genera in the Korup plot *Cola* Schott & Endl., *Diospyros* L., *Psychotrya* L., *Rinorea* Aubl. and *Garcinia* L. have 23, 14, 13, 13 and 10 species respectively [33]. Such closely related species are more likely to hybridize, have incomplete lineage sorting and share haplotypes, all of which can lessen the ability of barcode loci to discriminate among them. At the other end of the spectrum, 165 (33%) species in Korup are represented by a single species.

The Ngel Nyaki plot had a relatively low diversity, with only 105 species in 92 genera. The most speciose genera here are *Ficus* L. and *Psychotria* L., each having three species. Five other genera have two species each, while the remaining 85 species (81%) are represented each by a single species. This species-to-genus (S/G) ratio is not specific to the Ngel Nyaki montane forest. In fact, most Afromontane forests are characterized by a low diversity of trees and low S/G ratio. For example, in Woodbush–De Hoek montane forest in South Africa, 50 species of trees with dbh > 5 cm and dbh > 10 cm in 46 genera (S/G = 1.09) were recorded within 1.5 ha circular plots [34]. Similarly, [35] in a study on trees with dbh $\geq$ 5 cm in dry Afromontane forests of Awi Zone, northwestern Ethiopia, recorded 18 species in 18 genera, 21 species in 21 genera, 20 species in 20 genera, 16 species in 16 genera and 23 species in 23 genera in 0.6 ha of Bari, Apini, Dabkuli, Tsahare Kan, and Kahtasa forests respectively.

We further explored the relationship between the S/G ratio and elevation, by comparing the Ngel Nyaki data other African forest sites for trees with dbh $\geq$ 10 cm (Table A1). The S/G ratio decreases with increasing elevation, with a correlation coefficient of -0.722 (Figure 1A). The Lambi 2 and Ngovayang mid-elevation plots in Cameroon had the highest S/G ratio (1.55 and 1.51 respectively) while higher elevation plots Bwindi 1 and Bwindi 4 had the lowest. The Lambi the Ngovayang plots seem to be outliers in our dataset. In fact, a stronger relationship with $r = -0.80$ is shown when these plots are removed. Higher S/G ratio of 2.6 and 3 have been reported elsewhere in the Manu forest (Peru) and Yasuni forest (Ecuador) respectively for trees with the same diameter cutoff [36]. The S/G ratio increases when smaller diameter size classes are considered and the correlation with elevation is stronger ($r = -0.84$, p-value = 0.007). A highest S/G ratio of 1.64 is observed for the lowland Rabi plot and 1.15 for the Ngel Nyaki plot for all trees with dbh $\geq$ 1 cm were measured (Figure 1B). In fact, the understory of most African forests are stocked with speciose genera of small-statured trees that never attain large size diameter classes [37,38]. Several studies have shown the decrease in tree species diversity with elevation, e.g., [39,40]. Our data also shows a decrease of generic diversity with increasing elevation ($r = -0.84$). This means that the low diversity in higher elevations is also due to the decrease in the number of genera,

but coupled with the decrease in the number of species per genera. This result is consistent with Jaccard's observations in the Alps [41], who noted that "with increasing altitude, the number of genera decreases less rapidly than the number of species".

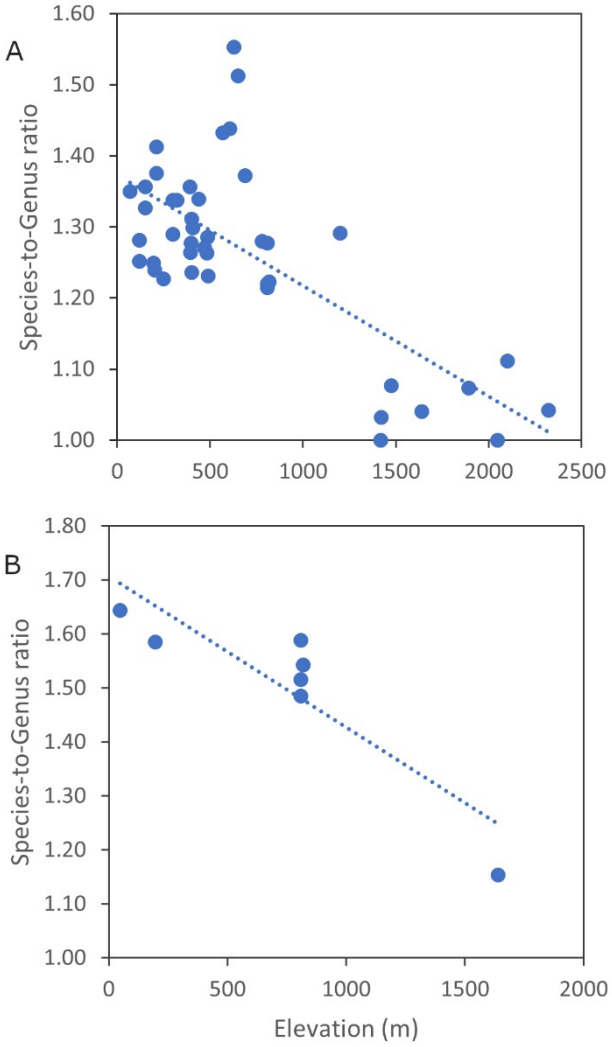

Figure 1. Correlation between the species-to-genus (S/G) ratio and elevation, (**A**) for trees with dbh > 10 cm in forty three 1-ha African forest plots, The correlation coefficient $r = -0.722$, p-value = 0.00000004635; (**B**) for trees with dbh > 1 cm in seven large (10–50-a) census plots, correlation coefficient $r = -0.88$, p-value = 0.007.

5. Conclusions

Our study highlighted how DNA barcoding can be efficient in identifying tree species in an Afromontane Forest. As in lowland forests, identification success is higher at genus than at species level. Identification success was higher than in lowland forest, due to the non-prevalence of highly diverse genera in this habitat. The comparison of species-to-genus among other sites with comparable data showed that Afromontane forests tend to have a low S/G ratio for tree species, which is an advantage for the use of DNA barcode in these forests.

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Data Availability Statement: Full census data for the Ngel Nyaki plot is available upon reasonable request from the ForestGEO data portal http://ctfs.si.edu/datarequest/ and the full plant DNA barcode library is available on BOLD (http://www.boldsystems.org/) (accessed on 10 February 2022).

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Species-to-Genus ratio (S/G) among 43 African forest 1-ha plots for trees with dbh $\geq$ 10 cm. * denote large plots (10–50 ha) of the Forest Global Earth Observatory (ForestGEO) network. The data for each large plot was obtained by averaging the values in 1-ha subplots within the plot. S = number of species, G = number of genera. TEAM = Tropical Ecology Assessment and Monitoring.

Site	Country	Elevation (m)	S	G	S/G	Source
Bwindi-1	Burundi	1474	42	39	1.08	TEAM Network
Bwindi-2	Burundi	1419	28	28	1.00	TEAM Network
Bwindi-3	Burundi	1893	44	41	1.07	TEAM Network
Bwindi-4	Burundi	2049	27	27	1.00	TEAM Network
Bwindi-5	Burundi	2101	30	27	1.11	TEAM Network
Bwindi-6	Burundi	2321	25	24	1.04	TEAM Network
Bidjouka-1	Cameroon	392	99	73	1.36	[42]
Bidjouka-2	Cameroon	605	105	73	1.44	[42]
Korup 50-ha *	Cameroon	195	87.2	48.82	1.79	[33]
Lambi-1	Cameroon	396	106	83	1.28	[42]
Lambi-2	Cameroon	627	118	76	1.55	[42]
Ngovayang-1	Cameroon	650	121	80	1.51	[42]
Rumpi-hills-11	Cameroon	1450	32	31	1.03	[43]
Takamanda-10	Cameroon	210	108	78.5	1.38	[44]
Takamanda-11	Cameroon	210	113	80	1.41	[44]
Takamanda-12	Cameroon	150	105.5	79.5	1.33	[44]
Takamanda-13	Cameroon	150	118	87	1.36	[44]
Takamanda-14	Cameroon	120	87	69.5	1.25	[44]
Takamanda-15	Cameroon	120	91	71	1.28	[44]
Takamanda-6	Cameroon	320	103	77	1.34	[44]
Takamanda-7	Cameroon	400	97	74	1.31	[44]
Takamanda-8	Cameroon	780	64	50	1.28	[44]
Takamanda-9	Cameroon	1200	71	55	1.29	[44]
Dzanga-Sanga-1	Central African Republic	471	108	85	1.27	[45]
Dzanga-Sanga-2	Central African Republic	482	120	95	1.26	[45]
Dzanga-Sanga-3	Central African Republic	393	67	53	1.26	[45]
Dzanga-Sanga-4	Central African Republic	489	96	78	1.23	[45]
Dzanga-Sanga-5	Central African Republic	485	108	84	1.29	[45]
Edoro-1 (10-ha) *	DR Congo	808	65.4	53.6	1.22	[46]
Edoro-2 (10-ha) *	DR Congo	809	67.4	55.5	1.21	[46]
Lenda-1 (10-ha) *	DR Congo	808	60.4	47.3	1.28	[46]
Lenda-2 (10-ha) *	DR Congo	819	49.9	40.8	1.22	[46]

Table A1. *Cont.*

Site	Country	Elevation (m)	S	G	S/G	Source
Monts de Cristal-1	Gabon	400	89	72	1.24	[47]
Monts de Cristal-2	Gabon	300	89	69	1.29	[47]
Monts de Cristal-3	Gabon	300	99	74	1.34	[47]
Monts de Cristal-4	Gabon	200	88	71	1.24	[47]
Monts de Cristal-5	Gabon	250	108	88	1.23	[47]
Rabi 25-ha *	Gabon	47	84.6	62.68	1.35	[38]
Waka-10	Gabon	569	106	74	1.43	[48]
Waka-6	Gabon	438	83	62	1.34	[48]
Waka-7	Gabon	407	100	77	1.30	[48]
Waka-8	Gabon	687	107	78	1.37	[48]
Ngel Nyaki (20.28 ha) *	Nigeria	1639	41.1	39.5	1.04	[19]

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