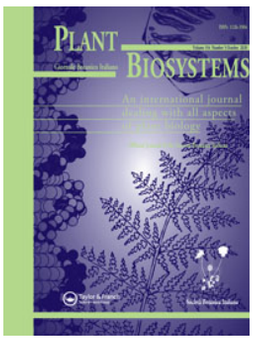

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Utility of iPBS retrotransposons markers for molecular characterization of African *Gnetum* species

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ABSTRACT

Species of African *Gnetum* are lianas used as vegetables, medicines and for generating income. Despite the taxonomic confusion, identification of new species and diverse morphological characters in African *Gnetum*, molecular markers on these plants are lacking. However, the inter-primer binding site (iPBS) retrotransposons markers could be simple and excellent molecular markers for African *Gnetum*. The objective of this study was to determine the efficiency of iPBS markers in detecting genetic differentiation in African *Gnetum* species. A set of 21 iPBS markers were analysed on 14 accessions including *G. africanum* Welw., *G. buchholzianum* Engl. and the recently identified species *G. latispicum*. Six best selected primers generated 103 bands in *G. africanum*, 95 in *G. buchholzianum* and 24 in *G. latispicum*. Cluster analysis divided the accessions into two major groups. The first group contained all the accessions of *G. africanum*, whereas the second group was further divided in two subgroups representing accessions of *G. buchholzianum* and *G. latispicum*. Additionally, the Jaccard similarity coefficient indicated a close relationship between accessions of *G. buchholzianum* and *G. latispicum*. The iPBS marker system revealed genetic differentiation within African *Gnetum* and could be useful for evaluating genetic diversity, conservation, taxonomy and evolution studies.

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African *Gnetum*; iPBS; molecular characterization; polymorphism; genetic differentiation; genetic diversity

Introduction

Gnetum L. (Gnetaceae) comprised approximately 50 species of lianas and trees distributed in the tropical areas of the world. Among this gymnosperm genus there are species of African *Gnetum* which are diocieous vines occurring only in the African continent.

Species of African *Gnetum* are slow growing vegetables highly prized for their edible leaves which have different taste depending on the strains. The leaves have cultural and socio-economic values and are traded locally and internationally. They are rich in mineral elements, essential amino acids and can constitute an important source of protein for the local populations (Sunderland and Clark 2001). In addition to their nutritional value, these plants are also reported to have very useful medicinal properties among which the treatment of nausea, enlarged spleen, sore throat and hypertension (Burkill 1994; Mensah et al. 2008) and the lowering of plasma cholesterol levels (Isong et al. 1999). Apart from the food source and primary health care that species of African *Gnetum* provide, they exhibit a long market chain with several steps between harvest and the final consumer (Ingram et al. 2012).

The wild population constitutes the main source of *Gnetum* lianas that are consumed locally or exported to other countries. In recent years, due to deforestation, overharvesting and increasing demand, decline of the wild stocks has been observed in certain natural habitat and countries. Therefore, African *Gnetum* species are now considered as near threatened species in International Union for Conservation of Nature (IUCN) red list. They are also identified by the World Agroforestry Centre as one of the most important species for domestication in the humid forest of Africa (Tchoundjeu et al. 2006). Consequently, several efforts based on sustainable cultivation were directed towards domestication and conservation of African *Gnetum* species. The introduction of *Gnetum* lianas into farming systems may reduce harvesting pressures on wild populations and total extinction of African *Gnetum* species can be averted (Ndam et al. 2001).

Morphologically, African *Gnetum* species are very similar making their identification difficult, even for commercial and utilization purposes. In general, two species exist: *G. africanum* and *G. buchholzianum* (Hutchinson and Dalziel 1954; Gillespie and Nowicke 1994; Hou et al. 2015). But there are cases that the names of *G. africanum* and *G. buchholzianum* have been applied interchangeably to similar morphological forms or on duplicates of specimens (Biye 2013, Biye et al. 2014). In some cases and studies only one species (Won and Renner 2003) or up to three species (*G. africanum*, *G. buchholzianum* and *Gnetum* sp.) have been distinguished (Onguene and Kuyper 2001). Thus, there is a confusion on taxonomic grouping and identification of these vines.

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Recently, Biye (2013) identified two new species thus making a total of four *Gnetum* species in Africa.

Furthermore, species of African *Gnetum* have a wide range of distribution across humid tropical forests of African countries including Angola, Cameroon, Central African Republic, Congo, Equatorial Guinea, Gabon, Democratic Republic of Congo and Nigeria. It appears that the taxonomic confusion coupled to the limited knowledge regarding genetic and geographical differentiation can limit domestication, conservation and the utilization strategies of African *Gnetum*. Research to define the identification, differentiation and genetic diversity of species of African *Gnetum* has depended mostly on the use of vegetative and reproductive morphological characters (Biye et al. 2016). However, little or no information exists on the successful use of molecular marker systems for African *Gnetum* characterization.

In this context, there is a need to identify a simple and efficient molecular marker that will supplement the morphological marker for the characterization, conservation and sustainable utilization strategies of African *Gnetum* species. Here, we were interested in exploiting the retrotransposon-based molecular marker inter-primer binding site (iPBS). This marker system has been shown to be a powerful fingerprinting technology for species without the need for sequencing or specific development of primers (Kalendar et al. 2010; Kalendar et al. 2019). It has also been used successfully for the characterization of *G. gnemon* (Kalendar et al. 2010). These qualities make iPBS ideal for African *Gnetum* species, which are still orphan crops. iPBS markers have recently been used to detect polymorphisms in closely related or clonally derived material, including *Phaseolus vulgaris* (Nemli et al. 2015), *Lens* (Baloch et al. 2015), *Camellia sinensis* (Phong et al. 2016) and *Vitis vinifera* (Milovanov et al. 2019).

In this study, we tested the efficiency of iPBS to discriminate between 14 accessions of African *Gnetum* species. The aims were to select simple and efficient markers that will be useful for delimiting species of African *Gnetum*, identifying genotypes and allowing in-depth analysis of population structure.

Materials and methods

Plant materials and DNA extraction

In this work we have used six accessions of *G. africanum*, six of *G. buchholzianum* and two of *G. latispicum*. Accessions were found in Cameroon from the IRAD Ekona Regional Research Centre and the Limbe Botanical Garden. These were grown from cuttings collected from South West, Littoral, South, Centre and East Regions, thus representing two agroecological zones of Cameroon.

Total genomic DNA of these genotypes was extracted from leaf samples following a modification of the cetyltrimethylammonium bromide (CTAB) extraction protocol (http://primerdigital.com/dna.html) with RNAse A treatment. The quality and concentration of genomic DNA was checked by gel electrophoresis and spectrophotometrically with Nanodrop (Thermo Fisher Scientific Inc.).

PCR protocol for iPBS

iPBS analysis was conducted as described by Kalendar et al. (2010) using iPBS primers. PCR reactions for iPBS analyses were performed in a 25-µl reaction containing 20–25 ng genomic DNA, 1× DreamTaq PCR buffer, 1 µM primer, 0.2 µM each dNTP and 1 U DreamTaq DNA polymerase. PCR reactions were performed in a Mastercycler Gradient (Eppendorf AG, Germany) in 0.2-ml tubes. After an initial denaturation step at 95 °C for 3 min, thermocycling was performed at 95 °C for 15 s, 50–60 °C for 30 s and 72 °C for 90 s for 32 cycles with a final extension at 72 °C for 5 min.

Each primer was tested singularly in PCR reactions using genomic DNA from all the accessions. The PCR products were separated by electrophoresis at 60 V for 8 h in a 1.5% agarose gel (RESolute Wide Range, BIOzym) with 0.5× Tris-Borate-EDTA (TBE) electrophoresis buffer. Gels were stained with EtBr and scanned using an FLA-5100 imaging system (Fuji Photo Film GmbH., Europe) with a resolution of 50 µm.

iPBS fingerprint analyses

Amplified bands were scored as present (1) or absent (0) in each sample for the analysis. Subsequently, from the binary code, iPBS primers were evaluated in two ways. First, a set of 21 primers were tested for efficiency in the yield of iPBS bands and for fingerprinting quality (ie for the possibility for the amplified loci to be distinguished and scored). These primers were selected based on their high performance in analysing barley genotypes and other crops such as *Spartina alterniflora* and *G. gnemon* which is closely related to African *Gnetum* (Kalendar et al. 2010; Smykal et al. 2011; Kalendar and Schulman 2014; Kalendar et al. 2019). Second, based on their efficiency in iPBS amplification, six excellent primers were retained for subsequent analysis related to the generated bands.

Genetic similarity and principal coordinate analysis among all accessions of African *Gnetum* were performed using GenAlex 6.5 (Peakall and Smouse 2012) and Fingerprint Analysis with Missing Data (FAMD) 1.31 programmes (Schlüter and Harris 2006). Genetic similarity was also calculated using the Jaccard coefficient (Jaccard 1912). The relationships among accessions of African *Gnetum* based on the data from all six chosen primers were defined by principal component analysis (PCA). The PCA was performed using a covariance matrix that was calculated from a pairwise genetic distance matrix.

A dendogram was constructed by the neighbour joining cluster analysis using PAUP software (Swofford 1998). Bootstrap operations with 1000 replicates were performed to support the tree.

Results

Initial screening of primers

The initial screening of the 21 iPBS primers across 14 accessions of African *Gnetum* produced variable polymorphic patterns depending on the primer tested (Table 1). All the

Table 1. 12-13-18-mer PBS primers and their efficiency in single-primer iPBS amplification as tested on African *Gnetum* species.

Primer ID[a]	Sequences	Tm (°C)[b]	Average iPBS efficacy[c]
2076	GCTCCGATGCCA	53.6	3
2078	GCGGAGTCGCCA	57.7	3
2080	CAGACGGCGCCA	58.2	5
2081	GCAACGGCGCCA	60.1	5
2385	CCATTGGGTCCA	48.2	2
2394	GAGCCTAGGCCA	51.2	3
2270	ACCTGGCGTGCCA	60.3	5
2271	GGCTCGGATGCCA	58.0	5
2272	GGCTCAGATGCCA	54.1	3
2221	ACCTAGCTCACGATGCCA	63.6	3
2228	CATTGGCTCTTGATACCA	57.6	3
2229	CGACCTGTTCTGATACCA	59.3	3
2230	TCTAGGCGTCTGATACCA	59.6	3
2231	ACTTGGATGCTGATACCA	58.4	2
2232	AGAGAGGCTCGGATACCA	62.0	3
2241	ACCTAGCTCATCATGCCA	60.9	4
2242	GCCCCATGGTGGGCGCCA	74.6	5
2243	AGTCAGGCTCTGTTACCA	60.4	5
2249	AACCGACCTCTGATACCA	60.1	3
2295	AGAACGGCTCTGATACCA	60.5	3
2298	AGAAGAGCTCTGATACCA	57.1	4

[a]Primers selected for further analyses are underlined.
[b]Oligonucleotide concentration of 1 μM, 1.5 mM Mg^{2+} (Kalendar and Schulman 2014).
[c]iPBS amplification efficacy rating scale: 0, no bands; 1, few and weak bands; 2, a few strong bands; 3, ≥10 strong bands; 4, many bands (good primer); 5, many strong and equally amplified bands (excellent primer).

tested primers amplified bands but six of them (2080, 2081, 2242, 2243, 2270 and 2271) gave best and highly polymorphic patterns. Only these primers were selected to investigate the utility of iPBS for interspecific relationships and genetic variation among the 14 accessions.

iPBS profiles (genetic differentiation in African Gnetum accessions)

The iPBS technology was used to study genetic variability among 14 accessions of African *Gnetum* using the six chosen primers singly in PCR reactions. The PCR reactions produced a large number of bands. A total of 497 reproducible bands, ranging from 200 bp (primer 2271) to 8000 bp (primer 2243) were detected using the six selected primers. The majority of genotype fragments had a frequency over 5%. The number of private bands exclusively present in accessions of only one species showed a pattern similar to the total scored, from 24 (in *G. latispicum*) to 95 (in *G. buchholzianum*) and 103 (*G. africanum*). From these private bands, 17 could unambiguously distinguish between *G. africanum* (7), *G. buchholzianum* (4) and *G. latispicum* (6). An amplification profile of the primers 2080 and 2270 is shown in Figure 1.

Across all samples analysed, the primers displayed between 90% (primer 2270) and 100% (primer 2271) polymorphism. The Shannon index was on average 0.4 and ranged from 0.34 (primer 2242) to 0.45 (primer 2270). The percentage of polymorphic loci and the Shannon index of the primers are presented in Table 2.

To assess the genetic relatedness among the 14 African *Gnetum* accessions, the Jaccard coefficient of similarity was calculated and ranged from 0.14 to 0.66. The minimum genetic similarity (0.14) was between G1 (*G. africanum* accession) and G10/G14 (*G. buchholzianum* accessions) and the greatest (0.66) was between G5 and G6 (two accessions of *G. africanum*). The results of the Jaccard genetic similarity coefficients between each pair of accessions are presented in Table 3.

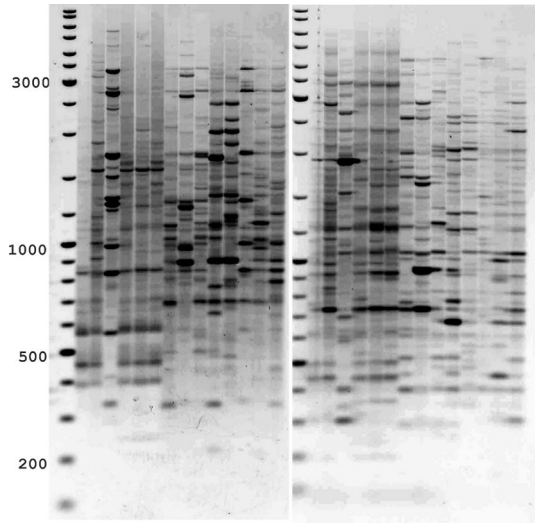

Figure 1. iPBS fingerprinting of African *Gnetum* species. Results for two primers 2270 (left) and 2080 (right) are shown. Lanes, left to right: 1, size ladder (bp); 2–7, *G. africanum* accessions; 8–9, *G. latispicum* accessions; 10–15, *G. buchholzianum* accessions.

Table 2. Percentage of polymorphic loci and Shannon index of the six iPBS primers in 14 African *Gnetum* accessions.

Primer ID	2080	2081	2270	2271	2242	2243
Polymorphic loci (%)	98.91	98.9	93.58	100	97.53	98.79
Shannon index	0.38	0.44	0.45	0.41	0.34	0.39

Multivariate analyses

In the dendrogram (Figure 2) resulting from the estimation of phylogenetic relationships among 14 accessions of African *Gnetum*, accessions of *G. africanum* and *G. buchholzianum* were found separately in two major clusters. In the first major cluster, the accessions of *G. africanum* were further grouped according to their geographic distribution. The second major cluster resolved two subgroups representing accessions of *G. buchholzianum* and accessions of *G. latispicum*, which is one of the recently identified species (Biye 2013).

PCA was performed to further analyse the variability among African *Gnetum* accessions in three-dimensional mode and to confirm the clustering pattern obtained from the dendrogram. When the combined iPBS data of the six primers was subjected to PCA analysis, the accessions formed three groups separated by the first three principal components, which accounted for 55% of the total variance detected. This consisted of 33, 11 and 10% from the first, second and third component, respectively. An ordination plot of the first two components (Figure 3) showed that *G. africanum* accessions were distantly separated from *G. buchholzianum* and *G. latispicum* accessions. This is congruent with the dendrogram from neighbour-joining analysis (Figure 2).

Table 3. Jaccard genetic similarity among pairwise comparisons for 14 African *Gnetum* accessions based on iPBS analysis using the set of six primers.

	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14
G1	1													
G2	0.57	1												
G3	0.25	0.32	1											
G4	0.51	0.51	0.27	1										
G5	0.47	0.47	0.24	0.68	1									
G6	0.52	0.47	0.23	0.63	0.66	1								
G7	0.19	0.21	0.23	0.20	0.20	0.22	1							
G8	0.20	0.22	0.22	0.21	0.20	0.23	0.54	1						
G9	0.17	0.15	0.19	0.18	0.17	0.18	0.50	0.42	1					
G10	0.14	0.17	0.17	0.16	0.17	0.17	0.40	0.35	0.44	1				
G11	0.15	0.18	0.19	0.17	0.18	0.18	0.38	0.35	0.41	0.59	1			
G12	0.16	0.16	0.26	0.18	0.16	0.17	0.36	0.32	0.42	0.41	0.47	1		
G13	0.17	0.21	0.25	0.20	0.18	0.19	0.37	0.33	0.33	0.43	0.46	0.54	1	
G14	0.14	0.18	0.22	0.17	0.18	0.16	0.36	0.36	0.40	0.41	0.44	0.46	0.50	1

Discussion

iPBS markers and genetic variation

African *Gnetum* species are only found in the humid forests of Africa. These species are lianas that need to grow on a support plant. These plants are gradually disappearing due to deforestation and overharvesting of wild populations. As a result of anthropogenic factors, African *Gnetum* populations are decreasing and must be adapted to new environments. Therefore, an assessment of genetic diversity is crucial for managing and developing effective conservation strategies, especially for *Gnetum* vines that are endemic to the African humid forest, taxonomically ambiguous, endangered and under domestication.

This study represents the first use of iPBS, a retrotransposon-based molecular marker, to measure genetic variation and interspecific relationships in African *Gnetum*. Most studies related to the genetic variation of African *Gnetum* species focused only on morphological characterization. Although iPBS is a new technology, it has already been used successfully as an effective tool to evaluate genetic diversity and phylogenetic relationships in many plant species. The number of amplified fragments and the degree of polymorphism of the iPBS products using the six iPBS primers in African *Gnetum* species obtained in this work are high. They were sufficient to estimate genetic relationships among and within plant species. Thus, the results obtained suggest the usefulness of iPBS markers for quantifying the genetic diversity of species of African *Gnetum* and to identify polymorphisms between accessions. The large number of amplified DNA fragments obtained with iPBS primers could be explained by an abundance of retrotransposon-derived sequences in the African *Gnetum* genome. This allows the inserted elements of the same family to be in head-to-head or tail-to-tail orientation sufficiently close to produce amplification products.

The application of iPBS markers shows that *Gnetum* vines have high genetic diversity at the species level, and even among-species genetic differentiation exists. The geographical distribution and evolutionary history could account for the high diversity shown across species of African *Gnetum*. Though all the samples used in this study were collected in Cameroon, they represent accessions grown from cuttings collected from different regions with different agroecologies.

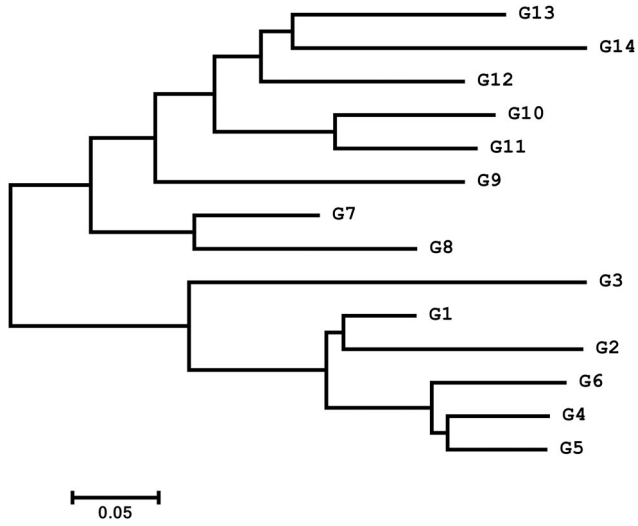

Figure 2. Neighbour-joining dendrogram showing the genetic relationships among 14 accessions of African *Gnetum* based on iPBS analysis. G1–G6, *G. africanum* accessions; G7–G8, *G. latispicum* accessions; G9–G14, *G. buchholzianum* accessions.

Among all species of the genus *Gnetum*, species of African *Gnetum* are found only in Africa. According to the evolutionary history suggested by Won and Renner (2006), the divergence of the extant clades of *Gnetum* began during the Upper Oligocene or a mid-Miocene based on relax clock and strict clock estimates, respectively. Moreover, these estimates implied that African *Gnetum* species are not a remnant of a once Gondwanan distribution, hence they have probably accumulated large quantities of genetic variations. This is eminent as long-lived lianas plants, species of African *Gnetum* have more opportunities to accumulate mutations.

Implication for conservation of wild resources and domestication

Species of African *Gnetum* can be propagated naturally by seeds. But due to the difficulty of the seed to germinate under nursery conditions, propagation methods through cuttings were developed to facilitate domestication. Thus far, studies on propagation have been performed on only one species separately (Shiembo et al. 1996) or on *Gnetum* spp. in bulk (Bongjoh et al. 2010) and just recently on *G. africanum* and *G. buchholzianum* (Doungous et al. 2019). Given that our study clearly demonstrates the existence of more than one species, it is important to develop efficient propagation methods considering the different species or accessions coming from different regions.

As high genetic differentiation resides among species, each of the species could also represent a large proportion of the genetic variation of the African *Gnetum* species. Therefore, major efforts should be directed to preserve the populations of African *Gnetum* in the wild and to establish a sustainable resource base sufficient to meet increasing demand. This seems to be possible through a combination of in situ and ex situ approaches. Efforts should thus be focused on sustainable exploitation of wild resources to avoid destruction of the full plant. This can only be done by

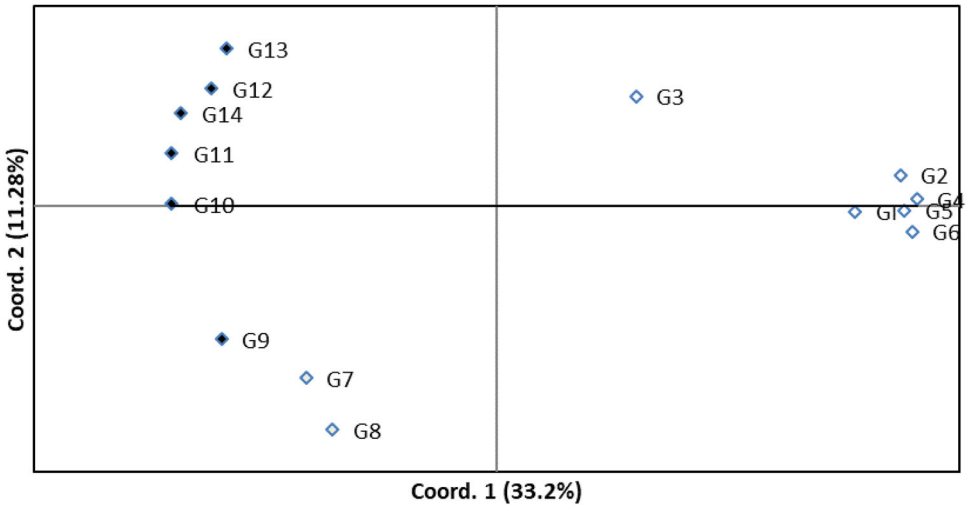

Figure 3. PCA plot of 14 African *Gnetum* accessions using the six primers. White dot, *G. africanum* accessions; grey dot, *G. latispicum* accessions; black dot, *G. buchholzianum* accessions. The percentage of variation accounted by each axis is reported.

addressing ecological, educational, policy and other issues that will build sustainable harvest and management systems. Ex situ conservation by the creation of germplasm should be given priority in each country. Accessions from each ecological zone should be considered, and samples should be collected from as many populations as possible. This is very important as species of African *Gnetum* are under domestication and their wild resources are subject to deforestation and excessive harvesting. It would be ideal if African *Gnetum* domestication can be accelerated to establish sufficient artificial plantations and to meet market demand. Only by these means can excessive harvesting from existing wild populations be reduced.

Implication for taxonomy

All species of African *Gnetum* are very similar for many characteristics and are collectively and commonly called under one vernacular name depending on the location or country. This has made identification challenging. The use of leaf shape as a characteristic to identify these species is usually judged subjectively. Recently, diverse morphological characters were used to determine the relationship between species. However, no molecular marker has been applied successfully to African *Gnetum* species. We used the iPBS technique to investigate if morphological data characteristics of African *Gnetum* species are confirmed by genome-level genetic differentiation. The dendogram and the PCA analysis indicated that accessions of *G. africanum* and *G. buchholzianum* are well separated, confirming the taxonomical treatment of these lianas as separate species. iPBS markers can also detect and differentiate *G. latispicum*, one of the recently identified species of African *Gnetum* (Biye et al. 2014). Therefore, these markers will be useful for complementing the morphological markers and resolving taxonomic questions within African *Gnetum*. Though a more comprehensive collection is needed to verify all these classifications and to clarify the taxonomic situation, this study showed that iPBS-based markers provide an alternative approach to examine the relationship of species of African *Gnetum*. Future works will be based on more samples collected from many countries where African *Gnetum* species are found.

Conclusion

Overharvesting and habitat destruction are responsible for the endangered status of African *Gnetum* species. It has been demonstrated that the iPBS marker system provides a simple and useful approach for studying the genetic diversity of African *Gnetum* species. The high percentage of polymorphisms and the large number of bands obtained per assay using a single primer show that iPBS is an informative marker that can be used for population studies, taxonomy, conservation and domestication of African *Gnetum* species.

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Disclosure statement

The authors declare that they have no conflict of interests.

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