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The African timber tree *Entandrophragma congoense* (Pierre ex De Wild.) A.Chev. is morphologically and genetically distinct from *Entandrophragma angolense* (Welw.) C.DC

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Abstract

Interpreting morphological variability in terms of species delimitation can be challenging. However, correcting species delineation can have strong implications for the sustainable management of exploited species. Up to now, species delimitation between two putative timber species from African forests, *Entandrophragma congoense* and *E. angolense*, remained unclear. To investigate their differences, we applied an integrated approach which combines morphological traits and genetic markers. We defined 13 morphological characters from 81 herbarium specimens and developed 15 new polymorphic microsatellite markers to genotype 305 samples (herbarium samples and specimens collected in the field across the species distribution ranges). Principal component analysis (PCA) of morphological data and the Bayesian clustering analyses of genetic data were used to assess differentiation between putative species. These analyses support two well-differentiated groups ($F_{ST} = 0.30$) occurring locally in sympatry. Moreover, these two groups present distinct morphological characters at the level of the trunk, leaflets, and seeds. Our genetic markers identified few individuals (4%) that seem to be hybrids, though there is no evidence of genetic introgression from geographic patterns of genetic variation. Hence, our results provide clear support to recognize *E. congoense* as a species distinct from *E. angolense*, with a much lower genetic diversity than the latter, and that should be managed accordingly. This work highlights the power of microsatellite markers in resolving species boundaries.

Keywords Species delimitation · Microsatellite markers · The Bayesian assignment · Africa rainforest · Tiama · *Entandrophragma* · Meliaceae

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Introduction

Identification and delimitation of species is of particular importance for species conservation, notably in the light of global change (Dayrat 2005; Schlick-Steiner et al. 2010). Defining species has long been contentious, leading to the development of many methods and concepts (e.g., Le Guyader 2002; De Queiroz 2007). Two species concepts focus the attention of most plant taxonomists (Le Guyader 2002). First, the "typological species concept" defines species as a group of individuals whose members share common characteristics that differ from other species (Mayr 1992, Le Guyader 2002, De Queiroz 2007). Unfortunately, classifying individuals sharing similar morphological traits is not always obvious, especially when those individuals represent cryptic species (Janzen et al. 2017) or come from contrasted habitats (Tarasjev et al. 2009). In these cases, it may not be easy to separate species-specific traits from individual phenotypic variability, leading to an excessive splitting or lumping of species. Second, the "biological species concept" defines species based on the interfecondity of individuals, thus the absence of reproductive isolation mechanisms (Mayr 1942). This concept can nowadays be easily investigated with the help of genetic markers able to identify interbreeding individuals using population genetics principles (Duminil and Di Michele 2009).

Tropical African rainforests exhibit a high richness of tree species (Slik et al. 2015) but comprising still many groups with a weak taxonomic framework (Sosef et al. 2017). In this context, species delimitation based on morphological characters might be difficult. Main factors such as environment, phenology, and growth stage can affect phenotypic variability among species (Poorter 1999; Tarasjev et al. 2009). Using molecular methods can help but they have their own drawbacks, for example, hybridization can blur the delineation of species boundaries (Duminil and Di Michele 2009; Ley and Hardy 2017; Weber et al. 2017). Accordingly, applying an approach integrating morphological and genetic data is generally necessary to unravel species delimitation. Such an approach has been successful in the resolution of several plant species complexes of African forests, in some cases resulting in the description of new species (e.g., Ley and Hardy 2010; Duminil et al. 2012; Dainou et al. 2016; Ikabanga et al. 2017).

Correct species delimitation is a fundamental issue for the sustainable management of timber tree species populations (e.g., Tosso et al. 2015). The genus *Entandrophragma* (Meliaceae), described in 1894 by Casimir De Candolle, includes economically important timber species, both from rain and dry forests. The genus has undergone important taxonomic revisions, which resulted in more than 44 taxonomic names published. Today, only 10 or 11 species are recognized depending on the database which recognizes, or not, *E. congoense* (Pierre ex De Wild.) A.Chev. as a synonym of *E. angolense* (Welw.) C.DC (African Plant Database 2018; The Plant List 2013). This uncertainty of the taxonomic status of an important timber species,

possibly due to wide intra-specific phenotypic variability, also relates to market issues (Kasongo-Yakusu et al. 2018).

Entandrophragma angolense (Welw.) C.DC. was first described as *Swietenia angolensis* Welw. but after new observations, de Candolle (1894) declared it was not a *Swietenia* and transferred it to his new genus *Entandrophragma*. Taxonomic revisions conducted within *Entandrophragma* consider many taxa as synonyms of *E. angolense* (Chevalier 1909; Staner 1941). Up to now, more than 14 taxa, including *E. congoense*, have been assigned to this species (Kasongo-Yakusu et al. 2018). *Entandrophragma congoense* was firstly described as *Leioptyx congoensis* Pierre ex De Wild in 1908 (Sprague 1910). One year later, the species was transferred to *Entandrophragma* by Chevalier (1909) and subdivided into two distinct species: *Entandrophragma pierrei* A.Chev. and *Entandrophragma congoense* A.Chev. Staner (1941) considered these two species as synonyms of *E. angolense*. Later, Liben and Dechamps (1966) and Liben (1970) recognized *E. congoense* again as different from *E. angolense*, based on morphological characters such as the absence of developed buttresses at the base, the presence of scaly rhytidome, generally glabrous ribs, acute-apiculate leaflet apex, much smaller capsules (18 cm long, 2 cm wide, and about 3.5 cm thick), and seeds with truncated base and narrower than the wings. More recently, in a revision of the Meliaceae family in the Flora of Gabon series, de Wilde (2015) considered *E. congoense* as a distinct species and described new diagnostic floral characters. Furthermore, in logging concessions, foresters use differences in trunk aspect to distinguish individuals belonging to each species (white tiama for *E. angolense* and black tiama for *E. congoense*) (Meunier et al. 2015; J-L Doucet, pers. comm). It is worth noting that *E. congoense* is exclusively distributed in the Congo basin region, while *E. angolense* is more widely distributed throughout the African rain forest (Liben 1970; de Wilde 2015; Meunier et al. 2015). Nevertheless, doubt still persists on the status of *E. congoense* and many authors are still considering *E. congoense* as a synonym of *E. angolense* (for example, Klopper et al. 2006).

In the present study, we combined morphological data and molecular markers to assess the taxonomic status of *E. congoense* and *E. angolense*. More specifically, we address the following questions. (i) Do they form distinct morphological and genetic entities suggesting the presence of two distinct species? (ii) If yes, do they hybridize and/or is there some evidence of genetic introgression that could explain the difficulty to delimit them in previous taxonomical works?

Material and methods

Sampling

For morphological analyses, we used 81 herbarium vouchers from different herbaria (BR and WAG—now at L) attributed

to *E. angolense* or *E. congoense* (Table 1). Samples were visually separated in two morphogroups (morphogroup A for "*E. angolense*" and morphogroup C for "*E. congoense*") based on leaflet and seed characters following Liben and Dechamps (1966) and Liben (1970) (Appendix).

For genetic analyses, we used a piece of leaflet from each successfully amplified herbarium voucher. We also collected leaf or cambium (dehydrated with silica gel) from 261 adult individuals sampled in the field across the Guineo-Congolian forest area (Fig. 1a), among which 88 specimens sampled within the Forest Stewardship Council (FSC)-certified logging concession granted to "Pallisco" in Eastern Cameroon (mean coordinates: 13.37° E, 3.29° N; Fig. 1b) where both taxa would occur (de Wilde 2015; J-L Doucet, comm. pers.). These specimens were also separated in two morphogroups based on trunk aspect, slash characters, and leaflet shape (Appendix). Genetic analyses were performed at two spatial scales: the whole Guineo-Congolian forest (all 261 individuals, maximal distance between samples c. 4000 km) and within the Pallisco forest concession (88 individuals, maximal distance between samples c. 5 km).

Morphometric traits and analyses

To confirm objectively the morphological differentiation between morphogroups, for each of the 81 herbarium samples, we observed and measured 13 morphological traits indicated by previous authors as being diagnostic: (i) three qualitative traits: apex (acute-apiculate or obtuse and exceptionally retuse and mucronate), median vein (glabrous or pubescent), and domatia (thick tuft or generally absent); (ii) three quantitative variables related to leaflets size and numbers; (iii) seven traits associated to fruits, but which were available for only five samples of each species (Table 2, Appendix). We performed a Hill-Smith ordination, an extended principal component analysis (PCA) for datasets containing both qualitative and quantitative variables, on the vegetative traits of the 81 samples applying the function "dudi.hillsmith" of the Ade4 package available in R 3.1.2 (Chessel et al. 2004; Dray and Dufour 2007). For all quantitative leaf and fruit variables, we tested the difference between morphogroups by the Welch two sample *t* tests using the package MASS available in R 3.1.2.

Table 1 Samples used for the morphometric analyses. The letters in voucher identification tags indicate the herbarium in which specimens were collected: L Leiden, BR Meise, WAG Wageningen, and GEM Gembloux greenhouse

Taxon name	Geographic origin	Voucher
Entandrophragma angolense	Central African Republic	WAG1096893, BR000005862887, BR0000013596002
	Cameroon	GEM10[a]
	Democratic Republic of the Congo	L2158046, WAG1096868, WAG1096871, BR0000013596576, BR0000013266974, BR0000013946036, BR0000013266981, BR0000013946081, BR0000013646104, BR0000013946128, BR0000013946180, BR0000013946277, BR0000013946364, BR0000013946371, BR0000013946401, BR0000013946418, BR0000013596422, BR0000013946050
	Gabon	WAG1096873, WAG1096895
	Ghana	WAG1096874, BR0000013592738
	Guinea-Bissau	WAG1096864, WAG1096865, WAG1488141
	Ivory Coast	L2158048, WAG1096837, WAG1096844, WAG1096878, WAG1096879, BR0000013596095
	Liberia	WAG1096851, WAG1096854, WAG1096858, WAG1096861, BR0000013592714
	Nigeria	WAG1096834, WAG1096843, WAG1096862, WAG1097368
	Sierra Leone	L2158047, L2158049, WAG1096857
Entandrophragma congoense	Central African Republic	WAG1096927
	Democratic Republic of the Congo	L2158016, L2158017, L2158018, L2158019, L2158020, L2158022, WAG1096928, WAG1096930, WAG1096931, WAG1096932, WAG1096935, WAG1096937, WAG1096938, WAG1096940, WAG1096942, WAG1096944, WAG1096945, WAG1096946, WAG1096947, WAG1096949, WAG1096950, WAG1096951, WAG1096953, BR0000013977382, BR0000013977504, BR0000013977511, BR00000672842, BR0000013977566, BR0000013977733, BR0000013977764
	Gabon	WAG1096956, BR00000586403, BR00000586280
	Nigeria	WAG1096838

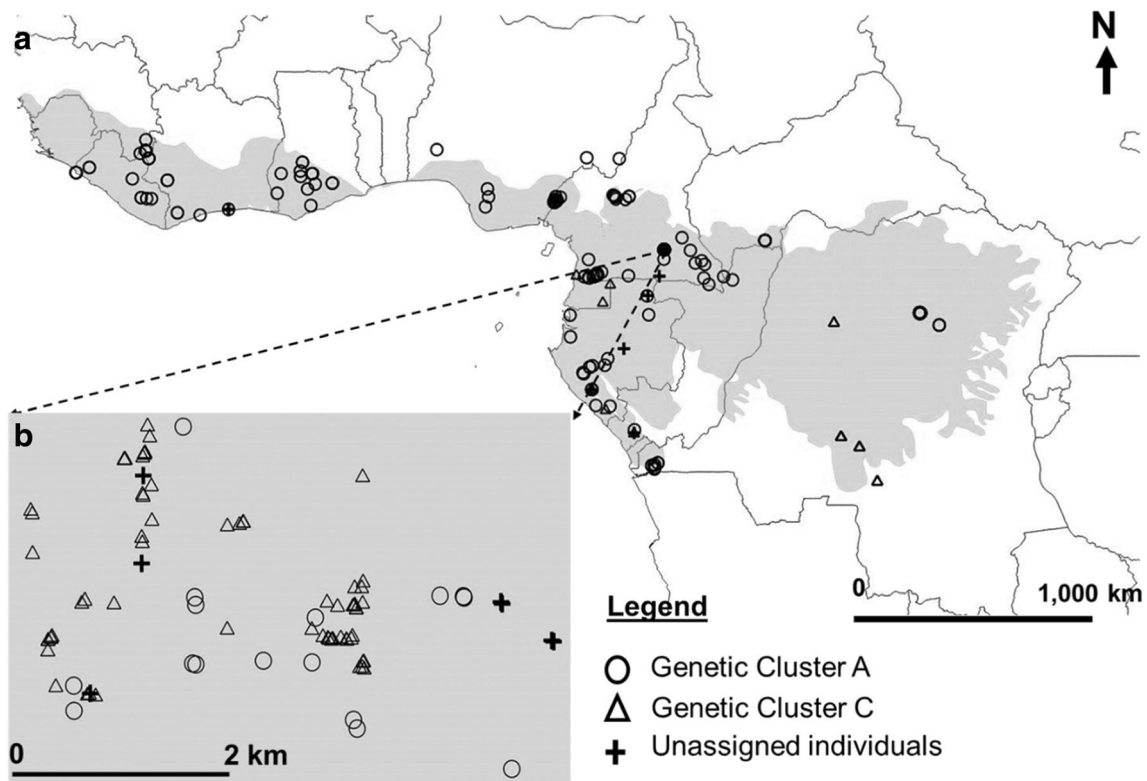[a] Sample used for genomic libraries available from Dr. Olivier Hardy collection (ULB, EBE team)

Fig. 1 Spatial distribution of genotyped samples of *Entandrophragma angolense*/*E. congoense* (**a**) across the Guineo-Congolian forest (gray area) and (**b**) in a forest from Eastern Cameroon. The symbols represent the output of the clustering algorithm (STRUCTURE) which assigned each sample to one of two genetic clusters (**a** or **c**) or left them unassigned when both clusters contributed to > 10% of the genome

Molecular genetic analyses

DNA extraction, microsatellite markers development and genotyping

Total genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1987) for herbarium specimens and the NucleoSpin 96 Plant II kit (Macherey-Nagel, Duren, Germany) for dry plant material collected in the field. Fifteen microsatellite loci were developed from a genomic library of a sample of *E. angolense* (GEM10; Table 1) using the protocol described in Monthe et al. (2017) developed for two other *Entandrophragma* species. The microsatellite loci were amplified in three multiplexes developed following the protocol of Micheneau et al. (2011). These multiplexes named

Table 2. Comparison between morphogroups A and B for nine quantitative variables measured from leaves from 81 samples and from seeds of 10 samples. Mean, standard deviation, and [minimum-maximum] values are reported, as well as *P* values of the Welch two-sample *t* tests

	Characters	Morphogroup A (n = 46 for leaves, n = 5 for seeds)	Morphogroup C (n = 35 for leaves, n = 5 for seeds)	*P*
Leaves	NF (number of leaflets per leaf)	7.64 ± 2.23 [3–13]	9.37 ± 2.97 [5–18]	0.01
	LF (leaflet length)	12.43 ± 3.93 [5.2–25.5]	11.93 ± 1.75 [7.5–16.5]	0.43
	wF (leaflet width)	4.77 ± 1.49 [2.9–9]	3.66 ± 0.91 [2.3–6.4]	< 0.01
	RLF (ratio of LF/wF)	2.62 ± 0.41 [1.48–4.25]	3.38 ± 0.69 [1.87–4.8]	< 0.01
Seeds and capsules	Lcp (capsule length)	16.32 ± 1.4 [14.6–17.5]	12.9 ± 1.60 [10–14]	0.01
	wcp (capsule width)	2.78 ± 0.7 [2–3.5]	2 ± 0.00 [2–2]	0.06
	Rcp (ratio of Lcp/wcp)	6.13 ± 1.75 [4.17–8.5]	6.75 ± 0.82 [5–7]	0.77
	Lsd (seed length)	8.74 ± 1.52 [6.5–10.2]	7.58 ± 0.75 [6.4–8.4]	0.18
	wsd (seed width)	1.62 ± 0.32 [1.2–2.1]	1.3 ± 0.12 [1.2–1.5]	0.09
	Rsd (ratio of Lsd/wsd)	5.42 ± 0.38 [4.85–5.88]	5.86 ± 0.68 [4.92–6.46]	0.25

"Mix 1," "Mix 2," and "Mix 3" were, respectively, composed of six (EnA-ssrEnA-ssr7, EnA-ssr2, EnA-ssr35, EnA-ssr23, EnA-ssr14, EnA-ssr48), five (EnA-ssr5, EnA-ssr34, EnA-ssr21, EnA-ssr42, EnA-ssr36), and four (EnA-ssr3, EnA-ssr29, EnA-ssr15 and EnA-ssr44) microsatellite markers (Table 3). PCR amplification was performed in a total volume of 15 μL containing 0.3 μL of the reverse (0.2 μM forming 100 μM initial concentration) and 0.1 μL of the forward (0.07 μM forming 100 μM initial concentration) primers with a Q1–Q4 universal sequence at the 5′ end (see Table 3), 0.15 μL of Q1–Q4 labeled primer (0.2 μM each), 7.5 μL of Type-it Microsatellite PCR Kit (QIAGEN), 15 μL of H_2O, and 1.5 μL of DNA extract. PCR program conditions were as follows: 95 °C for 3 min; 30 PCR cycles of 95 °C for 30 s/ 57 °C for 90 s/72 °C for 1 min; and 60 °C for 30 min. Using 1 μL of PCR product, 12 μL of Hi-Di Formamide (Life Technologies, Carlsbad, California, USA), and 0.3 μL of MapMarker 500 labeled with DY-632 (Eurogentec, Seraing,

Belgium). Loci were successfully cross-amplified between morphogroups. Among the 81 herbarium samples, only 44 were successfully amplified, so that a total of 305 individuals were genotyped using an ABI3730 sequencer (Applied Biosystems, Lennik, The Netherlands; ULB-EBE platform). The data generated for each individual were scored using the microsatellite plugin in Geneious 9.1.6 (Kearse et al. 2012). The first screening revealed that all samples were diploid as no more than two alleles per individual and per locus were found.

Population genetic analyses

The genetic structure was investigated through (i) a Bayesian clustering algorithm implemented in STRUCTURE v.2.3.4 (Pritchard et al. 2000) and (ii) a principal coordinate analysis (PCoA) on pairwise genetic distances between samples, performed using GenAlEx v.6.5 (Peakall and Smouse 2012).

Table 3 Characterization of 15 polymorphic nuclear microsatellite loci isolated from *Entandrophragma angolense*

Locus[a]	Primer sequences (5′–3′)	Fluorescent label[b]	Repeat motif	Allele size range (bp)	GenBank accession no.
EnA-ssr2	F: TGTGGAGAAACTGAGGGACC R: CGAATTGCAGATTGAGAGCTT	Q1-6-FAM	$(AG)_{16}$	212-253	MH382769
EnA-ssr3	F: CCCACCAATCCCTCTCAAA R: CCCTGCAGATGAAACCCTAA	Q1-6-FAM	$(AG)_{18}$	184-226	MH382770
EnA-ssr5	F: CTAGTGGGCGAACACAAACA R: CAAATTCAAGTCTGCTTTCGG	Q1-6-FAM	$(AT)_{15}$	152-186	MH382771
EnA-ssr7	F: GCCACGACATTATTTCCACC R: CAGTTGTTGCGGTCACAATC	Q1-6-FAM	$(AG)_{14}$	141-173	MH382772
EnA-ssr14	F: AACTCTGACACGTGCGGTTA R: GCTGCCAGCATTGATAGTGA	Q2-NED	$(AG)_{17}$	184-226	MH382773
EnA-ssr15	F: CCATGGGTAAGCTCTCAACAA R: GGAGTTTGGCCTCTCACCTT	Q2-NED	$(AG)_{15}$	159-219	MH382774
EnA-ssr21	F: TTGAGCATGGTTTATGTATCCG R: AACGTGAAGGTACAGGTTGTATCA	Q2-NED	$(AT)_{14}$	122-156	MH382775
EnA-ssr23	F: TGCTAACATCTGGTTGCATCA R: AAGTGCCTACCAGCCTTACTTT	Q2-NED	$(AC)_{12}$	115-182	MH382776
EnA-ssr29	F: AGATGGGCGACTAAAGCTGA R: ACAGGCACAGTACACCTGGA	Q3-VIC	$(AG)_{15}$	135-201	MH382777
EnA-ssr34	F: CATAGAGATTTGGGACATGGG R: ATGGCATACAGATGCAACGA	Q3-VIC	$(AC)_{12}$	157-190	MH382778
EnA-ssr35	F: CAGCATTTGAGTGTATGTTCCC R: TAATAGGGCAGACGGCTTGT	Q3-VIC	$(AG)_{11}$	121-158	MH382779
EnA-ssr36	F: TCTTTCCCACCAATTCAAGG R: TGAGGGTCTGAAACAAAGTGAA	Q4-PET	$(AAG)_{12}$	216-302	MH382780
EnA-ssr42	F: ACGGAAACCATTACCACACC R: TTTCATCGGGAAGAAGGC	Q4-PET	$(AC)_{16}$	145-186	MH382781
EnA-ssr44	F: AGAAGAATAAACAACACCACCC R: CTGTTCTTATGATGTCCATGGTG	Q4-PET	$(AG)_{18}$	119-157	MH382782
EnA-ssr48	F: TTGTTGTTCTGCAAGGATGG R: GGCCGAAGTCCCTTCTAATC	Q4-PET	$(AG)_{11}$	136-166	MH382783

[a] Optimal annealing temperature was 57 °C for all loci

[b] *Q1* TGTAAAACGACGGCCAGT, *Q2* TAGGAGTGCAGCAAGCAT, *Q3* CACTGCTTAGAGCGATGC, *Q4* CTAGTTATTGCTCAGCGGT (Q1 after Schuelke 2000; Q2–Q4 after Culley et al. 2008)

Considering all 305 samples, we applied the Bayesian clustering using the admixture and the correlated allele frequency model, declaring the presence of null alleles for all loci, without any location or population priors. We tested $K = 1$ to 10 genetic clusters with runs of 500,000 MCMC generations (burn-in period of 100,000 generations) and 10 runs for each K value. The online application STRUCTURE HARVESTER (http://taylor0.biology.ucla.edu/structureHarvester/) was used to compute and plot the deltaK statistics against the range of K values (Evanno et al. 2005; Earl et al. 2012). The optimum number of genetic clusters ($K = 2$, see the "Results" section) was identified considering the important gain in likelihood as K increases. Each individual was assigned to a genetic cluster when its probability of assignment to the most likely cluster, q, was higher than 0.9, while the remaining individuals were considered as unassigned.

For each identified cluster (hereafter called A and C given their correspondence with the A and C morphogroups), the following genetic diversity indices were computed for each locus using all samples (global scale): the number of alleles (A), the observed heterozygosity (H_o), the expected heterozygosity (H_e), the inbreeding coefficient (F), using SPAGeDi 1.5 (Hardy and Vekemans 2002). Null allele frequencies (r) were estimated for each genetic cluster in STRUCTURE. We used INEST 2.0 under a population inbreeding model to estimate $F_{(null)}$, an unbiased estimator of inbreeding coefficient robust to the presence of null alleles (Chybicki and Burczyk 2009). These analyses were repeated at the local scale (Pallisco) to factor out the potential impact of a phylogeographic structure occurring within each genetic cluster. We also assessed the differentiation between genetic clusters through the estimation of fixation indices (F_{ST} and R_{ST}) and tested whether stepwise mutations contributed to genetic differentiation (test if $R_{ST} > F_{ST}$; Hardy et al. 2003), using SPAGeDi 1.5 (Hardy and Vekemans 2002).

To evaluate whether unassigned individuals could represent hybrids, we simulated new genotypes under random mating based on the allele frequencies inferred by the STRUCTURE algorithm under $K = 2$. To this end, we generated 151 genotypes from cluster A and 77 genotypes from cluster C (sampling randomly two alleles per locus following the allele frequencies of cluster A or C, respectively), with respect to the same proportions as in the real data set, and 50 hybrid genotypes (sampling randomly one allele from cluster A and one from cluster C following the respective allele frequencies). The 278 simulated genotypes were then analyzed in STRUCTURE under $K = 2$ using the same parameters as described above to assess the distribution of q values for each category of genotypes. To further verify the occurrence of hybrids only at a local scale (88 individuals from Pallisco), we applied the NewHybrids method (Anderson and Thompson 2002) under the "Jeffreys prior" settings assuming six genotype frequency categories: purebred cluster A (A-A),

purebred cluster C (C-C), F1 hybrids (F1), F2 hybrids (F2), backcrossed F1 to purebred cluster A (BckA-A), and backcrossed F1 to purebred cluster C (BckC-C).

To test introgression between genetic clusters, we computed pairwise kinship coefficients between 208 individuals sampled in Central Africa (where the two clusters are sympatric), keeping only 17 random samples attributed to cluster C in Pallisco to better balance samples sizes and excluding unassigned samples. To this end, we used SPAGeDi 1.5 (Hardy and Vekemans 2002; estimator of J. Nason) to describe patterns of isolation by distance from the kinship-distance curves computed (i) within each genetic cluster and (ii) between the two clusters, using the mean allele frequencies observed between the two clusters as reference to estimate kinship coefficients.

Results

Morphometric differentiation between species

Considering the quantitative and qualitative traits observed on the 81 herbarium specimens, the first two axes of the Hill-Smith ordination summarized 53% of the total variance and allowed to distinguish two groups of samples segregating along the first axis (Fig. 2). All but three samples of morphogroup A showed negative scores along axis 1, while all but two samples of morphogroup C showed positive scores along axis 1. The Welch two-sample t test revealed significant differences between morphogroups for two quantitative leaf traits (number of leaflets per leaf and the length/width ration of leaflets were higher in samples attributed to *E. congoense*) and two fruits traits (the length and the width of capsules were higher in samples attributed to *E. angolense*) (Table 2). We also observed an important difference in seed base and wing (straight in morphogroup C and more curved in morphogroup A). Concerning leaflet characteristics, the specimens of morphogroup A are characterized by pubescent veins, a generally rounded apex, obovate leaflets, and an absence of pilosity between the main and secondary veins, and they showed high contribution on the first component (Table S1). Specimens of morphogroup C exhibited glabrous veins, acute apex, long leaflets and carrying thick tufts of hairs (domatia) between the main and secondary veins.

Development of microsatellite markers

In *E. angolense*, 15 highly polymorphic microsatellite markers were successfully developed. We observed 10 to 21 alleles per locus, with A ranging from 10 to 21 alleles and H_E from 0.72 to 0.91 (mean $H_E = 0.85$) among samples attributed to *E. angolense* (Table 4). These markers globally amplified on individuals attributed to *E. congoense* but four of them

Fig. 2 The Hill-Smith ordination of 81 *Entandrophragma angolense/E. congoense* specimens of morphogroups A (circles) and C (triangles) for six quantitative and qualitative leaf traits, using the two first axes (53% of variance explained)
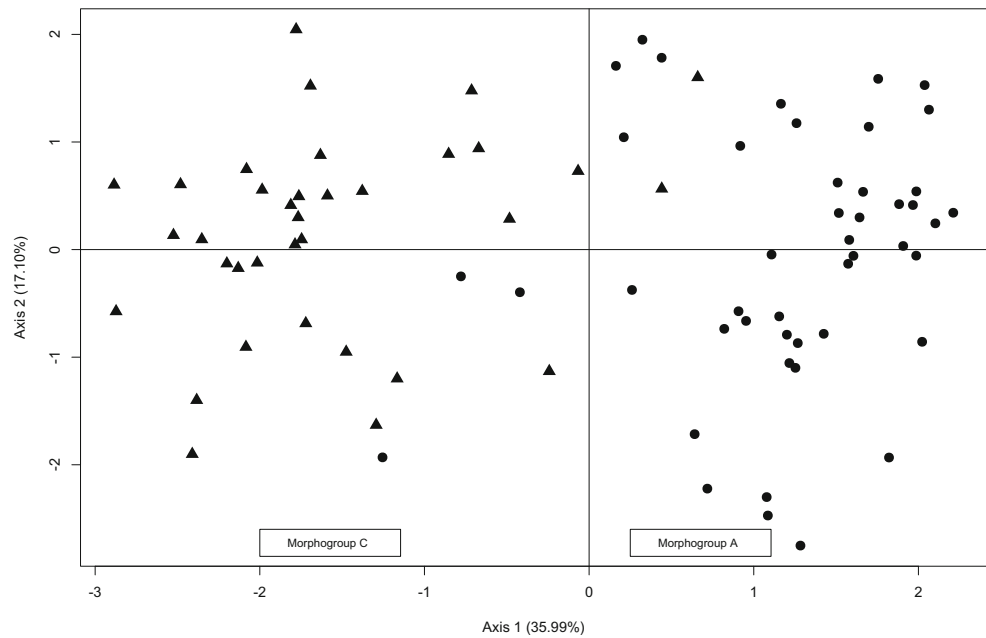
were monomorphic and genetic diversity was globally much lower, with A ranging from 1 to 12 alleles and H_E from 0 to 0.87 (mean $H_E = 0.31$; Table 4). Substantial heterozygote deficit was observed at most loci in both taxa and were at least partially explained by the presence of null alleles (Table 4). However, an analysis performed at the local scale (Pallisco) showed that the inbreeding coefficient was null in each taxon after factoring out the impact of null alleles (Table S2).

Inferred genetic clusters and correspondence with morphogroups

The Bayesian clustering analysis indicated that the likelihood of the data increased substantially from $K = 1$ to $K = 2$ and moderately at higher K, hence we conclude that the most likely number of clusters is two, consistent with the maximum deltaK statistic at $K = 2$ (Fig. S1). There was a 96%

Table 4 Genetic characterization of 15 polymorphic microsatellite loci for the two genetic clusters (individuals assigned at $q \geq 0.90$) at the scale of the Guineo-Congolian forests

Locus[a]	Cluster A ($n = 190$)					Cluster C ($n = 100$)				
	A	H_e	H_o	F	r	A	H_e	H_o	F	r
EnA-ssr2	21	0.89	0.48	0.45	0.18	6	0.63	0.55	0.13	0.01
EnA-ssr3	15	0.89	0.70	0.21	0.05	2	0.03	0.01	0.66	0.09
EnA-ssr5	10	0.81	0.22	0.73	0.32	3	0.50	0.56	−0.11	0.01
EnA-ssr7	17	0.91	0.47	0.48	0.21	3	0.26	0.06	0.77	0.19
EnA-ssr14	18	0.90	0.42	0.53	0.24	1	0.00	0.00	–	0.03
EnA-ssr15	16	0.80	0.71	0.16	0.06	1	0.00	0.00	–	0.29
EnA-ssr21	12	0.77	0.32	0.58	0.25	2	0.01	0.01	0.00	0.02
EnA-ssr23	23	0.88	0.77	0.12	0.05	12	0.87	–	0.15	0.07
EnA-ssr29	21	0.91	0.44	0.51	0.24	2	0.01	0.01	0.00	0.15
EnA-ssr34	15	0.72	0.25	0.64	0.27	4	0.51	0.23	0.53	0.17
EnA-ssr35	14	0.72	0.40	0.44	0.18	4	0.55	0.58	−0.05	0.00
EnA-ssr36	13	0.87	0.43	0.49	0.22	1	0.00	0.00	–	0.15
EnA-ssr42	18	0.90	0.45	0.5	0.00	6	0.55	0.58	−0.05	0.22
EnA-ssr44	18	0.91	0.51	0.44	0.18	7	0.76	0.43	0.43	0.20
EnA-ssr48	17	0.91	0.36	0.60	0.27	1	0.00	0.00	–	0.29
Multilocus average	16.53	0.85	0.46	0.45		3.67	0.31	0.25	0.19	

A number of alleles, F fixation index, H_e expected heterozygosity, H_o observed heterozygosity, n number of individuals, r null allele frequency according to the Bayesian structure analysis (STRUCTURE)

correspondence between a priori identification of morphogroups and genetic clusters. At a threshold of $q \geq 0.9$, we observed that 15 individuals, representing 4% of the total sample, were not assigned to a genetic cluster (Fig. 3). At a local scale in S-E Cameroon ("Pallisco"), where the two genetic clusters sampled are distributed in sympatry (Fig. 1), seven of the 88 (8%) individuals were unassigned.

The STRUCTURE analysis applied on the simulated genotypes at $K = 2$ showed that the thresholds of q values for pure parental species were $q > 0.80$ and $q < 0.20$ and that all F1 hybrids showed $0.20 < q < 0.80$ (Fig. 3b). The NewHybrids approach applied in the contact area ("Pallisco") correctly identified the two groups at $q > 0.90$; 94% of individuals belonging to category A-A were all assigned to the expected group except for one individual ($q > 0.72$). In the C-C category, all individuals were correctly assigned at $q > 0.90$. The seven putative hybrid individuals according to STRUCTURE

analysis were identified as F2 hybrids, whereas no F1 and no backcrossed individuals were identified by NewHybrids.

Results from PCoA analysis performed with GenAlEx were consistent with the Bayesian clustering analysis: the two main genetic clusters were segregated along axis 1 while 15 unassigned samples ($0.1 < q < 0.90$) had intermediate scores (Fig. 4).

The genetic differentiation between clusters was high ($F_{ST} = 0.30$), and the corresponding index accounting for microsatellite allele sizes was even higher ($R_{ST} = 0.48$). Allele size permutation tests (Hardy et al. 2003) revealed that four loci (EnA-ssr3, EnA-ssr23, EnA-ssr5, EnA-ssr42) showed significant shift in allele sizes between the two clusters (single-locus R_{ST} significantly larger than single-locus F_{ST}). Four other loci (EnA-ssr14, EnA-ssr48, EnA-ssr36, EnA-ssr15) were polymorphic in cluster A but mostly monomorphic in cluster C (see Fig. S2). These main differences are supported by the much higher genetic diversity and allelic richness in cluster

Fig. 3 **a** Histogram of genetic assignment of 305 individuals genotyped at 15 microsatellite loci at $K = 2$ genetic clusters, according to a Bayesian clustering analysis. Each bar indicates the proportion of the genome (q) of an individual being assigned to each genetic cluster. **b** Identical analysis performed on 278 simulated genotypes (151 pure cluster A, 77 pure cluster C, 50 F1 hybrids) to identify the q value thresholds corresponding to hybrids between the two genetic clusters ($0.2 < q < 0.8$)
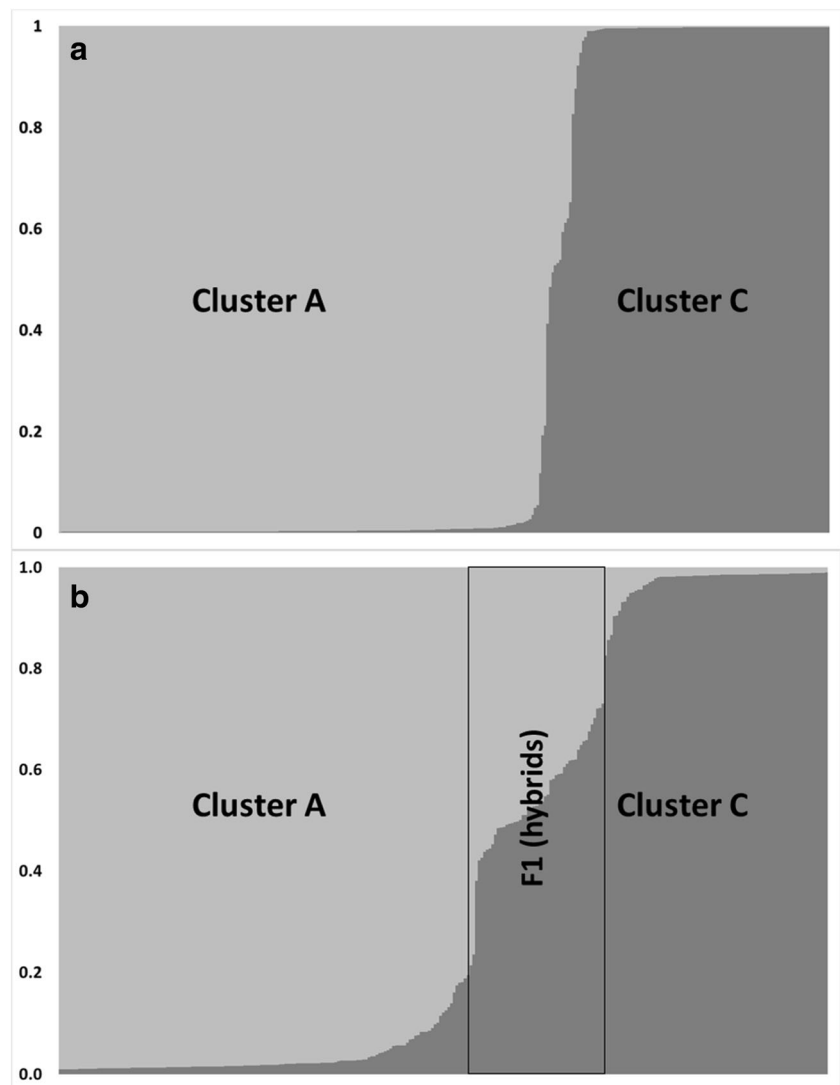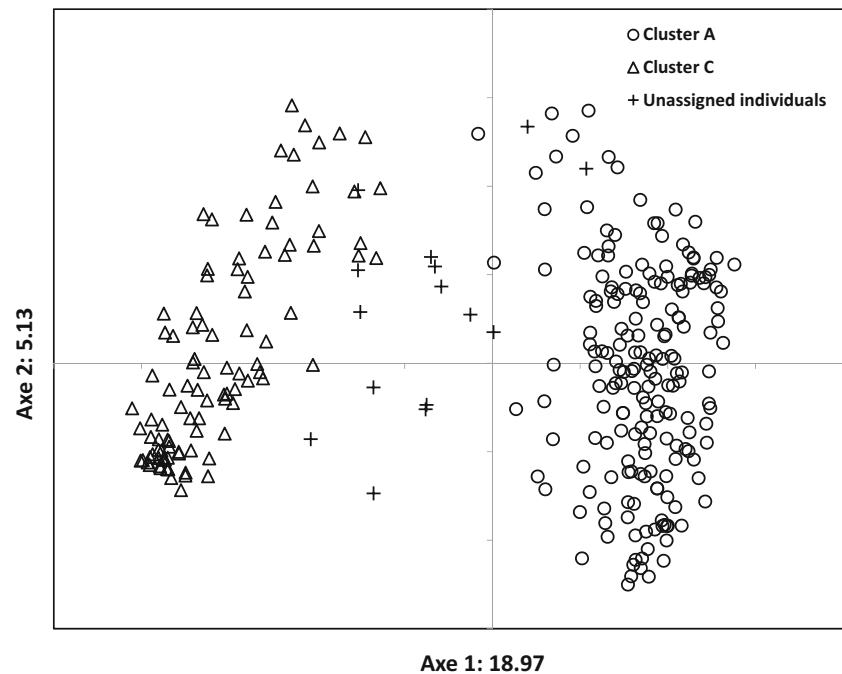
Fig. 4 Principal coordinate analysis (PCoA) of pairwise genetic distances between 305 individuals, with cluster assignments according to a Bayesian clustering analysis

A compared to cluster C (see Fig. S2, Table 4). On the other hand, identification of diagnostic alleles (frequency ≥ 0.30 in one cluster and below 0.10 in the other cluster) reveals that cluster C has more diagnostic alleles (14) than cluster A (4).

The kinship-distance curves for pairs of samples of the same genetic cluster (A-A or C-C pairs) decay with distance, indicating isolation by distance within each cluster (Fig. 5). However, the curve for pairs of samples from different clusters (A-C pairs) shows negative pairwise kinship coefficients without any trend with spatial distance (Fig. 5). Regression slopes of pairwise kinship coefficients on the logarithm of spatial distance equal $b \pm SE = -0.009 \pm 0.005$ for C-C pairs, -0.018 ± 0.002 for A-A pairs, and 0.001 ± 0.003 for A-C pairs, where A and C indicate the genetic clusters of pairs of samples compared.

Discussion

Morphological differentiation

Our analysis of morphological traits collected on herbarium specimens assigned to *E. angolense* or *E. congoense* confirmed that they can be morphologically differentiated based on characters of leaves and fruits (flowers were not observed in our study) (Fig. 2, Table 2). According to Liben and Dechamps (1966), other diagnostic traits concern the maximal dimensions of the tree (up to 50 m high and 200 cm in diameter at breast height (DBH), in *E. angolense*, compared to ≤ 45 m high and ≤ 90 cm in

DBH in *E. congoense*) and the trunk base which is smooth to scaly in *E. angolense* and generally cracked leading to rectangular elongated scales in *E. congoense*. The seeds are described as larger in *E. angolense*, although we did not observe significant differences, probably due to our low sample sizes. However, the wing shape appears to be different (straight in *E. congoense* and more curved in *E. angolense*; Fig. S3). Moreover, in a recent revision for "Flore du Gabon," de Wilde (2015) mentions differences in the staminal tube length (3–4 mm in *E. angolense*, 2–3 mm in *E. congoense*). Despite these observations, the presence of intermediate individuals (Fig. 2) can be explained by the limiting discriminating power of the variables used and/or the presence of hybrids. Unfortunately, none of the herbarium samples showing intermediate scores on axis one of the Hill-Smith ordination (Fig. 2) was successfully genotyped so that we cannot confirm if they corresponded to genetic hybrids.

Population genetics-based species delimitation

The present study confirms the validity of morphological characters described in Liben and Dechamps (1966) to distinguish the two taxa. However, in many cases morphological characteristics have showed their limits to confirm the distinction of a taxon at species level (Edwards and Knowles 2014).

We developed 15 nuclear microsatellite markers to test the differentiation between the putative species. Interestingly, the markers developed from a sample attributed to *E. angolense*
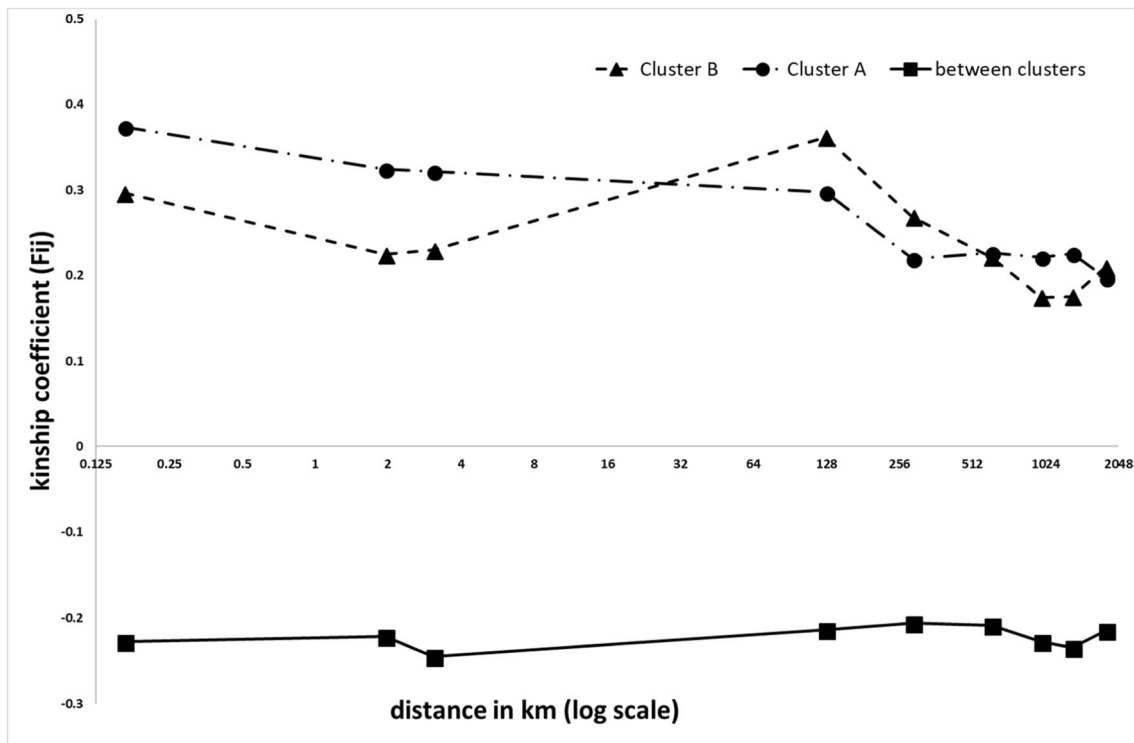
Fig. 5 Average kinship coefficients (F_{ij}) between pairs of individuals plotted against the logarithm of geographical distance for different pairwise comparisons between genetic clusters (**a** or **c**): A-A (dashed line), C-C (broken line), and A-C (solid line)

amplified on *E. congoense* samples, while cross-amplification of microsatellite markers between other *Entandrophragma* species often failed (Monthe et al. 2017), indicating the comparatively close phylogenetic relationship between *E. angolense* and *E. congoense*. However, microsatellite markers show heterozygote deficit due to null alleles (Table S2) and probably also due to the Wahlund effect considering the wide distribution range of our samples.

The Bayesian clustering and PCoA analysis support two well-differentiated genetic clusters corresponding to the two putative species (Figs. 3 and 4). Cluster A (*E. angolense*) displayed more alleles and much higher genetic diversity indices ($H_E = 0.85$) than cluster C (*E. congoense*; $H_E = 0.31$; Table 4). This difference in levels of polymorphism probably reflects a difference of genetic diversity between species but could also result from an ascertainment bias given that microsatellites were developed from an *E. angolense* individual. However, while allele sizes were on average smaller in cluster C than in cluster A at five loci, the reverse pattern occurred at four other loci where polymorphism was generally lower in cluster C (Fig. S2). Hence, a significant ascertainment bias (i.e., a selection of longer and more variable microsatellite loci in *E. angolense*) seems unlikely. The origin of the relatively low genetic diversity of *E. congoense* should probably be searched in its demographic history and would justify further research.

We observe a high genetic differentiation between the two clusters ($F_{ST} = 0.30$). Moreover, the genetic differentiation is also well marked at a local scale as we can distinguish the two

clusters distributed in sympatry in a forest concession (Fig. 4). While most loci displayed several alleles shared between species, with the notable exception of locus EnA-ssr21 which was fixed in *E. congoense* for an allele not found in *E. angolense*, many loci also showed alleles at high frequency in one cluster and (near) absent in the other (Fig. S2). Some loci also displayed a global shift of allele size ranges between species (e.g., EnA-ssr 3, 5, 14, 21, 23, 42; Fig. S2), resulting in a signal whereby R_{ST} is significantly larger than F_{ST}, which implies long-term differentiation due to the accumulation of stepwise mutations (Hardy et al. 2003). Overall, morphological and genetic analyses give strong support for the recognition of two species: *E. congoense* and *E. angolense*.

Evidence of hybridization but not of introgression between species

Despite the strong genetic differentiation between the clusters, our genetic clustering analyses indicate the presence of putative hybrid individuals that were found only in regions where the two species co-occur (Fig. 1). The presence of hybrids was observed in STRUCTURE analysis ($0.11 < q < 0.88$) and confirmed by applying the NewHybrids method at a local scale, where around 8% of individuals appear to represent a hybrid (Fig. 3). This is relatively low compared to hybridization rates reported for other contact zones of congeneric African plant species: 13–40% for *Haumania* (Ley and Hardy 2017), 12% for *Milicia* (Daïnou et al. 2017). Unfortunately, we were not able to assess the

morphological characteristics of genetic hybrids, as the latter were individuals collected in the field without herbarium vouchers. Additional investigations on hybrid specimens are needed to find out if they are morphologically intermediate.

Hybrids observed here may result from occasional hybridization events between the clusters, a phenomenon frequently observed between other closely related plant species with overlapping distribution ranges (Haselhorst and Buerkle 2011; Duminil et al. 2012; Dainou et al. 2016; Ikabanga et al. 2017). The presence of hybrids does not necessarily imply gene flow between clusters (i.e., introgression) because hybrids may be sterile or unable to back-cross with either parental cluster. Surprisingly, according to NewHybrids analyses, the seven hybrids detected appeared to be second-generation hybrids (F2 hybrids). The absence of F1 hybrids may be due to our limited sample size; however, the absence of back-crosses with a parental cluster is consistent with the absence of evidence of genetic introgression. Indeed, if gene flow occurred regularly between the clusters in contact zones, we would expect pairs of individuals from different clusters to be on average more genetically related when sampled in the same contact zone than when sampled far apart (Hardy and Vekemans 2001), which is not the case (Fig. 5). Hence, further research is needed to understand the underlying mechanisms (e.g., pre-zygotic isolation due to phenological shift, selection against introgressed genotypes) explaining such observation.

Conclusion

Our combined morphological and genetic approach confirmed that morphogroups A and C constitute distinct taxa that can be identified using the morphological characters described by Liben and Dechamps (1966). Our work therefore also confirms the correctness of the differentiation made between *E. angolense* and *E. congoense* in the recent revision of Meliaceae in Flore du Gabon (de Wilde 2015). Although occasional hybridization events do occur, these do not cause significant genetic introgression. Hence, because of the fair number of morphological differences and the strong genetic signal, we conclude a distinction at species level is most appropriate. The recognition of *E. congoense* as a distinct species implies that its populations must be managed separately from those of *E. angolense*. This can be easily implemented as field technicians in forestry concessions are already used to distinguish these species. The much lower genetic diversity found in *E. congoense* may also have management implications, but the origin of this feature must still be understood.

Appendix

Table 5 Morphological characters differentiating *Entandrophragma* species as reported by Liben and Dechamps (1966) and by de Wilde (2015)

	E. congoense	*E. angolense*
Trunk		
Tree dimensions	Up to 45 m high and 90 cm diameter.	Up to 50 m high and 200 cm diameter
Base	Straight more or less thickened, sometimes sub-winged, but without developed buttresses	Sometimes simply thickened, but usually has well-developed aliform buttresses.
Rhytidome	Suberous peeling off into regular plaques (square or rectangular)	Non-suberous, peeling off by irregular plaques
Leaflet		
Form	Elliptical oblong, rarely ovate	Generally obovate, rarely elliptical or oblong
Base	Generally acute, rarely obtuse, asymmetrical	Generally acute, rarely obtuse or round
Apex[$]	Acute-apiculate, more rarely bluntly rounded and apiculate	Obtuse to rounded, with long deciduous acumen, rarely acute, exceptionally retuse and mucronate
Midrib[$]	Glabrous, exceptionally pubescent when young	Pubescent, rarely glabrous below
Domatia[$]	Present, a thick tuft of hairs	Generally absent

Table 5 (continued)

	E. congoense	E. angolense
Capsules		
Valves	(9.5−) 11–16 (− 18) cm long (1−) 1.5–2 cm wide (1−) 1.5–3 (− 3.5) mm thick	(12−) 15–19 (− 22) cm long 2–2.7 (− 3) cm wide 4–6 (− 7) mm thick
Columella	Almost straight and subcircular section	Curved at the extremity and with a long elliptical section
Seeds		
Base	Straight, narrower than the wing	Wavy, as wide as the wing
External teguments	Violet blue	Brown

$^\$$ Qualitative variables used for morphological analyses

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