Research Article

Interspecific comparisons with chloroplast SSR loci reveal limited genetic variation in Nigerian montane forests: A study on *Cordia millenii* (West African Cordia), *Entandrophragma angolense* (tiama mahogany), and *Lovoa trichilioides* (African walnut).

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Abstract

The montane forests of south-eastern Nigeria are of immense conservation value due to their high levels of biodiversity and endemism. Yet despite increasing anthropogenic disturbance and forest fragmentation, little is known about the genetics of resident tree populations. We used a set of conserved chloroplast simple sequence repeat (SSR) primers to quantify and directly compare genetic diversity in three tree species: *Cordia millenii*, West African Cordia; *Entandrophragma angolense*, tiama mahogany; and *Lovoa trichilioides*, African walnut, within a single montane forest. Additionally, we assessed the diversity of West African Cordia between forests at a local and regional scale. Results indicate that for our focal loci, in all three species, there is a general lack of chloroplast genetic diversity. Our study is particularly relevant because it considers genetic diversity among multiple tree species simultaneously. This work represents the first study of its kind in the region, and will pioneer the way for future conservation genetic studies in montane Nigeria.

Keywords: Afromontane, chloroplast microsatellites, interspecific population genetic comparisons, Mambilla Plateau, Nigeria.

Les forêts de montagne du sud-est Nigerien sont d'une grande valeur pour la conservation immense en raison de leurs niveaux élevés de biodiversité et d'endémisme. Pourtant, malgré l'augmentation de perturbations et de fragmentations (d'origine) anthropique de la forêt, peu est connu sur la génétique des populations d'arbres résidents. Nous avons utilisé un ensemble de répétition de séquences chloroplaste simples conservées (SSR) amorces pour quantifier et comparer directement la diversité génétique de trois espèces d'arbres (*Cordia millenii*, Cordia Afrique de l'Ouest; *Entandrophragma angolense*, tiama acajou; *Lovoa trichilioides*; noyer africaine) au sein d'une seule forêt de montagne. En outre, nous avons évalué la diversité de Cordia Afrique de l'Ouest entre les forêts à l'échelle locale et régionale. Les résultats indiquent que pour notre loci focal, pour les trois espèces, il y a un manque général de diversité génétique des chloroplastes pour les trois espèces. Notre étude est particulièrement pertinente car elle estime que la diversité génétique à travers les espèces d'arbres multiples simultanément. Ce travail représente la première étude de ce genre dans la région, et pionnier façonne la voie à de futurs travaux de conservation dans montagnarde Nigeria.

Disclosure: Neither Tropical Conservation Science (TCS) or the reviewers participating in the peer review process have *any* editorial influence or control over the content that is produced by the authors that publish in TCS.

Introduction

The consequences of deforestation on tree population genetics are diverse [1–6] and are intimately related to how different tree species respond to fragmentation [7, 8]. Unlike other organisms, trees are sessile, so long distance dispersal generally occurs during the reproductive cycle as pollen flow or seed dispersal [8, 9]. Fragmentation influences plant population evolution by impacting the interplay among ecological traits, ecological interactions, gene-flow, and the underlying population genetic variation [10].
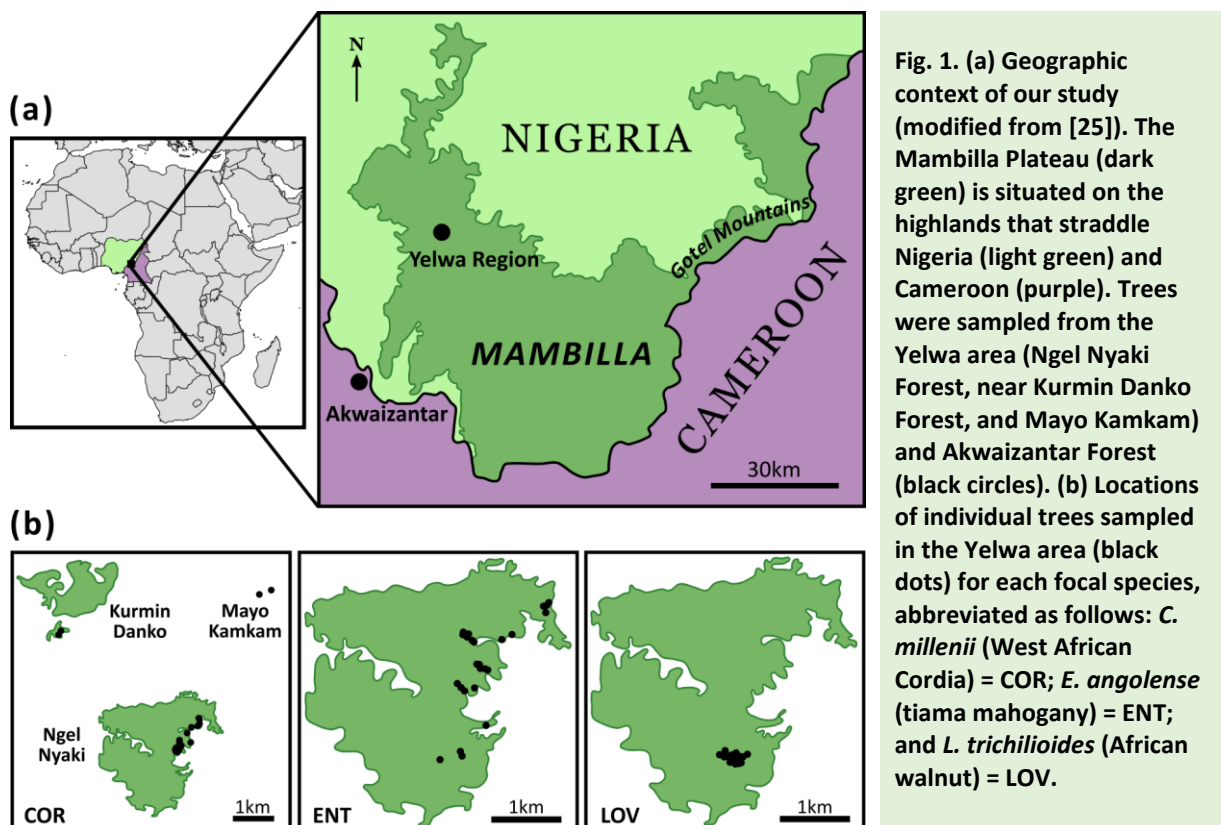
Studies that investigate population genetic patterns in multiple co-occurring tree species are rare [11–14], and there are issues with finding molecular markers that provide directly comparable measurements across species [15]. While the use of simple sequence repeats (SSRs, *i.e.* microsatellites) from the nuclear genome is a popular method of assessing population genetic parameters, primer sets often have limited transferability amongst taxa, making it difficult to directly compare species [15]. Chloroplast (cp) SSRs, therefore, are an attractive alternative, because primers can be anchored in conserved regions of the chloroplast genome and be used to compare interspecific genetic patterns [16, 17].

The montane forests of Africa have experienced fluctuations in size and distribution due to historic cycles of global warming and cooling [18–22]. However, growing anthropogenic pressure in West Africa is creating unprecedented forest loss, and relative lack of knowledge of the region's biodiversity and ecology demands a greater effort from conservation biologists [23, 24]. In particular, the Mambilla Plateau on the Nigeria-Cameroon border (and part of the Cameroon Highlands) is of high conservation priority due to its high levels of biodiversity in flora and fauna [25, 26] (Fig. 1a).

Three tree species were selected for this study based on their differences in ecology and conservation status: West African Cordia (*Cordia millenii*), tiama mahogany (*Entandrophragma angolense*), and African walnut (*Lovoa trichilioides*). Tiama Mahogany (Welw.) and African walnut (Harms.) are both shade-tolerant, wind-dispersed Meliaceae species [27] that are listed as Vulnerable on the IUCN Red List [28, 29]. West African Cordia (Bak.) is a shade-intolerant, animal-dispersed Boraginaceae species [27, 30], considered to be of Least Concern on the IUCN Red List [31]. All three tree species are under pressure from logging in Nigeria [32–34]. These three species

share a similar geographic range (from West to East Africa), though the distribution of African walnut is not as extensive as that of West African Cordia or tiama mahogany [28, 29, 31].

Our objective was to provide a preliminary investigation into interspecific patterns of maternally-inherited genetic diversity of trees on the Mambilla Plateau. Our hypotheses pertain to the distribution of diversity within and amongst patchily distributed forests. Considering all three species, we expected that: (1) at the within-forest scale, our three focal species would exhibit different genetic patterns, related to their different dispersal ecologies. Focusing solely on West African Cordia, we also expected that: (2) between forests at a local scale (<10km apart) some small genetic differences might exist between forests; and (3) at a broader regional scale (>40km apart) significant genetic differences would exist between forests.

Fig. 1. (a) Geographic context of our study (modified from [25]). The Mambilla Plateau (dark green) is situated on the highlands that straddle Nigeria (light green) and Cameroon (purple). Trees were sampled from the Yelwa area (Ngel Nyaki Forest, near Kurmin Danko Forest, and Mayo Kamkam) and Akwaizantar Forest (black circles). (b) Locations of individual trees sampled in the Yelwa area (black dots) for each focal species, abbreviated as follows: *C. millenii* (West African Cordia) = COR; *E. angolense* (tiama mahogany) = ENT; and *L. trichilioides* (African walnut) = LOV.

Methods

Our main site for interspecific comparisons at the 'within forest' scale was Ngel Nyaki Forest Reserve (07°05′N and 011°05′E), a forest fragment of approximately 5.5km^2 on the western escarpment of the Mambilla Plateau. Elevation ranges from 1,400–1,600m. The mean annual rainfall is 1,800mm, occurring mainly between mid-April and mid-October. Mean maximum/minimum monthly temperatures for the wet/dry season are 26.1/13.1°C, and 23.1/16.1°C (respectively) based on Nigerian Montane Forest Project (NMFP) weather records. Sampling occurred during the years 2012 and 2013. Where possible leaf tissue was collected, otherwise a cambium core was taken [35].

We were able to sample Ngel Nyaki Forest relatively extensively. The NMFP has established a network of transects throughout the east side of the forest. We sampled all adult individuals, from each tree species, known to occur on those transects. A tree was classed as an adult when its DBH (diameter at breast height) was >10cm and its height was >4m. The distribution of trees in all sites is illustrated in Fig. 1b. West African Cordia are found in small stands throughout the forest. Tiama

mahogany is dispersed throughout the forest. All known African walnut occur in the southern part of Ngel Nyaki Forest and form a single large stand. Based on DBH measurements, we sampled a broad age range for each species—our collection was thus not biased towards older or younger trees.

Additional samples of West African Cordia were also obtained from a small forest fragment bordering Kurmin Danko Forest (~07°06'N 01°01'E), and from degraded riparian forest bordering the Mayo Kamkam (~07°07'N 011°04'E). These three sites are proximal to Yelwa Village and were also classed as being sampled from the Yelwa area. In addition we obtained a sample from the more distant Akwaizantar Forest (~06°52'N 10°55'E), ~43km away (Fig. 1a), as a herbarium specimen (31795) from the Royal Botanic Gardens, Kew. These additional sample sites allowed us to assess the distribution of genetic variation in populations of West African Cordia at the 'between forests' scale at both the local and regional level. Total samples for each species and each site are detailed in Table 1.

Table 1. Sample sizes for each of our focal species. The proximally close sites, Ngel Nyaki Forest, Kurmin Danko Forest, and Mayo Kamkam, were grouped as the Yelwa area to be compared to a sample sourced from Akwaizantar Forest at the regional scale. Species abbreviations: *C. millenii* (West African Cordia) = COR; *E. angolense* (tiama mahogany) = ENT; and *L. trichilioides* (African walnut) = LOV.

Species	Ngel Nyaki	Kurmin Danko	Mayo Kamkam	Akwaizantar	TOTAL
COR	37	2	2	1	41
ENT	25				25
LOV	26				26
	Sampled from the Yelwa area				

DNA extractions were prepared from 5–6mg ground cambial or leaf tissue using a modified CTAB method sourced from Brunner *et al.* (2001) or a PowerPlant® DNA Isolation Kit (MoBio Laboratories). KAPA3G Plant PCR Kit (Kapa Biosystems) was used for PCR reactions. A 20μL reaction volume was used with the reagent mix: 10μL KAPA Plant PCR Buffer (2×), 0.3μM each primer, 5mM additional $MgCl_2$, ~20ng DNA, 0.2μL KAPA3G Plant DNA Polymerase, and PCR water as required. Some reactions required addition of KAPA3G Enhancer solution at 1× final concentration. Primers implemented in this study were sourced from a conserved set of chloroplast SSR primers for dicotyledonous angiosperms [16]. After screening all 10 loci it was determined ccmp2, ccmp3, ccmp4, ccmp5, ccmp6, ccmp7, and ccmp10 would amplify in our study species (Appendix 1).

The PCR conditions were: 3 minutes at 95°C (initial denaturing); 40 cycles of 20 seconds at 95°C (denaturing), 15 seconds at 50°C (annealing), and 45 seconds at 72°C (extension); completed with 30 seconds at 72°C (final extension). For some particularly difficult samples, increasing the annealing time to 20 seconds and the extension time to 50 seconds, assisted successful amplification. Genotyping was carried out using an ABI Prism 3130xl Genetic Analyzer. For all species, loci could be pooled into two groups for genotyping: (i) ccmp2, ccmp3, ccmp4, and ccmp5; and (ii) ccmp6, ccmp7, and ccmp10. Each genotyping reaction contained 0.6μL PCR product per locus, 12uL HiDi™ (Applied Biosystems) and 0.3μL 500 LIZ™ (Gene Scan™; an internal size standard). The resulting chromatrograms were aligned with the internal size standard and analyzed with Gene Marker v1.97 (SoftGenetics LLC, CA, USA). As in Weising & Gardner [16], alleles for each ccmp locus were scored as the second to largest peak. We were able to resolve alleles to 1bp and any ambiguous alleles were re-run on the sequencer. Because the genotypes produced by cpSSRs are haploid, there was no need to assess for null alleles as should be done for nSSRs [37].

The effective number of haplotypes (n_e) and the haplotypic diversity (H_E) were calculated for each population of tree species, following Nei [38]. Average genetic distances amongst individuals within each tree species' respective population were measured using the metric $\overline{D}_{SH}^2$ [39, 40]. $\overline{D}_{SH}^2$ is based on a step-wise mutational model [41] and measures the mean squared pair-wise haplotype differences amongst all possible pairs of individuals in the population using absolute size differences in their alleles. If n is the number of individuals, L is the number of loci, a_{ik} and a_{jk} are the allele sizes for individuals i and j at locus k, $\overline{D}_{SH}^2$ is calculated as follows:

$$\overline{D}_{SH}^2 = \frac{1}{[n(n-1)]/2} \times \frac{1}{L} \times \sum_{i=1}^{n} \sum_{j=i+1}^{n} \left[\sum_{k=1}^{L} | a_{ik} - a_{jk} | \right]^2$$

$\overline{D}_{SH}^2$ is a useful and commonly used statistic because it provides a simple way to interpret measures of genetic dissimilarity among individuals at cpSSR loci [39, 40, 42, 43].

In order to compare our results to previous investigations into genetic diversity within adult tropical/subtropical tree populations, we reviewed 16 papers that measured cpDNA diversity in 20 species. Diversity measures included cpSSRs, restriction sites, indels, and substitutions, and covered a range of spatial scales (Appendix 2). It was not always possible to extract the same genetic diversity statistics from these studies, so we focused on the mean number of haplotypes per population for each species in each study. To understand how the number of cpDNA loci and number of populations sampled might impact the mean number of haplotypes observed, we conducted linear regressions on these data; models were fit using R (R Core Team 2014).

Results

The results from the ccmp SSR analysis showed incredibly low cpDNA genetic variation in each focal tree species, within Ngel Nyaki Forest. Of the seven loci examined, all were monomorphic in West African Cordia and tiama mahogany; African walnut was only polymorphic at one locus, ccmp6 (Table 2). One haplotype was therefore observed in West African Cordia and tiama mahogany, and two in African walnut. The lack of genetic diversity at the ccmp cpSSRs used in this study is consequently reflected in the three genetic diversity indices used. Both West African Cordia and tiama mahogany had values of n_e = 1.00, H_E = 0.00 and $\overline{D}_{SH}^2$ = 0.00. African walnut exhibited slightly different values due to the small amount of variation present at ccmp6. In this population: n_e = 1.17, H_E = 0.15, and $\overline{D}_{SH}^2$ = 0.02, which are very small and suggest almost no variation.

We also observed complete genetic homogeneity of West African Cordia samples between forests at both the local and regional scale. From all the sampled sites, only a single haplotype was recovered (Table 2). This was surprising, because we expected at least some variation to exist between forests, particularly when comparing trees from Akwaizantar Forest and those in the Yelwa area.

Our review of other tropical tree cpDNA diversity studies provided some interesting insights into studying population genetic patterns in the chloroplast genome. We considered how both number of loci and number of populations sampled might impact the mean number of haplotypes observed. However, neither the number of loci (P = 0.807), nor the number of populations (P = 0.984), were a statistically significant predictor of the mean number of haplotypes observed within populations of the 20 tree species investigated (Appendix 3). One species, *Dysoxylum malabaricum* (white cedar)[45] was an outlier in our analysis with an average of 7.667 haplotypes per sampled population, notably more than in all the other species studied (Appendix 3). However, removing *D. malabaricum* from the analysis did not alter the statistical non-significance of the relationship

between the number of loci, and the number of populations sampled, with the mean number of haplotypes.

Table 2. Frequency of haplotypes (written in the numeric order of ccmp loci, *i.e.*: ccmp2/ccmp3/ccmp4/ccmp5/ccmp6/ccmp7/ccmp10) for the focal species**.** Alleles are represented by number of base pairs. Frequency and sample size (*n*) for *C. millenii* sampled from within Ngel Nyaki Forest only, the Yelwa area (Ngel Nyaki + Kurmin Danko + Mayo Kamkam), and Akwaizatar Forest, are separated with a "/" (respectively). Species abbreviations: *C. millenii* (West African Cordia) = COR; *E. angolense* (tiama mahogany) = ENT; and *L. trichilioides* (African walnut) = LOV.

Species	Haplotype	Allelic composition	Frequency	*n*
COR	C1	185/106/127/102/70/125/109	1.00/1.00/1.00	37/41/1
ENT	E1	213/104/120/92/124/136/107	1.00	25
LOV	L1	206/105/120/90/120/137/107	0.08	26
	L2	206/105/120/90/121/137/107	0.92	

Discussion

The primary objective of this study was to assess levels and distribution of cpDNA diversity in three tree species in the montane forest Ngel Nyaki, but also in forests on the Mambilla Plateau. We chose seven conserved ccmp primers for a directly comparable measure of genetic variation among species. Our diversity estimates were very low compared to some other studies that had used cpSSRs [39, 40, 43, 46] as well as the ccmp primers used by us [45, 47, 48]. In those studies, population H_E was found to be substantial in many cases (*i.e.* >0.50).

However, relative to many comparable studies on tropical tree species, our low cpDNA diversity was not unusual. All but three (*Dysoxylum malabricum*, white cedar [45]; *Dicorynia guianensis*, Angelique batard [49]; and *Ficus insipidia* subsp. *insipidia*, swamp fig [50]) of the 20 species, in the 16 studies we reviewed, had populations fixed for a single haplotype. While it is possible that our results reflect our choice of universal cpSSRs, which can produce a lack of observable polymorphisms from ascertainment bias in the original subject species [15, 37], two observations support our conclusion that our cpSSR diversity estimates reflect general cpDNA trends in our focal species. First, ccmp primers have revealed considerable variation—where it exists—in tropical trees [45, 51, 52]. Indeed, using a single ccmp primer, Bodare *et al.* [45] observed an average of 7.667 haplotypes per population of white cedar. Second, our West African Cordia cpSSR dataset corroborates a cpDNA and nDNA sequencing study conducted by Thia [53] on these exact same individuals. Sequencing of various chloroplast intergenic spacers and the nuclear ITS1 showed that all individuals had the same sequences. Given that cpSSRs should be more mutable, the lack of genetic variation observed in this present study is even more poignant.

The chloroplast genome is highly susceptible to genetic drift. Because chloroplasts are almost always uniparentally inherited, they have a much smaller effective population size relative to the nuclear genome, increasing the rate of drift. Fixation of particular haplotypes creates the scenario where most of the genetic diversity occurs among populations [54, 55], and it is common for nDNA diversity to exceed cpDNA diversity [54, 56–58]. However, in cases where seed dispersal exceeds the distance of pollen dispersal, cpDNA diversity may be greater [45] because adequate dispersal helps to buffer the effects of small population size.

The montane forests of the Mambilla Plateau are highly fragmented; patches of forest habitat are nested in a surrounding matrix of grassland. The ability to disperse between forest patches in our three focal species differs. Tiama mahogany and African walnut are both wind-dispersed and are unlikely to disperse between forests in a single generation. In contrast, West African Cordia is primate-dispersed and seeds could theoretically be moved considerable distances. However, the identical patterns of limited cpDNA diversity in all three species imply that dispersal mode is not a major contributor to apparent genetic patterns.

There are three possible scenarios that may explain the low cpDNA diversity in our three focal species: (1) tree populations are only recently colonized (on evolutionary timescales) from a limited number of sources; (2) tree populations have been isolated for a long period of time, perhaps representing relict populations; and (3) natural selection has fixed tree populations at the chloroplast. The first two scenarios propose that genetic patterns have been influenced by fluctuations in forest cover as a result of climatic changes associated with glacial-interglacial cycles [14, 18, 59]. Recent analysis of pollen cores from Lake Bambili in the Cameroon Highlands suggest that forest cover in these mountainous areas might have been very low during the last glacial maximum, which was approximately 20 kya [60]. At the glacial-interglacial transition, some 12–18 kya, an increased presence of arboreal pollen indicates the colonization and expansion of forest at Lake Bambili around this time [60]. Given that the Mambilla Plateau is also part of the Cameroon Highlands, if the pattern of forestation at Lake Bambili is representative of that across this montane region, forests on Mambilla may have been seeded relatively recently in evolutionary time as a result of post-glacial forest expansions. Recent colonization from a limited number of sources could create low contemporary patterns of genetic diversity [50, 59]. However, forests on the Mambilla Plateau might instead be relict populations that have been isolated for considerable time. If this is the case, isolation and drift likely explain low genetic diversity. Unfortunately, to truly tease apart these two scenarios, we would need either fossil pollen data (to determine the floristic composition history of Mambilla) and/or genetic data from populations at the continental scale (to estimate patterns of isolation/migration).

A final possibility that selection has contributed to low cpDNA diversity cannot be ruled out. Regardless of whether tree species are recent colonists, or represent relict populations, strong selection on the chloroplast genome could drive haplotypes to fixation. Testing this scenario would, again, require extensive sampling throughout each species' respective range to correlate haplotype distribution with environmental variables. A rigorous test of selection would also involve reciprocal transplants between populations.

Implications for Conservation

While we cannot rule out that greater diversity might have been observed had we sampled more extensively from forests dispersed throughout Mambilla, or used different loci, our data do support the possibility that chloroplast lineages may be constrained in their diversity on Mambilla. Within a single forest, three separate species showed identical genetic trends of complete (or nearly complete) genetic homogeneity. Furthermore, the fact that all West African Cordia in the Yelwa area had the same haplotype as a single individual found >40km away in Akwaizantar Forest implies that regional genetic homogeneity could be on the scale of tens of kilometers.

Whether or not the low cpDNA diversity observed is due to demographic history or selection has different consequences for management. Low genetic diversity and resulting inbreeding effects can reduce a tree population's fitness: *e.g.* pollen incompatibility [61–63], abortion of seeds [62], or greater juvenile mortality [64, 65]. Plant populations that lose genetic diversity will likely have increased extinction risk, especially if they are self-incompatible [61, 62]. If low cpDNA diversity is a

product of either founder effects or long-term isolation, populations may benefit in the future from the addition of new genetic variants. However, if selection on the chloroplast has been strong, addition of non-locally adapted genetic variants would create outbreeding depression and decrease fitness [66].

Conservation efforts will need to focus on identifying what variation does exist and at what scale; how populations are connected across the landscape; how human presence may disrupt gene dispersal; and how this may impact fitness and recruitment patterns. Whether the analogous patterns of low cpDNA diversity in our focal species are due to the same or different mechanisms also needs to also be tested. Our study provides an initial stepping stone for future tree population genetic studies on the Mambilla Plateau and highlights the need for more such studies in montane Africa.

Acknowledgements

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1 **Appendix 1.** Location, repeat motif, and primer sequences for each cpSSR. Dye choice for each primer was based on differences in product size
2 (as observed from gel electrophoresis) to facilitate pooling. Repeat motifs mentioned here are those deduced from the original source species,
3 *i.e.* tobacco [16], and are not necessarily the same in our focal species.
4

Primer	Location	Repeat motif	Primer sequence	Dye
ccmp2	5' to *trn*S	$(A)_{11}$	5'–GATCCCGGACGTAATCCTG–3' 5'–ATCGTACCGAGGGTTCGAAT–3'	6-FAM
ccmp3	*trn*G intron	$(T)_{11}$	5'–CAGACCAAAAGCTGACATAG–3' 5'–GTTTCATTCGGCTCCTTTAT–3'	PET
ccmp4	*atp*F intron	$(T)_{13}$	5'–AATGCTGAATCGAYGACCTA–3' 5'–CCAAAATATTBGGAGGACTCT–3'	6-FAM
ccmp5	3' to *rps*2	$(C)_7(T)$ $(T)_5C(A)_{11}$	5'–TGTTCCAATATCTTCTTGTCATTT–3' 5'–AGGTTCCATCGGAACAATTAT–3'	VIC
ccmp6	ORF77–ORF82 intergenic	$(T)_5C(A)_{17}$	5'–CGATGCATATGTAGAAAGCC–3' 5'–CATTACGTGCGAACTATCTCC–3'	NED
ccmp7	*atpB*–*rbc*L intergenic	$(A)_{13}$	5'–CAACATATACCACTGTCAAG–3' 5'–ACATCATTATTGTATACTCTTTC–3'	PET
ccmp10	*rpl*2–*rps*19 intergenic	$(T)_{14}$	5'–TTTTTTTTTAGTGAACGTGTCA–3' 5'–TTCGTCGDCGTAGAAATAG–3'	VIC

5   **Appendix 2.** Review of 16 cpDNA diversity studies in 20 tropical and subtropical tree species across different continents. The mean and total
6   number of haplotypes observed in these studies is listed. The total number of populations sampled is also given. Methods of cpDNA diversity
7   estimates were obtained in different ways: cpSSRs = chloroplast microsatellites; restriction sites = digestion of PCR products; indel sites =
8   sequence insertions and deletions; substitution sites = nucleotide base changes. The number of loci used is also indicated. The spatial scale is
9   with respect to kilometers. Exploratory analyses of this data can be found in Appendix 3. We report measures of adult genetic diversity only
10 from those studies that considered different age classes.
11

Species	Common name	Continent	Mean haplotypes	Total haplotypes	Populations sampled	Method	Loci	Scale (km)	Reference
Dysoxylum malabaricum	White cedar	Asia	7.667	24	12	cpSSRs	1	100	Bodare *et al.* [45]
Dicorynia guianensis	Angelique batard	South America	3.6	12	5	cpSSRs; restriction sites	10	100	Caron *et al.* [49]
Vouacapoua americana	Brownheart	South America	1.214	6	14	Restriction and indel sites	5	100	Dutech *et al.* [67]
Vitellaria paradoxa	Shea butter tree	Africa	1.333	7	12	cpSSRs	4	1000	Fontaine *et al.* [54]
Bruguiera gymnorrhiza	Burmese mangrove	Asia	2	2	1	cpSSRs	6	0.01	Islam *et al.* [68]
Ficus insipida subsp. *insipida*	Swamp fig	South/Central America	1.648	19	54	Restriction, indel, and substitution sites	1	1000	Coronado *et al.* [50]
Swietenia macrophylla	Big leaf mahogany	South/Central America	3.563	31	16	cpSSRs	6	1000	Lemes *et al.* [51]

Quercus semiserrata	Wú chǐ Qīnggāng	Asia	2	16	10	cpSSRs	9	100	Pakkad *et al.* [58]
Croton floribundus	Velame	South America	1.375	4	8	cpSSRs	6	1	Silvestrini *et al.* [69]
Castanopsis fargesii	Kǎo	Asia	2.778	47	27	cpSSRs	8	1000	Sun *et al.* [52]
Neobalanocarpus heimii	Chengal	Asia	2.344	15	32	Substitution sites	5	100	Tnah *et al.* [70]
Irvingia gabonensis	African mango	Africa	1.182	2	11	Restriction sites	12	1000	Lowe *et al.* [59]
Macaranga capensis	Wild poplar	Africa	1.3	12	9	Indel and substitution sites	3	100	Jump *et al.* [14]
Newtonia buchananii	Forest Newtonia	Africa	0.9	5	7	Indel and substitution sites	2	100	Jump *et al.* [14]
Ocotea usambarensis	East African camphor	Africa	1.7	17	7	Indel and substitution sites	3	100	Jump *et al.* [14]
Xymalos monospora	Lemon wood	Africa	2.4	23	7	Indel and substitution sites	2	100	Jump *et al.* [14]
Zanthoxylum gilletii	Large-leaved knobwood	Africa	1.4	14	6	Indel and substitution sites	2	100	Jump *et al.* [14]

Cordia africana	Large-leaved Cordia	Africa	1.637	22	22	cpSSRs	2	100	Derero *et al.* [57]
Caesalpinia echinata	Peachwood	South America	1.286	8	7	cpSSRs	3	1000	Lira *et al.* [55]
Milicia excelsa	Mvule	Africa	1.818	14	22	cpSSRs; indel and substitution sites	3	1000	Daïnou *et al.* [56]

12

13   **Appendix 3.** Mean number of haplotypes (sum of within population haplotype
14   count/number of sampled populations) for each species in reviewed studies (Appendix 2) as
15   a function of the number of loci used (a), and the number of populations sampled (b). Trend
16   lines were fit with a simple linear regression in the statistical package, R; R^2_{ajd} = adjusted R^2
17   value, P = statistical significance.

18
19

(a)

$P = 0.807$
$R^2_{adj} = -0.052$

(b)

$P = 0.984$
$R^2_{adj} = -0.056$