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ARTICLE

Relative genome size variation in the African agroforestry tree *Parkia biglobosa* (Fabaceae: Caesalpinioideae) and its relation to geography, population genetics, and morphology

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Abstract: Variation in genome size and in chromosome number can be linked to genetic, morphological, and ecological characteristics, and thus be taxonomically significant. We screened the relative genome size (RGS) and counted the number of mitotic chromosomes in the African agroforestry tree *Parkia biglobosa*, a widely distributed savannah species that shows conspicuous morphological clinal variation and strong genetic structure, and tested for linkage of RGS variation to geography, leaf morphology, and population genetic variation. An improved protocol for the preparation of chromosomes was developed. The study is based on 58 individuals from 15 populations covering most of the distribution range of the species. We observed differences in RGS among individuals of up to 10.2%, with some of the individuals differing statistically in RGS from the bulk of screened individuals. Most of the RGS variation was within populations, whereas variation was unrelated to any of the tested features of the species. Those chromosome numbers that could be exactly established were invariable $2n = 2x = 26$. In conclusion, there was no evidence from the karyological data for structured intraspecific taxonomic heterogeneity.

Key words: genome size, chromosome number, forestry, morphometry, population genetics.

Résumé : La variation quant à la taille du génome et au nombre de chromosomes peut être liée à des caractéristiques génétiques, morphologiques et écologiques, et peut donc s'avérer utile en taxonomie. Les auteurs ont estimé la taille relative du génome (RGS) et compté le nombre de chromosomes mitotiques chez le *Parkia biglobosa*, un arbre utilisé en agroforesterie et largement distribué dans la savane; celui-ci présente une variation morphologique évidente qui suit un gradient ainsi qu'une structure génétique affirmée. Les auteurs ont également vérifié l'association entre la variation pour le RGS et la géographie, la morphologie foliaire et la variation génétique au sein des populations. Un protocole amélioré pour la préparation des chromosomes a été mis au point. Cette étude a porté sur 58 individus provenant de 15 populations couvrant la majorité de l'aire de distribution de cette espèce. Les auteurs ont observé des différences en matière de RGS pouvant atteindre 10,2 % entre les individus, certains individus présentant un RGS significativement différent de la majorité des individus examinés. La plus grande partie de la variation pour le RGS existait au sein des populations tandis que cette variation n'était associée à aucun des caractères étudiés. Le nombre de chromosomes, établi de manière exacte, était invariable à $2n = 2x = 26$. En conclusion, aucune évidence d'une hétérogénéité taxonomique intra-spécifique n'a été prouvée à partir des analyses caryologiques. [Traduit par la Rédaction]

Mots-clés : taille du génome, nombre de chromosomes, foresterie, morphométrie, génétique des populations.

Introduction

Variation in genome size, i.e., variation in the DNA-content of cell nuclei, is a commonly observed phenomenon in plants (Bennett and Leitch 1995, 2011) and is generated by three principal mechanisms: polyploidization, aneuploidization, and deletion or amplification of DNA. Polyploidy refers to the presence of more than two basic chromosome sets (i.e., monoploid genomes: Greilhuber 2005*b*) within a cell nucleus and arises through the instantaneous addition of entire chromo-

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some sets (Ramsey and Schemske 1998). Aneuploidy results from chromosome missegregation and refers to the deviation of chromosome number from a multiple of the monoploid chromosome number due to nonbalanced gain or loss of whole chromosomes (e.g., Compton 2011). Finally, molecular mechanisms like transposon activation, unequal recombination between adjacent repeated DNA motifs, or specific elimination of genes and DNA sequences (Leitch and Bennett 2004; Soltis et al. 2009; Soltis and Soltis 1999) potentially change the DNA content of monoploid genomes (or of single chromosomes; e.g., Bennetzen et al. 2005; Grover and Wendel 2010). An additional cytological mechanism optionally involved in change of genome size is dysploidization, defined as the stepwise change of the haploid—the meiotically reduced — chromosome number achieved through fusion or fission of chromosomes. The resultant reduction or increase in the number of chromosomes (descending and ascending dysploidy, respectively) can either occur with or without loss of chromosomal fragments and therefore DNA (unbalanced and balanced rearrangements, respectively; see reviews by Lysák and Schubert 2013; Schubert 2007; Weiss-Schneeweiss and Schneeweiss 2013).

Genome size variation can be a driver or at least has been identified as an associate of evolutionary divergence. Each of the mentioned principal mechanisms causing genomic modification thus were shown to be linked to patterns of differentiation: monoploid genome size was found to vary with ecological preferences (Jakob et al. 2004; Reeves et al. 1998) or tolerances (Macgillivray and Grime 1995), although the causality of the association often remained elusive or speculative (Schmuths et al. 2004; Slovák et al. 2009; Šmarda and Bureš 2006; Suda et al. 2007*a*). Large differences in genome size among crossing partners furthermore can cause sterility of the progeny this way reducing gene flow (Levin 2002). Chromosomal structural rearrangements (with or without change of the number of chromosomes) and ploidy variation can both introduce barriers to gene flow and thus promote diversification of cytologically differentiated populations and contribute to speciation (Coyne and Orr 2004; Levin 1975, 2002). Finally, although aneuploidy is usually a transient condition—because it changes the genetic balance within a genome—, the presence of extra chromosomes like B chromosomes has been shown to be associated with the expression of specific functional plant traits of evolutionary importance like asexual reproduction via seeds (Sharbel et al. 2004).

Variation in genome size and (or) chromosome number becomes taxonomically significant if associated with some degree of morphological (or genetic) and ecological differentiation (Murray 2005). Genome size can both help to discover taxonomic heterogeneity and be a first clue to understand the mechanisms of associated cytological barriers to gene flow. For instance, two subspecies of the New Zealand grass *Lachnagrostis littoralis* (Hack.) Edgar (both with $2n = 8x = 56$ chromosomes) have been recognized based on a 23% difference in genome size (Murray et al. 2005), a variation which clearly coincided with morphological and ecological differences (Edgar 1995). Different dysploid chromosome numbers can serve as diagnostic characters in species that otherwise only show cryptic morphological divergence (e.g., the species pair *Pulmonaria obscura* Dumort. – *P. officinalis* L. from the Boraginaceae: Dobeš and Vitek 2000; Sauer 1975). Finally, the importance of difference in ploidy per se (i.e., autopolyploidy) only relatively recently has been fully acknowledged. Several examples demonstrate that the diploid parents and their autopolyploid descendants might qualify as species of their own right since they possess distinct geographic ranges (e.g., Cosendai et al. 2011; Soltis 1984), can be distinguished morphologically (Chansler et al. 2016; Hodálová et al. 2007; Marhold 1999), and are largely reproductively isolated via cytological and other barriers (Hardy et al. 2001; Zohary and Nur 1959).

In the following, we asked if morphological and molecular differentiation in the African agroforestry tree *Parkia biglobosa* is paralleled by divergence in relative nuclear genome size (RGS), i.e., if RGS could be used as a predictor of taxonomic heterogeneity. We (*i*) estimated RGS using flow cytometry for 15 populations from throughout most of the distribution range of the species and (*ii*) correlated estimates to the geographic origin of populations (distances among populations) and affiliation to major genetic groups as identified by Lompo et al. (2018) and to the leaf morphology of individuals, the latter analysed within the present study. We (*iii*) further aimed to optimize the protocol for preparing root tips for counting chromosomes in mitotic divisional plates and to provide new chromosome counts.

Materials and methods

Study system

Parkia biglobosa (Jacq.) R. Br. ex G. Don (Fabaceae, subfamily Caesalpinioideae, inclusively Mimosoideae: Stevens 2001), the African locust bean or in French néré, is one of the most important indigenous agroforestry trees of Western and Central Africa (Ouedraogo 1995). Although in the majority of the Caesalpinioideae genera karyotypes evolved without change of the base chromosome number $x = 13$ characterizing the subfamily (Santos et al. 2012), three different chromosome numbers have been reported for *P. biglobosa* that appear to be based on analyses of five accessions or reports ($2n = 22$, 24: Uyoh et al. 2011; $2n = 24$: Mangenot and Mangenot 1957; $2n = 26$: Miege 1960*a*). Additionally, spread citations either refer to these counts or do not contain the referenced information (Mangenot and Mangenot 1962; Miege 1960*b*; Santos et al. 2012; Sina and Traoré 2002). The Caesalpinioideae are characterized by relatively small chromosomes, usually in the range of 1–3 μm (Santos et al. 2012),

Table 1. Geographic origin of 17 studied populations of *Parkia biglobosa* from Western and Central Africa.

Country	Population	Latitude	Longitude	Analyses			Genetic cluster
				FCM	Morpho	CS	
Benin	P6	10.32	1.32	×	×		CWA
Burkina Faso	P7	10.33	–4.9	×	×		CWA
	P14	11.83	–1.25	×	×		CWA
	Vouza	11.94	–3.81	×		×	—
	Walley	11.21	–3.73	×		×	—
Cameroon	P2	9.42	13.37	×	×		CA
Ghana	P4	6.63	0.47	×	×		CWA
Guinea	P4	10.30	–12.23	×	×		EWA
	P7	10.50	–11.82	×	×		EWA
Côte d'Ivoire	P7	9.55	–4.00	×	×		CWA
	P10	9.42	–5.55	×	×		CWA
Mali	P10	11.02	–5.58	×	×		CWA
Niger	P2	12.56	2.79	×	×		CWA
Nigeria	P4	8.42	4.58	×	×		CWA
Senegal	P5	12.75	–15.10	×	×		EWA
	P6	12.63	–15.43	×	×		EWA
Togo	P5	9.27	0.78	×	×		CWA

Note: The kind of performed analyses is indicated as CS, determination of chromosome number; FCM, flow cytometric determination of RGS; Morpho, morphometric analyses of leaves. Populations studied for RGS were assigned to the three major genetic clusters identified by Lompo et al. (2018) in an admixture analysis based on nuclear microsatellite data: EWA, extreme West Africa; CWA, Centre of West Africa; CA, Central Africa. Population codes refer to Ouedraogo et al. (2012).

which render identification of the morphology of chromosomes or of the karyotype challenging. So far, the geographic origin of karyologically studied accessions of *P. biglobosa* has been provided only by these authors (three locations in northern Cross River State, Nigeria), rendering associations of karyological features with geography, ecology, or patterns of morphological and population genetic differentiation still elusive.

Parkia biglobosa covers a wide geographic distribution range extending over about 6000 km in African savannahs from Senegal to Uganda (Hall et al. 1997; Lompo et al. 2017). Most of the intraspecific variation occurs along the longitudinal extension of the distribution range of the species as seen from morphological and molecular studies performed on provenances from 15 West and Central African countries. Ouedraogo (2014) thus observed a significant clinal variation in leaf morphological characters with morphotypes from the very West and very East of the cline being conspicuously dissimilar from each other. An analogous West–East differentiation exists for seed size (Ouedraogo 1995). The geographical distribution of morphological variation corresponds to the dominant pattern of population genetic structure observed for the species: chloroplast haplotype variation is partitioned into a major eastern and western group, a pattern explained by the separation of populations during the last ice ages followed by secondary contacts in the central parts of the distribution range of the species (Ouedraogo 2014). A similar pattern emerged from nuclear DNA variation (Lompo et al. 2018). The existence of a strong phylogeographic signal in the maternally inherited chloroplast data further suggests that the current large-scale distribution of *P. biglobosa* has been shaped by natural forces rather than human activity. The divergence of populations, leading to the current genetic structure, thus happened approximately 160 000 years B.P. with a first split between populations located in extreme West Africa (e.g., Senegal, Guinea) and West Africa (e.g., Mali, Burkina Faso). A second split took place between West Africa and Central Africa (e.g., Cameroon) 60 000 years later, shortly before, or just at the beginning of the penultimate glacial period (Lompo et al. 2018).

Plant material

Individuals collected from 15 natural populations from a provenances trial at the experimental site Gonse (12°25′N, 1°20′W; elevation 280 m a.s.l.) in Burkina Faso, were included for RGS estimation and morphological analyses in the study (Table 1). Per population 3–4 individuals were analysed, 58 in total. For chromosome counting, seeds from 15 trees in two wild populations from Burkina Faso were used (Table 1). We tried but unfortunately failed to get seed material from other countries. Seeds were sampled and analysed separately for each tree (i.e., single-tree seed lot, referred to as seed lot in the following). Vouchers were deposited in WFBVA (Federal Research and Training Centre for Forests, Natural Hazards and Landscape, Vienna, Austria).

Morphometry

Morphometry was performed on a central pinna (i.e., leaflet of 1st order) of the bipinnate foliage leaf of

P. biglobosa (Fig. S1[1]), a morphological structure which was shown to strongly vary with geography in this species (Ouedraogo 2014). One foliage leaf per adult tree was collected from a randomly selected branch at a height of 1–1.5 m. Pinnas were scanned at 1200 dpi on a Scanjet 4570c (Hewlett Packard, Palo Alto, Calif., USA) and scans saved as grayscale in bitmap format. The program tpsDig264 version 2.25 (Rohlf 2016) was used to set landmarks at the very limits of the following characters of a middle leaflet (of 2nd order): length, width, distal width, proximal width, length of the rounded basis, length of the proximal tooth, distal extension of the basal notch (at which the leaflet is attached to the rachis), proximal extension of the basal notch, and distance to the neighboring distal leaflet. Characters have been derived from the set landmarks in calculating the linear distance among the coordinates defining a character using the R environment (R Development Core Team 2011). The following additional characters have been determined manually: angle of the leaflet to the rachis of the pinna; and length of the rachis and number of leaflets along one side of the pinna. We tested for correlation of characters among each other by pairwise comparison using the *cor* function in R and excluded one character per pair if highly correlated (≥0.90). Characters were Z-transformed before further analyses.

Flow cytometric determination of RGS

Sample: standard fluorescence ratios were inferred using a Partec CyFlow® space Ploidy Analyser (Partec, Münster, Germany) equipped with a 365 nm UV LED. Samples were prepared using the two-step Otto procedure (Otto 1990) following Doležel et al. (2007): silica gel-dried leaflets of *Parkia* and the internal standard *Solanum pseudocapsicum* L. (Temsch et al. 2010) were co-chopped with a razor blade in 500 μL Otto I buffer in a petri dish. After a lag phase of 30 min at 4 °C and subsequent ultrasonification of the Petri dish for 1 min, the suspension was filtered through a 20 μm nylon mesh (Partec Cell Trics), and 2 mL Otto II buffer containing 0.2 μg DAPI (4–6-diamidino-phenylindole) mL^{-1} as DNA-specific stain added. Samples were measured in linear registration mode after a lag phase of 5 min. Sample and standard fluorescent peaks were manually gated using FlowMax v2.7 (Partec, Münster, Germany). We used an upper quality limit of 5.0 for the CV of the seedlings and the standard, i.e., fresh material. We did not apply this limit to the CVs of the leaf samples, because silica gel-dried samples typically have poorer quality (Bainard et al. 2011; Suda and Trávnícek 2006). Because we were for logistic reasons restricted to silica gel-dried leaf material, we tested for a bias of higher CVs of the sample peaks on measurement precision. For that purpose we regressed—using the repeat measurements—averaged CVs of the silica gel-dried leaf samples against the variation among sample : standard fluorescence ratios. For that purpose we used the *lm* function of R. Sample : standard fluorescence ratios were calculated from the means of fluorescence histograms. Each sample was measured three times on different days. Samples were compared to each other in performing a pairwise t-test and applying a Bonferroni correction for multiple comparisons using the *pairwise.t.test* function implemented in R. We also calculated the measurement error expressed as 3.92 times the coefficient of variation inferred from the values obtained for the repeated measurements (corresponding to the 5%/95% confidence limits of the normal distribution or ±1.96 times its CV). The CVs were first calculated for each sample and then averaged. The sample : standard fluorescence ratio estimated for an individual was calculated as the average of the three replicates (henceforth we refer to this averaged values as RGS). Three pairs of individuals, which differed most in their RGS estimates, were co-chopped and co-measured to verify these differences. The correlation between RGS and chromosome number was established in measuring the leaves of seedlings of chromosome counted individuals. Finally, occurrence of fluorescence quenching was tested for by comparing the DAPI fluorescence intensity of the standard *Solanum* prepared and measured alone to its intensity when co-chopped with the *Parkia* sample. The gain was held constant and the percent difference in the fluorescence intensities of the standard obtained in the two measurements calculated. The inferred differences were tested for deviation from zero using the *t.test* function in R (normality distribution of values was first tested using the Shapiro–Wilk test).

Chromosome counting

Chromosome counts were made from mitotic late prophase and metaphase plates in apical root meristems. Root tips were obtained from seeds germinated on wetted filter paper. Because pretreatment is a critical phase in the preparation of chromosome counts the pretreatment protocol was optimized in varying parameters as follows: time of pretreatment 3 and 5 h; temperature: 4 °C, room temperature (18–22 °C), 30 °C, room temperature followed by 4 °C for half of the pretreatment time, and 30 °C followed by 4 °C for half of the pretreatment time; chemicals: 0.002 M 8-hydroxyquinoline, 0.2% colchicine, and 0.5% colchicine. Root tips were pretreated under all 30 possible parameter combinations. Suitability of pretreatment was evaluated based on the frequency of mitotic divisions, degree of condensation of chromosomes, and quality of spread of chromosomes (versus stickiness). Pretreated root tips were subse-

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2019-0069.

quently fixed in freshly prepared acetic acid – ethanol solution (1:3) for 24 h at room temperature and then stored at –20 °C till further preparation. Root tips were hydrolyzed in 5 N HCl for 30 min at 20 °C, meristems isolated beneath a binocular and squashed in a drop of 45% acetic acid between a glass slide and cover slide. Cells were fixed on the slide by floating slides on the surface of liquid nitrogen. Giemsa stock solution (Carl Roth, Karlsruhe, Germany) diluted 1:10 in deionized water was used for staining (for 3 min). Chromosome counts were performed using a Zeiss Axioskop 2 MAT light microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 100× oil immersion objective (Plan NeoFluoar numeric aperture 1.30) and an OPTOCAM-III colour camera (Optoteam, Vienna, Austria). We set five exactly countable divisional plates (late prophases or metaphases) as the lower threshold for unambiguous establishment of the chromosome number of a seed lot.

Statistical data analyses

We performed three types of analyses to understand the relationship of variation in RGS to patterns of morphological as well as genetic differentiation and geography. (*i*) Mantel tests to test for significant associations between RGS and geographic as well as morphological distance among individuals. Pairwise differences in RGS as well as in morphology among individuals were calculated as Euclidean distance using the *dist*-function in R. Spatial distances were calculated from the geographic coordinates of populations using the GeographicDistanceMatrixGenerator_v1.2.3 (Ersts 2016). Tests were performed using the function *mantel.rtest* implemented in the R package *ade4* (Dray et al. 2007). Significance of correlation between matrices was assessed in permuting one matrix 10 000 times. (*ii*) RGS was regressed against the longitude and latitude of populations using the *lm* function implemented in R. For comparison, we also regressed morphology against geography. To this end, we calculated the principal components from the covariance matrix of the morphometric characters using the *eigen* function in R and we regressed them against each of the two geographic coordinates. (*iii*) The hierarchical organization of RGS was explored using an ANOVA with populations and the three geographic clusters identified by a genetic admixture analysis based on nuclear microsatellites (Extreme West Africa, Central West Africa, and Central Africa (Lompo et al. 2018) as grouping variables. For comparison, the ANOVA was also run on the morphometric data. Arlequin version 3.5 was used for running the ANOVAs (Excoffier and Lischer 2010).

Results

Chromosomal analyses

A total of 119 chromosomal preparations were screened for performance under the 30 different pretreatments performed. The highest number of divisions was observed for pretreatment with 8-hydroxyquinoline, at room temperature, and lasting 3 h. A similar trend was observed for the quality of spread of chromosomes: 8-hydroxyquinoline and 0.5% colchicine, pretreatment at room temperature, and 3 h of pretreatment all performed best. Condensation (prevalence of meta- and late prophases), in contrast, was optimal for pretreatments with 0.2% colchicine, at 30 °C (in tendency higher temperatures promoted condensation), and lasting 5 h. We chose the parameter combination 8-hydroxyquinoline at room temperature for 3 h as the most suitable pretreatment (see Discussion).

We screened 133 preparations (i.e., root tips) pretreated using the optimized protocol. We were able to establish an exact chromosome number for four seed lots (invariably $2n = 26$; Fig. 1) out of 15 analysed. For two additional seed lots we narrowed down the estimate to $2n = 25–27$. This variation was due to uncertainties in determining an exact chromosome number encountered when analyzing the individual divisional plates. Endomitotic chromosome number duplications were observed in one seed lot (E251) from the population Vouza (Table 2; Fig. 1*b*). Condensation started in the proximal region of the chromosomes. The size of fully condensed metaphase chromosomes ranged from ~0.5 to 1.5 μm.

Flow cytometric results

A single class of sample : standard fluorescence ratios was identified for the silica gel-dried leaf material. Values were normally distributed (tested using the Shapiro–Wilk test, $W = 0.988$, $p = 0.132$) and ranged from 0.306 to 0.347 (mean 0.328 ± 0.009 SD, CV 2.59%, $N = 174$) corresponding to a maximum difference of 13.4%. Coefficients of variation (CVs) for the G0/G1 peak of the analyzed samples and the standard ranged from 3.57% to 8.18% (mean 5.07%) and from 1.50% to 3.40% (mean 2.26%), respectively (see Table S2[1] for statistical details of measurements). An exemplary histogram is provided in Fig. S2[1]. The CV of the sample peaks was unrelated to the variation in sample : standard fluorescence ratios (linear regression, $p = 0.710$, $F = 0.140$), indicating that the higher CVs did not negatively affect the precision of measurements. Measurement error estimated from the replicates was 5.91%. The DAPI fluorescence signal of the *Solanum* standard decreased by –12.3% ± 7.4% when co-processed with a *Parkia* sample relative to the value obtained when analyzed alone (non-normality of data was rejected by the Shapiro–Wilk test, $W = 0.814$, $p = 0.08$). The difference was statistically highly significant ($t = -6.0196$, df = 11, $p < 0.001$), indicating presence of an inhibitory effect of the sample on the intensity of the fluorescence signal.

RGS (i.e., the sample : standard fluorescence ratios averaged over repeated measurements) varied between 0.310 and 0.342 (mean 0.328 ± 0.007 SD, $N = 58$). Thirty-five pairs of individuals significantly differed from each other in the pairwise t-test (Table S3[1]). Differences were not structured by populations but involved single or in maximum two individuals per population. Although es-

Fig. 1. Mitotic chromosome plates from seedling root tips of *Parkia biglobosa*. (*a*, *c–f*) $2n = 26$. (*b*) Entomitotically duplicated chromosome number $2n = 51$ or 52. (*a*, *b*) Population Vouza seed lot E251, (*c*) Walley W4, (*d*) Vouza W9, (*e–f*) Vouza E8. The scale provided in *f* applies to all subfigures.

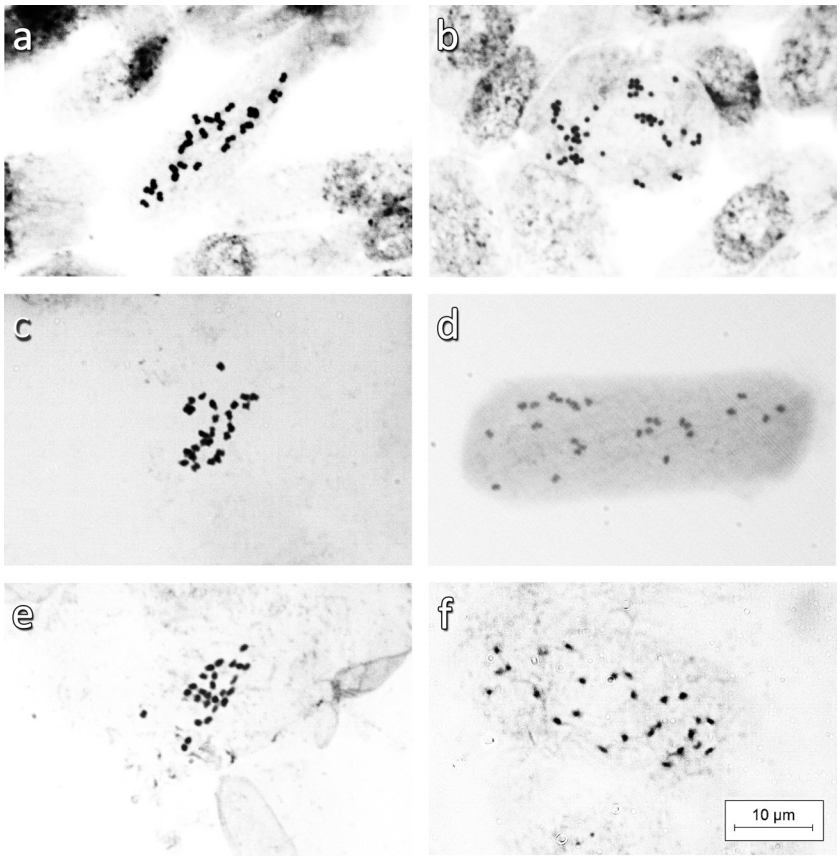

Table 2. Chromosome numbers established for six seed lots of *Parkia biglobosa* from Burkina Faso.

Population/ seed lot	Accepted number (2*n*)	Numbers determined in individual divisional plates (2*n*)						
		24–26	25–26	25–27	26	26–27	51–52	52
Vouza/E8	26	—	—	—	8	—	—	—
Vouza/E10	25–27	1	—	4	2	1	—	—
Vouza/E251	26	—	1	—	6	—	1	1
Vouza/W9	26	—	1	—	9	3	—	—
Vouza/W25	25–27	—	2	—	3	2	—	—
Walley/W4	26	—	1	—	5	—	—	—

Note: Chromosomes were counted in seedling root tips using light microscopy. Hyphens indicate uncertainties in chromosome number estimation. Numbers of divisional plates analysed are provided for each of the determined chromosome numbers. Numbers 51 and 52 represent endomitotic chromosome number duplications.

timates of RGS differed among individuals by up to 10.2%, we could not observe distinctly separated peaks when co-processing pairs of individuals that differed most from each other in the original measurements (Mali P10 individual 2 [RGS 0.310] versus Burkina Faso P7 individual 4 [0.343]; Senegal P5 individual 1 [0.314] versus Ivory Coast P7 individual 3 [0.341]; Niger P2 individual 4 [0.318] versus Togo P5 individual 3 [0.340]). However, these individuals were significantly different in 2 to 17 pairwise comparisons (Table S3[1]). In addition, the CVs of the sample peaks of the joined individuals (mean CV 10.3%) raised strongly compared to those from measurements of single individuals (2.12 ± 0.03 times). Values for sample : standard fluorescence ratios inferred from the seed lots were 0.328–0.342 (mean 0.335 ± 0.004 SD; 5.4% maximal difference; $N = 37$). The mean RGSs inferred from the chromosome counted seedlings was 0.335 and thus differed only by 2.4% from that of the adults, indicating that the adults are diploid. The quality of seedling sample peaks was in tendency better compared to the silica gel-dried leaf samples: CVs of these sample peaks ranged between 2.70% and 5.00% (mean 4.04%).

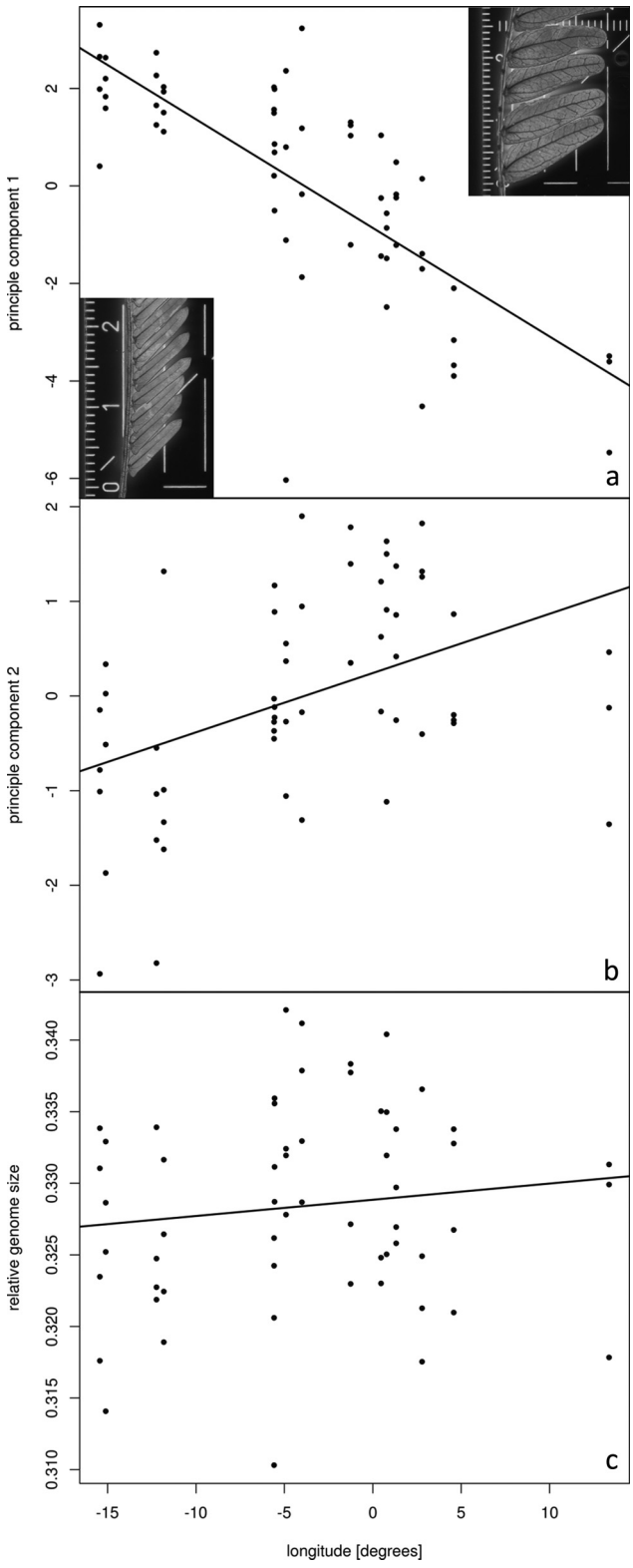

Fig. 2. The association of leaf morphology and relative genome size (RGS) of 58 individuals of *Parkia biglobosa* to the longitude of populations. (*a*, *b*) The principle components 1 and 2 of eight morphological characters. The conspicuous morphological differentiation of leaves with longitude is indicated by the inset scans of leaflets (left individual collected in Senegal from population 6; right from Cameroon, population 2) and was statistically highly significant (linear regression against PC1: F-value 72.150, $p < 0.001$; PC2: F-value 12.300, $p = 0.001$). (*c*) In contrast, the tendency of RGS to increase towards the East had only low statistic support (F-value 0.868, $p = 0.356$).

Morphometry

The measured morphological characters were all variable both within and among populations (Table S1[1]). Four characters—total, distal, and proximal width and length of the rounded basis of the leaflet — were omitted because they were highly correlated with leaflet length (Pearson correlation coefficient $r \geq 0.90$). The first three axes of the principal component analysis explained 86.9% of the total variance in the data (62.5%, 15.4%, and 8.9%, respectively). The first principal component was most highly correlated with the distance of leaflets to each other ($r = -0.923$) and the length of the leaflet (−0.916). The second principal component showed strongest correlation with the length (−0.892) and the number of leaflets (−0.636) of the pinna. The third principal component was most highly correlated with the distal extension of the basal notch of the leaflet (−0.520) and the angle of the leaflet to the rachis (−0.494).

Association of RGS to geography, morphology, and patterns of genetic differentiation

We could not observe a significant association between RGS and any of the tested predictors: geography, morphology, and patterns of genetic differentiation. This was in stark contrast to morphology that significantly and strongly varied for the screened characters with geography and the pattern of genetic variation. The probability that RGS and geography are uncorrelated was $p = 0.600$ according to the Mantel test, but p was <0.001 for morphology. Besides, the null hypothesis of the test that RGS and morphology are unrelated was not declined either ($p = 0.831$). Morphology was significantly related to both longitude and latitude in the regression analyses for the principle components 1 and 2, relationships which were insignificant for RGS (Table S4[1]; Fig. 2). According to the ANOVA, finally, variation in RGS occurred predominantly within populations (96.29% of the total variation, $p = 0.215$). In contrast, 66.97%, 28.20%, and 5.83% of the morphological variation was respectively assigned to the within populations, among regions, and among populations within regions components of variation. The pattern was significant for all hierarchical levels (Table 3).

Discussion

In agreement with earlier reports, we verified existence of a single ploidy level, DNA-diploidy, in *P. biglobosa* by means of flow cytometry. Within this ploidy level, estimates of RGS differed by up to 10.2%. This variation had different components: (*i*) Some of the observed vari-

Table 3. The results of hierarchical ANOVAs partitioning variation in (*a*) relative genome size (RGS) and (*b*) eight morphological characters to the levels of among regions, among populations within regions, and within populations of *Parkia biglobosa*.

Source of variation	d.f.	Sum of squares	Variance components	Probability	Percentage of variation
(*a*) RGS					
Among regions	2	0.012	0.00014	0.2151	3.38
Among populations within regions	12	0.047	0.00001	0.4682	0.33
Within populations	43	0.167	0.00389	0.3489	96.29
(*b*) Morphology					
Among regions	2	20.200	0.60588	<0.001	28.20
Among populations within regions	12	22.911	0.12532	0.0137	5.83
Within populations	43	60.956	1.41759	<0.001	65.97

Note: ANOVAs were run on matrices of pairwise distances among individuals. See Table 1 for assignment of populations to regions and sample sizes.

ation appears to be due to real genome size variation among individuals. A good indicator that inferred differences are due to real variation in genome size and not "technical noise" is recovery of co-processed and co-measured individuals as distinct fluorescence signals (Greilhuber 2005*a*; Greilhuber et al. 2007; Noirot et al. 2000). Although applying this strategy, we failed to distinguish distinct flow cytometric signals from individuals that differed most in the original estimates of RGS, the observed broadening of the joined sample peak compared to that of plain samples indicated existence of some cryptic genome size variation. This was likely because separation of peaks requires that their means differ by more than twice the standard deviation (or CV) (Doležel and Göhde 1995). The CVs of the *Parkia* leaf sample peaks ranged between 3.57% and 8.18% (mean 5.05%). Given the maximum estimated difference in RGS these relations may not have allowed to separate the signals of the genomically even most diverged individuals. (*ii*) The inferred measurement error of the applied flow cytometric method of 5.91% covered a major proportion of the variation observed among measurements (13.4%). The precision of plant genome size estimates is usually lower for silica gel-dried material compared to fresh material. Bainard et al. (2011) reported (their table 7) CVs for replicates that were in average about 50% higher for dry compared to fresh material in six plant species. Lower precision of single measurement can be compensated by averaging over replicates. A normally distributed measurement error thus reduces by $1/N^{0.5}$ (derived from the standard error of the mean = $SD/N^{0.5}$) with N being the number of replicates. We performed three replicates by which the error accordingly should have been reduced by ~58%. In addition, we tested for a possible relation of the quality of the sample peak and the precision of the measurement but found them unrelated for our data. Based on these measures we tried to guarantee that the silica gel-dried leaf material did not perform worse than fresh material. (*iii*) In plants, a likely additional source of measurement error pertains to secondary compounds that are known to vary among tissues, individuals, and with developmental stages (Greilhuber 1987). Endogenous metabolites such as phenolic substances (e.g., tannins, flavonoids, anthocyans, cumarins) are known to interfere with the staining of the DNA (i.e., fluorescence quenching) and can introduce serious stoichiometric error (Greilhuber 1987). A methodological measure recommended to minimize the effects of fluorescence inhibition by plant metabolites is co-extraction and simultaneous flow cytometric measurement of the sample and the standard, i.e., use of an internal standard (Greilhuber et al. 2007; Loureiro et al. 2007; Suda et al. 2007*b*). Inhibitory substances introduced by the sample should bind to the DNAs of both the sample and the standard nuclei during co-isolation, reducing the absolute fluorescence intensity of all nuclei and ideally having little or negligible influence on the sample : standard fluorescence ratio (e.g., Greilhuber et al. 2007). Despite taking this measure, secondary compounds of plants were reported to bias results (Greilhuber 1998). Co-processing of the *Parkia* sample with the standard inhibited the fluorescence signal by 12.3% in average. Although this reduction was moderate, fluorescence inhibition may have introduced "unreal genome size variation" into our data. For example, estimates of RGS were downwardly biased in *Potentilla puberula* Krašan (Rosaceae) by up to 2.82%, in repeated measurement of leaves taken from the same individual, which varied in strength of their inhibitory effects onto the fluorescence of an included internal standard (Myllynen 2017). Price et al. (2000) observed a decrease of the sample : standard fluorescence ratio of 16% in co-stained preparations of *Helianthus annuus* L. and *Pisum* upon a 4-fold increase in the amount of the sample, and in *Leonurus cardiaca* L. genome size estimates were significantly down-shifted in tissues sampled in summer compared to tissues collected in spring despite inclusion of an internal standard (Bainard et al. 2011).

We could not infer significant associations of RGS estimates neither with the geographic distribution, the major pattern of genetic differentiation, nor the leaf morphology of *P. biglobosa*, although there was a weak

West–East-directed tendency of increasing RGS (Fig. 2) and small components of among populations and among regions variation in RGS (Table 3). Thus, the RGS data did not provide evidence for taxonomic heterogeneity in *P. biglobosa*. Instead, they supported the notion of a—fairly—constant genome size that has been considered the norm for a long time (Bennett and Leitch 1995). Lack of a clear association between genome size and morphological, geographical, or ecological patterns of differentiation has also been found for two species of *Artemisia* (Asteraceae, Mas de Xaxars et al. 2016).

Chromosomal differences among crossing partners including differences in the size of chromosomes may impose meiotic irregularities on their progeny which reduces the potential for gene flow. In general, the smaller the difference in chromosome size, the less meiotic pairing is affected. Differences less than 25% may not disrupt the regular pairing of chromosomes (see review by Levin 2002), a value which by far exceeds the differences in RGS inferred here for *P. biglobosa*. The pronounced and relatively sharp population genetic patterning of the species (Lompo et al. 2018) therefore seems unlikely to have been caused by variation in genome size. Whether structural chromosomal rearrangements have contributed to that pattern remains an open question. The magnitude of a barrier to gene flow depends on the nature, number, and size of the chromosomal rearrangements (Levin 2002), modifications which have not been demonstrated for *P. biglobosa* yet. Chromosome counts from material of known geographic origin has become available from Nigeria ($2n = 22$ and 24: Uyoh et al. 2011) and Burkina Faso ($2n = 26$: this study). Both countries fell within one of the three genetic clusters (Central West Africa: Lompo et al. 2018). This constellation would indicate that the lines demarcating the areas of major genetic differentiation cannot be drawn between populations of individuals differing in chromosome number. However, published chromosome numbers are in need of verification.

In *P. biglobosa*, metaphase chromosomes are often sticky which additionally seriously complicates or precludes individualization of chromosomes (C. Dobeš, personal observation). These conditions, which are unfavorable for chromosomal analyses, may be a reason for the quite poor quality of hitherto published chromosome counts. Graphical presentations of counts were provided only by Uyoh et al. (2011), but chromosomes were hardly separated in the depicted mitotic plates. Mangenot and Mangenot (1957) performed counts on dissected root tips, a technique which is prone to underestimate chromosome number due to the danger of "cutting away" chromosomes during the preparation of the microscopic slides. We tried to optimize the protocol for the preparation of chromosomes. We only had access to seed material and used the apical meristems of root tips that are traditionally preferred over shoot meristems for this purpose. Although we varied five parameters critical for the quality of chromosome preparations (Singh 1993), we could not develop a protocol that we consider fully satisfactory. The combination of a pretreatment with 8-hydroxyquinoline at room temperature for 3 h usually ensured high numbers of mitotic divisions, which were in many cases at least moderately spread, but at the cost of suboptimal condensation of chromosomes. Applying this pretreatment, prophases dominated. Incomplete condensation, however, was suboptimal for individualization of chromosomes. Higher temperatures, longer pretreatment times, and use of 0.2% colchicine advanced condensation, as seen from considerably higher frequencies of metaphases, which, however, were rarely sufficiently spread to allow for the counting of chromosomes. This tradeoff precluded use of these settings. Because we could not provide an optimal protocol for *P. biglobosa*, the counting of chromosomes was still laborious although not impossible. Using the best pretreatment identified (8-hydroxyquinoline, room temperature, 3 h), we were able to determine the chromosome number with sufficient accuracy for 40% of the seed lots analysed.

Differentiation for genome size may be intimately related to chromosome number through loss or gain of chromosomes, the typical case of aneuploidy. We observed statistically supported differences in RGS for several pairs of individuals (Table S3[1]), the size of which might indicate variation in the number of single chromosomes. This interpretation would be in accordance with the observation that only one or two individuals per population in maximum differed from the bulk of individuals, an interpretation, however, which still needs verification. Alternatively, a change in chromosome number may be due to fusion or fission of chromosomes without a change of genome size, processes characteristic of the evolution of dysploidies (Lysák and Schubert 2013; Schubert 2007; Weiss-Schneeweiss and Schneeweiss 2013). We were able to verify the chromosome number $2n = 2x = 26$ for *P. biglobosa*, as previously reported by Miege (1960*a*). Although we have not found evidence for dysploidy in our data in the chromosome counted material, we cannot exclude, given limited sample size, the occurrence of this phenomenon in *P. biglobosa*. The majority of genera of the Caesalpinoideae have a base chromosome number of $x = 13$, conserved throughout their evolution including frequent cases of polyploidization of genomes. Chromosome numbers up- and (or) downwardly deviating from a multiple of $x = 13$ are documented for 12 genera out of 39 reviewed and considered dysploidies (Santos et al. 2012). In *Parkia*, descending dysploid numbers have been claimed—aside of *P. biglobosa*—for *Parkia bicolor* A. Chev. ($2n = 24$: Mangenot and Mangenot 1957), *Parkia javanica* (Lam.) Merr. ($2n = 24$: Yeh et al. 1986), and *Parkia pendula* (Willd.) Walp. ($2n = 22$: Barella and Karsburg 2007). For the last species, $2n = 26$ has been

found too (Santos et al. 2012). Both numbers are well documented for this species and thus indicate the existence of intraspecific chromosome number variation in the genus.

As for *P. biglobosa*, we are currently reluctant, keeping to the hard data, to accept the notion of intraspecific dysploid chromosome number variation, due to insufficient quality or documentation of previous results. A recent example provided by Walter et al. (2015), and largely revising earlier claims of chromosome number variation, points at the importance to use optimized protocols for chromosomal preparation and to thoroughly document observations to obtain correct results. To get a more sound understanding of the karyology of *P. biglobosa*, we therefore would like to encourage researchers to extant their efforts to more provenances—possibly making use of the protocols provided here—, ideally from throughout the ecological and geographic range of the species, while keeping to a stringent methodology.

Author contributions

D.L. organized the analysed plant material and revised the manuscript. C.D., I.S., and S.K. performed laboratory analyses. C.D. analysed data and wrote the manuscript.

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