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Insights on cytogenetic of the only strict African representative of genus *Prunus* (*P. africana*): first genome size assessment, heterochromatin and rDNA chromosome pattern

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Abstract. *Prunus africana* is a multipurpose evergreen species endemic to Africa and an endangered species because of overexploitation. The great importance of this species resides particularly in the use of its bark against benign prostatic hyperplasia. As for most tropical trees and generally woody species, cytogenetic studies are scarce. Standard and molecular cytogenetic approaches have been implemented for the first time to study *P. africana* from Cameroon. This is the tetraploid species with a chromosome number of 2n=4x=32. Genome size estimated by flow cytometry was 2C=1.44 pg. Five loci (ten signals) of 35S rRNA genes were observed after fluorescence *in situ* hybridization. Ten G-C rich DNA regions were detected by chromomycin A3 fluorochrome banding. All chromomycin positive bands were co-localized with 35 S rDNA signals. *Prunus africana*, the only strict African representative of genus *Prunus,* is in need of the conservation strategy and *in situ* management that we are also discussing in this work.

Keywords. Chromomycin fluorochrome banding, chromosome number and 2C DNA value, fluorescent in situ hybridization (FISH), heterochromatin and rRNA gene patterns.

INTRODUCTION

The genus *Prunus s. l.* comprise from 200 to 430 species according different authors: over 200 (Rehder 1940), approximately 250 (Wen et al. 2008), 400 (Maghuly et al. 2010), and 430 (according Wielgorskaya 1995 and Niklas 1997).

Prunus africana (Hook. f.) Kalkman, (Syn. *Pygeum africanum* Hook.f.) belonging to the Rosaceae family (subfamily Amygdaloideae), known as African Cherry (Kalkman 1965), is the only species of the genus *Prunus* endemic

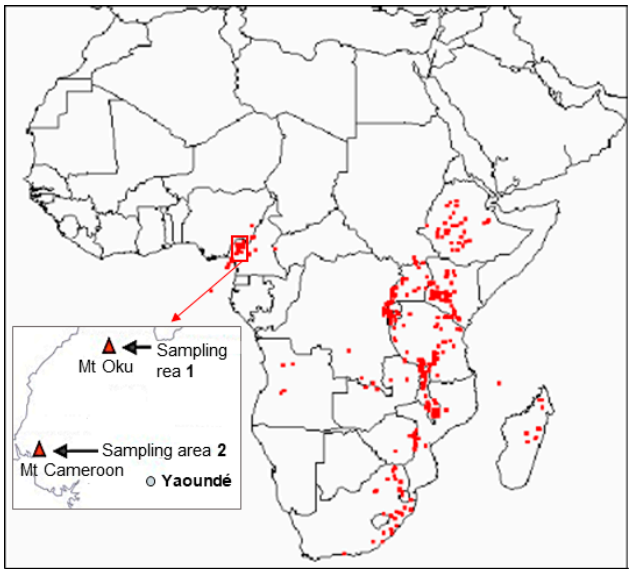

Fig. 1. Global distribution of *P. africana* according Hall et al. (2000) with geographic position of collection sites in Cameroon (box).

to Africa (Fig. 1). It belongs to subgenus *Laurocerasus* which comprises all evergreen species of the genus *Prunus* (Kalkman 1965; Bodeker et al. 2014). This species of the Afromontane flora is present along the central and southern part of Africa, as well as Madagascar and on the islands of Bioko, São-Tomé, and Grande Comore (Kalkman 1965; Cunningham et al. 1997; White 1983; Hall et al. 2000). The main habitats of this species are fallow land, primary and secondary forests. It grows in montane and sub montane forest at a relatively high altitude, from 600 m to 3000 m in the Tropical Africa according to Kalkman (1965) and Stewart (2003a, b). The eastern limit of the distribution of the species is in Cameroon, where *Prunus africana* is found on mountain ranges called "volcanic line of Cameroon" which includes the regions of Southwest, Northwest, West and Adamaoua, and in some sites of the Central region (Melle et al. 2016). This species has several vernacular names in Cameroon (Cunningham and Mbenkum 1993) but it is commonly called African pygeum which is the name attributed to this plant during its first description by Hooker (1864) as *Pygeum africanum* Hook.f. and later was moved to the genus *Prunus* subg. *Laurocerasus* by Kalkman (1965).

Prunus africana attains a height of 30 to 40 m (Stewart 2003; Kadu et al. 2012). Its mature bark resembles the skin of the crocodile and its wood is brown and red. However, the morphology of the species varies according to its habitat, especially concerning the diameter and the height of the trees. The flowers of *Prunus africana* are hermaphrodite (Hall et al. 2000) and the flowering period coincides with low seasonal rainfall and the lowest temperatures, including November to February. Its fruiting is irregular, two to three months intervening between flowering and fruit maturity, and occurs every 2 to 3 years (Geldenhuys 1981). The first flowering takes place when the tree is between 15 and 20 years old, when it is not exploited (Simons et al. 1998). Seeds are recalcitrant (Schaefer, 1990) and lose their germinative power after three weeks (Ondigui 2001). Both self-pollination and insect cross-pollination (Were et al. 2001) occur in *Prunus africana*; however, cross-pollination is the preferential reproductive system of this species (Tonye et al. 2000).

Prunus africana is used for multiple purposes, including artisanal and medicinal uses (Cunningham et al. 2002). The great importance given to this tree resides particularly in the use of its bark against benign prostatic hyperplasia, first discovered and patented by Debat (1966) and since studied by several authors (Njamnshi and Ekati 2008; Kadu et al. 2012). The *Prunus* leaves and roots are also used in the traditional pharmacopoeia as febrifuge for the treatment of upset stomach, pulmonary infection (chest pain), malaria, fever, sexually transmitted diseases and injuries (Carter 1992; Cunnigham and Mbenkum 1993). Apart from this medicinal use, its wood is used locally as firewood and for construction, especially the construction of wagons (Stewart 2003a and b). This plant gives important economic opportunities to rural communities, and over the past decades interest for *P. africana* has changed from traditional to international commercial use. Since 1972, Cameroon has been the major source of bark trade (Cunningham and Mbenkum 1993). Despite the multiplicity of its uses, the declining of *P. africana* populations has been observed in many areas due to the overexploitation of its bark, which threatens a gradual disappearance of the species mainly by commercial harvesting (Cameroon, Equatorial Guinea, Kenya, DRC, Uganda, Madagascar) and by habitat degradation and fragmentation accompanied by invasive alien species which is the case in the southern Africa (Jimu et al. 2011). This overexploitation led the World Alliance for Nature to classify *P. africana* as a vulnerable species which was listed in Appendix II of the Convention on International Trade in Endangered Species of Fauna and Flora (CITES) in 1994, becoming effective in 1995 (Sunderland and Tako 1999). To ensure sustainable utilization and management of *P. africana* in many African countries where it grows, exportation requires a permission.

Before other considerations, the conservation and *in situ* management of *P. africana* requires a good knowledge

of the genetic structure of this species with low potential of colonization. For *Prunus africana*, many studies have been carried out on genetic diversity (Barker et al. 1994; Dawson 1999; Avana et al. 2004; Muchugi et al. 2006; Atnafu 2007; Clair and Howe 2011; Kadu et al. 2013; Mihretie et al. 2015, Nantongo et al. 2016), biochemical property (Tchouakionie 2014; Nzweundji 2015) but cytogenetic studies are still lacking, hence the present study.

The karyological studies in the genus *Prunus* were principally limited on chromosome count. If we consider, as Wen et al. (2008), that the genus *Prunus* comprises about 250 species, the chromosome number is available for more than 76 percent of species (191/250 according to available chromosome databases). The genus presents the basic chromosome number of x=8 (Darlington 1927, 1928) and at least three ploidy levels, diploid, tetraploid and hexaploid (Oginuma 1987; Bennett and Leitch 1995; Iwatsubo et al. 2002; Maghuly et al. 2010). However, some species have been studied by molecular cytogenetics as *P. amygdalus* Stokes (Corredor et al. 2004), *P. persica* (L.) Batsch Peach (Yamamoto et al. 1999; Yamamoto 2012) and *P. subhirtella* Hook. (Maghuly et al. 2010).

Genome size is a fundamental parameter in many genetic and molecular biological studies. Knowledge of the genome size or the 2C value is important for basic and applied studies involving genome organization, species relationships, phylogeny and even taxonomy and biodiversity (Bennett 1984; Bennett and Leitch 2005). At present, flow cytometry is the main method for evaluation of nuclear DNA content because it is both rapid and precise, and reveals even the small differences in DNA content (Marie and Brown 1993; Doležel and Bartoš 2005). However, till today, and according to available databases the genome size of about only 25 species of *Prunus* has been estimated (Arumuganathan and Earle 1991; Dickson et al. 1992; Baird et al. 1994; Bennett and Leitch 1995; Loureiro et al. 2007; Siljak-Yakovlevet al. 2010; Gainza-Cortés 2014; Žabka et al. 2018), which presents 10% for the genus.

Despite the importance of genome size, to the best of our knowledge there is no report on the DNA amount of *Prunus africana,* which is the only species of *Prunus* originating from Africa. It is the same case for cytogenetical characterization of this species, despite the fact that for other species of this genus the banding techniques and Fluorescent *In Situ* Hybridization (FISH) has been developed in order to detect respectively the difference between morphologically similar chromosomes and localization of useful genes (Soodan et al. 1988; Corredor et al. 2004; Maghuly et al. 2010; Yamamoto 2012).

Therefore, we consider that it was necessary to characterize the genome of *P. africana:* 1) by assessment of DNA content using the flow cytometry technique; 2) chromosome counting for determination of ploidy level using the standard cytogenetic method; 3) distribution patterns of GC-rich heterochromatin using the fluorochrome banding; 4) chromosome localization of 18S-5.8S-26S (35S) rRNA genes using a fluorescent *in situ* hybridization. The results will be compared and discussed with those available for other species of the genus *Prunus*.

MATERIALS AND METHODS

Origin and conditioning of plant material

The seed samples (50 seeds from two populations) of *Prunus africana* were collected in Mont Oku at 2400 m and Mont Cameroon at 2500 m of altitude in Cameroon (Fig. 1, box). The hard endocarps of seeds were removed and seeds of uniform size (about 0.7 cm) were used for germination. They were germinated directly in glass dishes containing watered filter paper (Fig. 2C). Cultures were visually checked daily and irrigated with tap water when necessary under laboratory conditions at about 24°C. After 6 weeks, the root meristems of germinated seeds were used for cytogenetic studies. Germinated seeds were transferred to pots and after 4 weeks the leaves were collected for flow cytometry (Fig. 2D).

Genome size assessment by flow cytometry

The total nuclear DNA content was determined by flow cytometry according to Marie and Brown (1993). *Solanum lycopersicum*, 2C= 1.99 pg (Lepers-Andrzewski et al. 2011) was used as internal standard. Leaf tissue of *Prunus africana* and *Solanum lycopersicum* was placed in a plastic Petri dish and chopped together with a razor blade in 600 µL of cold Gif Nuclear Buffer which is slightly modified Galbraith's buffer (Galbraith et al. 1983): 45 mM $MgCl_2$, 30 mM sodium citrate, 60 mM 4-morpholinepropane sulfonate pH 7, 0.1 % (w/v) Triton X-100, 1% polyvinylpyrrolidone (~10,000M_r, Sigma P6755), 5 mM sodium metabisulfite and 10 µg/ml RNase (Sigma Aldrich, Saint Quentin, France). Nuclear suspensions were filtered through nylon mesh (pore size 50 mm, Cell Trics, Partec) and stained with 50 mg ml^{-1}propidiumiodide (PI: Sigma-Aldrich, France). After 5 min incubation on ice, nuclear suspensions were analyzed. Five individuals per accessions were analyzed in order to obtain the mean DNA content. At least 5000 to 10, 000 nuclei were analyzed for each sample using a Cyflow SL3, Partec, 532-nm laser cytometer (Munster, Ger-

Fig. 2. *Prunus africana:* A) 6-years-old tree; B) fruit - two-lobed drupe, with a seed in each lobe; C) germination of seeds in Petri dishes; D) seedlings.

many). Nuclear DNA content was estimated using the linear relationship between the fluorescent signals from stained nuclei of *P. africana* specimens and the internal standard (*S. lycopersicum*) according to the following equation:

2C DNA content/nucleus = [Sample 2C peak mean × Standard 2C DNA] (pg)

Standard 2C peak mean

The symbol C corresponds to the holoploid nuclear genome size (the whole chromosome complement with chromosome number *n*), 1C and 2C being, respectively, the DNA contents of the haploid (*n*) and diploid (2*n*) sets of chromosomes, irrespective of ploidy level (Greilhuber et al. 2005). The conversion from picograms (pg) to base

pairs (bp) was done as follows: 1 pg DNA = 978 Mbp (Doležel et al. 2003).

Determination of chromosome number

Root tips, obtained from potted plants were pretreated with 0.002 M 8-hydroxyquinoline at 16°C for 3h. Fixation was performed in freshly prepared 3:1(v/v) ethanol-acetic acid for at least 24h. Fixed root tips were stored for a few days in the first fixative, or several months in 70% ethanol at 4°C. For chromosome counting the root tips were hydrolyzed in 1N HCl for 10 min at 60°C and stained in Schiff reagent following the standard Feulgen method, or in an enzymatic mixture for 45 min at 37°C. The squash was performed in a drop of acetic carmine. The chromosome number was also verified on slides prepared for fluorochrome banding by chromomycin A_3 or for FISH for which they were counterstained with DAPI (4', 6-diamidini-2-phenylindole). Chromosome plates were observed under a Zeiss Axiophot microscope. The chromosome number was determined for at least 10 individuals, from several well-spread metaphases per root tip.

Preparation of protoplasts for fluorochrome banding and fluorescence in situ hybridization (FISH)

Fixed root tips were washed in 0.01 M citrate buffer pH 4.6 for 15 min, then incubated in an enzymatic mixture for 45 min at 37°C. This enzymatic mixture was composed of 4% cellulase R10 (Onozuka Yakult Honsha Co.), 1% pectolyase Y23 (Seishin Pharmaceutical, Co., Tokyo, Japan), and 4% hemicellulase (Sigma Chemical Co.) in 0.01M citrate buffer at pH 4.6.

These digested meristems were squashed onto a drop of freshly prepared 45% acetic acid and the preparations were observed at a phase contrast microscope. The best slides were frozen at -80° C over night and then the cover slips were removed and the slides were rinsed with absolute ethanol and air-dried.

Fluorochrome banding

For detection of GC-rich DNA regions, the chromosomes were stained with chromomycin A_3 (CMA_3) fluorochrome using Schweizer's (1976) method, with slight modifications, from Siljak-Yakovlev et al. (2002) concerning the concentration of chromomycin (0.2 mg/ml) and time of staining (60 min). After chromosome observation, the best slides were distained in 3 :1 ethanol-acetic acid, dehydrated in a graded ethanol series (70%, 90%,100%), air-dried for at least 12h at room temperature, and then used for FISH experiment.

Florescence in situ *hybridization*

FISH was performed for the detection of 35S rDNA loci. The 35S rDNA probe was a clone of 4-kb from *Eco*RI fragment, including 18S-5.8S-26S rDNA sequences from *Arabidopsis thaliana* (L.) Heynh. labelled with the direct Cy3 fluorochrome (Amersham, Courtaboeuf, France) by nick translation, according to the manufacturer's protocol. *In situ* hybridization was carried out following Heslop-Harrison et al. (1991) with some minor modifications. Slides were counter-stained and mounted in Vectashied medium (Vector Laboratories, Peterborough, UK) with DAPI. Chromosome plates were observed using an epifluorescence Zeiss Axiophot microscope with different combinations of excitation and emission filter sets (01, 07, 15 and triple filter set 25). Hybridization signals were analyzed and the best metaphase plates were photographed using a highly sensitive CCD camera (RETIGA 200R, Princeton Instruments, Every, France) and image analyzer (Metavue, Every, France).

RESULTS

Nuclear DNA content and chromosome number

In this study both the genome size and chromosome number of *Prunus africana* were determined for the first time. Nuclear DNA content of *P. africana* was 2C=1.44 pg or 1408 Mbp (1pg DNA = 978 Mbp according to Doležel et al. 2003), ranking the taxon in the category of very small genomes (2C ≤ 2.8 pg, according to Leitch et al. 1998). The chromosome number showed that *P. africana* is a tetraploid species with 2n=4x=32 small (~ 1 to 2 μm) chromosomes (Fig. 3) and basic chromosome number x=8. This number, which showed a high stability, has been verified with all used techniques: squash in acetic acid after staining in carmine acetic (Fig. 3A); protoplast technique with chromosome speeded in acetic acid without staining (Fig. 3B – phase contrast picture), after fluorochrome bandings with DAPI staining (Fig. 3C) and CMA_3 (Fig. 3D), and after FISH experiment (Fig. 3F).

rRNA genes and heterochromatin pattern

Fluorochrome banding revealed ten G-C rich heterochromatin regions (CMA^+ bands), which were always

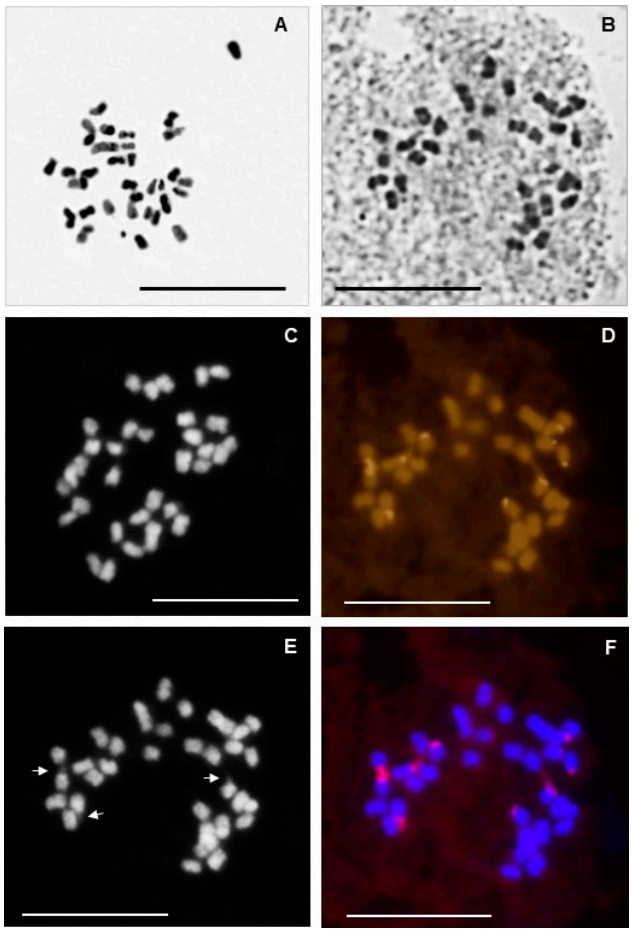

Fig. 3. A) Schiff stained metaphase plate showing 2n=32 chromosomes. B) Non-colored metaphase chromosome plate obtained after enzymatic hydrolysis photographed under microscope with phase-contrast. C) DAPI stained chromosomes without visible bands. D) The same metaphase plate (D, E and F) after the fluorochrome banding with chromomycin A_3. The CMA^+ bands corresponded to DNA regions rich in G-C bases. E) DAPI stained chromosome after FISH experiment showing DAPI negative bands (arrows) corresponding to A-T low DNA regions. F) FISH experiment revealed ten 35 rDNA (red) signals which corresponded to ten CMA^+ bands (see Fig. C). Bar scale = 10 μm.

associated with rDNA loci (Fig. 3D). The karyotype of *P. africana* is characterized by five loci of 35S rDNA (visible as ten red hybridization signals), which were located in terminal or subtelomeric positions. These strong red signals appeared as weakly stained regions after DAPI counterstaining which indicated their richness in G-C bases (Fig. 3E, arrows). The number and the position of 35S rDNA loci are presented in Fig. 3F. Some centromeric 35S signals were of weaker fluorescent intensity than terminal signals and do not appear in all observed metaphase plates. DAPI stained chromosomes did not display any visible bands (Fig. 3C).

DISCUSSION

Phylogenetic context

The subfamily Amygdaloideae comprises nine tribes and for several of them the relationships were uncertain in previous phylogenetic study (Potter et al. 2007). In recent work of Xiang et al. (2016) the tribe *Neillieae* is supported as the basal lineage of Amygdaloideae, followed by the tribe *Spiraeeae*. The only species of *Lyonothamneae*, previously placed as the basal lineage, becomes the sister to *Amygdaleae*. This phylogenetic context is also very well supported by the basic chromosome number. The two basal clades have x = 9, which is also the ancestral basic number of Rosaceae (Vilanova et al. 2008), and two tribes *Lyonothamneae* and *Amygdaleae,* formed the only clade with x = 8 in subfamily Amygdaloideae. It is, at the same time, the only events of decreasing dysploidy in this subfamily while in the subfamily Rosoideae this phenomenon occurred several times because x = 7 is the most frequent base number. The closest genera of *Prunus*, *Maddenia* and *Pygeum* also have x = 8.

In the phylogenetic frame *Prunus africana* is always in the same clade especially with the Asian tropical species of the genus *Pygeum* which shows that they are very close (Wel et al. 2008).

Chromosome number and nuclear DNA content

To verify the genome size and chromosome number data for the genus *Prunus* and to attribute the status of novelty for investigated species, we used updated databases: Kew plant DNA C-values database (http://data.kew.org/cvalues), FLOWer, a plant DNA flow cytometry database (http://botany.natur.cuni.cz/flower/index.php), Index to Plant Chromosome Numbers (IPCN) - Tropicos (http://www.tropicos.org/Project/IPCN) and The *Chromosome Counts Database* (*CCDB),* (http://ccdb.tau.ac.il/search/).

In the genus *Prunus*, which has been highly studied from many viewpoints, the number of species with available genome size information is very low, only about 25 (10 %) species have genome size records to date, whereas chromosome numbers have been reported for at least 76 % of the taxa. It is a very good report knowing that chromosome number has been determined to date for about only 25% of angiosperms taxa (Stuessy 2009) or for only 20% according to Rice (2015).

The basic chromosome number of *Prunus* genus is x=8 (Darlington 1927, 1928) and their ploidy level ranges from diploid ($2n$=$2x$=16) to tetraploid ($2n$=$4x$=32)

Table 1. *Prunus* species for which some molecular cytogenetic data are available. Comparison with our results of *P. africana*.

Species	2n (ploidy level)	2C DNA in pg	35S rDNA signals number (position)	$CMA^+/DAPI^-$ bands number	References
P. africana (Hook. f.) Kalkman	32 (4*x*)	1.44	10 (terminal, satellite)	10	Present work
P. amygdalus Stokes	16 (2*x*)	0.66	6 (terminal satellite)	-	Corredor et al. 2004
P. armeniaca L.	16 (2*x*)	0.60	-	6	Yamamoto 2012; Arumuganathan and Earle 1991
P. incisa x *serrula*	32 (4*x*)	1.22	12 (terminal satellite)	-	Maghuly et al. 2010
P. mume (Siebold) Siebold & Zucc	16 (2*x*), 32 (4*x*)	-	-	5 to 8	Yamamoto 2012
P. persica (L.) Batsch Peach	16 (2*x*)	0.55	6 (terminal, satellite, proximal)	6	Yamamoto et al. 1999, Yamamoto 2012
P. salicina Lindl.	16 (2*x*)	-	-	6	Yamamoto 2012
P. subhirtella Hook.	probably 16 (2*x*)	0.61	6 (terminal)	-	Corredor et al. (2004); Maghuly et al. 2010

and hexaploid (2*n*=6*x*=48) (Bennett and Leitch 1995; Maghuly et al. 2010; Siljak-Yakovlev et al. 2010). *Prunus africana* is a tetraploid species (2*n*=32) with genome size 2C=1.44 pg. Based on available bibliographic data genome size for diploid *Prunus* species ranges from 0.55 to 1.00 pg (Dickson et al. 1992) and for tetraploids from 1.14 to 1.30 (Arumuganathan and Earle 1991; Siljak-Yakovlev et al., 2010). The 2C values of *P. africana* (1.44 pg) was slightly bigger that in other tetraploids. The only hexaploid species measured until now is *P. domestica* L. (Darlington and Wylie 1955) with 2C=1.85 pg (Arumuganathan and Earle 1991; Bennett and Leitch 1995). Horjales et al. (2003) reported 2C=2.35 pg for *P. padus* L. and Zonneveld et al. (2005) 7.30 pg for *P. laurocerasus* L. which indicated the existence of higher ploidy levels that 6*x* in this genus.

Heterochromatin and 35S rDNA pattern recorded

In this study the data concerning heterochromatin and rRNA genes patterns for *Prunus africana* were reported for the first time. In Table 1 we presente available molecular cytogenetics data for some species of the genus *Prunus* and compare with the results obtained in this work. We observed 10 G-C rich DNA regions in all the samples used for this experiment. Previous studies performed for *Prunus persica* (Yamamoto et al. 1999; Yamamoto 2012), *P. salicina* Lindl. (Yamamoto 2012) and *P. armeniaca* L. (Yamamoto 2012; Arumuganathan and Earle 1991) revealed six and in *P. mume* five to eight G-C rich DNA regions (Yamamoto 2012). The GC-rich heterochromatin was always co-localized with 35S rDNA loci. The same observation has been reported by Yamamoto et al. (1999) and Yamamoto (2012) for *Prunus persica*. The co-localization of GC-rich heterochromatin and ribosomal genes has been frequently reported; e.g. in *Retama* (Benmiloud-Mahieddine et al. 2011); *Fraxinus* (Siljak-Yakovlev et al. 2014); *Tanacetum* L. (Olanj et al. 2015); *Eucalyptus* (Riberio et al. 2016); *Sclerocaria* (Bationo-Kando et al. 2016). Other rDNA patterns have been described in several *Prunus* species. This is the case of 5S and 18S-5.8S-25S ribosomal RNA genes which have been located in *Prunus persica* (Yamamoto et al. 1999), in *P. amygdalus* Batsch (Corredor et al. 2004) and in two others species of Cherry rootstock; *P. subhirtella* Miq. (Corredor et al. (2004); Maghuly et al. 2010) and *P. incisa* x *serrula* (Maghuly et al. 2010). In these last studies the diploid *P. subhirtella* presented six 35S rDNA signals as in the majority of diploid *Prunus* species (Yamamoto 2012), while the recent tetraploid hybrid *P. incisa* x *serrula* presented namely the double (12 signals). The diploid ancestor of *P. africana* probably had three loci of 35S rDNA whose numbers decrease from six to five loci during the chromosomal restructuring after polyploidisation.

CONCLUSION

The present paper has focused on the cytogenetic characterization of *Prunus africana*, contributing to the better knowledge of this useful African tree. How-

ever, future studies from populations collected in other regions would provide more information for the sustainable management of this endangered species. Currently, *P. africana* is on the IUCN Red List and has been classified as a priority for conservation by FAO. In Cameroon and at the international level, provisions have been made and laws have been drawn up to ensure the rational exploitation of this species. These investigations will make possible to identify the priority areas for the conservation of this species but also to establish a best management plans for the sustainability of genetic resources of *Prunus africana*.

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