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Cytogeography of *Cenchrus ciliaris* (Poaceae) in Tunisia

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Abstract Belonging to the genus *Cenchrus* with 16–22 species, *Cenchrus ciliaris* L. (syn. *Pennisetum ciliare* (L.) Link, buffelgrass) is a perennial, common in warmer regions of both hemispheres, growing as a C4 grass in a wide range of habitats. In the present study we determined chromosome number and nuclear DNA content (2C DNA) for 28 natural populations collected from northern to southern Tunisia. Three ploidy levels were found: one tetraploid population ($2n=4x=36$), three pentaploid ($2n=5x=45$), and 24 hexaploid populations ($2n=6x=54$). The hexaploid chromosome number has already been reported for Tunisian populations of *C. ciliaris* but tetraploid and pentaploid ($2n=45$) are new for this area. The tetraploid population was found in the semi-arid north; pentaploids were mostly on the northern side of the arid region, while the hexaploids were located mainly in the arid southern Tunisian and Saharan region. 2C DNA values, assessed using flow cytometry, correlated with chromosome counts. Nuclear DNA content ranged from 2C=3.03 to 4.61 pg, revealing three ploidy levels corresponding to 4*x*, 5*x*, 6*x*, and mean 2C DNA amounts were of 3.03, 3.7 and 4.48 pg, respectively. Each cytotype produced viable pollen. Flow cytometric seed screening neither proved nor disproved apomixis. The most frequent hexaploid populations seem best adapted to arid conditions in southern Tunisia. The monoploid value, 1Cx, was constant. The existence of pentaploid cytotype suggests hybridization ability between tetraploids and hexaploids. It appears that polyploidization is the major evolutionary mechanism in the speciation of *C. ciliaris.*

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Introduction

Under arid climates, the disturbance of the natural ecosystem is often assessed by the reduction of perennial grasses; overgrazing leads in particular to the extinction of species of high nutritional value (Le Houérou 1993). In this context, ecological restoration by reintroduction of extinct species should be based on choice of taxa optimal by their large ecological amplitude and phenological plasticity (Aronson et al. 1993; Daehler and Goergen 2005; Visser et al. 2007). Perennial herbaceous plants promote good development of the vegetation cover and are present in almost all seasons of the year. In several arid ecosystems, such restoration and rehabilitation processes are currently underway. For example, two perennial rangeland grasses such as *Enteropogon macrostachyus* (Hochst.) Munro and *Eragrostis superba* Peyr. have been used extensively to rehabilitate semi-arid land of Kenya (Mganga et al. 2010).

Cenchrus L., a genus belonging to the tribe Paniceae of Poaceae family, is an important component of the world's major grass covers, distributed throughout the tropical and warm temperate zones (Watson and Dallwitz 1992). The genus *Cenchrus* is morphologically closely related to the genus *Pennisetum*, and their relationship has also been analyzed using molecular phylogenies (Martel et al. 2004; Donadío et al. 2009). These authors favor the inclusion of *Cenchrus ciliaris* L. within the genus *Pennisetum* based on the study of chromosomal and genetic characteristics combined with phylogenetic relationships. Therefore they propose to reconsider the taxonomic position of *Cenchrus ciliaris* and treat it as *Pennisetum ciliare* (L.) Link.

Species of *Cenchrus* are mainly distributed in Africa, Asia and Australia and have a base chromosome number of $x=9$ (DeLisle 1963; Crins 1991). Most of the 22 species recognized by Clayton and Renvoize (1982) are considered undesirable and noxious weeds such as *C. echinatus* L. and *C. biflorus* Roxb. (Correll and Johnston 1970; Clayton and Renvoize 1986; Stieber and Wipff 2003). On the contrary, *Cenchrus ciliaris* is of economic significance as an important forage grass (Correll and Johnston 1970; Clayton and Renvoize 1986; Stieber and Wipff 2003) in many of the drier regions of the world. This species is native to the tropical and subtropical areas of Africa (Bogdan 1977) and has been widely introduced into subtropical arid regions of the world for its high pastoral value (Franklin et al. 2006). Following the introduction of *C. ciliaris*, it has invaded extensive areas of arid and semi-arid lands in Australia affecting fire regimes and loss of native species (Jackson 2005).

In Tunisia *C. ciliaris* ranges from the lower semi-arid to the Saharan limit, a broad bioclimatic gradient (Cuénod et al. 1954; Ziegler et al. 2000). However, overgrazing has decimated populations, and the species now contributes little to natural plant communities. Within protected areas (such as the National parks of Bouhedma and Sidi Toui) this species is still abundant and vigorous (Bousquet 1992). Considering its high adaptability to bioclimatic constraints of arid zones and its high pastoral value (Chaieb et al. 1996), *C. ciliaris* may be suitable for the restoration of degraded

arid ecosystems of Tunisia and of North Africa in general (Visser et al. 2008; Mseddi et al. 2002).

Cytoembryological investigations showed that *C. ciliaris* reproduces mainly by aposporous apomixis (Ozias-Akins et al. 2003). The non-reduced apomictic embryo sacs in this species originate mitotically from nucellar cells and contain four cells. However, pollen grains play a role by initiating the development of endosperm and seed (Snyder et al. 1955; Vielle-Calzada et al. 1996). Consequently *C. ciliaris* is considered as a pseudogamous apomictic species (Vielle et al. 1995). Most *C. ciliaris* populations are obligate apomicts but sexual forms have been occasionally identified (Fisher et al. 1954; Bray 1978; Sherwood et al. 1980). The species shows three ploidy levels: tetraploid ($2n=4x=36$), pentaploid ($2n=5x=45$) and hexaploid ($2n=6x=54$) (Fisher et al. 1954; Bashaw and Hignight 1990).

Given the paucity of information about genome size and chromosome number of *C. ciliaris* in natural populations, we have investigated 28 populations from Tunisia. The goals of this study were to establish the cytogeography of different ploidy levels for *C. ciliaris* in Tunisia, and to evaluate its reproductive mode by flow cytometric seed screening (FCSS). We also aimed to explore association between ploidy levels and climatic factors.

Material and Methods

Plant Material

Twenty-eight populations of *Cenchrus ciliaris* were collected from different regions in Tunisia, as listed in Table **1**. These populations represent a range of climate and soil conditions (Table **1**) and are supposed to be genetically the most distant and polymorph, according to Mseddi et al. (2004).

Flow Cytometry: Determination of 2C DNA

The internal standard used for flow cytometric analysis was *Petunia hybrida* Vilm. cv. PxPc6 (2C=2.85 pg) (seeds available from CNRS Gif-sur-Yvette) for pentaploids and hexaploids, and *Lycopersicon esculentum* Mill. cv. Roma (2C=1.99 pg, Marie and Brown 1993) for tetraploids. Leaves of one sample and an internal standard were chopped with a razor blade in a Petri dish with 600 μL of cold Galbraith buffer (Galbraith et al. 1983) supplemented with 1 % polyvinylpyrrolidone 10,000 and 50 μg/ml RNAse A (Sigma Chemical Co. St. Louis, USA). The nuclei suspension was then filtered through nylon mesh (pore size 48 μm) to remove fragments and large tissue debris and stained with 50 μg/ml propidium iodide (Sigma Chemical Co. St. Louis, USA). In total 168 samples were analyzed on a flow cytometer (CyFlow SL3, Partec, Munster, Germany) with a 532 nm laser. At least five individuals per population were analyzed to obtain the mean DNA content. At least 5,000 to 10,000 nuclei were analyzed for each sample. The 2C DNA value was calculated using the linear relationship between the fluorescent signals from stained nuclei of the unknown specimen and the known internal standard.

Table 1 Geographical origin and environmental conditions of the studied 28 Tunisian populations of *Cenchrus ciliaris*

Pop. code	Population	Geographic location	Latitude Longitude	Bioclimate[a]	Winter[a]	P (mm)[a]	Substrate[a]
MR01	Morneg-01	S of Tunis	36°73′ N 10°24′ E	less semi-arid	fairly cold	500–600	clay-loam soil
SA02	Sahel-02	E of Teboulta	35°56′ N 11°68′ E	more semi-arid	mild	200–300	calcareous muddy
SA03	Sahel-03	NW of Mahdia	35°46′ N 11°49′ E	less semi-arid	mild	200–300	silty-sand with alluvial deposit
ME04	Mezzouna-04	Meknassi Piste to Bou Hedma National Park	34°32′ N 10°06′ E	less arid	mild	150–200	soil with bigger sand on gypsum crust
ME05	Mezzouna-05	Bou Hedma National Park (centre)	34°28′ N 09°38′ E	less arid	moderate	150–200	alluvial sandy muddy soil
ME06	Mezzouna-06	Haddej National Park	34°26′ N 09°12′ E	less arid	moderate	150–200	chalky and deep soil with medium sand
ME07	Mezzouna-07	beginning of the Haddej Piste to Bou Hedma National Park	34°27′ N 09°39′ E	less arid	mild	150–200	alluvial sandy muddy soil
ME08	Mezzouna-08	middle of the Haddej Piste to Bou Hedma National Park	34°24′ N 09°29′ E	less arid	moderate	150–200	muddy soil with gypsum clay
ME09	Mezzouna-09	Gabès (97 km) – Sfax	34°10′ N 09°59′ E	less arid	mild	150–200	soil with bigger sand on gypsum crust
ME10	Mezzouna-10	El Hamma – Menzel Habib, jebel Haidoudi	34°02′ N 09°44′ E	less arid	mild	100–150	muddy soil with gypseus clay
JR11	Jerba-11	Jerba; road Taguemess – Midoun	33°47′ N 11°02′ E	more arid	lime	200–300	chalky and deep soil with medium sand

Table 1 (continued)

Pop. code	Population	Geographic location	Latitude Longitude	Bioclimate[a]	Winter[a]	P (mm)[a]	Substrate[a]
JF12	Jeffara-12	Gabès (45 km) – Medenin	33°37'N 10°28′ E	more arid	mild	150–200	chalky and deep soil with medium sand
JF13	Jeffara-13	Piste Metameur - Toujane	33°28′ N 10°19' E	more arid	mild	150–200	soil with gypsum crusting
JF14	Jeffara-14	Metameur (18 km) – Toujane	33°24′ N 10°16′ E	more arid	mild	200–300	brown soil associated with lithosol
JF15	Jeffara-15	Zarzis (22 km) – Medenine	33°23′ N 10°42′ E	more arid	mild	150–200	salt soil
JF16	Jeffara-16	Medenine, road of Tejra Mountain	33°23′ N 10°24′ E	more arid	mild	150–200	soil with gypsum crusting
JF17	Jeffara-17	Jendli	33°23′ N 10° 31′ E	more arid	mild	150–200	brown soil associated with gypsum crusting
JF18	Jeffara-18	Oued El kantra, upstream of Medenine	33°19′ N 10°28' E	more arid	mild	150–200	brown soil associated with gypsum crusting
JF19	Jeffara-19	Medenine Piste toward Beni Khedache	33°16′ N 10°23′ E	more arid	mild	150–200	brown soil associated with gypsum crusting
JF20	Jeffara-20	Raâs Jedir (Ktef) – Ben guerdane	33°08′ N 11°12′ E	more arid	mild	200–300	soil with eolian contribution
JF21	Jeffara-21	Medenine (11 km) – Tataouine	33°03′ N 10°28′ E	more arid	mild	150–200	soil on chalky crust
JF22	Jeffara-22	Kerchaou Piste (7 km) - Chahbania	33°02′ N 10° 51′ E	more arid	mild	100–150	soil on gypsum crust

Table 1 (continued)

Pop. code	Population	Geographic location	Latitude Longitude	Bioclimate[a]	Winter[a]	P (mm)[a]	Substrate[a]
JF23	Jeffara-23	Piste Smar (4 km) – Cherchara	32°57′ N 10°53′ E	more arid	mild	100–150	soil on gypsum crust
ST24	Sidi Toui-24	IRA of Ben Guerdane (35 km) – Sidi Mahdi	32°49′ N 11°20′ E	more arid	mild	100–150	brown soil associated with gypsum crusting
ST25	Sidi Toui-25	Sidi Toui National Park (east)	32°43′ N 11°14′ E	more arid	moderate	50–100	brown soil associated with lithosol on chalky crust
ST26	Sidi Toui-26	IRA of Ben Guerdane (50 km) – Sidi Mahdi	32°42′ N 11°18′ E	more arid	moderate	50–100	brown soil associated with lithosol on chalky crust
ST27	Sidi Toui-27	Sidi Toui National Park (west)	32°41′ N 11°12′ E	more arid	moderate	50–100	brown soil associated with lithosol on chalky crust
DH28	Dhibat-28	Remada-Dhibat (Oued el Anguar)	32°08′ N 10°32′ E	Saharan sup.	moderate	50–100	soil on chalky crust

[a] – Belkhodja et al. (1973).

Flow Cytometric Seed Screening (FCSS)

Reproductive mode was analyzed using methods similar to those described by Matzk et al. (2000) and Wieners et al. (2006) with slight modifications. One population for tetraploid, two for pentaploids and ten populations for hexaploids were examined. For each population three individuals were selected separately for analysis. Twenty seeds per individual were chopped with a razor blade in 600 μL of cold Galbraith buffer (Galbraith et al. 1983) as for leaves. No internal standard was added but for each ploidy level the leaf was re-measured to confirm the 2C peak position. According to Matzk et al. (2000), seeds produced through sexual reproduction result in a high 2C embryo peak and a small 3C endosperm peak resulting from the double fertilization. Seeds produced by pseudogamous obligate apomixis present a 2C embryo and 5C endosperm DNA peak, formed from the fusion of two unreduced polar cells with a haploid sperm nucleus.

Statistical Analysis

For evaluating variance in 2C DNA content of the whole dataset, one-way ANOVA was used. To assess potential differences among the populations of the same ploidy level, the Scheffé test was applied to the data for hexaploids only (omitting JF17 which had $2n=55$).

We were interested in knowing whether ploidy level was associated with climatic factors. A principal component analysis (PCA) was performed on the climatic variables that characterized the collection sites of each studied population, in relation with the ploidy level. Four climatic variables were obtained from the WorldClim data set (www.worldclim.org) that contains records from 1950 to 2000 with a 2.5-min resolution (Hijmans et al. 2005): annual mean temperature (AnnMT), maximum temperature of the warmest month (MaxTWarmM), minimum temperature of the coldest month (MinTColdM) and annual precipitation (AnnPmm).

Chromosome Preparation

Seeds from different individuals were germinated in Petri dishes at 28°C. After 3 days the root tips were excised from germinated seedlings and treated with 8-hydroxyquinoline solution for 3 h at 16°C and subsequently fixed in fresh ethanol : acetic acid (3 : 1, v/v). It was difficult to obtain good and entire metaphase plates with satisfactory spreading for precise counting of chromosomes when using classical squash techniques. Firstly, the root tips are hard and difficult to squash, and secondly the chromosomes of *C. ciliaris* tend to stick together and overlap. Therefore, we used the air-drying technique of Geber and Schweizer (1987), with slight modifications. After 24h−48h of fixation, root tips were washed in 0.01 M citrate buffer pH 4.6 for 10 min and then incubated in an enzyme mixture: 4 % R10 cellulase (Yakult Honsha Co. Tokyo, Japan), 1 % pectolyase Y23 (Seishin Co., Tokyo, Japan), 4 % hemicellulase (Sigma) for about 25 min at 37°C, depending on the root size. The protoplast suspension was washed with the same buffer, centrifuged twice (4,000 rpm for 5 min) and finally re-fixed in ethanol : acetic acid (3 : 1). The final pellet was resuspended in 50 μl of fixative solution. Protoplasts were dropped on a clean slide and kept at room temperature. A minimum of three individuals per population and about 10 cells per slide were counted.

Estimation of Pollen Viability

The pollen grains were extracted at anthesis and stained with Alexander reagent (Alexander 1969) that colours viable pollen red and aborted pollen green. The percent of viable and non-viable pollen grains was determined for four individuals per ploidy level on the basis of 100 pollen grains per individual.

Results

Genome Size Estimation

The results on nuclear DNA content are presented in Table 2. The 2C DNA value ranged from 3.03 to 4.61 pg and revealed existence of different ploidy levels among populations, confirmed by chromosome counts. One tetraploid population (MR01) with $2n=4x=36$ chromosomes had a mean 2C DNA content of 3.03 pg. Three pentaploid populations with $2n=5x=45$ (ME08, ME05 and ME04) presented 2C DNA contents of 3.63, 3.73, and 3.74 pg, respectively. For the remaining 24 populations which were hexaploid with $2n=6x=54$, their mean 2C DNA content ranged from 4.30 pg to 4.61 pg.

Figure 1 shows the relative nuclear DNA content found in individuals from three populations confirmed to be tetraploid, pentaploid and hexaploid. The mean pentaploid 2C value (3.70 pg) was, as expected, close to the mean (3.76 pg) between the tetraploid (3.03 pg) and the hexaploid populations (mean 4.48 pg). The insert in Fig. 1 represents nuclei from a broader logarithmic scale, and here it is apparent that all three individuals display endoreplication with values up to 8C.

Statistical analysis of 2C DNA content, computed for the whole dataset, predictably showed significant differences between the tetraploid, pentaploids and hexaploid levels (Table 2). No significant differences were observed among the pentaploid populations (indicated by the same letter “b”). Only one significant difference (denoted as “c” and “d”) appeared among the hexaploids: between the aneuploid population Jeffara-17 with the highest 2C DNA content (4.61 pg) and population Sidi Toui-26 with the lowest 2C DNA content (4.30 pg).

The Scheffé test was then applied to the most frequent and widespread hexaploid populations (except JF17 with $2n=55$). This identified two homogeneous groups. The first one contained all populations except Jeffara-12 (with the highest mean of 2C=4.57 pg), and the second one contained all populations except Sidi Toui-26 (with the lowest mean of 2C=4.30 pg). The only significant difference was thus between JF-12 and ST-26 populations.

For unknown reasons the resolution of the nuclear peaks was sometimes suboptimal. Nevertheless the peaks were symmetric and their relative position to the internal standard was particularly stable, the intrapopulational variance being low (mean square being of 0.005 pg). So, nuclear DNA content could be calculated with low variance. We present here these values with the precaution that a future high-resolution analysis might reveal marginal deviation of the absolute values from the present data and could reveal significant differences between populations of the same ploidy level. At any rate, an equivocal identification of the ploidy levels was possible. The monoploid value (1Cx) showed no significant variation.

Table 2 Chromosome number (2*n*), nuclear DNA content (2C) and monoploid genome size (1Cx) in the studied populations of *Cenchrus ciliaris*. *N* − number of flow cytometric measurements

Pop. code	Population	2*n*	DNA ploidy level	2C DNA (pg) ± SD[a]	ANOVA (2C DNA)[b]	Scheffé test of hexaploids[c]	1Cx (pg)	1Cx (Mbp)[d]	*N*	Previous counts 2*n*[e]
MR01	Morneg-01	36	4*x*	3.03±0.03	a	-	0.75	733	8	-
SA02	Sahel-02	54	6*x*	4.56±0.05	cd	A B	0.76	743	6	-
SA03	Sahel-03	54	6*x*	4.55±0.07	cd	A B	0.75	733	5	-
ME04	Mezzouna-04	45	5*x*	3.74±0.09	b	-	0.76	743	11	8*x*=72
ME05	Mezzouna-05	45	5*x*	3.73±0.03	b	-	0.75	733	5	6*x*=54
ME06	Mezzouna-06	54	6*x*	4.34±0.06	cd	A B	0.72	704	5	-
ME07	Mezzouna-07	54	6*x*	4.43±0.09	cd	A B	0.74	723	7	4*x*=36
ME08	Mezzouna-08	45	5*x*	3.63±0.03	b	-	0.73	713	5	-
ME09	Mezzouna-09	54	6*x*	4.55±0.03	cd	A B	0.76	743	5	2*n*=6*x*=54
ME10	Mezzouna-10	54	6*x*	4.46±0.09	cd	A B	0.74	723	7	2*n*=44
JR11	Jerba-11	54	6*x*	4.47±0.04	cd	A B	0.75	733	5	2*n*=6*x*=54
JF12	Jeffara-12	54	6*x*	4.57±0.05	cd	B	0.76	743	5	2*n*=6*x*=54
JF13	Jeffara-13	54	6*x*	4.40±0.07	cd	A B	0.73	713	5	-
JF14	Jeffara-14	54	6*x*	4.49±0.11	cd	A B	0.74	723	11	2*n*=48
JF15	Jeffara-15	54	6*x*	4.50±0.10	cd	A B	0.75	733	5	-
JF16	Jeffara-16	54	6*x*	4.51±0.11	cd	A B	0.75	733	5	2*n*=60
JF17	Jeffara-17	55	6*x*	4.61±0.13	d	-	0.77	753	14	2*n*=8*x*=72
JF18	Jeffara-18	54	6*x*	4.43±0.03	cd	A B	0.74	723	5	-
JF19	Jeffara-19	54	6*x*	4.52±0.10	cd	A B	0.75	733	5	2*n*=6*x*=54
JF20	Jeffara-20	54	6*x*	4.51±0.08	cd	A B	0.75	733	6	2*n*=8*x*=72
JF21	Jeffara-21	54	6*x*	4.46±0.03	cd	A B	0.74	723	5	-
JF22	Jeffara-22	54	6*x*	4.56±0.09	cd	A B	0.76	743	5	2*n*=48
JF23	Jeffara-23	54	6*x*	4.48±0.03	cd	A B	0.75	733	5	-
ST24	Sidi Toui-24	54	6*x*	4.47±0.04	cd	A B	0.75	733	5	-
ST25	Sidi Toui-25	54	6*x*	4.48±0.04	cd	A B	0.75	733	5	-
ST26	Sidi Toui-26	54	6*x*	4.30±0.08	c	A	0.72	704	5	2*n*=48
ST27	Sidi Toui-27	54	6*x*	4.45±0.04	cd	A B	0.74	723	5	-
DH28	Dhibat-28	54	6*x*	4.34±0.04	cd	A B	0.72	704	5	-

[a] − mean value ± standard deviation of *N* measurements; [b] − one-way ANOVA performed on the whole dataset, the same letter indicates no statistical differences, while different letters mean statistical differences at $P<0.05$; [c] − Scheffé test performed on hexaploid populations with 2*n*=54, A and B correspond to two homogeneous groups identified here; [d] −1 pg=978 Mbp (according to Doležel et al. 2003); [e] − Mnif et al. (2005).

Reproductive Mode Analysis

Seed samples of all tetraploid, pentaploid and hexaploid individuals examined by flow cytometry for their mode of reproduction yielded qualitatively the same profiles of histograms (Fig. 2). A dominant peak of 2C somatic embryonic nuclei was coupled

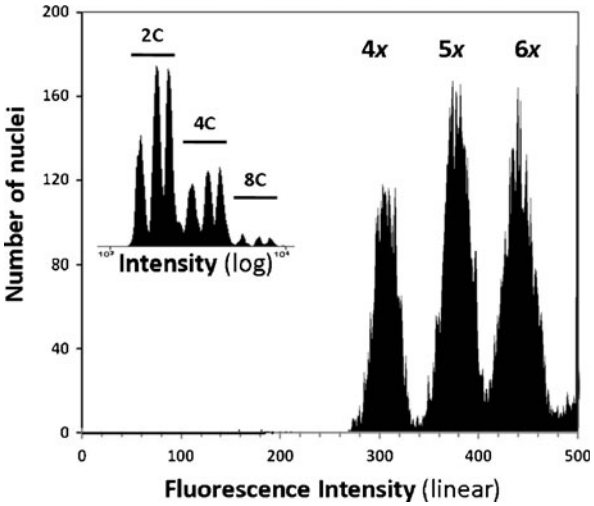


Fig. 1 Flow cytometric histogram of a propidium iodide-stained nuclear suspension prepared by chopping one leaf from each of tetraploid, pentaploid and hexaploid individuals of *Cenchrus ciliaris*. The three peaks correspond to the 2C nuclei; larger ones are off the linear scale. An inset shows data collected on a logarithmic scale. In this case, the 2C, 4C, and 8C nuclei are evident, resulting from endoreplication

with a smaller 3C peak corresponding to endosperm. Additional peaks with duplications of the basic values were also observed, such as 4C and 6C, resulting from endoreduplication of 2C and 3C nuclei, respectively.

Chromosome Counting

The ploidy level of at least 84 individuals was confirmed using chromosome counting in root tip meristems (Table 2). For each individual an average of ten mitotic metaphase plates was scored. For all measured plants, the nuclear DNA content completely agreed with the results obtained by chromosome counting. In this way three ploidy levels were identified. Only one population (MR01) was confirmed as tetraploid ($2n=4x=36$) after chromosome number determination (Fig. 3a). Three populations with mean 2C DNA content of 3.7 pg presented the pentaploid chromosome number $2n=5x=45$ (Fig. 3b). The remaining 24 populations whose DNA content ranged from 4.30 to 4.61 pg were classified as hexaploid with 54 chromosomes (Fig. 3c). Hyper-aneuploidy was also observed for a single population (JF17) with 55 chromosomes (Fig. 3d).

Geographical Distribution of Different Ploidy Levels

The distribution of different cytotypes of *C. ciliaris* in Tunisia is presented in Fig. 4. Hexaploid populations, being the most frequent, were widely distributed from the center to the south of the country, except two populations (SA02 and SA03) located in the northern part (Fig. 4). Despite thorough search in the northern region we found only one population in the extreme north, and this was tetraploid with $2n=4x=36$ (MR01).

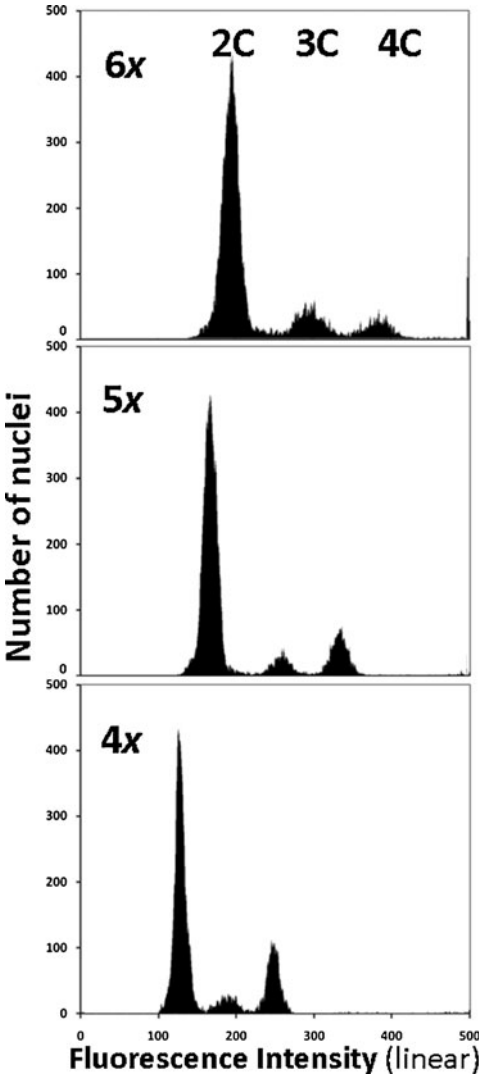


Fig. 2 Flow cytometric histograms of propidium iodide-stained nuclear suspensions prepared by chopping decorticated seeds from hexaploid, pentaploid and tetraploid individuals of *Cenchrus ciliaris* (here without internal standards). In each case, 2C and 4C peaks correspond to embryo tissue and the intermediate 3C peaks are from endosperm tissue. The average frequencies of 2C, 3C and 4C nuclei were 77 %, 9 % and 14 %, respectively. The average coefficient of variation of these peaks was 3.7 %

Pentaploid populations were clustered in the north of the arid region, in the center of Tunisia where some hexaploid populations (ME06, ME07, ME09 and ME10) also occurred.

The principal component analysis (PCA) performed on the climatic variables of the sites of collections (Fig. 5) showed that most hexaploid populations (22 out of 24) were associated with warmer climatic conditions on the positive side of axis 1

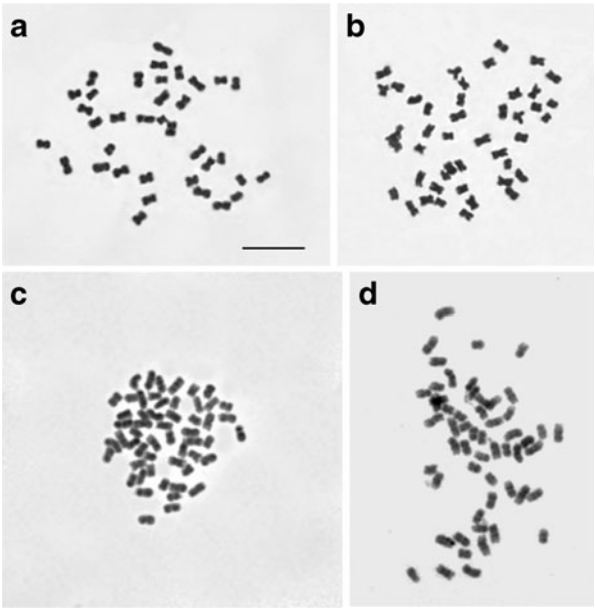


Fig. 3 Metaphase chromosome plates corresponding to different ploidy levels in *Cenchrus ciliaris*. **a** Tetraploid, $2n=4x=36$ (MR01 population); **b** Pentaploid, $2n=5x=45$ (ME08 population); **c** Hexaploid, $2n=6x=54$ (JF14 population); **d** Hyper-aneuploid, $2n=6x+1=55$ (JF17 population). Bar − 10 μm

(Fig. 5b), characterized by high values of thermal variables, annual mean temperature (AnnMT) and maximum temperature of the warmest month (MaxTWarmM) (Fig. 5a). The exceptions were two hexaploid populations (SA02 and SA03) correlated with the negative side of axis 1 (Fig. 5b) representing high values for annual precipitation (AnnPmm) and high values for the minimum temperature of the coldest month (MinTColdM) (Fig. 5b). The occurrence of hexaploids in colder and drier areas suggests that this cytotype can be adapted to different climatic conditions. Only one pentaploid population (ME04) was plotted on the negative side of axis 1, clearly separated from the other two pentaploid populations (ME05 and ME08) situated with hexaploid populations on the positive side of axis 1 characterized by warmer thermal variables. The tetraploid population (MR01) exclusively occupied the most humid area of the country; it was clearly associated with high annual precipitation and higher minimum temperature of the coldest month.

Pollen Viability

Pollen viability was different for the three ploidy levels. The tetraploid cytotype had the highest frequency of viable pollen (78 %), pentaploid had the lowest (65 %), and the hexaploid cytotype showed intermediate frequency (76 %). Reduced pollen viability for pentaploids is probably due to meiotic abnormalities. We observed regular meiotic behavior generally in the tetraploid and hexaploid cytotypes, but found occasional abnormalities in the pentaploid cytotype such as laggard chromosomes in anaphase I (data not shown).

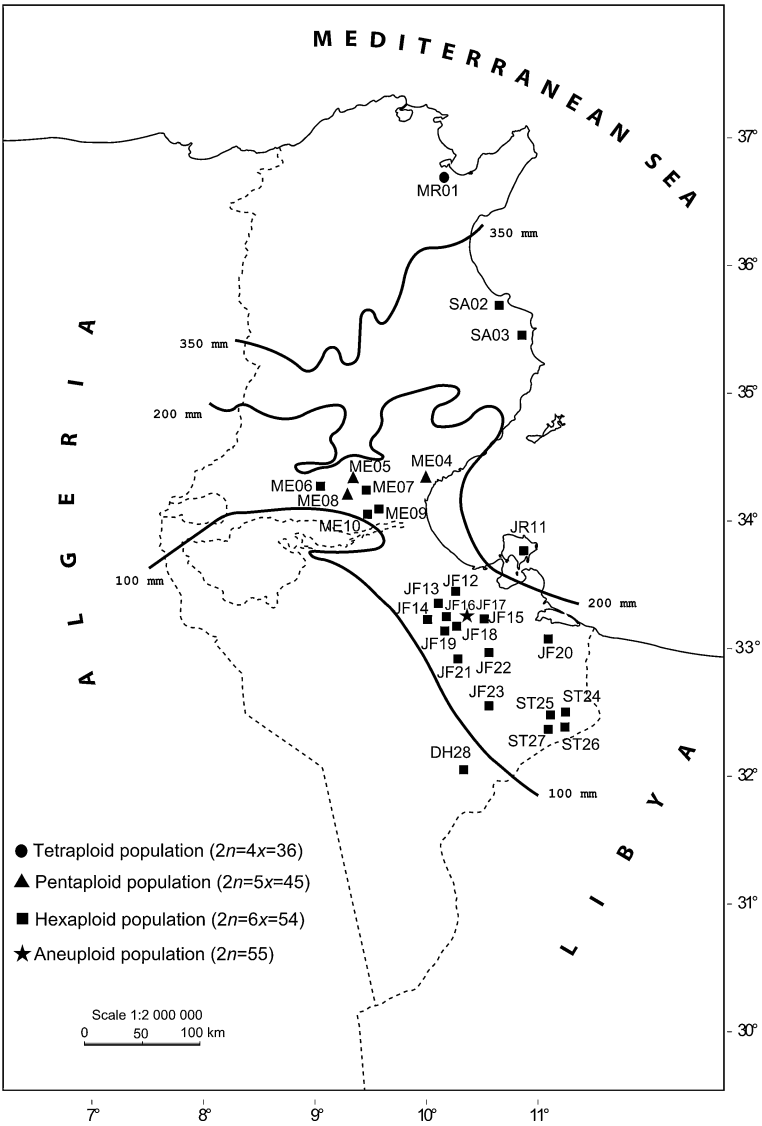


Fig. 4 Geographical distribution of different cytotypes of *Cenchrus ciliaris* in Tunisia. For details on localities, see Table 1

Discussion

Chromosomes Number Variation

In this study three cytotypes were identified in *Cenchrus ciliaris*: the hexaploid level ($2n=6x=54$) was found in 25 populations, the pentaploid one ($2n=5x=45$) in three populations (ME04, ME05 and ME08), and only one tetraploid ($2n=4x=36$) population (MR01) was identified, for the first time in northern Tunisia. Fourteen populations analyzed here were previously also studied by Mnif et al. (2005) (see

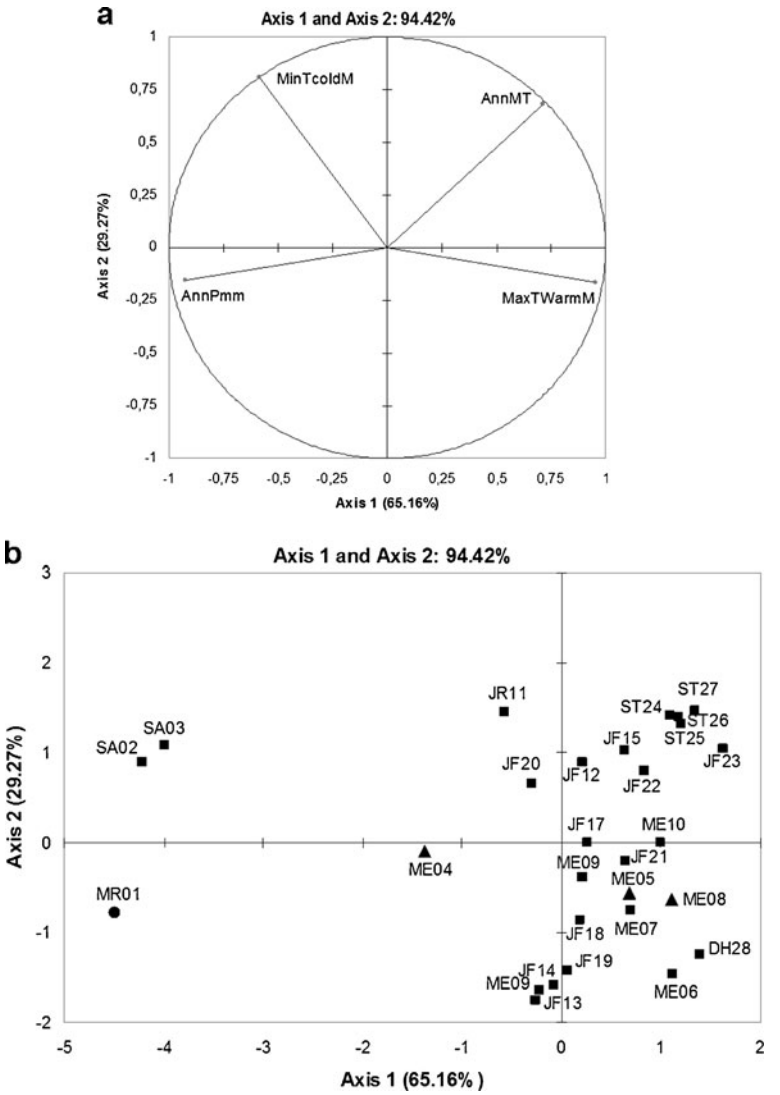


Fig. 5 Principal component analysis (PCA): **a** Correlations and variable weights of climatic variables; **b** Scatter plot of populations: hexaploid cytotype (square), pentaploid cytotype (triangle), tetraploid cytotype (circle). AnnMT – annual mean temperature; MaxTWarmM – maximum temperature of the warmest month; MinTColdM – minimum temperature of the coldest month; AnnPmm – annual precipitation. See Table 1 for population codes

Table 2). Our results concord with these authors in only four cases (ME09, JR11, JF12 and JF19), which were hexaploid (Table 2). For the other populations we found either lower chromosome number and ploidy levels (ME04, ME05, JF16, JF17 and JF20) or higher chromosome number and ploidy levels (ME07, ME10, JF14, JF22 and ST26, Table 2). Our counting was made on complete mitotic cells. To avoid erroneous counts on squash preparations, especially with small chromosomes of hexaploid *C. ciliaris* populations, we used a protoplast dropping technique (Geber and Schweizer 1987). Differences in chromosome number between our results

and those of Mnif et al. (2005) might also be attributed to the fact that the earlier work used meiotic cells for counting. Clearly, it is more robust to determine the chromosome number on root tip meristems than on meiotic cells (Gagnieu 1949; Gervais et al. 1993; Brandes et al. 1997). The formation of univalents and multivalents in meiosis of *C. ciliaris* is frequent (data not shown) and it is difficult to detect which type of multivalent (tri-, tetra- or pentavalents) occurs. For this reason the counting of chromosomes in meiosis for *C. ciliaris* is problematic.

Genome Size Variation

Total DNA content determined by flow cytometry showed intraspecific differences in *C. ciliaris* populations which could, however, be simply attributed to different ploidy levels. Mean DNA content was always in accordance with chromosome number and ploidy level. To our knowledge, this is the first report for genome size of natural pentaploid and hexaploid populations of *C. ciliaris*. However, Burson et al. (2002) analyzed the DNA amount of the $2n+n$ progeny of six populations (tetraploid and pentaploid) of *C. ciliaris* which were self-pollinated and cross-pollinated with Birdwood grass (*Cenchrus setigerus*; $2n=36$) to determine if $2n+n$ fertilization could be increased by early pollination. The genome size of the tetraploid population was estimated at 2C=3.08 pg (Burson et al. 2002), which is in agreement with the present study (2C=3.03 pg). As expected mathematically, the natural pentaploids studied here present 2C=3.7 pg, but this value is lower than that reported by Burson et al. (2002). Our result for hexaploid populations (4.48 pg), again very close to a simple mathematical extrapolation, showed lower values than those reported by Burson et al. (4.76 pg). For the same ploidy level these authors wrote incoherently that five self-pollinated plants had 2C=5.59 pg and were hexaploid: this DNA value would suggest octoploids ($2n=8x=72$) rather than hexaploids ($2n=6x=54$). Moreover, these authors used Buffelgrass (tetraploid *C. ciliaris*) as internal standard with 2C DNA=3.08 pg, which has the same genome size as any tetraploid population. In our study, we started with an internal standard (petunia) valid for pentaploid and hexaploid populations, and took another standard (*Lycopersicon esculentum*) once the tetraploid group was discovered as it was so close to petunia (although petunia nuclei can be discriminated on their light-scattering properties). In general, any standard should have DNA content similar but not equal to that of the sample (Roux et al. 2003).

The phenomenon of genome-downsizing (Leitch and Bennett 2004: decrease in monoploid genome size, 1Cx, according to Greilhuber 2005) is often observed in high polyploids. On the contrary, we did not observe this for *C. ciliaris*. The 1Cx values were similar for tetraploid, pentaploid and hexaploid cytotypes (0.757, 0.746 and 0.745 pg, respectively).

In the latest phylogenetic analysis (Donadío et al. 2009) *C. ciliaris* was included in the same clade as *Pennisetum purpureum* and *Cenchrus setigerus*. These species show differences in basic chromosome number: *P. purpureum* is an allotetraploid with the basic chromosome number $x=7$, while *C. ciliaris and C. setigerus* have a basic chromosome number $x=9$ (Donadío et al. 2009). Unfortunately, the genome size of *Cenchrus setigerus* is unknown. Tetraploid *P. purpureum* ($2n=4x=28$; 2C DNA=4.59 pg) has a much larger genome than tetraploid *C. ciliaris* ($2n=4x=36$; 2C DNA=3.03 pg): *P. purpureum* has fewer but twice larger chromosomes.

Cytogeography of Different Ploidy Levels of Cenchrus ciliaris *in Tunisia*

The distribution of *C. ciliaris* cytotypes in Tunisia seems to follow a north-south bioclimatic gradient. The hexaploid cytotype has a wide ecological amplitude because it is observed from the North African Saharan regions (case of Tunisia), where annual rainfall is below 100 mm, to the semi-arid region where annual rainfall is about 350 mm. The pentaploid populations have a more restricted occurrence, being situated in the center of the country (Mezzouna region near the National Park of Bou Hedma) and particularly in a region where rainfall is between 100 and 200 mm. The occurrence of hexaploids from the semi-arid to the Saharan limits suggests that this cytotype is better adapted to different environmental conditions. This is reflected in the current adaptive capacity of *C. ciliaris* to various habitats worldwide (Arriaga et al. 2003). Indeed, the results of the principal component analysis (PCA) performed on the climatic variables of the collection sites (Fig. **5**) indicate that the hexaploid cytotype can be adapted to different climatic conditions. Although no single strong correlation emerges between ploidy level and a climatic factor, hexaploid were most frequent in the warmer climatic conditions.

During our field investigations we noted that the tetraploid population was morphologically different from the other ploidy levels in terms of its purple inflorescence, in contrast to the other populations that had yellowish inflorescence. Recently we measured several phenotypic traits associated with productivity in a common garden environment to study the morphological variability between pentaploid and hexaploid plants of diverse provenance (Kharrat-Souissi et al. 2011). A considerable and significant variation was observed according to provenance for the morphological traits without, however, any relation to ploidy levels (Kharrat-Souissi et al. 2011).

The existence of pentaploid cytotype suggests hybridization ability between tetraploids and hexaploids. The pentaploids are morphologically more similar to hexaploids than to tetraploids. Although most *C. ciliaris* populations are obligate apomicts (Fisher et al. 1954; Snyder et al. 1955), sexual forms have also been reported (Bray 1978; Sherwood et al. 1980). Accordingly, we recently observed differences among individuals descending from the same mother plant for the Tunisian *C. ciliaris* by using AFLP markers (Kharrat-Souissi et al. 2011). This unexpected level of variability for an apomictic species suggests that the sexual mode of reproduction is not rare in native populations of *C. ciliaris*.

Mode of Reproduction

The apomictic mode of reproduction known as apospory is displayed by most *Cenchrus ciliaris* genotypes, but rare sexual individuals have been identified (Bashaw 1962; Bray 1978). The pseudogamous *Cenchrus ciliaris* forms an embryo sac of the Panicum type with only four unreduced nuclei at maturity (Snyder et al. 1955), as does *Panicum maximum* Jacq. (Warmke 1954). Matzk et al. (2000) concluded that for facultative apomictic species like *C. ciliaris* and *P. maximum*, flow cytometric seed screening (FCSS) could not differentiate sexual from apomictic genotypes. As the endosperm cells of both the aposporous and sexual plants yield 3C values, our observations using FCSS (high 2C embryo peak with a smaller 3C endosperm peak,

Fig. 2) do not clarify the reproductive mode of the investigated individuals from native Tunisian *C. ciliaris* populations. As expected, the distinctive obligate apomictic profile with 2C embryo and 5C endosperm DNA peak was never observed in our work, but both sexual and apomictic modes probably coexist. The traditional cytological technique of dissecting immature ovaries would appear more suitable to detect aposporous apomixis in the case of *C. ciliaris*, because the aposporous plants of the Panicum type, which produce a 4-nucleate sac(s) per ovule, can be easily differentiated from sexually reproducing plants that display a solitary 8-nucleate sac.

Concluding Remarks

Our cytogenetic analyses of native *C. ciliaris* populations shed light on the evolutionary and phytogeographic history of this species in arid regions of Tunisia. According to our investigations of nuclear DNA amounts and determination of chromosome number, the following conclusions can be reached. The hexaploid populations ($2n=6x=54$) with the highest 2C DNA value (4.48 pg) were in general the most frequent and are particularly representative in the southern region of Tunisia. It seems that individuals of this ploidy level can adapt to different conditions, especially arid environments. Consequently, this cytotype would seem best for the restoration of the degraded habitats in arid areas rather than the other ploidy levels. One tetraploid ($2n=4x=36$; 2C=3.03 pg) population occurred uniquely in the north, and pentaploids were only in the center. Flow cytometric seed screening could not discriminate between sexual and apomictic reproduction in our case.

The present results indicate that cytogenetical analyses should be broadened, involving more populations of all three ploidy levels, especially for the seemingly rare tetraploids. In this way it would be possible to elucidate to what extent the Tunisian populations remain microgeographically differentiated in overlapping zones of two ploidy levels as is the case for hexaploid and pentaploid populations. Thereafter, a solid explanation of the interpopulation differentiation in general and of the intrapopulation variation of genome size and chromosome number (aneuploidy) could be developed. The hypothesis of hybridization ability between tetraploids and hexaploids to give rise to a pentaploid cytotype should also be verified by laboratory crosses between these two ploidy levels.

These cytogenetic approaches should improve our understanding of the origin and phytogeographic significance of ploidy level changes as well as the relationships between the Tunisian and other Mediterranean populations of *C. ciliaris*.

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References

Alexander MP (1969) Differential staining of aborted and non-aborted pollen. *Biotech Histochem* 62:107–112

Arriaga L, Castellanos AE, Moreno E, Alarcocon J (2003) Potential ecological distribution of Alien invasive species and risk assessment: A case study of buffel grass in arid regions of Mexico. *Conservation Biol* 18:1504–1514

Aronson J, Floret C, Le Floc'h E, Ovalle C, Pontanier R (1993) Restoration and rehabilitation of degraded ecosystems in arid and semi-arid lands. II. Case studies in Southern Tunisia, Central Chile and Northern Cameroon. *Restor Ecol* 1:168–87

Bashaw EC (1962) Apomixis and sexuality in buffelgrass. *Crop Sci* 2:412–415

Bashaw EC, Hignight KW (1990) Gene transfer in apomictic Buffelgrass through fertilization of an unreduced egg. *Crop Sci* 30:571–575

Belkhodja K, Bortoli L, Cointepas JP, Dimanche P, Fournet A, Jaquinet JC, Mori A (1973) *Carte pédologique de la Tunisie 1/500 000. Sol de Tunisie n°5*. République Tunisienne, Ministère de l'Agriculture, Tunis

Bogdan AV (1977) *Tropical pasture and fodder plants (Grasses and legumes)*. Longman, London

Bousquet B (1992) *Guide des parcs nationaux d'Afrique (Afrique du Nord, de l'Ouest)*. Ed. Delachaux et Niestlé, Paris

Brandes A, Heslop-Harrison JS, Kamm A, Kubis S, Doudrick RL, Schmidt T (1997) Comparative analysis of the chromosomal and genomic organization of *Ty*1-*copia*-like retrotransposons in pteridophytes, gymnosperms and angiosperms. *Pl Molec Biol* 33:11–21

Bray RR (1978) Evidence for facultative apomixis in *Cenchrus ciliaris*. *Euphytica* 27:801–802

Burson BL, Hussey MA, Actkinson JM, Shafer GS (2002) Effect of pollination time on the frequency of 2n +n fertilization in apomictic Buffelgrass. *Crop Sci* 42:1075–1080

Chaieb M, Henchi B, Boukhris M (1996) Impact of clipping on root systems of 3 grasses species in Tunisia. *J Range Managem* 49:336–339

Clayton WD, Renvoize SA (1982) *Cenchrus*. In Polhill RM (ed) *Flora of Tropical East Africa (part 3)*. A.A. Balkema, Rotterdam, pp 691–695

Clayton WD, Renvoize SA (1986) *Genera Graminum: Grasses of the World*. Kew Bulletin Additional Series 13, HMSO, London

Correll DS, Johnston MC (1970) *Manual of the vascular plants of Texas*. Texas Research Foundation, Renner, Texas

Crins WJ (1991) The genera of Paniceae (Gramineae: Panicoideae) in the Southeastern United States. *J Arnold Arbor Suppl* 1:205–220

Cuénod A, Pottier-Alapetite G, Labbe A (1954) *Flore analytique et synoptique de la Tunisie : cryptogames vasculaires, gymnospermes et monocotylédones*. SEFAN, Tunis

Daehler CC, Goergen EM (2005) Experimental restoration of an indigenous Hawaiian grassland after invasion by buffel grass (*Cenchrus ciliaris*). *Restor Ecol* 13:380–389

DeLisle DG (1963) Taxonomy and distribution of the genus *Cenchrus*. *Iowa State J Sci* 37:259–351

Doležel J, Bartoš J, Voglmayr H, Greilhuber J (2003) Nuclear DNA content and genome size of trout and human. *Cytometry* 51:127–128

Donadío S, Giussani LM, Kellogg EA, Zuloaga FO, Morrone O (2009) A preliminary molecular phylogeny of *Pennisetum* and *Cenchrus* (Poaceae-Paniceae) based on the trnL-F, rp116 chloroplast markers. *Taxon* 58:392–404

Fisher WD, Bashaw EC, Holt EC (1954) Evidence for apomixis in *Pennisetum ciliare* and *Cenchrus setigerus*. *Agron J* 46:401–404

Franklin KA, Lyons K, Nagler PL, Lampkin D, Glenn EP, Molina-Freaner F, Markow T, Huete AR (2006) Buffelgrass (*Pennisetum ciliare*) land conversion and productivity in the plains of Sonora, Mexico. *Biol Conservation* 127:62–71

Gagnieu A (1949) *L'observation des chromosomes*. SEDES, Paris

Galbraith D, Harkins K, Maddox J, Ayres N, Sharma D, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 220:1049–1051

Geber G, Schweizer D (1987) Cytochemical hetero-chromatin differentiation in *Sinapis alba* (Cruciferae) using a simple air-drying technique for producing chromosome spreads. *Pl Syst Evol* 158:97–106

Gervais C, Trahan R, Moreno D, Drolet AM (1993) Le *Phragmite australis* au Québec: distribution géographiques, nombres chromosomiques et reproduction. *Canad J Bot* 71:1386–1393

Greilhuber J (2005) Intraspecific variation in genome size in angiosperms: Identifying its existence. *Ann Bot* (*Oxford*) 95:91–98

Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A (2005) Very high resolution interpolated climate surfaces for global land areas. *Int J Climatol* 25:1965–1978

Jackson J (2005) Is there a relationship between herbaceous species richness and buffel grass (*Cenchrus ciliaris*)? *Austral Ecol* 30:505–517

Kharrat-Souissi A, Baumel A, Torre F, Juin M, Siljak-Yakovlev S, Roig A, Chaieb M (2011) New insights into the polyploid complex *Cenchrus ciliaris* L. (*Poaceae*) show its capacity for gene flow and recombination processes despite its apomictic nature. *Austral J Bot* 59:543–553

Le Houérou HN (1993) Grazing lands of the Mediterranean Basin. In Coupland RT (ed) *Natural grasslands: Eastern Hemisphere*. Elsevier, Amsterdam, pp 197–220

Leitch IJ, Bennett MD (2004) Genome downsizing in polyploidy plants. *Biol J Linn Soc* 82:651–663

Marie D, Brown SC (1993) A cytometric exercise in plant DNA histograms, with 2C values for seventy species. *Biol Cell* 78:41–51

Martel E, Poncet V, Lamy F, Siljak-Yakovlev S, Lejeune B, Sarr A (2004) Chromosome evolution of *Pennisetum s*pecies (Poaceae): implications of ITS phylogeny. *Pl Syst Evol* 249:139–149

Matzk F, Meister A, Schubert I (2000) An efficient screen for reproductive pathways using mature seeds of monocots and dicots. *Plant J* 21:97–108

Mganga KZ, Musimba NKR, Nyangito MM, Nyariki DM, Mwang'ombe AW, Ekaya WN, Muiru WM, Clavel D, Francis J, Kaufmann R, Verhagen J, Muiru WM (2010) Dry matter yields and hydrological properties of three perennial grasses of semi-arid environment in east Africa. *Afr J Pl Sci* 4:135–141

Mnif L, Mseddi K, Chaieb M (2005) *Cenchrus ciliaris* L. en Tunisie aride: nombre chromosomiques, aptitudes germinatives et reproductives de quelques populations. *Acta Bot Gallica* 152:45–56

Mseddi K, Mnif L, Chaieb M, Neffati M, Roux M (2004) Aboveground phytomass productivity and morphologic variability of Tunisian populations of *Cenchrus ciliaris* L. *Afr J Range Forage Sci* 19:13–20

Mseddi K, Visser M, Neffati M, Reheul D, Chaieb M (2002) Seed and spike traits from remnant populations of *Cenchrus ciliaris* L. in South Tunisia: high distinctiveness, no ecotypes. *J Arid Environm* 50:309–324

Ozias-Akins P, Akiyama Y, Hanna WW (2003) Molecular characterization of the genomic region linked with apomixis in *Pennisetum*/*Cenchrus*. *Funct Integr Genomics* 3:94–104

Roux N, Tolaza A, Radecki Z, Zapata-Arias FJ, Doležel J (2003) Rapid detection of aneuploidy in *Musa* using flow cytometry. *Pl Cell Rep* 21:483–490

Sherwood RT, Young BA, Bashaw EC (1980) Facultative apomixis in buffelgrass. *Crop Sci* 20:375–379

Snyder LA, Alice RH, Warmke, HE (1955) The mechanisms of apomixis in *Pennisetum ciliare*. *Bot Gaz* 116:209–221

Stieber MT, Wipff JK (2003) *Cenchrus* L. In Barkworth ME et al. (eds) *Flora of North America north of Mexico, vol. 25, Magnoliophyta: Commelinidae (in part): Poaceae, part 2*. Oxford University Press, New York, Oxford, pp 529–535

Vielle JP, Burson BL, Bashaw EC, Hussey MA (1995) Early fertilization events in the sexual and aposporous egg apparatus of *Pennisetum ciliare* (L.) Link. *Plant J* 8:309–316

Vielle-Calzada JP, Nuccio ML, Budiman MA, Thomas TL, Burson BL, Hussey MA, Wing RA (1996) Comparative gene expression in sexual and apomitic ovaries of *Pennisetum ciliaris* (L.) Link. *Pl Molec Biol* 32:1085–1092

Visser N, Morris C, Hardy MB, Botha JC (2007) Restoring bare patches in the Nama-Karoo of Southern Africa. *Afr J Range Forage Sci* 24:87–96

Visser M, Mseddi K, Chaïeb M, Neffati M (2008) Assessing yield and yield stability of remnant populations of *Cenchrus ciliaris* L. in arid Tunisia: developing a blueprint for initiating native seed production. *Grass Forage Sci* 63:301–313

Warmke HE (1954) Apomixis in *Panicum maximum*. *Amer J Bot* 41:5–11

Watson L, Dallwitz MJ (1992) *The grass genera of the world*. CAB International, Wallingford

Wieners RR, Fei S, Johnson RC (2006) Characterization of a USDA Kentucky bluegrass (*Poa pratensis* L.) core collection for reproductive mode and DNA content by flow cytometry. *Genet Res Crop Evol* 53:1531–1541

Ziegler AD, Warren SD, Perry JL, Giambelluca TW (2000) Reassessment of revegetation strategies for Kaho'olawe Island, Hawai'i. *J Range Managem* 53:106–113

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