A single species, two basic chromosomal numbers: case of *Lygeum spartum* (Poaceae)

¹Abdeddaim-Boughanmi Katia, ²Garnatje Teresa, ²Vitales Daniel, ³Brown Spencer C., ¹Harche-Kaïd Meriem, ^{4*}Siljak-Yakovlev Sonja

¹ Université des Sciences et de la Technologie d'Oran Mohamed Boudiaf, BP 1505, El M'Naouer Oran 31000, Algérie. Laboratoire des productions végétales et valorisation microbienne

²Institut Botànic de Barcelona (IBB, CSIC-ICUB). Passeig del Migdia s/n, 08038 Barcelona, Catalonia, Spain

³Institute of Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Saclay, 91198, Gif-sur-Yvette, France

⁴Ecologie Systématique Evolution, Univ. Paris-Sud, CNRS, AgroParisTech, Université Paris-Saclay, 91400, Orsay, France

*Corresponding authors sonia.yakovlev@u-psud.fr

Abstract

The existence of two chromosome numbers ($2n=16$ and $2n=40$) in *Lygeum spartum* is confirmed in the Algerian steppe populations of Oran region. Chromosome counts were established for 11 Algerian populations and three supplementary populations from Italy, Spain and Greece. The karyotypes were characterized by chromosome markers obtained using fluorescence *in situ* hybridization and fluorochrome bandings. The organization of 5S and 18S-5.8S-26S (35S) rDNA was studied in both cytotypes. Six signals of 35S and 2 signals of 5S were observed in $2n=16$ population, while 10 signals of 35S and 4 signals of 5S were detected in the population with $2n=40$. All 35S loci were also strongly marked by chromomycin, but negatively stained by Hoechst, which indicates the presence of GC rich DNA in rDNA regions. The B chromosomes were found in both cytotypes, bearing a 35S locus in $2n=16$ population. Genome size, determined by cytometry of 10 populations, ranged from 9.27 pg for $2n=16$ to 26.63 pg for $2n=40$ populations. The sequencing of plastid and nuclear DNA markers did not reveal major differences among $2n=16$ and $2n=40$ populations. However, given the differences between two cytotypes and based on their morphological and cytogenetic characteristics, the $2n=16$ cytotype merits novel taxonomic treatment.

Kew words: B chromosomes, basic chromosome number, DNA sequencing, fluorochrome banding, genome size, *Lygeum spartum*, Poaceae, 35S-5.8S-26S and 5S rDNA

Introduction

The genus *Lygeum* L. (Poaceae, sub-family Pooideae, tribes *Nardeae* W.D.J.Koch or *Lygeae* J.Presl (following Schneider et al. 2009 and Soreng et al. 2017, respectively) is monospecific (Quézel & Santa 1962; Clayton & Renvoize 1986; Döring et al. 2007; Kellogg 2015), constituted by only species *Lygeum spartum* L. that is considered as a Mediterranean taxon (Hochreutiner 1904), growing spontaneously in North Africa, in the South of Spain and Portugal (Graells 1876; Pinto da Silva 1976), up to the South of Italy (Maire 1953) and of Greece (Brullo et al. 2002). In North Africa, *L. spartum* extends from Morocco to Egypt (Maire 1953; Quézel & Santa 1962).

Preliminary observations on two Algerian populations (Benmansour & Harche-Kaid 2001; Abdeddaïm-Boughanmi & Kaid-Harche 2009) revealed the existence of two cytotypes with different ploidy levels: $2n=2x=16$ and $2n=4x=40$. The $2n=40$ has already been described in Egypt (Ramanujam 1938; Amin 1972) and in Spain (Lorenzo-Andreu & García-Sanz 1950; Abdeddaïm-Boughanmi & Kaid-Harche 2009; Schneider et al. 2011). On the other hand, the cytotype $2n=16$ was found for the first time in the south-west Oran region (Benmansour & Harche-Kaid 2001; Abdeddaïm-Boughanmi & Kaid-Harche 2009). The finding of this very unusual chromosome number for the genus *Lygeum* deserves more thorough investigation.

Although both polyploidy and the existence of different base chromosome numbers, together with hybridization, are phenomena that have occurred frequently during the evolution of grasses (Hilu, 2004), we think that the finding of two different chromosome numbers within the same species deserves to be studied in depth, and even more if this could implicate the existence of different basic chromosome numbers.

In the present study, we aim to finely analyze two cytotypes of *Lygeum spartum* in order to establish their relationships by using the classic and molecular cytogenetic techniques

and molecular phylogeny. For this purpose, we have explored genome size variation, heterochromatin and rDNA distribution patterns in both cytotypes. In addition, genome size variation has been examined related to basic chromosome number and in relation to our earlier determinations of pollen grain size (Abdeddaim-Boughanmi & Kaid-Harche 2009). The main question has become: what is the evidence for two clearly differentiated taxa?

Materials and methods

Plant material and origin of samples

Plant material (leaves and seedlings) was collected in wild populations or was obtained from botanical gardens. The geographical origin of collected material is given in Table 1 and Figure 1. Populations from High Plateaus (highlands) steppes grow on silt-laden sandy soil, in an arid climate with harsh winter and hot summer. Littoral populations grow in semi-arid temperate climate conditions on coastal Oran region. Herbarium vouchers of the studied populations are deposited in the Herbarium of « Laboratoire des productions et valorisations végétales et microbiennes », Université des Sciences et de la Technologie d'Oran-Mohamed Boudiaf, and in the herbarium of the Botanical Institute of Barcelona [numbers BC-990301 (El-Keither) and BC-990302 (Ain Benkhelil 2') for diploid and polyploid cytotypes, respectively].

Chromosome preparation

For mitotic chromosome analysis root-tip meristems, obtained from seed germinations on moist filter paper in Petri dishes at constant temperature of 24°C in thermostat, were pretreated with 0.002 M 8-hydroxyquinolin for 4.5h at 16°C. Fixation was performed in 3/1

(v/v) ethanol/acetic acid at least 24-48 h. After hydrolysis in 1N HCl for 10 min at 60°C and staining in Schiff's reagent following standard Feulgen method (Feulgen & Rossenbeck 1924), the squash was performed in a drop of acetic carmine.

For fluorochrome banding and FISH experiments, protoplast dropping technique was used to obtain metaphase chromosomes (Geber & Schweizer 1988). Protoplasts were dropped on a clean slide and kept at room temperature for different staining techniques.

Karyotype analysis

Karyotype analyses were based on the measurement of length of long and short chromosome arms. The chromosomes were measured on five well spread metaphase plates for one population with $2n=16$ and two populations (one Algerian and one Italian) with $2n=40$. The chromosome types were characterized according to Levan *et al.* (1964) nomenclature. Idiograms were drawn from mean values of long and short arms for each chromosome pair.

Fluorochrome banding

GC-specific staining with chromomycin A₃ (CMA) was performed following Schweizer (1976) with minor modifications (Siljak-Yakovlev *et al.* 2002) concerning the concentration of chromomycin (0.2 mg/ml, Sigma) and time of staining (60 min). Dystamicin A₃ staining was avoided because it did not improve CMA staining. The slides were counterstained with methyl green (0.1% in McIlvaine's pH 5.5 buffer) for 10 min, rinsed in this buffer and mounted in Citifluor AF1 anti-fade agent (Agar Scientific, Stansted, Essex, UK). After microscopic observation and taking photographs, the best slides were destained in 2xSSC buffer, dehydrated in a graded ethanol series (70%, 90% and 100%) and used for Hoechst staining and FISH experiment.

AT-specific staining with bisbenzimidazole Hoechst 33258 (Ho) was carried out according to Martin and Hesemann (1988) with minor modification: slides were mounted in Citifluor AF1 (Agar Scientific, Stansted, Essex, UK).

Fluorescence in situ hybridization (FISH)

Double FISH experiment was carried out with two DNA probes: 18S-5.8S-26S (35S) rDNA probe was a clone of a 4 kb *Eco*RI fragment containing a part of 18S, 5.8S and 26S rDNA of *Arabidopsis thaliana* labelled with Digoxigenin-11-dUTP (Roche Diagnostics GmbH), and pTa 794 probe was a clone containing a 410-pb *Bam*HI fragment of 5S from wheat direct labelled with Cy3 (Amersham, Courtaboeuf, France). In the tetraploid cytotype, the 5S signals were only visible after the use of probe pTa 794 labelled with digoxigenin-11-dUTP. This is the reason why we used fluorescent labelling of the probes opposite to that for the diploid cytotype. Hybridization was performed following the Heslop-Harrison et al. (1991) technique with minor modifications. The slides were finally mounted in Vectashield mounting medium with DAPI (VECTOR).

Chromosome plates were observed using an epifluorescence Zeiss Axiophot microscope with different combinations of Zeiss excitation and emission filter sets (01, 07, 15). Hybridization signals were analysed using the highly sensitive CCD camera RETIGA 2000R (Princeton Instruments, Evry, France) and image analyser (MetaVue, Evry, France).

Estimation of nuclear DNA content and base composition by flow cytometry

The total DNA amount was assessed by flow cytometry using *Triticum aestivum* cv. Chinese spring (2C= 30.9 pg, 43.7% GC) as an internal standard according to a technique of Marie & Brown (1993).

DNA content of 5,000-10,000 stained nuclei was determined for each sample using an Elite ESP flow cytometer (Beckman-Coulter, Roissy, France) with a water-cooled argon laser. Total 2C DNA value was calculated using the linear relationship between the fluorescent signals from stained nuclei of sample and those of internal standard. Base composition (GC percentage) was calculated for two populations (one for each cytotype) using the nonlinear model established by Godelle et al. (1993). Each studied population comprised at least five individuals, measured separately and with repetition.

DNA extraction, amplification, sequencing strategies and analyses

Total genomic DNA was extracted following the CTAB method (Doyle & Doyle, 1987) with slight modifications (Soltis et al. 1991, Cullings, 1992), from silica gel dried leaves collected in the field. Nuclear rDNA (ITS) region was amplified and sequenced for six individuals belonging to three polyploid populations and one individual from the diploid population. One individual from each of these populations was sequenced for the trnL-F region from cpDNA (see Appendix 1 for the origin of populations and GenBank accession numbers).

Direct sequencing of the amplified DNA segment was performed using the Big Dye Terminator Cycle sequencing v3.1 (PE Biosystems, Foster City, California, U.S.A.). Nucleotide sequencing was carried out on an ABI PRISM 3700 DNA analyzer (PE Biosystems, Foster City, California, U.S.A.).

DNA sequences were edited by Chromas v.2.6.4 (Technelysium PTy, Tewantin, Queensland, Australia) and Bioedit v.7.0.9 (Ibis Biosciences, Carlsbad, California, USA) and aligned visually. The aligned matrix is available from the corresponding author. Nucleotide polymorphisms from ITS sequences were codified following IUPAC nomenclature. Taking into account the polymorphisms, the ITS matrix was analysed using SplitsTree v. 4.6 (Huson

& Bryant, 2006) to construct a Neighbor Net (NN) handling polymorphic sites as average states of the characters.

Results

Chromosome number and karyotype features

Two chromosome numbers were observed. Only two populations (restricted to Algerian High Plateau) present $2n=16$ (Figure 2A), while the 11 other studied populations possessed from $2n=36$ to $2n=40$ chromosomes (Table 2, Figures 2B and 2C and supplementary Figure, S1). In all polyploid populations the inter- and intra-individual variability of chromosome number were observed. The Italian population from Calabria presented mainly $2n=40$ except some rare aneuploid metaphases with $2n=38$ (Figure 2C). The presence of B chromosomes (Figure 2A and 2C, arrows) was confirmed in both cytotypes.

The detailed morphometric karyotype analyses were performed in order to compare the two cytotypes from same geographical area. The $2n=16$ karyotype is composed of 6 m and 2 sm chromosome pairs and chromosome length ranged from 6.04 to 8.15 μm (Figure 3F). The cytotype $2n=40$ presents 11 m and 9 sm chromosome pairs, and chromosome length ranges from 2.54 to 6.39 μm for littoral Oran population Sebkha (Figure 3G). For the Italian population, chromosome length ranged from 3.53 to 8.21 μm . This difference between two tetraploid populations could nevertheless be due to different level of chromosome condensation.

Genome size and base composition

In this study nuclear genome size was estimated for two populations of $2n=16$ and 7 populations of $2n=40$ cytotype (Table 2). Nuclear 2C DNA content for the first cytotype

range from 9.27 to 10.74 pg and for the second cytotype from 19.75 to 21.53 pg. The mean monoploid genome size (1Cx) was 4892 Mbp for diploid and 5203 Mbp for tetraploid cytotype. The statistical analysis (ANOVA test) shows highly significant ($p < 0.001$) differences between two cytotypes, however, among the seven populations of $2n=40$ cytotype, 2C DNA values were not significantly different (Table 2). Likewise, no significant differences in DNA content were observed between measurements obtained from stems, leaves and roots, or at different seasons. This result reinforces the reliability of our results. The base composition (GC %) was 43.93% in diploid and 42.95% in polyploid cytotype, but this difference was not significant.

GC and AT rich DNA regions; localisation and organization of rRNA genes

Chromomycin revealed positive bands (CMA^+) on three chromosome pairs and on B chromosome in the population of El-Kheiter $2n=16$ (Figure 3A₁), and on five pairs of chromosomes in the population of Sebkha $2n=40$ (Figure 3C).

Hoechst staining demonstrate no positive bands in either cytotypes. In each case, all CMA^+ bands appeared Hoechst-negative or very weakly stained which confirmed they were GC-rich (Figures 3A₂ and 3D, arrows).

The number of 35S and 5S rDNA loci was different in two studied cytotypes. The population of El-Kheiter ($2n=16$) presents seven 35S rDNA signals (six on chromosomes A and one on the chromosome B) and two 5S rDNA signals on the long arms of chromosome pair 3 (Figures 3A₃, 3B and 3F). The population of Sebkha ($2n=40$) showed ten 35S and four 5S rDNA signals (Figures 3E and 3G). In both cytotypes all 35S signals are located at telomeric region and co-localised with CMA^+ bands.

Diploid cytotype placement

The ITS matrix included 659 aligned positions, 16 of them showing intragenomic polymorphisms, being the only variability observed in this region. No variability among populations or cytotypes was observed in trnL-F region, which included 565 aligned positions. Figure 4 shows the NN obtained from ITS analysis.

Our results show the inclusion of the diploid cytotype amongst the tetraploid cytotypes. In NN reconstruction, diploid cytotype appears closely related with individuals from Sebkha population (Oran West). The individuals of populations from Oran East and Alicante cluster in two distinct groups according to their geographic origin (Figure 4).

Discussion

Chromosome number in Lygeum spartum

In our sample *Lygeum spartum* was characterized by two chromosome numbers which also reflected two very different basic chromosome numbers: $x=8$ for diploid $2n=16$ and $x=10$ for tetraploid $2n=40$. Indeed, this last cytotype presented intra-population and even intra-individual chromosome number variation causing by aneuploidy phenomenon. Losses of two or four chromosomes have been observed ($2n=40-2$ to 4 chromosomes). The $2n=16$ was reported for the first time by Benmansour & Harche-Kaid (2001) from the population growing in the region of Ain-Benkhelil on the Algerian High Plateau. These numbers were also verified in the pollen mother cells (Abdeddaim-Boughanmi & Kaid-Harche 2009; Abdeddaim-Boughanmi 2010), confirming that the chromosome number in metaphase I was $n=8$ for the diploid and $n=20$ for the polyploid cytotype. The hypo-aneuploid chromosome number (mainly $2n=36$ and $2n=38$) was probably maintained by vegetative reproduction. Sexual reproduction through a regular meiosis established and stabilized the $2n = 40$ chromosomes.

The chromosome number $2n=40$ has been found in numerous populations of the Mediterranean region (Ramanujam 1938; Lorenzo-Andreu & García-Sanz 1950; Amin 1972; Abdeddaim-Boughanmi & Kaid-Harche 2009; Schneider et al. 2011), while $2n=16$ was observed, until now, only in two Algerian populations from High Plateau.

The genus *Lygeum* has been consistently taken as monospecific (*L. spartum*) with $x=10$. It is all the more curious to find the existence of another very close taxon with a base number so different ($x = 8$). On the basis of this important characteristic, it is evident that these two cytotypes have reached a high level of differentiation. At this time, it is difficult to explain the existence of two such different basic chromosome numbers in the small *Lygeum* genus. Nevertheless, we will try to emit some hypotheses based on reflections of other authors who are interested in the evolution of basic chromosome number in the family of Poaceae (Hilu 2004, Schneider et al. 2011).

The base number $x = 11$ is considered ancestral in the family of Poaceae by Hilu (2004). Schneider et al. (2011) indicated that early diverging lineages of Pooidea have higher basic chromosome number than the terminal lineages as tribes *Triticeae* and *Aveneae*. Indeed, in the tribe *Nardeae* (including *Lygeae*), which is considered as early diverging lineages, two high basic chromosomes numbers are present: $x=13$ for *Nardus stricta* and $x=10$ for *Lygeum spartum*.

On the basis of our results, two hypotheses could be proposed regarding basic number in *Lygeum*:

1. Ancestral basic chromosome number $x=10$ could be occurred in ancestor like *Lygeum* in the region of Algerian High Plateau. Two major events could occur. On the one hand, after the chromosome duplication (polyploidy event), the tetraploid ($2n=40$) was spread in all Mediterranean. On the other hand, the diploid goes through several events of aneuploidy (decreasing dysploidy) up to $x=8$.

2. After a possible hybridization event between two taxa, one with $x=8$ and another with $x=12$, the hybrid doubling his chromosome set and become a taxon with $2n=40$ and $x=10$. This hypothesis seems less likely because it is not clear which species may have been the parents of the hybrid.

If the diploid had undergone several aneuploidy events, according to the $2C$ values, it did not lose a lot of nuclear DNA because its $1Cx$ is slightly smaller than that of tetraploid. That is to say, it has undergone numerous chromosomal rearrangements without deletion of parts of chromosomes.

However, we cannot rule out Stebbins' hypothesis (Stebbins 1982, 1985) that the basic chromosome number could be $x=5$ and that derived $x=10$ was already a paleopolyploid. In this case the loss of two pairs of chromosome would be less problematic than if it was the case in a diploid.

Although *Lygeum spartum* is a species traditionally used in the rope making and handicraft supplies (Halmy, M.W.A., 2015) among others, as fodder for livestock and also for the stabilization of dunes, we have no evidence of anthropogenic origin by domestication of the cytotype $2n = 40$ since the majority of populations occurring in its distribution area present this ploidy level.

Genome size variation

The only previous nuclear DNA assessment in the genus *Lygeum* gave $2C=27.60$ pg for a $2n=40$ *L. spartum* population (Bennett & Leitch 1995). That genome size was determined by Feulgen cytodensitometry and our present estimate is 26% lower.

According to the genome size classifications proposed by Leitch et al. (1998), both cytotypes belong to the category of intermediate ($3.5 \leq 14$ pg) $1C$ -values.

The DNA content was correlated with the chromosomes number (Table 2). However,

an inter- and intra-population variability was detected for the cytotype $2n=40$ which could be explained by the existence of variable chromosome number (aneuploidy) ranging from 36 to 40, while in the $2n=16$ cytotype, which seems more stable, the observed differences could be related to the presence or not of the B chromosomes. Such intra-specific and inter-population variations of C-value have been reported by numerous authors (Garnatje et al. 2004; Bogunic et al. 2007; Hidalgo et al. 2008; Pellicer et al. 2010; Pustahija et al. 2013) and have been variously attributed to environmental conditions, geographical situation, proliferation and elimination of dispersed repeats derived from mobile elements, and so forth (Pellicer et al. 2014).

The essential difference between our two Algerian cytotypes concerned their $1Cx$ values, higher in the polyploid cytotype. This, even though a reduction in monoploid genome size, “genome downsizing”, is clearly the most extended pattern in polyploid taxa (Leitch and Bennett 2004; Suda et al. 2007; Pellicer et al. 2010; Soltis et al. 2014). This is in favor of our hypothesis that first there was the occurrence of the entire genome duplication of the ancestor with $x=10$ (by polyploidy) and only later the reduction of the basic chromosome number by decreasing dysploidy from $x = 10$ to $x = 8$.

Curiously, the size of the pollen grains does not follow the genome size. On the contrary, diploid pollen diameter is 8% larger than that of tetraploids [mean diameters 68.82 and 63.96 μm , respectively, according to Abdeddaim-Boughanmi & Kaid-Harche, (2009)]. Thus, on the basis of pollen size and $1Cx$ values, these two cytotypes represent two well differentiated taxa.

Heterochromatin and rDNA organization

The number and position of the ribosomal genes are normally constant for a given species, but this pattern can be different even between very close species (Bogunic et al. 2011). So the

ribosomal genes are good cytogenetic markers for understanding chromosomal evolution in a complex of related species (Cerbah et al. 1998; Muratovic et al. 2010; Bogunic et al. 2011; Siljak-Yakovlev et al. 2013, 2014).

In both *L. spartum* cytotypes, fluorochrome banding revealed the heterochromatin bands rich in GC and poor in AT CMA⁺ bands (namely, GC-rich DNA) were always associated with 35S rDNA sites. This co-localization with ribosomal genes has often been reported (Zoldos et al. 1999; Bou Dagher-Kharrat et al. 2001; Muratovic et al. 2010; Bogunic et al. 2011; Siljak-Yakovlev et al. 2013, 2014). In both cytotypes two ribosomal gene families, 5S and 35S, were separated from each other and located in different chromosome pairs which present the good markers for recognition of some pairs in chromosome set. This organization of rDNA is known as an “S-type arrangement”, which is the most frequent amongst angiosperms (Garcia et al. 2012). The number of 5S rRNA gene copies is apparently lower in tetraploids, given that their localization was visible only after labelling of probe with digoxigenin-11-dUTP, more sensitive in FISH protocols. The exact positions of the 5S on the chromosome set was less certain, so we did not mark these on the idiogram of the $2n = 40$ cytotype (Figure 3).

Phylogenetic relationships between cytotypes

Our results do not support any differentiation of the diploid cytotype at the molecular level. More studies should be conducted to find out whether cytogenetic differentiation is reflected in nucleotide sequences and which processes of hybridization, introgression or others may have taken place during the evolutionary history of this small genus.

Concluding remarks

Results from previous studies concerning morphological, anatomical, biochemical and palynological aspects, extended here-in, point to diversity in this genus *Lygeum* which has always been considered as monospecific. The present results reveal important differences at morphological and cytogenetic level, and are in favor of the existence of two distinct taxa: the diploid one with $2n=16$, ancient and probably a relict species whose range is restricted to Algerian High Plateau in the southwest of Oran, and the paleo-polyploid species with $2n = 40$, widespread in the Mediterranean basin.

As has been observed, the new cytotype ($2n=16$) is more vigorous on Algerian High Plateau and consequently is more interesting for programs against desertification. For this reason, it is necessary to establish a good strategy for conservation and regeneration of $2n=16$ populations in their natural habitat (*in situ*), given its limited distribution and the excessive exploitation of this endangered species.

The diploid cytotype appears clearly included in the Neighbour-Net of ITS data together with the tetraploid cytotypes.

Taking into account all the differences found between the two *Lygeum* cytotypes, it is obvious that we are in the presence of two well differentiated taxa and that the diploid cytotype can be described as a new taxonomic entity.

Acknowledgements

The authors thank Dr. Simonetta Peccenini for help obtaining material, Odile Robin for technical assistance in cytogenetics and Professor Joan Vallès for critical reading of the manuscript. This study was supported by the CMEP (Le Comité Mixte d'Evaluation et de Prospective Franco-Algérien - Programme TASSILI, Nb. 01 MDU/530).

References

- Abdeddaim-Boughanmi K. 2010. Etude de deux cytotypes de *Lygeum spartum* L. par approches pluridisciplinaires : palynologie, cytogénétique classique et moléculaire. PhD Thesis. Université des Sciences et de la Technologie d'Oran-Mohamed Boudiaf.
- Abdeddaim-Boughanmi K, Kaid-Harche M. 2009. Structure, ultrastructure of the anther, pollen microsporogenesis and morphology of pollen grains of two populations of *Lygeum spartum* L. in Algeria. *Amer Journ of Agric and Biol Sci* 3: 201-205.
- Amin A. 1972. Seven chromosome numbers of Egyptian plants. *Bot. Notiser* 125 pp.
- Benmansour N, Harche-Kaid M. 2001. Etude caryologique de deux populations de *Lygeum spartum* L. (Gramineae) de l'ouest algérien. *Bocconeia* 13: 371-376.
- Bennett MD, Leitch IJ. 1995. Nuclear DNA amounts in angiosperms. *Ann of Bot* 76: 113-176.
- Bogunic F, Siljak-Yakovlev S, Muratovic E, Ballian D. 2011. Different karyotype patterns among allopatric *Pinus nigra* (Pinaceae) populations revealed by molecular cytogenetics. *Plant Biology* 13: 194-200.
- Bou Dagher-Kharrat M, Grenier G, Bariteau M, Brown S, Siljak-Yakovlev S, Savouré A. 2001. Karyotype analysis reveals interspecific differentiation in the genus *Cedrus* despite genome size and base composition constancy. *Theor Appl Genet* 103: 846-854.
- Brullo S, Giusso del Galdo G, Guarino R. 2002. Phytosociological notes on the *Lygeum spartum* grasslands from Crete. *Lazaroa* 23: 65-72.
- Cerbah M, Coulaud J, Siljak-Yakovlev S. 1998. rDNA organization and evolutionary relationships in the genus *Hypochoeris* (Asteraceae). *J Hered* 89: 312-318.
- Clayton WD, Renvoize SA. 1986. *Genera Graminum*. London: HMSO
- Cullings KW. 1992. Design and testing of a plant-specific PCR primer for ecological and evolutionary studies. *Mol. Ecol.* 1: 233-240.

Con formato: Español (alfab. internacional)

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

Con formato: Francés (Francia)

Döring E, Albrecht J, Hilu KW, Röser M. 2007. Phylogenetic relationships in the Aveneae/Poeae complex (Pooideae, Poaceae). *Kew Bull.* 62: 407-424.

Doyle JJ Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11-15

Feulgen R, Rossenbeck H. 1924. Mikroskopisch-chemischer Nachweis einer Nucleinsäure vom Typus der Thymonucleinsäure und die darauf beruhende elektive Färbung von Zellkernen in mikroskopischen Präparaten. *F. Hoppe-Seyler's Zeitschr. Physiol Chem* 135: 203-248.

Garcia S, Chrák KL, Kovařík A. 2012. Expression of 5S rRNA genes linked to 35S rDNA in plants, their epigenetic modification and regulatory element divergence. *BMC Plant Biology* 12: 95.

Garnatje T, Vallès J, Garcia S, Hidalgo O, Sanz M, Canela AM, Siljak-Yakovlev S. 2004 Genome size in *Echinops* L. and related genera (*Asteraceae*, *Carduaceae*): Karyological, ecological and phylogenetic implications. *Biology of the cell* 96: 117-124.

Geber G, Schweizer D. 1988. Cytochemical heterochromatin differentiation in *Sinapis alba* (Cruciferae) using a simple air-drying technique for producing chromosome spreads. *Pl Syst Evol* 158: 97-106.

Godelle B, Cartier D, Marie D, Brown SC, Siljak-Yakovlev S. 1993. Heterochromatin study demonstrating the non-linearity of fluorometry useful for calculating genomic base composition. *Cytometry* 14: 618-626

Graells MP. 1876. Les spartes, les joncs, les palmiers et les pites. *Soc. Acclim.* 8: 419-493.

Halmy, M.W.A. 2015. Traditional knowledge associated with desert ecosystems in Egypt. Pp: 107-145. In: Roué, M., Césard, N., Adou Yao, Y.C. and Oteng-Yeboah, A. (eds.).

Knowing our Lands and Resources. Indigenous and Local Knowledge of Biodiversity and Ecosystem Services in Africa. UNESCO. Paris.

Heslop-Harrison JS, Schwarzacher T, Anamthawat-Jonsson K, Leitch IJ. 1991. *In situ* hybridization with automated chromosome denaturation techniques. *Methods Cell Mol Biol* 3: 109-116.

Hidalgo O, Garcia-Jacas N, Garnatje T, Romashchenko K, Susanna S, Siljak-Yakovlev S. 2008. Extreme environmental conditions and phylogenetic inheritance: systematics of *Myopordon* and *Oligochaeta* (Asteraceae, Cardueae, Centaureae). *Taxon* 57: 769-778.

Hilu WH. 2004. Phylogenetics and chromosomal evolution in the Poaceae (grasses). *Australian journal of Botany* 13-22.

Hochreutiner B. 1904. Le sud-oranais. Etude floristique et phytogéographique. *Ann Conserv Jard Bot (Genève)* 7-8: 23-276.

Huson DH, Bryant D. 2006. Application of Phylogenetic Networks in Evolutionary Studies, *Mol. Biol. Evol.*, 23(2):254-267.

Kellogg EA. 2015. The Families and Genera of Vascular Plants. Vol. XIII. Flowering Plants. Monocots. Poaceae, edited by K. Kubitzki. Springer, Xv+416 pp. ISBN 978-3-319-15332-2. Hardback.

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

Leitch IJ, Chase MW, Bennett MD. 1998. Phylogenetic analysis of DNA C-Values provides evidence for a small ancestral genome size in flowering plants. *Ann of Bot* 82: 85-94.

Levan A, Fredga K, Sandberg AA. 1964. Nomenclature for centromeric position on chromosomes. *Hereditas* 52: 201-220.

Lorenzo-Andreu A, García-Sanz P. 1950. Chromosomas somaticos de plantas espontaneas en la estepas de Aragon II. *Ann Estac Exptl. Aula Dei* 2, 1: 12-20.

- Maire R. 1953. Flore de l'Afrique du nord 2, Monocotyledoneae, Gramineae sf., Pooideae. Le Chevalier Ed., Paris, France. p.12-13.
- Marie D, Brown SC. 1993. A cytometric exercise in plant DNA histograms, with 2C values of 70 species. Biol Cell 78: 41-51.
- Martin J, Hesemann CU. 1988. Evaluation of improved Giemsa C – and fluorochrome banding techniques in rye chromosomes. Heredity 6: 459-467.
- Muratovic E, Robin O, Bogunic F, Soljan D, Siljak-Yakovlev S. 2010. Speciation of European lilies from *Liriotypus* section based on karyotype evolution. Taxon 59: 165-175.
- Pellicer J, Garcia S, Canela MA, Garnatje T, Korobkov AA, Twibell JD, Vallès J. 2010. Genome size dynamics in *Artemisia* L. (Asteraceae): following the track of polyploidy. Plant biol 12: 820-830.
- Pellicer J, Kelly LJ, Leitch IJ, Zomlefer WB, Fay MF. 2014. A universe of dwarfs and giants: genome size and chromosome evolution in the monocot family Melanthiaceae. New Phytol 201: 1484–1497.
- Pinto Da Silva AR. 1976. De flora Lusitanica commentarii: plantas novas e novas areas para a flora de Portugal. Ad Norman Herbarii Stationis Agronomicae Nationalis 12: 167-188.
- Pustahija F, Brown CS, Bogunic F, Basic N, Muratovic E, Ollier S, Hidalgo O, Bourge M, Stevanović V, Siljak-Yakovlev S. 2013. Small genomes dominate in plants growing on serpentine soils in West Balkans, an exhaustive study of 8 habitats covering 308 taxa. Plant and soil 373: 427–453.
- Quézel P, Santa S, 1962. Nouvelle flore de l'Algérie et des régions désertiques méridionales. Tome 1, CNRS Ed. France, 1170 p.

- Pimentel M, Escudero M, Sahuquillo E, Minaya MÀ, Catalán P. 2017. Are diversification rates and chromosome evolution in the temperate grasses (Pooideae) associated with major environmental changes in the Oligocene-Miocene? PeerJ3815. DOI 10.7717.
- Ramanujam S. 1938. Cytogenetical studies in the Oryzae I. Ann of Bot. 2 (5): 107-125.
- Schneider J, Döring E, Hilu KW, Röser M. 2009. Phylogenetic structure of the grass subfamily Pooideae based on comparison of plastid matK gene-3'trnK exon and nuclear ITS sequences. Taxon 58: 405-424.
- Schneider J, Winterfeld G, Hoffmann M H, Röser M. 2011. Duthieae, a new tribe of grasses (Poaceae) identified among the early diverging lineages of subfamily Pooideae: molecular phylogenetics, morphological delineation, cytogenetics, and biogeography. Systematics and Biodiversity 9: 27-44.
- Schweizer D. 1976. Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma (Berl.) 58: 307-324.
- Siljak-Yakovlev S, Cerbah M, Coulaud J, Stoian V, Brown SC, Zoldos V, Jelenic S, Papes D. 2002. Nuclear DNA content, base composition, heterochromatin and rDNA in *Picea omorika* and *Picea abies*. Theor Appl Genet 104: 505-512.
- Siljak-Yakovlev S, Temunovic M, Robin O, Raquin Ch, Frascaria-Lacoste N. 2013. Genome size and physical mapping of rDNA and G-C rich heterochromatin in three European *Fraxinus* species. Tree Genet Genomes 10: 231-239.
- Siljak-Yakovlev S, Pustahija F, Vicic V, Robin O. 2014. Molecular cytogenetics (FISH and fluorochrome banding): resolving species relationships and genome organization. In: Methods in Molecular Biology, Besse P. Ed., John M. Springer-Verlag, Vol. 1115: 309-323.

Con formato: Español (alfab. internacional)

Con formato: Español (alfab. internacional)

Con formato: Español (alfab. internacional)

Soltis DE, Soltis PS, Collier TG, Edgerton ML. 1991. Chloroplast variation within and among genera of the *Heuchera* Group (Saxifragaceae): evidence for chloroplast transfer and paraphyly. *Amer. J. Bot.* 78: 1091-1112

Soltis SE, Visger CJ, Soltis PS. 2014. The polyploidy revolution then and now: Stebbins revisited. *Am J Bot* 101: 1–22.

Con formato: Español (alfab. internacional)

Soreng JR, Peterson MP, Romaschenko K, Davidse G, Teisher KJ, Clark GL, Barbera P, Gillespie JL, Zuloaga OF. 2017. A worldwide phylogenetic classification of the Poaceae (Gramineae) II: An update and a comparison of two 2015 classifications. *J Syst.Evol* 55: 259–290.

Stebbins GL. 1982 Major trends of evolution in the Poaceae and their possible significance. In 'Grasses and grasslands: systematics and ecology'. (Eds JR Estes, RJ Tyrl, JN Brunken) p. 3–36. (University of Oklahoma Press: Norman)

Stebbins GL. 1985. Polyploidy, hybridization and the invasion of new habitats. *Annals of the Missouri Botanical Garden* 72: 824–832.

Suda J, Krahulcová A, Trávníček P, Rosenbaumová R, Peckert T, Krahulec F. 2007. Genome size variation and species relationships in *Hieracium* sub-genus *Pilosella* (Asteraceae) as inferred by flow cytometry. *Ann of Bot* 100: 1323–1335.

Tutin TG. 1980. *Flora Europaea. Alismataceae to Orchidaceae.* Cambridge university press.UK.

Zoldos V, Papes D, Cerbah M, Panaud O, Besendorfer V, Siljak-Yakovlev S. 1999. Molecular cytogenetic studies of ribosomal genes and heterochromatin reveal conserved genome organisation among 11 *Quercus* species. *Theor Appl Genet* 99: 969-977.

Tables:

Table 1. Geographic origin, climatic type and chromosome number of *Lygeum* populations studied.

Table 2. Chromosome numbers, karyotype features, genome size (2C DNA) and number of CMA bands and 35S and 5S rDNA signals.

Figure captions:

Figure 1. Geographic origin of the populations of two *Lygeum* cytotypes sampled in Algeria, Italia, Greece and Spain. $2n=40$ (red squares) and $2n=16$ (green circles). Population numbers correspond to Table 1.

Figure 2. Feulgen staining of metaphase chromosome plates in *Lygeum* populations:

A) El-Kheiter (High Plateau, $2n=16+B$); B) Oran (Littoral - Sebkha, $2n=40$); C) Calabria (Italy, $2n=38+B$). Bar= $10\ \mu\text{m}$

Figure 3. The same *Lygeum* chromosome plate after chromomycin (**A₁**), Hoechst (**A₂**), and simple 35S FISH (**A₃**) experiment in El-Kheiter ($2n=16+B$) population; double FISH on metaphase plate with 35S (green) and 5S (red) signals in El-Kheiter ($2n=16+B$) population (**B**); CMA (**C**), Hoechst staining (**D**) and double FISH (**E**) in Sebkha ($2n=40$) population where, conversely, 5S is in green and 35S in red. Note co-localization of CMA⁺ bands and 35S signals. The negative signals after Hoechst staining appeared positive after CMA and FISH. Bar= $10\ \mu\text{m}$

Idiograms concerning $2n=16$ (**F**) and $2n=40$ (**G**): CMA staining (yellow), 35S (green) and 5S (red).

Figure 4. Neighbour-Net of ITS data obtained from diploid and tetraploid *Lygeum* cytotypes.

Figure S1. Feulgen staining of metaphase chromosome plates in *Lygeum* populations:

A) Ain-Benkhelil (High Plateau, $2n=16$); **B)** Kristel, $2n=40$; **C)** Saida, $2n=38$ +**B**; **D)** Alicante, $2n=40$; **E)** Crete, $2n=39$; **F)** Djelfa, $2n=38$. Bar=10 μm

Appendix 1. Origin and GenBank accession numbers of the sequenced individuals from *Lygeum spartum* populations.

Population	ITS	trnL-F
El-Kheiter (High-plateau)	MK014711	MK014715
Sebkha (Oran West)	Ind 1 (MK014706) Ind 3 (MK014708)	Ind 3 (MK014713)
Kristel (Oran East)	Ind 4 (MK014707) Ind 5 (MK014710)	Ind 4 (MK014714)
Alicante (Spain)	Ind 1 (MK014705) Ind 3 (MK014709)	Ind 3 (MK014712)

0.001

Table 1. Geographic origin, climatic type and chromosome number of *Lygeum spartum* studied populations

Species	Locality	Geographic data			Climatic type	Collectors
		Latitude	Longitude	Altitude		
<i>Lygeum spartum</i> 2n=16	1. El-Kheiter (high-plateau)	34°08'N	0°04'E	1003 m	dry	Abdeddaim K., Djabeur A.
	2. Ain-Benkhelil (high-plateau)	33°26'N	0°56'O	1045m	dry	Abdeddaim K., Djabeur A.
<i>Lygeum spartum</i> 2n=36-40	2'. Ain-Benkhelil (high-plateau)	33°26'N	0°56'O	1045m	dry	Abdeddaim K., Djabeur A.
	3. Sebkha (Oran West)	35°38'N	0°36'O	86 m	semi-dry	Abdeddaim K.
	4. Kristel (Oran Est)	35°48'N	0°29'O	60 m	semi-dry	Abdeddaim K.
	5.Oued El Hdjel (high-plateau)	32°22'N	0°35'E	815 m	dry	Abdeddaim K., Djabeur A.
	6. Tlemcen (high-plateau)	34°52'N	1°18'O	792 m	dry	Djabeur A.
	7. Saida (high-plateau)	34°51'N	0°08'E	853 m	dry	Djabeur A.
	8. Aflou (high-plateau)	34°06'N	2°05'E	1404 m	dry	Djabeur A.
	9. Djelfa (high-plateau)	34°40'N	3°14'E	1150 m	dry	Djabeur A.
	10. Rogassa (high-plateau)	34°26'N	0°28'E	1045 m	dry	Abdeddaim K., Djabeur A.
	11. Crete (Greece)	39°01'N	17°12'E	15 m	semi-dry	Garnatje T.
	12.Calabria (Italy)	35°08'N	26°12'E	407 m	semi-dry	Peccini S.
	13. Alicante (Spain)	38°21'N	0°29'O	58 m	semi-dry	Vallès J.

Table 2. Chromosome numbers, karyotype features, genome size (2C DNA) and number of CMA bands and 35S and 5S rDNA signals^a1pg=978 Mbp (according to Doležel *et al.* 2003); ^bSD=standard deviation; ^cmonoploid genome size; ^dpresence of B chromosome

Population	2n	Chromosome formula	2C DNA in pg ^a (SD) ^b	1Cx DNA ^c in Mbp	Number of CMA+ bands	Number of 35S signals	Number of 5S signals
1. El-Kheiter	16+1B ^d	12 m ² , 4 sm ³	9.27 (0.11)	4533	7	7	7
2. Ain-Benkheílil (2x)	16		10.74 (0.33)	5252	6	6	2
<i>Mean for 2x</i>			10.01	4892			
2' Ain-Benkheílil (4x)	38-40	22 m, 18 sm	19.39 (1.48)	4741	10	10	4
3. Sebkha	36-40		19.75(0.31)	4829			
4. Kristel	38-40		18.37 (1.27)	4491			
5.Oued El Hdjel	38-40		21.53 (0.58)	5264			
6. Tlemcen	36-40						
7. Saida	36-40+B		21.34 (0.56)	5218			
8. Aflou	38-40						
9. Djelfa	38-40		23.63 (1.53)	5778			
10. Rogassa	38-40						
11. Crete	38-40		23.18 (0.18)	5668			
12.Calabria	40	22 m, 18 sm					
13. Alicante	38-40		23.06 (2.11)	5638			
<i>Mean for 4x</i>			21.28	5203			