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Full Length Research Paper

Genome size unaffected by moderate changes in climate and phosphorus availability in Mediterranean plants

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Nuclear DNA amount has been assessed in a set of 6 Mediterranean plant species including subshrubs, shrubs and trees (*Dorycnium pentaphyllum* Scop*., Erica multiflora* L*., Fumana ericoides* (L.) Webb*, Globularia alypum* L*., Pinus halepensis* Mill and *Rosmarinus officinalis* L.). Genome size values have been assessed by flow cytometry from plants growing in their natural habitats, in plots with a particular experimental design to measure the effects of drought and warming and also with different phosphorus (P) concentration in soil. 2C values have been fairly constant in all the species studied under all conditions. These results, which provide first records of DNA content in all the studied species except for *P. halepensis*, suggest that moderate changes in climate such as a 0.73°C warming or a drought consisting of 19% decrease in soil moisture on an average of 7 years and the consequent changes in the soil availability of such an essential element as P (ranging from 80 to 160 µg/g) do not affect genome size stability, at least not by producing rapid and significant variations.

Key words: C-value, drought, Mediterranean plants, nuclear DNA amount, phosphorus, warming.

INTRODUCTION

Genome sizes of different organisms are usually expressed as C-values, as coined by Swift (1950) to indicate the DNA content of the haploid chromosome complement in an unreplicated gamete, where the C accounts for constant, as it is considered a fixed characteristic of every organism. Several studies have shown the existence of a relationship between plant nuclear DNA amount and ecological features of their habitats, such as altitude, latitude, human activities or nitrogen content in soil (Poggio et al., 1998; Torrell and Vallès, 2001; Vilhar et al., 2002; Garcia et al., 2004; Garnatje et al., 2004) and that genome size should be predictable, adaptive and of evolutionary significance, although there is no simple explanation for its variation in higher plants (Bottini et al., 2000).

The relationships between genome size and climatic variables have been the object of numerous studies, particularly in recent times, when climatic change became a concern and at the same time a focus for many researches (MacGillivray and Grime, 1995; Huntley et al., 1998; Dunnet and Grime, 1999). Most of these works have been conducted in areas outside Mediterranean climate regions. The Mediterranean regions present very special characteristics which, for the western Mediterranean area, may be summarized as winters with mild temperatures and dry and warm summers, with 2 rainfall maxima, in spring and autumn. Whereas drought stress

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is the climatic factor which conditions more plant growth in Mediterranean type regions, high summer temperatures represent an additional stress for plants (Larcher, 2000). Apart from the general difficulty for living under these climatic conditions, for which plants have developed numerous adaptation mechanisms, deficiencies in important elements, such as nitrogen or phosphorus are linked to water availability and transport through the plant, affecting photosynthetic capability (Lovelock et al., 2006).

Climate change projections indicate that towards the end of the current century mean global surface temperatures will increase by 1.8 - 6.4°C (IPCC, 2007). Warmer temperatures will increase potential evapotranspiration and will aggravate water stress in Mediterranean basin, where, in addition, precipitations are also predicted to decrease. These climatic changes affect plant development, growth and life cycle (Peñuelas and Filella, 2001), but it is not clear whether these variables may also affect genome size, that is nuclear DNA amount. Variation in DNA C-value has far-reaching biological consequences and can have considerable adaptive and hence ecological significance (Bennett, 1987). A relationship has been hypothesized between plant genome size and cold or hot conditions. Grime and Mowforth (1982) have shown that plant communities in Britain contain species which differ considerably in genome size according to environmental conditions. The short duration of the cell cycle in small genome species (Bennett, 1972) in warm conditions makes these plants unusually responsive to rising temperatures (Grime, 1989). MacGillivray and Grime (1995), while predicting frost resistance in herbaceous plants, suggested that genome size has implications in their potential responsiveness to global warming, which could be curtailed by the continued occurrence of late-frost events.

DNA content may change rapidly in response to the physiological state of the plant or to physical factors related to the environment (Walbott and Cullis, 1985; Price, 1988). Rapid alterations in the basic amount of nuclear DNA, which may occur during seed germination, can affect phenotypic characteristics at cellular and organismal levels and may be correlated with environmental factors (Caceres et al., 1998). Bottini et al. (2000) found a correlation between DNA amount and climatic conditions, particularly in xeric Patagonian habitats, concluding that C-values are linked to the capability of species to adapt to different growing conditions.

Apart from climatic characteristics, Hanson et al. (2001) suggested that a small genome size does not reflect the necessity for rapid growth but the lack of nutrients, especially phosphorus (P) needed for DNA biosynthesis. Phosphorus is an essential element constituent of nucleic acids (DNA and RNA) and membranes (phospholipids), and an integral part of energy metabolism. In plants it is found in low concentrations (ca. 0.2%, Schachtman et al., 1998) in several chemical forms.

Pierce (1937) reported a considerable increase in chromosomal and nuclear volume with P increase in the watering solution in *Viola conspersa* Rchb. Fusconi et al. (2005) found a negative correlation between P content and the proportion of 2C nuclei, leading to an earlier occurrence, in time and space of polyploid nuclei in plants of *Allium porrum* L. submitted to different phosphate nutrition.

Phosphorus is one of the least available of all essential nutrients in the soil and the deficiencies in this nutrient are a major factor limiting plant growth, and other functions, in many natural ecosystems (Margalef, 1998; Raghothama, 1999). Besides soil availability, warming and drought also affect P absorption by plants and leaf P concentrations have been reported to be inversely correlated to water availability (Sardans and Peñuelas, 2007). Drought, particularly dramatic in Mediterranean ecosystems with the increment in aridity due to climatic change, makes the absorption of inorganic P difficult. Different experiments (Sardans and Peñuelas, 2004; Sardans et al., 2006) have shown that the soil availability of P is more affected by drought than by warming.

In this context we have conducted an experimental work simulating climatic change by varying temperature and water availability in a Mediterranean shrubland, with the main goal of finding, if any, the effect of the future climate conditions on the variation of genome size of plants. In addition, the P content has been measured in each experimental plot, with the aim of evaluating the possible correlation between DNA amounts and P availability in the soil. DNA measurements have been conducted in 6 typical Mediterranean plant species in an innovative field experimental device in which we simulated the drought (19% decreased soil moisture) and warming (0.73°C increase) conditions projected in climatic and ecophysiological models for the immediate next 2 - 3 decades (Sabaté et al., 2002; Peñuelas et al., 2005; IPCC, 2007).

MATERIALS AND METHODS

Plant material

This study was carried out on 6 species (*Dorycnium pentaphyllum* Scop., *Erica multiflora* L., *Fumana ericoides* (L.) Webb, *Globularia alypum* L., *Pinus halepensis* Mill. and *Rosmarinus officinalis* L.), growing in a prelittoral Mediterranean shrubby community in a calcareous, karstic area situated in the massif of Garraf (Catalonia, Iberian Peninsula). Vouchers are deposited in the herbarium of the centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona (BCN). Nuclear DNA amount was measured in nine plots included in an experiment studying climate change (warming and drought) effects on this shrubland (Beier et al., 2004; Peñuelas et al., 2004, 2007), as described in detail below.

Experimental design

We performed field-scale night-time warming and drought in treatment plots and compared them with untreated plots (controls). Plots were 4 x 5 m^2 with a 3 x 4 m^2 actual study area after considering 0.5 m of buffer area at the perimeter. A total of nine plots were esta-

blished, 3 replicates per treatment. Treatments started in spring 1999 and have been active since then until measurements were done, in spring 2006. Climatic variables at the experimental site are provided in Figure 1.

Warming treatment

The warming treatment was performed as night-time warming by reflective curtains covering the vegetation at night (Beier et al., 2004). Solar energy is accumulated in the ecosystem during the day and a fraction of the energy is re-radiated back to the atmosphere at night as long wave infrared (IR)-radiation. The covering of the ecosystem during night with the reflective aluminium foil curtains (ILS ALU, AB Ludvig Svensson, Sweden) reduces the loss of IR radiation. The warming plots are covered by light scaffolding carrying the reflective aluminium curtain. The coverage of the study plots is activated automatically according to preset light (< 200 lux), rain and wind (< 10 ms^{-1}) conditions (Beier et al., 2004). In order to avoid influencing the hydrological cycle, the covers are automatically removed during rain events, triggered by rain sensors. The warming treatment has been applied since spring 1999 with a warming effect on soil and plants of ca. 1°C with slight variations depending on time of the year and meteorological conditions (Beier et al., 2004). Warming treatment increased average annual temperatures by 0.73°C across the 7 years of study.

Drought treatment

The drought treatment was performed for 2 to 3 month period in the spring and autumn growing seasons since 1999 by covering the vegetation with waterproof, transparent covers. The drought plots were constructed similar to the warming plots except that the curtain material is a transparent plastic and that the moving of the curtains is governed only by rain and wind. During drought periods, the rain sensors activate the curtain to cover the plots whenever it rains and remove the curtains when the rain stops. The curtains are removed automatically if the wind speed exceeds 10 ms^{-1}. Drought treatment was on in spring and fall seasons during which plants show growth. For the part of the year without drought treatment, the drought plots were run parallel to the control plots. The maximum decrease of soil moisture during the applied drought treatment was 33% while air and soil temperatures were not affected. For the rest of the year, the drought treatment was off and therefore, the soil water content was not affected (Beier et al., 2004). Drought treatments reduced average annual soil humidity by a 19% across the seven years of study.

Untreated control

3 untreated control plots with a similar light scaffolding as for the warming and drought treatments but without any curtain were installed for comparison.

Foliar and soil P analyses

Leaves were sampled in January 2005, 6 years after treatments beginning. All the plant and soil samples were taken to the laboratory and stored at 4°C until analysed. Plant leaves were washed with distilled water. Prior to analyses, samples were dried in an oven at 60°C until constant weight was reached and then ground in a CYCLOTEC 1093 (Foss Tecator, Höganäs, Sweden) (biomasses) or in a FRITSCH Pulverisette (Rudolstadt, Germany) (soils).

The concentrations of P in plant and soil samples were measured using ICP-OES (Optic Emission Spectroscopy with Inductively Coupled Plasma) in a JOBIN IBON JY 38 (Longjumeau, HORIBA Jobin Ibon S.A.S., France). Before the biomass ICP-OES analyses, an acid digestion of the samples was carried out with an acid mixture of HNO_3 (60%) and $HClO_4$ (60%) (2:1) in a microwave oven (SAMSUNG, TDS, Seoul, South Korea). 2 ml of the mixed acid solution were added to 100 mg of dry biomass for each sample. The digested solutions were taken to 10 ml final volume. During the acid digestion process, 2 blank solutions (2 ml of acid mixture without any sample biomass) were also analyzed. In order to assess the accuracy of biomass digestion and analytical procedures, we used standard certified biomass (DC73351). For the determination of total P concentration in soil samples, digestion was carried out with 0.25 g of ground sample in 9 ml of HNO_3 (65%) and 4 ml HF (40%) in a microwave oven at 120°C for 8 h. The digested solutions were taken to 50 ml final volume, filtered with a Millex 0.45 µm filter, and then stored at 4°C until analysis. The precision of the soil analyses, as verified by parallel analyses using an international (GSR-6) standard, was better than 5% for all the trace elements analyzed.

To calculate the labile P in soil, an extraction with 0.5 M $NaHCO_3$ (Olsen-P) was taken (Watanabe and Olsen, 1965). A total of 20 ml of extracting solution (0.5 M $NaHCO_3$, pH 8 with NaOH) was added to 2 g of sieved soil (< 2 mm) in a flask that was then shaken for 30 minutes. The suspension was filtered through a Whatman No 40 paper and a 5 ml aliquot was placed in a 50 ml volumetric flask and acidified to pH 5 with 5 N H_2SO_4. Then, 4 ml of colour reagent (ammonium paramolybdate-antimony tartarate-ascorbic acid solution) were added along with distilled water to take the solution to 50 ml. The absorbance was measured at 860 nm against a blank in a Spectronic 20 Genesys (Spectronic Instruments Inc, Rochester NY, USA) spectrophotometer.

Nuclear DNA content measurements

DNA 2C values were assessed by flow cytometry following the procedure of Marie and Brown (1993). *Petunia hybrida* Vilm. 'PxPc6' (2C = 2.85 pg), *Pisum sativum* L. 'Express long' (2C = 8.37 pg) and *Triticum aestivum* L. 'Chinese spring' (2C = 30.90 pg), obtained in the Institut des Sciences du Végétal (CNRS) at Gif-sur-Yvette (France), were used as internal standards for the range of 2C values found. Two different isolation buffers were prepared. On one hand, Galbraith buffer (Galbraith et al., 1983) with fresh 10 mM sodium metabisulphite and 1% polyvinylpyrrolidone 10.000 was used to measure the 2C value of *P. halepensis*. On the other hand, LB01 buffer (Doležel et al., 1989) containing 4% (w/v) Triton X-100 was used for the rest of species. Young healthy leaf tissues from the standards and the species to be studied were placed in a Petri dish and chopped with a razor blade in 600 µl of isolation buffer. In the case of *F. ericoides*, in which chopped leaves were particularly mucilaginous, 1,200 µl of isolation buffer were added to recover 600 µl at least. The suspension of nuclei in the isolation buffer was filtered through a nylon mesh with a pore size of 70 µm and stained for 20 - 25 min with 36 µl of propidium iodide (Sigma-Aldrich Química, Alcobendas, Madrid, 60 µg/ml) and supplemented with 100 µg/ml ribonuclease A (Boehringer, Meylan). Tubes were kept on ice during staining and then left at room temperature until measurement. For each taxon and environmental conditions, 5 individuals were analyzed and 2 replicates of each individual were measured.

Measurements were made at the Serveis Cientificotècnics generals de la Universitat de Barcelona using an Epics XL flow cytometer (Coulter Corporation, Hialeah, Fla.). The instrument was set up with the standard configuration: excitation of the sample was done using a standard 488 nm air-cooled argonion laser at 15 mW power. Forward scatter (FSC), side scatter (SSC), and red (620 nm) fluorescence for propidium iodide were acquired. Optical alignment

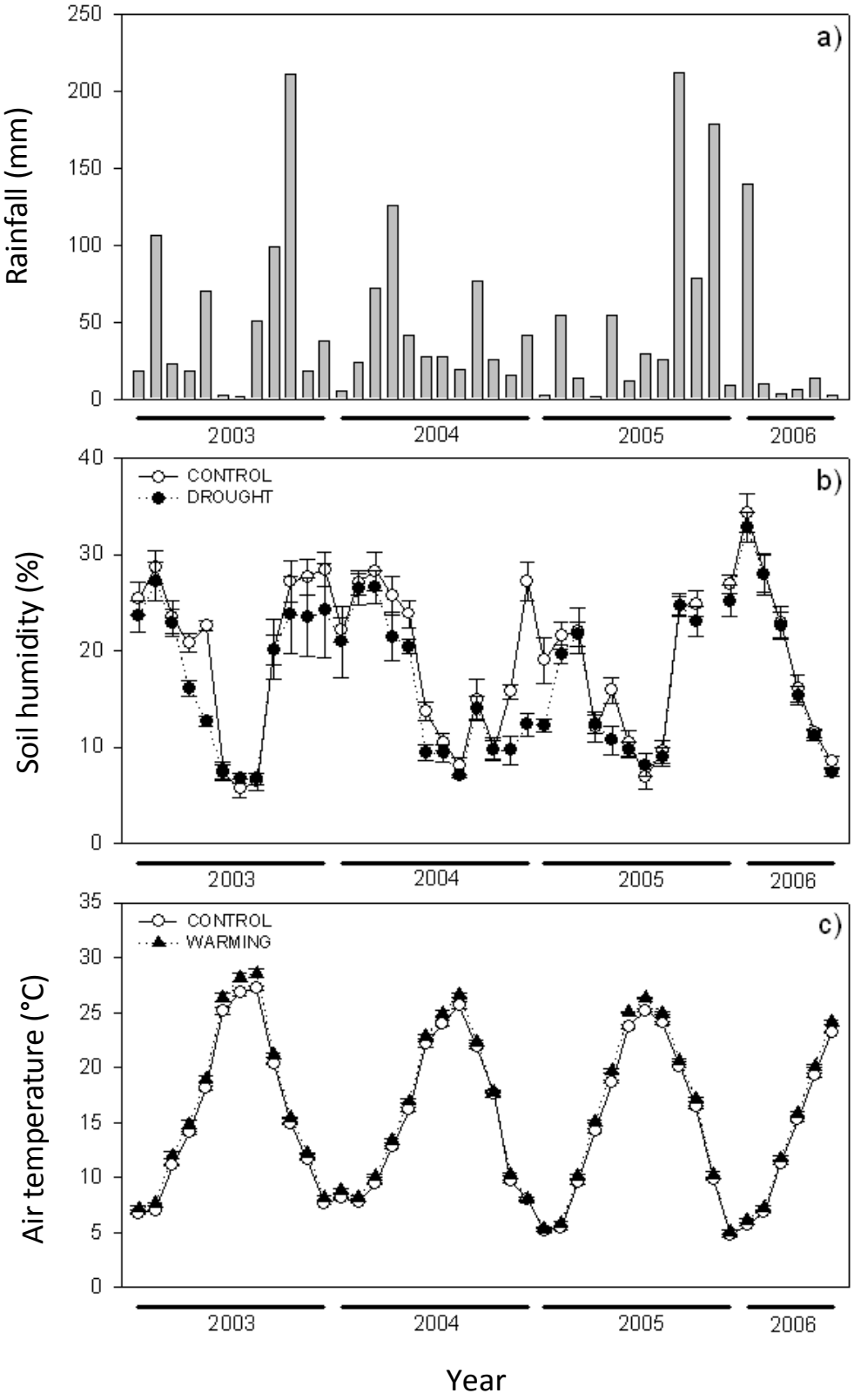

Figure 1. Climatic parameters at experimental site (for simplicity only the period January 2003-june 2006 is displayed). Values are monthly averages for a) Precipitation (mm), b) soil humidity (% v/v) (empty dots and continuous line are control treatment and filled dots and dotted line are drought treatment) and c) air temperature (ºC) (empty dots and continuous line are control treatment and filled triangles and dotted line are warming treatment).

Table 1. Nuclear DNA amount (2C values in pg, AVG ± SE) of the studied species. Different letters (highlighted in bold type) indicate significant statistical differences between species (P < 0.05).

Species	Control	Drought	Warming
P. halepensis	49.09 ± 0.92 a	51.73 ± 0.97	49.72 ± 1.39
G. alypum	0.80 ± 0.01 c	0.78 ± 0.02	0.79 ± 0.01
E. multiflora	0.95 ± 0.02 c	0.96 ± 0.02	0.93 ± 0.01
F. ericoides	2.55 ± 0.06 b	2.53 ± 0.05	2.53 ± 0.04
D. pentaphyllum	2.47 ± 0.03 b	2.46 ± 0.04	2.42 ± 0.01
R. officinalis	2.45 ± 0.03 b	2.41 ± 0.11	2.51 ± 0.11
P. halepensis	49.09 ± 0.92 a	51.73 ± 0.97	49.72 ± 1.39

was based on optimized signal from 10 nm fluorescent beads (Immunocheck, Epics Division, Coulter Corporation). Time was used as a control of the stability of the instrument. Red fluore-scence was projected on 1024 monoparametrical histograms. Gating single cells by their area versus peak fluorescence signal excluded aggregates. Acquisition was automatically stopped at 8000 nuclei. The total nuclear DNA content was calculated by multiplying the known DNA content of the internal standard used by the quotient between the 2C peak positions of the target species and the internal standard in the histogram of fluorescence intensities for the 10 runs, based on the assumption that there is a linear correlation between the fluorescence signals from stained nuclei of the unknown specimen and the known internal standard DNA amount. Mean values and standard deviations were calcu-lated based on the results of five individuals.

Statistical analyses

Treatment effects on P content in soils and plants was tested by one way ANOVA with transformations to achieve homoscedasticity when needed. Treatment effects on nuclear DNA amounts were performed independently for each species by means of a one way ANOVA. Differences among species in nuclear DNA amounts were tested by means of one way ANOVA on transformed data to achieve normality and homoscedasticity. Post-hoc Fisher's PLSD tests were performed after tests.

RESULTS AND DISCUSSION

P. halepensis is the species with the largest amount of DNA, having 2C values (49.09-51.73 pg) 20 times higher than *D. pentaphyllum*, *F. ericoides* and *R. officinalis*. (Table 1). *E. multiflora* and *G. alypum* have the lowest amount of DNA with 2C values (Table 1).

The data presented are the first records of nuclear DNA amount in all the studied species, except for *P. halepensis*, according to the plant genome size databases (Bennett and Leitch, 2004, 2005; Murray et al., 2004). The results obtained for *G. alypum* are close to the only previously reported in the genus (2C = 0.84 pg for *G. salicina*; Suda et al., 2005) and no reports exist for the other angiosperm genera studied here. For the only gymnosperm considered, *P. halepensis*, the currently assessed DNA amount is lower than the only preceding record (2C = 64.62 pg; Grotkopp et al., 2004), but is similar to the amounts of different pines, and agrees with genome size differences of 20% reported for a *Pinus* species (Bogunic et al., 2003).

There were no statistically significant differences between the nuclear DNA contents of each species in the different treatments (control, warming and drought). There was not either, any significant correlation between plant P concentration and genome size.

The present results indicate that a climatic change of 0.73°C warming or 19% lower soil moisture, as projections in this region suggest for the next immediate 2 - 3 decades (Sabaté et al., 2002; Peñuelas et al., 2005; IPCC 2007), does not directly and rapidly affect nuclear DNA amount in Mediterranean plants. In agreement with this, heat stress and/or water deficit have been reported not to increase variability in DNA content of *Helianthus annuus* L. varieties (Johnston et al., 1996). Similar results were found in other studied species, such as *Microseris douglasii* Sch. Bip. (Price et al., 1986), in which DNA amount only varied with changes in nitrogen concentration, but not with temperature or humidity variations. These data do not come from field experiments, but from warming and drought conditions simulated in a chamber. The results of the present paper on genome size under climatic change conditions, obtained for the first time with a field experimental design, corroborate these previous reports.

Soil total P ranged between 83 and 161µg/g with drought plots having significantly more total P than warming plots (Table 2). Soil immediate available P ranged between 1.3 and 2.4 µg/g which is a low level in temperate soils (Sardans et al., 2006). Drought had contrasting effects on leaf P concentration depending on species. It increased foliar P concentrations in *E. multiflora*, reduced them in *G. alypum*, and had no significant effect in the leguminous *D. pentaphyllum*. Warming had no effect in any of the soil and plant P concentrations monitored here.

These differences in P concentrations had no effect on genome size, showing that genome size is not directly and drastically conditioned by P content in the soil in the range studied here. A possible explanation for this is that in the case of P scarcity, plants would probably first reduces formation of ATP or other P-containing energetic compounds rather than structural and functionally important molecules, such as DNA. It is also possible that the

Table 2. Soil and plant P concentration (AVG ± SE) for the different treatments. Significant differences among treatments are indicated by different letters ($p < 0.05$) highlighted in bold.

Soil P (μg g^{-1})	Control	Drought	Warming
Total P	131 ± 18 ab	161 ± 29 b	83 ± 20 a
Olsen-P	1.50 ± 0.46	2.87±0.43	1.28 ± 0.48
Plant leaf P (mg g^{-1})			
E. multiflora	0.12 ± 0.03 a	0.13 ± 0.02 ab	0.18 ± 0.02 b
G. alypum	0.42 ± 0.05 a	0.33 ± 0.02 b	0.43 ± 0.01 a
D. pentaphyllum	0.53 ± 0.06	0.73 ± 0.10	0.55 ± 0.04

amount of P in DNA is too low (it has been for instance calculated 7% in tomato and 10% in cucumber according to Valenzuela et al. (1992)) to be affected by the treatment effects on P-availability in the soil. In addition, the inorganic P present in the cytoplasm and the vacuoles is probably enough for the processes of cell metabolism. In fact, nor drought neither warming had significant effects on aboveground accumulation of P after the 6 first years of experiment (Sardans et al., 2008). Moreover, as reported by several studies on ecological stoichiometry, the phenotypic and genetic responses (including genome size) to nutrient availability change would be related not only to the absolute content of N and P but also to its (N:P) ratio (Elser et al., 2000; Makino et al., 2003; Weider et al., 2003, 2004; Elser, 2006). Drought only changed N: P in stems of one of the three studied species, *E. multiflora* (Sardans et al., 2008).

Plants respond to P limitation through morphological changes (e.g. growing more roots), adjusting their physiology (like increasing phosphatase secretion to soil, increasing the rate of P uptake by roots, retranslocating Pi from older leaves, and at cellular level depleting the vacuolar stores of Pi (Schachtman et al., 1998) and by biochemical changes (like increasing Pase and RNase production to release Pi from organic compounds (Bariola et al., 1994; Duff et al., 1994). Among the different chemical forms of P within the plant cells (Pi, P-ester, P-lipid and nucleic acids), the proportion of P in each chemical form is variable with tissue type and age, except for P in DNA. For example, it has been described that the modification of the lipid composition of photosynthetic membranes could be achieved by increasing sulpholipids and decreasing phospholipids under P deficiency (Yu et al., 2002). The changes observed in P content were likely a result of phenotypic adaptations to drought affecting some P cellular pool other than changes in DNA.

However, irrespective of the fact that limitations in P are decisive for plant structural and functional aspects and that these limitations may be related to drought, our results suggest that nuclear DNA amount is not significantly influenced by these conditions. Hanson et al. (2001) stated that a P-poor life style may tend to minimize genome size, but our results cannot confirm this. Conversely, they agree with those from Bennett and Rees (1969), who obtained *Allium cepa* L. plants with chromosomes 50% larger when treated with P-rich solutions, but showing the same nuclear DNA amount than those plants with smaller chromosomes, the increase in volume being basically attributable to changes in protein amount.

To sum up, the stability of genome size suggested by the present data is not a strange condition and does not imply stasis in genome evolution or rigidity in structure. Apart from a fundamental constancy, changes in genome size occur, although it is not clear whether the discontinuous C-value distributions arise in a continuously varying distribution or whether the molecular mechanisms generating saltatory increases and/or decreases in DNA content also play a role (Narayan, 1998). Among other possible factors, some transposable elements, which in principle remain quiescent, may be activated (McClintock 1965) and might mediate the restructuring of the genome promoting increases or decreases in nuclear DNA amount (Federoff, 1984).

The present study has shown stability in genome size in 6 Mediterranean species (including subshrubs, shrubs and trees) irrespective of the variations in temperature, humidity (simulating climatic change conditions) and also independently of P concentration variation in soil.

Thus, no immediate and dramatic variations in nuclear DNA content are caused by moderate climatic change or by the consequent moderate changes in P availability. Further studies with a larger temperature variation and, including other Mediterranean plant species are warranted to complement the present survey.

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