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Nuclear rDNA instability in *in vitro*-generated plants is amplified after sexual reproduction with conspecific wild individuals

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Using micropropagation through tissue culture has become the most used approach worldwide for mass production for the conservation of endangered species. However, the screening of somaclonal variations generated using *in vitro* culture is usually restricted to the first generation of micropropagated plants, when they have not yet been released in the field. Accordingly, the fate of genetically modified regenerants after sexual reproduction is usually not assessed and changes in the genetic structures of species are unknown. In this work, we assess the cytogenetic stability of two rDNA gene families in the offspring of experimental crosses between accessions generated after *in vitro* culture and wild individuals of *Cistus heterophyllus* (Cistaceae). The cytogenetic rDNA profiles (45S rDNA, 5S rDNA) of 118 accessions including wild and *in vitro* micropropagated individuals and bi-directional artificial crosses between wild and *in vitro*-generated plants were assessed by fluorescence *in situ* hybridization (FISH) and Ag-NOR staining. Plants regenerated by micropropagation showed a lower size of the FISH signals in a 45S rDNA site, but this condition was not present in the wild accessions. Three new cytogenetic and cytological variants were present in 36% of the experimental progeny, involving the amplification of one additional 45S rDNA site and the presence of heteromorphic nucleoli. rDNA-based genomic instability was present after sexual reproduction between wild and *in vitro*-generated plants. The results of this study discourage the use of micropropagated materials for plant conservation unless comprehensive surveys of the genetic integrity and stability of the regenerants are performed after crossing between wild and micropropagated plants. *Botanical Journal of the Linnean Society*, 2016, **181**, 127–137

ADDITIONAL KEYWORDS: 45S rDNA – 5S rDNA – Cistaceae – *Cistus* – micropropagation – rDNA families.

INTRODUCTION

Management of the diversity of endangered plants is a biological challenge of global interest that is inherently linked to the current worldwide biodiversity crisis. Prioritizing species for conservation and restoration efforts is not free from controversy, but there is ample agreement that, all things being equal, threatened species with restricted distribution areas and reduced population effective size should be prioritized (Heywood & Iriondo, 2003; Gauthier, Debussche & Thompson, 2010; Arponen, 2012).

One of the ultimate goals of plant conservation is the maintenance of natural self-sustaining wild populations, but when this appears to be unattainable, several approaches aiming to facilitate species recovery in the wild (including translocation, population reinforcement and reintroduction) can be envisaged

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(Ashton, 1987; Falk, 1990; Given, 1994; Kell *et al.*, 2008; IUCN/SSC, 2013). Independently of a restoration approach, the increase of plant stocks for species recovery is a prerequisite for releasing enough *ex situ*-managed germplasm samples in natural habitats.

Using micropropagation through tissue culture has become the most widely used approach worldwide for *in vitro* mass production of endangered species (Fay, 1992; Iriondo, 2001; Rao, 2004; Engelmann, 2010; Cruz-Cruz, González-Arnao & Engelmann, 2013). Thus, hundreds of *ad hoc* protocols have been implemented for the rapid cultivation of at-risk species using a minimal amount of starting material that may potentially originate from virtually any vegetative or reproductive plant organ.

In vitro approaches for plant conservation are methodologically similar to those developed early on for the clonal propagation of agricultural, ornamental and medicinal plants. Thus, although the benefits of mass propagation are highly diverse, they show the same potential drawbacks and shortages inherent to using tissue culture in artificial media and that are subjected to environmental stresses.

Changes in ploidy, chromosome number, nuclear DNA amount, chromosome repatterning, distribution and abundance of highly repeated sequences, and transposition of ribosomal gene families are the most frequently reported mutational variations detected in the *in vitro* culture of plant tissues (Gernand *et al.*, 2007; Bairu, Aremu & Van Staden, 2011; Neelakandan & Wang, 2012; Rosato, Galián & Rosselló, 2012). In addition, the formation of *in vitro*-derived individuals showing abnormal morphological, anatomical, chemical and physiological features is one of the conspicuous epigenetically induced consequences of plant micropropagation (reviewed by Hazarika, 2006).

The production of genetically modified or genetically unstable regenerants not present in the original genotype stock is undesirable when addressing the conservation of endangered plant species. Most importantly, such regenerants could compromise the successful reintroduction of *in vitro*-propagated plants and the ethical guidelines linked to restoration programmes.

The screening of somaclonal variations is usually restricted to the first generation of micropropagated plants, when the plants have not yet been released in the field. Accordingly, the fate of genetically modified regenerants after sexual reproduction is usually not assessed and thus the long-term effects of possible genetic variants in the genetic structure of the species are unknown, as are their influence over ecosystems.

In this work, we assess the stability of two nuclear ribosomal gene families (nr 5S and 45S) through molecular cytogenetic techniques and Ag-NOR staining in the offspring of F1 experimental crosses generated between *Cistus heterophyllus* Desf. (Cistaceae) accessions generated after *in vitro* culture and wild individuals. The key role of 45S rDNA in the cell is not merely to provide the mature rRNAs for assembling the two subunit backbones of the eukaryotic ribosomes together with the 5S rRNA (Hemleben & Werts, 1988; Shaw & Jordan, 1995). In fact, other structural and functional aspects, including the maintenance of genome stability and modulating cellular homeostasis, are also thought to be of relevance in the eukaryote cell (Kobayashi, 2008; Hein *et al.*, 2012).

Our results indicate that *in vitro* culture generates 45S rDNA changes in micropropagated plants. However, rDNA instability is amplified after sexual reproduction with conspecific wild individuals, producing novel genotypes linked to the number and functionality of the 45S rDNA locus.

MATERIAL AND METHODS

Species background

Cistus heterophyllus is a narrowly distributed western Mediterranean species present in North Africa (Morocco, Algeria) and the Iberian Peninsula (Spain) (Crespo & Mateo, 1988; Démoly & Montserrat, 1993). Like other species of *Cistus* L., *C. heterophyllus* is self-incompatible (Boscaiu & Güemes, 2001), although fruits containing seeds are rarely produced (E. Laguna & P. Ferrer-Gallego, pers. comm.). The European individuals are at risk and are extremely endangered because of the species' rarity (only two populations, with about 26 and a single individual, respectively, have been reported), and threats caused by abiotic (fires, severe drought) and biotic factors (habitat transformation) (Güemes, Jiménez & Sánchez-Gómez, 2004). In addition, nuclear and plastid DNA markers and morphological evidence suggest strongly that ongoing gene flow with the related *C. albidus* L. is occurring in European and North African populations (Jiménez, Sánchez-Gómez & Rosselló, 2007; Navarro *et al.*, 2009). Plants from south-eastern Spain (Cartagena, Murcia) were reported to be present at the beginning of the 20th century, but the species was not found again until 1993 (nine individuals; Robledo *et al.*, 1995). These individuals disappeared shortly afterwards (1998) due to a fire (Navarro & Rivera, 2001; Navarro, 2002). Spontaneous regeneration from seeds was later observed, but the few dozens of individuals recovered were identified as hybrids with *C. albidus*

(Navarro, 2002; Sánchez-Gómez *et al.*, 2002). In 1987, a single individual showing no signs of interspecific hybridization was found in eastern Spain (Pobla de Vallbona, Valencia) (Crespo & Mateo, 1988) and recovery plans were designed to create a new population in Valencia. This specimen was multiplied through *in vitro* culture (Arregui *et al.*, 1993; González-Benito & Martín, 2011) to obtain accessions suitable for reintroduction at a new site (Tancat de Portaceli, Valencia, Spain) (Laguna *et al.*, 1998; Aguilella, Fos & Laguna, 2010).

Plant materials

Wild *C. heterophyllus* samples were obtained from propagated stems (rooted cuttings) from a European individual (Pobla de Vallbona, Valencia, Spain) and from germinated seeds obtained in a North African population (Targuist-Alhucemas, Morocco). *In vitro*-multiplied plants from *in vitro* culture and plant regeneration techniques (Arregui *et al.*, 1993) were (1) from samples produced during the first micropropagation culture originating from the wild European individual (denominated first *in vitro* generation) and (2) from two second *in vitro* generation lines obtained by micropropagation of two spontaneous new plants of a translocated population (Tancat de Portaceli, Valencia, Spain). Overall, 12 wild samples, 16 samples from the first *in vitro* generation and 20 from the second *in vitro* generation lines were analysed (Table 1). In addition, 50 individuals obtained from the progeny between *in vitro*-generated European *C. heterophyllus* and wild North African plants were analysed (see below). The related species *C. albidus* and *C. creticus* L. were sampled from wild populations (ten plants each) and were used for comparative purposes (Table 1).

Table 1. Origin of the samples used in the karyological study of *Cistus heterophyllus* and related species

Accessions and origin	Sample size
Wild samples	
C. heterophyllus	
Morocco, Targuist-Alhucemas	10
Spain, Valencia, Pobla de Vallbona	2
C. albidus	
Spain, Valencia, Llombai	10
C. creticus	
Spain, Valencia, Jalance	10
In vitro regenerated plants	
C. heterophyllus	
Spain, Valencia, Tancat de Portaceli	
First generation	16
Second generation	20
Artificial crosses	
C. heterophyllus ♀ (wild, Morocco) × *C. heterophyllus* ♂ (*in vitro* first generation, Spain)	22
C. heterophyllus ♀ (*in vitro* first generation, Spain) × *C. heterophyllus* ♂ (wild, Morocco)	28

Experimental crosses

Adult reproductive plants from European *C. heterophyllus* obtained by *in vitro* culture (first generation) and North African individuals from Targuist-Alhucemas (Morocco) were grown in a greenhouse to serve as parents to obtain bi-directional artificial crosses. Although the breeding system of *C. heterophyllus* has been reported to be self-incompatible (Boscaiu & Güemes, 2001), flowers acting as the female progenitor were emasculated prior to anthesis. All flowers from each maternal parent were crossed with pollen from single flowers of the paternal parent. Crosses were conducted by brushing pollen from flowers at anthesis with a fine paintbrush onto receptive stigmas and immediately bagging them with paper bags. Fertilized flowers were allowed to reach maturity and seeds from each cross were collected. One year after conducting the experimental crosses, seeds were cleaned with sodium hypochlorite, scarified with hot water and germinated on agar plates at 20 °C using a 12-h light/dark photoperiod. We analysed the parental individuals used in the crosses, 28 individuals obtained from the progeny between *in vitro*-generated European *C. heterophyllus* (maternal plant) and wild North African plants (pollen donor), and 22 individuals from the reciprocal crosses (Table 1).

Cytogenetic analysis

Cytological preparations

Living plants were cultivated in pots at the CIEF greenhouses. The root tips from the plants were excised and pre-treated with 2 mM 8-hydroxyquinoline for 2 h at 4 °C, then 2 h at room temperature, fixed in an ethanol/glacial acetic acid (3:1) mixture and stored at −20 °C until required. For mitotic chromosome spreads, we followed the protocols described by Rosato, Castro & Rosselló (2008).

Fluorescence in situ hybridization (FISH)

The 45S and 5S rDNA multigene families were localized using the pTa71 (Gerlach & Bedbrook, 1979) and pTa794 (Gerlach & Dyer, 1980) clones, respectively, according to the *in situ* hybridization protocols of Rosato *et al.* (2008), except for the proteinase K pre-treatment, which was performed following

Schwarzacher & Heslop-Harrison (2000). Probe detection was conducted using the method of Zhong *et al.* (1996) with modifications according to Galián, Rosato & Rosselló (2014). Cytogenetic analyses were made for at least ten well-spread metaphase plates for each accession.

Ag-NOR staining

Silver impregnation was carried out on 1- to 2-day-old chromosome preparations according to the protocol described by Rosato & Rosselló (2009). Ag-NOR activity was analysed in at least five interphase nuclei and five well-spread metaphase plates for each accession.

Karyotype analysis

Chromosome measurements were made on digital images using the processing image software IMAGETOOL v.5.0 and the freeware application MICROMEASURE v.3.3 (available at http://www.colostate.edu/depts/biology/micromeasure). For FISH analysis, each fluorochrome was captured separately and chromosomes were pseudo-coloured (grey) to enhance rDNA signals. Idiograms were obtained from chromosome measurements of at least five well-spread metaphase plates for each accession. Chromosome pairs were identified by their size and their centromeric index, and ordered accordingly for the construction of idiograms.

RESULTS

Chromosome features of wild plants

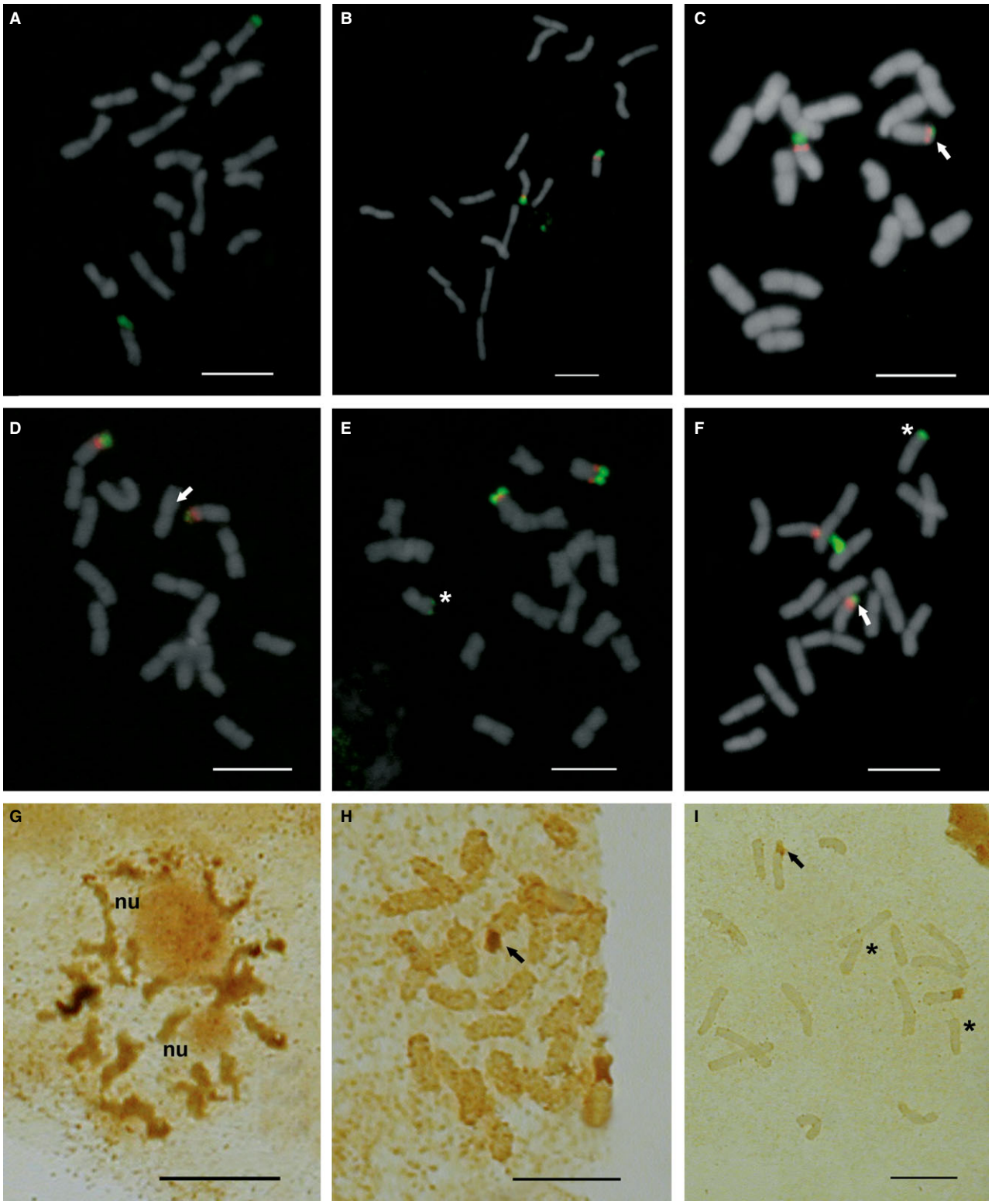
All accessions analysed showed a somatic chromosome number of $2n = 18$. Overall, the karyotype was constituted by chromosomes of similar size and shape, including metacentric (14), submetacentric (2) and metacentric–submetacentric (two) chromosomes. The metacentric–submetacentric pair (chromosome pair 8) showed a subterminal secondary constriction on the short arm and was inferred to carry the active NOR locus (Fig. 1). FISH results confirmed this finding and showed the presence of a single NOR locus in wild *C. heterophyllus* plants. Additionally, a single 5S rDNA locus was located adjacent (co-linear) to the 45S rDNA locus (Figs 1, 2). Activity of the NOR locus was assessed by silver staining, and the results showed similar active Ag-NOR sizes for the two 45S sites. At interphase, individuals showed one nucleolus or two nucleoli of similar size, prior to the nucleolar fusion occurring at the end of interphase, indicating that both chromosomes bear active Ag-NOR at secondary constrictions. Cytogenetic features of the related *C. albidus* and *C. creticus* were identical to those shown by *C. heterophyllus*. Thus, a single 45S and 5S rDNA locus was detected and two nucleoli of similar size were present at interphase.

In vitro-generated plants are heteromorphic for the 45S rDNA locus

All accessions of *C. heterophyllus* produced by *in vitro* culture showed identical karyological features, irrespective of belonging to the first- or second-generation lines of micropropagated plants. Their karyotypes were similar, but not identical, to those presented by the wild plants. After DAPI (4′,6-diamidino-2-phenylindole) staining, only one chromosome of the metacentric–submetacentric pair carrying the active NOR locus showed a conspicuous secondary constriction. FISH experiments using the

Figure 1. A–F, 45S and 5S rDNA patterns identified on mitotic metaphase chromosomes in wild, *in vitro*-micropropagated and progeny accessions of *Cistus heterophyllus* assessed through FISH and Ag-NOR analysis. 45S rDNA sites are shown as green fluorescent signals and 5S rDNA sites as red signals. Chromosomes are counterstained by DAPI staining and pseudocoloured (grey). A, B, karyological phenotype I. Wild accessions from Morocco (A) and Spain (B). C, karyological phenotype II. *In vitro*-micropropagated plant from the wild Spanish accession. The chromosome bearing the minor 45S rDNA site is arrowed. D, karyological phenotype II. Progeny from a cross between wild (Morocco, ♀) and *in vitro*-micropropagated plants (♂) as progenitors. The chromosome bearing the minor 45S rDNA site is arrowed. E, karyological phenotype IV. Progeny from a cross between wild (Morocco, ♀) and *in vitro*-micropropagated plants (♂) as progenitors. The additional 45S rDNA cluster is identified by an asterisk. F, karyological phenotype V. Progeny from a cross between *in vitro*-micropropagated plants (♀) and wild (Morocco, ♂) as progenitors. The chromosome bearing the minor 45S rDNA site is arrowed and the additional 45S rDNA cluster is identified by an asterisk. G–I, Ag-NOR staining in prophase and metaphase chromosomes. G, unequal size of nucleoli (nu) resulting from the differential expression of the NOR chromosome pair in micropropagated plants. H, karyological phenotype II. Metaphase chromosomes showing two Ag-NOR sites of unequal size (the minor site is arrowed). Progeny from a cross between *in vitro*-micropropagated plants (♀) and wild (Morocco, ♂) as progenitors. I, karyological phenotype V. Metaphase chromosomes showing two unequal Ag-NOR sites (the minor site is arrowed). The additional minor 45S rDNA cluster shown by FISH (identified by an asterisk) does not show Ag-NOR staining. Progeny from a cross between wild (Morocco, ♀) and *in vitro*-micropropagated plants (♂) as progenitors. Scale bars = 10 μm.

45S rDNA probe revealed that one of the chromosomes has a major 45S rDNA site similar in size to that of the wild plants. However, a strong size reduction of the fluorescence signals in one 45S rDNA site was detected. This heteromorphic condition as revealed by both DAPI staining and FISH



was consistently present in all cells and accessions studied and it was a fixed feature characterizing the plants regenerated by *in vitro* micropropagation. Ag-NOR staining of the 45S rDNA sites agreed with these findings, showing size differences between both homologous sites. These observations were associated with a different size of the two nucleoli, probably as a result of the differential rDNA gene expression between homologous chromosomes (Fig. 1).

New cytogenetic and nucleolar variants are present in intraspecific experimental crosses

In contrast with the two chromosomal patterns found in wild and micropropagated plants (I and II, Fig. 2), five karyological phenotypes, including three novel ones, were detected in the progeny resulting from the experimental crosses (50 plants analysed; Tables 2, 3). The three novel cytogenetic phenotypes (III, IV and V; Fig. 2) showed the shared presence of one additional 45S rDNA site. This new site was located in a distal position in the same short arm of the submetacentric chromosome pair 9 in 18 out of 50 (36%) F1 plants (cytogenetic phenotypes III–V, Table 3). FISH signals from this new site were even smaller and lower in intensity than the minor-size site observed in the micropropagated plants used as progenitors. The three new karyological patterns were characterized (Fig. 2) by the presence of one pair of homologous chromosomes carrying equivalent NOR sizes plus an additional rDNA site and had two homomorphic nucleoli (III): one pair of homologous chromosomes carrying equivalent NOR sizes plus an additional site and showing two heteromorphic nucleoli sometimes containing one micro-nucleolus (IV); and one pair of homologous chromosomes showing unequally sized NOR plus an additional site and showing two heteromorphic nucleoli sometimes containing one micro-nucleolus (V).

Finally, to test whether the distribution of the additional 45S rDNA site in the progenies was similar when the *in vitro*-propagated plant was used as pollen or ovule donor in the experimental crosses, the weighted distributions between both crosses were calculated and compared with a chi-square contingency test. The differences were not significant ($\chi^2 = 1.524$, $P = 0.2170$) and clearly suggested that the distribution of number of rDNA sites was irrespective of the direction of the crosses. Additionally, the Mendelian segregation of both progenitors was estimated. The segregation of crosses '♀ wild × ♂ *in vitro*' progenitors was 12:10 conforming to Mendelian expectations (11:11; $\chi^2 = 0.18$, $P = 0.6630$). However, in the reciprocal crosses, ♀ *in vitro* × ♂ wild progenitors, the segregation was 20:8 and significantly deviated from Mendelian expectations (14:14; $\chi^2 = 5.14$, $P = 0.0234$). These results suggest a preferential distortion of the segregation favouring the absence of the additional rDNA cluster in the progenies. This indicates that unexpected progenies with an additional 45S rDNA site were affected by which plant was used as the female or male progenitor.

DISCUSSION

Loss of rDNA repeats or changes in differential expression in *in vitro*-regenerated plants

Three lines of evidence (the presence of a conspicuous secondary constriction in only one of the chromosomes, the strong size reduction of the FISH signals in one 45S rDNA site and the different size of the two nucleoli) assessed by independent experimental techniques are in agreement that the 45S rDNA loci in *in vitro*-regenerated plants suggest a contrasting number of copies or a differential expression of the rRNA genes from those present in wild plants. These two mechanisms (genome restructuring and epigenetic control) are suggested to be influenced by *in vitro* culture affecting rRNA genes (Lee & Phillips, 1988; Bairu *et al.*, 2011) .

Assessing which of the two processes is responsible for this variation in the 45S rDNA locus is beyond the scope of this paper and, in fact, is of marginal relevance for the main aims of the research. However, the following reasoning may be useful for additional research on this topic. The observed 45S rDNA heteromorphism is a constant feature for all analysed cells and individuals of the regenerated plants. It has even been recorded in different phases of the cellular cycle at which the 45S rDNA sites are not active. Assuming that the constant unequal size and intensity of hybridization signals observed are due to a differential rDNA chromatin condensation associated with epigenetic mechanisms affecting the differential expression of rRNA genes would imply a remarkable cytological behaviour not so far reported in the organization of plant rDNA chromatin (Caperta *et al.*, 2007). For these reasons, we speculate that our data more probably suggest that the permanent heteromorphism linked to the condensation of the NOR locus is associated with a loss of number of rDNA units.

The 45S rDNA loci are reputed to be frequent hotspots of chromosome breakage that are involved in somaclonal variation rearrangements and in plant chromosome evolution in general (Lee & Phillips, 1988; Phillips, Kaeppler & Olhoft, 1994; Schubert, 2007; Kovařík *et al.*, 2012). Accordingly, our observations (that all regenerated *Cistus* plants so far

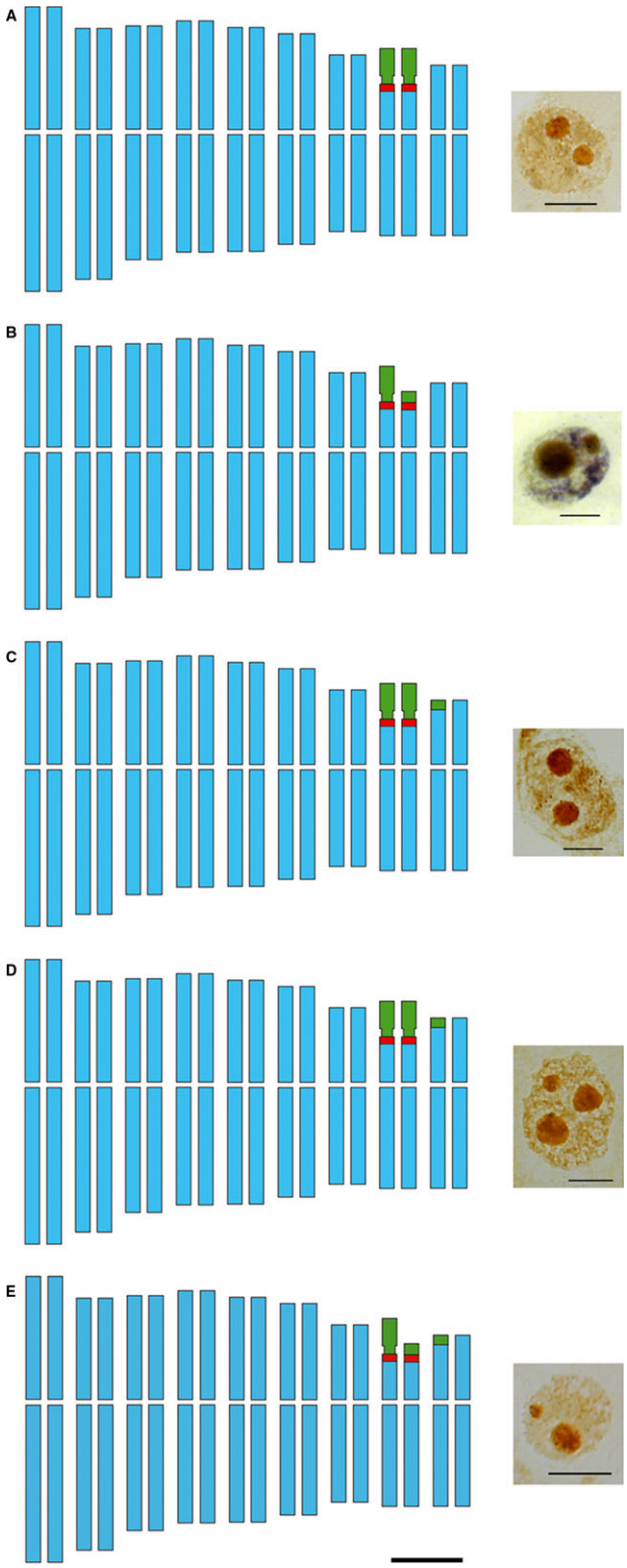


Figure 2. Idiograms and Ag-NOR staining of interphase nuclei in *Cistus heterophyllus* characterizing the five ribosomal phenotypes (I–V) described in Table 2. Left: localization of 45S rDNA (green) and 5S rDNA sites (red) are mapped on the chromosomes (scale bars = 2 μm). Right: Ag-NOR staining shows the expression of individualized NOR sites as independent nucleoli (scale bars = 10 μm).

Table 2. Characteristics of the 45S rDNA sites and nucleoli (rDNA phenotype) observed in *Cistus heterophyllus* accessions

Phenotype	No. of 45S rDNA sites		No. of nucleoli and size
	Ch. pair 8	Ch. pair 9	
I	2 equivalent	–	2 homomorphic
II	2 unequal	–	2 heteromorphic
III	2 equivalent	1 minor	2 homomorphic
IV	2 equivalent	1 minor	2–3 heteromorphic
V	2 unequal	1 minor	2–3 heteromorphic

The heteromorphism was scored by visual inspection of the metaphase plates.
Ch, chromosome.

analysed have suggested a drastic reduction in copy number or expression of ribosomal genes in one homologous rDNA site and that this tissue culture-induced somaclonal variation remains somatically stable and heritable by horizontal transmission) are in agreement with previous observations on behaviour of ribosomal loci during tissue culture propagation (Brettell *et al.*, 1986; Breiman *et al.*, 1987).

rDNA-based genomic instability is amplified after sexual reproduction

The assessment of the extent and degree of somaclonal variation induced by *in vitro* culture is usually restricted to the first generation of micropropagated plants and few studies have monitored the genomic changes produced in later generations. These reports used crops as case studies and the later generations were obtained after self-fertilization, precluding the study of genetically modified regenerants after sexual reproduction with unrelated genotypes. The analysis of progeny between *in vitro* and wild *C. heterophyllus* has shown that the heterozygous condition of the single NOR locus from the *in vitro*-regenerated plant progenitor has segregated properly through sexual reproduction, producing the two expected Mendelian genotypes. In addition, novel unexpected chromosomal and cytological variants associated with the ribosomal genes were observed in 36% of progeny plants. Surprisingly, the mode of inheritance of the novel minor rDNA cluster agreed with a Mendelian segregation only when the male progenitor is used in the artificial crosses.

In theory, the increase in the number of 45S rDNA loci could be explained by an ectopic recombination between non-homologous chromosomes (two bivalents), causing inter-chromosomal interchange of rDNA gene copies. However, this process would cause a concomitant reduction of 45S rDNA copies in the NOR chromosome pair (Schubert, 2007), a fact not observed in the analysed material. Alternatively, the origin of the new 45S rDNA site could be attributed to transposition mediated by the activation of mobile elements in the germ line that were probably induced by the *in vitro* culture process, and genomic effects of which were heritable to the sexual progeny.

The intragenomic mobility of rDNA genes as a consequence of transposon activity has been widely reported in plants and is thought to be one of the major forces driving rDNA locus evolution (Dubcovsky & Dvorák, 1995). The activation of retrotransposons has been reported to be present in *in vitro*-propagated plants and new transposon insertions are involved in somaclonal variation derived from tissue culture (Gao *et al.*, 2009). Thus, it is

Table 3. Distribution of ribosomal phenotypes found in the accessions of *Cistus heterophyllus* used in this study

Accession and origin	rDNA phenotype					Sample size
	I	II	III	IV	V	
C. heterophyllus						
Morocco, wild	10	–	–	–	–	10
Spain, wild	2	–	–	–	–	2
Spain, *in vitro* (first generation)	–	16	–	–	–	16
Spain, *in vitro* (second generation)	–	20	–	–	–	20
C. heterophyllus ♀ (wild, Morocco) × *C. heterophyllus* ♂ (*in vitro* first generation, Spain)	6	6	2	5	3	22
C. heterophyllus ♀ (*in vitro* first generation, Spain) × *C. heterophyllus* ♂ (wild, Morocco)	10	10	1	5	2	28
C. albidus	10					10
C. creticus	10					10

Phenotype characterization is given in Table 2.

likely that during sexual reproduction (either during gamete formation or after zygote formation), activated mobile elements derived from the *in vitro* parental generation would produce a transposition of rDNA copies to a new genomic location. However, it could be argued that transposon activation may not be necessarily linked to the *in vitro* propagation process. As the parents involved in the crossing experiments come from different populations that are likely to be genetically differentiated, the observed cytogenetic changes may rather be induced by genomic stress resulting from fusion of divergent genomes. Although we cannot rule out this hypothesis, it should be stressed that hybridization has been reported to be an active process in *Cistus* (Grosser, 1903), but the number of 45S rDNA loci appears to be highly conserved in the purple-flowered lineage in which all species, except the early-diverging *C. crispus* L., had one locus (C. Totta, M. Rosato, P. Ferrer-Gallego F. Lucchese & J.A. Rosselló, unpubl. data).

Implications for conservation

The dual and antagonist perception of the appearance of somaclonal variation in plant propagation based on biotechnology has a history nearly as long as the first report of its occurrence. Thus, somaclonal variation has been perceived either as the opportunity to establish convenient plant systems not found in nature amenable for plant cell experimentation and bioassays or as undesirable traits departing from the explant source that should be rejected. Although specific applications of somaclonal variation of academic and agronomic importance have been reported (Bairu *et al.*, 2011), one of the great concerns for any micropropagation system is to obtain genetically uniform propagated plants showing a genetic integrity with regard to the explant source (Rani & Raina, 2000). This concern is exacerbated and may remain a major problem if the populations derived from tissue culture and showing somaclonal variation are not confined to artificial environments, but are instead released to the wild where cross-compatible, conspecific true-to-type plants and congeneric relatives are present.

Our research has demonstrated that the *in vitro*-generated plants and a non-negligible portion of the progeny between wild and *in vitro* plants have new ribosomal and cytological variants absent from the wild plants. On the one hand, the NOR heteromorphism in regenerated plants and progeny has implications for the cell phenotype, as the two nucleoli had contrasting sizes, suggesting that at least differential rDNA gene expression between 45S rDNA sites is in operation. On the other hand, the new rDNA locus present in the cross progenies was sometimes expressed, forming a micro-nucleolus. This suggests the presence of canonical rDNA units with a proper structure and epigenetic stability that do not affect its functionality.

There may not be any selective bias in plants to a loss or gain of nuclear rDNA copies, because nuclear genomes show a number in excess of rDNA repeats that are needed for transcription, and epigenetic factors are responsible for their inactivation (Waters & Schaal, 1996). However, although nuclear 45S rDNA has been long regarded as merely involved in ribosome and nucleolar biogenesis, recent evidence has dramatically changed this perception, suggesting that it plays more key roles in the biology of the cell.

Thus, it has been hypothesized that rDNA constitutes a central factor in the maintenance and organization of the genome, modulating cellular homoeostasis by: acting to preserve genome stability, triggering cell ageing and senescence, and regulating genome damage resistance (Kobayashi, 2008; Hein *et al.*, 2012); maintaining genome-wide chromatin structure (Paredes & Maggert, 2009); and modulating variation in gene expression across the genome (Paredes *et al.*, 2011). It has even been suggested that rDNA variation in copy number has a significant impact on the evolutionary ecology of all organisms, mediated through increased phosphorus demand in organisms with high rRNA content (Weider *et al.*, 2005). Additional research would be necessary to assess the effects of the new ribosomal genotypes on the fitness of these newly generated genotypes.

CONCLUSIONS

The cytogenetic data presented in this paper have led to the conclusion that the *Cistus* material from *in vitro* culture should not be used for restoration purposes. Not only have new genotypes departing from the explant source been generated during *in vitro* culture, but new genomic rearrangements have also been produced after crossing with wild plants. If the offspring plants were randomly crossed among each other, then the expected generation of new rDNA genotypes (without taking into account the hypothetical appearance of new genotypes by transposition) would significantly increase after a few generations, distorting the original genetic signal present in the natural populations. Furthermore, the presence of additional 45S rDNA loci in hemizygosis would increase the likelihood of ectopic recombination at meiosis that could generate unbalanced gametes, thus affecting their fertility.

Overall, we discourage the use of the micropropagated material for release into the field unless

comprehensive surveys (in addition to studies of the somaclonal variation generated) of the genetic integrity and stability of the regenerants are performed after several generations of crossing between wild and micropropagated plants. Only with this ancillary knowledge could the role of biotechnology in plant conservation reach its goals.

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