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Karyological evolution in Cistaceae, with emphasis on *Cistus* and *Helianthemum*

Tutor: Prof . Fernando Lucchese
Co-tutor: Prof. Josep A. Rosselló

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Evoluzione cariologica in Cistaceae,
con particolare attenzione ai generi *Cistus* e *Helianthemum*

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Front cover: Iconographies of *Cistus heterophyllus* (purple-flowered individual), *Cistus monspeliensis* (white-flowered individual) and *Helianthemum nummularium* (yellow-flowered individual).

Thesis defense 15th February 2016 in front of the following jury of committee members:
Prof. Ferdinando Boero
(Università del Salento)

Prof. Giovanna Abbate
(Università Sapienza di Roma)

Prof. Romolo Fochetti
(Università della Tuscia)

“Wenn [...] ich dann im hohen Grase am fallenden Bache liege, und näher an der Erde tausend mannigfaltige Gräschen mir merkwürdig werden [...] mein Freund! Wenn's dann um meine Augen dämmert, und die Welt um mich her und der Himmel ganz in meiner Seele ruhn”

“Quando [...] mi stendo nell'erba alta accanto al torrente e, cosí vicino alla terra, scopro le piante più diverse e più singolari [..] oh, amico mio!, i miei occhi si smarriscono in questa vertigine e l'universo e il cielo riposano nella mia anima”

Johann Wolfgang von Goethe (1774)

CONTENTS

PREFACE

This thesis encompasses a general introduction, three independent researches, and general conclusions.

CHAPTER 1. General introduction

The first chapter gives an overview of the basic cytogenetic concepts, followed by the description of the study systems with notes of their systematic, distribution and karyological features. For the last study system, some information about the conservation strategy are also given. At the end of the chapter, the aims of the thesis are explicitly stated.

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Marcela Rosato, Pablo Ferrer-Gallego, Chiara Totta, Emilio Laguna and Josep A. Rosselló. *Botanical Journal of the Linnean Society*, accepted for publication January 2016.

CHAPTER 5. Conclusions

The chapter includes brief synthesis of the major outcomes of this thesis, in the light of the aims stated.

CHAPTER 1

GENERAL INTRODUCTION

Understanding how species interact with each other, and when/where the diversification of clades has taken place provides hints about the process of speciation in plants. A more detailed view can only be achieved by looking at the mechanisms responsible for the reproductive isolation of species. Although the role of chromosomal rearrangements as mechanisms for plant speciation is still debated (Faria and Navarro, 2010), studies on the distribution of ribosomal RNA gene families and changes in chromosome numbers have begun to shed light about the evolutionary significance of chromosomal changes (Weiss-Schneeweiss and Schneeweiss, 2013).

1.1 Genome evolution in plants

Eukaryotic chromosomes are organized linear structures carrying the majority of the genetic material of the organisms and are located within the nucleus of all eukaryotic cells. They differ in size, shape and composition of DNA and proteins (Schubert, 2007). All of these features can be affected during evolution and participate at chromosomal diversification, accompanying taxa diversification and eventually speciation (Stebbins, 1971; Rieseberg, 2001; Levin, 2002; Schubert, 2007; Leitch and Leitch, 2008). Chromosomal rearrangements involving inversions, translocations, duplications, deletions, fusions and fissions result in changes in chromosome number (dysploidy and polyploidy) and chromosome structure (Schubert, 2007; Lysak and Schubert, 2013). Genome size changes and more subtle changes in sequence composition of the repetitive fraction of the genome, most commonly involving expansions or reductions of repetitive DNA sequence amounts, constitute additional sources of variation occurring during the evolutionary history of taxa (Weiss-Schneeweiss and Schneeweiss, 2013). Scientists have long argued over whether – and to what degree – changes in chromosome structure contribute to reproductive isolation and, ultimately, speciation.

Despite polyploidy is much more studied and often viewed as the most common type of chromosome evolution in plants and the main chromosomal driver of plant diversification, dysploidy, defined as the stepwise change of the haploid chromosome number among related species (Stebbins, 1971), is also very common across angiosperms and may frequently co-occur with polyploidy within lineages (Escudero *et al.*, 2014). Although chromosome number change may involve the loss or gain of entire chromosomes, it is much more frequently

achieved via genome restructuring, resulting in centromere/chromosome number reduction or increase with or without significant loss of genetic material (unbalanced and balanced rearrangements, respectively). The majority of earlier studies considered descending dysploidy as the main trend of chromosomes number change in angiosperms (Weiss-Schneeweiss and Schneeweiss, 2013).

1.2 Organization of plant chromosomes

In short, in most angiosperm species the shape of monocentric chromosomes is determined by a centromere (primary constriction), comprised of regions of condensed chromatin, flanked by pericentromeric regions rich in heterocromatin, and telomeres that mark the ends of chromosomes and protect them from degradation (Gill *et al.*, 2008). Based on morphological observation, quantitative chromosome maps called "ideograms" can be constructed (Fukui *et al.*, 1998).

The genome of most higher organisms consists of a large amount of DNA motifs repeated in hundreds or thousands of copies (Britten, 1969). For instance, the *Arabidopsis* genome is 25% repetitive DNA (Leutwiler *et al.*, 1984) whereas in pea it has been estimated to reach up the 95% of the total DNA (Thompson and Murray, 1980). Some sequence motifs are extremely variable while other are highly conserved from one species to another (rRNA genes), and information about their number and chromosomal distribution provide tools to assess potential functions of particular aspects of genome architecture and evolution and for studying inter-organism relationships.

Molecular cytogenetic methods offer a powerful system for looking at the organization of DNA repeat motifs along a chromosome using *in situ* hybridization (ISH). With this technique, labeled probe sequences are able to localize a specific sequence to the denatured DNA of chromosomes spread on microscope slides. In plants, the use of radioactive tracer or modified nucleotides (attached to biotin, digoxigenin, or flourophores) to make ISH probes allows the microscopic visualization and localization of complementary sequences within cells and nuclei, and on individual chromosomes (Figueroa and Bass, 2010). Direct and indirect fluorescence *in situ* hybridization (FISH) has been broadly used over the last 30 years to localize repetitive sequences. Moreover, it can be used to observe genomic organization and chromosome structure, and to look for landmarks that allow the identification of genes, their clustering and orientation.

1.3 45S and 5S rDNA

Tandemly repeated genes encoding 45S (18S-5.8S-25S) and 5S rRNAs (rDNA) are ubiquitous and highly conserved over long evolutionary timescales (Volkov

et al., 2007; Richard *et al.*, 2008 Weiss-Schneeweiss *et al.*, 2008), therefore, their variation in number and distribution can be easily detected and represents a useful model to study and compare chromosome evolution and genome organization in different taxonomic groups (Shan *et al.*, 2003; Volkov *et al.*, 2007; Heslop-Harrison and Schwarzacher, 2011). The 45S rDNA cistron, coding for 18S, 5.8S, and 25S rRNAs, also includes external transcribed spacer (ETS), two internal transcribed spacers (ITS1 and ITS2) and the non-transcribed intergenic spacer (IGS). Numerous 45S rDNA repeats are tandemly arranged in one or more loci in the genome known as nucleolar-organizer regions (NORs). Cytologically, the 45S rDNA appears in the nucleolus in interphase nuclei and forms secondary constrictions in mitosis/meiosis (Volkov *et al.*, 2007).

While FISH detects all 45S rDNA signals, including small and/or inactive ones, the silver staining (Ag-NOR) allows to identify only those loci transcriptionally active in the preceding interphase of the cell cycle (Jiménez *et al.*, 1988), staining both the decondensed NOR-associated proteins on chromosomes or the nucleoli, the nuclear organelle assigned to ribosomes synthesis (Medina *et al.*, 2000), whose maximum number stained corresponds to the maximum number of active NORs in the diploid chromosome complement.

The 5S rDNA repeated unit is comparatively simple: each repeat is composed of a conserved, ca. 121 bp long coding region separated by variable length Non-Transcribed Spacer (NTS). In angiosperm, both the 45S and 5S loci mostly map independently from each other, despite exceptions are known (Sone *et al.*, 1999; Garcia *et al.*, 2010; Garcia and Kovařík, 2013).

Moreover, the ITS and ETS of the 45S rDNA cistron and the NTS of the 5S rDNA array are often use as molecular markers for phylogenetic analysis. Hence, the combination of such genetic and cytogenetic approaches enables the direct correlation of chromosomal loci evolution with phylogenetic relationships (Weiss-Schneeweiss *et al.*, 2007, 2008; Weiss-Schneeweiss and Schneeweiss, 2013; Mahelka *et al.*, 2013).

1.4 Chromosome changes in plant tissue culture

Plant tissue culture is one of the fundamental tools of plant science research and it is extensively employed in the production, conservation and improvement of plant resources. Micropropagation through plant tissue culture has become the most widely used approach for *in vitro* mass production of endangered species (Fay 1992; Iriondo 2001; Rao 2004; Engelmann 2010; Cruz-Cruz *et al*. 2013).

The growth of plant cells *in vitro* and their regeneration into whole plants is an asexual process, involving only mitotic division of the cell and, theoretically, it should not cause any variation. However, the special *in vitro* culture environments can be mutagenic and plants regenerated from organ

cultures, calli, protoplasts and via somatic embryogenesis sometimes exhibit phenotypic and DNA variations (Bouharmont 1994; Orbović *et al.*, 2008), defined as somaclonal variations. Somaclonal variants may differ from the source plant permanently (heritable variation due to a genetic mechanism) or temporarily (changes result from epigenetic or physiological effects and are non-heritable and reversible) (Kaeppler *et al*., 2000).

Changes in ploidy level, 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 aberrations detected in the *in vitro* culture of plant tissues (Al-Zahim *et al.*, 1999; Hao and Deng, 2002; Mujib *et al.,* 2007).

Transposable and retrotransposable elements are mobile DNA sequences in a genome that can induce gene mutations and contribute to genome rearrangements. Transposons account for significant portions of most plant genomes. Activation of cryptic transposable elements is another source of chromosome based somaclonal variation (Grandbastien, 1998). For instance, protoplast isolation or *in vitro* cell or tissue cultures disrupt normal cell function and may activate transposable elements, stress-induced enzymes or other products (Pietsch and Anderson, 2007). It is not unexpected, therefore, that repeated sequence variation has been detected among tissue culture regenerants and that this variation may be responsible for some of the observed phenotypic variability.

The *in vitro* culture induces major modifications of cell metabolism and gene expression, by the activation of growth- and stress-related genes. Hence, transposon research has implications for a better understanding of various areas as the genome evolution and speciation, and the possibility that the activity of plant retrotransposons is directly linked to defense responses.

1.5 Ancestral state reconstruction

To elucidate the evolutionary dynamics of rDNA site numbers in plants, they can be used as character states to trace changes during the history of organisms evolution.

Ancestral state reconstruction is an important approach to understanding the origins and evolution of key features of different living organisms (Liberles, 2007). A variety of ancestral reconstruction including parsimony and likelihood-based methods exist for biomolecular sequencing (Yang *et al.*, 1995; Koshi and Goldstein, 1996; Elias and Tuller, 2007), multistate discrete data (Pagel, 1999, Schultz *et al.,* 1996; Mooers and Schluter, 1999) and continuous data (Martins, 1999). Likelihood-based methods of state reconstruction (both maximum likelihood and Bayesian) have more advantages over parsimony methods. Maximum parsimony reconstruction method finds the ancestral states that minimize the number of steps of character change given the tree and

observed character distribution, while the likelihood reconstruction finds, for each node, the state assignment that maximizes the probability of arriving at the observed states in the terminal taxa, given the model of evolution, and allowing the states at all other nodes to vary (Schluter *et al.*, 1997; Pagel, 1999).

1.6 The study systems

1.6.1 Cistaceae

The family of Cistaceae consists typically of heliophyte shrubs, subshrubs and herbs occurring in open areas on poor soils. Distributed in temperate and subtropical regions of the northern hemisphere, the family shows the highest genus and species diversity in the Mediterranean floristic region. Usually, Cistaceae has been divided into eight genera (ca. 200 species); five of them (*Cistus*, *Fumana*, *Halimium*, *Helianthemum*, *Tuberaria*) are native to the Mediterranean area while the remaining three (*Crocanthemum*, *Hudsonia*, *Lechea*) inhabit temperate regions in America (Arrington and Kubitzki, 2003). The two genera *Cistus* and *Halimium* were recognized as distinct by most botanists, although they share some morphological, palynological and karyological characters (Sánchez Anta *et al.*, 1986; Ukraintseva, 1993). They hybridize in wild and in cultivation, making genus delimitation more tedious. Phylogenetic analysis (Civeyrel *et al.*, 2011; Guzmán and Vargas, 2005) are congruent with the monophyly of the assemblage *Cistus-Halimium* supporting the hypothesis that the separation between *Halimium* and *Cistus* was not appropriate. Therefore Demoly (2006) proposed a new taxonomy, combining the two groups of species in the unique *Cistus* genus.

In literature there are many studies providing details about chromosome number of the Mediterranean species of Cistaceae, which seems to be in line with dysploid chromosome differentiation: *Cistus* (x=9), *Helianthemum* (x=5, 10, 11, 12), *Fumana* (x=8), *Tuberaria* (x=6, 7) (Proctor, 1955; Markova, 1975; Sánchez and Gallego, 1985; Sánchez *et al.*, 1986, Gallego and Aparicio, 1992). These multiple shifts in chromosome number reflect active cytological differentiation in Cistaceae (Guzmán and Vargas, 2009b).

1.6.2 *Cistus*

The genus *Cistus* is a relatively small genus, although shows a noteworthy morphological diversification, such as to be considered one of the most characteristic genera of the Mediterranean flora. The genus (*Cistus-Halimium*) actually counts about 28 species, with the highest taxa diversity occurring in the western Mediterranean, particularly on both sides of the Straits of Gibraltar (Guzmán and Vargas, 2005). The Canary Islands host ten *Cistus* species, of which eight are endemic. *Cistus* is self-incompatible and outcrossing favors the

inter-individual and inter-specific production of hybrids. Consequently, natural hybrids are quite common when *Cistus* species co-occur (Danserau, 1940; Demoly and Montserrat, 1993), and intermediate morphological characters are discernible. Hence, it was hypothesized that the hybridization can be the main evolutionary force in *Cistus* (Dansereau, 1940; Demoly, 1996). Nonetheless, all species display a uniform diploid chromosome number of $2n$=18 and variation of DNA content is not significant among them (Ellul *et al.*, 2002).

Phylogenetic analyses performed with both nuclear and plastidial markers (Guzmán and Vargas, 2005, 2009a, 2009b; Guzmán *et al.*, 2009; Civeyrel *et al.*, 2011; Fernandez-Mazuecos and Vargas, 2010) always confirm the monophyly of the entire assemblage *Cistus-Halimium* and separate *Cistus* lineage into two monophyletic groups: the white-flowered lineage, including all species with white flowers, except *C. parviflorus* with pale-pink petals, and the purple-flowered lineage, mainly including species endemic to the Canary Islands. Taxa formerly classified as *Halimium* fall in three separate clades. The geographical distribution and the genetic analysis of the haplotypes suggest that dispersion and colonization across the Mediterranean basin (Europe, North Africa) have taken place successfully after divergence and species formation, resulting from the onset of the Mediterranean climate change. The history of the Canarian purple-flowered *Cistus* has implied a single mainland-to island invasion event, followed by a recent rapid geographic radiation. Conversely, the white-flowered *Cistus* landed on the islands have not been involved in radiation processes, whereas adaptive radiation occurred throughout the Mediterranean where the group is best represented.

1.6.3 *Helianthemum*

Helianthemum Miller is the biggest genus in the Cistaceae family and currently includes about 110 heliophytic species and subspecies concentrated in the Mediterranean region, the bulk of which occurring in and about the Iberian Peninsula, though a few occur in the Canary and Cape Verde Islands, a single species found in the Asian steppes, and some species extend into Central and Nord Europe (Proctor, 1956; Arrington and Kubitziky, 2003). The genus is divided into two subgenera, *Helianthemum* and *Plectolobum*, whose differences led several authors to consider them as separate genera, *Helianthemum* and *Rhodax* (Löve and Kjellqvist, 1964; Holub, 1970, Markova, 1975). As *Cistus*, also *Helianthemum* is self-incompatible, and frequent events of hybridization and introgression make the taxonomy still uncertain, due to the high even intra-population morphological variability. Thus, each subgenus has further been divided into different sections (Lopez-Gonzalez, 1992).

Cytologically, the subgenus *Helianthemum* shows different base chromosome numbers, where the most represented is x=10 (sect. *Brachypetalum*, *Eriocarpum, Helianthemum, Lavandulaceum*), but also x=5

(sect. *Argyrolepis*) and x=12 (sect. *Caput-felis*) have been registered. In contrast, the subgenus *Plectolobum* (sect. *Atlanthemum* and *Pseudocistus*) presents the uniform base chromosome number of x=11 (Markova, 1975, Sánchez *et al.*, 1986; Arista *et al.*, 1990; Izuzquiza *et al.*, 1998). Only one tetraploid has been reported for the Bulgarian and Armenian populations of *H. lasiocarpum* (2*n*=4x=40) with probably allopolyploid origin (Markova, 1975). Despite these observations and the multitude of reports of *Helianthemum* chromosome numbers, a hypothesis about the pattern of this chromosome number transition is still not available. Moreover, while *Cistus* has been widely investigated in terms of phylogeny and divergence times, studies about the whole *Helianthemum* evolutionary history are in short supply. Nevertheless, the few available data provide the monophyly of *Helianthemum* genus, thus rejecting the possibility of raising the subgenus *Plectolobum* to the *Rhodax* genus rank and supported the section division made by Lopez-Gonzalez (1992).

1.6.4 *Cistus heterophyllus*

Cistus heterophyllus is a narrowly-distributed W-Mediterranean species present in North Africa (Morocco, Algeria) and in the Iberian Peninsula (Spain) (Crespo and Mateo, 1988; Demoly and Montserrat, 1993). The European individuals are extremely endangered because of their 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 *et al*., 2004). In addition, nuclear and plastid DNA markers, together with morphological evidences, strongly suggest that ongoing gene flow with the related *C. albidus* L. is occurring in European and North African populations (Jiménez *et al*., 2007; Navarro *et al*., 2009). In 1987, a single individual showing no signs of interspecific hybridisation was found at E Spain (Valencia) (Crespo and 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 and Martín, 2011) to obtain accessions suitable for reintroduction in a new site (Tancat de Portaceli, Valencia, Spain) (Laguna *et al*., 1998; Aguilella *et al*., 2010). Here, new plants arising from *in vitro*-generated individuals were spontaneously produced between 2012 and 2013. Actually the species is listed as Critically Endangered (CD), according to the Red List criteria of the International Union for Conservation of Nature and Natural Resources, IUCN (Moreno, 2008).

1.7 Aims of the study

Modern molecular cytogenetic offers a number of tools suitable for investigating chromosomal architecture such as nucleolar organizer regions (NORs) and 5S rDNA, centromere and telomeres, as well as for identifying

locations of genes and repetitive elements and for molecular karyotyping, providing interesting information about plant speciation processes. Cytogenetic features may also be useful when studying threatened or rare species, in case of their management is entrusted to micropropagation protocols, since the stress conditions generated by the *in vitro* tissue culture are responsible of chromosomal changes. Despite patterns of chromosome evolution using molecular cytogenetic have been established in several plant genera, studies mainly focus on the relationships within polyploid lineages, whereas little is still known about the evolutionary patterns of chromosome changes in predominantly diploid plant groups.

Cytogenetic analyses, using FISH and AgNOR staining methods, have been employed in an attempt to gather information about the karyotypes of a number of selected species and to elucidate the evolutionary dynamics of 45S rDNA site number and position within the homoploid genus *Cistus* (Chapter 2) and a species sample representative of the whole chromosome base number variation of the genus *Helianthemum* (Chapter 3). With this purpose the rDNA site number features were mapped onto available phylogenies. Furthermore, the same cytogenetic approach has been used to assess the stability of two nuclear ribosomal DNA families (5S rDNA and 45S rDNA) in the offspring of F1 experimental crosses between *Cistus heterophyllus* Desf. (Cistaceae) accessions generated after *in vitro* culture and wild individuals (Chapter 4).

The information collected serve to:

- ascertaining whether changes in the 45S rDNA site features are phylogenetically-correlated within the genus *Cistus*, and whether the organismal evolutionary history may affects the patterns of rDNA site changes;
- evaluating whether the 45S rDNA site changes reflect the taxonomic infrageneric ranks and the chromosome base number variations, and whether they are phylogenetically-correlated within the genus *Helianthemum*;
- verifying whether the *in vitro*-propagated plants of *C. heterophyllus* have produced genetically modified or genetically unstable regenerants not present in the original genotype stock, and drowing conclusions with respect to the use of micropropagated material for the management of endangered species.

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CHAPTER 2

Temporal frames of 45S rDNA site-number variation in homoploid plant lineages: lessons from the rock rose genus (*Cistus*, Cistaceae)

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INTRODUCTION

Genes encoding ribosomal RNA (rDNA) are universal constituents of cell genomes and are essential for organismal growth and integrity, since their products form the backbones of cytoplasmic, plastidial, and mitochondrial ribosomes (Hillis and Dixon, 1991). In contrast with the single or low-copy number of rDNA genes present in the plastidial and mitochondrial genomes, the nuclear genome harbours hundreds to several thousand copies of each ribosomal species (18S, 5.8S, 25S/26S, 5S) that are usually arranged in distinct arrays of tandemly-repeated units (Srivastava and Schlessinger, 1991).

Although rDNA is the most abundant gene family and occupies a large fraction of the nuclear genome, it is also the most unstable genomic region (Kobayashi, 2008). The reasons for this instability are not fully appreciated; however, it has been reported that rDNA loci are the predominant sites of repeated recombination (Kobayashi and Ganley, 2005). Thus, unequal recombination between homologous and homoelogous loci may trigger both intragenomic fluctuation in rDNA copy number and amplification of new arrays (Cronn *et al.,* 1996; Tsang and Carr, 2008). Furthermore, it has been shown that rDNA arrays and neighbouring regions are one of the frequent targets for mobile element insertions (Raskina *et al.*, 2008). Transposition may promote the evolutionary dynamics of rDNA loci not only across species radiation but also during intraspecies differentiation and domestication, producing karyological rearrangements that may be the onset of speciation processes (Pedrosa-Harand *et al.,* 2006).

In plants, detailed knowledge about the number of rDNA loci, their genomic location, and rDNA linkage are known for a substantial number of species (Garcia *et al.* 2012, 2014; Roa and Guerra, 2012). However, information concerning the dynamics of rDNA loci among closely related species only becomes biologically relevant when put into an evolutionary or phylogenetic framework.

One of the best-studied systems in plants for assessing the dynamic turnover of rDNA loci is polyploidy. The rapid rearrangement of parental genomes after polyploid formation is now a well-established paradigm in plant genome evolution (Wendel, 2000). The available knowledge suggests that the fate of 45S rDNA units in plant polyploids after their genesis does not follow a uniform evolutionary pattern (Volkov *et al.*, 2007). Because the ancestors of many polyploids have been fully identified, the synthetic reconstruction of the polyploids has been possible in many cases, and the temporal frame and exact origin of polyploid formation are usually known, the dynamic distribution patterns of rDNA loci have been confidentially assessed.

In sharp contrast with polyploids, assessing the evolutionary patterns of rDNA loci change in predominantly diploid lineages has usually received less

attention (e.g., Datson and Murray, 2006). Unfortunately, the conclusions drawn from previous studies lack explicitly temporal frames for rDNA loci changes. This is unfortunate since the rates of chromosomal diversification and rDNA dynamics could not be assessed, and their comparison with those known for polyploid lineages could not be established. In addition, eventual correlations between rDNA changes and known significant palaeoecological and palaeoclimatic events could be missed.

The genus *Cistus* (including *Halimium*; Cistaceae) provides a suitable case study for assessing the temporal patterns of rDNA site evolution in entirely diploid lineages. Congruent phylogenies based on a suite of plastidial and nuclear DNA markers have been obtained, and dated nodes for virtually all known species (ca. 28) are available (Guzmán and Vargas, 2005, 2009a, 2009b, 2010; Guzmán *et al*., 2009; Fernández-Mazuecos and Vargas, 2010, 2011; Civeyrel *et al*., 2011). In addition, several important evolutionary patterns and processes have been revealed for *Cistus* (Table 2), including colonization and asymmetric diversification of insular oceanic lineages, synchronous evolutionary histories in Mediterranean and Macaronesian species, adaptative radiation in non-insular lineages, and long-distance colonization events despite the absence of effective dispersal mechanisms.

In this study, the number and position of 45S rDNA loci have been determined in *Cistus* species using fluorescent *in situ* hybridisation (FISH) studies and Ag-NOR staining, and mapped onto dated phylogenies in an attempt to ascertain whether (a) a phylogenetically-correlated variation in rDNA loci features is present within the genus, (b) rates of rDNA site evolution are equivalent in all lineages, and (c) organismal evolutionary history may have an effect on the patterns of rDNA site or family change.

MATERIALS AND METHODS

Plant materials

Thirty-five taxa of the Cistaceae family belonging to *Cistus, Tuberaria* and *Fumana* genera were analyzed in this study. Seeds were obtained from field sampling, botanical gardens, plant breeding stations and research centers. The origin of the plant analyzed material is provided in 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 4ºC, then 2 h at room temperature, fixed in an ethanol/glacial acetic

acid (3 : 1) mixture and stored at –20ºC until required. For mitotic chromosome spreads, we followed the protocols described in Rosato *et al.* (2008).

Fluorescence in situ hybridisation

The 45S rDNA multigene family was localised using the pTa71 (Gerlach and Bedbrook, 1979) clones. The pTa71 probe was labelled with digoxigenin-11dUTP through a nick translation procedure (Roche, Germany). We followed the *in situ* hybridisation protocols of Rosato *et al.* (2008), except for the proteinase K pre-treatment, which was performed following Schwarzacher and Heslop-Harrison (2000). Probe detection was conducted using the method of Zhong *et al.* (1996) with modifications according to Galián *et al.* (2014).

Ag-NOR staining

Silver impregnation was carried out on 1–2 day-old chromosome preparations according to the protocol described in Rosato and Rosselló (2009).

Karyotype analysis

Chromosome measurements were made on digital images using the freeware software MICROMEASURE v.3.3 (available at http://www.colostate.edu/depts/biology/micromeasure). Idiograms were obtained from chromosome measurements of at least five well-spread metaphase plates.

rDNA site-number evolution

Mapping of cytogenetic features onto a phylogenetic framework was carried out following the likelihood reconstruction methods in Mesquite, version 3.04 (Maddison and Maddison, 2015), assigning to each internal node the state that maximizes the probability of obtaining the observed states in the terminal taxa under the specified model of evolution (Mk1 model, in this study). In addition, the most parsimonious reconstruction of the ancestral character states for the number of rDNA sites were also estimated using the Mesquite software.

The phylogenetic tree used as an evolutionary framework for *Cistus* was the backbone consensus tree obtained by several plastidial and nuclear markers, e.g. *rbcL*/*trnL-trnF* (Guzmán and Vargas, 2009a), *rbcL*/*trnK-matK* (Guzmán and Vargas, 2009b), *trnL-trnF*/*trnS-trnG*/*trnK-matK*/*rbcL*/*ITS*/*ncpGS* (Guzmán *et al.*, 2009), *trnL-trnF*/*matK* (Fernández-Mazuecos and Vargas, 2010), *trnS-trnG*/*trnK-matK* (Guzmán and Vargas, 2010), and *trnS-trnG*/*trnL-trnF* (Civeyrel *et al.*, 2011). *Fumana thymifolia* and *T. lignosa* were used as outgroups (Guzmán and Vargas, 2005, 2009a, 2009b, 2010; Guzmán *et al.*, 2009; Fernández-Mazuecos and Vargas, 2010). Dated nodes obtained by relaxed clock methods by Guzmán *et al.* (2009) and Guzmán and Vargas (2009a) were indicated in the consensus tree.

Table 1 - Cistaceae studied taxa, sources of material and seed accessions. BGB-D, Botanischer Garten Berlin-Dahlem; CBNM, Conservatoire Botanique National Méditerranéen de Porquerolles; CIEF, Centro para la Investigación y Experimentación Forestal; IJBB, Jardí Botànic de Barcelona; JBC, Jardín Botánico de Córdoba; JBCA, Jardín Botánico El Castillejo; JBCVC, Jardín Botánico Canario Viera y Clavijo; JBDO, Jardín Botánico Dunas del Odiel; MHN, Muséum d'Histoire Naturelle; RJB-CSIC, Real Jardín Botánico de Madrid.

Taxon	**Origin**	**Accession**
Cistus		
C. albidus L.	Portugal, Algarve, Lagoa	BGB-D. 460**.** PT-0.B-0100208
C. atriplicifolium Lam.	Spain, Alicante, Villena	CIEF A26E
C. calycinus L.	Spain, Cádiz, Trafalgar	JAR-1
C. chinamadensis Bañares & Romero subsp. *gomerae* Bañares & Romero	Spain, Canary Islands, La Gomera	JBCVC 2674-B
C. clusii Dunal	Spain, Valencia, Villamarchante	CIEF V24B
C. creticus L. subsp. *creticus*	Cyprus, Paphos, Polis	BGB-D. 463. CY-0-B-2400300
subsp. *eriocephalus* (Viv.) Greuter & Burdet	Italy, Grosseto, Marina di Grosseto	BGB-D. 469. IT-0-B-1971186
C. crispus L.	Spain, Valencia, Torreblanca	CIEF C67A
C. grancanariae Marrero-Rodríguez *et al.*	Spain, Canary Islands, Gran Canaria, Cabo Verde-Moya	JBCVC 3567-B
C. halimifolius L. subsp. *multiflorus* (Salzm. ex Dunal) B. Bock	Spain, Huelva, Mazagón	JBDO without number
C. heterophyllus Desf.	Morocco, Targuist-Alhucemas	CIEF M6A
C. horrens Demoly	Spain, Canary Islands, Gran Canaria, Mogana	JBCVC 3580-B
C. ladanifer L. subsp. *ladanifer*	Spain, Valencia, Sinarcas	CIEF V51A
C. lasianthus Lam. subsp. *alyssoides* (Lam.) Demoly	Spain, Burgos, Torres de Abajo	IJBB 1370-JBB-Uribe
C. laurifolius L. subsp. *laurifolius*	France, Prades, Nyer	MHN 96-307
subsp. *atlanticus* (Pitard) Sennen	Morocco, Taza, Tazekka	IJBB 3372-Marroc
C. libanotis L.	Spain, Huelva	JBDO Hu 800-450

Table 1 (Continued)

Taxon	Origin	Accession
C. monspeliensis L.	Spain, Canary Islands, Gran Canaria, Artenara	JBCVC 2880/B
	France, Montpellier, Saint-Mathieu-de-Tréviers	BGB-D. 474. FR-0-B-2042205
C. ochreatus Chr. Sm. ex Buch	Spain, Canary Islands, Gran Canaria, Artenara	JBCVC 1402/B
C. ocymoides Lam.	Spain, Huelva, Aracena	CIEF Hu1A
C. osbeckiifolius Webb ex Christ.	Spain, Canary Islands, Tenerife, La Orotava	JBCVC 3641/B
C. palmensis Bañares & Demoly	Spain, Canary Islands, La Palma, San Andrés y Sauces	JBCVC 3617/B
C. parviflorus Lam.	Greece, Crete, Akrotiri, Chania	BGB-D. 475. GR-0-B-2680597
C. populifolius L.	Spain, Valencia, Serra	CIEF V92A
C. pouzolzii Delile	France, Alès, Saint Jean du Gard	CBNM without number
C. psilosepalus Sweet	Spain, Toledo, Velada	RJB-CSIC JGF.030
C. salviifolius L.	Greece, Dodecanese, Karpathos	BGB-D. 476. GR-0-1270207
C. symphytifolius Lam.	Spain, Canary Islands, Gran Canaria Artenara	JBCVC 3656/B
C. umbellatus L.		
subsp. *micranthus* Demoly	Greece, Messinía, Spárti	BGB-D. 580**.** GR-O-B-1901502
subsp. *viscosum* (Willk.) Demoly	Spain, Valencia, Castielfabib	CIEF A65A
Tuberaria		
Tuberaria lignosa (Sweet) Samp.	Spain, Valencia, Sinarcas	CIEF V141A
Fumana		
F. clausonis Pomel	Morocco, Beni Mellal	IJBB 165-Marroc
F .ericifolia Wallr	Spain, Valencia, Sot de Chera	CIEF V273A
F. fontanesii Pomel	Spain, Murcia, Sierra de Espuña	CIEF Mu4A
F. thymifolia (L.) Spach	Spain, Balearic Islands, Formentera	CIEF IB6A

RESULTS

Karyotype analysis

The chromosome numbers of thirty-five species and subspecies belonging to *Cistus* (30), *Tuberaria* (1), and *Fumana* (4) were determined and the idiograms for *Cistus* and *Tuberaria* samples were assessed. No departures from the previous known chromosome counts were found and the chromosome number of *Cistus grancanariae* (2*n*=18) was determined for the first time. For most species, the karyotype was composed by seven metacentric, one submetacentric and one metacentric-submetacentric chromosome pairs with slight variations in *C. clusii* and *C. umbellatus* (Table 2). In contrast, the *Tuberaria lignosa* karyotype (2*n*=14) was characterized by the presence of two metacentric and five submetacentric chromosome pairs. In *Fumana* species (2*n*=32) idiograms could not be assessed due to difficulties in defining the centromere position of most chromosomes.

Variation in the number and localization of 45S rDNA loci

45S rDNA site-number variation in *Cistus* ranged from one to four, whereas two loci were present in *Tuberaria*, the sister clade of *Cistus*, and in the four analyzed species of *Fumana* (Table 2).

Most species of *Cistus* showed one or two 45S rDNA loci, accounting for the 43.3% and 46.7% on the entire sample analyzed, respectively. Higher numbers of loci were rarely present; three loci were found in *C. monspeliensis* and *C. umbellatus* subsp. *viscosum*, while the maximum number of 45S rDNA loci, four, was present exclusively in *C. grancanariae* (Figure 1).

The 45S rDNA sites were mostly localized at the sub-terminal regions of chromosomes and were associated to secondary constrictions. In a few cases, a satellite portion was observed attached to the chromosome body by a de-condensed string of labeled chromatin. No interstitial or proximal rDNA signals were observed.

According to the localization of the 45S rDNA sites along the chromosome, four landmarks (types I-IV) were defined (Figure 1, *g*). Type I represented chromosomes bearing a sub-terminal 45S rDNA site on the short arm. Type II consisted of a chromosome showing one 45S rDNA site on the short arm followed by a minor satellite region. Type III was characterized by the presence of a sub-terminal 45S rDNA site on the long arm and type IV by a 45S rDNA locus located on the long arm attached by a conspicuous satellite region.

Chromosome type I was present in all *Cistus* accessions, whereas type III co-occurred in only seven accessions. The less represented rDNA locations

were observed exclusively in *C. lasianthus* subsp. *alyssoides* and *C. psilosepalus* (type II), and in *C. libanotis* and *C. salviifolius* (type IV) (Table 2).

For those species showing more than a single rDNA locus their NOR activity was assessed by silver staining, and the maximum number of interphase nucleoli was recorded. In all but one the analyzed taxa all 45S rDNA loci were transcriptionally active, since the number of the FISH signals equated the maximum number of nucleoli detected. In *C. grancanariae*, the maximum number of nucleoli observed was six, suggesting that one of the four 45S rDNA loci was silenced. Often odd numbers of heteromorphic nucleoli were observed (Figure 2).

Table 2 - Karyotypic features of the analyzed species. The chromosome number, the haploid karyotype formula, the total number of 45S rDNA loci with their distribution in each rDNA-bearing chromosomal type and maximum number of nucleoli observed are indicated.

Taxon	2*n*	Haploid karyotype formula	45S rDNA loci	Chromosome type				Max. no. of nucleoli
				I	II	III	IV	
Cistus								
C. albidus L.	18	8m + 1sm	1	1	-	-	-	
C. atriplicifolium Lam.	18	7m + 2sm	1	1	-	-	-	
C. calycinus L.	18	8m + 1sm	2	2	-	-	-	4
C. chinamadensis Bañares & Romero								
subsp. *gomerae* Bañares & Romero	18	8m + 1sm	1	1	-	-	-	
C. clusii Dunal	18	3m + 6sm	2	2	-	-	-	4
C. creticus L.								
subsp. *creticus*	18	8m + 1sm	1	1	-	-	-	
subsp. *eriocephalus*								
(Viv.) Greuter & Burdet	18	8m + 1sm	1	1	-	-	-	
C. crispus L.	18	6m + 3sm	2	1	-	1	-	3
C. grancanariae Marrero-Rodríguez *et al.*	18	8m + 1sm	4	3	-	1	-	6
C. halimifolius L.								
subsp. *multiflorus* (Salzm. ex Dunal) B. Bock	18	7m +-2sm	2	2	-	-	-	4
C. heterophyllus Desf.	18	7m + 2sm	1	1	-	-	-	
C. horrens Demoly	18	7m + 2sm	1	1	-	-	-	
C. ladanifer L.	18	7m + 2sm	1	1	-	-	-	
C. lasianthus Lam.								
subsp. *alyssoides* (Lam.) Demoly	18	7m + 2sm	2	1	1	-	-	3
C. laurifolius L.								
subsp. *atlanticus* (Pitard) Sennen	18	8m + 1sm	2	1	-	1	-	4
subsp. *laurifolius*	18	7m + 2sm	2	1	-	1	-	3

Table 2 (Continued)

Taxon	2*n*	Haploid karyotype formula	45S rDNA loci	Chromosome type				Max. no. of nucleoli
				I	II	III	IV	
Cistus								
[1]*C. monspeliensis* L.	18	8m + 1sm	3	2	-	1	-	6
[2]*C. monspeliensis* L.	18	8m + 1sm	3	2	-	1	-	5
C. ochreatus Chr. Sm. ex Buch	18	-	1	1	-	-	-	
C. ocymoides Lam.	18	7m + 2sm	2	2	-	-	-	4
C. osbeckiifolius Webb ex Christ.	18	8m + 1sm	1	1	-	-	-	
C. palmensis Bañares & Demoly	18	8m + 1sm	1	1	-	-	-	
C. parviflorus Lam.	18	7m + 2sm	2	1	-	1	-	3
C. populifolius L.	18	7m + 2sm	2	2	-	-	-	4
C. pouzolzii Delile	18	7m + 2sm	2	2	-	-	-	4
C. psilosepalus Sweet	18	8m + 1sm	2	1	1	-	-	3
C. salviifolius L.	18	8m + 1sm	2	1	-	-	1	4
C. symphytifolius Lam.	18	7m + 2sm	1	1	-	-	-	
C. umbellatus L.								
subsp. *micranthus* Demoly	18	6m + 3sm	1	1	-	-	-	
subsp. *viscosum* (Willk.) Demoly	18	3m + 6sm	3	3	-	-	-	5
Tuberaria								
Tuberaria lignosa (Sweet) Samp.	14	2m + 5sm	2	1	1	-	-	4
Fumana								
F. clausonis Pomel	32	-	2	2				
F. ericifolia Wallr	32	-	2	2				3
F. fontanesii Pomel	32	-	2	2				
F. thymifolia (L.) Spach	32	-	2	2				na

na, not analysed.

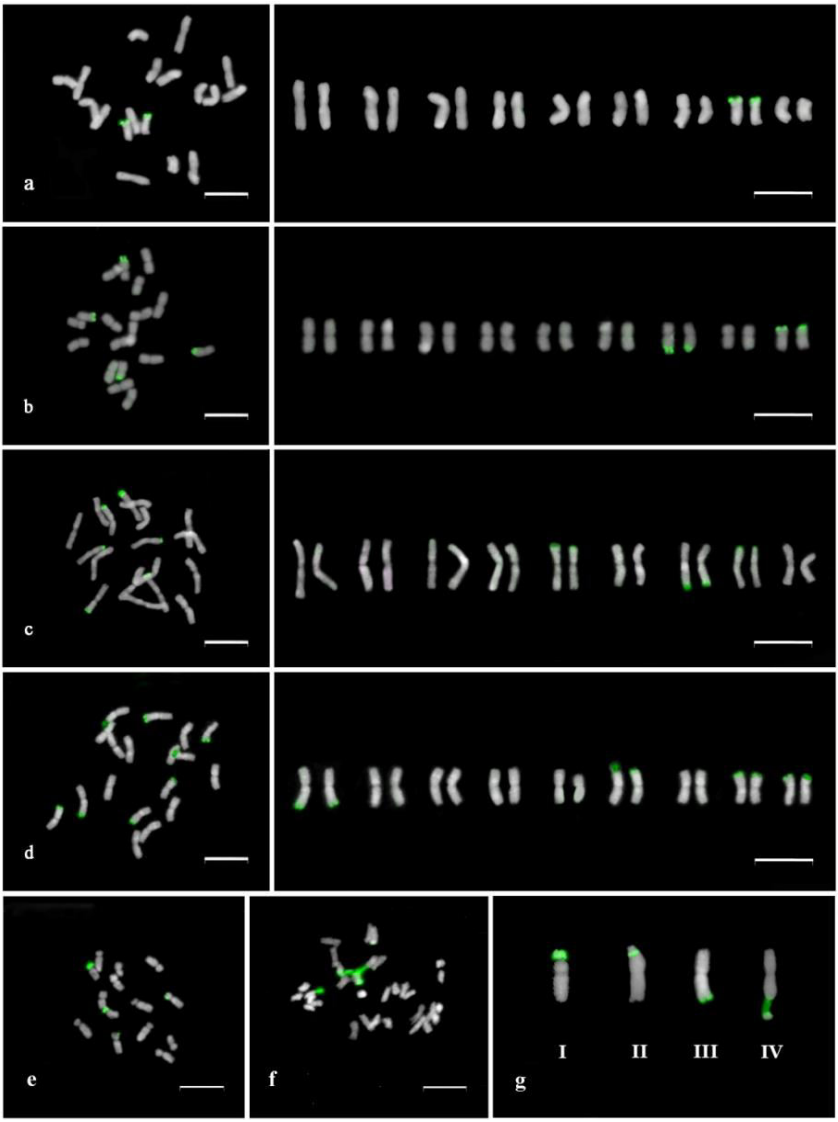

Figure 1 - Arrangement of chromosomes in metaphase plates and idiograms showing the location of 45S rDNA sites in (*a*) *C. creticus*, with one single 45S rDNA locus (*b*) *C. libanotis* with two 45S rDNA loci (*c*) *C. monspeliensis* with three 45S rDNA loci and (*d*) *C. grancanariae* with four 45S rDNA loci, and in the related genera *T. lignosa* (*e*) and *F. thymifolia* (*f*) both with two 45S rDNA loci. The four chromosome landmarks based on the different position of 45S rDNA loci are indicated (*g*). Scale bars represent 10 μm.

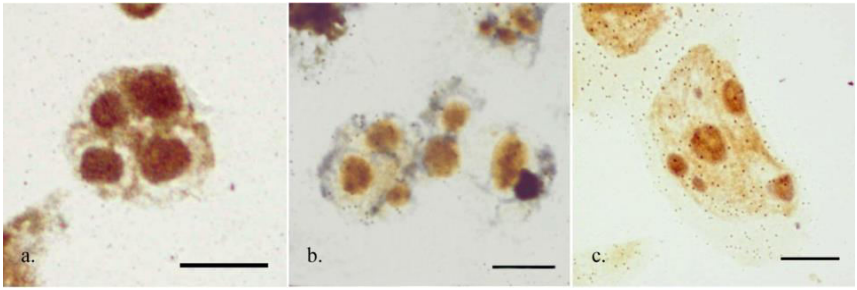

Figure 2 - Maximum number of silver-stained interphase nucleoli on non-pretreated root tips. (*a*) *Cistus libanotis*, (*b*) *Cistus monspeliensis*, and *(c) Cistus grancanariae*. Scale bar represents 10 μm.

Evolutionary trends in rDNA site-number change

The results of the maximum likelihood and maximum parsimony reconstructions of the ancestral 45S rDNA site number state for each node are indicated in Table 3. Both the methods inferred on the backbone phylogenetic chronogram (Figure 3) yielded identical results with a single exception. Overall, the analysis showed multiple shifts between the number of the rDNA site of *Cistus* taxa as independent events occurred belatedly during the evolution of the group dated starting from the Pliocene-Pleistocene about 2 Ma (Guzmán *et al.*, 2009). The most encountered states were two and one 45S rDNA sites. The two-loci state was unambiguously optimized as the ancestral condition and a stasis was inferred at the common ancestor of most of the main clades diversified during the Pleistocene. The first loss event of one rDNA locus has been inferred in the common ancestor of the lineage including the purple-flowered species - whose diversification took place recently in the Mid Pleistocene - after the splitting of the basal *C. crispus* (node 9). The loss event has been supported by a high likelihood score (0.93) and involved the whole clade without further changes. The one-site state appeared independently from the two-site ancestral state also at several terminal nodes. In contrast, rDNA loci amplification have evolved independently only twice at the *C. monspeliensis* and *C. umbellatus* subsp. *viscosum* terminal nodes. The inferred ancestral state for the *C. umbellatus* subsp. *micranthus* and *C. umbellatus* subsp *viscosum* clade (node 13) was ambiguous in the parsimony with all the recorded state numbers as equally parsimonious. However, the maximum likelihood method placed the one-locus state as the most likely.

Table 3- Likelihood scores and Parsimony results of the three possible states of 45S rDNA loci number at each node indicated in Figure 3.

Node	Maximum Likelihood 45S rDNA			Maximum Parsimony 45S rDNA		
	1 locus	2 loci	3 loci	1 locus	2 loci	3 loci
1	0.01	0.98	0.01	-	+	-
2	0.00	1.00	0.00	-	+	-
3	0.00	1.00	0.00	-	+	-
4	0.01	0.99	0.00	-	+	-
5	0.00	1.00	0.00	-	+	-
6	0.01	0.99	0.00	-	+	-
7	0.06	0.93	0.01	-	+	-
8	0.12	0.87	0.01	-	+	-
9	0.93	0.07	0.00	+	-	-
10	1.00	0.00	0.00	+	-	-
11	1.00	0.00	0.00	+	-	-
12	1.00	0.00	0.00	+	-	-
13	0.37	0.31	0.32	+	+	+
14	0.04	0.96	0.00	-	+	-
15	0.11	0.89	0.00	-	+	-
16	0.00	1.00	0.00	-	+	-
17	0.00	1.00	0.00	-	+	-

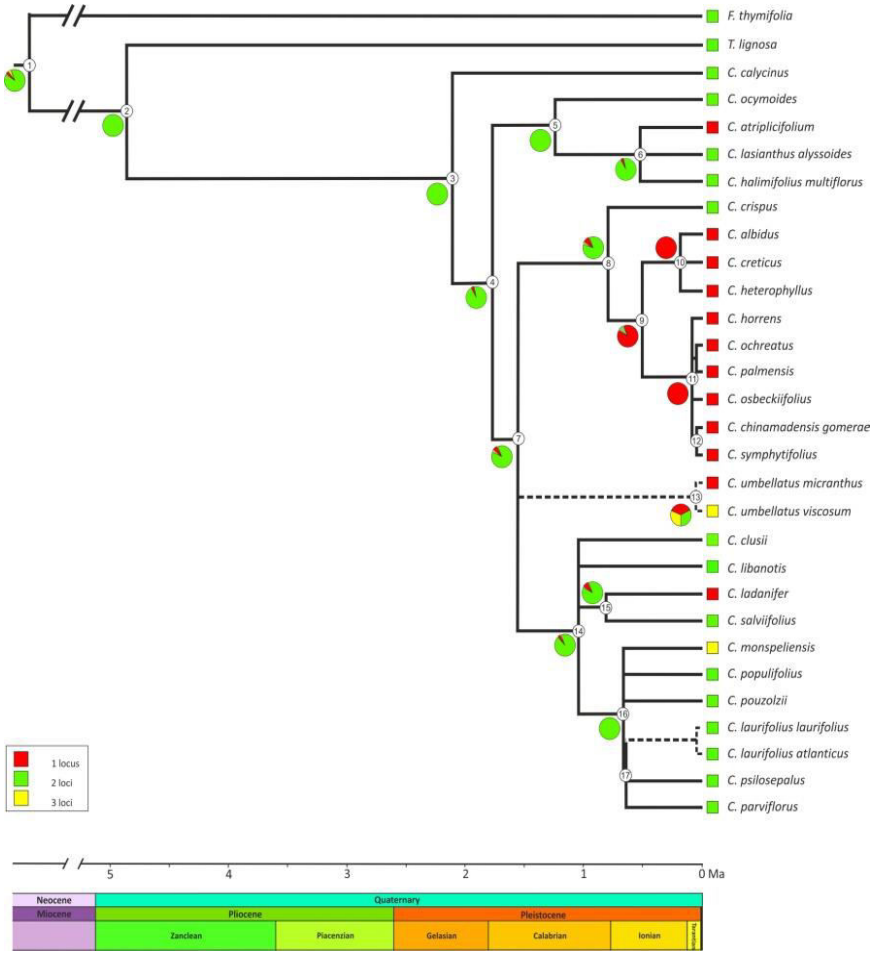

Figure 3 - Ancestral 45S rDNA site number reconstruction mapped on the backbone *Cistus* chronogram. Pies illustrate relative likelihood estimates of the three possible states of 45S rDNA loci at each node. Numbers in the circles correspond to the node numbers indicated in Table 3. Dotted line indicates absence of age estimate.

DISCUSSION

Karyotype stasis

The present study confirms previous reports indicating that *Cistus* species are very uniform in chromosome number (2*n*=2x=18). Moreover, karyological uniformity is also detected in the karyotype patterns, mainly characterized by metacentric and sub-metacentric chromosome pairs, with little interspecific chromosome variability. *Cistus grancanariae*, not previously analyzed, has shown the same chromosome number and the karyotype was highly similar to that observed in the most related species *C. monspeliensis*. The uniformity in chromosome number and coarse karyotype features may be the result either of the two ways used to explain the karyotype evolution among related taxa: the karyotype orthoselection and the karyotype conservation (White, 1973). Karyotypic orthoselection is a process wherein the same type of chromosomal rearrangement is established repeatedly in the same lineage whereas karyotype conservation is the maintenance of similar chromosome morphology in different taxa through a lack of structural mutations. Similar conserved chromosome pattern has been reported for homoploid species like *Helianthus* (Lai *et al.*, 2005) and *Nemesia* (Datson and Murray, 2006).

This karyotype stasis agrees with the young age of the genus inferred from molecular clock analyses (Guzmán & Vargas, 2009a, 2009b, 2010; Guzmán *et al*. 2009; Fernández-Mazuecos & Vargas, 2010), by which the diversification of *Cistus* species took place not earlier than the Pliocene, and mainly in the Pleistocene, largely driven by the Mediterranean climate changes occurred around 3.4-2.8 Ma ago (Suc, 1984; Palamarev, 1989; Fiz-Palacios and Valcárcel, 2013).

The karyotype conservatism displayed by *Cistus* and most of the paraphyletic *Halimium* group support the monophyletic origin of the assemblage *Cistus-Halimium* (Guzmán & Vargas, 2009a).

Changes in 45S rDNA sites in a genus characterized by conserved karyotype

When polyploidy is not taken into account and no chromosomal repatterning is evident, evolution of the number and location of the 45S rDNA is thought to proceed by gains and losses of sites. Different mechanisms have been postulated to account for the mobility and polymorphism of numbers, sizes and positions of rDNA sites, such as transposition, and chromosome rearrangements (translocation, inversion, duplication, deletion) caused by homologous or non-homologous unequal crossing-over (Raskina *et al*, 2008; Schubert and Lysak, 2011). These processes could act alone or in combination,

and they do not necessarily imply changes in overall chromosome morphology (Hall and Parker, 1995; Datson and Murray, 2006). In *Cistus*, the mapping of 45S rDNA family on chromosomes allows to identify a restricted variation concerning both number and position of sites in a homoploid series. The number and location of 45S ribosomal sites follows the common distribution pattern found in most diploid angiosperm species (Roa and Guerra, 2012), occurring mainly in a number of one or two loci at the sub-terminal chromosome regions on short arm and sometimes on long arm, rarely showing a satellite portion after the FISH signal. The location of rDNA sites in the terminal chromosome region in most angiosperms has been explained as the result of homologous recombination constrains, which play a fundamental role in the homogenization of intralocus and interloci repeats (Wendel *et al.,* 1995). Homogenization is an essential process to reduce the nuclear variability of this key molecule, ensuring the removal of non-functional units from the clusters. The sub-terminal position of rDNA sites allows the occurrence of rearrangements without deleterious effects related to gene balance and meiotic segregation (Hanson et al., 1996; Pedrosa-Harand et al., 2006; Roa and Guerra, 2012). Higher loci number have been also represented but in a rather lower percentage. *C. grancanariae* has exhibit a unique karyological phenotype being the only species with four 45S rDNA sites, one of which was silenced. The transcriptional inactivity of the site detected in the sample could be explained with the nucleolar dominance phenomenon, as a manifestation of rRNA gene dosage control, which was mainly described for hybrid species (Costa-Nunes *et al.*, 2010) but also occurs in non-hybrids, regulating the number of active rRNA genes according to the cellular demand for ribosomes and protein synthesis (Tucker *et al.*, 2010). This mechanism could be also responsible of the heteromorfism observed between nucleoli, indicating that different pattern of transcriptional expression between rDNA genes is operating (Caperta *et al.*, 2002). The odd nucleoli numbers observed in many species may be the result of nucleoli fusion, a phenomenon that has been reported for many plant species (Schubert and Kunzel 1990, Bustamante *et al.*, 2014; Rocha *et al.*, 2015).

Phylogenetic interpretation of rDNA site-number change

Despite the observed changes in 45S rDNA sites may appear unenlightening, the information concerning the dynamics of the loci number becomes more relevant when analyzed into the phylogenetic scenario.

Cistus actually includes species formerly classified in two separate taxonomic groups, *Cistus* and *Halimium* (Willkomm, 1956). Demoly (2006) suggested that morphological, palynological and biomolecular data are convergent to indicate that separation between the two genera is not appropriate. Therefore, he proposed to transfer *Halimium* into the genus *Cistus*. The consensus tree obtained by several plastidial and nuclear markers (Guzmán

and Vargas, 2009a, 2009b, 2010; Guzmán *et al*., 2009; Fernández-Mazuecos and Vargas, 2010; Civeyrel *et al.*, 2011) is congruent with the monophyly of the assemblage *Cistus-Halimium* and groups the species into three well-supported clades named yellow-flowered species, consisting of species formerly included in the genus *Halimium*, (Civeyrel *et al.*, 2011), purple-flowered species and white-flowered species (Guzmán and Vargas, 2005). The *Cistus umbellatus* clade clusters with *Cistus* s.s. species despite the analysis does not allow to clarify the relationship between *C. umbellatus* and the other groups (Civeyrel *et al.*, 2011).

The mapping of 45S rDNA loci number as character state onto the backbone *Cistus* species consensus tree seems to show a congruence of the changes to the tree topology. Two 45S rDNA loci has been the most likely ancestral state for the Cistaceae studied sample and a stasis has been mainly inferred during the evolution of the genus. Indeed, the diversification of the yellow- and of the white-flowered species lineages has not implied any 45S rDNA number change and the few observed variations have occurred as independent events or as changes in more recent lineages. This is the case of *C. monspeliensis*, whose differentiation between canarian and mainland populations took place about 0.90 Ma (Fernández-Mazuecos and Vargas, 2011). In this group it has been observed a tendency toward an amplification of loci, with the gain of one locus in *C. monspeliensis*, irrespective of the accessions provenience, and with a further increase in *C. grancanariae*. Conversely, the diversification of the purple-flowered species lineage has been characterized by a single event of loss of one NOR after the splitting of *C. crispus* about 1.23 Ma and no further changes from this loci number condition have occurred in the most recent lineages. Therefore, the rapid species radiation responsible of the differentiation of the two sub-lineages of mainland (0.66 ± 0.18 Ma) and Canary islands taxa (0.33 ± 0.14 Ma) (Guzmán and Vargas, 2010) does not appear associated to an evident karyotype diversification.

In the clade of the two *C. umbellatus* studied subspecies has been observed a remarkable difference in the nuclear ribosomal loci number, implying the occurrence of both amplification and loss events. The ancestral loci number state inferred with the analysis shows a loss of one locus at the common ancestor of the lineage in comparison to the most ancestral state of *Cistus*. This condition changes again in *C. umbellatus* subsp. *viscosum* through the amplification of two 45S ribosomal loci. Moreover, *C. umbellatus* has also been the only species showing slight intra- and inter-specific karyotype variation, suggesting that the 45S rDNA site number changes may have be associated to major chromosomes rearrangements. The higher karyotype variation found in this lineage seems to agree with previous taxonomic thoughts which considered *C. umbellatus* subsp. *micrantus* and *C. umbellatus* subsp. *viscosum* as separate taxonomic identities, *Halimium voldii* and *Halimium umbellatum* subsp *viscosum*, respectively (see Demoly, 2006).

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CHAPTER 3

Trends in site-number change of 45S rDNA loci during karyological evolution in *Helianthemum* (Cistaceae). A genus with distinct chromosome base numbers

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INTRODUCTION

Chromosomes carry the genetic information of the organism and changes in their number (poly- and dysploidy) and structure (rearrangements such as inversions, deletions, or translocations) are the most recognized potential mechanisms of evolution, diversification and speciation (Stebbins, 1971; Levin, 2002; Raskina *et al*., 2008; Weiss-Schneeweiss *et al.*, 2009; Schubert and Lysak, 2011). Chromosome evolution is often assessed through the physical mapping of different target sequences. The 45S ribosomal nuclear DNA (rDNA) loci are localized at one or more sites per chromosome set and their physical mapping using fluorescence *in situ* hybridisation (FISH) provide an excellent tool to determine the evolutionary patterns and processes resulting in chromosome changes (Weiss-Schneeweiss and Schneeweiss, 2013). Nuclear rDNA has been long regarded as merely involved in the biogenesis of both ribosomes and nucleoli (Hemleben and Werts, 1988; Shaw and Jordan, 1995), although recent evidence has dramatically changed this perception, suggesting that it plays additional roles in the biology of the cell, acting to preserve genome stability and trigger aging (Kobayashi, 2008).

A cytogenetic and phylogenetic integrated approach aimed to infer patterns of chromosome evolution has comprehensively been established mainly in polyploid lineages of plant genera analyzing the physical location of ribosomal DNA loci using FISH (e.g., *Aloe*, Adams *et al.*, 2000; *Paspalum*, Vaio *et al.*, 2005; *Hepatica*, Weiss-Schneeweiss *et al.*, 2007; *Anemone*, Mlinarec, 2012). Conversely, assessing the evolutionary patterns of 45S rDNA loci change in predominantly diploid lineages has usually received less attention, although limited examples are also available (e.g., *Passiflora*, Melo *et al*., 2001; Melo and Guerra, 2003; *Nemesia*, Datson and Murray, 2006; *Prospero,* Jang *et al.*, 2013).

Helianthemum Miller (Cistaceae), a predominant Mediterranean genus including about 110 species and subspecies of small herbs or subshrubs, is cytologically divided into four groups according to their chromosome base numbers, including x=5, x=10, x=11, and x=12 representatives (Sánchez Anta *et al.*, 1986; Arista *et al.*, 1990; Izuzquiza *et al.*, 1998). Some cytotypes appear to be correlated with infrageneric taxonomic boundaries (e.g., López-González, 1993) (Table 1). Virtually all species for which chromosome numbers are know are diploid, even if tetraploid levels have been reported for the Bulgarian and Armenian populations of *H. lasiocarpum*, with $2n$=4x=40 (Markova, 1975).

The taxonomy of *Helianthemum* is a challenge, due to a noticeable high intra-population morphological variability. Thus, most diagnostic features are useless because of putative frequent events of hybridization and introgression (López-González, 1993). Nevertheless, the only available phylogenetic hypothesis (Parejo-Farnés *et al.*, 2013) based on species of the Iberian

peninsula, mostly converges with morphological and karyological data giving strong support to the taxonomic synthesis above species level.

Therefore, the genus *Helianthemum* provides a suitable case study to assess the rDNA sites evolution in a predominant diploid scenario characterized by chromosome base number differentiation.

In this study, the number, physical mapping, and nucleolar activity of 45S rDNA loci have been assessed in representatives of all chromosome base numbers so far reported in *Helianthemum* using FISH and Ag-NOR staining. The results have been mapped onto a phylogenetic framework, in order to assess the evolutionary trends of 45S rDNA site changes.

MATERIAL AND METHODS

Plant materials

Thirty-two taxa of the Cistaceae family belonging to *Helianthemum and Fumana* genera were analyzed in this study. Seeds were obtained from botanical gardens, plant breeding stations and research centers. Information on the origins of this plant material is provided in Table 1.

Cytogenetic analysis

Cytological preparations
Living plants were cultivated in pots at the CIEF greenhouses. For mitotic chromosome preparations, the protocols described in Rosato *et al*. (2008) were followed with minor modifications.

Fluorescence in situ *hybridisation*
The 45S rDNA multigene family was localised using the pTa71 (Gerlach and Bedbrook, 1979) clones. The pTa71 probe was labelled with digoxigenin-11dUTP through a nick translation procedure (Roche, Germany). We followed the *in situ* hybridisation protocols of Rosato *et al.* (2008), except for the proteinase K pre-treatment, which was performed following Schwarzacher and Heslop-Harrison (2000). Probe detection was conducted using the method of Zhong *et al*. (1996) with modifications according to Galián *et al.* (2014).

Ag-NOR staining
Silver impregnation was carried out on 1-2 day-old chromosome preparations according to the protocol described in Rosato and Rosselló (2009).

Karyotype analysis

Chromosome measurements were made on digital images using the freeware software MICROMEASURE v.3.3 (available at http://www.colostate.edu/depts/biology/micromeasure). Idiograms were obtained from chromosome measurements of at least five well-spread metaphase plates.

Chromosome number evolution analysis

In order to determine the direction of chromosomal changes mapping of cytogenetic features onto a phylogenetic framework was carried out following the likelihood and parsimony reconstruction methods in Mesquite version 3.04 (Maddison and Maddison, 2015), thus assigning to each ancestral node the state that maximizes the probability of obtaining the observed states in the terminal taxa under the specified model of evolution (Mk1 model, in this study).

The phylogenetic tree based on nuclear ribosomal ITS sequences (Parejo-Farnés *et al.*, 2013) was used as an evolutionary framework to assess the evolution of 45S rDNA site number in 16 Iberian studied *Helianthemum* species. The genus *Fumana*, known to be basal in Cistaceae, was selected as the outgroup.

Table 1 - *Helianthemum* and *Fumana* studied taxa, sources of material and seed accessions. BGB-D, Botanischer Garten Berlin-Dahlem; CIEF, Centro para la Investigación y Experimentación Forestal; IJBB, Jardí Botànic de Barcelona; JBB, Jardin Botanique de Bordeaux; JBC, Jardín Botánico de Córdoba; JBCA, Jardín Botánico El Castillejo; JBCVC, Jardín Botánico Canario Viera y Clavijo; JBS, Jardí Botànic de Sóller; JBVAL, Jardí Botànic de la Universitat de València.

Taxon	Origin	Accession
Helianthemum		
Subgen. *Helianthemum*		
Sect. *Argyrolepis*		
H. squamatum (L.) Dum. Cours.	Spain, Valencia, Cofrentes	CIEF V3E
Sect. *Lavandulaceum*		
H. syriacum (Jacq.) Dum. Cours.	Israel, Judean Mountains	BGB-D 495. IL-0-B-1670602
H. bystropogophyllum Svent.	Spain, Canary Islands, Gran Canaria, La Aldea de San Nicolás	JBCVC 4383-INAG-B
Sect. *Helianthemum*		
H. viscarium Boiss. & Reut.	Spain, Alicante, Orihuela	JBVAL-BG 127B2014
H. almeriense Pau	Spain, Murcia, Saladar de Calarreona	CIEF MU1B
H. violaceum (Cav.) Pers.	Spain, Valencia, Villargordo del Cabriel	CIEF V261A
H. edetanum Mateo *et al.*	Spain, Valencia, Torrente	CIEF V191A
H. apenninum (L.) Mill.	Spain, Huesca, Colungo	CIEF H2A
H. croceum (Desf.) Pers.	Spain, Castellón, Vistabella del Maestrazgo	CIEF C120A
H. nummularium (L.) Mill.	Spain, León, Puerto de Somiedo	BGB-D 485. ES-0-B-1416479
H. grandiflorum (Scop.) DC.	France, Lozère, Mas-Saint-Chély	JBB N209-ES0150
H. hirtum (L.) Mill.	Spain, Valencia, Llombai	CIEF V280B
H. guerrae Sánchez Gómez *et al.*	Spain, Valencia, Petrer	CIEF A24H
H. scopulicolum L. Sáez *et al.*	Spain, Balearic Islands, Mallorca, Cap Fabioler	JBS without number
H. inaguae Marrero Rodr. *et al.*	Spain, Canary Islands, Gran Canaria, La Aldea de San Nicolás	JBCVC 4360-INAG-B

Infrageneric divisions are based on Proctor and Heywood (1968), Marrero A. (1992), López-González (1993), Sáez *et al.* (1999) and Mateo *et al.* (2009).

Table 1 (continued)

Taxon	Origin	Accession
Sect. *Eriocarpum*		
H. lippii (L.) DC.	Morocco, Guercif, Saka	IJBB 260-Maroc
H. stipulatum (Forssk.) C.Chr.	Greece, Crete, Nomos Lasithiou	BGB-D 1234. GR-0-B-2841299
H. canariense (Jacq.) Pers.	Spain, Canary Islands, Gran Canaria, Telde	JBCVC 4263-B
H. thymiphyllum Svent.	Spain, Canary Islands, Lanzarote, Tinajo	JBCVC 3199-B
Sect. *Brachypetalum*		
H. ledifolium (L.) Mill.	Spain, Navarra, El Charcal	IJBB 1303-JBB-Uribe
H. salicifolium (L.) Mill.	Spain, Alava, Fontecha	IJBB 1312-JBB-Uribe
Sect. *Caput-felis*		
H. caput-felis Boiss.	Spain, Alicante, Santa Pola	CIEF A9F
Subgen. *Plectolobum*		
Sect. *Pseudocistus*		
H. oelandicum (L.) DC.		
subsp. *alpestre* (Jacq.) Breistr.	Spain, Lleida, La Vall de Boí	CIEF LL1A
subsp. *canum* (L.) Bonnier	Greece, Macedonia, Nomos Dramas	BGB-D 493. GR-0-B-2293094
H. marifolium (L.) Mill.		
subsp. *origanifolium* (Lam.) G.López	Spain, Castellón, Penyagolosa	CIEF C169A
subsp. *molle* (Cav.) G.López	Spain, Castellón, Vistabella del Maestrazgo	CIEF C119A
H. cinereum (Cav.) Pers.		CIEF V260A
subsp. *rotundifolium* (Dunal) Greuter & Burdet	Spain, Valencia, Pobla de Vallbona	CIEF V260A
Sect. *Atlanthemum*		
H. sanguineum (Lag.) Dunal	Spain, Valencia, Cofrentes	CIEF V278A

Infrageneric divisions are based on Proctor and Heywood (1968), Marrero A. (1992), López-González (1993), Sáez *et al.* (1999) and Mateo *et al.* (2009).

Table 1 (Continued)

Taxon	Origin	Accession
Fumana		
F. lacidulemiensis Güemes	Spain, Cádiz, Grazalema	JBCA 33370
F. paradoxa Heywood	Spain, Córdoba	JBC 50763-06
F. procumbens (Dunal) Gren. & Godr.	Spain, Teruel, Maicas	IJBB 2345-JBB
F. scoparia Pomel	Morocco, Guercif, Mezguitem	IJBB 430-Marroc

Infrageneric divisions are based on Proctor and Heywood (1968), Marrero A. (1992), López-González (1993), Sáez *et al.* (1999) and Mateo *et al.* (2009).

RESULTS

Comparative Karyotyping

The chromosome number of thirty-two species and subspecies, belonging to *Helianthemum* (28) and *Fumana* (4) were determined and their idiograms were constructed. The chromosome number for *Helianthemum viscarium* (2*n*=20) was indicated for the first time. In the *Helianthemum* taxa analyzed all known cytotypes (x=5, 10, 11 and 12) were represented (Table 2). The species possessed very similar karyotypes, with mostly meta- and submetacentric chromosome pairs. In detail, *H. squamatum*, with 2*n*=10, showed three metacentric and two submetacentric chromosome pairs, accessions with 2*n*=20 possessed mostly six metacentric and four submetacentric chromosomes pairs and accessions with 2*n*=22 showed six metacentric and five submetacentric chromosome pairs. *H. caput-felis*, the only species with 2*n*=24, showed seven metacentric and five submetacentric chromosome pairs. The karyotype of *Fumana* species (2*n*=32) consisted of twelve metacentric and four submetacentric (*F. lacidulemiensis*) or ten metacentric and six submetacentric (*F. paradoxa*) chromosome pairs.

45S rDNA sites distribution

Total number and position of 45S rDNA sites in the somatic chromosomes of all the analyzed accessions of *Helianthemum* are summarized in Table 2. The number of 45S rDNA loci ranged from one to five. Figure 1 (*a-f*) illustrates selected chromosomal distribution and 45S rDNA site number variation in six *Helianthemum* accessions. Conversely, in *Fumana* (Figure 1, *g*) only two 45S rDNA loci were observed.

In *Helianthemum*, the 45S rDNA site number seemed to be associated to the chromosome base number variation. A single 45S rDNA locus was observed in four sections characterized by x=5 (*Argyrolepis*) and x=10 (*Brachypetalum*, *Eriocarpum*, *Lavandulaceum*) base numbers, while in the only section *Helianthemum* (x=10) most species showed two 45S rDNA loci. x=11 (section *Pseudocistus*) showed three and four 45S rDNA sites, with the exception of *H. sanguineum* (section *Atlanthemum*) where in addition to three main 45S rDNA sites, two minor proximal loci were also observed. In x=12 (section *Caput-felis*) three 45S rDNA loci were found.

The rDNA sites were mostly localized at sub-terminal regions of chromosomes and associated to the secondary constrictions. Signals with a satellite portion attached to the rDNA site and proximal site positions on chromosomes were also observed in a single accession. According to the chromosome type bearing 45S rDNA sites, it was possible to describe their

chromosomal localization. These chromosome landmarks, numbered I-IV, were defined by Totta *et al.* (Chapter 2; Figure 1, *g*) for describing the trends of 45S rDNA evolution in *Cistus*. Additionally, two new chromosome landmarks, numbered V-VI, were described for *Helianthemum* referring to the proximal site localizations on short and long arms, respectively. Type I and type III (with sub-terminal site position on short and long arms, respectively) were the most cytogenetic landmarks identified within the whole studied sample, whereas type II (with interstitial site position on short arm of chromosome) together with type V and VI showed a restricted distribution within the genus, being observed in *H. sanguineum* (Figure 1, *h*). However, the type IV was absent in *Helianthemum*.

Ag-NOR staining performed in those taxa showing two or three 45S rDNA loci revealed that all sites were active, as the number of FISH signals equated the maximum number of nucleoli detected in interphase nuclei (Table 2). In contrast, for those accessions showing four and five 45S rDNA loci, the maximum number of nucleoli found was six suggesting that not all ribosomal DNA sites presented transcriptional activity. Several accessions showed heteromorphic nucleoli and odd numbers (Figure 2).

Table 2 - Karyotypic features of the analyzed species. The chromosome number, the haploid karyotype formula, the total number of 45S rDNA loci with their distribution in each rDNA-bearing chromosomal type and maximum number of nucleoli observed.

Taxon	2*n*	Haploid karyotype Formula	No. of 45S rDNA loci	Chromosome Type					Max.no of nucleoli
				I	II	III	V	VI	
Helianthemum									
Subgen. *Helianthemum*									
Sect. *Argyrolepis*									
H. squamatum (L.) Dum. Cours.	10	3m + 2sm	1	1	-	-	-	-	-
Sect. *Lavandulaceum*									
H. syriacum (Jacq.) Dum. Cours.	20	8m + 2sm	1	-	-	1	-	-	-
H. bystropogophyllum Svent	20	-	1	-	-	1	-	-	-
Sect. *Helianthemum*									
H. viscarium Boiss. & Reut.	20	-	1	-	-	1	-	-	-
H. almeriense Pau	20	6m + 4sm	2	2	-	-	-	-	4
H. violaceum (Cav.) Pers.	20	-	2	2	-	-	-	-	3
H. edetanum Mateo *et al.*	20	7m + 3sm	2	1	-	1	-	-	4
H. apenninum (L.) Mill.	20	6m + 4sm	1	1	-	-	-	-	-
H. croceum (Desf.) Pers.	20	6m + 4sm	2	2	-	-	-	-	4
H. nummularium (L.) Mill.	20	6m + 4sm	2	2	-	-	-	-	4
H. grandiflorum (Scop.) DC.	20	6m + 4sm	2	2	-	-	-	-	4
H. hirtum (L.) Mill.	20	6m + 4sm	2	1	-	1	-	-	4
H. guerrae Sánchez Gómez *et al.*	20	6m + 4sm	2	1	-	1	-	-	4
H. scopulicolum L. Sáez *et al.*	20	6m + 4sm	2	1	-	1	-	-	3
H. inaguae Marrero *et al.*	20	6m + 4sm	1	1	-	-	-	-	-
Sect. *Eriocarpum*									
H. lippii (L.) DC.	20	6m + 4sm	1	-	-	1	-	-	-
H. stipulatum (Forssk.) C.Chr.	20	7m + 3sm	1	-	-	1	-	-	-
H. canariense (Jacq.) Pers.	20	-	1	-	-	1	-	-	
H. thymiphyllum Svent.	20	-	1	-	-	1	-	-	-

Infrageneric divisions are based on Proctor and Heywood (1968), Marrero A. (1992), López-González (1993), Sáez *et al.* (1999) and Mateo *et al.* (2009).

Table 2 (Continued)

Taxon	2*n*	Haploid karyotype Formula	No. of 45S rDNA loci	Chromosome Type					Max.no. of nucleoli
				I	II	III	V	VI	
Sect. *Brachypetalum*									
H. ledifolium (L.) Mill.	20	6m + 4sm	1	1	-	-	-	-	-
H. salicifolium (L.) Mill.	20	6m + 4sm	1	1	-	-	-	-	-
Sect. *Caput-felis*									
H. caput-felis Boiss.	24	7m + 5sm	3	1	-	2	-	-	5
Subgen. *Plectolobum*									
Sect. *Pseudocistus*									
H. oelandicum (L.) DC. subsp. *alpestre* (Jacq.) Breistr.	22	6m + 5sm	4	3	-	1	-	-	6
subsp. *canum* (L.) Bonnier	22	6m + 5sm	3	2	-	1	-	-	5
H. marifolium (L.) Mill. subsp. *origanifolium* (Lam.) G. López	22	6m + 5sm	4	3	-	1	-	-	5
subsp. *molle* (Cav.) G. López	22	-	4	3	-	1	-	-	6
H. cinereum (Cav.) Pers. subsp. *rotundifolium* (Dunal) Greuter & Burdet	22	6m + 5sm	3	2	-	1	-	-	5
Sect. *Atlanthemum*									
H. sanguineum (Lag.) Dunal	22	6m + 5sm	5	1	1	1	1	1	6
Fumana									
F. lacidulemiensis Güemes	32	12m + 4sm	2	1	1				4
F. paradoxa Heywood	32	10m + 6sm	2	2					4
F. procumbens (Dunal) Gren. & Godr.	32	-	2	1	1				
F. scoparia Pomel	32	-	2	2					na

na, not analyzed

Infrageneric divisions are based on Proctor and Heywood (1968), Marrero A. (1992), López-González (1993), Sáez *et al.* (1999) and Mateo *et al.* (2009).

45S rDNA changes in a phylogenetic framework

Changes of 45S rDNA loci number based on the maximum likelihood and maximum parsimony reconstructions are shown on the phylogenetic tree in Figure 3 and the probabilities for each node of both the methods are indicated in Table 3. Maximum likelihood and maximum parsimony methods rendered identical results, with a single exception. Overall, the analysis detected few changes in the number of the 45S rDNA sites. The inferred ancestral state in the common ancestor of the whole studied sample (node 1) was ambiguous in the parsimony with two-site and three-site state equally parsimonious. However, the likelihood reconstruction considered the two-site as the most probable ancestral state. Three 45S rDNA sites were inferred to originate in the common ancestor of *Helianthemum* (node 2) and no changes were inferred at the basal node of the subgenus *Plectolobum* (node 8). Four- and five-site states originated independently both from the three-loci state once in *H. marifolium* subsp. *origanifolium* (section *Pseudocistus*) and in *H. sanguineum* (section *Atlanthemum*), respectively. Conversely, the reduction from three 45S rDNA sites to one site was assumed to occur at the base of the paraphyletic lineage of subgenus *Helianthemum* (node 3). Two 45S rDNA sites originated once in the common ancestor of the section *Helianthemum* (node 6), even though two terminal nodes (*H. apenninum* and *H. viscarium*) showed a single 45S rDNA site. The section *Caput-felis* did not showed changes respect to the ancestral three-loci state.

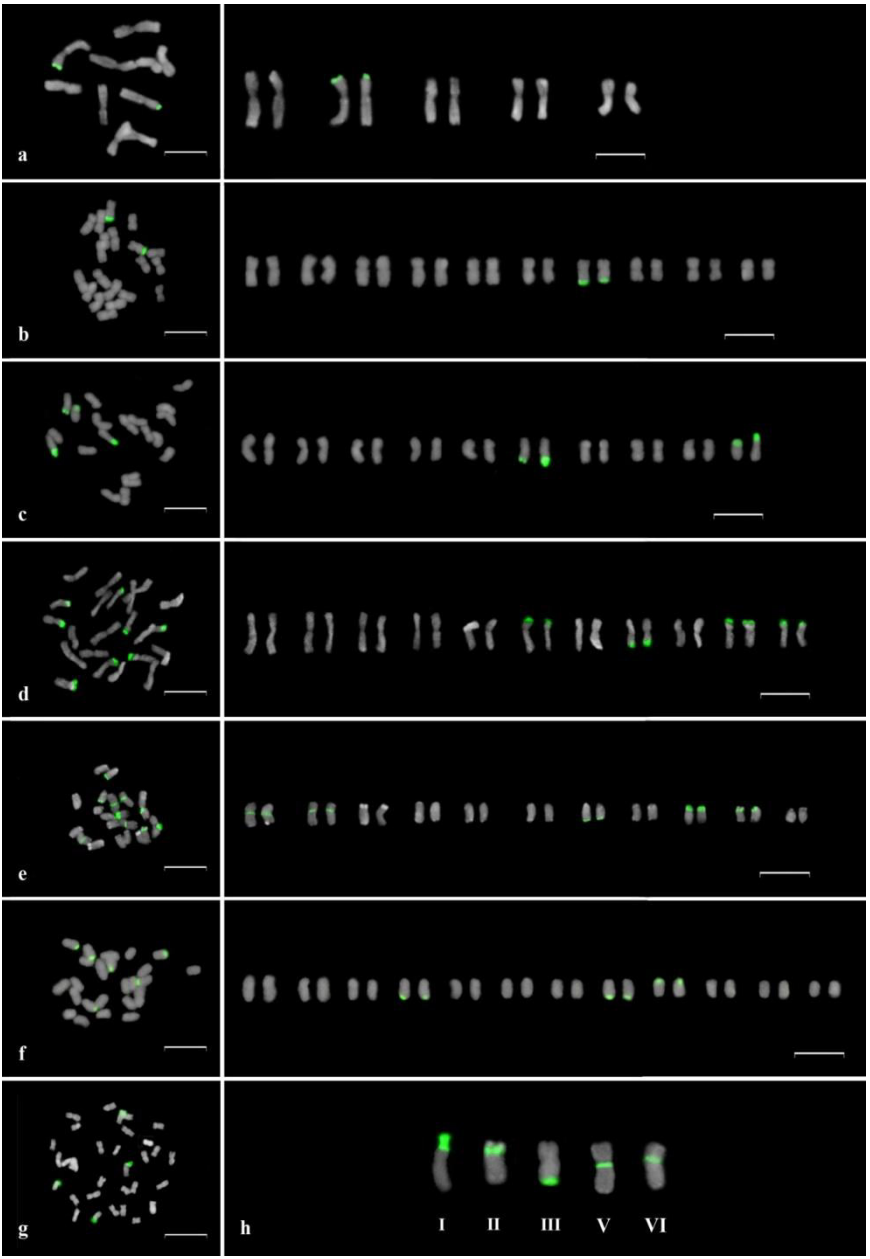
a
b
c
d
e
f
g
h
I
II
III
V
VI

Figure 1 - Arrangement of chromosomes in metaphase plates and idiograms showing the location of 45S rDNA sites in (*a*) *H. squamatum* (2*n*=10), with a single 45S rDNA locus, (*b*) *H. syriacum* (2*n*=20), with one 45S rDNA loci (*c*) *H. guerrae* (2*n*=20) with two 45S rDNA loci, (*d*) *H. marifolium* subsp. *origanifolium* (2*n*=22), with four 45S rDNA loci, (*e*) *H. sanguineum* (2*n*=22), with five 45S rDNA loci, (*f*) *H. caput-felis* (2*n*=24), with three 45S rDNA loci and (*g*) in the related species *F. lacidulemiensis* (2*n*=32) with two 45S rDNA loci. The five chromosome landmarks based on the different position of 45S rDNA sites are indicated (*h*). Scale bars represent 10μm.

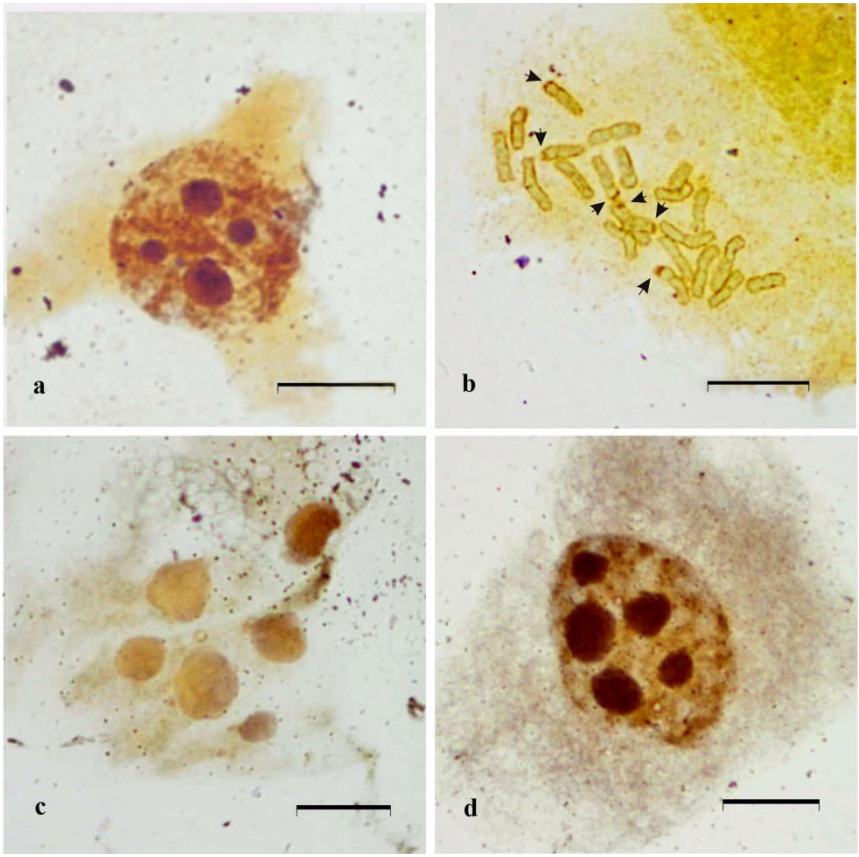

Figure 2 - Maximum number of silver-stained interphase nucleoli on non-pretreated root tips in (*a*) *H. grandiflorum*, (*c*) *H. sanguineum* and (*d*) *H. caput-felis*, and NOR signals on metaphase chromosomes in (*b*) *H. oelandicum* subsp. *alpestre*. Scale bars represent 10µm.

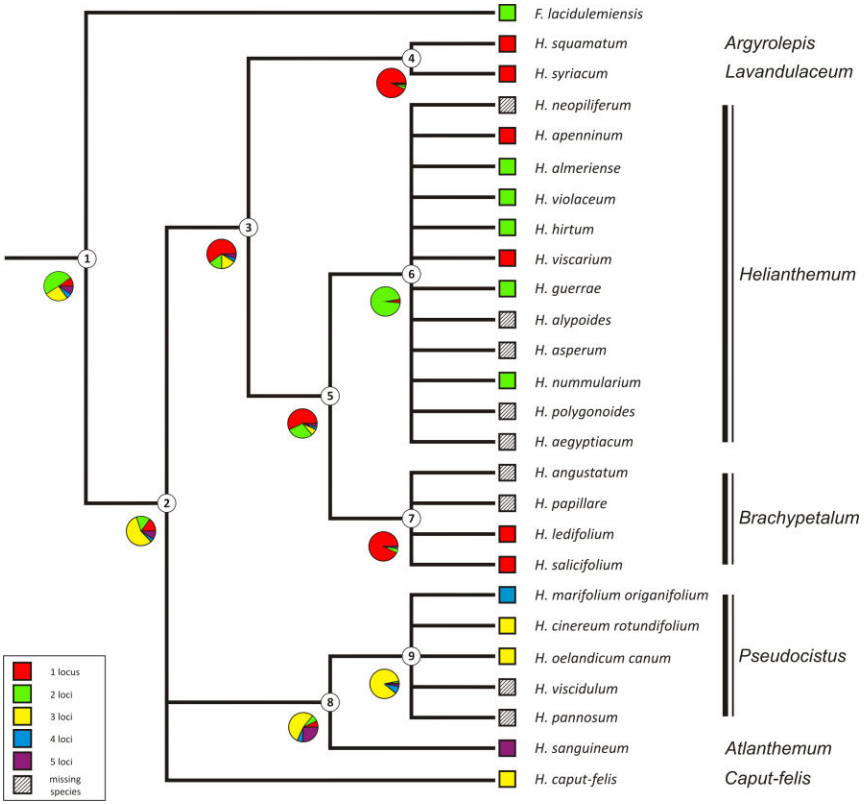

Figure 3 - Ancestral 45S rDNA site number reconstruction mapped on the backbone *Helianthemum* ITS tree. Pies illustrate relative likelihood estimates of the five possible states of 45S rDNA loci at each node. Filling lines in the square indicate missing species in the analysis. Numbers in the circles correspond to the node numbers indicated in Table 3. On right the sections to which belongs each accession are indicated.

Table 3 - Likelihood scores and Parsimony results of the five possible states of 45S rDNA loci number at each node indicated in Figure 3.

	Maximum Likelihood 45S rDNA					Maximum Parsomony 45S rDNA				
Node	**1 locus**	**2 loci**	**3 loci**	**4 loci**	**5 loci**	**1 locus**	**2 loci**	**3 loci**	**4 loci**	**5 loci**
1	0.10	0.49	0.26	0.06	0.09	-	+	+	-	-
2	0.15	0.15	0.57	0.04	0.09	-	-	+	-	-
3	0.60	0.15	0.16	0.04	0.05	+	-	-	-	-
4	0.93	0.03	0.02	0.01	0.01	+	-	-	-	-
5	0.57	0.29	0.07	0.03	0.04	+	-	-	-	-
6	0.01	0.99	0.00	0.00	0.00	-	-	-	-	-
7	0.93	0.04	0.01	0.01	0.01	+	-	-	-	-
8	0.07	0.07	0.54	0.06	0.26	-	-	+	-	-
9	0.01	0.02	0.86	0.08	0.03	-	-	+	-	-

DISCUSSION

Karyotype changes

All chromosome numbers confirmed previous reports remarking the presence of different cytotypes in the genus *Helianthemum*, whose distribution seems to be congruent with the infrageneric ranks. The chromosome count for *H. viscarium*, agreed with the cytotype of the section to which belongs (section *Helianthemum*). Within sections showing the same chromosome number it was detected a high karyological uniformity, with a reiteration of the same karyological pattern mainly characterized by metacentric and sub-metacentric chromosome pairs. This structure was found in all cytotypes. Hence, by the evidence none of the karyotypes showed any indication of evident simple fusion or fission as evolutionary relics derived from chromosomal rearrangements, suggesting that the mechanisms of chromosome number change may be more complex (Lysak *et al*, 2006) or, alternatively, may involve whole-chromosome loss or gain (Levin, 2002).

Variation in numbers and chromosomal locations of 45S rDNA

Variation in number and distribution patterns of rDNA loci among related species is commonly observed in many different plant genera, including Rosaceae (Mishima *et al.*, 2002), Brassicaceae (Hasterok *et al.*, 2006), Asteraceae (Moscone *et al.*, 1999; Garcia *et al.,* 2010), Cyperaceae (Da Silva *et al.*, 2010), Fabaceae (Malinska *et al.*, 2010), and Orchidaceae (Lan and Albert, 2011).

In *Helianthemum* the physical positions of 45S rDNA loci was highly conservative, in agreement with the highly conserved karyotype structure. In all but one analyzed species, 45S rDNA signals were located in sub-terminal chromosome positions following the common distribution pattern found in most diploid angiosperm species (Roa and Guerra, 2012). The two 45S rDNA loci observed at the proximal positions in *H. sanguineum* may be the result of insertion of mobile elements to which pericentromeric regions are prone, mediating the amplification of rDNA in a genome (reviewed by Raskina *et al*., 2008).

Conversely, albeit little differences were found in the site number of ribosomal DNA (one to five loci distributed on one to five chromosome pairs). The changes in 45S rDNA site number seemed related to the different cytotypes, since sections with lower chromosome base number showed one and two sites while sections with higher chromosome base number showed three, four and five sites. When the results are discussed with respect to the taxonomic infrageneric division, a correspondence with the loci number changes is highlighted. The main number of 45S rDNA loci in the subgenus *Helianthemum* was one, and two and three sites were rather well restricted to separate sections (sections *Helianthemum* and *Caput-felis*, respectively). Similarly, in the subgenus *Plectolobum*, five 45S rDNA sites were observed only in one section, while three and four sites were well distributed throughout the section *Pseudocistus*. Hasterok *et al.* (2006) and Malinska *et al.* (2010) found variation in the number and distribution of the 45S rDNA sites among close diploid relatives, which the authors explained by chromosomal rearrangements and dynamic double-strand break repair processes that characterize hotspots in pericentromeric and telomeric regions (Schubert and Lysak, 2011). This could also be the case of *Helianthemum* in which no polyploids are known, except for a not relevant isolate case.

The lower number of nucleoli detected by silver staining in species with four and five 45S rDNA loci suggests that one and two site were respectively silenced. Plant cells usually have more copies of rDNA than necessary for the synthesis of ribosomes (Phillips 1978; Rogers and Bendich 1987). The level of transcription of rRNA genes in eukaryotic cells is tightly regulated according to synthesis of proteins required in cells. The dosage compensation regulation can occur by varying the number of active sites or by decreasing the activity of all sites in response to factors such as nutrition and growth (Mcstay 2006, 2008). Therefore, the different sizes of nucleoli observed may be related to a pattern of differential transcriptional expression between genes of *Helianthemum* (Caperta *et al.*, 2002). The fusion of nucleoli is a phenomenon that has been reported for some plant species, among them barley (Schubert and Kunzel 1990) and *Lolium* (Bustamante *et al*., 2014; Rocha *et al.*, 2015). In *Helianthemum*, many species showed odd numbers of nucleoli which may be explained as a nucleolar fusions phenomenon.

Evolutionary trends in 45S rDNA loci according to intrageneric ranks

According to the ancestral state reconstruction the numbers of 45S rDNA site appeared to be consistent with the ITS tree.

As proposed by Adams *et al.* (2000) for the genus *Aloe*, this scenario may have two possible explanations: (1) the ITS sequence data reflect the history of 45S rDNA chromosome distributions in *Helianthemum* arising by amplifications and reductions of different 45S rDNA units at individual loci rather than a phylogeny of species relationships; (2) the ITS sequence data do reflect a phylogeny of evolutionary relationships, and related species have similar distributions and sequence composition of 45S rDNA.

In the most likely reconstruction the three-loci ancestral state showed different pattern of 45S rDNA number evolution in the two main lineages of *Helianthemum*. The diversification of the monophyletic group of subgenus *Plectolobum* was not characterized by loci number changes; the further numbers observed in the subgenus (four and five sites) originated in *H. marimifolium* subsp. *origanifolium* and in *H. sanguineum* as single independent events of loci amplification both from the three-loci state. This result is supported by the evidence that the additional locus in the section *Pseudocistus* was always located at sub-terminal position on chromosomes, while the two extra sites observed in *H. sanguineum* were found in proximal positions. Conversely, the evolution of the whole paraphyletic subgenus *Helianthemum* was characterized by the loss of two loci, and involved all sections irrespective of chromosome number, except the section *Helianthemum*, whose diversification was associated to a single amplification event of one locus. Consequently, according to the analysis, the one-loci state observed in several taxa of the section *Helianthemum* originated through the loss of one site as independent events. However, the polytomies in the clade rendered by the ITS phylogeny and the small species sample do not allow to assess a certain rDNA number change direction, and the hypothesis that one-loci state has been the ancestral condition could not be excluded. As regard *H. caput-felis*, the species was formerly included in the section *Polystachium* (subgenus *Helianthemum)*, together with *H. syriacum* and *H. squamatum* (Proctor and Heywood, 1968) while López-González (1992) proposed for the species a new section within the subgenus *Helianthemum*, although some morphological characters led him to hypothesized a separate intermediate position between the two subgenera, being *H. caput-felis* the unique representative of an ancient lineage. The phylogenetic tree provided support for the section *Caput-felis* on its own *sensu*, although does not allow inferring the phylogenetic relationship of this species with regard to any of both subgenera (Parejo-Farnés *et al*, 2013). According to the molecular cytogenetic results provided by this study, *H. caput-felis* shared the ancestral three-loci state with the subgenus *Plectolobum* lineage indicating a more probable relationship with this group than the subgenus *Helianthemum*.

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CHAPTER 4

Latent nuclear rDNA instability in *in vitro*-generated plants is activated after sexual reproduction with conspecific wild individuals

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INTRODUCTION

Management of the diversity of endangered plants is a biological challenge of global interest that is inherently linked to the current world-wide biodiversity crisis. Prioritising species for conservation and restoration efforts is not free from controversy. However, there is ample agreement that, all things being equal, threatened species showing restricted distribution areas and reduced population effective size should be prioritised (Heywood and Iriondo, 2003; Gauthier *et al*., 2010; Arponen, 2012).

One of the ultimate goals of plant conservation is to maintain 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) should be envisaged (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 pre-requisite 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 *et al*., 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 very 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 level, 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 *et al*., 2011; Neelakandan and Wang, 2012; Rosato *et al*., 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 in Hazarika, 2006).

The production of genetically modified or genetically unstable regenerants not present in the original genotype stock are 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 programs.

Unfortunately, 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 structures of 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 obtained between *Cistus heterophyllus* Desf. (Cistaceae) accessions generated after *in vitro* culture and wild individuals. The key role of 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 and Werts, 1988; Shaw and Jordan, 1995). In fact, other structural and functional aspects, including the maintenance of genome stability and modulating cellular homeostasis, are also credited to be of relevance in the eukaryote cell (Kobayashi, 2008; Hein *et al.*, 2012).

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

MATERIALS AND METHODS

Organismal background

Cistus heterophyllus is a narrowly-distributed W Mediterranean species present in North Africa (Morocco, Algeria) and the Iberian Peninsula (Spain) (Crespo and Mateo, 1988; Démoly and Montserrat, 1993). Like other *Cistus* species, *C. heterophyllus* is self-incompatible (Boscaiu and Güemes, 2001), although a small fruit production containing seeds is rarely produced (E. Laguna and P. Ferrer-Gallego, pers. comm.). The European individuals are at risk and are extremely endangered because of their 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 *et al.*, 2004; Jiménez and Sánchez-Gómez, 2004). In addition, nuclear and plastid DNA markers together with morphological evidence strongly suggest that ongoing gene flow with the related *C. albidus* L. is occurring in European and North African populations (Jiménez *et al.*, 2007; Sánchez-Gómez and Rosselló, 2007; Navarro *et al.*, 2009). Plants from SE 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 and Rivera, 2001; Navarro, 2002). Spontaneous regeneration by 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 hybridisation was found at E Spain (Pobla de Vallbona, Valencia) (Crespo and 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 and Martín, 2011) to obtain accessions suitable for reintroduction in a new site (Tancat de Portaceli, Valencia, Spain) (Laguna *et al.*, 1998; Aguilella *et al.*, 2010; 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 by *in vitro* culture and plant regeneration techniques (Arregui *et al.*, 1993) were (i) from samples produced during the first micropropagation culture originating from the wild European individual (denominated first *in vitro* generation) and (ii) 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* were sampled from wild populations (10 plants each) and were used for comparative purposes (Table 1).

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 and Guemes, 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. Fertilised 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 20ºC using a 12 h light/dark photoperiod. We analysed 28 individuals obtained from the progeny between *in vitro*-generated European *C. heterophyllus* (maternal plant) and wild North African plants (pollen donor), as well as 22 individuals from the reciprocal crosses (Table 1).

Cytogenetic analysis

Cytological preparations
Living plants were cultivated in pots at the CIEF greenhouses. For mitotic chromosome preparations, the protocols described in Rosato *et al.* (2008) were followed with minor modifications.

Fluorescence in situ hybridisation
The 45S and 5S rDNA multigene families were localised using the pTa71 (Gerlach and Bedbrook, 1979) and pTa794 (Gerlach and Dyer, 1980) clones, respectively, according to the *in situ* hybridisation protocols of Rosato *et al.* (2008), except for the proteinase K pre-treatment, which was performed following Schwarzacher and Heslop-Harrison (2000). Probe detection was conducted using the method of Zhong *et al.* (1996) with modifications according to Galián *et al.* (2014).

Ag-NOR staining
Silver impregnation was carried out on 1–2 day-old chromosome preparations according to the protocol described in Rosato and Rosselló (2009).

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). Idiograms were obtained from chromosome measurements of at least five well-spread metaphase plates.

Table 1 - Origin of the samples size used in the karyological study in *C. 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) x *C. heterophyllus* ♂ (*in vitro* first generation, Spain)	22
C. heterophyllus ♀ (*in vitro* first generation, Spain) x *C. heterophyllus* ♂ (wild, Morocco)	28

RESULTS

Chromosome features of wild plants

All accessions analyzed showed a somatic chromosome number of $2n = 18$. Overall, the karyotype was constituted by chromosomes of similar size and shape, including metacentric (fourteen), submetacentric (two), and metacentric-submetacentric (two) chromosomes. The latter (chromosome pair 8) showed a terminal secondary constriction at the short arm and was inferred to carry the active NOR locus (Figure 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-lineal) to the 45S rDNA locus (Figures 1, 2). The activity of the NOR locus was assessed by silver staining, and the results showed similar active Ag-NOR sizes for the two 45S sites. In interphase, individuals showed one nucleolus or two similar-size nucleoli, previous 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 pink roses *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 similar-size nucleoli were present at interphase.

In vitro-generated plants are heterozygous 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. In fact, after DAPI staining, only one chromosome of the metacentric-submetacentric pair carrying the active NOR locus showed a conspicuous secondary constriction. FISH experiments using the 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 fluorescent signals in one 45S rDNA site was detected. This heterozygous condition as revealed by both DAPI staining and FISH was consistently present in all cells and accessions studied, and it was a fixed feature characterising 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, most likely as a result of the differential rDNA gene expression between homologous chromosomes (Figure 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, Figure 2), five karyological phenotypes, including three novel ones, were detected in the progeny resulting from the experimental crosses (50 plants analysed; Tables 2 and 3). The three novel cytogenetic phenotypes (III, IV and V; Figure 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 in size and intensity than the minor-size site observed in the micropropagated plants used as progenitors. The three new karyological patterns were characterised (Figure 2) by the presence of one pair of homologous chromosomes carrying equivalent NOR sizes plus an additional rDNA site, and showing 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 ($X^2 = 1.524$, $P \geq 0.05$) 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 x ♂ *in vitro*" progenitors was 12 : 10 conforming to Mendelian expectations (11 : 11; $X^2 = 0.18$, $P \geq 0.05$). However, in the reciprocal crosses, ♀ *in vitro* x ♂ wild progenitors, the segregation was 20 : 8 and significantly deviated from Mendelian expectations (14 : 14; $X^2 = 5.14$, $P \leq 0.05$). 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.

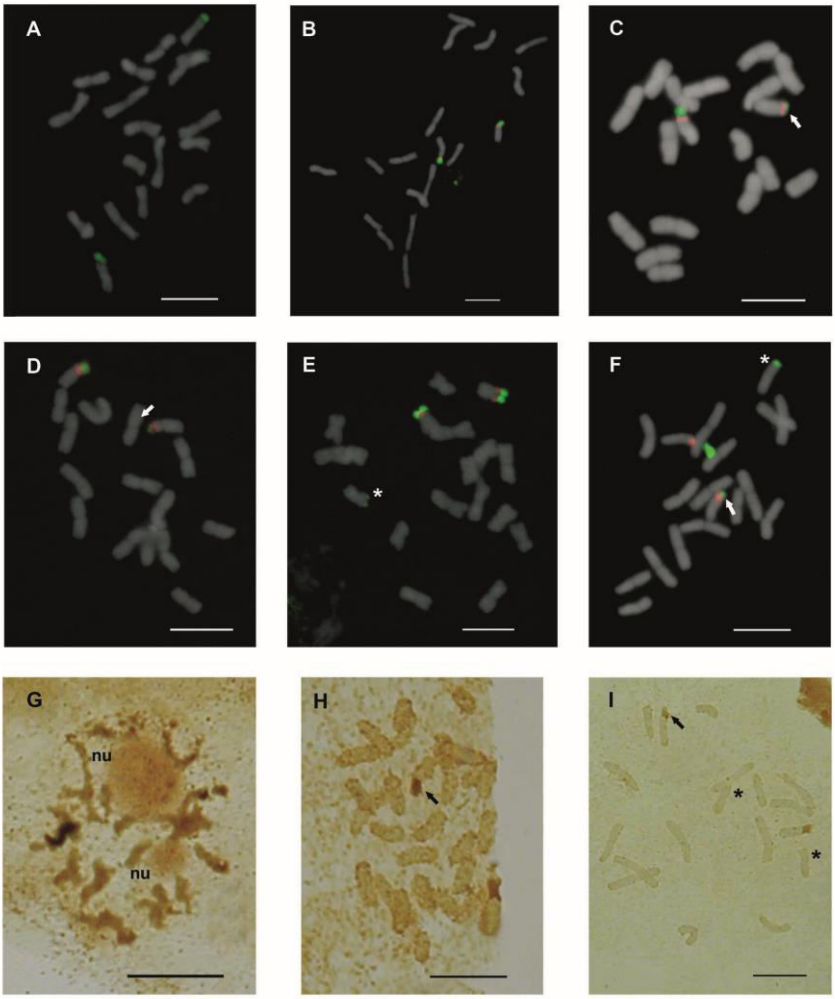
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Figure 1 - (*A-F*) 45S and 5S rDNA patterns identified on mitotic metaphase chromosomes according to the karyological phenotype (I to V) in wild, *in vitro*-micropropagated and progeny accessions of *Cistus heterophyllus* assessed through FISH and Ag-NOR analysis. 45S rDNA sites are shown as green fluorescent signals and 5S rDNA sites as red signals. Chromosomes are counterstained by 4′,6-diamidino-2-phenylindole (DAPI)-staining (grey colour). (*A*) karyological phenotype I in wild accession from Morocco. (*B*), karyological phenotype I in wild accession from Spain (Pobla de Vallbona). (*C*) karyological phenotype II in *in vitro*-micropropagated plant from the wild Spanish accession. The chromosome bearing the minor 45S rDNA site is arrowed. (*D*) progeny from a cross between wild (Morocco) as female and *in vitro*-micropropagated plants as male progenitors showing the paternal ribosomal phenotype II. The chromosome bearing the minor 45S rDNA site is arrowed. (*E*) progeny from a cross between wild (Morocco) as female and *in vitro*-micropropagated plants as male progenitors showing a new ribosomal phenotype not found in the parental accessions (IV). The additional 45S rDNA cluster is identified by an asterisk. (*F*) progeny from a cross between *in vitro*-micropropagated plants as female and wild (Morocco) as male progenitors showing a new ribosomal phenotype not found in the parental accessions (V). 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 (II). (*H*) metaphase chromosomes showing two Ag-NOR sites of unequal size (the minor site is arrowed) in progeny plants (II).(*I*) 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 in progeny plants (V). Scale bars represent 10 µm.

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

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

Table 3 - Distribution of ribosomal phenotype found in the accessions of *Cistus heterophyllus* used in this study. Phenotype characterization is given in Table 2.

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

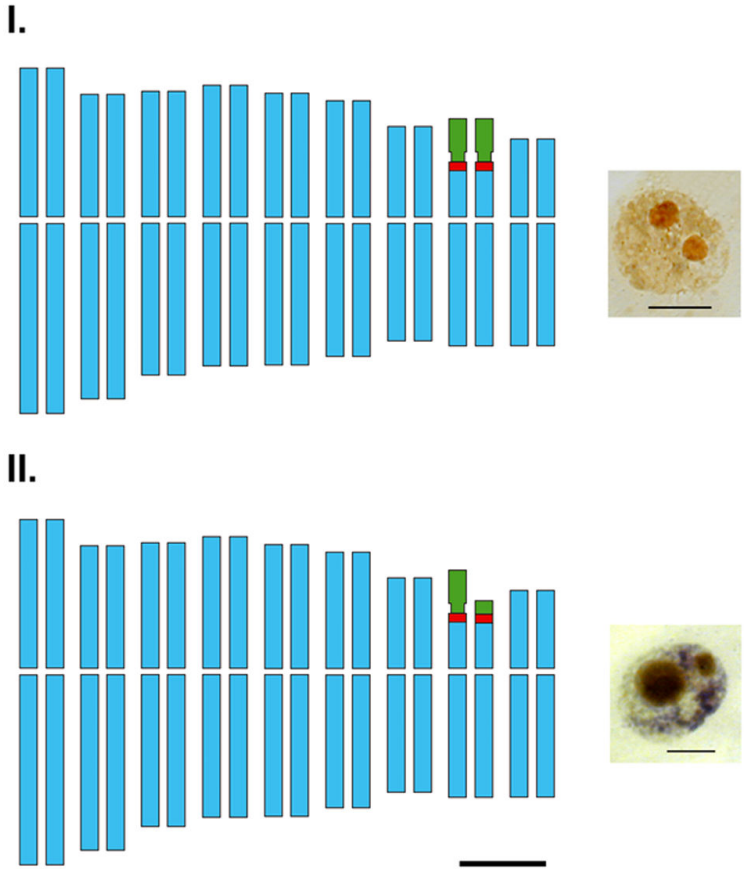

Figure 2 - Idiograms and Ag-NOR staining of interphase nuclei in *Cistus heterophyllus* characterising the five ribosomal karyological phenotypes (I to V) described in Table 1 are shown. Left: The localization of 45S rDNA (green) and 5S rDNA sites (red) are mapped on the chromosomes (scale bar represent 2 µm). Right: Ag-NOR staining shows the expression of individualized NOR sites as independent nucleoli (scale bar represents 10 µm).

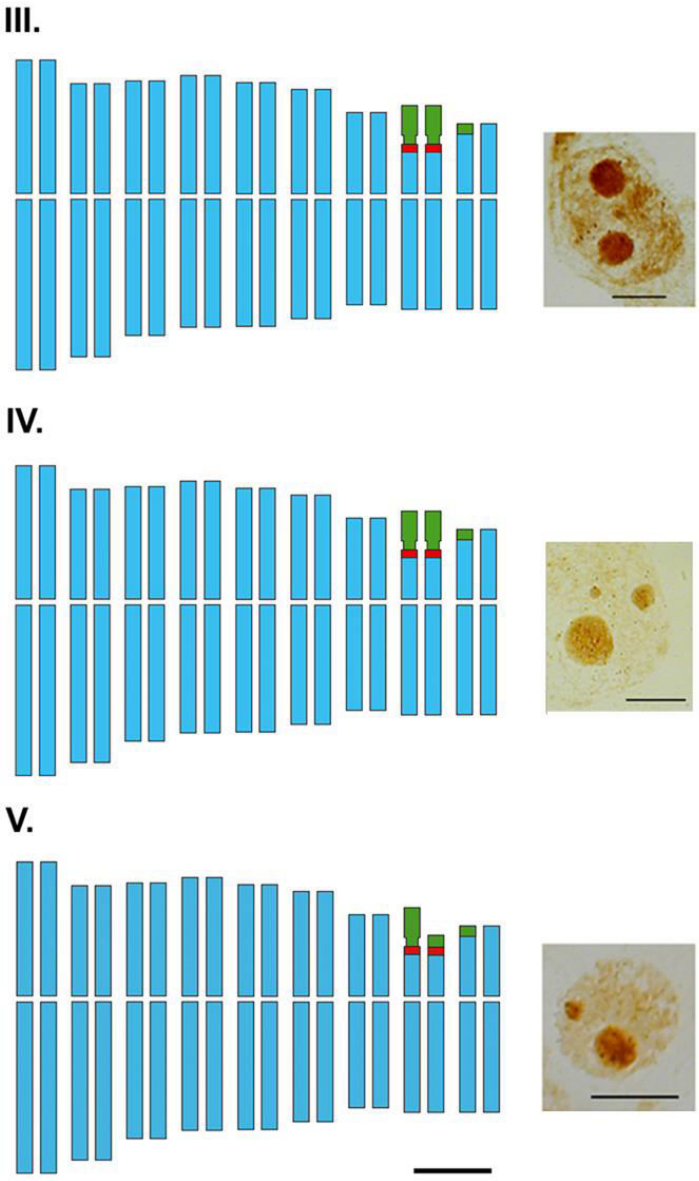

Figure 2 (Continued)

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 fluorescent FISH signals in one 45S rDNA site, and the different size of the two nucleoli) assessed by independent experimental techniques, concur that the 45S rDNA loci in *in vitro*-regenerated plants show a contrasting number of copies or a differential expression of the rRNA genes from those present in wild plants. These two mechanisms (genome restructuration and epigenetic control) are suggested to be influenced by *in vitro* culture affecting rRNA genes (Lee and 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 analyzed cells and individuals of the regenerated plants. Even, it has been recorded in different phases of the cellular cycle where 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 to epigenetic mechanisms affecting the differential expression of rRNA genes would imply a remarkable cytological behavior not reported so far in the organization of the plant rDNA chromatin (Caperta *et al*., 2007). For these reasons, we speculate that our data suggest more likely that the permanent heteromorphism linked to the condensation of the NOR locus is associated to a loss of number of rDNA units.

The 45S rDNA loci are reputed as frequent hot spots of chromosome breakage that are involved in somaclonal variation rearrangements and in plant chromosome evolution in general (Lee and Philipps, 1988; Schubert, 2007). Accordingly, our observations assessing (i) that all regenerated *Cistus* plants so far analysed suggested a drastic reduction of the copy number or expression of ribosomal genes in one homologous rDNA site and (ii) that this tissue culture-induced somaclonal variation remains somatically stable and heritable by horizontal transmission, agree with previous observations on ribosomal loci behaviour during tissue culture propagation (Breiman *et al*., 1987).

rDNA-based genomic instability is activated 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 very few studies have monitored the genomic changes produced in later generations. These reports used crops as case studies, and the new generations were obtained after self-fertilisation, precluding the study of genetically modified regenerants after sexual reproduction with unrelated genotypes. The analysis of progeny between *in vitro* and wild *Cistus heterophyllus* has shown that the heterozygous condition of the single NOR locus from the *in vitro*-regenerated plant progenitor has properly segregated 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 likely induced by the *in vitro* culture process and whose genomic effects 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 and 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 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.

Implications for conservation

The dual and antagonist perception of the appearance of somaclonal variation in biotechnological plant propagation, 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, has a history nearly as long as the first report of its occurrence.

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 and 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 assessed that both 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 and progeny plants 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 operating. 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.

It may be argued that 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 and 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 hypothesised that rDNA constitutes a central factor in the maintenance and organisation of the genome, modulating cellular homeostasis by acting to preserve genome stability, triggering cell aging and senescence, and regulating genome damage resistance (Kobayashi, 2008; Hein *et al.*, 2012); maintaining genome-wide chromatin structure (Paredes and 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 is necessary to assess the effects of the new ribosomal genotypes in the fitness of these newly generated genotypes.

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CHAPTER 5

CONCLUSIONS

Thanks to the methodology employed in this study – which has involved multiple kinds of molecular cytogenetic methods (FISH and AgNOR staining), image processing and statistical analysis – it has been possible to draw conclusions about karyotype features and patterns of distribution of rDNA sites in the family of Cistaceae at different taxonomic levels.

In *Cistus*, the detailed description of chromosomes indicates that the differentiation in the genus has not been associated to an evident chromosome restructuration, being the karyotypes highly uniform both in number and chromosome morphology. Also, it was noticed that there is little variability in number of the 45S rDNA sites: the result is an evidence of more subtle rearrangements involving loss and amplification of 45S rDNA sites, without distorting the shape of chromosomes. Analyzing the transcription activity, it was found that it is not modified within the sample, being all NOR loci active, with a single exception. It was also checked that there is a variability in the size of nucleoli, as well as the occurrence of nucleolar fusion. Tracing the 45S rDNA site number as character state on the backbone consensus tree it was observed a congruence with the loci number changes to the tree topology with well-supported clades, indicating that the organismal evolutionary history has had an effect on the patterns of 45S rDNA sites distribution. Moreover, the differences in cytogenetic features found in the two *C. umbellatus* subspecies reopen the debate about the definition of the taxonomic boundaries in the genus *Cistus*.

In *Helianthemum* karyotypes were more heterogeneous, being the genus characterized by four different cytotypes even though this variability was not accompanied by a structural relevant repatterning. Indeed, it was observed a tendency to preserve the karyotypic structure and the coarse chromosome morphology within and between the different cytotypes. As pointed in *Cistus*, also in *Helianthemum* the 45S rDNA site number and position did not vary much between the accessions. All NOR loci were transcriptionally active except species with higher 45S rDNA sites, phenomenon explained as the result of a gene dosage control mechanism. The ancestral state reconstruction put in evidence a relation between the 45S rDNA loci number changes to the ITS phylogenetic hypothesis, whose clades corresponded to the sectional taxonomic division. Therefore, all results indicate that species diversification of *Helianthemum* are, at least partly, driven by extensive chromosomal restructuring; this led to evident changes in chromosome number and to more subtle rearrangments highlighted by the pattern of distribution of the 45S rDNA

sites, indicating that differences in genome structure may be more complex (caused by several subsequently occurring events). The definition of which kind of rearrangement has promoted the chromosome base number differentiation observed within the genus is beyond the scopes of this thesis, but of course it can be the subject of further studies investigation.

At smaller scale, such as single-species level, the molecular cytogenetic approch proved to be a powerful tool for the identification of genetic variation in micropropagated plants, leading to the conclusion that the *Cistus* material originated by *in vitro* culture should not be used for restoration purposes. Not only it had 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, the study discourages the use of the micropropagated material for release into the field unless comprehensive surveys (in addition 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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